Catalysis of Adenovirus DNA Synthesis in vitro by DNA Polymerase γ

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SUMMARY

DNA replication complexes were purified from adenovirus type 5 (Ad5)-infected HeLa cells. DNA synthesis by these complexes in vitro was extremely sensitive to the inhibitors dideoxythymidine triphosphate, N-ethyl maleimide and p-hydroxymercuribenzoate. The bound DNA polymerase was released from the complexes by limited digestion with micrococcal nuclease. This released polymerase preferred poly(rA):(dT)12-18 as template over activated calf thymus DNA. These results are compatible with the major polymerase in the replication complex being of the γ class.

Although a number of virus-coded functions are required for the replication of adenovirus DNA, there is no evidence for any of these being a DNA polymerase and it has been assumed that host enzymes are involved in some way. DNA polymerase α has been implicated on the basis of the increase in activity of this enzyme in infected cells following adenovirus infection (de Jong et al. 1977) and on the inhibitory effect of aphidicolin (Longiaru et al. 1979) on virus replication. In contrast, experiments examining the effect of 2′3′ dideoxythymidine 5′ triphosphate (ddTTP) on adenovirus DNA synthesis in isolated nuclei (Van der Vliet & Kwant, 1978) have pointed to DNA polymerase γ playing a crucial role in replication. On the other hand, studies of the properties of putative replication complexes isolated from adenovirus-infected cells have suggested that cellular polymerases of both the α and γ classes are involved (Ito et al. 1976; Arens et al. 1977; Brison et al. 1977; Frenkel, 1978; Abboud & Horwitz, 1979).

We have previously described (Shaw et al. 1978, 1979; Shaw, 1979) a soluble subnuclear fraction (the S200 fraction) which synthesizes adenovirus DNA in vitro. This relatively crude fraction contains adenovirus DNA replication complexes, with a template-bound DNA polymerase, and also a vast excess of non-template bound DNA polymerase which can be effectively separated by sucrose gradient centrifugation. The nature of the DNA polymerase responsible for adenovirus DNA replication in this system has been studied.

The S200 fraction was prepared using the method of Wilhelm et al. (1976), by treating nuclei isolated from adenovirus type 5 (Ad5)-infected HeLa cells at 17 to 18 h p.i. with 200 mm-ammonium sulphate. Removal of the nuclear pellet by centrifugation produced a supernatant (the S200 fraction) containing most of the replicating DNA and the major portion of the in vitro DNA synthetic activity found in the infected cell. This fraction was then layered on to a 10 to 58% (w/v) sucrose gradient containing 20 mm-tris-HCl, pH 7-9, 2 mm-EDTA and 200 mm-ammonium sulphate, and centrifuged for 3 h at 41000 rev/min in a Beckman SW41 rotor. Fractions were collected dropwise from the bottom of the tube and analysed for DNA polymerase activity in the presence or absence of activated calf thymus DNA. Fractions in the middle third of the gradient containing the Ad5 DNA replication complex, and the non-template-bound DNA polymerase towards the top, were pooled separately for further analysis. The DNA replication complex thus isolated synthesized full length adenovirus DNA in vitro and accounted for the major portion of the
Table 1. Template preferences of DNA polymerases

(a) Micrococcal nuclease-treated replication complex

<table>
<thead>
<tr>
<th>Template</th>
<th>Incorporation with 5 mM-Mg^{2+}</th>
<th>Incorporation with 0.8 mM-Mn^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0 (153.5)†</td>
<td>0 (47.5)†</td>
</tr>
<tr>
<td>Poly(rA):(dT)$_{12-18}$ (1:1)</td>
<td>28.1</td>
<td>38.2</td>
</tr>
<tr>
<td>Activated calf thymus DNA</td>
<td>21.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Poly(rA):(dT)$_{12-18}$ (7:3)</td>
<td>12.8</td>
<td>ND‡</td>
</tr>
<tr>
<td>Poly(dA):(dT)$_{12-18}$ (7:3)</td>
<td>4.1</td>
<td>ND</td>
</tr>
</tbody>
</table>

