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CHARACTERIZATION OF AN RNA-DIRECTED DNA POLYMERASE FROM MOUSE SPLEEN INFECTED WITH LEUKAEMIA VIRUS ACTIVATED DURING N-METHYL-N-NITROSO UREA-INDUCED LEUKAEMOGENESIS

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Summary. — An RNA-directed DNA polymerase was purified from mouse spleen infected with leukaemia virus activated during N-methyl-N-nitroso urea- (MNU-) induced leukaemogenesis. The enzyme was isolated from the microsomal fraction and purified by successive chromatography of Sephadex G-200 and phosphocellulose. Estimation of molecular weight from the sedimentation rate of the purified enzyme in a sucrose gradient gave a value of 70,000. The enzyme had a pH optimum of 7.4, a KCl optimum of 50 mmol/l, an Mn^{2+} optimum of 0.2 mmol/l, and a temperature optimum of 25° C, when $(rA)_n \cdot (dT)_{10}$ was used as the template-primer. It preferred $(rA)_n \cdot (dT)_{10}$ as the template-primer and transcribed $(rC)_n \cdot (dG)_{12}$ and $(OMeC)_n \cdot (dG)_{12}$. A comparison of the properties of this DNA polymerase with the enzyme purified from murine type C retroviruses showed that the MNU-activated virus enzyme was both biochemically and biophysically indistinguishable from murine leukaemia virus DNA polymerases.

Key words: N-methyl-N-nitroso urea-induced leukaemogenesis; murine leukaemia virus; reverse transcriptase; DNA polymerase purification

Introduction

The RNA-directed DNA polymerase ("reverse transcriptase", deoxynucleoside triphosphate: DNA nucleotidyltransferase, E.C.2.7.7.7) plays an essential role in the multiplication of retroviruses and is a useful biochemical marker (Temin and Baltimore, 1972). Fey *et al.* (1980) demonstrated a significantly higher expression of murine leukaemia virus (MuLV) in tissues of N-methyl-N-nitroso urea- (MNU-) induced leukaemia of mice as compared to corresponding tissues of untreated control animals by reverse transcriptase activity assay, XC cell assay and indirect immunofluorescence. Furthermore, Fey *et al.* (1981) showed that the combined action of activated MuLV iso-

lated from MNU-induced leukaemias and suboptimal doses of MNU yields a significantly high incidence of leukaemias and tumours. Treatment with one agent alone was little effective. The investigation of interactions between chemical carcinogens and viral activities should promote our understanding of the mechanisms by which multiple aetiological factors take part in a syn-carcinogenic process.

In the present study we characterized the reverse transcriptase as a marker of virus association, using extracts from the enlarged spleen of mice infected with leukaemia virus activated during MNU-induced leukaemogenesis (MNU-virus). We found that the enzyme activities correspond to the criteria of the reverse transcriptase from murine type C retroviruses, as e.g. behaviour during column chromatography, molecular weight, transcription of specific template-primers, and catalytic properties.

Materials and Methods

Materials. The following were used: Sephadex G-200 (Pharmacia); phosphocellulose P-11 (Whatman); $(rA)_n \bullet (dT)_{10}$ (Boehringer); $(dA)_n \cdot (dT)_{12}$ (Calbiochem); $(rC)_n \cdot (dG)_{12}$, $(OMeC)_n \cdot (dG)_{12}$ (P. L. Biochemicals); Triton X-100 (Serva); deoxynucleoside triphosphates dGTP, dATP, dCTP, dTTP (Boehringer); tritium-labelled dTTP (Rossendorf) and dGTP (Radiochemical Centre, Amersham) (555 GBq/mmol and 925 GBq/mmol respectively); membrane filters (Sartorius 0.45 μm); bovine serum albumin (BSA; Serva); and polyethylene glycol 6000 (Serva).

