Macromolecular Content of Inclusions Produced by a Canine Adenovirus

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Early inclusions induced by a canine adenovirus in a canine cell line, appearing before the formation of infectious virus particles, were purified by differential centrifugation in sucrose followed by CsCl density gradient centrifugation. Chemical analysis of these inclusions revealed that they contained deoxyribonucleic acid (DNA), ribonucleic acid, and protein. On the basis of density gradient centrifugation, the DNA extracted from the inclusions was found to be viral DNA. Electron microscope autoradiography showed that these inclusions were the sites of DNA synthesis. In addition, association of DNA polymerase activity with the inclusions was detected by incorporation of radioactivity from 3H-thymidine triphosphate into a DNA product. The in vitro product of the enzyme had a density equal to that of viral DNA rather than host DNA. The level of DNA polymerase activity in exponentially growing infected and uninfected whole cells was similar, but in cells in stationary phase the enzyme activity of infected cells was twice that in noninfected cells. Furthermore, nuclei isolated from infected cells showed a fourfold increase in DNA polymerase activity over the noninfected cells.


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After adenovirus infection, various kinds of intranuclear inclusions appear at different times. These inclusions have been studied extensively with the light and electron microscopes (3, 6, 13, 15). Reports with regard to the nature of these inclusions have been based on cytochemical and autoradiographic studies (12, 13, 15).

Similar sequential studies of the inclusions have also been reported for a canine adenovirus (24). These inclusions were (i) the early granular inclusions and ring-form bodies which appeared before the formation of infectious virus particles and (ii) the dark-staining and light-staining inclusions which were formed after the appearance of virus particles. Histochemical and autoradiographic studies (27) showed that the early granular inclusions and the ring-form bodies were similar in their composition, both types being composed of deoxyribonucleic acid (DNA), protein, and ribonucleic acid (RNA). On the basis of morphological and cytological studies, it was concluded that the ring-form bodies developed from the early granular inclusions.

The present study further elucidates the nature of the early granular and ring-form inclusions and their role in virus multiplication as determined by purification of these inclusions and characterization of them with regard to their macromolecular content.

MATERIALS AND METHODS

Virus and virus assay. The strain of infectious canine laryngotracheitis (ICL) virus was previously characterized (25, 26) and was propagated and assayed as described (27).

Tissue culture and infection of cells. The Madin-Darby canine kidney (MDCK) cell line (CCL-34), obtained from American Type Culture Collection Cell Repository, Rockville, Md., was grown in monolayer at 37°C in Roux bottles. Growth medium was Eagle minimal essential medium supplemented with 5% calf serum penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were infected with ICL adenovirus at a multiplicity of 100 plaque-forming units (PFU)/cell. The virus was allowed to adsorb at 37°C for 1.5 hr. After the adsorption time, unadsorbed virus was removed, and the infected cells were incubated in growth medium at 37°C.

Purification of inclusions from infected cells. Light microscope examination of the cells infected under the above conditions revealed that at 10 hr after infection more than 90% of the cells contained early granular and ring-form inclusions. For the purpose of purification of the inclusions, approximately 6 × 10⁶ cells were used. Ten hours after infection, the cells were scraped off the Roux bottles with a rubber policeman and separated from the medium by low-speed centrifugation. The cells were suspended in 8 ml of reticuloocyte standard buffer (0.01 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane [Tris]-hydrochloride, 0.0015 M MgCl₂, 6H₂O,
pH 7.4) and allowed to swell for 15 min at 0°C. The nuclei were purified by homogenization followed by detergent treatment (17). The purified nuclei containing the granular and ring-form inclusions were suspended in 4 ml of 0.25 M sucrose, 0.01 M Tris-hydrochloride (pH 7.5), and 3 mM MgCl₂. The suspension was sonically treated with a Bronwill Biosonic III (Bronwill Scientific, Rochester, N.Y.) at a setting of 40 for a period of 10 to 15 sec in an ice bath. The extent of breakage of the nuclei was determined with a phase-contrast microscope after each 5-sec period of sonic treatment. After a total of 15 sec, over 98% of the nuclei were found to be broken. The sucrose concentration in the sonicated treated material was raised to 1.3 M in a total volume of 8 ml and homogenized in a Sti-R homogenizer (Tri-R Instruments, Rockville Center, N.Y.) for 3 min. The suspension was centrifuged at 650 × g for 20 min in a Sorvall RC-2B centrifuge using an HB swinging-bucket rotor. The pellet which consisted mainly of unbroken nuclei was discarded and the supernatant fluid was again centrifuged at 2,500 × g for 20 min. The pellet obtained was suspended in 6 ml of 0.88 M sucrose in Tris-hydrochloride (pH 7.5) containing 3 mM MgCl₂, homogenized for 3 min, and centrifuged at 500 × g for 10 min. At this stage of preparation, electron and light microscope examination of the pellet revealed that the preparation contained early granular and ring-form inclusions. Occasionally, some nucleoli (about 1–2%) were present in this preparation. To remove this nucleolar fraction, the pellet was suspended in 2 ml of 1.0 M sucrose homogenized to disperse the aggregates. The suspension was layered on a 4-ml linear CsCl gradient with densities from 1.6 to 1.2 g/cm³ and centrifuged at 30,000 rev/min for 3 hr using an SW 39 rotor and a Beckman model L-2 preparative ultracentrifuge. The inclusions located in a region with a density of 1.38 g/cm³ were collected with a syringe from the top of the tube and CsCl was removed by dialysis against 0.5 M Tris-hydrochloride, pH 7.5. To determine the purity of the inclusions, samples from the final preparation were fixed and embedded for electron microscopy as described previously (24).

