Mitochondrial DNA polymerase, deoxyribonuclease and ribonuclease H activities from brain of chick embryo

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ABSTRACT

R-DNA polymerase, D-DNA polymerase, DNase and RNase H activities in mitochondria from chick embryonic brain were studied by ion-exchange chromatography. Two main fractions were separated according to their chromatographic behaviour: a fraction M Ib which is eluted with the washing buffer from two successive DEAE-cellulose columns and a fraction M IV which is eluted at 400 mM KCl from a phosphocellulose column. Although the two fractions contain both the DNA polymerase and the degrading activities, all the specific activities are higher in fraction M IV than in fraction M Ib. Heat inactivation experiments have shown that R-DNA polymerase is inactivated in both fractions, whereas RNase H and DNase are not affected. Thus, degrading activities and R-DNA polymerase activity seem to be catalyzed by different molecular entities. However the fact that in most cases these activities co-chromatograph suggests that the corresponding molecules form rather stable complexes.

INTRODUCTION

Avian myeloblastosis viruses contain a DNA polymerase which catalyzes dGMP polymerization by transcription of polyribocytidylate sequences of either (C)\textsubscript{n}-(dI)\textsubscript{n} or (C)\textsubscript{n}-(I)\textsubscript{n} duplex structures\textsuperscript{1}. This activity has also been observed in other oncogenic RNA viruses by using (dG)\textsubscript{12-18}-(C)\textsubscript{n} as primer-template\textsuperscript{2,3}.

One of us\textsuperscript{4} has shown for the first time in eukaryotes the presence of a DNA polymerase which polymerizes dGMP when (dG)\textsubscript{n}-(C)\textsubscript{n} is provided as primer-template. This activity was probably due to a contamination of chick material with virus C-type particles, since a similar activity was observed by using (dG)\textsubscript{12-18}-(C)\textsubscript{n} as primer-template with cultured mouse fibroblast L/929 cells\textsuperscript{5} which were in fact probably contaminated with an endogenous murine leukaemia virus\textsuperscript{6,7}. On the other hand, three recent reports mention the presence
of (dG)$_{12-18}$·(C)$_n$-dependent DNA polymerase activity, in leukocytes from a childhood acute lymphoblastic leukaemia$^3$, in a human breast tumour cell line$^8$, and in murine plasmocytomas$^9$. These cellular enzymes are considered to be identical to reverse transcriptase from oncogenic RNA viruses.

Ribonuclease H, an enzyme which specifically degrades the RNA strand of DNA-RNA hybrids to acid-soluble products, was originally discovered in calf thymus$^{10,11}$. Subsequently, its presence was recorded in KB cells and chick embryo$^{12}$. Special attention has also been given to the association of DNA polymerase and ribonuclease H activities in several oncogenic RNA viruses$^{13,14}$.

It has been shown that neither exonuclease nor pyrophosphorolysis activity is present in purified calf thymus 3.4 S DNA polymerase$^{15}$. It is interesting to compare these results with those obtained with E. coli DNA polymerase which contains two associated exonuclease activities and also catalyzes pyrophosphorolysis and a pyrophosphate exchange reaction$^{16}$. However, the existence of degrading activities associated with DNA polymerase in other eukaryotic systems cannot be excluded since the presence of an endonuclease associated with a mitochondrial DNA polymerase has recently been shown$^{17}$.

