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Evidence that Reverse Transcriptase Is a Component of Murine Epididymal Fluid (41859)

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Abstract. The sperm-free fluid fraction of epididymal semen obtained from males of four mouse strains contained a DNA polymerase activity with properties of murine retrovirus reverse transcriptase (RT). Epididymal fluids from two of the strains of mice, NIH Swiss and New Zealand Black (NZB), had an order of magnitude more enzyme activity per microgram of protein than did the fluids of C57Bl/6 and Balb/c males. Most of the enzyme activity in the Swiss and NZB males, but not in the C57Bl/6 and Balb/c males, banded in isopycnic sucrose gradients at the buoyant density of retrovirus particles. The males were fertile and free of tumor or other detectable disease up to 17 months of age. The evidence suggests RT activity is ubiquitous in the male reproductive tract of mice and may or may not be associated with virus particles.

DNA polymerases β and γ have been isolated from mammalian spermatozoa by several investigators, but not DNA polymerase α, the most abundant DNA polymerase in mammalian somatic cells (1–5). Consistent with the presence of mitochondria in the midpiece of sperm tails, mitochondrial DNA polymerase (DNA polymerase γ (6)) has been isolated from bull sperm tails. The DNA polymerase γ class of enzymes effectively utilizes the synthetic RNA polymer, Poly(A), as a template for DNA synthesis in the presence of oligo(dT) primer. The ability to utilize poly(A)·oligo(dT) as a template-primer for DNA synthesis *in vitro* is a property that DNA polymerase γ has in common with reverse transcriptase (RT), the DNA polymerase in retroviruses that reverse transcribes virus RNA genomes into DNA copies.

During the purification of mouse sperm DNA polymerase γ in this laboratory, it became apparent that epididymal semen from Swiss males contained additional poly(A)·oligo(dT)-stimulated DNA polymerase activity that was not associated with the sperm. DNA polymerase activities in murine epididymal fluids have not been previously reported. The enzyme activity was characterized and the work extended to other mouse strains. The results of these studies, reported here, provide evidence that the enzyme activity was RT, it was present in epididymal secretions *in vivo*, and it may be a ubiquitous component of murine epididymal fluids that is independent of the presence of virus particles.

Materials and Methods. *Epididymal fluid collection and separation from sperm.* Epididymides from mature males were dissected free of adnexa, and the luminal contents (15–30 μl/epididymis) were extruded by gently milking each epididymis and a portion of the ductus deferens with dissecting forceps into 50–100 μl of buffer A (0.01 *M* Tris–HCl, pH 7.4; 0.1 *M* KCl; 1 mg/ml bovine serum albumin) plus 0.67 *M* sucrose. Sperm suspensions obtained in this manner were contaminated with fewer than 1% extraneous cells as determined by direct count in a hemocytometer. The suspensions were centrifuged at 8000*g* for 3 min to obtain a supernatant fraction and a sperm pellet which was resuspended in one original volume of fresh buffer A plus 0.67 *M* sucrose. Less than 0.1% of the sperm remained in the supernatant fraction under these fractionation conditions. Enzyme to be stored was adjusted to 0.25% Nonidet-P40 (NP-40, Shell), 5 m*M* dithiothreitol (DTT), and 40% ethylene glycol and stored at −20°C. Enzyme preparations were stable under these storage conditions for at least 9 months.

Fluorescence determinations. Fluorochromasia of the sperm was assessed as previously described (7). Briefly, a 10 m*M* stock solution of dicarboxyfluorescein diacetate (DCFA) in dimethylsulfoxide, a generous gift of Dr. Donner Babcock, was diluted to 5 μM in buffer A immediately prior to the addition of 5 to 10 μl of epididymal sperm suspension yielding a final sperm count of approximately 1×10^7/ml. Wet mount preparations of the sperm were

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viewed at a magnification of 1000× with a Zeiss Photomicroscope III set initially on dark-field illumination. Fluorescence was viewed and photographed by switching to an Osram HBO-200 ultraviolet light source using primary filter BG12 and secondary filter "50."

