Evidence for two forms of reverse transcriptase in human placenta of a patient with breast cancer

Purification and biochemical characterization of the enzymes*

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(Received 15 January 1981/Accepted 29 April 1981)

Two DNA polymerases with properties of viral RNA-directed DNA polymerase were found in the placenta of a patient with breast cancer. Both enzyme activities were purified by column-chromatographic procedures or by preparative isoelectric focusing. The most distinguishing feature of the two enzymes is their specificity to transcribe $(rA)_n \cdot (dT)_{12}$ or $(rC)_n \cdot (dG)_{18}$. The two enzymes differ with respect to their elution profiles from the phosphocellulose column, isoelectric point, molecular weight, bivalent-cation requirements and thermal stability. Serological analysis of the $(rA)_n \cdot (dT)_{12}$ -activated enzyme showed that this enzyme is immunologically not related to DNA polymerase- γ , or to any of the reverse transcriptases purified from retroviruses of avian, murine and subprimate origin. However, the activity of this enzyme was neutralized by antibodies to reverse transcriptase purified from human spleen of a patient with myelofibrosis [Chandra & Steel (1977) *Biochem. J.* **167**, 513–524]. Attempts to purify reverse transcriptase from normal human placenta were repeatedly unsuccessful. Once the crude homogenate of normal human placenta was freed from endogenous nucleic acids, no $(rC)_n \cdot (dG)_{18}$ -dependent activity could be detected.

Purification of reverse transcriptase from human tumour tissues and cells has been reported from several laboratories (Chandra & Steel, 1977, 1980; Chandra *et al.*, 1978, 1980; Ebener *et al.*, 1979; Gallo *et al.*, 1975; Ohno *et al.*, 1977; Poiesz *et al.*,

1980; Welte *et al.*, 1979; Witkin *et al.*, 1975). Unlike the enzyme from extracellular virions, the intracellular reverse transcriptase is difficult to purify because of the existence of several DNA polymerases (EC 2.7.7.7) in the cell and the presence of proteinases. In spite of these difficulties, the purification of reverse transcriptase from a human source and its biochemical and immunological characterization is important to allow one to look for the retroviral information in such tissues and cells. In fact, the discovery of reverse transcriptase in human leukaemic cells (Gallo *et al.*, 1970) was the only tangible evidence for the expression of retroviral information in human malignancy. Since then, we have purified reverse transcriptase from different human tumour tissues and shown that these enzymes are biochemically similar to reverse transcriptase from the known retroviruses, but distinct from their respective cellular DNA polymerases α , β and γ (Chandra & Steel, 1977, 1980; Chandra *et al.*, 1978, 1980; Ebener *et al.*, 1979; Welte *et al.*, 1979). Serological characterization of reverse transcriptase and the purified cellular DNA polymerases supports this conclusion and, in addition, indicates that reverse transcriptases from different human tumours

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Abbreviations used: $(dA)_n \cdot (dT)_{10}$, DNA–DNA hybrid of polydeoxyadenylic acid and oligodeoxythymidylic acid (chain length 10); $(rA)_n \cdot (dT)_{12}$, RNA–DNA hybrid of a polyriboadenylic acid and oligodeoxythymidylic acid (chain length 12); $(rC)_n \cdot (dG)_{18}$, RNA–DNA hybrid of a polyribocytidylic acid and oligodeoxyguanylic acid (chain length 18); $(rCm)_n \cdot (dG)_{18}$, RNA–DNA hybrid of poly(2'-O-methylribocytidylic acid) and oligodeoxyguanylic acid (chain length 18); SDS, sodium dodecyl sulphate; AM virus, avian myeloblastosis virus; BE virus, endogenous baboon type-C virus; RD-114 virus, rhabdomyosarcoma virus; FL virus, Friend leukaemia virus; RL virus, Rauscher leukaemia virus; MPM virus, Mason–Pfizer monkey virus; SS virus, simian sarcoma virus; GaL virus, gibbon-ape leukaemia virus; IgG, immunoglobulin G.

possess a sub-group immunological specificity (Chandra *et al.*, 1980).

The present paper describes the purification of two distinct forms of reverse transcriptase from the placenta of a patient with breast cancer. Attempts to purify this enzyme from normal human placenta were repeatedly unsuccessful, though in crude homogenates we observed a small $(rC)_n \cdot (dG)_{18}$ -catalysed activity. However, after the removal of endogenous nucleic acids on DEAE-cellulose columns, no $(rC)_n \cdot (dG)_{18}$ -dependent activity was found. We believe that the activity in crude homogenates, reported in the literature (Nelson *et al.*, 1978), is due to some non-specific transcription of oligomeric nucleic acids, and does not constitute any proof for the presence of reverse transcriptase in normal placenta. The experimental data presented here favour this conclusion.