In the present paper we have analyzed the DNA polymerase, deoxyribonuclease and ribonuclease H activities present in mitochondria isolated from 11-day-old chick embryonic brain. We have distinguished two separate DNA polymerase activities, a D-DNA polymerase and an R-DNA polymerase (nomenclature according to FRY and WEISSBACH$^5$). The former is characterized by its ability to catalyze either dGMP or dAMP polymerization when provided with (dG)$_n$·(dC)$_n$ and (dT)$_n$·(dA)$_{12-18}$ respectively, the latter by the polymerization of dTMP when using (dT)$_n$·(A)$_n$ or (dT)$_{12-18}$·(A)$_n$ as primer-template. Deoxyribonuclease and ribonuclease H activities (hereafter DNase and RNase H) are tested by using as substrates [$^3$H] (dT)$_n$·(A)$_n$ and (dT)$_n$·(dA)$_{12-18}$·[$^3$H]·(A)$_n$ respectively.
MATERIAL AND METHODS

A) Preparation of enzymes

About 250-300 brains from eleven-day-old chick embryos are removed as previously described. Mitochondria are isolated according to a slight modification of the method of CHAPPELL and HANSFORD. Brains are homogenized in buffer A (50 mM Tris-HCl pH 7.6, 25 mM KCl, 5 mM Mg(CH₃COO)₂, 1 mM dithiothreitol (DTT), 0.88 M sucrose). After filtration on gauze, the homogenate is centrifuged at 600 x g for 15 min, then the supernatant is centrifuged at 10,000 x g for 15 min. The pellet, which contains the mitochondria, is washed three times with buffer A and is then suspended in buffer B (50 mM Tris-HCl pH 7.8, 500 mM KCl, 1 mM EDTA, 1 mM DTT) and Nonidet P 40 (Shell) is added to a final concentration of 0.4% (v/v). The extract is then incubated 15 min at 37° and after centrifugation at 10,000 x g for 30 min the supernatant is recovered and (NH₄)₂ SO₄ is added in two steps: up to 25% saturation and between 25 and 80% saturation. In the second precipitation step most of the DNA polymerase and degrading activities are recovered. This precipitate is dissolved in a small volume of buffer B and dialyzed against 400 volumes of buffer C (50 mM Tris-HCl pH 8.3, 50 mM KCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT). After concentration of the sample by ultrafiltration (Amicon membrane UM 20 E), it is adsorbed on a DEAE-cellulose column. Protein concentration is determined by the method of LOWRY et al., and correction for the colour contribution of Tris-containing buffers is made.

B) Ion-exchange chromatography

6 ml of crude extract (11.5 mg protein/ml) are loaded on to a column (10 mm x 260 mm, volume 20 ml) of DEAE-cellulose (exchange capacity 0.82 meq/g) equilibrated with buffer C. After washing with 6 column-volumes of the equilibrating buffer, 200 ml of 175 mM KCl are applied. Mitochondrial fraction M Ia (Fig. 1), eluted with the washing buffer, is concentrated by ultrafiltra-
tion and loaded on to a second DEAE-cellulose column, which is equilibrated with buffer C. After washing with 100 ml of equilibrating buffer, 200 ml of 350 mM KCl are applied. 10 ml-fractions are collected from both DEAE-cellulose columns at a flow-rate of 15-20 ml/hr. Mitochondrial fraction M II (Fig. 1), eluted with 175 mM KCl, is concentrated and then loaded on to a phosphocellulose column (the same dimensions as the DEAE-cellulose column; exchange capacity 7.4 meq/g) which is equilibrated with buffer D (the same as buffer C except that it contains 175 mM KCl). After washing with 100 ml of the equilibrating buffer, 100 ml of 400 mM KCl are applied and 5-ml fractions are collected at the above-mentioned flow-rate.

Fractions eluted with the washing buffer from the second DEAE-cellulose column (mitochondrial fraction M Ib, Fig. 3), with the washing buffer from the phosphocellulose column (mitochondrial fraction M III) and at 400 mM KCl (mitochondrial fraction M IV, Fig. 2) are concentrated and stored at -20° in glycerol (50 % v/v).

C) Templates and substrates.