(b) Non-template-bound DNA polymerase

<table>
<thead>
<tr>
<th>Template</th>
<th>Incorporation with 5 mM-Mg^{2+}</th>
<th>Incorporation with 0.8 mM-Mn^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Poly(rA):(dT)$_{12-18}$ (1:1)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Activated calf thymus DNA</td>
<td>5.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Poly(rA):(dT)$_{12-18}$ (7:3)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Poly(dA):(dT)$_{12-18}$ (7:3)</td>
<td>0.15</td>
<td>0.05</td>
</tr>
</tbody>
</table>

* 20 μl samples were incubated in a reaction vol. of 100 μl which contained 4 mM-ATP, 5 mM-MgCl$_2$, 50 μm each of dATP, dCTP, dGTP, 0.5 μm-$^{3}$H-dTTP (sp. act. 30 Ci/mmol) 20 mM-Hepes/KOH, pH 7.9, 40 mM-(NH$_4$)$_2$SO$_4$, 44 mM-NaCl, 0.2 mM-CaCl$_2$, 0.4 mM-dithiothreitol and 20% glycerol. After 60 min incubation at 37 °C, the reactions were stopped by adding 2 ml of cold 20% trichloroacetic acid (TCA). The precipitates were collected by filtration on to Whatman GF/C discs, which were washed with 200 vol. of cold TCA and 50 vol. of cold ethanol. The discs were air dried and counted in 5 ml of toluene scintillation fluid containing 0.5% PPO and 0.01% POPOP. Template concentration used in all assays was 50 μg/ml. Figures in parentheses indicate the ratio of adenosine-containing polymer to thymine-containing polymer by molarity of nucleotides. Incorporation is expressed in pmol $^{3}$H-dTMP incorporated in 60 min. Poly(rA):(dT)$_{12-18}$ (1:1) was obtained from Boehringer (Lewes, Sussex), poly(rA), poly(dA) and (dT)$_{12-18}$ were obtained from P. L. Chemicals (Windsor, Berks.) and the appropriate primer-template prepared by mixing and incubating at 37 °C for 15 min, followed by rapid chilling.

† Incorporation with intact replication complex as template.
‡ ND, Not done.

endogenous DNA synthetic activity of the S200 fraction (Shaw et al. 1979b). The nontemplate-bound DNA polymerase could also be isolated in a similar manner from uninfected cells.

The template preferences of purified eukaryotic DNA polymerases have been used as a means of differentiating these enzymes. Thus, DNA polymerase γ prefers deoxyribopoligomeroligomerotidie-primed polynucleotideprimed polynucleotides, whereas the α- and β-polymerases prefer natural DNA activated by limited DNase digestion (Weissbach, 1977). To assess the template preference of the DNA polymerase already bound to the replication complex the enzyme was released by treatment of the replication complex with micrococcal nuclease (10 μg/ml) for 15 min at 37 °C then inhibiting the nuclease by addition of 2 mM-EGTA (Pelham & Jackson, 1976). Such micrococcal nuclease-treated replication complexes were dependent on added template DNA for synthetic activity and exhibited negligible residual nuclease activity.

Table 1(a) shows the results of assaysing micrococcal nuclease-treated replication complexes for DNA polymerase activity in the presence of various templates, utilizing Mg$^{2+}$ or Mn$^{2+}$ as the divalent cation. Using Mg$^{2+}$ as divalent cation, activity was greatest with poly(rA):(dT)$_{12-18}$ (1:1) followed closely by activated calf thymus DNA and poly(rA):(dT)$_{12-18}$ (7:3). A small amount of synthesis was detectable using poly(dA):(dT)$_{12-18}$ (7:3). However, using Mn$^{2+}$ as divalent cation, poly(rA):(dT)$_{12-18}$ was greatly preferred to activated calf thymus, activity on the former being 19-fold greater. In addition, Mg$^{2+}$ was
the preferred cation for copying activated calf thymus DNA. These results are all consistent
with the replication complexes containing mainly DNA polymerase γ.