Materials and methods

Materials

Labelled deoxynucleoside triphosphates were from NEN Chemicals G.m.b.H., Dreieichenhain, Germany; non-labelled deoxynucleoside triphosphates and all the template-primers were from P.L. Biochemicals G.m.b.H., St. Goar, Germany, or from Boehringer Mannheim, Tutzing, Germany. Nonidet P-40 (Shell) and all other chemicals (reagent grade) were obtained from Bethesda Research Laboratories, Neu Isenburg, Germany, or from Serva Finechemicals G.m.b.H., Heidelberg, Germany. DEAE-cellulose (DE 23 and DE 52), phosphocellulose and GF/C discs were bought from Whatman (Kontron G.m.b.H., St. Leon, Germany).

Antibodies (IgG) to purified RT from GaL virus, SS virus, RL virus, rhabdomyosarcoma (RD-114) virus and AM virus were kindly provided by Dr. Jack Gruber (Chief, Office of Programme Resources and Logistics, National Cancer Institute, Bethesda, MD, U.S.A.). Antibodies to purified DNA polymerase from BE virus and to DNA polymerase γ were gifts from Dr. Robert C. Gallo (Chief, Tumor Cell Biology Department, National Cancer Institute, Bethesda, MD, U.S.A.). Antibodies to RT purified from human spleen of a patient with myelofibrosis were prepared as described by Chandra & Steel (1977).

Tumour material. The placenta of the patient (M.K.) was obtained from the Gynaecological Hospital of the University of Mainz, Germany. The patient was diagnosed as having a primary breast cancer (left mammary gland) in 1978 and the tumour was operated. Some months later, the patient conceived, and was delivered to the hospital on May 17, 1979. At this time, the patient was in the 31st week of pregnancy and complained of pains in the region of the left thorax, in the vicinity of the

operated gland. A small palpable nodule was detected and immediately operated. The histology of this specimen indicated metastasis of a solid carcinoma. The child, a normal healthy girl, was delivered by primary section on July 5, 1979. The histological examination of a post-operative specimen indicated metastasis of the left ovary, and tumour cells were detected in the placenta. The patient died on December 30, 1979.

Enzyme preparation

Extraction of the enzyme. Placental tissue (50 g) was mixed with 200 ml of buffer [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol / 0.5 mM-EDTA / 5 mM-MgCl₂] and homogenized under ice/water in a Waring Blendor for 5 min at low speed and 10 min at high speed. The suspension was then filtered through a monolayer of nylon stocking. The filtered homogenate was centrifuged for 30 min at 12000 g in a Sorvall centrifuge; the supernatant was decanted and the pellet was discarded. The supernatant was layered on to a 25% (w/w) saccharose cushion (in the above buffer) and centrifuged at 170000 g in a Damon IEC ultracentrifuge for 2 h. The microsomal pellet obtained was combined with 30 ml of buffer [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/0.1 mM-EDTA/0.3% Nonidet P-40/1.0 M-KCl/10% (v/v) glycerol] homogenized with a hand-driven homogenizer and stirred slowly for 1 h at 4°C. The homogenate was centrifuged at 18000 rev./min for 30 min in a Sorvall centrifuge, and the supernatant was decanted and kept for further processing.

Sephadex G-15 chromatography. The solubilized 170000 g pellet (30–35 ml) was applied to a column (2.5 cm × 25 cm) of Sephadex G-15 (Pharmacia, Uppsala, Sweden) equilibrated with buffer A [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/10% glycerol]. The column was eluted with the same buffer and 4 ml fractions were collected.

DEAE-cellulose (DE 23) chromatography. DEAE-cellulose (DE 23; fibrous form) was equilibrated with buffer A and poured into a column (diam. 2.5 cm; vol., 1 ml of wet-packed cellulose per 5–6 mg of protein). The desalted protein fractions eluted from Sephadex G-15 were pooled. The pooled fraction was adsorbed on the column and eluted with a linear KCl gradient (0–0.5 M) in buffer A. Fractions (2 ml) were collected and assayed for the reverse transcriptase activity as described below. The peak fractions (0.25–0.28 M-KCl) were pooled and concentrated 3-fold by dialysis against poly(ethylene glycol) buffer [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/1 mM-EDTA/20% glycerol/30% poly(ethylene glycol)]. The dialysis residue was then dialysed against buffer B [0.05 M-Tris/HCl (pH 7.9)/1 mM-dithiothreitol/10% glycerol].

DEAE-cellulose (DE 52) chromatography. DEAE-cellulose (DE 52; microgranular) was equili-

brated with buffer B and poured into a column (diam., 1.6 cm; vol., 1 ml of wet-packed cellulose per 5 mg of protein). The concentrated and dialysed fraction from the DEAE-cellulose (DE 23) column was adsorbed on the column, and the column was eluted with a 200 ml linear gradient of KCl (0–0.5 M) in buffer B. The fractions (2 ml) were collected and assayed for the reverse transcriptase activity as described below. The peak fractions (0.078–0.09 M-KCl) were pooled, concentrated 2–3-fold against poly(ethylene glycol) buffer, and dialysed against buffer C [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/0.02% Triton X-100/10% glycerol].