Buffers. The homogenizing buffer HB and high salt buffer HSB were used according to Yang *et al.* (1972). HB: 0.4 mol/l sucrose, 50 mmol/l Tris.HCl, pH 8.0, 50 mmol/l KCl, 4 mmol/l $MgCl_2$ and 0.2% β-mercaptoethanol. HSB: 15% glycerol, 50 mmol/l Tris.HCl, pH 8.0; 500 mmol/l KCl; 4 mmol/l $MgCl_2$; 0.2% β-mercaptoethanol; and 0.5% Triton X-100. Sephadex buffer KEM: 15% glycerol; 500 mmol/l KCl; 4 mmol/l $MgCl_2$; 0.4 mmol/l EDTA; 0.2% β-mercaptoethanol, pH 7.2; and 0.2% Triton X-100. Phosphocellulose buffer PC: 30% glycerol; 10 mmol/l sodium phosphate, pH 6.8; 1 mmol/l EDTA; 0.2% β-mercaptoethanol; and 0.2% Triton X-100.

Animals and treatment. Lymphatic leukaemias or thymomas or both were induced in newborn mice of strain Balb/c as well as (VII × AKR) and (C57B1 × Balb/c) F1 hybrids by injection of MNU as described (Fey *et al.*, 1980). MNU-induced thymomas were minced with scissors and washed with isotonic NaCl solution. The pieces were injected intramuscularly into approximately 12-week-old mice of the same strain and sex. Spleens were obtained from the resulting transplantable generalized leukaemia of the (C57B1 × Balb/c) F1 hybrid mice. The mice were killed ten days after infection. The weight of the spleen was 30-50 times higher as compared with the normal weight.

DNA polymerase assay. Reverse transcriptase assays were carried out at 25° C for 60 min in 0.1 ml of a standard reaction mixture which contained 25 mmol/l Tris.HCl, pH 7.5; 50 mmol/l KCl; 10% polyethylene glycol; 2 mmol/l dithiothreitol; ^{3}H-dTTP (17000 dpm/pmol); 0.02 OD_{260} $(rA)_n \cdot (dT_{10})$; and 0.2 mmol/l $MnCl_2$. ^{3}H-dGTP (25000 dpm/pmol) was used for the assays with $(rC)_n \cdot (dG)_{12}$ and $(OMeC_n) \cdot (dG)_{12}$ used as templates. Syntesis was terminated by the addition of 5% ice-cold trichloroacetic acid containing 0.01% sodium pyrophosphate. Acid-insoluble material was collected onto membrane filters (0.45 μm), washed with 5% trichloroacetic acid, dried and counted using toluene-based scintillation fluid.

Protein concentration was measured by the method of Popov *et al.* (1975).

Cell fractionation. Spleens were homogenized in a Potter glass homogenizer in 4 volumes (v/w) of HB. Nuclear and mitochondrial fractions were sedimented by centrifugation at 10 000 × g for 20 min. The supernatant was then centrifuged at 100 000 × g for 90 min. The microsomal pellet was resuspended in 8 ml HSB, incubated at 37° C for 10 min, and centrifuged at 100 000 × g for 90 min.

Gel filtration. The solubilized polymerase in the supernatant was passed through a 2.5 × 100 cm column of Sephadex G-200, previously equilibrated with KEM. The flow rate was maintained at 8-10 ml/hr. Fractions of 8 ml each were collected and assayed for enzyme activity with $(rA)_n \cdot$

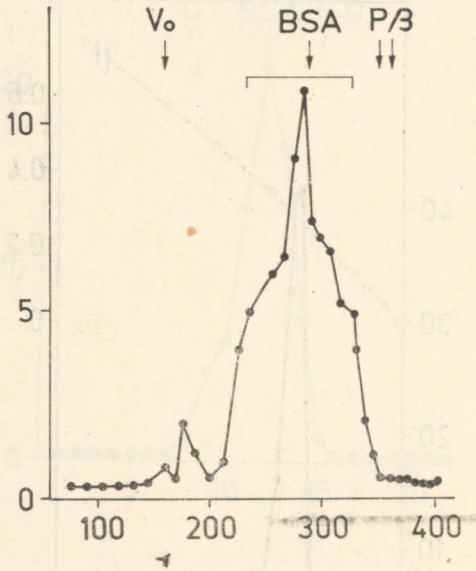

Fig. 1.