(dG)n-(dC)n (S20,W=16.3), (dT)n-(A)n (S20,W=8.7, Emax(λ) = 6.7, n=300 nucleotides) are obtained from Miles Laboratories, (dT)12-18-(A)n, (dT)n-(dA)12-18, are from Collaborative Research. The extinction coefficient of (dT)n-(A)n is that communicated by the manufacturer. For (dG)n-(dC)n, (dT)12-18-(A)n and (dT)n-(dA)12-18 the extinction coefficients are calculated on the basis of published values (RILEY et al.20, BOLLUM21) and the hypochromicity indicated for each one by the supplier. [3H] deoxyguanosine 5'-triphosphate (11 Ci/m mole), methyl [3H] thymidine 5'-triphosphate (28 Ci/m mole) and adenosine [3H] 5'-triphosphate (20.5 Ci/m mole) are supplied by the Radiochemical Centre, Amersham.

Ribonuclease H substrate.

(dT)n-(dA)12-18-[3H] (A)n is synthesized in a 1500-μl reaction mixture
containing: 100 mM Tris-Cl pH 7.6, 2.5 mM MnCl₂, 1 mM DTT, 0.4 pM adenosine
5'-triphosphate, 48.3 pmol of (dT)ₓ(dA)₁₂₋₁₈, 45 µg of purified yeast RNA
polymerase (a gift from Dr. A. SENTENAC) 10 µCi adenosine [³H] 5'-triphosphate.
After 15 min incubation at 37°, sodium dodecyl sulfate (SDS) is added to a final concentration of 0.2% (v/v). The sample is dialyzed against 50 mM Tris-
Cl pH 7.6 containing 1 mM EDTA. The specific activity of the complex is 70 cpm/pmol. Ribonuclease A (Worthington) has no action on this substrate.

Deoxyribonuclease substrate.

[³H] (dT)ₓ(A)ₓ is prepared in a reaction mixture of 1500 µl containing:
50 mM Tris-Cl pH 8.3, 60 mM KCl, 1mM DTT, 1 mM MnCl₂, 280 pmol of (dT)ₓ(A)ₓ,
100 µg of mitochondrial fraction M 1b (See Fig. 3), 15 µCi methyl[³H]
thymidine 5'-triphosphate. After 60 min incubation at 37°, SDS is added to a final concentration of 0.2% (v/v) and the sample is dialyzed against 50 mM
Tris-Cl pH 8.3, 60 mM KCl, 1 mM EDTA. After recovery of the complex by ethanol
precipitation, it is dried in vacuo and dissolved in 150 mM NaCl. The specific
activity of the duplex is 14 cpm/pmol.

1 unit of either RNase H or DNase is the amount of enzyme which
transforms 1 nmole of the respective [³H] substrate into acid-soluble form in
20 min. This time is chosen to define the unit since under our experimental
conditions the rates of degradation are linear up to 20 min and can be taken
as initial velocities.

D) Standard DNA polymerase assays.

In a final volume of 100-150 µl the reaction mixture contains 40 mM
Tris-Cl pH 8.3, 60 mM KCl, 1 mM DTT, either 1 mM MgCl₂ (when (dG)ₓ(dC)ₓ or
(dT)ₓ(dA)₁₂₋₁₈ is provided) or 1 mM MnCl₂ (when (dT)ₓ(A)ₓ or (dT)₁₂₋₁₈(A)ₓ
is used), one of the synthetic primer-templates and the enzyme fractions in
the amounts indicated for each experiment, one of the three[³H] deoxynucleoside
5'-triphosphates: 7.2 nmoles [³H]dGTP (specific activity 62 cpm/pmol),
Fig. 1. DEAE-cellulose column chromatography of mitochondrial extract. Fig. 2. Fraction M II from the first DEAE-cellulose column is concentrated and applied to a phosphocellulose column. Fig. 3. Fraction M Ia from the first DEAE-cellulose column is concentrated and applied to a second DEAE-cellulose column. For details see Material and Methods. 100 μl aliquot of each fraction is used to test R-DNA polymerase (62.1 nmoles of (dT)ₙ.(A)ₙ) (---), DNase (193 pmoles of [³H]-(dT)ₙ.(A)ₙ) (–Δ–) and RNase H (45 pmoles of (dT)ₙ.(da)₁₂₋₁₈–[³H]-(A)ₙ) (–△–). Time of incubation for R-DNA polymerase 60 min. Time of incubation for DNase and RNase H 210 min.