Phosphocellulose chromatography. Phosphocellulose (P) was prepared by the procedure described in the Whatman Manual, and equilibrated with buffer C. The column (diam., 1.6 cm; vol., 20 ml of wet-packed phosphocellulose) was prepared. The concentrated and dialysed peak fraction from the DEAE-cellulose column was applied to the column. After adsorption, the column was eluted with a 200 ml linear gradient of KCl (0–1.0 M) in buffer C. Fractions (2 ml) were collected and every second tube was assayed for reverse transcriptase activity.

Isoelectric focusing. Preparative electrofocusing in a pH gradient was carried out at 4°C on 110 ml electrofocusing columns (LKB: 8100–1). The sample (400–500 µg of protein) was distributed throughout the whole column by mixing it with the gradient solutions. The pH gradient was prepared by mixing carrier ampholytes (Ampholine; LKB) of pH ranges 3–10 (1 part) and 5–8 (2 parts) in a 10–60% (w/v) glycerol gradient. Electrofocusing was performed for 17 h at 1600 V. Fractions were collected and assayed for reverse transcriptase activity as described below.

DNA polymerase assays

The DNA polymerases were assayed with $(rA)_n \cdot (dT)_{12}$, $(rC)_n \cdot (dG)_{18}$, $(rCm)_n \cdot (dG)_{18}$, $(dT)_{12}$, $(dG)_{12}$, $(dA)_n \cdot (dT)_{12}$ and 70S RNA (AM virus) as primer–template. Fractions were assayed for the presence of DNA polymerase activity by adding 20 µl of the test fraction to a volume of 30 µl, which gave final concentrations of 50 mM-Tris/HCl (pH 8.0 for reverse transcriptase and DNA polymerase β ; 7.8 for polymerase γ), KCl (50 mM for reverse transcriptase, 80 mM for polymerase β and 20 mM for polymerase γ), $MnCl_2$ [0.6 mM for reverse transcriptase in the presence of $(rC)_n \cdot (dG)_{18}$; 1.2 mM for polymerase β ; 0.2 mM for polymerase γ] or 4 mM- $MgCl_2$ [reverse transcriptase in the presence of $(rA)_n \cdot (dT)_{12}$], 1 mM-dithiothreitol; 20 µM each of the unlabelled deoxyribonucleoside triphosphates, or 3H -labelled deoxyribonucleoside triphosphates (as indicated), and 1.25 µg of indicated primer–template, or primer alone. Reaction mixtures were incubated for 30 min at 37°C. Each assay was carried out in

triplicate. The reaction was terminated by the addition of 0.36 mg of bovine serum albumin and 3 ml of cold trichloroacetic acid (10%, w/v) containing 20 mM-sodium pyrophosphate. Acid-precipitable material was collected on Whatman (GF/C) glass-fibre discs, washed three times with 5 ml of 5% (w/v) trichloroacetic acid containing 20 mM-sodium pyrophosphate, dried at 100°C for 30 min, then suspended in 10 ml of toluene scintillator fluid (Quickszint, Zinsser Co., Frankfurt, Germany) and counted for radioactivity in a liquid-scintillation spectrometer (Nuclear Chicago, Mark III).

Antibody-inhibition studies

The purified reverse transcriptase from placental tissue was challenged with antibody (IgG) fractions prepared against purified reverse transcriptase from different sources. A portion of the enzyme (10 µl) was incubated for 4 h at 4°C with an equal volume of immune or preimmune IgG fraction (5–50 µg of protein). The remaining enzyme activity was measured with the assay system in a total volume of 50 µl. The effect of antibody fraction against DNA polymerase γ was studied under similar conditions, except that the tubes were centrifuged (20 000 g, 10 min) after the 4 h incubation period. Samples (10 µl) of the preincubated enzyme were used in the assay system.

Preparation of 70S RNA from AM virus

RNA from virus (National Cancer Institute, U.S.A.) was isolated as follows: 10 ml of the virus suspension (0.5 g wet wt.) was incubated with 5 mg of nuclease-free Pronase (Calbiochem, Giessen, Germany) for 30 min at 37°C. The virus particles were then disrupted by the addition of SDS (final concn. 1%) and were incubated at 37°C for 30 min. The suspension was sonicated for 2 min at 4°C (Branson), and then deproteinized with phenol/cresol solution (prepared as described by Wu *et al.*, 1974, and equilibrated with Tris/NaCl/EDTA buffer). After the third extraction, RNA was precipitated from the aqueous phase with 67% (v/v) ethanol containing 0.3 M-NaCl, at –20°C overnight. The precipitated RNA was collected by centrifugation at 30 000 g for 30 min and dissolved in TNE buffer [0.01 M-Tris/HCl (pH 7.0)/0.1 M-NaCl/1 mM-EDTA], and fractionated after centrifugation in a discontinuous glycerol gradient (Wu *et al.*, 1974). The fractions containing most of the RNA (approx. 90%) were pooled, and RNA was reprecipitated as described above.

