Viral DNA Synthesis In Vitro with the Inclusions Isolated from Adenovirus 12-Infected Cells

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(Received for publication, May 20, 1976)

ABSTRACT

A fraction defined as the inclusions was isolated by banding in CsCl gradients from nuclei of adenovirus 12-infected KB cells. When examined by electron microscopy, the isolated inclusions were relatively homogeneous, finely granular materials of moderate electron density, possibly representing the disintegrated type II or IV inclusions. The conditions of endogenous DNA synthesis in vitro with the inclusions were determined. The product of DNA synthesis in vitro with the inclusions was mainly viral and scarcely cellular, as revealed by DNA-DNA hybridization and methylated albumin kieselguhr column chromatography. However, viral DNA synthesized in vitro was smaller (18S, 22S) than viral DNA in virions (31S, 34S) in neutral and alkaline sucrose gradients. Effects of various treatment of the inclusions on the DNA-synthesizing activity showed that phospholipase C inhibited the activity efficiently. The in vitro DNA synthesis was stimulated by addition of the cytoplasmic extract from adenovirus 12-infected cells and not that from uninfected cells. The analysis of the composition of the inclusions showed that the inclusions contained DNA, protein, phospholipid and a small amount of RNA and carbohydrate.

Human adenovirus 12 (H12) DNA replicates in association with types II and IV inclusions in the nucleus of the infected cells [10, 20]. The analysis of viral DNA synthesis with temperature-sensitive mutants of H12 suggests that the inclusions are the viral DNA replication machinery [16]. Shahrabadi et al [15] reported viral DNA synthesis in vitro with the inclusions isolated from canine adenovirus-infected cells.

The present communication describes the examination of properties of a fraction defined as the isolated inclusions and viral DNA synthesis with the isolated inclusions to confirm the above suggestion. The results show viral DNA synthesis in vitro with the isolated inclusions and suggest the involvement of phospholipid in the reaction.

MATERIALS AND METHODS

Cell and virus. Monolayer cultures of human KB cells and the prototype strain of H12 were used. The routine procedures of cell culture and virus assay were described previously [17, 19]. The preparation of purified virions and 32P-labeled virions was also described [28]. 32P-H12 DNA was extracted from labeled virions by papain digestion followed by phenol extraction [7].

Isolation of inclusions from infected cells. This was performed according to Shahrabadi et al [13] with some modifications. Monolayer cultures of KB cells were infected with H12 at 10 PFU (plaque-forming units) per cell. Approximately 2 x 10^8 cells were harvested at 32-hr post infection (p.i.) and collected by low-speed centrifugation. The cells were washed with phosphate-buffered saline (PBS), suspended in 6 ml of reticulocyte standard buffer (RSB, 0.01 M NaCl, 0.01 M Tris-HCl pH7.4, 1.5 mM MgCl2) and allowed to swell for 15 min at 0°C. The nuclei were collected by centrifugation after Dounce homogenization and washed with detergent (sodium deoxycholate and Tween 40) as described by Penman et al [14].
isolated nuclei containing inclusions were suspended in 2 ml of 0.25 M sucrose, 0.01 M Tris-HCl pH 7.4, 0.05 M NaCl and 3 mM MgCl₂. The suspension was sonically treated by a sonicator (Sonore 150, Umeda Electrics, Tokyo) for a period of 60 to 90 sec in an ice bath. The destruction of over 90% of nuclei was confirmed by microscopic examination. The concentration of sucrose was raised to 1.3 M. The suspension was Dounce-homogenized again and centrifuged at 650 × g for 20 min. The supernatant was again centrifuged at 2500 × g for 20 min. The supernatant was layered on a linear sucrose gradient (15-30%, w/w) on a cushion of 2.5 M sucrose and centrifuged at 48 000 rpm for 45 min at 4°C in an SW 50.1 rotor. The inclusions located on the cushion were dialyzed against 0.01 M Tris-HCl pH 7.4, 0.05 M NaCl, 3 mM MgCl₂, 0.1 mM dithiothreitol (DTT) and 20% (v/v) glycerol at 4°C overnight. The dialyzed sample was layered on 4-ml linear CsCl-preformed gradient (density 1.2-1.6 g/cm³) and centrifuged at 30 000 rpm for 3 hr at 20°C in an SW 50.1 rotor. The band of inclusions (see Results) was collected and dialyzed against 0.01 M Tris-HCl pH 8.1, 1 mM MgCl₂, 0.25 mM KCl, 0.1 mM DTT and 30% (v/v) glycerol at 4°C overnight. The dialyzed inclusions were stored at −80°C.

