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Epididymis is a principal site of retrovirus expression in the mouse

ANN ANDERSON KIESSLING*†, RICHARD CROWELL*, AND CECIL FOX‡

*Department of Obstetrics, Gynecology and Reproductive Biology, and Laboratory for Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, MA 02115; and ‡National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

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ABSTRACT **High levels of retrovirus particles are present in the reproductive tract of male mice. In this report epithelial cells that line the lumen of the epididymis are shown to be a principal site of virus synthesis. Aggregates of free virus were evident in the epididymal lumen in addition to the sperm-associated virus previously reported. Large intraluminal cells with characteristics of macrophages and engorged with virus particles were also seen. Virus particles were not detected in testis, liver, brain, or spleen. Thus, the epididymal epithelium is a principal reservoir for retrovirus expression. The virus would be ejaculated as free, cell-associated, and sperm-bound particles. The high level of expression and the relative isolation of epididymal virus from the immune system may relate to venereal transmission of retrovirus infections in mice and humans.**

Retrovirus particles are present at high concentrations (10^5–10^8 particles per μg of protein) in the epididymal fluids of several strains of mice (1, 2). The significance of the reproductive tract retroviruses to the host male or to sexual partners exposed to ejaculated epididymal viruses is unknown. The venereal transmissibility of infectious human retroviruses, such as human immunodeficiency virus, has created an urgent need to fully understand retrovirus interactions with reproductive tract cells. The high levels of virus in the mouse epididymis provides a sensitive model for studies of expression and transmission of reproductive-tract retroviruses.

The ubiquity of the epididymal virus suggests that in most cases this occurrence is probably due to the expression of endogenous provirus(es) (for review of endogenous viruses, see ref. 3). However, evidence has been presented that the epididymis may also be a site of expression of exogenous, infectious retroviruses (4). This evidence supports the possibility that the epididymis may be a generally permissive site for retrovirus synthesis. Epididymal fluids could thus be an important vehicle for virus transmission. The association of epididymal virus particles with sperm (2) provides a route of virus exposure to cells not in direct contact with semen, such as the upper regions of the female reproductive tract, including oocytes. This possible exposure increases the likelihood of venereal and congenital infection. Infection of oocytes or early-cleavage stage embryos could lead to new germ-line insertions of the epididymal provirus. Such newly inserted provirus could then be expressed at high levels in the epididymis. The potential impact of this latter possibility on human immunodeficiency virus expression in the human population underscores the importance of investigating reproductive-tract retroviruses.

To further understand the murine epididymal viruses, we undertook studies to determine the source of the epididymal virus particles and to compare virus expression in reproductive-tract cells with that in somatic cells. The results reported here show that the specialized epithelial cells that line the lumen of the epididymis are a principal site of synthesis of retrovirus particles.

MATERIALS AND METHODS

Mouse Strains and Castration Experiments. Mature males were from virus-antibody free (VAF) stocks. Strains NZB [previously shown to have high concentrations of epididymal retrovirus (1, 2)] and CBA (breeding colony males) were from The Jackson Laboratories. Strain CD1 males (also from the breeding colony) were from Charles River Breeding Laboratories. For the castration experiments, CBA males were anesthetized and castrated unilaterally to deprive one epididymis of sperm but not testosterone; a suture was applied to tag the castrate epididymis. Six weeks later the castrate and intact epididymides were removed, and epididymal semen from the respective sides were pooled for assay of reverse transcriptase activity as described (1, 2). Seminal vesicles were removed, weighed, and compared with those from three normal males as a measure of testosterone levels.

Immunoblots. Tissues from three animals were pooled and homogenized in buffer A (0.01 M Tris·HCl, pH 7.4/0.1 M KCl/bovine serum albumin at 100 μg/ml) supplemented with the protease inhibitors leupeptin and soybean trypsin inhibitor at 100 μg/ml. The homogenates were centrifuged at 10,000 × *g* for 5 min to remove debris, and the supernatant fluids were assayed for protein as described (1, 2). Protein denaturation, electrophoresis, transfer to nitrocellulose, incubation with antibodies, and enzyme conjugates to develop the blot were as described (2).

