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REVERSE TRANSCRIPTASE OF RAT LIVER ASSOCIATED WITH THE ENDOGENOUS RETROVIRUS RELATED TO THE MOUSE INTRACISTERNAL A-PARTICLES

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SUMMARY: Reverse transcriptase activity was found in rat liver enclosed in virus-like particles. Through hybridization with DNA probes of A- and C-type retroviruses and with the help of electron microscopy the virus-like particles have been identified as endogenous retroviruses related to the mouse intracisternal A-particles. Blot hybridization revealed the provirus DNA in the rat genome. The enzyme was isolated from the virus-like particles, purified and characterized. The main properties of the enzyme resemble those of the mammalian retrovirus reverse transcriptase.

A number of data demonstrates the RT activity in animal cells generated either by the infection of the cells with retrovirus or via the expression of proviral genes (1). The RT in normal animal cells may have important biological functions. This notion is supported by the finding of pseudogenes and repetitive DNA sequences in animal genome which are suggested to originate by reverse transcription (2). The enzyme might also provide the amplification of extragenomic and intragenomic DNA sequences to ensure the intensive gene expression.

In this paper we demonstrate the RT in rat liver enclosed in virus-like particles. The RT was isolated from the retrovirus particles and its properties were studied. The virus-like particles have been identified as endogenous retroviruses related to the mouse IAP. The putative functions of RT in normal cells are discussed.

Abbreviations: RT, reverse transcriptase; IAP, intracisternal A particles; Mo-MuLV, Moloney murine leukemia virus; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; SSC, 0.15 M NaCl, 0.015 M sodium citrate.

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METHODS

Cell-fractionation procedures. The rat livers (25 g from 7 - 8 males of Wistar rats, 120-150 g) were used for each experiment. Livers were homogenized in buffer A (0.05 M Tris-HCl, pH 7.5; 1 mM DTT; 0.1 M KCl) and centrifuged for 20 min at 20,000 g to remove nuclei and mitochondria. The supernatant was centrifuged for 1 h at 165,000 g (SW 50.1, Beckman). The pellet was resuspended in buffer A and layered on a sucrose linear gradient (20-50 %) prepared with the same buffer containing 0.14 M KCl. After centrifugation for 17 h at 165,000 g (SW 50.1, Beckman) the fractions of 150-200 µl were collected and monitored for RT activity. The fractions were also studied by electron microscopy.

Enzyme assays. A 50 µl reaction mixture contained 50 mM Tris-HCl, pH 7.8 at 37° C; 1 mM DTT; 50-200 mM KCl; 10 mM $MgCl_2$; 1 µg of appropriate synthetic template and 0.5 µg of the corresponding primer or 2.5 µg of activated calf thymus DNA, or 1 µg rat liver poly(A)$^+$mRNA-$(dT)_{6-8}$; 0.02 mM [^{3}H]dGTP or [^{3}H]dTTP (sp. act. 1 Ci/mmole) when poly(C)-$(dG)_{12-18}$ or poly(A)-$(dT)_{6-8}$ were used, respectively; 5 nmol each of dATP, dCTP, dTTP and 0.02 mM [^{3}H]dGTP when heteropolymeric templates were used; 30 µl aliquot taken from each fraction of sucrose gradient. When RT activity was monitored during the enzyme purification 30 µl aliquots of the eluates taken from DEAE and phosphocellulose columns were added to the reaction mixture. To inhibit DNA dependent DNA synthesis actinomycin D (80 µg/ml) was added to the reaction mixture when endogenous retrovirus RNA was used as a template. After 30 min at 37° C the reaction was stopped by ice cooling. Aliquots were transferred to DE-81 (3).

One unit of RT activity is defined as the amount of enzyme which converts 1 pmol of [^{3}H]dGMP to an acid-insoluble form in 30 min at 37° C when poly(A)$^+$mRNA is used as a template.

Electron microscopy. Aliquots of sucrose gradient were applied on Formvar-coated grids and negatively stained in 2 % uranyl acetate. For thin sectioning the samples were fixed for 30 min at 4° C in 3 % glutaraldehyde in 0.1 M Tris-HCl, pH 7.8. After rinsing in the same buffer the pellets were postfixed in 2 % OsO_4 for 3 h at 4° C, dehydrated in ethanols of increasing concentration and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-100 C electron microscope.

