RNA-Instructed DNA Polymerase Associated with C-Type Particles Produced \textit{in vivo} by Murine Myeloma Cells

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(Accepted 5 April 1976)

SUMMARY

C-type particles secreted \textit{in vivo} by MOPC-315 myeloma cells were characterized. These particles localize at a density of 1.16 g/ml in sucrose and possess a 60 to 70S RNA and an RNA-instructed DNA polymerase. Endogenous enzyme activity requires manganese and is inhibited by ribonuclease or by the omission of any of the deoxynucleoside triphosphates. The enzyme utilizes the virus 60 to 70S RNA as a template to synthesize DNA molecules which specifically hybridize to the homologous RNA.

INTRODUCTION

Murine myelomas grown in tissue culture have been reported to produce virus particles with a density of 1.20 to 1.22 g/ml, containing RNA with a sedimentation coefficient of 74S and possessing the murine leukaemia group-specific antigen (Watson et al. 1970). The production of C-type particles by cultured myeloma tumour cells has also been demonstrated by electron microscopic examination, by the resistance-inducing factor (RIF) test (Karpas & Cawley, 1972a, b), and by XC cell cytopathogenicity test (Volkman & Krueger, 1973a).

The existence of an RNA-instructed DNA polymerase (reverse transcriptase) associated with the murine myeloma C-type particles, and its biochemical characteristics, has not yet been determined except in one case of Volkman & Krueger (1973b); they found that the murine myeloma cell line, FLOPC-1, derived from a lucite filter-induced tumour, produced C-type particles that lacked this activity. The FLOPC-1 particles also exhibited other properties which are different from those of oncornaviruses: they were unstable in NET (0.1 M-NaCl, 0.01 M-tris-HCl, pH 7.4, and 0.001 M-EDTA) sucrose buffer and contained only 18S and 28S species of RNA.

The present investigation demonstrates that MOPC-315 tumour cells release C-type particles \textit{in vivo} which possess RNA-instructed DNA polymerase and other biochemical features common to oncogenic RNA viruses.

METHODS

Buffers. TNE: 0.01 M-tris-HCl (pH 7.4), 0.1 M-NaCl, 0.001 M-EDTA. PBS: phosphate-buffered saline, pH 7.2.

Reagents. Unlabelled deoxynucleoside triphosphates and dithiothreitol were products of P-L Biochemicals. Polydeoxythymidylate-polyriboadenylate [poly(dT).poly(rA)] and polydeoxyadenylate-oligodeoxythymidylate [poly(dA).oligo(dT)$_n$] were supplied by Miles Laboratories. Tritiated thymidine triphosphate (40 to 50 Ci/mm) was purchased from...
New England Nuclear Corp., Boston, Mass. Actinomycin D and Cs₂SO₄ were products of the Sigma and Merck companies, respectively. Ribonuclease A was obtained from Worthington Biochemical Corporation and ribonuclease T₁ came from Calbiochem. Sephadex G-50 was obtained from Pharmacia Fine Chemicals.

Cells. Tumour MOPC-315 was initially induced in a heterozygous BALB/c-2 mouse by three intraperitoneal injections of Bayol F (Potter & Lieberman, 1967), and was kindly provided by Dr Reuven Laskow, Hadassah Medical School, The Hebrew University of Jerusalem. The tumour cells were transplanted intraperitoneally (i.p.) at weekly intervals into BALB/c mice from a local breeding colony.

Source and purification of C-type particles. Ascitic fluids collected from mice bearing tumours were clarified at 2000 g for 10 min and further centrifuged at 10000 g for 10 min. The supernatant fluid was placed on a 13 ml column of 20 % (v/v) glycerol in TNE and sedimented in a Beckman SW27 rotor at 95000 g for 1 h at 4 °C. The resulting pellet was resuspended in 2 ml TNE, layered over a pre-formed 20 to 55 % (w/w) sucrose gradient and centrifuged in an SW27 rotor at 95000 g for 16 h at 4 °C. The gradient was fractionated and each fraction was tested for DNA polymerase activity using poly(dT).poly(rA) template. Fractions corresponding to the peak of DNA polymerase activity were pooled, diluted with TNE and centrifuged at 95000 g for 60 min. The sedimented particles were resuspended in 0.01 M-tris, pH 8.2, and stored at −70 °C until used. Protein determinations were performed by the method of Lowry et al. (1951). Densities of the fractions were calculated from refractive index measurements.