Electron Microscopy. Tissues were fixed, embedded, sectioned, and stained for electron microscopy as described (2). Some of the tissues examined ultrastructurally were the same as those used for *in situ* hybridization.

***In Situ* Hybridization.** Tissues from NZB and CD1 males were dissected and transferred immediately to freshly prepared tissue fixative [2% (wt/vol) paraformaldehyde/1% glutaraldehyde in phosphate-buffered saline], minced, and, after 1-hr fixation, transferred to phosphate-buffered saline before routine dehydration and paraffin embedding. Tissue sections were collected onto prepared glass slides, and alternate slides were hybridized with either the positive- or negative-sense RNA probes. To prepare the template for RNA probe synthesis, a *Kpn* I fragment from the *pol* region of pMov9 murine leukemia virus provirus (5) was inserted into the *Kpn* I site of the multiple cloning-site region of pGEM-4 plasmid between the T7 and SP6 RNA polymerase promoters. The orientation of the fragment was such that linearization of the construct with *Eco*RI for the T7 promoter yielded antisense RNA strand relative to the retroviral RNA, and linearization with *Pst* I for the SP6 promoter yielded a sense RNA strand. Thus, the T7-synthesized strand positively hybridized to intracellular viral RNA, whereas the SP6-synthesized strand provided the negative experimental control. The ^{35}S-labeled T7 or SP6 RNAs were treated with base and RNA strands in the range of 10^2–10^3 bases selected

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†To whom reprint requests should be addressed at: Harvard Medical School, 250 Longwood Avenue, Room 204, Boston, MA 02115.

for use as probes. The slides were hybridized and washed at 45°C and coated with radiographic emulsion. After a 3-day exposure, the emulsion was developed, and the slides were counterstained with eosin.

RESULTS

Influence of Sperm Production on the Presence of Virus in the Epididymis. The reproductive-tract tissues investigated as potential sources of retrovirus synthesis were testis, epididymis, and ductus deferens. Spermatozoa exit the testis through the ductus efferens and enter the epididymis (Fig. 1). Specialized epithelial cells line the tubules of the epididymis (6, 7); lymphocytes and macrophages are found in the lumen and within the epididymal epithelium of several mammals, including humans (8–10). During transit through the three regions of the epididymis (caput, corpus, and cauda) the spermatozoa mature into motile, fertile sperm (refs. 11 and 12; for review of epididymis and functions, see refs. 13 and 14). Sperm are concentrated and stored in the cauda epididymis to be released with epididymal semen by smooth muscle contraction into the ductus deferens at ejaculation.

Because of the sperm–virus association described (2), we undertook studies to determine whether virus was synthesized in the testis and whether sperm was necessary to transport the virus to the cauda epididymis. Mature CBA males were unilaterally castrated to deprive one epididymis of sperm but not of testosterone, which would be supplied by the contralateral testis. Six weeks after castration, the sperm concentration decreased by three orders of magnitude in the epididymis of the castrate side. However, no decrease in the amount of reverse transcriptase activity was detected in the fluids from the castrate epididymides of these animals (Table 1). These results ruled out the testis as a significant source of virus, as well as any necessity for sperm to transport the virus to the cauda epididymis and ductus deferens.

Relative Levels of Virus Synthesis in Reproductive and Somatic Tissues. To compare virus expression in the reproductive tract with that in somatic tissues, proteins from the testis, each region of the epididymis, and the ductus deferens were compared with proteins from spleen, liver, and brain by immunoblot analyses with antibody directed against murine leukemia virus p30 core protein (Fig. 2). Seroreactive viral p30 protein was readily detectable in the epididymis and ductus deferens (Fig. 2, lanes 2–5). Two other proteins from the epididymis and ductus deferens that were recognized by the anti-p30 antibody (the protein bands of ≈65 kDa and ≈55 kDa) correspond to the reported molecular masses of uncleaved gag precursor protein and the gag precursor core protein after cleavage of the carboxyl-terminal 10-kDa protein (15, 16). A 65-kDa band was also detected in the spleen, testis, liver, and brain, and a trace 30-kDa band was detected in the spleen, but the major viral core protein bands present in the epididymis and ductus deferens were absent in the somatic tissues. The 65-kDa protein was not detected in previous studies of viral proteins in cell-free epididymal fluids (2). Although preabsorption of the anti-p30 antibody with testis proteins removes the 65-kDa band from the somatic tissue lanes (data not shown) without altering the epididymal viral bands, it is still not clear whether the 65-kDa band represents a proviral-encoded protein present at low levels in all tissues or a cross-reacting cellular protein. The lanes containing NZB virus as controls also exhibited multiple protein bands, in contrast to the single p30 band of Rauscher leukemia virus (2). This result may represent inclusion of viral protein precursors into the NZB virus or expression of multiple proviruses by the NZB-producing cell line (17).