In contrast, Table 1(b) shows that when assayed in the same manner, the non-template-bound DNA polymerase from Ad5-infected (or uninfected) cells exhibited a 40 to 70-fold preference for activated calf thymus DNA over poly(dA):(dT)₁₂₋₁₈, with negligible synthesis occurring on poly(rA):(dT)₁₂₋₁₈. This data is consistent with the non-template-bound DNA polymerase being mainly DNA polymerase α.

The nature of the replication complex and non-template-bound DNA polymerase was investigated further by the use of specific inhibitors of DNA synthesis. The three classes of eukaryotic DNA polymerase respond differently in vitro to ddTTP. The α-polymerase is largely resistant to ddTTP/dTTP ratios of up to 10, whereas the β-polymerase is 80 to 95% inhibited at this concentration (Edenberg et al. 1978; Van der Vliet & Kwant, 1978; Waqar et al. 1978). DNA polymerase γ is the most sensitive, being more than 95% inhibited at ratios of less than 1 (Edenberg et al. 1978; van der Vliet & Kwant, 1978). The effect of this inhibitor on DNA polymerase activity in the pooled fractions, in the nuclease-treated replication complex and in the S200 fraction was investigated, and the results plotted in Fig. 1(a). The values determined for the purified DNA polymerases α, β and γ by Edenberg et al. (1978) and Waqar et al. (1978) are also shown (solid lines).

Endogenous DNA synthesis in the replication complexes was extremely sensitive to ddTTP, 90% inhibition occurring at a ddTTP/dTTP ratio of 1:20. Micrococcal nuclease-treated replication complexes copying activated calf thymus DNA were slightly less sensitive, 90% inhibition occurring at a ratio of 1:3. Both these values are lower than those previously described for DNA polymerase γ (Edenberg et al. 1978; Van der Vliet & Kwant, 1978; Waqar et al. 1978). In neither case did any significant residual activity remain above a ddTTP/dTTP ratio of 1:1, even when copying activated calf thymus DNA. This is the preferred template for DNA polymerases α and β and, if significant amounts of these polymerases were present, they should have been detected by such an analysis. Since no ddTTP-resistant DNA polymerase activity was detectable under these conditions it would seem to preclude an involvement of DNA polymerase α or β in this in vitro DNA synthesis system (assuming that the results obtained with the purified enzymes are equally applicable to this relatively crude system).

In contrast, the non-template-bound DNA polymerase from both Ad5-infected (and uninfected) cells was largely resistant to ddTTP and retained 40% activity at ratios up to 100:1. The slightly greater sensitivity to low ddTTP concentrations indicates that there may be some β- or γ-polymerase in this fraction.

Endogenous DNA synthesis in the S200 fraction was sensitive to low concentrations of ddTTP. Up to a ratio of 1:10, the activity resembles DNA polymerase γ. However, above this value the activity remains resistant to ddTTP and retains 25% activity at a ratio of 10:1. Thus, DNA polymerase α probably accounts for 20 to 30% of the endogenous DNA synthetic activity of the S200 fraction. Whether this is due to DNA polymerase α loosely bound to the replication complexes which is subsequently removed by centrifugation, or simply adventitious synthesis by non-template-bound DNA polymerase on fragments of DNA is not known.

