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# Pattern of Expression of Ecotropic Murine Leukemia Virus in Gonads of Inoculated SWR/J Mice

JEAN-JACQUES PANTHIER,[1]* PIERRE GOUNON,[2] HUBERT CONDAMINE,[1] AND FRANÇOIS JACOB[1]

*Unité de Génétique Cellulaire du Collège de France et de l'Institut Pasteur, Unité Associée 1148 du Centre National de la Recherche Scientifique, Groupe de Génétique du Développement de l'Université Paris 7,[1] Station Centrale de Microscopie Electronique,[2] Institut Pasteur, 25 rue du Docteur Roux, 75724, Paris Cedex 15, France*

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**An ecotropic murine leukemia virus (MuLV) isolate has recently been shown to be able to infect the germ line or the early embryo or both when inoculated at birth to SWR/J females (J. J. Panthier, H. Condamine, and F. Jacob, Proc. Natl. Acad. Sci. USA 85:1156–1160, 1988). We have used this isolate to further study this phenomenon. By using the techniques of RNA-RNA in situ hybridization, immunocytochemistry, and transmission electron microscopy, the identities of two important cell types that are infected by ecotropic MuLV in the gonads of inoculated mice were determined. These cells are the thecal cells surrounding the follicles in the ovary and the Leydig cells in the testis. Both types actively synthesize viral RNA and express a viral antigen. Furthermore, we documented the production of viral particles by the thecal cells. The expression of ecotropic MuLV by nonlymphoid cells in vivo may play a key role in the vertical transmission of these viruses by females as well as in their horizontal transmission.**

DNA copies of the RNA genomes of ecotropic murine leukemia viruses (MuLVs) are present in the chromosomes of most inbred strains of mice (20). These endogenous proviruses are stably transmitted from one generation to the next as classical Mendelian loci (35), and the distribution of these viral DNA sequences suggests that in general, viral DNA integration preceded the establishment of inbred strains of mice (20). However, further acquisition of endogenous ecotropic MuLV DNA sequences has been documented repeatedly (i) during the breeding of one low- and two high-ecotropic-virus producing strains (6, 14, 22, 25, 34, 35) and (ii) during the obtention of two AKR-derived recombinant inbred strains (19, 38). Furthermore, Jenkins and Copeland (18) have reported the high-frequency germ line integration of new ecotropic proviruses in SWR/J-RF/J hybrid mice.

As first noted by Rowe and Kozak (35), proviral acquisitions seem to be specifically associated with viremic females, since no new proviruses are observed in the progeny of nonviremic females even after mating with viremic males. The same conclusion was drawn from the studies made on SWR/J-RF/J hybrid mice: the only crosses productive for new proviral loci involve female virus carriers (2, 18).

Various observations suggest that such acquisitions result from the infection of germ cell precursors or early embryos or both rather than from mere intracellular transposition of proviral sequences. (i) As noted above, the acquisition occurs in viremic mice. (ii) Provirus integration is not observed in SWR/J-RF/J hybrid females that are nonpermissive, at the Fv-1 locus, for the expression of ecotropic MuLVs (18). (iii) Provirus acquisition correlates with the presence of ecotropic MuLV RNA in the ovaries of SWR/J-RF/J mice (28). Furthermore, we have recently shown that SWR/J females inoculated at birth with an ecotropic MuLV derived from an SWR/J-RF/J hybrid female have progeny with new endogenous ecotropic proviruses, thus demonstrating that infection of the germ line by an exogenous virus may occur in vivo (29). Since the efficiency of this infection process is rather high, i.e. about 10% of the progeny of inoculated SWR/J females carry new viral insertions, we anticipated that this experimental system might provide a good model to study the germ line integration of the RNA genomes of exogenous retroviruses in mammals.

Although previous studies have dealt with the presence of either an ecotropic MuLV antigen (23) or infectious ecotropic MuLVs (36) in murine tissues, little is known about the infection of the gonads by ecotropic MuLVs. As a first step to understanding the mechanism of new proviral integration, we have undertaken a study of MuLV particle production in the gonads. It is known that transcription of ecotropic sequences occurs in the ovaries of inoculated mice (29). Here we show further that viral RNAs are translated, leading to the production of viral ecotropic particles in the ovaries of the inoculated SWR/J females. We also show that upon inoculation at birth ecotropic MuLVs are expressed in the gonads of SWR/J males. In both cases, MuLV expression occurs selectively in specialized cell subpopulations.

## MATERIALS AND METHODS

**Mice.** SWR/J inbred mice, which do not carry endogenous ecotropic MuLV proviruses (20), were obtained from the Jackson Laboratory (February 1985) and were bred at the Institut Pasteur thereafter.

**Virus.** SWR-RF MuLV, isolated from an SWR/J-RF/J hybrid female, was propagated in a simian virus 40-transformed SWR/J cell line as previously described (29). Cell-free viral stocks were prepared by filtration of subconfluent infected cell culture supernatant through a Millipore membrane (0.22 μm). SWR/J mice (age, one-half day) were inoculated intraperitoneally with 0.1 ml of viral stock (about $2 \times 10^5$ focus-forming units per ml as determined by the $S^+L^-$ assay [41]).