DNA, RNA, and protein determination. DNA was determined by the diphenylamine reaction (1) with calf thymus DNA as a standard. RNA was determined by the orcinol reaction (1) with yeast RNA as a standard. Protein was assayed by Lowry method (11) with bovine serum albumin as a standard.

Virus purification. ICL adenovirus was purified from the infected cells by centrifuging twice in CsCl (14).

DNA extraction. Extraction of DNA from infected cells, uninfected cells, or from inclusions was identical. Samples were lysed in a solution of 1% sodium dodecyl sarcosinate, 0.05 M Tris-hydrochloride, 0.1 M ethylenediaminetetraacetic acid, and 100 µg of self-digested Pronase per ml. The mixture was incubated at 37°C for 3 hr. Residual protein was removed by extraction (five times) with water-saturated phenol.

The aqueous solution from the final extraction was brought to 0.1 M NaCl, and the DNA was precipitated with ethanol. The precipitate was dissolved in 1 × SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and dialyzed against 3 liters of 1 × SSC for 24 hr (5 liter change). DNA from ICL adenovirus was extracted by papain digestion followed by phenol extraction (6).

Equilibrium sedimentation analysis of DNA in CsCl. To 2.3 ml of DNA solution in SSC was added 2.9 g of CsCl in 5-ml nitrocellulose tubes. The tops of the tubes were filled with mineral oil, and the tubes were centrifuged at 38,000 rev/min at 25°C in an SW50.1 rotor for 50 hr. Three-drop fractions (about 0.05 ml) were collected from the bottom of the tubes. Density of some fractions was measured with a refractometer, and the DNA in each fraction was precipitated with 5% trichloroacetic acid and collected on membrane filters (Millipore Corp.). The filters were washed in ethanol, placed in Bray solution, and counted in a liquid scintillation counter (Nuclear-Chicago). In some cases, the fractions were diluted with 0.3 ml of distilled water, and the optical densities at 260 nm were determined.

For analytical ultracentrifugation, approximately 3 to 4 µg of DNA was dissolved in CsCl solution with a density of 1.724 g/cm³ and centrifuged at 25°C in a Beckman model E analytical ultracentrifuge at 44,000 rev/min for 24 hr. Photographs of the ultraviolet-absorbing bands in the centrifuge cell were traced with a Beckman Analytrol, and the density of DNA was calculated by the method of Chervenka (4).

Isotopic labeling of DNA. To obtain a mixture of labeled host and viral DNA, MDCK cells grown in monolayer were exposed to a medium containing 0.2 µCi of [methyl-3H]thymidine (specific activity 15.5 Ci/m mole) per ml at 37°C for 24 hr. Cells were then infected with ICL adenovirus, and the virus DNA in infected cells was labeled by incubating in fresh medium containing 0.2 µCi of 3H-thymidine per ml. Sixteen hours after infection, these infected cells were harvested, and DNA was extracted. To label DNA in the early granular and ring-form inclusions, cells 8 hr after infection were labeled by adding 0.5 µCi of 3H-thymidine per ml for 2 hr. The inclusions were then purified as described, and their DNA was extracted.