3.2 nmoles [³H]dTTP (specific activity 147 cpm/pmol), 3.2 nmoles [³H]dATP (specific activity 165 cpm/pmol). All incubations are carried out at 37°. The time of incubation is 60 min. 20-50 μl are withdrawn and processed for determination of acid-precipitable radioactivity as described by SPIEGELMAN et al.¹²

1 unit of R-DNA or D-DNA polymerase is defined as the quantity of enzyme which catalyzes the incorporation of 1 nmole of [³H]dNMP into acid-insoluble form in 60 min.

RESULTS

A) DEAE-cellulose and phosphocellulose chromatography.

The elution profiles of R-DNA polymerase, DNase and RNase H of the mitochondrial extract on DEAE-cellulose and phosphocellulose are shown in Fig. 1, 2 and 3. Fig. 1 shows that R-DNA polymerase (checked by using (dT)ₙ.(A)ₙ) is resolved into two peaks: the first one (fraction M Ia) is eluted with the washing buffer and the second one (fraction M II) at 175 mM KCl. In spite of a widespread distribution of DNase and RNase H activities, one can see that
M Ia and M II fractions contain both the degrading activities. Fig. 3 shows that R-DNA, DNase and RNase H activities co-chromatograph when fraction M Ia is concentrated and applied to a second DEAE-cellulose column. All three activities are eluted with the washing buffer. This indicates that no overloading of the first DEAE-cellulose column has occurred.

When fraction M II is concentrated and applied to a phosphocellulose column (Fig. 2), one can see that R-DNA polymerase, DNase and RNase H are eluted as a single peak at 400 mM KCl (fraction M IV). However, a part of R-DNA polymerase and RNase H activities is also eluted with the washing buffer (fraction M III).

Fig. 4 and 5. Kinetics of DNase (Fig. 4) and RNase H (Fig. 5) activities with fractions M Ib (4 µg) (○), M III (12 µg) (■) and M IV (0.5 µg) (▲). In a final volume of 200 µl the reaction mixture contains 50 mM Tris-HCl pH 7.6, 50 mM KCl, 1 mM DTT, 2 mM MgCl₂, either 3.2 nmoles of [³H]n-(dT)ₙ-(dA)ₙ or 1.5 nmoles of [³H]n-(dT)ₙ-(dA)₁₀. At the end of the indicated times 20 µl aliquots are withdrawn and processed for determination of the remaining acid-precipitable radioactivity.

Fig. 6. Heat-inactivation experiment. Fractions M Ib (full symbols) and M IV (empty symbols) are incubated at 50° for the indicated times in the presence of 50 µg/ml of bovine serum albumin. 0.5 µg of each heated fraction M Ib and M IV is used to check (dT)ₙ-(A)ₙ and (dA)₁₂-1₈-(A)ₙ activities. 100% of R-DNA polymerase is 82 and 195 pmoles of dTMP incorporated per 60 min by non-heated fractions M Ib and M IV respectively. 100% of either DNase or RNase H activities is 260 and 78 pmoles released from the respective [³H]n-substrates in 120 min by non-heated fractions M Ib and M IV.
B) R-DNA, D-DNA polymerase, DNase and RNase H activities in purified mitochondrial fractions.

Table I shows that the highest levels of enzyme activities are found in fraction M IV, where specific activities of R-DNA polymerase, DNase and RNase H indicate 60-70-fold purification. Fraction M Ib, which represents another molecular species, since it has a different chromatographic behaviour, also shows the three described activities but with lower specific activities.