**Antisera.** Goat antiserum to Rauscher MuLV gp70 antigen was obtained through the Office of Program Resources and Logistics, Viral Oncology Division, National Cancer Institute, Bethesda, Md. (a gift of F. Plata, Institut Pasteur, Paris, France). Fluorescein isothiocyanate and rhodamine-

* Corresponding author.

labeled rabbit anti-goat immunoglobulin sera were purchased from Biosys and Cappel Laboratories, respectively. For electron microscopy, peroxidase-labeled anti-goat immunoglobulin serum (Institut Pasteur Production) was used.

**Histological preparations.** Ovaries and testes were excised from 4- to 6-month-old SWR-RF MuLV-inoculated SWR/J mice and fixed immediately. For in situ hybridization, gonads were fixed in 3% paraformaldehyde (PFA) in 1× phosphate-buffered saline (PBS) at 4°C for 20 h. For indirect immunofluorescence, ovaries were fixed in 3% PFA in 1× PBS at 20°C for 30 min, while testes were fixed in 3% PFA–0.25% glutaraldehyde in 1× PBS at 20°C for 3 h. Frozen sections of 5- to 7-μm thickness were cut and stuck onto gelatin-coated slides.

**In situ hybridization.** RNA-RNA in situ hybridization experiments were performed using the 330-base-pair *Sma*I fragment specific for ecotropic MuLV DNA sequences (10) to synthesize an RNA probe under previously described conditions (28).

**Immunodetection procedure.** The cryostat sections were sequentially incubated in a humid box with drops of the following antisera: goat anti-gp70 serum diluted 1:100 in 1× PBS added with 0.2% gelatin (30 min at 20°C) and rabbit anti-goat immunoglobulin antibodies coupled with either fluorescein isothiocyanate or rhodamine diluted 1:50 in 1× PBS added with 0.2% gelatin (20 min at 20°C). After each serum incubation, the sections were washed in three changes of 1× PBS added with 0.2% gelatin over a period of 5 min. The sections were mounted in Mowiol 4-88 (Hoechst) for examination in light microscopy. Sections of ovaries and testes from uninoculated SWR/J mice were included in each experiment as negative controls. The use of either conjugate without the goat serum gave no detectable tissue staining.

**Electron microscopy. (i) Detection of virus particles in the gonads of inoculated mice.** Small pieces of gonads were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 2 h at room temperature. The samples were postfixed for 1 h with 1% osmium tetroxide ($OsO_4$) in cacodylate buffer and then stained en bloc with 1% uranyl acetate in 50% ethanol for 30 min, washed in 50% ethanol, and further processed for embedding in Epon. Thin sections were conventionally stained and observed with a Philips CM12 electron microscope.

**(ii) Immunodetection of ecotropic MuLV gp70 antigen.** After fixation (see above), the samples were infiltrated overnight with sucrose (0.5 M in 1× PBS). Cryostat sections 15 to 50 μm thick were made, adhered to a small strip of gelatinized Terphane, and air dried. The sections attached to the Terphane sheet were rehydrated in small tubes with 1× PBS added with 0.2% gelatin and processed for immunocytochemistry as described above. Peroxidase activity was detected with 0.05% diaminobenzidine (DAB) in 0.05 M Tris hydrochloride–10 mM imidazole–0.03% hydrogen peroxide (pH 7.4) for 5 min (39). This method was used in order to increase the sensitivity of the cytochemical reaction of peroxidase at neutral pH. The method leads to well-limited and contrasted deposits of black DAB (39). The sections were then fixed with glutaraldehyde (2.5% in 1× PBS, 5 min), postfixed with $OsO_4$ (1% in 1× PBS, 10 min). The sections were embedded by simply pouring an inverted gelatin capsule filled with Araldite over the sections that were stuck to the Terphane sheet at the end of the dehydration process. Thin sections were observed unstained.

**Detection of ecotropic MuLV in mouse sera and epididymal semen.** Blood samples from inoculated SWR/J mice were collected by puncture at the retro-orbital sinus. Semen was collected from the cauda epididymis and ductus deferens and diluted in 1× PBS. Serial dilutions of serum or of semen were analyzed by the $S^+L^-$ test (41) as previously described (29).