Sucrose density gradient centrifugation. Epididymal fluid enzyme fractions were diluted with one equal volume of Buffer A and layered atop a 10–70% linear gradient of sucrose in TKE (0.01 *M* Tris–HCl, 0.1 *M* KCl, 0.001 *M* EDTA). Isopycnic centrifugation was carried out at 40,000 rpm in a SW 50 rotor for 16 hr and the gradients fractionated as previously described (8). Aliquots of each gradient fraction were adjusted to 0.25% NP-40, 5 m*M* DTT, and assayed for DNA polymerase activity in the presence of DNA and poly(A) · oligo(dT) as described below.

DEAE-cellulose chromatography. A 1-ml column of DEAE-52 cellulose (Whatman) was equilibrated in PB (0.015 *M* potassium phosphate, pH 8.0; 35% ethylene glycol; 1 m*M* DTT). Enzyme activity in supernatant and pellet fractions was solubilized by exposure to 1% NP-40, 20 m*M* DTT, 0.5 *M* KCl, and 40% ethylene glycol for 20 min at 4°C. The enzyme samples were diluted 1:5 with the equilibration buffer, applied to the column, and the column washed with 1 vol of equilibration buffer. Enzyme activity was eluted by the stepwise addition of 0.1, 0.2, and 0.4 *M* potassium phosphate in PB.

Gel filtration. Molecular size estimates were made by chromatography on a 100-ml column of Sephadex G-150 equilibrated with 0.05 *M* potassium phosphate in PB. Aldolase (mol wt = 160,000), bovine serum albumin (mol wt = 64,000), and ovalbumin (mol wt = 43,000) were used as molecular size standards.

Protein measurements. A modification of the amido schwarz dye binding assay for protein (11) was used to estimate protein concentrations. Protein aliquots were adjusted to 2% sodium dodecyl sulfate (SDS) and spotted directly onto nitrocellulose filters (Millipore HA 0.45 μm) in 1- to 10-μl amounts. The filter was submerged in cold 10% trichloroacetic acid (TCA) for a minimum of 10 min followed by a quick rinse in cold distilled water. The filters so treated could be stored at 4°C for up to 2 weeks without introducing error into the protein determination. The protein spots were stained with 0.1% amido black 10B in methanol:acetic acid:distilled water (45:10:45) and destained as described (9). The individual spots were removed from the filter with a cork borer or paper punch and eluted into 200 μl of freshly prepared eluant (25 m*M* NaOH, 50 m*M* EDTA in 50 volume % aqueous ethanol) for concentration estimates at 630 nm. The protein assay was linear from 2 to 40 μg protein/ml and reproducible to a standard error of less than ± 3.3%.

DNA polymerase assays. All enzyme assays were performed in the wells of microtiter trays as previously described (10, 11). Briefly, the assays with DNA were 30 m*M* Tris–HCl (8.0 at 37°C), 2 m*M* dithiothreitol (DTT), 5% ethylene glycol, 20 m*M* KCl, 10 m*M* $MgCl_2$, 0.5 mg/ml "activated" (10) calf thymus DNA (Sigma), 0.2 m*M* dATP, dCTP, dGTP, and 5–6 μ*M* dTTP (4 μ*M* dTTP plus 1–2 μCi of [^{3}H]dTTP, 58 Ci/m*M*, New England Nuclear, or 67 Ci/m*M* International Chicago Nuclear). Standard assay incubations was for 30 min at 37°C.

The assays with poly(A) · oligo(dT) were 30 m*M* Tris–HCl (pH 8.3 at 25°C), 2 m*M* DTT, 5% ethylene glycol, 80 m*M* KCl, 0.35 m*M* $MnCl_2$, 60 μg/ml poly(A) · oligo(dT) (P–L Biochemicals), 0.2 m*M* dATP, and 4 μ*M* dTTP plus 1–2 μCi of [^{3}H]dTTP. The standard assay incubation was for 30 min at 30°C.

The assays with poly(C) · oligo(dG) were 30 m*M* Tris–HCl (pH 8.0 at 40°C), 2 m*M* DTT, 5% ethylene glycol, 0.5 m*M* $MnCl_2$, 10 m*M* KCl, 60 μg/ml poly(C) · oligo(dG), 0.2 m*M* dCTP, and 4 μ*M* dGTP plus 5 μCi of [^{32}P]dGTP (580 Ci/mmole, New England Nuclear). The standard assay incubation was for 30 min at 40°C (10).