Isolation of virus RNA. Purified virus was diluted in TNE and incubated with 1 % sodium dodecyl sulphate (SDS) and 500 μg/ml self-digested Pronase (free of nucleases, Calbiochem) at 37 °C for 30 min. The reaction mixture was then extracted twice with equal vol. of phenol-cresol-8-hydroxyquinoline (equilibrated with TNE, pH 7.4), and purified on a gradient of 10 to 30 % glycerol in TNE. The gradient was centrifuged in a Beckman SW50.1 rotor at 234000 g for 90 min at 4 °C, and then fractionated from below. The fractions containing the 60 to 70S RNA were pooled and alcohol precipitated. After 24 h at −20 °C the RNA was collected by centrifugation and dissolved in a small vol. of 5 mM-EDTA.

Standard polymerase assay. Reaction mixtures (0.1 ml) for homopolymer-templated reactions contained the following: tris-HCl (pH 8.2), 50 mM; potassium chloride, 48 mM; magnesium chloride, 8 mM; dithiothreitol, 0.4 mM; dATP, 0.2 mM; TTP, 0.1 mM; ³H-TTP to yield 300 ct/min/pmol; and synthetic poly(dT).poly(rA) or poly(dA).oligo(dT)₉₀, 0.4 μg. Virus particles in 0.01 M-tris-HCl, pH 8.2, were disrupted in the presence of 0.05 % Nonidet P-40 at 0 °C for 10 min and then the reaction mixture was added. Reactions were incubated at 37 °C and terminated by addition of cold trichloroacetic acid to a final concentration of 15 %. The acid-precipitable radioactivity was collected on nitrocellulose filters and counted in 0.4 % 2,5-bis-2-(5-tert-butylbenzoazoxolyl) thiophene (BBOT) in toluene.

Endogenous assay of high mol. wt. RNA and RNA-instructed DNA polymerase. The assay was performed essentially by the method of Slhom & Spiegelman (1971). Suspended viruses in 0.01 M-tris, pH 8.2, were incubated for 10 min at 0 °C in 0.05 % Nonidet P-40, 25 mM-dithiothreitol, and then added to a reaction mixture of RNA-instructed DNA polymerase (125 μl final vol.) containing 100 μg/ml actinomycin D, 50 mM of tris-HCl (pH 8.2), 10 mM-NaCl, 4 mM-MnCl₂, 1:2 mM each of dGTP, dCTP, dATP, and 600 μCi/ml of ³H-TTP (40 to 50 Ci/mmol). After 10 min incubation at 37 °C, the reaction was terminated by the addition of NaCl and SDS to final concentrations of 0.4 M and 1 %, respectively. An equal volume of phenol-cresol-8-hydroxyquinoline was added, and the mixture was shaken for 5 min and centrifuged at 5000 g for 5 min at room temperature. The aqueous phase was
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then layered over a 5 ml linear glycerol gradient (10 to 30 % in TNE) and centrifuged at 234000 g for 90 min at 4 °C in a Beckman SW50.1 rotor. External markers were 18 and 28 S 3H-ribosomal RNA. Fractions were collected from below and assayed for acid precipitable radioactivity.