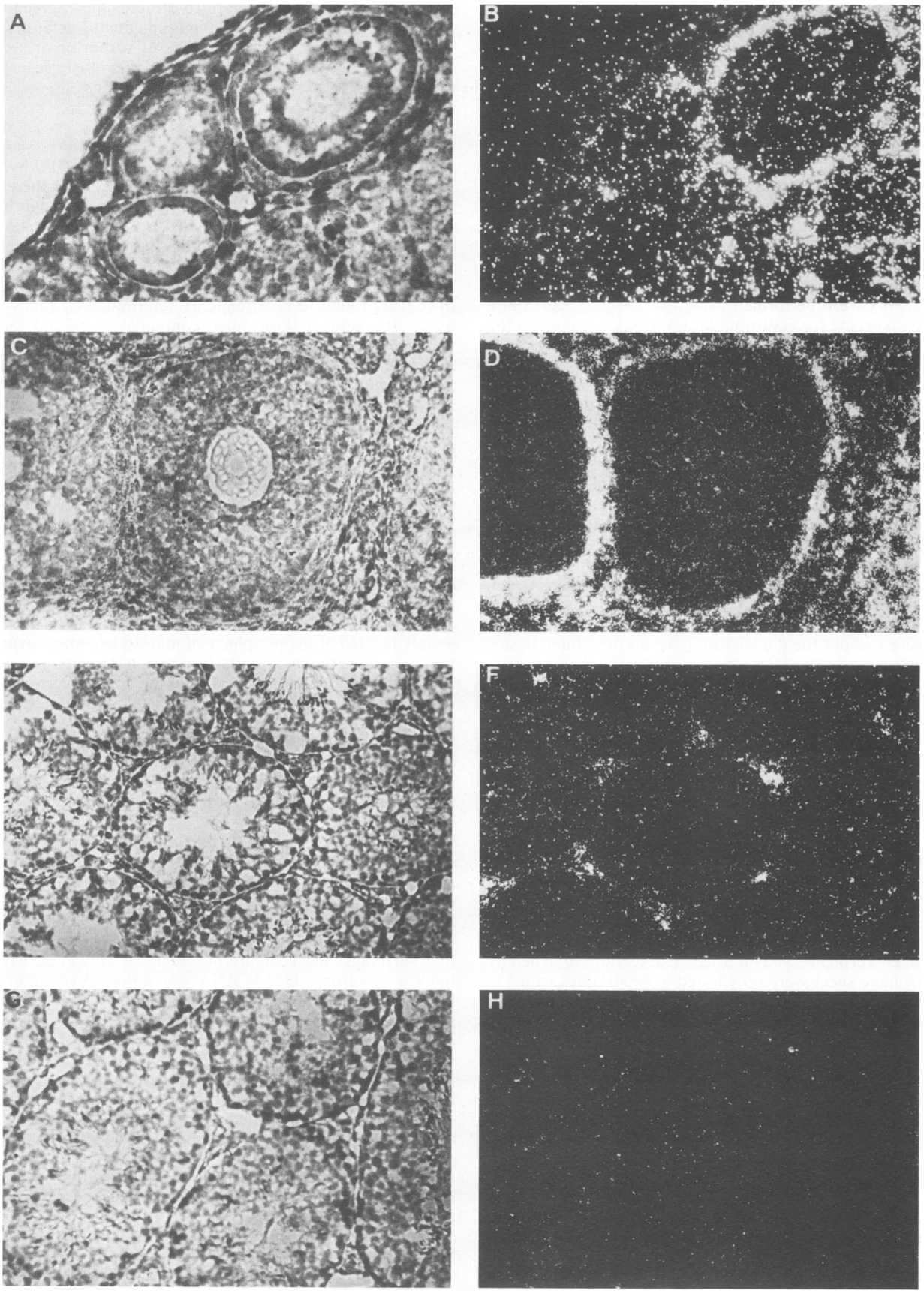
## RESULTS

An ecotropic MuLV, isolated from an SWR/J-RF/J hybrid female, has been shown to infect the germ cells or the early embryos or both of SWR/J females when inoculated at birth (29). We have used this virus isolate, named SWR-RF MuLV, to inoculate 10 newborn SWR/J females and males. Two months later, blood samples were taken from these mice and the sera were analyzed for their viral content. All mice were found to be viremic as determined by the $S^+L^-$ assay: the sera of these mice contained 10 to 200 focus-forming units per ml.

**Detection of ecotropic MuLV RNA in gonads of inoculated SWR/J mice.** Two females and two males were sacrificed, and RNA-RNA in situ hybridization was used to look for possible ecotropic MuLV viral RNA expression in the gonads from these inoculated mice. Frozen tissue sections of ovaries and testes were prepared and hybridized with a radioactive ecotropic MuLV-specific probe complementary to 330 nucleotides of the ecotropic MuLV RNA (10). The presence of viral RNA was revealed in both male and female gonads by silver grains overlying some of the gonadal cells. Preparations treated with RNase prior to hybridization with the probe, as well as ovary and testis sections from uninoculated SWR/J mice, failed to react (Fig. 1G and H). As previously observed (29), the grains in the ovaries were mainly located at the periphery of mid- to late-stage ovarian follicles on the highly vascularized region that composes the theca. Neither the follicular cells nor the oocyte were labeled (Fig. 1A to D). A strong sparse labeling was also detected on some areas of the stroma, which forms the interstitial connective tissue of the ovary. Only the follicles with at least three rows of granulosa cells surrounding the oocyte had a strongly labeled thecal envelope, while the theca of smaller follicles did not appear to be labeled significantly (Fig. 1A and B).

In the testis, a specific pattern of silver grains was also observed. Ecotropic MuLV RNA was detected in the connective tissue that fills the interstices among the seminiferous tubules. The labeling was especially strong in the angular interstices among the seminiferous tubules. Some labeling could also be seen on cells along the basement membrane that surrounds the seminiferous tubules. However, essentially no ecotropic MuLV RNA was detected either in the basement membrane itself or on the seminiferous epithelium where spermatogenesis takes place (Fig. 1E and F). The connective tissue of testis is known to contain fibroblasts, macrophages, mast cells, and the so-called Leydig cells (5). Attributing the hybridization signal more specifically to any of these cell types was not possible. Furthermore, the regions of the testis where ecotropic MuLV RNA was detected are provided with numerous blood vessels, as was previously noted with the ovaries. These results clearly show that transcription of the inoculated virus occurs in both female and male gonads according to a specific cell pattern.