Isolation and fractionation of RT. The RT was purified from the sucrose gradient fractions (1.16-1.18 g/cm^3) which contained retrovirus-like particles and possesed high RT activity. An equal volume of disruption buffer was added to the collected fractions. The buffer included the following components: 50 mM Tris-HCl, pH 7.5; 0.8 M KCl; 20 mM DTT; 20 % glycerol; 0.6 % Triton X-100. The lysate was dialysed against buffer B (50 mM Tris-HCl, pH 7.5; 1 mM DTT; 10 % glycerol). The dialysed sample was applied to the DEAE cellulose (DE 23) column to remove nucleic acids and eluted with buffer B containing 0.3 M KCl. The further steps of RT isolation were performed according to Lewis et al. (4) (Table 1).

Extraction of RNA and DNA. RNA was extracted from the sucrose gradient fractions containing retrovirus-like particles and RT activities (ρ = 1.16-1.18 g/cm^3) (5). RNA preparations were diluted in buffer C (10 mM Tris-HCl, pH 7.5; 1 % SDS) layered on a sucrose gradient (5-20 %) prepared on the buffer C plus 5 mM EDTA and centrifuged for 18 h at 20,000 rpm (SW 27.1, Beckman);

Table 1. Purification of RT from retrovirus-like particles

Purification steps	Total protein (mg)	Total activity (UA)	Specific activity (UA/mg of protein)	The degree of purification	Yield (%)
Liver homogenate	4,000	$18 \cdot 10^4$	45	1	100
Postmitochondrial supernatant	2,000	$18 \cdot 10^4$	90	2	100
165,000 g pellet of postmitochondrial supernatant	330	$24 \cdot 10^3$	73	1.6	12.6
Peak of $\rho = 1.18$ g/cm^3 particles	110	$18 \cdot 10^3$	164	3.6	10
DE 52 chromatography	0.1	$7.6 \cdot 10^3$	$76 \cdot 10^4$	1,670	4.2
Phosphocellulose P 11 chromatography	N.T.	$18 \cdot 10^3$	N.T.	N.T.	10

UA - unit of RT activity. N.T. - not tested.

20° C. E. coli rRNA was used as a marker. The high molecular weight RNA ($S_{20} > 23$ S) was collected and precipitated with 2.5 volumes of ethanol. The RNA was diluted in 10 mM Tris-HCl, pH 7.5 and applied to the poly(U)-Sepharose column equilibrated with the same buffer. Poly(A)$^+$RNA was eluted with 10 mM Tris-HCl, pH 9.0 at 37° C.

The rat liver DNA was prepared according to (5).

Filter hybridizations. RNA was dotted into nitrocellulose filters as described (6). The endonuclease digestion of DNA, the electrophoresis on agarose gels, the transfer to nitrocellulose membranes and the labelling of probes were performed as described (5). The hybridization and the washing procedure after hybridization of nitrocellulose membranes varied and are specified in Fig. 3.

RESULTS AND DISCUSSION

Previous papers from this laboratory have demonstrated that the postmitochondrial supernatant of the rat liver homogenate posseses RT activity (7, 8). To define the source of the activity the postmitochondrial supernatant of the rat liver homogenate was centrifuged at 165,000 g. The pellet was layered onto the preformed linear 20-50 % sucrose gradient and centrifuged for 17 h at 165,000 g in SW 50.1 rotor. High RT activity was found in the fractions with a density of 1.16-1.18 g/cm^3 (Fig. 1). The enzyme activity at its peak was greatly enhanced by adding Triton X-100 to the incubation mixture (Fig. 1). The data suggests that the RT activity is enclosed in particles in which the enzyme is protected by an envelope which may be destroyed by detergent.