Molecular-weight determination

Molecular weights of the purified reverse transcriptase fractions from human placenta were determined by SDS/polyacrylamide-gel electrophoresis, as described by Weber & Osborn (1969)

and Lotz *et al.* (1976). Marker protein or enzyme fractions were dissolved in 0.2 ml of a solution containing 24 mg of Tris, 30 mg of dithiothreitol, 20 mg of SDS, 0.2 ml of glycerol and 1.8 ml of double-distilled water by heating for 2 min at 100°C. A polyacrylamide gel (10%) was made 0.2% with respect to SDS in a slab-gel apparatus. Gels were stained with Coomassie Blue, as described by Weber & Osborn (1969), and destained electrophoretically. The electrophoretograms were scanned in a Quick-Scan Densitometer (Desaga, Heidelberg, Germany).

Results and discussion

Purification of reverse transcriptase

Scheme 1 outlines the overall technique of reverse transcriptase purification from the placental tissue of the patient with breast cancer. The enzyme was purified by successive chromatography on DEAE-cellulose (DE 23 and DE 52) and phosphocellulose, followed by preparative isoelectric focusing on a pH-gradient column. The enzyme activity in the column effluent was monitored in the presence of various template–primers, by the standard assay.

DEAE-cellulose (DE 23) chromatography of the desalted (Sephadex G-15) solubilized microsomal extract showed a major peak of activity eluted in 0.25–0.28 M-KCl. This step removed almost 85–90% of the endogenous nucleic acids, as determined by the A_{254} of input and effluent material. DEAE-cellulose (DE 52) chromatography of the peak fraction from DEAE-cellulose (DE 23) chromatography resulted in the elution of peak activity at around 0.08 M-KCl.

When the 0.08 M-KCl eluate was chromatographed on phosphocellulose, the reverse transcriptase activity was resolved into two peaks. Fig. 1 shows the profile of these activities in the presence of $(rC)_n \cdot (dG)_{18}$ and $(rA)_n \cdot (dT)_{12}$. One peak of major activity appeared in the 0.26–0.33 M-KCl eluate (pool I), and the other peak of activity was eluted in the gradient at 0.5–0.6 M-KCl (pool II). As the elution profile shows, both the major peaks of activity differ in their response towards the template–primer used in the assay system. The same pattern of elution with two distinct reverse transcriptase activities was observed in three independent isolations of the enzyme from the same placental tumour tissue.

Scheme 1. Schematic presentation of the methods used to isolate reverse transcriptase from human placenta of a patient with breast cancer

170 000 g centrifugation was carried out for 2 h and 12 000 g centrifugation for 30 min. Abbreviation: IEF, isoelectric focusing.

Fig. 1. *Phosphocellulose chromatography of the material eluted from a DEAE-cellulose (DE 52) column*
The peak of activity eluted in the 0.078–0.09 M-KCl wash was pooled and dialysed against buffer containing poly(ethylene glycol), as described in the Materials and methods section. The dialysis residue was applied to the phosphocellulose column and eluted with a linear KCl gradient (0–1.0 M; 100 ml each); 2 ml fractions were collected. Assay conditions are described in the Materials and methods section, with (▲) $(rC)_n \cdot (dG)_{18}$ or (●) $(rA)_n \cdot (dT)_{12}$.

Table 1. *Purification of reverse transcriptase activities from human placenta of patient with breast cancer*
Reaction conditions are described in the Materials and methods section.

Stage of purification (see Scheme 1)	Total protein (mg)	Specific activity (pmol/mg of protein)	
		$(rA)_n \cdot (dT)_{12}$	$(rC)_n \cdot (dG)_{18}$
Sephadex G-15 (desalting)	14.94	26.0 (40.16)*	34.1 (39.1)†
DEAE-cellulose (DE 23)	8.34	46.7 (7.19)*	35.9 (14.38)†
DEAE-cellulose (DE 52)	1.96	255.0 (5.10)*	168.3 (11.20)†
Phosphocellulose			
Pool I	0.08	1675 (0)*	550 (0)†
Pool II	0.078	2410	5307

* Endogenous incorporation of $[^3H]dTMP$.

† Endogenous incorporation of $[^3H]dGMP$.

The purification of reverse transcriptase through various column-chromatographic steps is summarized in Table 1. As shown by the specific activities of reverse transcriptase in pools I and II, 155-fold purification of the $(rC)_n \cdot (dG)_{18}$ -catalysed activity and approx. 92-fold purification of the $(rA)_n \cdot (dT)_{12}$ -catalysed activity was achieved.