Table 1. Reverse transcriptase activity and sperm concentration in epididymal fluids of intact and unilaterally castrated male mice

	Control	Unilateral castrate	
		Intact	Castrate
Sperm per ml	8×10^7	6×10^7	5×10^4
Reverse transcriptase activity*	24	32	56
Seminal vesicles, mg	92	121	

Seminal vesicle weights were measured to verify testosterone production by the remaining testis in the unilateral castrates. Reverse transcriptase activity is in fmol of [^{3}H]dTMP incorporated into acid-precipitable product per μg of protein with poly(A)·oligo(dT) template primer, as described (1).

*The threshold level of reverse transcriptase detection in this assay system was 4 fmol/μg per 30 min; assay background subtracted was acid-precipitable counts in the presence of antibody against murine leukemia virus reverse transcriptase (1).

Indirect immunofluorescence of frozen sections of reproductive tract and somatic tissues with the antibody against the p30 core protein revealed bright staining of the lumen and

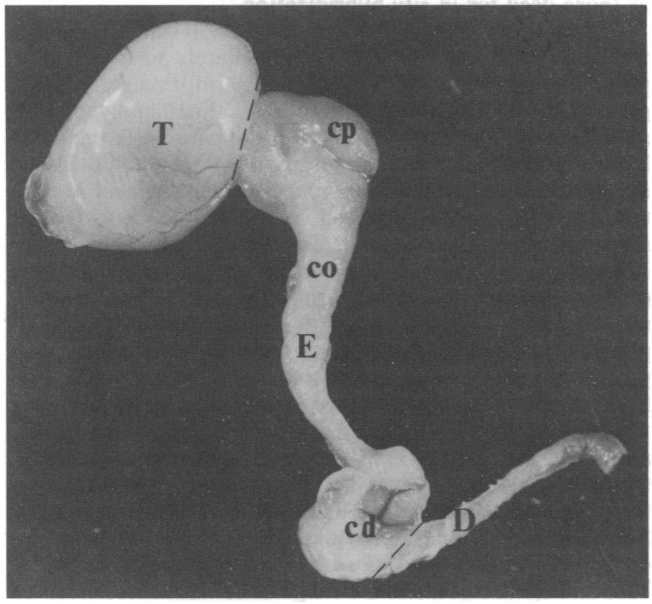

FIG. 1. Reproductive tract tissues of the male mouse. Testes (T), epididymis (E), ductus (vas) deferens (D); regions of the epididymis: caput (cp), corpus (co), cauda (cd). (×8.)