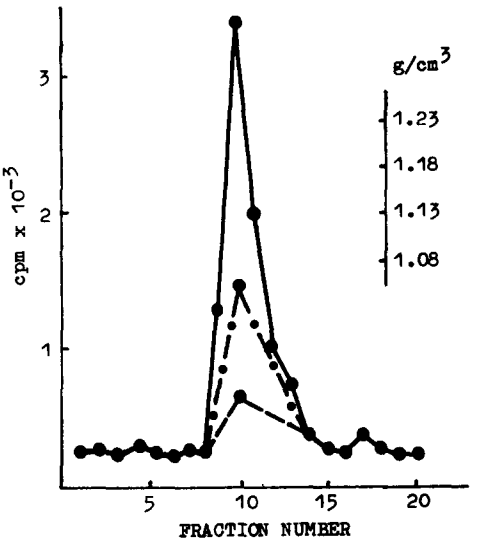

Figure 1. Distribution of RT activity of pellet of rat liver microsomes through equilibrium density sucrose gradient.
——●—— complete reaction mixture
—•—●—•— complete minus Triton X-100
— —●— — complete plus RNAse

Electron microscopy study of the linear sucrose gradient fractions has shown that in the fractions with high RT activity retrovirus-like particles 80-120 nm in diameter were present. Negative staining and ultrathin sections demonstrated that the particles have smooth outer membranes, nucleoids and resemble retroviruses of the A and C types (Fig. 2).

In other fractions of the linear sucrose gradient lacking RT activity retrovirus-like particles were absent. In all fractions with and without RT activity microsomal vesicles of various sizes and forms were seen.

The retrovirus-like particles appeared to contain endogenous RNA which serves as a template for the RT. We assumed this because after the incubation of Triton X-100 pretreated peak with RNAse A a sharp decrease of the DNA synthesis occurred (Fig. 1).

As a result of the endogenous synthesis of nucleic acids, DNA-RNA hybrid molecules were produced: after nuclease S1 treatment they banded as a single peak at 1.76 g/cm^3 density of CsCl which is characteristic of the hybrids.

Hybridization of the poly(A)$^+$RNA ($>$ 23 S) which had been isolated from the peak containing the virus-like particles, to the cloned nick-translated DNA probes of the mouse IAP or Mo-MuLV revealed homology of the poly(A)$^+$RNA with the mouse IAP

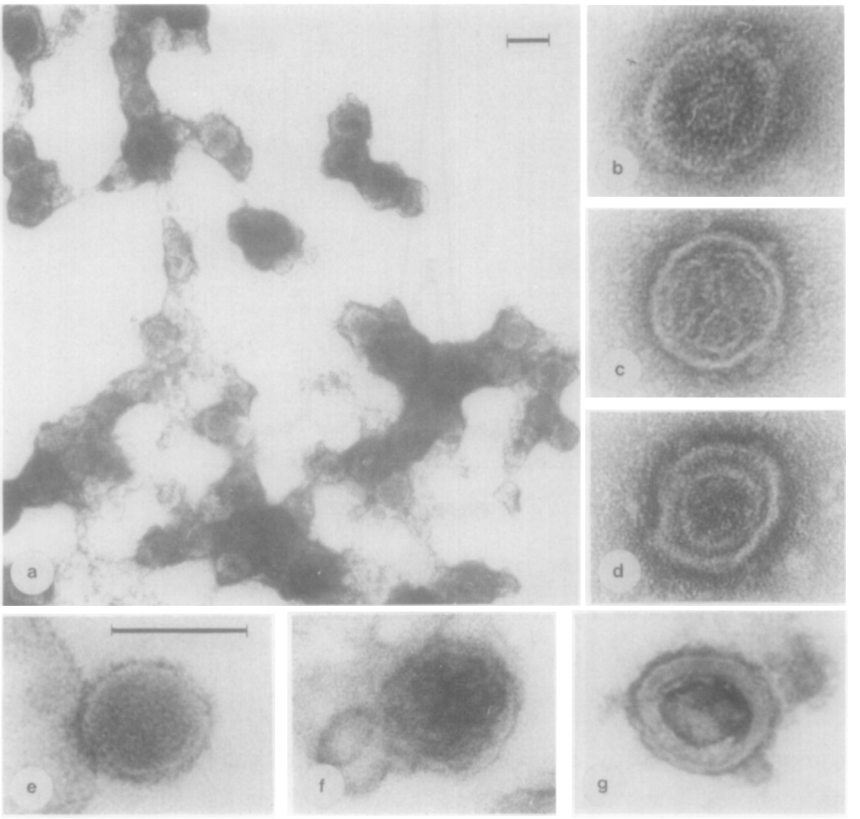

Figure 2. Virus-like particles from fraction of density sucrose gradient (ρ = 1.17 g/cm^3). a) Cluster of virus-like particles; b, c, e, f) virus-like particles of A type; d, g) virus-like particles of C type (a - d negative staining; e - g ultrathin sections). Bars, 0.1 μm.