Electron microscopy. The fraction of inclusions was examined as described previously [20].

DNA synthesis in vitro with inclusions. The reaction mixture [total volume 100 μl, 10 mM Tris-HCl pH 8.1, 1 mM MgCl₂, 0.25 mM KCl, 0.1 mM DTT, 20 μM dATP, dGTP, dCTP, 2 μM dTTP, 0.2 mM ATP, 1 μCi ³H-TTP (26.4 Ci/mmol)] received the isolated inclusions. The reaction was performed at 37°C for 50 min. The reaction was stopped by addition of cold 5% trichloroacetic acid (TCA) and the TCA-insoluble radioactivity trapped on a membrane filter (Millipore) was counted.

DNA-DNA hybridization. DNA synthesized in vitro was incubated at room temperature overnight after addition of sodium dodecyl sulfate (SDS, final 0.3%), layered on neutral sucrose gradients (15-30%) in 0.1 M NaCl, 0.1% SDS, 0.01 M Tris-HCl pH 7.4, and centrifuged at 22 000 rpm for 15 hr at 20°C in an SW 25.1 rotor. The same material was first treated with 0.5 N NaOH, 0.1 M EDTA, layered on alkaline sucrose gradients (5-20%) in 0.3 N NaOH, 0.5 M NaCl, 10 mM EDTA and centrifuged at 22 000 rpm for 16 hr at 4°C in an SW 25.1 rotor. Fractions were collected from the bottom and the TCA-insoluble radioactivity in each fraction was counted.

Analysis of the contents of the inclusions. DNA was determined by diphenylamine reaction [6], RNA by orcinol reaction [12], protein by Lowry's method [9], lipid by the method of Folch et al [3] and Gerlach and Beutick [5] and carbohydrate by the colorimetric method of Dubois et al [2].

RESULTS

Isolation of the Inclusions

Two bands (densities 1.380 and 1.402, respectively) and floating materials over the upper band became visible after centrifugation for 3 hr in a preformed CsCl gradient (Fig. 1). After fractionation and dialysis, the endogenous DNA-synthesizing activity in each fraction was examined. The activity was found only in the upper band (1.380) fraction. When the mock-infected cells were treated in the same way, only a thin band (density 1.33) and floating materials were observed. No band was observed at the density equivalent to either
Fig. 1. Isolation of the inclusions in a preformed CsCl gradient. KB cells were infected with H12 at 10 PFU/cell. At 32-hr p.i., the cells were harvested and the inclusions were isolated as described in Materials and Methods. Two bands and floating materials over the upper band became visible after centrifugation for 3 hr in a preformed CsCl gradient.

of the above two bands and no DNA-synthesizing activity was detected in any fractions. This observation indicated that these bands were specific for H12-infected cells and we defined operationally the upper band as the isolated inclusion, since it was active in DNA synthesis in vitro and its density was equal to the inclusions isolated from canine adenovirus-infected cells (1.38) [15]. The lower band was not examined further, since it was inactive in DNA synthesis in vitro. The viral DNA-synthesizing activity in the inclusions (0.32 mg protein) was about 50% of that in the isolated nuclei (119 mg) and the specific activity of the inclusions was approximately 200 times higher than that of the isolated nuclei. Centrifugation for a longer time resulted in the loss of this activity. Electron microscopic examination revealed that the upper band fraction consisted of relatively homogeneous finely granular material of moderate electron density (Fig. 2) and did not retain the integrity of inclusions as observed in the interior of the nuclei of H12-infected cells [10, 11, 20], although occasionally condensed bodies similar to the type IV inclusions and less dense aggregates of granular material suggestive of type II inclusions were present (picture not shown). This granular materials may be inclusions disintegrated during centrifugation in a preformed CsCl gradient, since the inclusions types II and IV as well as various cellular materials were observed in the fraction above the sucrose cushion. This observation shows that the form of the inclusions type II or IV is disintegrated with the isolation procedure, although the endogenous DNA-synthesizing activity is preserved. It shows further that the isolated inclusions are incompletely pure. Under this restricted condition, we used the upper band fraction as the isolated inclusions with the reservations described above.