The assays of activity with endogenous templates were 30 m*M* Tris–HCl (pH 8.0 at 37°C), 2 m*M* DTT, 5% ethylene glycol, 0.35 m*M* $MnCl_2$, 10 m*M* KCl, 0.2 m*M* dATP, dCTP, and either 0.2 m*M* dGTP and 4 μ*M* dTTP plus 10 μCi of [^{3}H]dTTP or 0.2 m*M* dTTP and 4 μ*M* dGTP plus 5 μCi of [^{32}P]dGTP. The standard assay incubation was for 30 min at 37°C.

DNA products were collected directly into GFC papers (Whatman), acid precipitated, washed, and the radioactivity counted in a liquid scintillation counter as previously described (13). One unit of enzyme activity was equal to 1 fmole of dTTP incorporated in 30 min. Assay variability was less than 10% of

the mean for duplicates assayed consecutively over a period of 7 months.

Antigen–antibody studies. Goat antibody against RT purified from Rauscher Leukemia virus (RLV) was previously characterized (10). The anti-RT-RLV IgG, purified by chromatography on protein A Sepharose, was diluted with nonimmune goat IgG to maintain a constant level of total IgG in all assays which also contained 1 mg/ml bovine serum albumin. Antigen and antibody were allowed to react for 18 hr at 4°C in a total volume of 10 μl in a portion of the assay mixture (45 m*M* Tris–HCl, pH 8.3, 2 m*M* DTT, 120 m*M* KCl, 5–10% ethylene glycol, 2 mg/ml BSA, 0.1% NP-40). Residual enzyme activity was measured in the standard assay conditions following the addition of the remaining assay components in a final volume of 15 μl containing 30 m*M* Tris–HCl, pH 8.3, 80 m*M* KCl, 0.5 m*M* $MnCl_2$, 1.5 mg/ml BSA, 0.1 m*M* dATP, 10 μ*M* [^{3}H]dTTP (21 Ci/mMole), 70 μg/ml poly(A)·oligo(dT), 5% ethylene glycol, and 0.08% NP-40. The antibody titers obtained in this way were reproducible to ±15% over a period of several months with IgG preparations stored in 50% ethylene glycol at −20°C.

Results. *Source of the DNA polymerase activity in epididymal fluid.* Initial sperm purification procedures confirmed that the amount of enzyme activity with poly(A)·oligo(dT) remaining in supernatant fluid fractions could not be accounted for by residual sperm or sperm tails. Sperm lysis and release of mitochondrial enzyme into the supernatant fluids was a possibility, although somewhat unlikely in light of the report by Phillippe and Chevallier (3) of the difficulty encountered in solubilizing sperm DNA polymerases. However, to determine whether or not the DNA polymerase activity was being released from sperm in the buffer conditions employed, the integrity of the sperm membranes was assessed by fluorochromasia (7). Following exposure to DCFA, the head-to-tail fluorescence of ≧95% of sperm suspended in buffer A for up to 6 hr at room temperature indicated the sperm membranes were intact. Hence, the enzyme in supernatant epididymal fluid fractions could not be accounted for by sperm degradation.

DEAE–cellulose chromatography of the DNA polymerase activities (Fig. 1) provided additional information that the enzyme ac-