Sephadex G-200 chromatography of MNU-virus DNA polymerase

The supernatant of the lysed microsomal fraction (crude extract) was loaded onto a 2.6 cm × 100 cm column of Sephadex G-200 equilibrated with KEM buffer. The column was eluted at a flow rate of 8-10 ml/hr and 8-ml fractions were collected. Ten-microliter portions from each fraction were assayed with (rA) . . $(dT)_{10}$ as template-primer. The fractions pooled for further purification are indicated by a bar. The column was calibrated separately by filtration of BSA and pepsin (P). V_o exclusion volume; B DNA polymerase B.

Abscissa: volume eluted (ml); ordinate: ^{3}H-dTMP incorporated (dpm × 10^{-3}).

. $(dT)_{10}$ as template-primer. The fractions containing DNA polymerase activity were pooled and concentrated in an Amicon cell with UM-10 filter.

Phosphocellulose chromatography. The concentrated enzyme was diluted tenfold in PC and applied to a 0.9 × 15 cm columns equilibrated with PC. The column was washed with PC and then eluted (4-5 ml/hr) with a 160 ml linear gradient of 0-0.6 mol/l KCl in PC. Fractions of 1.5 ml were collected and portions assayed for enzyme activity with $(rA)_n$. $(dT)_{10}$ as template-primer.

Concentration of phosphocellulose eluate. The pooled enzyme fractions were diluted fivevold in PC, and passed through a 0.9 × 1 cm phosphocellulose column. All enzyme activity was eluted with 0.5 mol/l KCl in PC in a volume of 2 ml.

Rate zonal centrifugation. The enzyme preparation was centrifuged in gradients of 5-20% sucrose in 50 mmol/l Tris, pH 7.4, 2 mmol/l β-mercaptoethanol, and 0.5 mol/l KCl. BSA as a protein marker was run in a parallel gradient and determined by measuring absorbance at 280 nm. Enzyme activity was assayed as described above.

Results

In previous experiments we detected a MuLV activity during MNU-induced leukaemogenesis (Fey *et al.*, 1980). The resulting amounts of virus material were not sufficient for further studies (Ehm, unpublished). We tried, therefore, to increase the MNU-activated virus expression by rapid in vivo passages of the thymomas as described in Materials and Methods. After injection of the minced thymomas various transplantable solid tumours developed in the mice. In one case a transplantable generalized leukaemia developed in the (C57Bl × Balb/c) F1 hybrids. The highest virus expression was demonstrated by reverse transcriptase assay in the lymph node cells of the transplantable generalized leukaemia (Ehm *et al.*, in preparation). The generalized leukaemia was therefore used in our subsequent experiments.

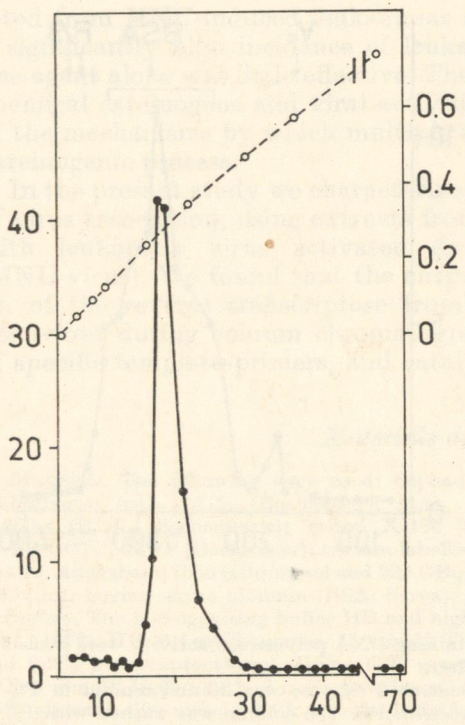

Fig. 2.
Chromatography of DNA polymerase on phosphocellulose
The peak of enzymatic activity eluted from Sephadex G-200 column was concentrated as described in „Materials and Methods" and adsorbed to phosphocellulose (Whatman P-11). Elution was performed with a gradient of 0-0.6 mol/l KCl'. Samples (10 µl) were taken for assay of enzyme activity with (rA) . . $(dT)_{10}$ as template-primer.
Abscissa: fraction number; left ordinate: ^{3}H-dTMP incorporated (dmp × 10^{-3}; ●), right ordinate: KCl concentration mol/l; ○)

The spleen cells were processed to yield a high-speed particulate fraction, which on analysis in a sucrose gradient (20—60% w/v) showed an activity peak around 1.17 g/ml with the template-primer $(rA)_n$. $(dT)_{10}$ (data not shown). The microsomal pellet was prepared as described in Materials and Methods. We were able to elute the DNA polymerase β by washing this pellet with a low salt buffer as described earlier (Drescher *et al.*, 1978). The RNA-directed DNA polymerase was extracted from the microsomal pellet with HSB containing nonionic detergent.