Electron microscope autoradiography. Monolayers of cells grown in 3-oz (ca. 0.09 liter) bottles were infected with ICL adenovirus. Ten hours after infection, cells were labeled with 100 µCi of 3H-thymidine per ml for 4 min at 37°C. After the labeling period, the radioactive medium was removed, and the cells were washed five times in chilled medium. The cells were then fixed and embedded as described earlier (24).

Thin sections with the same interference color (gold) were picked up and placed on collodion-coated slides (20). The slides were dipped in Ilford L-4 emulsion diluted 1:6 and kept in a light-proof box in a nitrogen gas atmosphere for 2 months. The slides with the emulsion were developed in Kodak D-19 developer, and the sections were examined in the electron microscope. Approximately 112 cells showing distinct nuclear inclusions were examined. The number of grains over the various areas of the cells were counted directly on the microscope screen. In addition, the number of grains located in the section outside the...
cell, but equivalent to the cell area, was also counted to obtain an estimate of background grains.

**DNA polymerase assay.** Tritiated thymidine triphosphate (TTP), specific activity 18.5 Ci/m mole, was obtained from New England Nuclear, Boston, Mass. Other deoxynucleoside triphosphates were purchased from Calbiochem, Los Angeles, Calif. The reaction mixture for polymerase assay contained 200 to 400 \( \mu \)g of protein from freshly prepared inclusions, 0.05 mm each, of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate (dCTP), and 0.3 \( \mu \)m 3H-TTP. The mixture was made to a total volume of 1 ml with a solution containing 50 mm Tris-hydrochloride (pH 8.3) containing 10 mm MgCl\(_2\), 50 mm KCl, and 2 mm dithiothreitol.

In some cases, 200 \( \mu \)g of nicked calf thymus DNA was added to the reaction mixture as an exogenous DNA template. The mixture was incubated at 37°C. At intervals, samples were removed, and the trichloroacetic acid-precipitable material was collected on membrane filters (Millipore Corp.), washed in ethanol, and counted.

**DNA polymerase assay in infected and uninfected cells.** Exponentially growing cells in monolayer were infected with ICL virus (multiplicity of infection [MOI], 100 PFU/cell). A portion of cells was also kept uninfected. Ten hours after infection, both infected and uninfected cells were harvested and suspended in the buffer used for the DNA polymerase assay. Cells were sonically treated for 90 sec (sufficient to break over 99% of the cells). Samples from both uninfected and infected cells were directly assayed for DNA polymerase activity in the presence of 200 \( \mu \)g of nicked calf thymus DNA. In other experiments, cells in stationary growth phase were similarly infected and assayed for polymerase activity. To determine the level of the enzyme in the nuclei, a portion of cells in exponential growth phase were infected as above. Ten hours later, the nuclei from both the infected and uninfected cells were purified. They were sonically treated for 30 sec and assayed for DNA polymerase activity.

**RESULTS**

**Isolation of the early granular and ring-form inclusions.** The time of appearance of the early granular and ring-form inclusions in infected cells was found to be dependent on the MOI. At high multiplicity (100 PFU/cell), the early granular inclusions and ring-form bodies appeared in infected cells by 10 hr after infection. At this time, no infectious virus particles were present in infected cells, as determined by plaque assay. The inclusions in 10-hr infected cells were purified by the procedures described above. Morphological examination of the final preparation of the inclusions stained with hematoxylin and eosin showed the presence of less than one nucleolus per 500 inclusions. Thin sections of the purified inclusions were also examined in the electron microscope. It was found that the inclusions appeared to be free from cytoplasmic material and their morphological integrity was well preserved during the purification procedures (Fig. 1). Even though the early granular inclusions and ring-form bodies were heterogeneous in size, both types were present in the final preparation. To estimate the degree of purity, the amount of host chromatin present in the final preparation of inclusions was determined by prelabeling the cells with 3H-thymidine (0.2 \( \mu \)Ci/ml for 24 hr). The labeled cells were divided into two groups. In the first group, the cells were infected with ICL virus and incubated in fresh medium, and the second group was left uninfected. Ten hours after infection inclusions from the infected cells were purified. DNA from the inclusions and from the uninfected cells was extracted, and the specific activity of the labeled DNA from each preparation was determined. The specific activity of DNA from cells was \( 5.5 \times 10^6 \) counts per min per \( \mu \)g, whereas the specific activity of DNA from the inclusions was \( 2 \times 10^5 \) counts per min per \( \mu \)g. Therefore the association of host chromatin with the purified inclusions was less than 0.5%. From this it was concluded that the preparation was over 99% pure with respect to cellular DNA.