**Detection of ecotropic MuLV viral antigen in gonads of SWR/J inoculated mice.** It is known that the 70-kilodalton glycoprotein gp70, encoded by part of the *env* gene of ecotropic MuLVs, can be expressed as a surface component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in MuLV-infected cells (7). Indirect immunofluorescence tests were thus performed to look for the presence of an ecotropic MuLV viral antigen on sections of the gonads from four inoculated SWR/J mice, two females and two males. The goat antiserum to the envelope glycoprotein gp70 of Rauscher MuLV which we used has been shown to precipitate an ecotropic MuLV 70-kilodalton protein exclusively from Friend-MuLV-induced murine leukemia cells (32). Preliminary indirect immunofluorescence tests showed that while a simian virus 40-transformed SWR/J cell line, the so-called SWRSV cells (29), failed to react with this serum, these cells were strongly labeled following infection with SWR-RF MuLV, indicating that a virus-induced antigen is present in these infected cells (data not shown). With this serum, ovarian sections from the inoculated SWR/J females were labeled according to a specific pattern. The circumferentially oriented cells immediately surrounding the follicular cells were fluorescent, while the follicular cells themselves and the oocytes displayed essentially no fluorescence. All the follicles containing a growing oocyte enveloped by at least one ring of granulosa cells were surrounded by fluorescent thecal cells (Fig. 2A through F). However, young follicles with only one layer of follicular cells had theca that were more weakly stained than older follicles (Fig. 2; compare panels B, D, and F). Some fluorescence was also detected in patches in the ovarian stroma. Sections from uninfected SWR/J females were not stained (Fig. 2G and H). Thus, virus expression in SWR/J ovaries results in the specific detection of an antigen recognized by goat anti-gp70 antibodies, presumably the gp70 *env* protein itself. This antigen is abundant around all ovarian follicles that have developed beyond the so-called type 3b of Peters' classification of follicle types (30). This detection of a virus-induced antigen around all stage 3b follicles, where significant amounts of viral RNA could not be detected (see above), is likely to reflect the higher sensitivity of indirect immunofluorescence tests compared with RNA-RNA hybridization experiments.

On testis sections from inoculated males, only cells located in the intertubular areas were stained. Clusters of cells located in the angular interstices between the seminiferous tubules were strongly fluorescent (Fig. 3A and B), as was also the case occasionally for interstitial cells isolated between two tubules. However, no viral antigen was detected on sections of the seminiferous tubules themselves. Sections from uninoculated SWR/J males remained unstained (not shown).

Thus, in situ hybridization and indirect immunofluorescence experiments indicate that specific areas in both male and female gonads of SWR/J mice were infected by the exogenous ecotropic MuLV, actively synthesized viral RNA, and expressed a viral antigen.

**Identification of the infected cells in gonads of inoculated mice.** As noted above, the regions of both female and male gonads where viral RNA and antigen were detected are composed of cells closely associated with blood vessels. Thus, it was important to check that the infected cells seen with the former techniques were not merely blood cells present in the vessels irrigating the gonads. To further identify the productively infected cells, ultrastructural studies and immunocytochemical analyses were carried out.

Systematic observations were first performed on gonads which were conventionally fixed and processed in order to detect viruses in inoculated SWR/J mice. C-type particles, clearly recognizable by their budding pattern and their average diameter (approximately 100 nm), were observed in thecal cells at the periphery of ovarian follicles (Fig. 4A through D). These thecal cells were identified by (i) their position at the periphery of the ovarian follicle and (ii) their long extended shape (data not shown). C-type particles were not detected in other cell types of the ovary; however, C-type particles are difficult to find, and their presence in nonthecal cells cannot be conclusively excluded. The ovaries of control individuals that had not been inoculated with the ecotropic MuLV were negative for C-type particles. Taken together, these results agree with those obtained by in situ hybridization and indirect immunofluorescence; they suggest that viral transcription in thecal cells leads to the production of mature virus particles.

C-type particles were not detected in the testis of an infected SWR/J male. Therefore, immunoperoxidase localization of the viral antigen detected by goat anti-gp70 antibodies was carried out to identify the productively infected cells of the male gonad more precisely. In the testis, deposits of black DAB were found in interstitial cells only as discrete patches generally localized close to the Golgi apparatus (Fig. 5A and B). This intracellular distribution of discrete labeled patches agrees with the localization of retrovirus antigens as visualized by others (1), although no association with a cytoplasmic organelle or cytoskeletal elements was detected. The labeled cells were identified as Leydig cells. This conclusion is based on their morphology, with their cytoplasm characteristically displaying an extensive smooth endoplasmic reticulum (Fig. 5B and D) (5). A discrete layer of DAB occasionally observed on the cell membrane of the Leydig cells (Fig. 6) shows that the virus antigen detected is also distributed on the membrane. This localization of viral material, both intracellular and in the cell membrane, agrees with the known processing events to which viral glycoproteins are submitted (31). Peroxidase labeling was rare in other interstitial cells. These results thus indicate that the cells most effective in producing gp70 viral antigen in the testis are the Leydig cells, as defined by the particular ultrastructure of these cells.