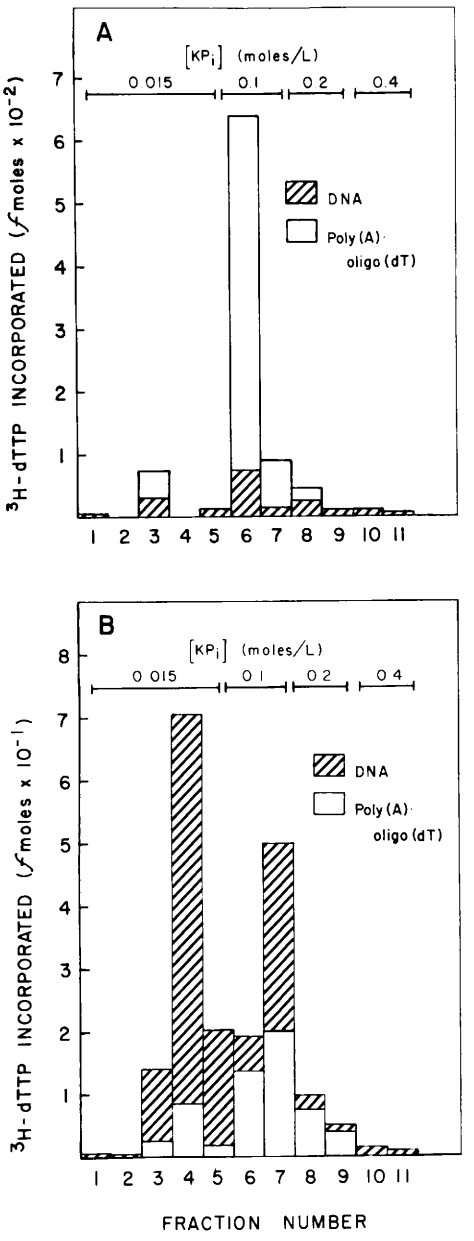

FIG. 1. DEAE–cellulose chromatography of DNA polymerases in NIH Swiss epididymal semen. Epididymal semen from 5–10 mature NIH Swiss males was pooled, separated into fluid and sperm fractions, treated with detergent and reducing agent, and chromatographed as described in Materials and Methods. Supernatant fluid enzyme was chromatographed on column A and pelleted sperm enzyme on column B. Two-microliter aliquots of each fraction (0.5 ml) were assayed for DNA-dependent and poly(A)·oligo(dT)-stimulated DNA polymerase activities. Essentially identical enzyme activity profiles were obtained by chromatography of similar samples from NZB, Balb/c and C57Bl/6 males.

tivity in epididymal supernatant fluid was not due to sperm lysis in the semen preparation. The supernatant fluid DNA polymerase (Fig. 1A) eluted as a single dominant peak of enzyme activity that was more stimulated by poly(A)·oligo(dT) than by DNA. In contrast, the DNA polymerase activities in the pelleted sperm (Fig. 1B) eluted as two dominant peaks that were each more active with DNA than with poly(A)·oligo(dT).

Optimal assay conditions for the epididymal fluid DNA polymerase activity. Mono- and divalent metal ion concentrations for maximal enzyme activity were determined for epididymal fluid DNA polymerase, before and after DEAE-cellulose chromatography, as previously described (10). Briefly, DNA synthesis was measured in each assay of a matrix of Mg^{2+} concentrations at different KCl concentrations with DNA template, and a similar matrix with varying Mn^{2+} and KCl concentrations with poly(A)·oligo(dT) template-primer. The enzyme activity in the epididymal fluid supernatant, and the enzyme activity eluted from the DEAE-cellulose column were maximally active at the same ion concentrations, namely, 10 m*M* Mg^{2+}, 20 m*M* KCl with DNA, and 0.5 m*M* Mn^{2+} and 80 m*M* KCl with poly(A)·oligo(dT). These optimal ion concentrations are the same as those for murine retrovirus RT (10), and unlike those for DNA polymerase γ (12). Optimal incubation temperatures were 37 and 25°C for the assays with poly(A)·oligo(dT) and DNA, respectively.

Buoyant density measurements of the DNA polymerase activity in epididymal fluid. Most of the poly(A)·oligo(dT)-stimulated DNA polymerase activity in the epididymal fluid from Swiss males banded at the buoyant density of retrovirus particles (Fig. 2A) in linear sucrose gradients (8). This was a somewhat surprising finding because of the relatively low incidence of tumors or virus expressed by NIH Swiss, and the males used in this study were members of an active breeding colony with no evidence of disease for their useful lifespan (up to 10–15 months of age). The DNA-dependent DNA polymerase activity at buoyant densities of 1.08–1.10 g/cc did not have the response to poly(A)·oligo(dT) characteristic of RT, and varied in amount relative to the particulate RT activity in each preparation.