3H-DNA product synthesis. 3H-DNA product was prepared from detergent disrupted viruses using identical reaction mixture and concentrations as described for the assay of high mol. wt. RNA and RNA-instructed DNA polymerase. After phenol extraction, the reaction product was applied to a column (0.7 by 31 cm) of Sephadex G-50 equilibrated with 1 mM-EDTA containing 0.2 M-sodium bicarbonate and the nucleic acids chromatographed. Fractions corresponding to 3H-DNA were pooled and lyophilized. Following lyophilization, the 3H-DNA product was dissolved in a small vol. of 5 mM-EDTA with 0.3 M-NaOH and the RNA template was hydrolysed for 10 h at room temperature. The solution was neutralized with 0.3 M-HCl and stored at -70 °C.

Hybridization reaction and its analysis. Annealing reactions were performed as previously described (Yaniv et al. 1973). In brief, 2000 ct/min of virus 3H-DNA transcript (2 × 10^7 ct/min/#g) and 60 to 70 S virus RNA (1 #g) were mixed in 50 #l of reaction mixture containing 50 % formamide, 0.4 M-NaCl, 20 mM-EDTA, and 0.1 % SDS. The samples were heated at 80 °C for 2 min, quickly chilled to 0 °C, and incubated for 18 h at 37 °C. The hybrids were layered over a 5 ml pre-formed Cs2SO4 gradient (1.34 to 1.78 g/ml) and centrifuged at 35000 rev/min for 30 h at 15 °C in a Beckman SW50.1 rotor. Fractions were collected and assayed for acid precipitable radioactivity.

Electron microscopy. Virus particles purified by two subsequent sucrose equilibrium centrifugations were fixed in 2.5 % glutaraldehyde. After post fixation with 1 % osmium tetroxide, the sample was dehydrated in ethanol and propylene oxide and embedded in Epon-Araldite. Sections were cut from these blocks and stained with uranyl acetate and lead citrate.

RESULTS

Isolation of MOPC-315 virus particles and their buoyant density

BALB/c mice inoculated i.p. with MOPC-315 tumour cells developed large abdominal tumours within 7 to 10 days. High speed pellets of cell-free ascitic fluids revealed, following non-ionic detergent treatment, significant levels of DNA polymerase activity. However, washings of the peritoneal cavity of control BALB/c mice injected with PBS did not exhibit that enzymatic activity.

An experiment was performed to determine whether the detected DNA polymerase activity is encapsulated in a structure possessing the buoyant density characteristic of RNA tumour viruses. Ascitic fluids collected from mice bearing tumours were clarified and sedimented through a column of 20 % glycerol. The resulting pellet was subjected to sucrose equilibrium centrifugation on a linear gradient of 20 to 55 % sucrose and fractions were tested for DNA polymerase activity using the synthetic template poly(dT).poly(rA).

The data presented in Fig. 1 demonstrate that the enzymic activity bands at a density of 1.16 g/ml, the density characteristic of oncornaviruses. Thin section examinations of the peak fractions by electron microscopy revealed the presence of virus particles with a C-type morphology (Fig. 2).

General properties of the virus polymerase

The detection of an enzymic activity coinciding with particles possessing a C-type morphology enabled us to proceed and to characterize this activity further by using virus particles purified by sucrose equilibrium centrifugation.
Fig. 1. Isopycnic sucrose density gradient centrifugation of MOPC-315 virus and detection of DNA polymerase activity. Following centrifugation, 20 μl samples of gradient fractions were tested for DNA polymerase activity in a standard reaction mixture, containing poly(dT).poly(rA), for 30 min at 37 °C and assayed for acid-precipitable radioactivity.

Fig. 3 demonstrates the kinetics of ³H-TMP incorporation into acid-insoluble product observed with MOPC-315 virus polymerase in a templated reaction. The virus polymerase responds to the synthetic RNA-DNA hybrid, poly(dT).poly(rA), and the kinetics of the reaction is linear for more than 60 min. However, the enzyme cannot utilize at all the synthetic homopolymer-oligomer template, poly(dA).oligo(dT)₁₀.