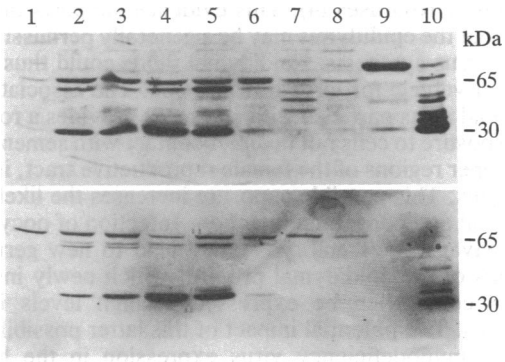

FIG. 2. Immunoblot analyses of tissue proteins from NZB and Swiss males. Tissues were from two males of each strain. Twenty micrograms of total protein was electrophoresed, transferred, and immunoblotted with anti-Rauscher leukemia virus p30 antibody (Microbiological Associates) as described. Lanes: 1, liver; 2, caput epididymis; 3, corpus epididymis; 4, cauda epididymis; 5, ductus deferens; 6, spleen; 7, liver; 8, brain; 9, molecular mass markers; 10, NZB virus (1 μg of protein). (*Upper*) Proteins from Swiss (CD1) males. (*Lower*) Proteins from NZB males. Molecular-mass estimates are based on the molecular masses of the colored marker proteins fructose phosphate kinase (86 kDa), bovine serum albumin (66 kDa), and lactatedehydrogenase (36 kDa).

lining of the epididymis, no detectable virus protein staining in the testis, and patchy staining in the spleen (data not shown). These results supported the evidence obtained from the immunoblots that virus protein concentration is highest in the epididymis and ductus deferens and minimal or nonexistent in the testis and somatic tissues. These observations agree also with the detection of reverse transcriptase activity in fluids from all three regions of the epididymis and the ductus deferens (data not shown).

Localization of Virus-Producing Cells. To determine which cell in the epididymis synthesized the virus particles, viral RNA expression was examined by hybridization *in situ* with RNA probes to the *pol* region of murine leukemia viruses. Hybridization of antisense viral probe was intense in the epididymal epithelial cells of the cauda epididymis (Fig. 3*a*), undetectable in cells of testes (Fig. 3*c*), and localized to patchy areas of the spleen (Fig. 3*e*). The same tissue specificity of hybridization patterns were seen with both NZB (Fig. 3 *a–f*) and Swiss (Fig. 3*g*; spleen and testis data not shown) males. These results demonstrated that the epithelial cells lining the lumen of the epididymis are a principal site of virus synthesis. Virus RNA was not detected in the testis. The patchy areas of the spleen that hybridized to the RNA probe are consistent with the low level of detection of virus p30 in the immunoblot analyses of spleen proteins.

To verify virus particle synthesis by the epididymal cells and to determine whether the evidence of virus in the spleen was due to virus production by spleen cells or to the presence in the spleen of virus-laden cells from the epididymis, we further investigated virus particle expression by ultrastructural analyses. Electron microscopy of sections of the cauda epididymis revealed aggregates of virus within the epididy-