Preparative isoelectric focusing of the pooled enzymes

By the procedure described in the Materials and methods section, electrofocusing of reverse transcriptase was carried out with peak fractions obtained after DEAE-cellulose (DE 52) and phosphocellulose chromatography. Fig. 2(b) illustrates the electrofocusing of combined activities from Pools I and II eluted from the phosphocellulose

column. Again, the reverse transcriptase activity was resolved into two peaks, one at pH 5.5 in the gradient, and the other at pH 7.2. The fractionation profile of the isoelectric focusing is very similar to the elution profile of the phosphocellulose column. Both the activities are split according to their activity towards $(rC)_n \cdot (dG)_{18}$ or $(rA)_n \cdot (dT)_{12}$. The $(rA)_n \cdot (dT)_{12}$ -specific peak is separated at pH 7.2, whereas the $(rC)_n \cdot (dG)_{18}$ -activated peak has pI 5.5. Fig. 2(a) shows the electrofocusing of reverse transcriptase purified from spleen of mice infected with Friend leukaemia virus. The procedure employed for the purification of this enzyme was similar to that used for human placenta. The fractionation profile shows a single peak of activity at pH 5.6 in the gradient. This shows that the presence of two distinct reverse transcriptase acti-

Fig. 2. Profile of DNA polymerase activities after electrofocusing phosphocellulose-column eluates of enzymes from FL-virus-infected mouse spleen (a) and from human placenta (b)

Pool-I and pool-II enzyme activities eluted from the phosphocellulose column were combined and subjected to electrofocusing, as described in the Materials and methods section. O, FL-virus enzyme with $(rA)_n \cdot (dT)_{12}$; ●, enzyme from placenta with $(rA)_n \cdot (dT)_{12}$; ▲, enzyme from placenta with $(rC)_n \cdot (dG)_{18}$; △, pH gradient.

vities, as found in the human placenta of a cancer patient, is not a general feature of oncornaviral expression in malignant cells.

The two peaks of reverse transcriptase activity could also be resolved by electrofocusing the 0.08M-KCl eluate from the DEAE-cellulose (DE 52) column (Fig. 3). The fractionation profile of the two activities is similar to that obtained for the purified pools from the phosphocellulose column (cf. Fig. 2b). The $(rA)_n \cdot (dT)_{12}$ -activated peak is eluted at pH 7.0 in the gradient, and the $(rC)_n \cdot (dG)_{18}$ -activated peak shows pI 5.2. However, the isoelectric focusing, as a purification step, is more useful if employed after the phosphocellulose chromatography.

Fig. 3. Profile of DNA polymerase activities after electrofocusing DEAE-cellulose (DE 52) eluate of the enzyme from human placenta of a patient with breast cancer

Experimental details are described in the text and in the Materials and methods section: ●, with $(rA)_n \cdot (dT)_{12}$; ▲, with $(rC)_n \cdot (dG)_{18}$; △, pH gradient.

Fig. 4 illustrates the activity profiles of different fractions obtained by the electrofocusing of reverse transcriptase after the DEAE-cellulose (DE 52) step (Fig. 4a), and after the phosphocellulose chromatography (Fig. 4b). It is evident that although the electrofocusing of the DEAE-cellulose (DE-52) eluate leads to the resolution of two distinct reverse transcriptase activities, both the major peaks (pH 7.0 and pH 5.2) are associated with activities other than reverse transcriptase.

Biochemical properties of the two forms of reverse transcriptase

(a) *Template properties.* Among the various features of retroviral reverse transcriptase which distinguish them from mammalian DNA polymerases is the ability of reverse transcriptase to utilize the heteropolymeric sequences of the viral genomic RNA (70S) to direct the synthesis of a complementary DNA. The response of pool-I and pool-II enzymes to 70S RNA and oligodeoxynucleotide primers is shown in Table 2. The results

Fig. 4. DNA polymerase activity pattern after electrofocusing of DEAE-cellulose column eluate (a) and of phosphocellulose column eluates (b) Electrofocusing of the DEAE-cellulose (DE 52) eluate was carried out with the peak fractions in the 0.078–0.09 M-KCl wash. Electrofocusing of the phosphocellulose eluate was carried out as described in Fig. 2; the numbers designate enzyme activities in the presence of: 0, (rCm)_n·(dG)₁₈; 1, (rC)_n·(dG)₁₈; 2, (rA)_n·(dT)₁₂; 3, (dA)_n·(dT)₁₂; 4, activated DNA; 5, endogenous [³H]dGTP incorporation; 6, endogenous [³H]dTTP incorporation.

Table 2. Effect of 70S RNA (AM virus) and oligodeoxynucleotides on the activity of purified reverse transcriptases from placental tissue

Reaction conditions are described in the Materials and methods section. Abbreviation: NT, not done.