In contrast to the Swiss, New Zealand Black (NZB) mice have a high incidence of spontaneous virus expression and the reproductive tract of males has been shown to contain "C"-

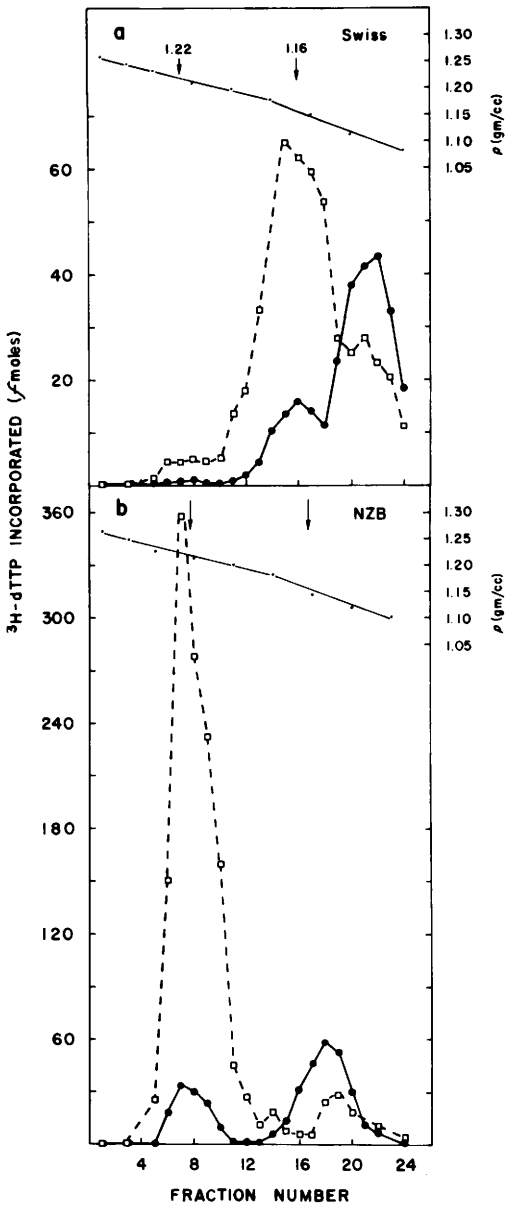

FIG. 2. Isopycnic banding in sucrose density gradients of epididymal fluid DNA polymerases from NIH Swiss and NZB males. Epididymal fluids from five to eight males were pooled and centrifuged to equilibrium as described in Materials and Methods. Three-microliter aliquots of each gradient fraction (0.2 ml) were adjusted to 0.25% NP-40, 5 m*M* DTT, and assayed for enzyme activity with poly(A)·oligo(dT) (□) or DNA (●).

type retrovirus (13). In keeping with these reports, the epididymal fluid from these males contained RT which banded in linear sucrose gradients at the buoyant density of retrovirus particles (Fig. 2B). The measured equilibrium density of 1.22 g/cc was more characteristic of retrovirus particle "cores" that have been freed of outer membrane envelopes. This finding is under further investigation, however, due to the fact that pretreatment with detergent was necessary for expression of peak NZB RT activity in the sucrose density gradients, a characteristic of whole, enveloped virus and unlike the detergent-independent expression of RT activity in virus "cores" (8).

Similar sucrose density gradient measurements of the DNA polymerases in epididymal fluids from Balb/c and C57Bl/6 males revealed multiple peaks of RT activity at buoyant densities below retrovirus particles, between 1.12 and 1.10 g/cc. The enzyme activity was always present in males of these two mouse strains, was stimulated by detergent and reducing agent, but the peaks of activity on the sucrose gradients were not reproducible (data not shown).

Assay characteristics of epididymal fluid DNA polymerases. The responses (Table I) of Swiss epididymal fluid enzyme activity in peak sucrose density gradient fractions confirmed the similarity to RT: the enzyme was more active with synthetic templates, more inhibited by phosphate ion and *N*-ethylmaleimide, and less inhibited by dideoxythymidine, than is characteristic of the DNA polymerase γ class of enzymes (12, 14). In addition, endogenous enzyme activity was detectable in the absence of added exogenous templates; the endogenous activity was not affected by actinomycin D, but was inhibited by ribonuclease, indicating the endogenous template was probably RNA (10). These assay characteristics are indistinguishable from those of murine retrovirus RT and essentially identical results were obtained with similar enzyme fractions from NZB males. C57Bl/6 and Balb/c enzyme responded similarly to the templates, DNA, poly(A) · oligo(dT) and poly(C) · oligo(dG), but activity on endogenous templates was not consistently observed (data not shown).