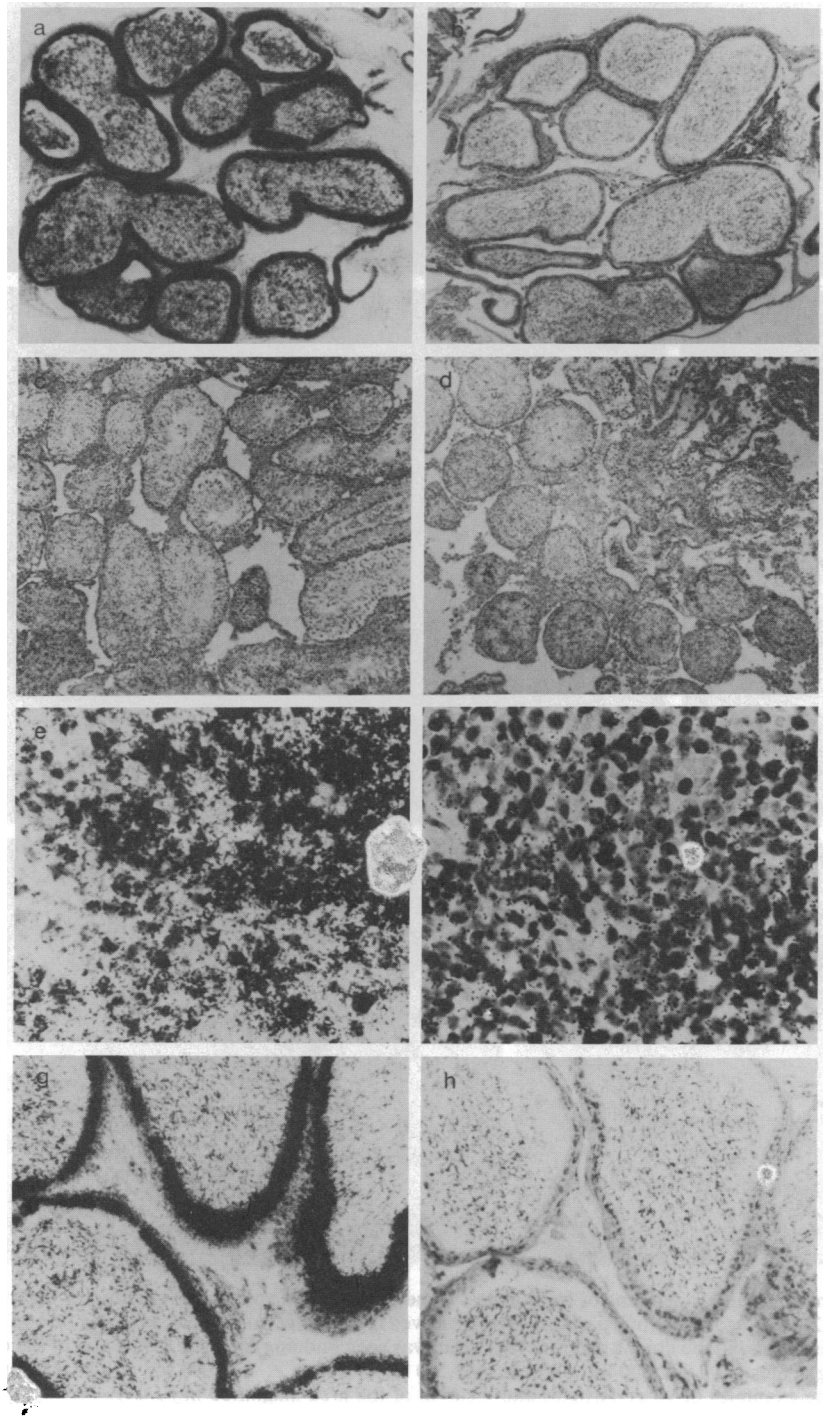

FIG. 3. *In situ* hybridization of retroviral RNA in reproductive and somatic tissues of male mice. NZB cauda epididymis (*a* and *b*), testis (*c* and *d*), and spleen (*e* and *f*) were hybridized to RNA probes synthesized by T7 RNA polymerase (positive) (*a*, *c*, and *e*) or SP6 RNA polymerase (negative) (*b*, *d*, and *f*). (×104.). The hybridization scheme was repeated with CD1 cauda epididymis (positive) (*g*) and negative (*h*). (×400.)

mal lumen (Fig. 4*a*), clustered around sperm heads (Fig. 4*b*), associated with sperm in contact with cells or cell processes (Fig. 4*c*), and concentrated within structures with characteristics of lymphocytes or macrophages (8–10) (Fig. 4*d*). Cell-associated and free virus clusters were also present in the ductus deferens (Fig. 4*e*). Budding virus, evidence for viral synthesis, was observed rarely in the epithelial cells lining the cauda epididymis and the ductus deferens. In contrast, ultrastructural studies of the upper regions of the epididymis revealed numerous virus particles budding from the epithelial cells of the corpus epididymis (Fig. 4*f*), particularly from the stereocilia of the epididymal cells (Fig. 4*f*, *Inset*). Virus particle aggregates in a rosette around an amorphous material were common in the lumen of the corpus epididymis (Fig. 4*g*). No particles were seen budding from the membranes of the intraluminal cells encasing the large aggregates of virus. Virus particles were not detected in sections of the testis or spleen of either strain of mouse.