**Analysis of macromolecular content of the inclusions.** Chemical analysis of the isolated inclusions revealed that they contained DNA, RNA, and protein. DNA was a major constituent of these bodies (51% of 0.440 \( \mu \)g), whereas RNA was present in comparatively small amount (3% of 25 \( \mu \)g); protein was 46% (400 \( \mu \)g). (Purified inclusions were suspended in 1 ml of 0.05 mm Tris-hydrochloride, pH 7.5. The data are the average of three experiments.) These results agree with the results of the previous electron microscope study which showed that early granular inclusions and the ring-form bodies were composed of nucleoprotein (27). DNA from the inclusions...
was extracted and analyzed by isopycnic centrifugation in CsCl. The first attempt was to differentiate the ICL adenovirus DNA and MDCK cell DNA on the basis of their buoyant densities in CsCl. Labeled DNA extracted from ICL adenovirus-infected cells was centrifuged to equilibrium in CsCl. Drops were collected, and radioactivity in the acid-precipitable material was determined. It was found that the radioactivity was localized in two bands with densities of 1.718 and 1.700 g/cm³ (Fig. 2). The radioactivity in DNA from the inclusions banded in a region with a density of 1.718 g/cm³. This experiment was repeated three times, and similar results were obtained.

The buoyant densities of viral DNA and MDCK cell DNA were also determined separately after equilibrium centrifugation in CsCl. Viral DNA had a buoyant density of 1.718 g/cm³, and MDCK cellular DNA had a density of 1.700 g/cm³. When a mixture of cell and viral DNA and DNA extracted from the inclusions was centrifuged in CsCl to equilibrium in the analytical ultracentrifuge, viral and cellular DNA localized in two bands with densities of 1.718 and 1.701 g/cm³. The DNA from the inclusions localized in one band with a density of 1.718 g/cm³ (Fig. 3). Repeated CsCl centrifugation of DNA from the ICL adenovirus and from the inclusions revealed that they banded at a region with the same buoyant density. The results indicated that the early granular and ring-form inclusions contained a DNA species, which, on the basis of buoyant density, was similar to viral DNA.

Inclusions as the site of DNA synthesis. In a previous autoradiographic study (27), it was found that exposure of infected cells to tritiated thymidine for 1 hr would result in incorporation of this DNA precursor into the early granular and ring-form inclusions. Therefore, it was suspected that these inclusions were the site of DNA synthesis. In the present study, the infected cells were exposed to ³H-thymidine for a short pulse of 4 min and then used for autoradiography as described in Materials and Methods. Electron microscope examination of the autoradiograms showed that silver grains were mainly located over the early granular and ring-form inclusions (Fig. 4). Although the number of grains per unit area was not determined, from distribution of grains over different cell areas it was found that 88% of them were located over the early granular and ring-form inclusions (Table 1). The back-

![Fig. 2. CsCl density gradient equilibrium centrifugation of DNA from the inclusions and infected cells. ³H-labeled DNA from infected cells was centrifuged in CsCl in separate tubes. Centrifugation was performed in a Beckman model L ultracentrifuge at 38,000 rev/min for 50 hr at 25 C using an SW50.1 rotor. Fractions were collected, and their density was determined. ³H-DNA was collected and counted as described. Location of viral DNA after equilibrium centrifugation in CsCl is shown by an arrow.](http://jvi.asm.org/)

![Fig. 3. CsCl density gradient equilibrium centrifugation of DNA from ICL adenovirus, MDCK cells, and inclusions. Samples of mixed cell and viral DNA (upper) and DNA from the inclusions (lower) were centrifuged in CsCl in a Beckman model E ultracentrifuge using an An-D rotor. Centrifugation was performed at 44,000 rev/min for 24 hr at 25 C. Photographs of ultraviolet-absorbing bands were taken and traced with a Beckman Analytrol. The DNA from this inclusion corresponds to the viral DNA.](http://jvi.asm.org/)
ground grains were slightly less than those for the cytoplasm and nucleoplasm together but not significantly so. These results indicated that thymidine is rapidly transported from the cytoplasm into the early inclusions which are therefore the probable site of DNA synthesis. The lack of any significant radioactivity in the cytoplasm as in the nucleoplasm outside the inclusion would indicate the lack of a thymidine pool in either of these areas. However, the actual grains in the cytoplasm due to thymidine and those due to background was not determinable due to the relatively small numbers of grains observed.