## DISCUSSION

The results presented here document the expression of ecotropic MuLV sequences inside the gonadal tissues of both male and female SWR/J mice which have been inoculated at birth. Although the presence of MuLV particles in many tissues of AKR/J mice was recognized a long time ago (36), little is known about the distribution of virus-producing cells in various tissues. Our study demonstrates that the production of such particles can be highly patterned and that different subsets of cells in both ovaries and testes have

FIG. 1. In situ hybridization experiments with an ecotropic MuLV RNA-specific probe were performed on gonads from SWR/J mice inoculated at birth with SWR-RF MuLV (29). (A, C, E, and G) Phase-contrast micrographs and (B, D, F, and H) the same fields seen in dark field. (A through D) Ovarian sections through three small (A and B; magnification, ×380) and two mature follicles (C and D; magnification, ×152). Thecal cells that surround follicles beyond stage type 5a (30) as well as some stromal cells are labeled. Younger follicles remain unlabeled. (E through H) Transverse sections (toluidine blue stain) through seminiferous tubules of testis from both inoculated (E and F) and uninoculated (G and H) males showing labeled clusters of cells between the seminiferous tubules (magnification, ×152).

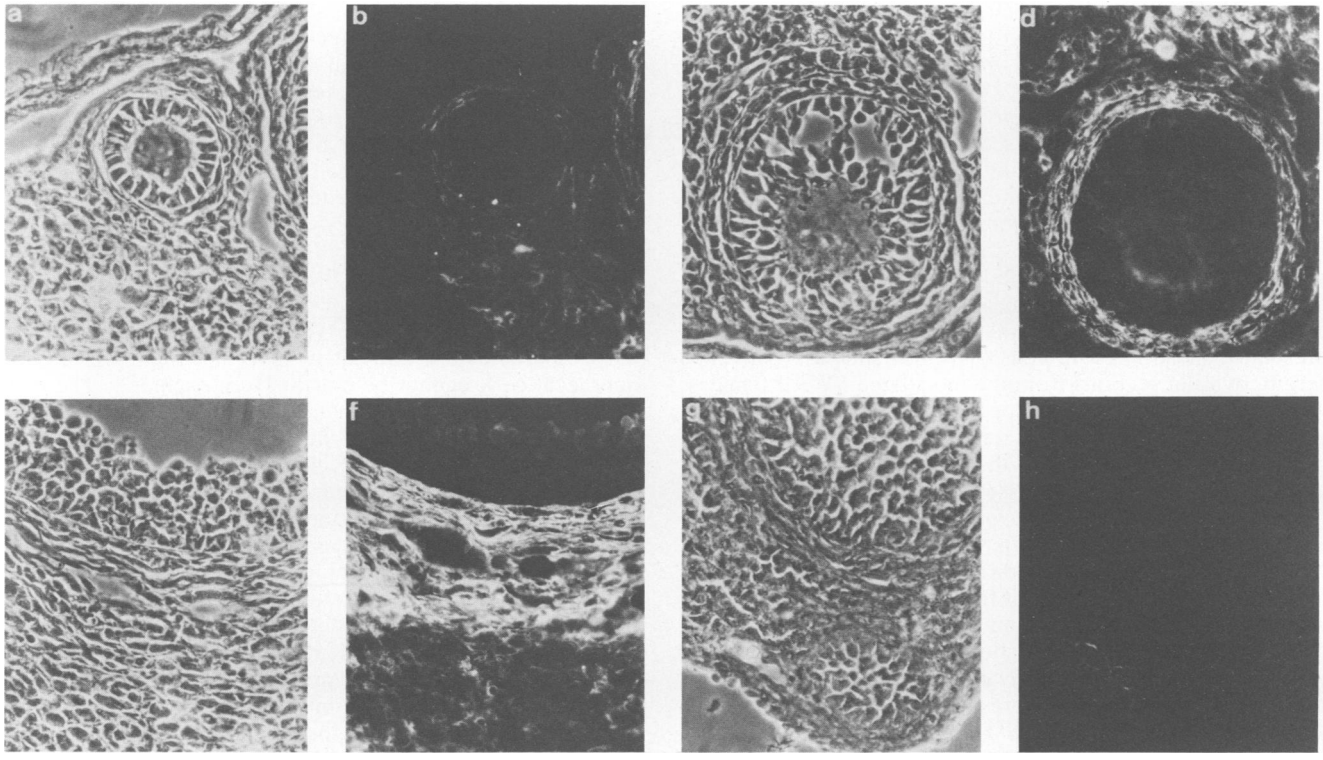


FIG. 2. (a through f) Sections through the ovaries of SWR/J females inoculated at birth with SWR-RF MuLV. (g and h) Section through a mature follicle of an uninoculated SWR/J female (negative control). Shown are phase-contrast micrographs (a, c, e, and g) and the same fields stained in indirect immunofluorescence with antibodies against gp70 (b, d, f, and h). (a through f) Sections through ovarian follicles at different stages of development showing thecal cells and some stromal cells with anti-gp70 staining (type 3b [a and b], type 6 [c and d], and type 8 [e and f]) (30). The exposure time for panel h was adjusted to be the same as that for panels b, d, and f. Magnification, ×400.

widely different levels of virus expression. Male and female gonads thus appear as heretofore unsuspected targets for MuLV infection. Although the mechanisms which make thecal and interstitial cells such efficient targets for virus expression are unknown, it is striking that both cell types are also steroid hormone producers. Whether such a correlation is merely fortuitous is unknown, however.