Template–primer	Enzyme fraction (pool)	³ H]dNMP incorporation (pmol/mg of protein)	
		dTMP	dGMP
70S RNA	I	256.5	63.2
	II	108.3	435.2
70S RNA + (dT) ₁₂	I	498.4	276
	II	577.9	1013
(dT) ₁₂	I	0	NT
	II	0	NT
(dG) ₁₂	I	0	NT
	II	0	NT

with both the enzymes show a pattern of activities consistent with that obtained with reverse transcriptase purified from retroviruses (see Gillespie *et al.*, 1975). Interestingly, the two enzymes show some preferences in their ability to incorporate dGMP or dTMP. Thus the (rC)_n·(dG)₁₈-specific peak (pool II) shows the highest incorporation of dGMP in the presence of 70S RNA alone, or in the presence of 70S RNA and (dT)₁₂. In the presence of 70S RNA alone, the dTMP incorporation by pool-I enzyme is more than double the amount incorporated by pool-II enzyme. However, the difference was not so much when (dT)₁₂ was added in the same reaction. One possibility is that the purified enzyme fractions may have associated terminal deoxynucleotidyl-transferase activity, which can utilize (dT)₁₂ as initiator. However, this possibility is ruled out by the fact that none of the purified enzymes was able to utilize (dT)₁₂ or (dG)₁₂ as initiators when the oligodeoxynucleotides were added alone. This is evident from the data shown in the lower half of Table 2.

The template–primer preference of the two polymerases is further evident from the Lineweaver–Burk plot (Fig. 5) of the kinetic data obtained in the presence of (rC)_n·(dG)₁₈. The K_m value for the dGMP incorporation by pool-I enzyme is 10 μM, whereas that for the pool-II enzyme is 4.5 μM.

(b) Bivalent-cation requirements. The cation requirement of the pool-I and pool-II enzymes for transcription of (rC)_n·(dG)₁₈ and (rA)_n·(dT)₁₂ was examined. The activity of pool-I enzyme showed a strong preference for Mg²⁺ ions over Mn²⁺ ions in transcribing (rC)_n·(dG)₁₈. However, the pool-II enzyme did not exhibit such a strong preference for either of the bivalent ions, though the incorporation of dGTP in the presence of Mn²⁺ ions was significantly higher. The optimum concentration of Mn²⁺ ions for the transcription of (rC)_n·(dG)₁₈ by pool-II enzyme was 0.6 mM. The optimum concen-

Fig. 5. Lineweaver–Burk plot of the kinetic data for (rC)_n·(dG)₁₈-catalysed incorporation of [³H]dGMP by reverse transcriptase pools I (O) and II (Δ), eluted from the phosphocellulose column

tration of Mg^{2+} ions for the transcription of $(\text{rC})_n \cdot (\text{dG})_{18}$ by pool-I enzyme was 4 mM, and for the pool-II enzyme 2 mM. In contrast, the transcription of $(\text{rA})_n \cdot (\text{dT})_{12}$ showed a strong dependency on Mg^{2+} ions for both the enzymes. The optimum concentration of Mg^{2+} ions for pool-I enzyme was 4 mM, and for pool-II enzyme 2 mM. These values are identical with the Mg^{2+} concentrations required to transcribe $(\text{rC})_n \cdot (\text{dG})_{18}$.

Molecular-weight determination

The molecular weights of pool-I and pool-II enzymes were determined on SDS/polyacrylamide gels with bovine serum albumin as molecular-weight marker. Molecular weights of all the human reverse transcriptases purified so far have been reported to be 68 000–70 000. For this reason, bovine serum albumin (mol.wt. 68 000) was chosen as marker. Scans of the gels of pool-I and pool-II enzymes are illustrated in Fig. 6. The $(\text{rC})_n \cdot (\text{dG})_{18}$ -specific enzyme (Fig. 6c) migrates very close to the marker (Fig. 6b), indicating a molecular weight of about 70 000, consistent with the value reported for all the human reverse transcriptases (Chandra & Steel, 1977, 1980; Ebener *et al.*, 1979; Gallo *et al.*, 1975; Ohno *et al.*, 1977; Witkin *et al.*, 1975). More recently, Poiesz *et al.* (1980) have reported that reverse transcriptase from a T-cell lymphoma of man has a molecular weight of about 98 000. However, the $(\text{rA})_n \cdot (\text{dT})_{12}$ -specific enzyme (Fig. 6a) does not migrate as far as the pool-II enzyme. Thus the pool-I enzyme is heavier than the pool-II

enzyme; the rough estimation for the molecular weight of the pool-I enzyme is 98 000.

Thermal stability of the two forms of reverse transcriptase

During the isolation procedures, we observed that pool-II enzyme was very unstable. We therefore undertook studies of the thermal stability of purified reverse transcriptase in pools I and II. The enzyme fractions from pools I and II were incubated at 41°C; 10 μl samples were withdrawn from the incubated enzyme solution at time intervals as indicated in Fig. 7 (abscissa), and the remaining activity of the enzyme fraction was determined by the standard assay procedure, described in the Materials and methods section.