DNA polymerase activity associated with early granular and ring-form inclusions. The results obtained from the autoradiography experiment encouraged the search for the presence of enzymes involved in DNA synthesis. Freshly purified inclusions were assayed for DNA polymerase as described. The preparation incorporated radioactivity from \(^{3}H\)-TTP into an acid-insoluble product (Fig. 5). The product could be degraded into acid-soluble material by treatment with 5 µg of pancreatic deoxyribonuclease. The rate of incorporation of radioactivity from \(^{3}H\)-TTP by the inclusions was constant for about 30 min and then declined. The amount of radioactivity incorporated was approximately proportional to the amount of added inclusions (Fig. 6). The results indicated the association of DNA polymerase activity with the inclusions. The enzyme required all four deoxynucleoside triphosphates for full activity, but some activity was present in the absence of dCTP (Fig. 7). Since the inclusions in the above experiments were rich in DNA, it was not necessary to add DNA as template for the enzyme activity to the reaction mixture. To examine the enzyme activity in the presence of exogenous template, 200 µg of nicked calf thymus DNA was added to the reaction mixture. It was found that incorporation of radioactivity from \(^{3}H\)-TTP into acid-insoluble material increased about 60% (Fig. 8). It is possible that the enzyme could use both the DNA in inclusions and calf thymus DNA. In preliminary experiments, treatment of inclusions with protamine

**Table 1. Distribution of silver grains on thin sections of infected cells**

<table>
<thead>
<tr>
<th>Cell region</th>
<th>Total no. of grains</th>
<th>Percent total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>582</td>
<td>100</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>29</td>
<td>4.6</td>
</tr>
<tr>
<td>Nucleoplasm</td>
<td>41</td>
<td>7.4</td>
</tr>
<tr>
<td>Inclusions</td>
<td>512</td>
<td>88</td>
</tr>
</tbody>
</table>

* MDCK cells were infected with ICL adenovirus. Ten hours after infection, they were labeled with \(^{3}H\)-thymidine for 4 min, then used for autoradiography. A total of 112 cells showing distinct early granular and ring-form inclusion were examined, and the number of grains over the entire cell areas was counted. Background grains on the glycol-methacrylate section approximated those found over the cytoplasm and the nucleoplasm.
Characterization of the DNA polymerase activity product. The sensitivity of the in vitro product to digestion with deoxyribonuclease indicated that the product had properties similar to DNA. To further characterize the DNA product and to determine its relationship with respect to viral DNA, it was analyzed by isopycnic centrifugation in CsCl.

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

**FIG. 7.** Requirement of DNA polymerase activity in the inclusions for the four deoxynucleoside triphosphates. The assay mixtures, each containing 150 µg of inclusion protein, were prepared as described in Fig. 5. One was the complete system, and in each of the others one of the unlabeled deoxynucleoside triphosphates was omitted. Radioactivity from ³H-TTP incorporated into the acid-insoluble material was determined.

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

**FIG. 8.** Effect of calf thymus DNA on ³H-TTP incorporation. Two assay mixtures, each containing 150 µg of inclusion protein, were prepared as in Fig. 5. To one of the reaction mixtures 200 µg of nicked calf thymus DNA was added. The mixtures were incubated at 37 C. At intervals samples were removed, and ³H-TTP incorporation into acid-precipitable material was determined.