Fig. 4 and 5 show the kinetics of DNase and RNase H activities when fraction M Ib, M III and M IV are used. It can be seen that fractions M Ib and M IV contain both DNase and RNase H activities but fraction M III shows no DNase and only traces of RNase H.

Table II shows the D-DNA and R-DNA polymerase activities of fractions M Ib, M III and M IV, when different synthetic primer-template are provided.

Two remarks can be made:
1) \((dG)_n \cdot (dC)_n\)-dependent and \((dT)_{12-18}\)-dependent DNA polymerase activities are higher in fraction M IV than in fraction M Ib (12.4 and 5.9 times respectively).
2) \((dT)_{12-18} \cdot (A)_n\)-dependent DNA polymerase activity is considerably higher in fraction M IV than in fraction M Ib (ratio : 76.3). In contrast, \((dT)_{n}\).\((A)_{n}\)-dependent DNA polymerase activity is 2.3-fold higher in fraction M IV than in fraction M Ib.

C) Heat-inactivation.

Fig. 6 shows the inactivation pattern of R-DNA polymerase in both fractions M Ib and M IV when samples are heated at 50° at the indicated times in the presence of 50 μg/ml of bovine serum albumin. In contrast to the polymerase neither RNase nor DNase is affected by heating.
<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Total protein mg</th>
<th>R-DNA polymerase</th>
<th>DNase</th>
<th>RNase H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total activity units</td>
<td>Specific activity</td>
<td>Recovery %</td>
</tr>
<tr>
<td>1</td>
<td>Crude extract</td>
<td>69.300</td>
<td>443</td>
<td>6.4</td>
<td>100</td>
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<tr>
<td></td>
<td>Amm. Sulfate</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>Fraction C.E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>1st. DEAE-cell</td>
<td>1.400</td>
<td>156</td>
<td>112.0</td>
<td>35.3</td>
</tr>
<tr>
<td></td>
<td>Fraction M Ia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction M II</td>
<td>5.540</td>
<td>40</td>
<td>7.3</td>
<td>9.1</td>
</tr>
<tr>
<td>2b</td>
<td>2nd. DEAE-cell</td>
<td>0.100</td>
<td>17</td>
<td>176.8</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Fraction M Ib</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Phospho cell.</td>
<td>2.900</td>
<td>3</td>
<td>1.3</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Fraction M III</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fraction M IV</td>
<td>0.013</td>
<td>5</td>
<td>403.3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

For details see Material and methods. R-DNA polymerase is detected by using 62.1 nmoles of \((dT)_n\cdot(A)_n\) and \((^3\text{H})\)-dTTP. DNase and RNase H activities are tested as indicated in Fig. 4.
**TABLE II**

Primer-template utilization by purified mitochondrial-fractions

<table>
<thead>
<tr>
<th>Template</th>
<th>Substrate</th>
<th>Fraction Mlb</th>
<th>Fraction MIII</th>
<th>Fraction MIV</th>
<th>Ratio MIV/Mlb</th>
</tr>
</thead>
<tbody>
<tr>
<td>(dG)ₙ(dC)ₙ</td>
<td>(3H)dGTP</td>
<td>362±10</td>
<td>2±0.05</td>
<td>4486±187</td>
<td>12.4</td>
</tr>
<tr>
<td>(dT)ₙ-(dA)₁₂₋₁₈</td>
<td>(3H)dATP</td>
<td>14±0.01</td>
<td>0.1±0.01</td>
<td>83±2.2</td>
<td>5.9</td>
</tr>
<tr>
<td>(dT)ₙ-(A)ₙ</td>
<td>(3H)dTTP</td>
<td>176±6</td>
<td>1.3±0.05</td>
<td>403±12.2</td>
<td>2.3</td>
</tr>
<tr>
<td>(dT)₁₂₋₁₈(A)ₙ</td>
<td>(3H)dTTP</td>
<td>3±0.4</td>
<td>0.1±0.01</td>
<td>229±5.5</td>
<td>76.3</td>
</tr>
</tbody>
</table>