N-ethyl maleimide (NEM) and p-hydroxymercuribenzoate (pHMB) are sulfhydryl group blocking agents which are extremely inhibitory to DNA polymerase α and γ, but DNA polymerase β is resistant to up to 5 mM-NEM and 30 μM-pHMB (Weissbach et al. 1971; Wang et al. 1974; Matsukage et al. 1975). The effect of these inhibitors on DNA polymerase activity was thus investigated and the results expressed in Fig. 1(b) (NEM) and 1(c) (pHMB) in which the values determined for a purified β-polymerase by Matsukage et al. (1975) are shown as solid lines.
Fig. 1. Effect of specific inhibitors on DNA polymerase activity. DNA polymerase assays were performed as described in the legend to Table 1. (a) ddTTP. ●, Non-template-bound DNA polymerase from Ad5-infected cells, copying 50 µg/ml activated calf thymus DNA: 100% activity = 12.5 pmol 3H-dTMP incorporated/60 min. ○, Non-template-bound DNA polymerase from mock-infected cells, as above: 100% activity = 7.8 pmol 3H-dTMP incorporated/60 min. ▲, Endogenous DNA synthesis in the S200 fraction: 100% activity = 1 pmol 3H-dTMP incorporated/60 min. ■, Endogenous DNA synthesis in the replication complexes: 100% activity = 220 fmol 3H-dTMP incorporated/60 min. □, Micrococcal nuclease-treated replication complexes copying 50 µg/ml activated calf thymus DNA: 100% activity = 13 fmol 3H-dTMP incorporated/60 min. In all assays the dTTP concentration was adjusted to 1 µM. (b) NEM. ○, Endogenous DNA synthesis in the replication complexes: 100% activity = 93 fmol 3H-dTMP incorporated/60 min. (c) pHMB: symbols as in (b).

Both NEM and pHMB were extremely inhibitory to the endogenous DNA synthetic activity of the replication complexes, pHMB being effective at 1 µM. The non-template-bound DNA polymerase was somewhat less sensitive to both inhibitors, but still considerably more sensitive than a purified β-polymerase. These results indicate that it is unlikely that DNA polymerase β is involved in Ad5 DNA synthesis in vitro, although small amounts of this enzyme may be present among the non-template-bound DNA polymerase.
However, it should also be considered that other enzymes involved in DNA synthesis containing a functional sulphydryl group could also be inhibited under these conditions.

The results presented here amplify previous studies on this problem and indicate that within the limits of this analysis the major DNA polymerase activity present in partially-purified Ad5 DNA replication complexes was the γ-polymerase, although the participation of other polymerases could not be completely excluded. It is possible that a complex could exist containing both α- and γ-polymerases, in which each enzyme was dependent on the other and inhibition of one enzyme would inhibit the other. If this were so, treatment with micrococcal nuclease which could conceivably dissociate such a complex should reveal the two activities. It is also worth noting in the light of the method used to prepare the replication complex that the γ-polymerase appears to bind more strongly to DNA than the α- or β-polymerase under high salt conditions (B. Otto, personal communication). Since the replication complexes described here appear to elongate DNA chains presumably initiated in vivo (Shaw, 1979) it is probable that certain components essential for complete adenovirus DNA replication are removed during their preparation. If one of these components were DNA polymerase α, this could account for the relative resistance of endogenous DNA synthesis in the S200 fraction to ddTTP and also the finding that aphidicolin, a specific inhibitor of this enzyme, inhibits adenovirus DNA synthesis in intact cells (Longiaru et al. 1979).

The results presented in this report are in agreement with those of Van der Vliet & Kwant (1978), who showed that ddTTP inhibited adenovirus DNA synthesis in isolated nuclei. On the other hand, Abboud & Horwitz (1979), analysing replication complexes prepared from a fraction similar to the S200 fraction, detected more α-polymerase than γ-polymerase. It should be noted, however, that these workers separated the replication complexes by sedimentation on to a sucrose cushion and thus could not exclude contamination by other aggregates in this fraction.

The function of the γ-polymerase is not known for certain, although it does seem to be the sole DNA polymerase detectable in mitochondria (Bolden et al. 1977). The mitochondrial genome, although circular (Borst & Grivell, 1978), has several features in common with adenovirus DNA. The genome is not complexed with cellular histones (D. Williamson, personal communication) and DNA replication proceeds via a displacement mechanism (Robberson et al. 1972). A further interesting observation is that adenovirus infection inhibits host nuclear DNA synthesis (Mantyjarvi & Russell, 1969) but not mitochondrial DNA synthesis (Fisher & Horwitz, 1977). Although the results presented in this report may not be applicable to the in vivo situation, which is certainly more complex, they suggest that DNA polymerase γ is intimately involved in the replication of adenovirus DNA.

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