The pH of early granular samples shows that the nucleoprotein the triphosphates, dCTP, and deoxyadenosine triphosphate remain intact into the enzyme end of incubation. Different sulfate and ammonium sulfate failed to liberate the enzyme from the inclusions. Therefore, it appeared that the enzyme was tightly bound to the nucleoprotein complex of the inclusions.
A reaction mixture containing inclusions representing 1.0 mg of protein was incubated for 1 hr under the conditions described above. The DNA product was extracted and centrifuged in CsCl to equilibrium. DNA from MDCK cells was also centrifuged in CsCl to determine the position of host cell DNA. The DNA product which contained radioactivity banded at a region with a buoyant density of 1.719 g/cm³ (Fig. 9). Cellular DNA, which could be detected by determining the absorbancy at 260 nm, banded at a region with a density of 1.701 g/cm³. This experiment was repeated twice, and results similar to those in Fig. 9 were obtained. Since the DNA product had a buoyant density close to that of viral DNA, it was suggested that the enzyme copied viral DNA present in the inclusions as a natural template. The breadth of the peak of radioactivity suggests that the newly synthesized chains of DNA were relatively short.

DNA polymerase activity in infected and noninfected cells. To determine whether there was an increase in the level of DNA polymerase in cells after being infected with ICL adenovirus, the activity of this enzyme was determined in infected cells and compared with that in noninfected cells. Cells either in the exponential phase or the stationary phase were infected with ICL adenovirus. Ten hours after infection, the activity of DNA polymerase in both infected and noninfected cells was determined as described. In exponentially growing cells, there was no increase in DNA polymerase activity after infection (Table 2). In contrast, when the cells in the stationary phase were infected, the enzyme activity doubled over that of noninfected cells. Furthermore, when the activity of DNA polymerase in the purified nuclei from exponentially growing infected and noninfected cells was measured, a four-fold increase in the enzyme activity was found in infected nuclei over that of noninfected nuclei.

**DISCUSSION**

Formation of inclusion bodies inside the nuclei of infected cells is a common feature of adenovirus infection (6). Information with regard to the nature of adenovirus-induced inclusions has been largely based on cytochemical and autoradiographic studies (12, 13, 15). A detailed chemical analysis of the inclusions requires a method by which these bodies can be purified from the infected cells. In this study, the early inclusions which appear before the formation of infectious virus particles were isolated, and the purity of the preparation was examined both morphologically and by isotopic labeling. On the basis of previous autoradiographic studies (27), it was assumed that the early granular inclusions and ring-form bodies did not contain cellular DNA. With this assumption, the presence of cellular DNA in the purified inclusions was determined and used as a criterion for purity from contaminating cell material. On this basis, the preparation of the purified inclusions con

![Fig. 9. CsCl gradient equilibrium centrifugation of DNA product. A reaction mixture containing 1 mg of protein from inclusions was prepared as described in the text, except that a double concentration of each of the four nucleoside triphosphates was used. The mixture was incubated at 37 C for 1 hr. 3H-labeled DNA product was extracted. Approximately 20 µg of 3H-labeled DNA product was mixed with 120 µg of MDCK cellular DNA and centrifuged in CsCl to equilibrium as described in Materials and Methods. Fractions were collected, and the density was determined. Optical density at 260 nm and radioactivity in the acid-insoluble material of each fraction were determined.](http://jvi.asm.org/)

**Table 2. DNA polymerase in infected and uninfected cells**

<table>
<thead>
<tr>
<th>State of cells</th>
<th>Source of enzyme</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exponentially grown</td>
<td>Infected cells</td>
<td>7,684</td>
</tr>
<tr>
<td>Exponentially grown</td>
<td>Uninfected cells</td>
<td>7,544</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>Infected cells</td>
<td>8,148</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>Uninfected cells</td>
<td>3,744</td>
</tr>
<tr>
<td>Exponentially growing</td>
<td>Infected nuclei</td>
<td>25,376</td>
</tr>
<tr>
<td>Exponentially growing</td>
<td>Uninfected nuclei</td>
<td>6,528</td>
</tr>
</tbody>
</table>

* MDCK cells in either exponentially growing phase or in the stationary phase were divided into two groups. One group of cells was infected with ICL virus for 10 hr. The other group of cells was kept uninfected as controls. Cells were sonically treated and assayed for DNA polymerase activity as described in the text. Nuclei of infected and uninfected cells in the stationary phase were isolated and similarly assayed for enzyme activity. The protein concentration of each sample was determined, and the enzyme activity was calculated as counts per minute of 3H-TTP incorporated into the acid-insoluble material per milligram of protein of sample per hour.
tained 0.4% cellular DNA. This association of cell DNA with the purified inclusions could be due either to the incorporation of small amounts of host DNA into the inclusions, or to contamination by cell material.