The thermal effects on the $(\text{rC})_n \cdot (\text{dG})_{18}$ -catalysed activity of pool-I and pool-II enzymes are shown in Fig. 7. This activity of pool-II enzyme is inhibited by 60% already in the first 5 min of incubation at 41°C. In contrast, the pool-I enzyme loses only 25% of activity in the first 5 min of incubation. The thermal-inactivation patterns of pool-I and pool-II enzymes for $(\text{rC})_n \cdot (\text{dG})_{18}$ transcription are different; the pool-II enzyme is by far more sensitive to thermal inactivation than the pool-I enzyme. Using identical experimental conditions, we observed a rapid fall of $(\text{rA})_n \cdot (\text{dT})_{12}$ -catalysed activity of pool-I and pool-II enzymes in the first 20 min of incubation, leading to about 40–45% loss of activity. Prolonged incubation led to a further loss of enzyme

Fig. 6. Scan of SDS/polyacrylamide gels after Coomassie Blue staining

(a) Pool-I enzyme eluted from the phosphocellulose column at 0.26–0.33 M-KCl. (b) Bovine serum albumin (marker). (c) Pool-II enzyme eluted from the phosphocellulose column at 0.50–0.60 M-KCl.

Fig. 7. Thermal effects on the enzyme activity of pool-I (●) and pool-II (▲) reverse transcriptases eluted from the phosphocellulose column

Enzyme fractions were incubated at 41°C and 10 μl samples were withdrawn at time intervals shown on the abscissa, pipetted into the reaction mixture (40 μl) and the remaining reverse transcriptase activity was assayed, as described in the Materials and methods section. The enzyme activities were measured in the presence of $(\text{rC})_n \cdot (\text{dG})_{18}$.

activity. However, the thermal-inactivation pattern of both forms of the enzyme was similar.

Serological characterization of pool-I enzyme

The data presented so far are quite convincing that the the pool-I and pool-II enzymes represent two different activities, and are biochemically related to reverse transcriptases of retroviruses. However, the very high specificity of pool-I enzyme for $(rA)_n \cdot (dT)_{12}$ and its molecular weight of approx. 98000 lead to the question of whether the purified enzyme is a true reverse transcriptase, or whether it has DNA polymerase γ associated activity. To investigate this problem, we examined the effect of anti-(DNA polymerase- γ) IgG on the $(rA)_n \cdot (dT)_{12}$ -catalysed activity of pool-I enzyme. As a control, similar experiments were carried out with a highly purified DNA polymerase γ from human ovarian tumour tissue. As shown in Fig. 8, the activity of pool-I enzyme fraction is completely insensitive to antibodies against DNA polymerase γ , whereas a concentration-dependent inhibition of the DNA polymerase γ was observed with the same IgG fraction. This proves that there is no polymerase γ activity in the pool-I fraction.

To substantiate further that the pool-I enzyme is a retroviral-like reverse transcriptase, antibody-inhibition studies were carried out with antibodies to purified reverse transcriptase from authentic retroviruses, and from human spleen of a patient with myelofibrosis (Chandra & Steel, 1977). The antibody-inhibition studies are reported in Fig. 9.

Fig. 8. Inhibitory effect of antibody to DNA polymerase- γ on reverse transcriptase from human placenta (pool-I enzyme) (●) and on DNA polymerase- γ activity from human ovarian tumour tissue (▲)

The enzyme activities were measured in the presence of $(rA)_n \cdot (dT)_{12}$. Preimmune IgG served as control for each concentration of immune IgG used. Each set of experiments was carried out in triplicate.

Each experiment included the same amount of preimmune IgG as control (100%). No inhibition of the placental reverse transcriptase was observed when challenged with anti-polymerase IgG from AM virus, SS virus, RD-114 virus, BE virus and RL virus. Interestingly, the placental enzyme was inhibited strongly by the anti-polymerase IgG fraction of a human spleen from a patient with myelofibrosis. The curve for inhibition of placental polymerase activity with increasing amounts of this latter IgG is similar to that for the reverse transcriptase from myelofibrotic spleen (Chandra & Steel, 1977). Under identical experimental conditions, antibodies used in our study neutralized the activity of homologous enzyme systems.

The serological relatedness of placental pool-I reverse transcriptase to the reverse transcriptase from human myelofibrotic spleen is strange for two reasons. Firstly, as previously reported by us (Chandra & Steel, 1977; Chandra *et al.*, 1980), the purified reverse transcriptase from human myelofibrotic spleen cross-reacts immunologically with the enzyme from SS virus. Since the placental enzyme does not cross-react with the enzyme from SS virus (Fig. 9), its cross-reaction with reverse transcriptase from myelofibrotic spleen was unexpected. This result would indicate that immunological relatedness between polymerases from placenta and myelofibrotic spleen is due to a common determinant between the two enzymes, different from the determinant recognized by SS-virus polymerase.