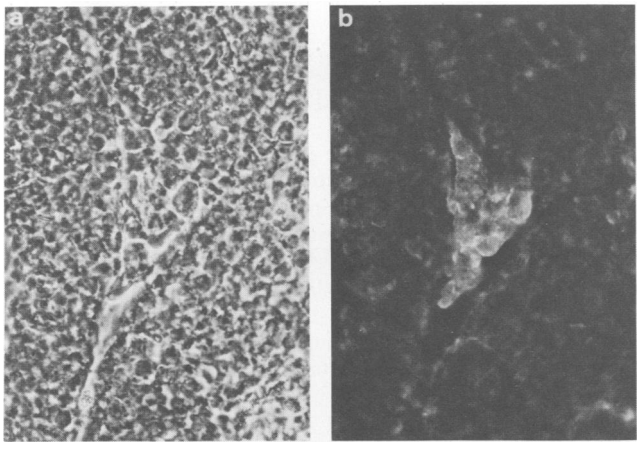


FIG. 3. Section through the testis of a SWR/J male inoculated at birth with SWR-RF MuLV. Phase-contrast micrograph (a) and the same field stained in indirect immunofluorescence with antibodies against gp70 (b). The section shows a cluster of interstitial cells located between the seminiferous tubules positive for gp70 antigen (magnification, ×400).

It remains to be determined whether the pattern of expression disclosed here is exclusively specific for the SWR-RF MuLV isolate used throughout our study. However, we believe that this is not likely for various reasons. First, this virus is derived from the endogenous ecotropic proviruses carried by RF/J mice (designated *Emv-16* and *Emv-17*) which are structurally related to AKR/J endogenous ecotropic proviruses (20). Second, ecotropic MuLV RNAs are also detected in the ovaries of AKR/J mice (28). This suggests that the ecotropic MuLV produced in AKR/J mice might display a pattern of infectivity in SWR/J gonads very similar if not identical to SWR-RF MuLV. The nucleotide sequence determination of a molecular clone encoding SWR-RF MuLV (in progress in our laboratory) should clarify the structural relationships between these viruses.

One may ask whether the virus reservoir in the gonads leads to oncogenesis in infected mice. The possibility that the virus inoculated into SWR/J mice will eventually trigger leukemogenesis remains to be documented. In this context, however, it is worth pointing out that tumors of the ovary have been occasionally detected in our stock of SWR/J-RF/J hybrid females carrying *Emv-16-Emv-17*. These females also express high levels of ecotropic MuLV RNA in their ovarian thecal cells (28). However, these tumors have been characterized as granulosa cell tumors (J. Gaillard, personal communication), i.e., they derive from follicular cells that do not appear to be productively infected by ecotropic viruses, and it should be noted that spontaneous malignant granulosa cell tumors are found in SWR/J and SWR-derived mice (3). As far as AKR/J mice are concerned, it is intriguing that McEndy et al. (24) have shown that ovariectomy of AKR