DNA Synthesis In Vitro with the Inclusions

The conditions for the reaction were examined without addition of the exogenous DNA template. The pH optimum was broad, ranging from 7.0 to 9.0 (Fig. 3c). Although the reaction proceeded without addition of KCl, 0.25 mM KCl gave the maximum value (Fig. 3b). ATP and MgCl₂ stimulated the reaction at 0.2 mM and 1 mM, respectively (Fig. 3a and d). The kinetic analysis of DNA synthesis showed a linear increase of the reaction product up to 50 min at 37 C (Fig. 4). The increase of radioactivity incorporated is approximately
Fig. 3. The conditions for DNA synthesis in vitro with the isolated inclusions. The rates of DNA synthesis in vitro were compared at various concentrations of ATP (a), KCl (b), and MgCl₂ (d) and at various pH (Tris-HCl buffered) (c) in the reaction mixture.

Fig. 4. The kinetics of DNA synthesis in vitro with the isolated inclusions. The reaction was performed as described in Materials and Methods and the TCA-insoluble radioactivity at the indicated time was counted.

The DNA synthesized in vitro hybridized predominantly with viral DNA. The lower hybridization of the in vitro product compared with viral DNA may be due to the large excess of cold H₁₂ DNA in the inclusion preparation. This result showed that DNA synthesized in vitro was mainly viral and scarcely cellular. The conclusion was confirmed by MAK column chromatography (Fig. 6). The in vitro product eluted at the concentration of NaCl of viral DNA and not of cellular DNA.

Table 1. Requirement of deoxyribonucleoside triphosphates for DNA synthesis in vitro)

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>cpm</th>
<th>% of complete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1327</td>
<td>100</td>
</tr>
<tr>
<td>-dATP</td>
<td>688</td>
<td>51.8</td>
</tr>
<tr>
<td>-dGTP</td>
<td>1052</td>
<td>79.3</td>
</tr>
<tr>
<td>-dCTP</td>
<td>627</td>
<td>47.3</td>
</tr>
<tr>
<td>-dATP, dGTP, dCTP</td>
<td>660</td>
<td>49.7</td>
</tr>
</tbody>
</table>

a) DNA synthesis in vitro was carried out with deoxyribonucleoside triphosphates shown above and compared to the complete system.

when one to three of them were omitted, probably due to the large nucleotide pool in the inclusion preparation. A similar result has been reported by Yamashita and Green [27].

Nature of DNA Synthesized In Vitro

The DNA synthesized with the inclusions hybridized predominantly with viral DNA (Table 2). The lower hybridization of the in vitro product compared with viral DNA may be due to the large excess of cold H₁₂ DNA in the inclusion preparation. This result showed that DNA synthesized in vitro was mainly viral and scarcely cellular. The conclusion was confirmed by MAK column chromatography (Fig. 6). The in vitro product eluted at the concentration of NaCl of viral DNA and not of cellular DNA.
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Fig. 6. MAK column chromatography of DNA synthesized in vitro with the inclusions. DNA synthesized in vitro with the inclusions was analyzed by MAK column chromatography as described in Materials and Methods. a, 3H-labeled in vitro product (○) and 14C-labeled KB cell DNA (●). b, 3H-labeled in vitro product (○) and 32P-labeled H12 DNA (●).