D-DNA and R-DNA polymerase assays are performed according to the standard DNA polymerase assay described in Material and methods. Volume of the reaction mixture 100 µl. The following amounts of the primer-templates are used: 11.3 nmoles of (dG)ₙ(dC)ₙ, 5.8 nmoles of (dT)ₙ-(dA)₁₂₋₁₈, 62.1 nmoles of (dT)ₙ-(A)ₙ, 5.6 nmoles of (dT)₁₂₋₁₈-(A)ₙ. The results are expressed in units/mg of the corresponding fractions. Standard error is calculated on the basis of 18 determinations.

**DISCUSSION**

In a previous report, we have shown that there are at least two D-DNA polymerases in the mitochondrial preparation from chick embryonic brain, since on DEAE-cellulose chromatography, although the bulk of the enzyme is eluted at 150-175 mM KCl, a small but significant amount is eluted with the washing buffer. The study of an R-DNA polymerase (by using (dT)₁₂₋₁₈-(A)ₙ) revealed that all of this activity is eluted together with the bulk of D-DNA polymerase, and that no (dT)₁₂₋₁₈-(A)ₙ-dependent activity could be detected.
in the washing buffer fractions. A similar result has recently been obtained with HeLa mitochondrial DNA polymerase. In this case, the bulk of D-DNA polymerase activity was eluted with the washing buffer and no \((dT)_12\cdot(A)_n\)-dependent activity could be detected in this fraction. From these and our previous results, one could be tempted to think that the DEAE-cellulose washing buffer fraction of chick embryonic brain and HeLa mitochondrial DNA polymerase contains exclusively D-DNA polymerase activity. In fact, according to the results presented in this paper, mitochondrial fraction M Ib (eluted with the washing buffer of the second DEAE-cellulose column, Table II) shows a high D-DNA polymerase activity \((dG)_n\cdot(dC)_n\)-dependent activity) but also a low R-DNA polymerase activity when \((dT)_12\cdot(A)_n\) is used as primer-template. In contrast, when \((dT)_n\cdot(A)_n\) is provided, fraction M Ib shows a considerable R-DNA polymerase activity. Thus, it appears that in chick embryonic brain mitochondria D-DNA and R-DNA polymerase activities cannot be separated. This feature of the partially purified mitochondrial enzyme is not unexpected, since it is observed in other highly purified cellular DNA polymerases.

We have seen that DNase and RNase H are found together with DNA polymerase in fractions M Ib and M IV. Recent reports show that exonuclease activity is not essential for DNA polymerization in eukaryote systems, and the presence of degrading activities in partially purified mitochondrial fractions could be interpreted as contamination. However, the association of a nuclease activity with mitochondrial DNA polymerase cannot be excluded since an endonucleolytic activity has been found in HeLa mitochondrial DNA polymerase. On the other hand, the presence of an RNase H activity associated with DNA polymerase I of E. coli and DNA polymerase II from KB cells has been described.

The differential thermal lability of R-DNA polymerase and both degrading activities could suggest that RNase H and DNase activities are catalyzed by molecular species different from R-DNA polymerase and for this reason...
are to be considered as contaminants. However, the fact that R-DNA polymerase and both degrading activities co-chromatograph under various conditions suggests that the corresponding enzymes might form rather stable complexes and that they present a high affinity for each other. On the other hand, the possibility of association of degrading and polymerizing activities to the same molecule cannot be completely excluded merely on the basis of their different thermal inactivation profiles.

A fact which deserves a final comment is the comparison of the kinetic patterns of DNase activity (Fig. 4) in fractions M Ib (sigmoid curve) and M IV (hyperbolic curve). The apparent differences observed could suggest the existence of different DNase activities contaminating or associated with R-DNA polymerase. We are presently trying to analyze these DNase activities in more detail.

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BIBLIOGRAPHY


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