Fig. 9. Effects of antibodies to various purified reverse transcriptases on the reverse transcriptase activity of pool-I enzyme eluted from the phosphocellulose column

The enzyme activity was measured in the presence of $(rA)_n \cdot (dT)_{12}$. The experimental conditions were as for Fig. 8. The symbols designate antibodies to DNA polymerases from AM virus (▲), SS virus (□), RD 114 virus (○), BE virus (■), RL virus (△) and from the spleen of a patient with myelofibrosis (●).

Secondly, the source of this placenta was a patient with metastatic breast cancer, and by analogy with similar neoplasia in animals, one would expect the involvement of B-type virus particles. Thus one would expect a strong immunological homology between the placental enzyme and the enzymes from human breast-cancer tissue, or from MPM virus. It would be of immediate interest to look for the possible cross-reactivity of the placental enzyme(s) with other reverse transcriptases.

To our knowledge, the first indication of two reverse transcriptases in a human tumour tissue emerged from studies on human breast cancer particles, reported by Ohno *et al.* (1977). These authors reported two reverse transcriptase peaks, eluted from the phosphocellulose columns at 0.08 M- and 0.18 M-phosphate buffer. The major peak of activity, eluted at 0.18 M-phosphate, was reported to be absent from normal breast tissues. For this reason, no attempt was made by Ohno *et al.* (1977) to characterize the enzyme activity eluted at 0.08 M-phosphate. In this regard, the studies reported here constitute the first evidence of the two distinct forms of reverse transcriptase in a human tissue from a cancer patient.

The data reported here stemmed from three independent isolations of the enzymes from the same placental tissue. All yielded enzyme activities with the same biochemical and biophysical properties. We have examined normal human placenta by the same fractionation technique, using identical experimental conditions. Table 3 summarizes the results of an isolation procedure at various steps of purification. The solubilized microsomal pellet (170 000 g) shows a high endogenous incorporation of [³H]dGMP and [³H]dTTP; it also shows a significant incorporation of dTMP and dGMP in the presence of (rA)_n·(dT)₁₂ and (rC)_n·(dG)₁₈ respectively. However, the template-primer-catalysed incorporation is lower than the endogenous incorporation of the precursors. Column chromatography of the solubilized extract on DEAE-cellulose (DE 23) leads to an overall inhibition of enzyme activity

catalysed by (rA)_n·(dT)₁₂ or (rC)_n·(dG)₁₈. However, the (dA)_n·(dT)₁₀-catalysed incorporation shows a 3-fold increase of activity. Further purification of the enzyme activity from normal human placenta on DEAE-cellulose (DE 52) column leads to a total loss of (rC)_n·(dG)₁₈-dependent activity. The endogenous incorporation of [³H]dGMP, at this purification step, was also zero. From these data, it is evident that the (rC)_n·(dG)₁₈-dependent activity in crude preparations from normal human placenta is due to incorporation of precursors catalysed by the nucleic acids present endogenously. Once the fractions are freed from endogenously present nucleic acids, the (rC)_n·(dG)₁₈-catalysed activity is completely lost. We therefore believe that the reverse transcriptase activity in crude preparations from normal human placenta, reported in the literature (Nelson *et al.*, 1978), is due to non-specific transcription of endogenous nucleic acids, and does not constitute any proof for the presence of reverse transcriptase activity in normal human placenta. The experimental data presented in Table 3 support this conclusion.

Note Added in Proof (Received 15 June 1981)

Consistent with the results presented here, Lowenstein *et al.* (1980) have reported that they were unable to detect any DNA polymerase activity eluted between 0.05 M- and 0.30 M-NaCl on phosphocellulose chromatography of normal human placenta (cf. Table 1).

We thank Dr. Robert C. Gallo (National Cancer Institute, Bethesda, MD, U.S.A.) for communicating unpublished results to us and for valuable discussion. We are grateful to Dr. Jack Gruber (Chief, Office of Programme Resources and Logistics, National Cancer Institute, U.S.A.) for providing antisera against the DNA polymerases from RNA tumour viruses. We thank Dr. Rolf Kreinberg (Gynaecological Hospital, Mainz, Germany) for providing the placenta specimen and the clinical and histological reports on the patient. This work was supported by grant from Stiftung Volkswagenwerk (14-0305).

Table 3. Attempts to purify reverse transcriptase activity from normal human placental tissue
Reaction conditions are described in the Materials and methods section.

Stage of purification (cf. Scheme 1)	³ H]dNMP incorporation (pmol/mg of protein) in the presence of		
	(rC) _n ·(dG) ₁₈	(rA) _n ·(dT) ₁₂	(dA) _n ·(dT) ₁₂
170 000 g (solubilized pellet)	7.66 (19.1)*	20.68 (26.8)†	19.92
DEAE-cellulose (DE 23) (0.25–0.35 M-KCl pool)	5.36 (7.66)*	11.87 (9.96)†	30.65
DEAE-celulose (DE 52) (0.05–0.25 M-KCl pool)	0 (0)*	2.29 (1.53)†	90.91

* Endogenous incorporation of dGMP.

† Endogenous incorporation of dTMP.

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