Mouse Mammary Tumour Virus RNA-dependent DNA Polymerase: requirements and Products

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SUMMARY

Mouse mammary tumour virus contains an RNA-dependent DNA polymerase. The enzyme requirements and physical properties of the reaction products are similar to those of other RNA tumour viruses, despite the unique characteristics of the virus in other properties.

The discovery of an RNA-dependent DNA polymerase in RNA tumour viruses (Baltimore, 1970; Temin & Mizutani, 1970) has led to a rapid accumulation of data confirming and extending these reports (Fujinaga et al. 1970; Green et al. 1970; Hatanaka, Huebner & Gilden, 1970; Scolnick, Aaronson & Todaro, 1970; Spiegelman et al. 1970). The mouse mammary tumour virus (MTV) holds a somewhat unique position within the RNA tumour virus group, as demonstrated by its morphology (B-type particle) and its inability to cross react immunologically with other RNA tumour viruses (Hilgers et al. 1972). This suggested that the relationship of MTV with the rest of the group could be further investigated by an examination of the requirements and products of its RNA-dependent DNA polymerase.

The virus was isolated from mammary tumours arising in C3H/Avy and BALB/cfC3H mice strains by the method of Calafat & Hageman (1968 and personal communication). Negative-stain electron microscopy of the preparations demonstrated that at least 90% of the material consisted of characteristically spiked virus particles. The total protein was estimated by the method of Lowry et al. (1951).

DNA polymerase assays were performed in reaction mixtures of 125 μl or multiples thereof by a procedure adapted from Spiegelman et al. (1970), and contained 50 mM-tris-HCl, pH 8.3; 40 mM-KCl; 5 mM-MgCl₂; 20 mM-dithiothreitol; 0.8 mM-dATP, dCTP, dGTP, 0.04 mM-TPP with 5 or 10 μCi of [²H]-TPP and a nonionic detergent (usually Nonidet P 40, 0.02%, v/v). The reaction was started by the addition of virus, incubated at 37°C, and stopped with 5 ml of cold 10% trichloroacetic acid (TCA) containing 10% saturated tetra-sodium pyrophosphate, 10% saturated tri-sodium orthophosphate and 0.4 mg of carrier DNA to facilitate precipitation. After 20 min at 4°C the precipitates were filtered on to glass-fibre discs (2.4 cm GF/C Whatman) and washed with 10 vol. of cold 10% TCA. The discs were dried at 120°C and counted in a toluene-based scintillant. The efficiency of counting [²H] immobilized on glass-fibre discs was estimated to be approximately 26%.

The MTV-associated DNA polymerase was shown to require a divalent cation, all four deoxynucleotide triphosphates, dithiothreitol and a nonionic detergent to obtain incorporation of [²H]-TPP into a TCA-insoluble product (Table 1). Both Mg²⁺ and Mn²⁺ fulfill the requirement for a divalent cation, Mg²⁺ giving the higher activity at a concentration of 2 to 5 mM as compared to Mn²⁺, which showed a sharp dependence on concentration with an optimum of 0.5 mM.

The RNA dependence of the reaction was demonstrated by preincubation of the disrupted virus with 20 μg of pancreatic ribonuclease A (RNase) (preheated to 85°C for 10 min to
**Table 1. Requirement of the MTV-associated DNA polymerase**

<table>
<thead>
<tr>
<th>Contents of reaction mixture*</th>
<th>Incorporation of [PH]-TTP (ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>11214</td>
</tr>
<tr>
<td>—KCl</td>
<td>10308</td>
</tr>
<tr>
<td>—MgCl₂</td>
<td>149</td>
</tr>
<tr>
<td>—Dithiothreitol</td>
<td>556</td>
</tr>
<tr>
<td>—dATP</td>
<td>272</td>
</tr>
<tr>
<td>—dGTP</td>
<td>975</td>
</tr>
<tr>
<td>—dCTP</td>
<td>553</td>
</tr>
<tr>
<td>—Detergent</td>
<td>301</td>
</tr>
<tr>
<td>—MTV</td>
<td>84</td>
</tr>
</tbody>
</table>

* The reaction mixtures contained 10 μCi of [PH]-TTP at a final concentration of 0.04 mM, 0.1% Triton X 100, 30 μg of virus protein, and were incubated at 37 °C for 90 min.

Fig. 1. Analysis of the DNA polymerase reaction products by rate zonal centrifuging. From a 625 μl reaction mixture containing 180 μg of virus protein; 100 μl samples were withdrawn at (a) 10 min, (b) 30 min, (c) 60 min, (d) 4 h and treated as described in the text. r and s refer to the regions of the gradient profile considered as rapidly and slowly sedimenting, respectively.

inactivate any deoxyribonuclease activity) for 20 min at 37 °C. The reaction showed an 85% decrease in incorporation over a similarly pre-incubated control without RNase.

The optimum concentration of TTP was found to be in the order of 0.04 mM. However, for optimal incorporation of label as used in product analysis experiments, no cold TTP was added. Under these conditions the utilization of artificial templates by the enzyme was the same as at optimum TTP concentrations and therefore specificity of the reaction was considered to be comparable.