Analysis of the early granular inclusions and ring-form bodies revealed that they were composed of nucleoprotein containing DNA as a major constituent. Although the nature of protein content of the inclusions was not determined, on the basis of immunoferritin staining (20a) it can be concluded that part of this protein(s) is fiber antigen. Histochemical studies (19) on inclusions in adenovirus type 5-infected HEK cells revealed in ring-form inclusions the presence of an arginine-rich basic protein similar to virus core proteins. It is probable that some basic proteins are associated with the RNA in the inclusions of canine adenovirus-infected cells.

The guanine plus cytosine (GC) content of DNA in adenoviruses ranges from 48 to 61% (9). The buoyant density of ICL adenovirus of 1.718 g/cm³ corresponds to a mole fraction of GC of 59%, whereas the buoyant density of MDCK cells was 1.700 g/cm³ which corresponds to a mole fraction of GC of 41%. This difference in density of host and viral DNA made it possible to differentiate them by isopycnic centrifugation in CsCl. Since the DNA content of the early granular and ring-form inclusions had a buoyant density equal to that of viral DNA, it was concluded that these inclusions contained viral DNA. The presence of DNA polymerase activity associated with the inclusions and rapid incorporation of ³H-thymidine into these bodies provided evidence that viral DNA is synthesized and accumulated at the site of early granular and ring-form inclusions. Recently, DNA-synthesizing complexes have been isolated from adenovirus type 12-infected cells (16). These complexes were nucleoprotein containing newly synthesized viral DNA. It is most likely that such DNA replicating complexes are present in the inclusions of ICL adenovirus-infected cells. Furthermore, the rate of synthesis of viral DNA is considerably greater than that of the host DNA during the period of inclusion formation (Fig. 2).

The origin of the enzyme associated with early granular and ring-form inclusions is not known. It could be the host enzyme which becomes involved in synthesis of viral DNA or a totally new virus-coded enzyme which is synthesized in the cells after infection. It has been reported that in adenovirus type 2-infected HeLa cells, DNA polymerase had properties similar to that in noninfected cells (10). Regardless of the origin of the DNA polymerase in inclusions of ICL adenovirus-infected cells, it appears that the enzyme is tightly bound to the nucleoprotein complex of the inclusions. This could explain the increase in DNA polymerase activity in infected nuclei over that of noninfected nuclei.

The level of DNA polymerase activity in adenovirus-infected cells has been reported to depend on the cell type and its physiological state (8). In exponentially growing KB cells infected either with adenovirus type 2 (7) or with adenovirus types 12 and 31 (18), the activity of DNA polymerase did not increase over that of noninfected cells. In other systems using HEK cells infected with adenovirus types 2 and 12, a 1.5- to 3-fold increase in the level of DNA polymerase activity has been reported (1). We also found that in exponentially growing MDCK cells the level of DNA polymerase activity did not change after infection with ICL adenovirus, whereas in cells infected during the stationary phase the enzyme activity increased twofold. This could be due to the low enzyme level of cells in stationary phase, but following infection there is an induction of enzyme synthesis for increased DNA synthesis. The increase in DNA polymerase activity in purified, infected nuclei over noninfected nuclei is not surprising since similar results have been reported in KB cells infected with adenovirus type 2 (7, 21). The low enzyme activity in the nuclei of uninfected cells has been suggested to be due to the leakage of DNA polymerase from the nuclei into the cytoplasmic fraction during cell fractionation in aqueous solution (2, 21, 22). We suggest that in MDCK cells infected with ICL adenovirus the DNA polymerase is bound to the nucleoprotein complex of the early granular and ring-form inclusions, thus preventing its leakage into the cytoplasmic fraction. Whether the preexisting host enzyme binds to the viral DNA in the inclusions or whether a new enzyme is induced after infection remains to be determined. Although the inclusions in ICL adenovirus-infected cells contained RNA, further studies are required to elucidate the relationship of this RNA to the process of virus multiplication. Recent studies (23) have shown that viral nucleoprotein complexes isolated from adenovirus type 2-infected HeLa cells were active in RNA synthesis. It is tempting to speculate that RNA in ICL adenovirus inclusions is transcribed from the viral DNA and is probably viral messenger RNA.

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