Table 2. DNA-DNA hybridization of DNA synthesized in vitro

<table>
<thead>
<tr>
<th>Input DNA</th>
<th>DNA immobilized</th>
<th>Blank filter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viral (5 μg)</td>
<td>Cellular (50 μg)</td>
</tr>
<tr>
<td>Product</td>
<td>1 880 cpm</td>
<td>515 cpm (27.4%)</td>
</tr>
<tr>
<td>Viral</td>
<td>25 280</td>
<td>14 588 (57.7)</td>
</tr>
<tr>
<td>Cellular</td>
<td>1 800</td>
<td>46 (2.5)</td>
</tr>
</tbody>
</table>

DNA synthesized in vitro with the inclusions was alkali-denatured and hybridized to viral and cellular DNA immobilized on membrane filter as described in Materials and Methods. The result is expressed in cpm hybridized. As controls, 3H-labeled H12 DNA from purified virions and 3H-labeled DNA from uninfected KB cells were used.

The size of DNA synthesized in vitro was analyzed by the rate zonal sedimentation in neutral and alkaline sucrose gradients (Fig. 7). A major portion of DNA synthesized in vitro sedimented at about 16 to 18 S in neutral sucrose gradients, with a small portion co-sedimenting with mature H12 DNA (31S). In alkaline sucrose gradients, the in vitro product sedimented at 22 to 24 S. For comparison, H12 DNA synthesized in
Fig. 7. Sedimentation of DNA synthesized in vitro in neutral and alkaline sucrose gradients. The analysis was carried out as described in Materials and Methods. a, Neutral sucrose gradients. b, Alkaline sucrose gradients. \(^3\)H-labeled in vitro product(○). \(^32\)P-labeled H12 DNA(△).

**Table 3. Effects of enzymes and inhibitors on DNA synthesis in vitro with the inclusions**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>cpm</th>
<th>% of complete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>3342</td>
<td>100</td>
</tr>
<tr>
<td>+DNase 50 µg/ml</td>
<td>1095</td>
<td>33.0</td>
</tr>
<tr>
<td>+RNase 50 µg/ml</td>
<td>2995</td>
<td>90.2</td>
</tr>
<tr>
<td>+pronase 250 µg/ml</td>
<td>980</td>
<td>29.5</td>
</tr>
<tr>
<td>+actinomycin D 10 µg/ml</td>
<td>2244</td>
<td>67.5</td>
</tr>
<tr>
<td>+α-amanitin 1.0 µg/ml</td>
<td>3297</td>
<td>99.9</td>
</tr>
<tr>
<td>+phospholipase Cb) 100 µg/ml</td>
<td>365</td>
<td>11.0</td>
</tr>
<tr>
<td>+NP-40 0.5 %</td>
<td>1136</td>
<td>34.2</td>
</tr>
</tbody>
</table>

a) Enzymes and inhibitors were added to the reaction mixture and DNA synthesis in vitro with the inclusions were carried out. The TCA-insoluble radioactivity in each reaction mixture was counted, compared to the complete system without enzyme or inhibitor and expressed as % of the complete system.

b) Phospholipase C was purified to protease-free from phospholipase C type I (Sigma) and given by Dr. S. Nojima, Faculty of Pharmaceutical Sciences, University of Tokyo.

**Effects of Enzymes and Inhibitors on DNA Synthesis In Vitro**

Various enzymes and inhibitors were added in the reaction mixture and DNA synthesis in vitro was carried out (Table 3). Of the reagents tested, phospholipase C inhibited the reaction most efficiently. The result suggested the involvement of phospholipid in the reaction. Moderate inhibition by NP-40 also supported the
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Fig. 8. The effect of phospholipase C on DNA synthesis in vitro. DNA synthesis in vitro with the inclusions (10 μg protein) was carried out without and with the addition of phospholipase C (Sigma Chemicals) at the indicated concentration. After incubation, the TCA-insoluble radioactivity in each reaction mixture was counted.