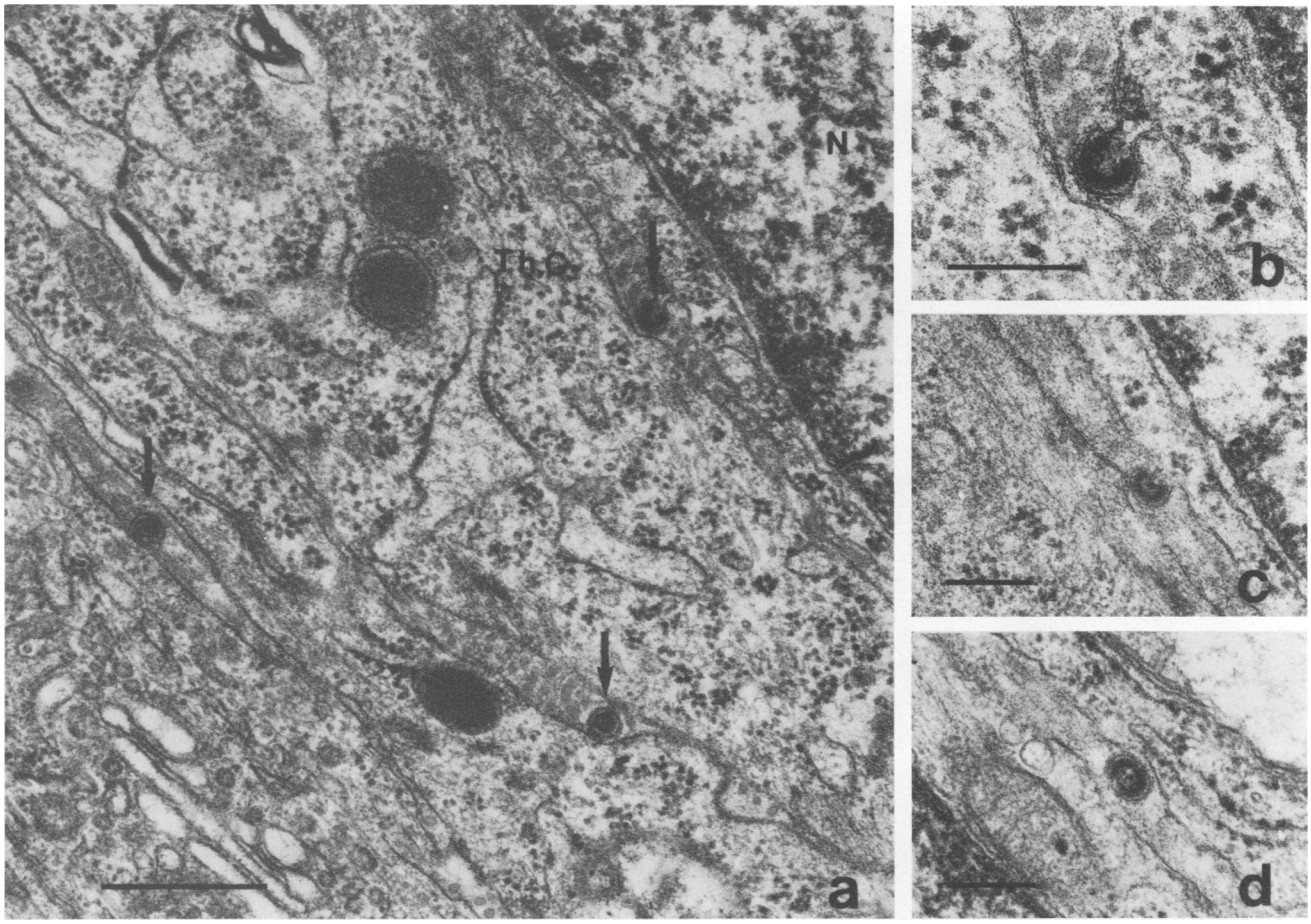


FIG. 4. (a) Production of virus by thecal cells from the ovary of inoculated SWR/J females. Budding particles (arrows) are clearly visible in the intracellular space occupied by collagenous fibrils between the thecal cells (magnification, ×42,500; bar, 0.5 μm). N, Nucleus; Th.C., thecal cell. (b) Same cell as in panel a, seen at a higher magnification (×87,500; bar, 0.2 μm). (c and d) Other viruses observed around thecal cells. The average diameter of these particles is 100 nm (bar, 0.2 μm).

mice reduces the incidence of leukemia from 74% among the controls to 45% among the ovariectomized females, thus pointing out the influence of the ovary on the incidence of spontaneous leukemia. In contrast, orchidectomy has been shown to increase the incidence of leukemia in AKR mice from 65 to 84% (37). Obviously, the endocrine functions and the capacity to produce ecotropic MuLV inherent in the gonads were not separated in these experiments, preventing us from assessing the relative importance that these elements may have individually in leukemogenesis. Nevertheless, the results presented indicate that both male and female gonads might be a particular site where continuously produced ecotropic MuLVs would be available to recombine with endogenous xenotropic or mink cell focus-forming (MCF)-like sequences, resulting in recombinant MCF viruses (8, 9, 15, 16, 33, 40). The production of such recombinant viruses is thought to be a prerequisite for the development of T-cell lymphomas (8, 26), which occur with a high incidence in AKR/J mice. The existence of such reservoirs appears to be especially important if one recalls that thymic lymphocytes have been reported to be resistant to infection by ecotropic virus (4, 13, 17), and one of the models for T-cell lymphomagenesis assumes that the generation of MCF viruses is needed in order to infect a specific target cell which is refractory to infection by ecotropic MuLV (11). Oncogenesis would result from the activation of some distinct cellular genes (for a review, see reference 27) by either an MCF virus or by a pseudotyped MuLV (MCF envelope, ecotropic genome) (12).

Finally, it is not clear whether the MuLV pattern of expression in the ovaries of inoculated SWR/J mice bears any relevance to the integration of MuLV sequences in the progeny. We demonstrated that ecotropic MuLV particles are produced in the vicinity of the oocytes; however, this does not imply that the target cells for the virus infection are the oocytes themselves. More studies involving manipulation of the oocyte or egg transfer experiments or both should allow to understand the infectivity process which results in transgenic pups from infected SWR/J mothers.

It has been recently shown that sperm-associated retroviruses are common in mouse epididymal semen, indicating that retroviruses are expressed in the male reproductive tract (21). Our results show that Leydig cells express viral messenger and protein product; moreover, infectious ecotropic MuLV particles can be recovered from the semen of infected SWR/J males (J.-J. Panthier, unpublished data). In apparent contradiction, male virus carriers do not transmit endogenous ecotropic proviruses to their progeny after mating with nonviremic females. This implies either that spermatozoa are not infected or that the infection efficiency