DISCUSSION

These results show that the epithelial cells of the epididymis are a principal site of retrovirus synthesis. The high levels of

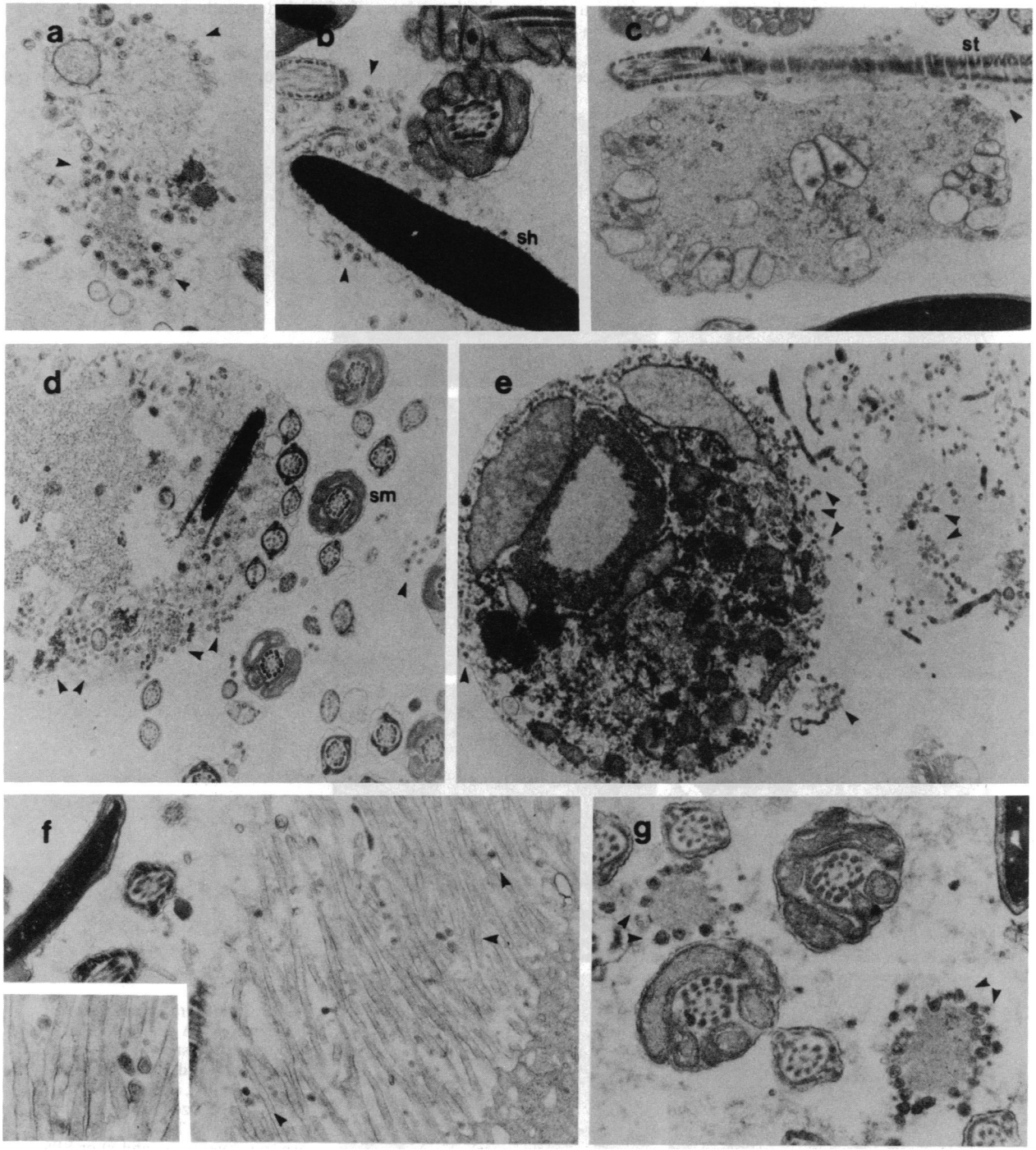

FIG. 4. Retrovirus particles in the epididymis and ductus deferens of NZB males. Cauda and corpus epididymis and ductus deferens were prepared for electron microscopy as described (2). Aggregates of virus particles were observed free (*a*), sperm-associated (*b* and *c*), and enclosed within intraluminal cells in the cauda epididymis (*d*) and in the ductus deferens (*e*). Virus was seen budding from the stereocilia of the epithelial cells of corpus epididymis (*f*) and as virus aggregates (*g*). (×22,500 for *a* and *b*; ×13,500 for *c*, *d*, and *e*; ×18,000 for *f*; ×36,000 for *f inset*; and ×27,000 for *g*.) Small arrowheads designate examples of virus particles and location of the area magnified in *f Inset*.