Fig. 9. Effect of the cytoplasmic extract on DNA synthesis in vitro with the inclusions. H12-infected (32-hr p.i.), uninfected non-growing and growing KB cells were washed with PBS, swollen with RSB for 15 min, homogenized with a tight-fitting Dounce homogenizer and centrifuged at 800 × g for 10 min. The supernatants were centrifuged at 30,000 × g for 2.5 hr at 4°C. The supernatants were dialyzed against 10 mM Tris-HCl, pH 8.1, 1 mM MgCl₂, 0.25 mM KCl, 0.1 mM DTT and used as the cytoplasmic extracts. DNA synthesis in vitro with the inclusions was carried out with or without addition of the cytoplasmic extract. The cytoplasmic extract from infected cells (○), from uninfected non-growing cells (△), from uninfected growing cells (●).

Fig. 10. The late enhancement of DNA synthesis in vitro by the cytoplasmic extract. The reaction was carried out as in Figure 4. When the reaction reached its plateau (60 min, indicated by an arrow), a half of the reaction mixtures received the cytoplasmic extract from H12-infected KB cells, incubated further and examined. Reaction with (△) and without the cytoplasmic extract (○).

suggestion. Moderate inhibition by DNase and pronase may be reasonable, since both DNA and protein in the inclusions must be involved in the reaction. Little or no inhibition by RNase and α-amanitin suggests little involvement of RNA in the reaction. The effect of phospholipase C was examined quantitatively (Fig. 8). Al-
Table 4. Macromolecular contents in the isolated inclusions

<table>
<thead>
<tr>
<th>Component</th>
<th>Inclusions</th>
<th>Nuclear membrane 1.16–1.18 g/ml</th>
<th>Nuclear membrane 1.18–1.20 g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>39.4%</td>
<td>3.5%</td>
<td>3.8%</td>
</tr>
<tr>
<td>RNA</td>
<td>2.4</td>
<td>7.0</td>
<td>6.8</td>
</tr>
<tr>
<td>Protein</td>
<td>22.5</td>
<td>58.2</td>
<td>59.9</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>30.6</td>
<td>25.9</td>
<td>25.2</td>
</tr>
<tr>
<td>Neutral sugar</td>
<td>3.6</td>
<td>3.5</td>
<td>2.6</td>
</tr>
<tr>
<td>Unidentified residue</td>
<td>2.1</td>
<td>1.9</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Contents of the isolated inclusions were determined as described in Materials and Methods and expressed in % of the dry weight of the inclusions. As controls, the nuclear membrane fractions (inner density 1.18–1.20 and outer density 1.16–1.18) were isolated from uninfected KB cells by the method of Kashing and Kasper [8] and similarly analyzed.

Effect of the Cytoplasmic Extract on the Reaction

Since the reduction in DNA-synthesizing activity was experienced during the isolation procedure of the inclusions, the effect of the cytoplasmic extract on the reaction was examined (Fig. 9). The cytoplasmic extract from H12-infected KB cells stimulated the activity about threefold, while those from uninfected cells showed no enhancing effect. The extract alone did not show DNA synthesis in vitro. This observation indicated that a factor(s) necessary for viral DNA replication may exist in the cytoplasm of infected KB cells. When the reaction reached a plateau, the addition of the cytoplasmic extract stimulated the reaction again with a lag for 10 min (Fig. 10). It is not known whether this resumption of DNA synthesis is due to the reinitiation, to the stimulation of the disturbed reaction, or to the repair synthesis.