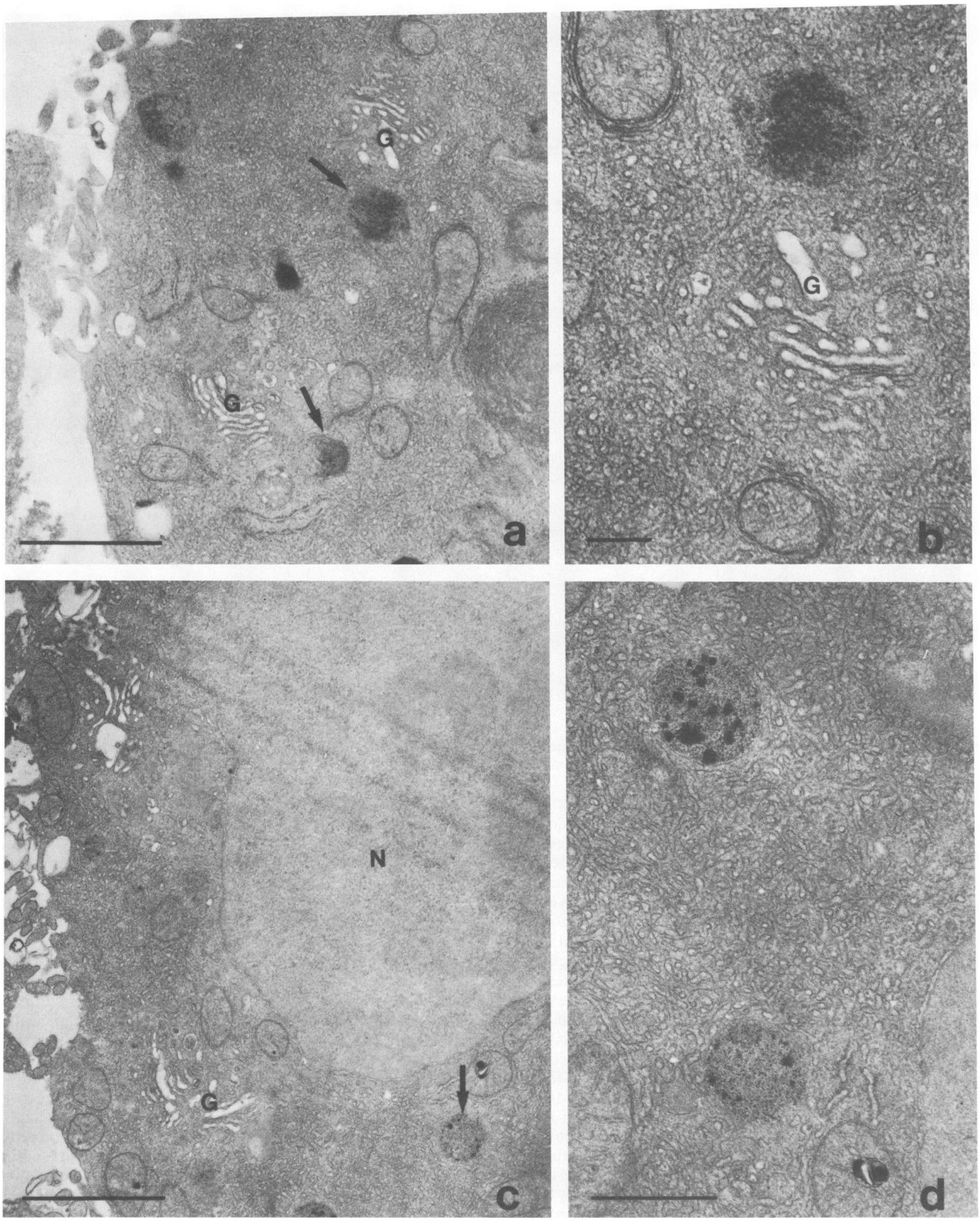


FIG. 5. Immunoperoxidase localization of gp70 viral antigen in Leydig cells of inoculated SWR/J males. Sections are observed unstained. Patches of black deposits were often seen near the Golgi apparatus. The granular aspect of the intense black deposit is typical of the enhancing method used (39) (see Materials and Methods). Note the extensive smooth endoplasmic reticulum characteristic of Leydig cells in panels b and d. Magnifications: panel a, ×25,000 (bar, 1 μm); panel b, ×55,000 (bar, 0.5 μm); panel c, ×25,000 (bar, 1 μm); panel d, ×42,000 (bar, 0.5 μm).

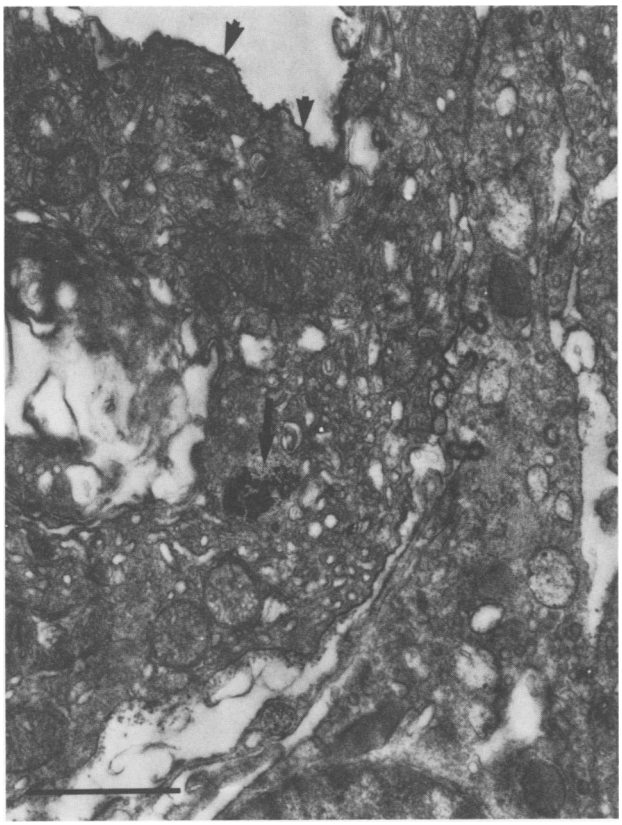

FIG. 6. Immunoperoxidase localization of gp70 viral antigen in testis of inoculated males. A black deposit of DAB was often observed at the surface of interstitial cells (arrowheads) and in the cell itself (arrow). Magnification, ×20,000.

of free virus particles present in sperm is too low in utero to be easily detected by progeny testing or both.

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