RNA but not with the RNA of C type retrovirus (Fig. 3). These observations seem to indicate that in the livers of Wistar rats an endogenous retrovirus related to the mouse IAP is present.

Rat liver DNA was digested with EcoRI, PstI or BamHI and analysed by the Southern blotting technique using the [^{32}P]-labelled poly(A)$^+$RNA of the IAP-like rat retrovirus and the cloned nick-translated DNA of the mouse IAP as probes. Nucleotide sequences homologous to those of the probes were found in the rat genomic DNA. It appeared that both probes hybridized with the same sequences of the rat DNA (Fig. 4). The data indicate that the proviral DNA of the rat IAP-like retrovirus homologous to the mouse IAP RNA is present in the rat genome. However, when the rat IAP RNA was used as a probe additional fragments after EcoRI and BamHI digestion were revealed. Their absence when the mouse IAP DNA was applied as a probe is due, evidently, to the

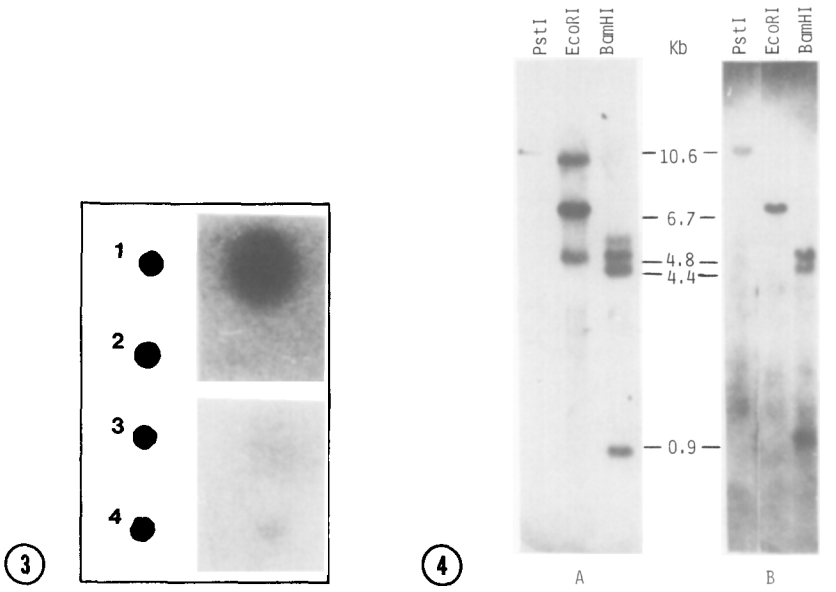

Figure 3. Identification of the virus-like particles from the rat liver by dot hybridization. Aliquots of poly(A)$^+$RNA (5 µg) from the sucrose gradient fractions (ρ = 1.16 g/cm^3) containing virus-like particles (dots 1, 3) and from the rat liver cells (dots 2, 4)(control) were dotted onto nitrocellulose filters as described (7) and hybridized with [^{32}P]DNA of the mouse IAP (dots 1, 2) or Mo-MuLV (dots 3, 4). Conditions of hybridization were as follows: 50 % formamide; 5 x SSC; 0.02 % Ficoll; 0.02 % bovine serum albumin; 0.02 % polyvinylpyrrolidone; 100 µg/ml salmon sperm DNA; mouse IAP [^{32}P]DNA or Mo-MuLV [^{32}P]DNA ($5 \cdot 10^7$ cpm/µg). Hybridization was carried out for 36 h at 20° C and followed by washing in 2 x SSC; 0.1 % SDS (3 x 30 min at 20° C) and in 0.1 SSC; 0.1 % SDS (3 x 30 min at 20° C). Sources of DNA: pBR322 DNA with integrated BamHI-EcoRI fragment of mouse IAP DNA 2,200 bp long (9) was obtained from Dr D.A. Kramerov, while pBR322 with integrated full cDNA of Mo-MuLV RNA (15) was obtained from Dr I.M. Chumakov.