Table 1. Purification of RNA-directed DNA polymerase from MNU-virus-infected spleen

Step	Protein (mg)	Total activity* (units)	Specific activity (units/mg)	Relative specific activity
Crude extract	224.0	2.1	0.009	1
Sephadex pool	6.1	23.9	3.9	415
Phosphocellulose pool	0.75	8.2	10.9	1160

* Total activity is the total number of nmol of ^{3}H-dTMP incorporated into trichloroacetic acid-precipitable product in 60 min with $(rA)_n$. $(dT)_{10}$ as a template-primer. DNA polymerase assays were carried out as described in Materials and Methods. The calculation refers to 20 g of spleen.

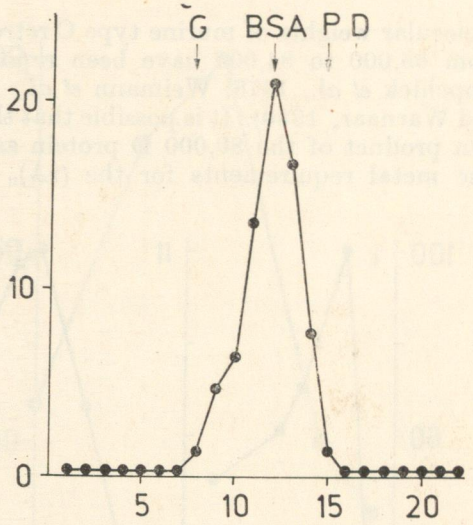

Fig. 3.
Rate-zonal centrifugation of DNA polymerase
DNA polymerase as eluted from phosphocellulose was centrifuged in a density gradient of 5—20% sucrose in buffer containing 0.5 mol/l KCl. Human gamma-globulin (G), bovine serum albumin (BSA), pepsin (P), and DNase I (D) served as sedimentation reference. Centrifugation was carried out in an SW 60 rotor at 45 000 rev/min for 16 hr at 2° C. Activity was measured in a standard reaction mixture with (rA) . $(dT)_{10}$ as template-primer.
Abscissa: fraction number (from bottom to top); ordinate: ^{3}H-dTMP incorporated (dpm $\times$ 10^{-3}).

Fig. 1 shows the elution pattern of the lysed microsomal fraction on a Sephadex G-200 column. The RNA-directed DNA polymerase activity measured by $(rA)_n$. $(dT)_{10}$-directed poly(dT) synthesis was eluted in the molecular weight range of BSA and was well separable from the DNA-directed DNA polymerase activity by this procedure as previously described (Drescher *et al.*, 1978). Further purification of the reverse transcriptase by phosphocellulose chromatography showed a single peak, eluted at a salt concentration of 0.28 mol/l KCl (Fig. 2), and assayed with $(rA)_n$. $(dT)_{10}$ as template-primer. This salt concentration is characteristic of the elution of RNA-directed DNA polymerase from spleens of Rauscher leuakemia virus-infected mice (Drescher *et al.*, 1978).

When calculations were made on the basis of the specific activity, more than 1000-fold purification of the MNU-virus DNA polymerase was obtained by two column-chromatographic steps. These results are summarized in Table 1. It is obvious that the enzyme activity of crude extract was strongly inhibited.