Molecular weight determinations. Molecular weights in the range of 65,000 to 75,000

TABLE I. ASSAY CHARACTERISTICS OF EPIDIDYMAL FLUID ENZYME

Assay condition[a]	DNA	Enzyme activity (units)[b]		
		Poly(A) · oligo(dT)	Poly(C) · oligo(dG)	Endogenous
Complete	62	202	113	16
−KCL	49	42	—	—
40 m*M* KCl	53	146	—	—
160 m*M* KCl	29	71	—	2
+10 m*M* *N*-ethylmaleimide	27	65	42	3
+25 m*M* ethidium bromide	58	62	—	—
+20 m*M* phosphate	41	124	—	—
+80 m*M* phosphate	23	41	—	—
+d_2TTP	30	61	—	4
−dATP, dCTP, dGTP	19	—	—	5
−oligo dT	—	12	—	—
+Actinomycin D	22	212	111	17
+Ribonuclease	64	—	—	6

[a] Assays were conducted as described in Materials and Methods. Optimal conditions of incubation temperature and assay ingredients were employed for each template-primer. Dideoxythymidine triphosphate (d2TTP) was added at a mole ratio of 1.5 that of the dTTP employed in the assay. Actinomycin D was added at a level of 100 μg/ml and ribonuclease at a level of 20 μg/ml.

[b] One enzyme unit is defined as 1 fmole of total nucleotide incorporated/μg of protein/30 min of incubation at the optimal temperature for the template-primer employed. Each value listed is the average of two to three determinations for each of three enzyme preparations. The standard error for each value was less than ±17% of the mean. The limit of assay sensitivity was 0.5 units, and the assay error in a standard enzyme preparation assayed as a control in each experiment was less than ±8% of the mean. The values reported were determined in the range of linear enzyme response. Enzyme activity in the absence of pretreatment with detergent and reducing agent was 7–33% of the activity with each template-primer tested. Enzyme preparations stored at −20°C exhibited no loss of activity for up to 9 months when adjusted to 50% ethylene glycol, 5 m*M* dithiothreitol (DTT).

Da (data not shown), in good agreement with the molecular weight of murine retrovirus RT (5), were estimated for enzyme from all mouse strains by gel filtration of enzyme fractions that had been partially purified by sucrose density gradients and DEAE chromatography.

Antigenic similarities of epididymal fluid RTs and Moloney murine leukemia virus. The effect of anti-RT antibody on the activity of enzymes from Swiss, NZB, and two retroviruses is shown in Fig. 3. The titration curves of antibody inhibition of the enzyme activity from the Swiss and NZB males were nearly identical to the inhibition curve of M-MuLV RT. The control avian myeloblastosis virus RT was not affected. Balb/c and C57Bl/6 enzyme was inhibited 72 and 66%, respectively, by 200 ng of anti-RLV IgG under the same assay conditions.

Reverse transcriptase activity in epididymal semen fractions from four mouse strains. The concentration of sperm and protein (2.5–3.8 mg protein/10^8 sperm) was comparable for epididymal fluid preparations from the four mouse strains examined (Table II). The level of enzyme activity varied between individual males, but the greater variation was between strains (Table II). Enzyme activity was consistently higher in the supernatant fluid fractions than in the sperm pellets, including the strains with relatively low concentrations of enzyme, Balb/c and C57Bl/6. Pretreatment of enzyme aliquots with detergent and reducing agent (Materials and Methods) was required for maximum expression of enzyme activity in all fractions.