viral RNA detected by hybridization *in situ* and the large aggregates of virus visualized ultrastructurally agree with the previously reported estimates of 10^{11}–10^{12} virus particles per ml of epididymal fluid (1, 2). These estimates are further supported by reports several years ago that, although attributed to partial proviral expression instead of virus-particle production, retroviral *env* glycoprotein (Gp70) accounted for 10% of the epididymal protein in NZB males (18, 19). This profound tissue-specific expression of retroviruses in male reproductive tract cells has several important implications.

The first implication relates to the spread of retroviruses. The male reproductive tract is protected from the immune surveillance operational in somatic tissues (20). Exogenous retroviruses could be chronically expressed in the epididymis after infection. That this occurs in male mice infected as neonates with a neurotropic retrovirus has been reported (4). Epididymal retroviruses would be disseminated to other male tissues by virus-laden macrophages or lymphocytes by means of the major lymphatics that drain the epididymis (21, 22). This fact may account for the patchy areas of viral RNA detected in the spleen in the *in situ* hybridization studies and the traces of viral proteins detected in the spleen by the immunoblot analyses. This possibility is supported by the fact that the spleens of both NZB, a chronically viremic strain, and CD1, a nonviremic Swiss strain, exhibited similar evidence of viral presence.

Epididymal viruses could also be spread by ejaculation. Sperm transit time through the epididymis during maturation affords ample exposure to virus particles for absorption (2). The presence of specific virus receptors on the surface of sperm has not been demonstrated, but the persistence of the binding (2) and the suggestion that sperm–cell interaction may be mediated by cell virus receptors (23) indicate that specific virus–sperm interaction is possible. Our results show that epididymal viruses would be ejaculated as free particles as well as cell- and sperm-associated particles. That human retroviruses occur in seminal fluids as free, as well as cell-associated, particles has recently been discussed (24). Such diverse presentation of virus to a host increases the likelihood of successful infection. Female exposure to the retroviruses would occur at copulation and would include even the upper regions of the female reproductive tract contacted by sperm-bound virus, including oocytes. Horizontal, possibly venereal, spread of murine retroviruses has been reported (4, 25, 26).

A second consideration relates to the tissue-specificity of the proviral expression. Perhaps the high level of virus expression in the epididymis relates to the androgen influence on epididymal cells, which synthesize specific classes of proteins in response to testosterone stimulation (27, 28). The absence of virus expression in the testis suggests, however, that if androgens are involved, their influence is mediated through tissue-specific regulation. Understanding epididymal viral expression and its regulation may provide information important to designing therapies to control retroviral expression and spread. It is also possible that the epididymal epithelial cells could simultaneously express more than one retrovirus—e.g., exogenous as well as endogenous viruses. In this event, recombination between the viral genomes is likely, resulting in recombinant viruses with potentially increased virulence. On the other hand, endogenous provirus expression could confer resistance to superinfection by exogenous viruses. This possibility has been suggested to explain the resistance of AKR strain females to venereal infection by neurotropic virus (4).

Oocyte exposure to virus is a third important consideration. The oocyte could support provirus synthesis after fertilization, thus affording an opportunity for new chromosomal integrations to occur. The consequences to the embryo of proviral integration are difficult to predict but probably range from embryonic demise to stable chromosomal, possibly germ-line, insertion. Stable chromosomal insertion of infectious provirus could lead to constitutive expression in the epididymis. This fact has profound implications with respect to infectious human retroviruses. For example, human immunodeficiency virus or human T-cell leukemia type I infection of human oocytes at fertilization could result in similar chromosomal integrations of provirus(es). Individuals arising from such a conception could have the potential for chronic epididymal expression of the virus. This viral expression would not only increase the expression of infectious retroviruses in the human population but also provide an opportunity for recombination between old and new proviruses. All of these possibilities underscore the importance of further studies on the interactions between retroviruses and reproductive tract cells.

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