We were unable to analyse the purity of the DNA polymerase by polyacrylamide gel electrophoresis because of insufficient amount of enzyme material. The enzyme eluted from the phosphocellulose column was pooled, concentrated as described in Materials and Methods and used for further biochemical characterization. The molecular weight of MNU-virus DNA polymerase was estimated by sedimentation in sucrose gradients (Fig. 3). The DNA polymerase had the same sedimentation coefficient as BSA (4.3 S). Similar molecular weight estimations (70,000) have been reported for reverse Manscriptase from Rauscher leukaemia virus (Abrell and Gallo, 1973; trodak and Marcus, 1977; Verma, 1977). On the other hand, values for the

molecular weights of murine type C retrovirus reverse transcriptases ranging from 65,000 to 84,000 have been reported (Moelling, 1974; Verma, 1975; Kopchick *et al.*, 1978; Weimann *et al.*, 1978; Sarin *et al.*, 1979; van Muijen and Warnaar, 1980). It is possible that the 70,000 D polypeptide is a degradation product of the 80,000 D protein as was suggested by Moelling (1976). The metal requirements for the $(rA)_n \cdot (dT)_{10}$-directed poly(dT) synthesis

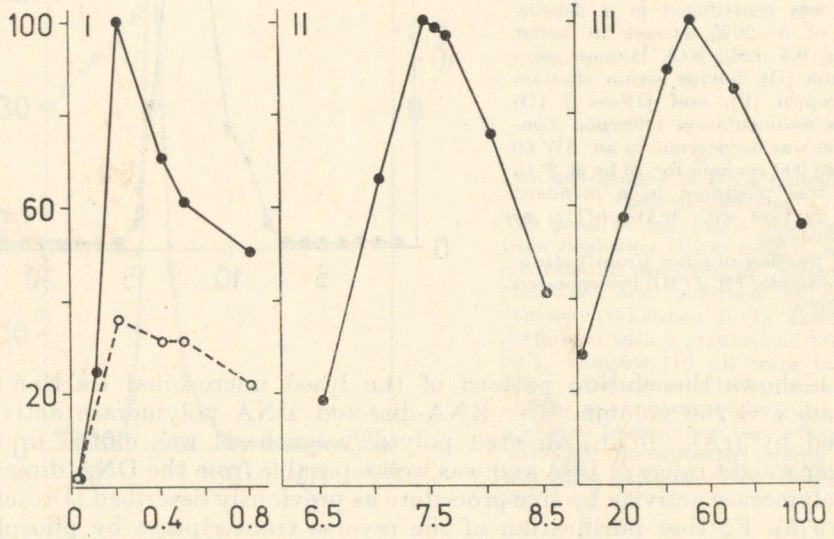

Fig. 4.

Determination of optimum reaction conditions for MNU-virus DNA polymerase
DNA polymerase assays were carried out in a standard reaction mixture as described in "Materials and Methods" with $(rA)_n \cdot (dT)_{10}$ as template-primer. I — Effect of Mn^{2+} concentration (abscissa; mmol/l) on ^{3}H-dTMP incorporation (●), Mg^{2+} at a concentration of 2 mmol/l added (○). II — Effect of pH (Tris-HCl buffers; abscissa) on ^{3}H-dTMP incorporation. III — Effect of KCl concentration (abscissa; mmol/l) on ^{3}H-dTMP incorporation.
Ordinate: relative activity (%).

were 0.2 mmol/l Mn^{2+} and 50 mmol/l KCl (Fig. 4-I, III). Similar requirements of divalent and monovalent cations and their concentrations have been reported (Sarin *et al.*, 1979). Mg^{2+} did not stimulate the enzyme activity. Mg^{2+} at a concentration of 2 mmol/l and Mn^{2+} used together were not synergistic. This is in contrast to an earlier report on a synergistic effect of Mn^{2+} and Mg^{2+} on reverse transcriptase activity from Rauscher leukaemia virus (Waters and Yang, 1974). The pH optimum for this enzyme was around 7.4 (Fig. 4-II) and the temperature optimum was at 25° C when $(rA)_n \cdot (dT)_{10}$ was the template-primer (Fig. 5) according to previous publications concerning Rauscher murine leukaemia virus DNA polymerase (Waters and Yang, 1974; Modak and Marcus, 1977).