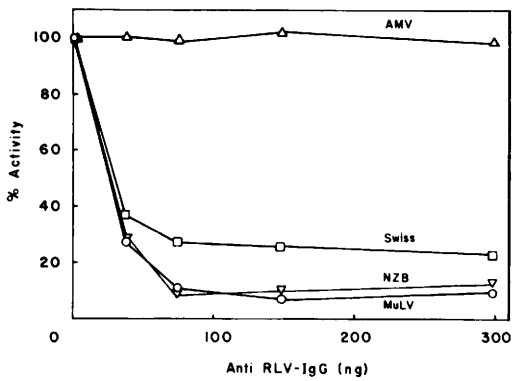

FIG. 3. Titration of epididymal fluid RT activity with goat IgG against RT purified from Rauscher Leukemia virus. Antigen (11 μg, Swiss; 14 μg, NZB; 4 μg MuLV; 8 μg AMV) and antibody were allowed to react overnight and residual enzyme activity assayed as described in Materials and Methods.

TABLE II. REVERSE TRANSCRIPTASE ACTIVITY IN EPIDIDYMAL FLUIDS AND SPERM PELLETS

Mouse strain	Enzyme activity (units)[a]	
	Fluid	Pellet
Balb/c	11 ± 1	10 ± 2
C57Bl/6	14 ± 2	10 ± 3
Swiss	189 ± 37	81 ± 24
NZB	354 ± 104	78 ± 17

[a] Enzyme units and assay conditions are as described in Table I with poly(A) · oligo(dT) template-primer. Values shown are averages of two to three determinations for each of 3–11 enzyme preparations. Enzymes assayed were aliquots of supernatant preparations solubilized with detergent, dithiothreitol, and ethylene glycol (Materials and Methods). Omitting the solubilization reduced measured enzyme activity to 5 to 16% in the Swiss and NZB preparations and 23 to 47% in the Balb/c and C57Bl/6 preparations.

Discussion. These studies provide evidence that RT is present in epididymal fluids of four mouse strains, including high (NZB) and low (C57Bl/6) spontaneous producers of retrovirus. Whether or not the RT is associated with retrovirus remains to be determined, although the known occurrence of C-type retrovirus in NZB males (13) is assumed to account for at least part of the RT activity observed in this strain. The concentration of RT activity in the NZB and Swiss males was equivalent to 10^{11} to 10^{12} virus particles/ml (12) of epididymal fluid, a remarkably high titer for nonmoribund animals. Two possible explanations are (i) the RT is present independent of virus and/or (ii) the RT is associated with endogenous xenotropic virus. The sucrose density gradient profiles of enzyme activity from the Balb/c and C57Bl/6 males support the former possibility as does the work on glycoprotein Gp70, another retrovirus protein known to be present in the male reproductive tract of mice (15, 16). Reports of endogenous xenotropic C-type retrovirus in mouse uteri, particularly NIH Swiss females (17), provides support for the latter possibility, that of xenotropic virus expression in male reproductive tracts. The two possibilities are not mutually exclusive. RT could be present in both viral and nonviral forms simultaneously in the epididymal lumen.

In the context of endogenous virus expression, it is interesting to note that the two strains in this study with the highest level of RT activity in epididymal fluid, NZB and Swiss, are known to express only type III endogenous C-type virus. Proteins of the type III class of virus are expressed in embryo cells of several mouse strains (18). The presence of endogenous virus in both male and female reproductive tracts as well as developing embryos is consistent with the suggestion that the viruses have a normal biological role during embryonic development (19–21). Such circumstantial evidence does not, however, rule out the possibility that virus expression accompanies, but does not influence, cell differentiation.

The contention that reverse transcription of RNA to DNA is a common event in cells of higher organisms is gaining in credibility (22). Whether the process plays a role in cell commitment during early embryogenesis, as originally postulated by Temin (20), and/or serves other roles in cells (23) is under investigation. Although there is evidence that RT is present in normal cells, such as human (8), chick embryo (24), early cleavage stage mouse embryo (25), rabbit uteri (26), and mouse uteri and placenta (27, 28), no unequivocal demonstration of reverse transcription or RT has been associated with normal cell events.

A DNA polymerase with some characteristics of RT was detected in human seminal fluid and sperm (29, 30). The RT activity in epididymal fluids described here is an indication that RT may be associated with the male gametes of mice. Additional information on the presence of virus particles and the site of synthesis of the RT-like enzyme activity present in epididymal fluids is necessary to understand the role of the enzyme and any associated virus in the male reproductive tract of mice. Preliminary findings of electromicrographic studies in progress indicate the presence of retrovirus particles in Swiss and NZB males, but not in the C57Bl/6 males.

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