Rate zonal sedimentation of the product was performed by taking samples from a reaction mixture at intervals and dissociating by the addition of 1/5 vol. 10% SDS and 1/5 vol. 0.25 M-acetate buffer, pH 5.0, containing 0.05 M-EDTA. The samples were centrifuged in 5 ml 15 to 30% sucrose-density gradients containing 0.1 M-NaCl and 0.5% SDS at 105,000 g for 165 min at 20 °C (Fig. 1a–d), and the gradients unloaded in 6 drop fractions. The initial products obtained after 10 or 30 min of incubation consisted of a heterogeneous mixture of
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Fig. 2. Caesium sulphate equilibrium sedimentation analysis of (a) rapidly and (b) slowly sedimenting fractions from a 120 min reaction mixture. The positions at which mouse RNA and DNA band in parallel gradient are indicated.

rapidly sedimenting nucleic acid species. After 60 min, additional, more slowly sedimenting species were obtained and by 4 h, these smaller components were predominant.

In order to determine the nature of both the slow and rapidly sedimenting material, the appropriate fractions were combined, made 0·2 M with respect to NaCl, and the nucleic acid isolated by precipitation with 2 vol. of cold ethanol in the presence of 200 μg of yeast RNA as carrier. After a minimum of 16 h at −20 °C the precipitate was collected by centrifuging at 4000 g for 20 min and then resuspended in a small vol. of 1·5 × concentrated SSC (0·15 M-sodium chloride, 0·015 M-sodium citrate). The isolated nucleic acid from both the rapid and slow sedimenting material was examined by equilibrium sedimentation in caesium sulphate. The gradients were formed by mixing 2·5 ml of caesium sulphate dissolved in 0·01 M-tris-HCl buffer, pH 7·0, and 0·01 M-EDTA to a density of 1·95 g/ml, with 0·2 ml of 0·01 M-phosphate buffer, pH 7·0, and 2·0 ml of sample and formed by centrifuging at 82 000 g for 65 h at 20 °C. The tubes were unloaded by bottom puncture and 4 drop fractions collected. The density profile was determined by refractive-index measurements at 25 °C on selected fractions. All fractions were TCA-precipitated as described for the polymerase assay. Fig. 2 shows the radioactivity profiles of rapidly and slowly sedimenting fractions from 120 min reaction mixture. The rapid fraction banded at a density of 1·62 g/ml and the slow fraction at 1·43 g/ml; the rapidly sedimenting fraction for a 30 min reaction also banded at a density of 1·62 g/ml, indicating that there was no change of density at the later times. Treatment of the rapidly sedimenting fraction with 200 μg of RNase for 20 min at 37 °C prior to centrifuging with caesium sulphate resulted in the material banding with a major peak of density 1·485 g/ml, while pretreatment with heat at 100 °C for 10 min resulted in a single band at a density of 1·43 g/ml.

RNase digestion or heat treatment of the slow sedimenting fraction had little effect on its position in caesium sulphate gradients.

Analysis of the rapidly sedimenting fraction in caesium sulphate gradients showed that it bands near to the density of RNA, although prior treatment of this fraction with heat caused the labelled material to band at the density of DNA. These observations are consistent with the results obtained with other RNA tumour viruses (Garapin et al. 1970; Rokutanda et al. 1970; Spiegelman et al. 1970; Fanshier et al. 1971; Faras et al. 1971; Manly et al. 1971) and with the hypothesis that the initial product is the high mol. wt. RNA genome with short lengths of newly synthesized DNA attached, these pieces of DNA being released by the denaturing effect of heat. The digestion of samples with RNase was expected to allow the
formation of fully double-stranded RNA:DNA hybrids with a density midway between that of RNA and DNA. In these experiments the RNase-digested, rapidly sedimenting fraction banded at a density of 1.485 g/ml and not at the expected density of 1.54 g/ml. The reason for this discrepancy is uncertain, but could be explained by partial digestion of the RNA in the RNA:DNA hybrid which occurred under the conditions of the experiment.

The formation of several intermediately sedimenting peaks and the absence of the rapidly sedimenting peak at late times is thought to result from the action of nucleases, present either as contaminants in the preparation or as inherent nuclease of the type described by Mölling et al. (1971) for avian myeloblastosis virus. Further evidence of nuclease degradation is the density of the rapidly sedimenting fraction (1.62 g/ml), indicating a lower RNA:DNA ratio than reported by other authors (Bishop et al. 1971).

In summary, these results are similar to those obtained with other RNA tumour viruses, showing an enzyme requirement for a divalent cation, all four deoxynucleotide triphosphates, dithiothreitol and a nonionic detergent to elicit activity. The initial product appears to be a rapidly sedimenting RNA:DNA hybrid, followed after longer incubation by a predominantly slow sedimenting DNA product.

During the preparation of this manuscript, Dion & Moore (1972) published some results on the MTV RNA-dependent DNA polymerase, which apart from some minor differences regarding product size, are similar to those reported here.

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