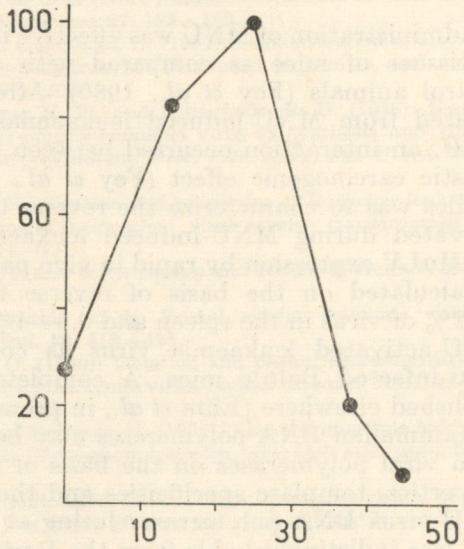

Fig. 5.
Determination of optimum temperature for MNU-virus DNA polymerase with $(rA)_n \cdot (dT)_{10}$ as template-primer
Abscissa: temperature (°C); ordinate: relative dTMP incorporation (%).

The enzyme activity was stimulated threefold by the addition of polyethylene glycol 6000 at a concentration of 10% as proposed by Chan *et al.* (1980).

A comparison of the transcription of various templates by MNU-virus DNA polymerase is shown in Table 2. The purified DNA polymerase utilized efficiently $(rA)_n \cdot (dT)_{10}$, $(rC)_n \cdot (dG)_{12}$, and $(OMeC)_n \cdot (dG)_{12}$ as template-primers; $(rA)_n \cdot (dT)_{10}$ was the preferred template. As expected for a viral RNA-directed DNA polymerase, we observed a very low activity in response to $(dA)_n \cdot (dT)_{12}$ as template-primer. This pattern of template-primer responses is consistent with that observed for DNA polymerases purified from other murine retroviruses (Sarin *et al.*, 1979).

Table 2. Template-primer activities

Template-primer	Substrate	pmol dNMP incorporated
$(rA)_n \cdot (dT)_{10}$	^{3}H-dTTP	100
$(dA)_n \cdot (dT)_{12}$	^{3}H-dTTP	2.9
$(rC)_n \cdot (dG)_{12}$	^{3}H-dGTP	8.26
$(OMeC)_n \cdot (dG)_{12}$	^{3}H-dGTP	6.88

All assays were carried out for 60 min at 25° C as described in Materials and Methods.

Discussion

Administration of MNU was effective in increasing the expression of MuLV in tissues of mice as compared with corresponding tissues of untreated control animals (Fey *et al.*, 1980). After inoculation of activated MuLV isolated from MNU-induced leukaemias followed by suboptimal doses of MNU, an interaction occurred between these components provoking a synergistic carcinogenic effect (Fey *et al.*, 1981). The purpose of the present studies was to characterize the reverse transcriptase of the leukaemia virus activated during MNU-induced leukaemogenesis. Therefore, we increased the MuLV expression by rapid in vivo passages of the induced thymomas.

Calculated on the basis of reverse transcriptase activity, we detected 1—2% of virus in the spleen and 0.5—1% in the plasma of mice infected with MNU-activated leukaemia virus as compared with Rauscher leukaemia virus-infected Balb/c mice. A complete account of these results will be published elsewhere (Ehm *et al.*, in preparation).

Mammalian DNA polymerases may be distinguished from each other and from viral polymerases on the basis of their subcellular topology, physical properties, template specificities and their optimal reaction conditions. The MNU-virus DNA polymerase eluting at 0.28 mol/l KCl from phosphocellulose was indistinguishable from the Rauscher leukaemia virus reverse transcriptase as judged by its elution pattern from Sephadex G-200 and phosphocellulose, and by its size (70,000 D) as obtained after centrifugation in linear sucrose gradients. In addition to $(rA)_n \cdot (dT)_{10}$ and $(rC)_n \cdot (dG)_{12}$, the enzyme was able to transcribe $(OMeC)_n \cdot (dG)_{12}$, a template-primer shown to be specific for retrovirus polymerases. In principle our findings on the catalytic properties of the purified enzyme are in agrement with those reported for Rauscher murine leukaemia virus reverse transcriptase (Modak and Marcus, 1977). A comparison of the biochemical and biophysical data (Sarin *et al.*, 1979) of the MNU-virus DNA polymerase with DNA polymerases from other murine type C retroviruses makes clear that various murine retroviruses have similar biochemical properties.

Further experiments are in progress to (i) characterize the host range of the MNU-activated MuLV and (ii) clarify whether or not different MuLV strains or recombinations between different MuLVs are activated in MNU leukaemogenesis.

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