The template preference of the virus polymerase does not provide sufficient information to claim that an RNA-instructed DNA polymerase reaction has been followed. The performance of an endogenous reaction and the determination of its requirements to synthesize a DNA molecule on the virus RNA template would be more indicative. Table 1 summarizes the requirements of the MOPC-315 virus DNA polymerase utilizing the endogenous template. The enzyme possesses an absolute requirement for Mn²⁺, and Mg²⁺ does not substitute for Mn²⁺, as the divalent cation. Omission of any of the deoxynucleoside triphosphates from the reaction mixture abolishes the incorporation of TMP. Pre-incubation of the detergent-disrupted virions with ribonuclease A and T₁ inhibits the DNA polymerase activity. The optimal concentration of Nonidet P-40 required for maximum enzymatic activity was found to be 0.05 % both for the endogenous reaction as well as for the synthetic template.
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Fig. 2. Thin section of a purified pellet obtained from the 1.16 g/ml region of the second sucrose equilibrium gradient, showing immature and mature type-C particles. Note typical C-type budding.

Table 1. Requirements of MOPC-315 virus DNA polymerase*

<table>
<thead>
<tr>
<th>Conditions</th>
<th>³H-TMP incorporated (ct/min)</th>
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</thead>
<tbody>
<tr>
<td>Complete</td>
<td>10519</td>
</tr>
<tr>
<td>Minus Mn²⁺ plus Mg²⁺</td>
<td>944</td>
</tr>
<tr>
<td>Minus dATP</td>
<td>1486</td>
</tr>
<tr>
<td>Minus dCTP</td>
<td>1561</td>
</tr>
<tr>
<td>Minus dGTP</td>
<td>1970</td>
</tr>
<tr>
<td>Complete plus ribonuclease A and T₁</td>
<td>975</td>
</tr>
</tbody>
</table>

* Endogenous DNA polymerase assays were performed using 50 μg virus protein with omissions and additions as indicated. Ribonuclease A and ribonuclease T₁ (50 μg/ml each, heated at 100 °C for 10 min) were added to detergent-disrupted particles. The mixture was incubated for 15 min at 37 °C and then added to a complete reaction mixture. The reactions were incubated at 37 °C for 30 min, and then assayed for acid-insoluble radioactivity.

The kinetics of the endogenous reaction (Fig. 4) is linear for 30 min and then levels off.

Sedimentation property of virion RNA

The sedimentation property of virion RNA was determined by the concomitant test (Schlom & Spiegelman, 1971) for the detection of high mol. wt. RNA and RNA-instructed DNA polymerase.

Detergent-disrupted MOPC-315 virus particles were subjected for 10 min to an endogenous RNA-instructed DNA polymerase reaction and the product of the reaction was analysed on a glycerol velocity gradient. The results presented in Fig. 5 demonstrate that part of the radioactively labelled DNA is travelling with a sedimentation coefficient of 60
characterization of the $^3$H-DNA product

To determine further that an RNA-directed DNA polymerase had synthesized DNA molecules using the virus RNA as a template, the purified DNA was tested for its complementarity to MOPC-315 virus RNA and to avian myeloblastosis virus (AMV) RNA.

$^3$H-DNA was prepared in an endogenous RNA-instructed DNA polymerase reaction and separated from residual substrate on a Sephadex G-50 column. The $^3$H-DNA was then subjected to extensive alkali digestion to remove all RNA present. 60 to 70S RNA extracted from purified MOPC-315 virus particles and 70S RNA from AMV were annealed to the
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Fig. 5. Sedimentation analysis of the 10-min products of MOPC-315 virus DNA polymerase; non-treated (●—●) and RNase treated (○—○). Two samples of 100 µg of virus protein were subjected to an endogenous RNA-instructed DNA polymerase reaction. The products from both samples were extracted by phenol and one was treated with boiled RNase A and T1 (50 µg/ml each) for 15 min at 37 °C. The nucleic acids of the two samples were then analysed separately on glycerol gradients as described in Methods.