DISCUSSION

Contradictory reports that adenovirus DNA replicates in association with the nuclear membrane [13, 27, 29] and that adenovirus DNA replicates at the inclusions without association with the nuclear membrane [10, 11, 20, 21] can be consistent with each other, when the inclusions are contained in the nuclear membrane fraction [20]. To confirm the suggestion, a fraction defined as the inclusions was isolated from H-12-infected KB cells by the method similar to that described by Shahrabadi et al [15]. The isolated inclusions retained the viral DNA-synthesizing activity, although their morphological form was mostly disintegrated (Fig. 2). The analysis of the contents of the inclusions showed a high content of phospholipid (Table 4). The content of phospholipid in the inclusions higher than that in the nuclear membrane excludes the possible contamination of phospholipid from the membrane, and explains the reason why the inclusions are contained in the nuclear membrane fractions. Moreover, DNA synthesis in vitro with the inclusions was inhibited efficiently by phospholipase C (Table 3, Fig. 8). The preparation of phospholipase C is sometimes contaminated with proteases. However, phospholipase C used in this experiment was purified to protease-free and inhibited the reaction more strongly even at a concentration of 20 μg/ml.
than pronase. This observation suggests the involvement of phospholipid in DNA synthesis. It is not known, however, whether the phospholipid is involved in DNA synthesis directly or indirectly by preserving the conformational integrity of the DNA replication machinery. It has been reported that a holoenzyme of DNA polymerase III from \textit{E. coli} needs phospholipid for the conversion of the phage \textphi X174 single-stranded DNA to the replicative form [25] and that DNA replication in \textit{E. coli} is initiated at a particular lipid site on the membrane [4]. Further studies are necessary to clarify the role of phospholipid in adenovirus DNA synthesis. Shahrabadi et al [15] isolated the inclusions from canine adenovirus-infected cells. However, they did not show the existence of phospholipid in the inclusions. The reason for this discrepancy is not known. When cells are labeled with $^3$H-cholin, infected with \textit{H5} and examined by electron microscope autoradiography, grains are found at or on the nuclear membrane [23]. This result may be reasonable and not be contradictory to the presence of phospholipid in the inclusions, since the radioactivity in the inclusions is low when cells are labeled with $^3$H-cholin only early after infection and is pretty high when the cells are labeled at the late stage of infection (Ariga, unpublished observation). Electron microscope autoradiography of cells labeled with $^3$H-cholin during the late stage of infection is now in progress.

The inclusions contained DNA, protein, phospholipid and small amounts of RNA and carbohydrate (Table 4). The ratio of DNA, protein and RNA is similar to that in the inclusions from canine adenovirus-infected cells [15]. Although the role of RNA in the inclusions is not known, it is tempting to speculate the viral transcription in the inclusions, since it is reported that the viral nucleoprotein complex isolated from H2-infected cells is active in viral RNA synthesis in vitro [24]. Further studies are now in progress to confirm the idea.

Viral DNA synthesized in vitro with inclusions was smaller (18 S) than viral DNA in virions (31 S) in neutral sucrose gradients. The same result is reported by Yamashita and Green [27], showing that the 18 S DNA is formed by a specific endonuclease present in the nuclear membrane complex from H2-infected KB cells. When in vitro reaction with H12-infected KB cell nuclei was performed, viral DNA synthesized in vitro was also small (18 S). However, viral DNA synthesized in vitro with H5-infected KB cell nuclei was the mature form (31S) ([22], Ariga, unpublished observation). It is known that H5 has no endonuclease. Yamashita observed that in vitro product with the replication complex extracted from H2-infected KB cells was mature (31 S, 34 S) in neutral and alkaline sucrose gradients, due to the lack of the endonuclease (Yamashita, personal communication). It is not known, however, whether or not this endonuclease is identical to that reported by Burlingham and Doerfler [1]. The analysis of viral genome with EcoR1 cleavage indicates that adenovirus DNA replication starts at two points and the distance between these points and the both ends of the genome is about one fourth of the viral genome (Winacker, personal communication). The 18 S DNA is about one fourth of 31 S DNA. The significance of this coincidence cannot be evaluated, however.

Viral DNA synthesis in vitro was analyzed with the inclusions as described above. However, many problems remained undetermined, such as the role of phospholipid in viral DNA synthesis, the lack of the formation of mature viral DNA, the role of the cytoplasmic extract in stimulating the in vitro reaction etc. Further studies are necessary to clarify these problems.

**ACKNOWLEDGMENT**

This work was supported by grants from Ministry of Education, Science, and Culture of Japan and from the Mitsubishi Foundation.

**REFERENCES**