Figure 4. Identification of sequences homologous to rat IAP (A) and mouse IAP (B) genes in restriction fragments in rat genomic DNA. Aliquots of the indicated DNAs were digested with EcoRI, PstI or BamHI, separated in an agarose gel, transferred to a nitrocellulose filters. Hybridization and washing were carried out as indicated in the legend to Fig. 3 for A) at 37° C, B) at 20° C.

fact that the latter comprises only the central part of the IAP proviral DNA (9). The DNA sequences related to the mouse IAP genes have been found previously in the rat genome by Lueders and Kuff (10).

The RT activities from the peak with a density of 1.16-1.18 g/cm^3 containing the retroviral particles (Fig. 1) were purified by DEAE and phosphocellulose chromatography as described in the Methods. The enzyme was characterized by its chromatographic behaviour, pH optimum, sedimentation constant and preference for

Table 2. The main properties of the RT from rat liver and retroviral particles

Template-primers	Enzyme activity			
	Retroviral particles		Rat liver	
	pmoles/30 min	%	pmoles/30 min	%
Poly(C)·$(dG)_{12-18}$ + Mg^{2+}	12.0 ± 0.8	100	6.1 ± 0.6	100
+ Mn^{2+}	8.2 ± 0.7	68	-	
Poly(A)$^+$mRNA·$(dT)_{6-8}$ + Mg^{2+}	4.5 ± 0.3	37.5	2.7 ± 0.2	44
+ Mn^{2+}	3.5 ± 0.2	29	1.8 ± 0.1	29
Poly(A)·$(dT)_{6-8}$ + Mg^{2+}	4.0 ± 0.6	33	-	
+ Mn^{2+}	4.7 ± 0.4	39	2.1 ± 0.2	35
Activated DNA + Mg^{2+}	2.9 ± 0.2	24	3.2 ± 0.2	52
$(dT)_{6-8}$ + Mg^{2+}	0	0	0	0
Poly(dT)·$(rA)_{8-10}$ + Mg^{2+}	0.3	2.5	-	-

Both enzymes are similarly eluted from phosphocellulose P 11 by 0.1-0.15 M KCl; their pH optimum is 7.8. S_{20} for enzyme of retroviral particles is 4.6.

definite template-primers (Table 2). RT elutes at 0.1 M KCl from DEAE cellulose (DE 52) and at 0.1-0.15 M KCl from phosphocellulose (P 11) columns. Its pH optimum is at 7.8. The RT preferred poly(C)-oligo(dG) over all other synthetic template-primers. The enzyme accepted poly(A)$^+$RNA-oligo(dT) that is the main characteristic feature of the reverse transcriptases (11). It also preferred Mg^{2+} to Mn^{2+}. The sedimentation constant of the enzyme is 4.6 S_{20}. Therefore, the isolated enzyme with respect to its patterns of utilization of template-primers, its preference for Me^{2+}, sedimentation constant, and its chromatographic behaviour is very close to the characteristics of the RT of the mammalian retroviruses which have been studied (1).

RT activity has also been found in rat liver in a particle-free form. The characteristics of this enzymatic activity are similar to those found in the RT isolated from the rat liver IAP-like retroviral particles. A free form of RT prevails in the rat liver. Clearly, free RT is coded for by the proretroviral genes. One can not exclude the possibility that the enzyme provides for not only the replication of the retroviral nucleic acids but also the amplification of some of cellular RNAs as was suggested earlier (8). The synthesis of extrachromosomal DNA copies of genes intensely expressed under induction stimuli may be

important for cell differentiation and adaptation to special conditions. This assumption is supported by a number of data. The induction of the myeloid cell differentiation is accompanied by the production of ecotropic viruses of the C type and an enhancement of the RT activity (12). The increase of the expression of endogenous retrovirus has been found in the mouse uterus in response to the administration of estrogens (13). Our data have previously demonstrated that the transcription and translation which in rat liver ensure the synthesis of various sets of adaptive enzymes under the effect of administration of glucocorticoids, amino acids or galactose are accompanied by the increase of RNA-dependent DNA synthesis (14).

While the suggestions outlined above are still speculative they provide models for further experiments.

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