Fig. 6a presents the outcome of an annealing reaction between the 3H-DNA product and RNA from MOPC-315 virus particles. It is evident that almost all of the DNA product is shifted from the DNA region to the RNA region of the gradient due to DNA-RNA hybrid complexes that form during the annealing reaction. When the 3H-DNA is annealed with an equivalent amount of RNA isolated from AMV, no evidence of hybrid formation is detected (Fig. 6b).

DISCUSSION

The MOPC-315 tumour cells were found to release particles possessing DNA polymerase activity into the intraperitoneal fluid of mice bearing tumours. Equilibrium centrifugation analysis performed in TNE-sucrose gradient determined that these particles localize within a density of 1.16 g/ml, the density characteristic for the oncornaviruses. The buoyant
density of these particles and their stability in TNE-sucrose solution indicate that MOPC-315 particles are different from those isolated from the P3 myeloma (Watson et al. 1970) and FLOPC-1 C-type particles (Volkman & Krueger, 1973b).

Electron microscopy examination of the MOPC-315 particles banding at a density of 1.16 g/ml revealed a typical C-type morphology coinciding with the DNA polymerase activity determined by the synthetic template, poly(dT).poly(rA).

The C-type particles isolated from cultured FLOPC-1 tumour cells were found to be deficient in RNA-instructed DNA polymerase (Volkman & Krueger, 1973b). It was therefore of interest to test whether the C-type particles produced by the MOPC-315 tumour cells do contain an RNA-instructed DNA polymerase which fulfils the established criteria (Goodman & Spiegelman, 1971) for identification.

The MOPC-315 virus polymerase was found to share many of the general characteristics common to the oncornavirus reverse transcriptases (Temin & Baltimore, 1972). It exhibits a positive response to the synthetic RNA-DNA hybrid poly(dT).poly(rA), and does not accept the poly(dA).oligo(dT)_{10} template known to be preferred by cellular DNA polymerases (Goodman & Spiegelman, 1971). The enzyme is active in an endogenous reaction requiring the presence of the four deoxyriboside triphosphates and manganese, and is sensitive to ribonuclease. Further, the DNA synthesized by the MOPC-315 C-type particles, on its endogenous nucleic acid template, hybridizes specifically to MOPC-315 virus RNA, thus providing strong evidence of a relevant RNA-instructed DNA polymerase activity.

In performing the concomitant test for the detection of high mol. wt. RNA and RNA-instructed DNA polymerase with and without ribonuclease, we demonstrated that MOPC-315 virus particles contain a high mol. wt. RNA with a sedimentation coefficient of 60 to 70S. Since the procedure employed for RNA size determination is limited in its ability to reveal low mol. wt. RNA species, we cannot exclude their presence from the MOPC-315 virus genome. Further investigation with radioactively labelled virus RNA is required to resolve this issue. In any event, the presence of a high mol. wt. RNA in the MOPC-315 C-type particles, as well as in those isolated from the P3 myeloma cultured cells (Watson
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et al. 1970) distinguishes them from the FLOPC-1 viruses, which contain only low mol. wt. RNA species (Volkman & Krueger, 1973b).

The biological functions of the RNA virus particles, isolated so far from myeloma tumour cells, are unknown. No transformation or tumourigenic activities have been demonstrated (Dalton, Potter & Merwin, 1961; Merwin & Redmon, 1963; Kuff, Wivel & Lueders, 1968). A direct relationship between the presence of C-type particles in myeloma cells and their ability to synthesize immunoglobulin, although suggested (Schubert & Horibata, 1968; Watson et al. 1970), has not yet been established. Investigations along these lines are now in progress.

We thank Dr Kalman Perk of the Hebrew University of Jerusalem, Rehovot Campus, Israel, for the electron microscopic examinations. The excellent technical assistance of M. Berkovitz is appreciated. This research was supported by the Advancement of Mankind Foundation. E.E. is an Established Investigator of the Chief Scientist’s Bureau, Ministry of Health.

REFERENCES


(Received 1 December 1975)