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Expression of Human Endogenous Retrovirus K Elements in Germ Cell and Trophoblastic Tumors

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Antibodies against proteins encoded by human endogenous retrovirus (HERV)-K family genes are consistently found in the sera of patients with classical seminoma. Furthermore, HERV-K Gag-encoded protein could be detected in corresponding tumor biopsies. Addressing questions as to the extent of HERV gene expression in biologically related lesions, we studied various testicular and ovarian germ cell tumors (GCTs), GCT precursor lesions, and gestational trophoblastic disease (GTD) for the presence of HERV-K gene transcripts in tissue sections. By* in situ *hybridization using four non-overlapping, isotopically labeled RNA probes specific for HERV-K* gag *and* env *sequences on archival tissue samples, consistent HERV-K expression of* gag *and* env *genes was found to be common to all GCTs and their testicular precursor lesions with the exception of teratomas, mature and immature, and spermatocytic seminomas. HERV-K expression was also found in malignant GTD (choriocarcinoma) but not in benign GTD (noninvasive molar pregnancy). There was no evidence for HERV-K expression in differentiated embryonal and adult tissues as well as a total of 53 tumors other than GCT or GTD. The findings point to a common molecular pathoetiology of GCT and malignant GTD, have implications for the classification of GCTs, and support the concept of carcinoma* in situ *as a precursor lesion common to all forms of testicular GCT. (Am J Pathol 1996, 149:1727–1735)

Many malignant tumors occurring in the second to fourth decade of life are associated with potentially oncogenic viruses. Examples include Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma, all of which are associated with Epstein-Barr virus infection, hepatocellular carcinoma and hepatitis B virus, genital cancers and papilloma viruses, or adult T-cell lymphoma/leukemia and human T-lymphotropic virus type-1. Another tumor group with high incidence in young adults comprises testicular and ovarian germ cell tumors (GCTs). The association of these tumors with a distinct virus may therefore be conceivable. No such association has as yet been established, however. Here we report on the detection of gene transcripts encoded by a human endogenous retrovirus (HERV) that are common to all GCTs with the exception of teratomas and that are also found in testicular GCT precursor lesions as well as in gestational choriocarcinoma.

Retroviruses are a family of RNA viruses that produce a DNA intermediate, the provirus, designed to integrate into the host cell genome. In the rare event of provirus integration into germ cell genomes, the integrated provirus turns into an inheritable trait of the host. Such endogenous retroviruses have been found in several mammalian species including man.[1] The human genome carries numerous loci corresponding to endogenous retroviral sequences, only few of which, however, represent complete proviral genomes. A family of complete human endogenous retrovirus elements carrying a primer binding site for lysine-specific tRNA (HERV-K) was described a few

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years ago.[2] The members of the HERV-K family comprise full-length proviruses displaying two long terminal repeats flanking intact open reading frames for *gag*, *pol*, and *env* genes characteristic for retroviruses.[3,4] One of the HERV-K sequences encodes a full-length Gag-homologous 73-kd protein and a functional protease, Prt.[4] Furthermore, spliced RNA transcripts encoding nonstructural proteins related to the Rev protein of human immunodeficiency virus-1 (HIV-1) may originate from HERV-K genomes.[5]

Expression of endogenous retroviral sequences has been noted in some animal tumors whereas screening of human tumors for HERV has yielded inconsistent results. Recently, using a baculovirus expression system for the generation of HERV-K Gag polypeptide and subsequently developed Gag-specific antibodies, a highly specific immunofluorescence assay system has been applied to analyze human sera for the presence of HERV-K Gag-specific antibodies.[6] Anti-Gag antibodies were consistently found with high titers in sera from seminoma patients but not in significant proportions in sera of patients with other tumors[6,7]. These results prompted us to investigate various testicular and ovarian GCTs as well as trophoblastic gestational disease (GTD) for the expression of HERV-K *gag* and *env* genes by *in situ* hybridization using non-overlapping probes. We also tested lesions considered precursors of testicular GCTs, which are variably called carcinoma *in situ* of the testis (CIS),[8] intratubular germ cell neoplasia,[9] or testicular intraepithelial neoplasia (TIN),[10] among other names. Immunoblot assays were used to screen representative tissue samples for the presence of polypeptides corresponding to those gene transcripts detected by *in situ* hybridization.

Materials and Methods

Tissues

Archival tissues were used that had been fixed in neutral-buffered formalin and embedded in paraffin wax by routine procedures. The 5-μm sections were cut and mounted onto glass slides pretreated with 3-aminopropyl-triethoxysilane. Histological diagnosis followed the criteria published by the World Health Organization for testicular, ovarian, and female genital tract tumors.[11–13] The diagnoses are listed in Table 1. Immunohistological demonstration of placental alkaline phosphatase, α-fetoprotein, cytokeratin, CD30 antigen, or β-human choriogonadotropin (β-HCG) served as a diagnostic adjunct.

Table 1. *Summary of* in Situ *Hybridization Results*

	HERV-K RNA$^+$/ cases tested
Testicular GCT	
Seminoma, pure	23/23
Spermatocytic seminoma	0/2
Embryonal carcinoma, pure	5/5
Yolk sac tumor, pure	3/3
Immature teratoma	0/2
Mixed testicular GCT	15/15
Seminoma	7/7
Embryonal carcinoma	11/11
Yolk sac tumor	13/13
Choriocarcinoma	3/3
Immature teratoma	0/7
CIS/TIN	
Ipsilateral to testicular GCT	13/13
In maldescended testis	1/1
In dysgenetic gonads	1/1
Ovarian GCT	
Dysgerminoma	3/3
Yolk sac tumor	2/2
Immature teratoma	0/2
Mature teratoma	0/8
GTD	
Choriocarcinoma	2/2
Partial molar pregnancy	0/12
Other tissues	
Placenta	0/5
Differentiated embryonal tissues*	0/5
Breast carcinoma	0/15
Prostate carcinoma	0/10
Hodgkin's disease	0/20
Other malignancies†	0/8

Concordant results were obtained with all four non-overlapping, independently applied probes.

*Embryonal tissues included cerebrum, eye, lung, heart, liver, stomach, gut, kidney, skeletal muscle, cartilage, and skin but not gonads.

†Cases included one uterine carcinosarcoma, one rhabdomyosarcoma, two small-cell carcinomas, one serous adenocarcinoma of the ovary, and three colonic adenocarcinomas.

Cell Lines

The embryonal carcinoma cell line, Tera 1, was obtained from the American Type Culture Collection, Rockville, MD (ATCC HTB105). The B95-8 cell line was originally established by infecting marmoset B lymphocytes with Epstein-Barr virus.[14] Formalin-fixed and paraffin-embedded cell pellets were sectioned like tissues to serve as positive control.[15]

Probes

The non-overlapping 522-bp *Bgl*II/*Eco*RV and the 815-bp *Eco*RV/*Pst*I fragments corresponding to 5′ and 3′ regions of the HERV-K *gag* gene, respectively (positions 1276 to 1798 (5′-*gag*) and 1798 to 2613 (3′-*gag*) of the published HERV-K sequence[2,3] were subcloned into the run-off expression vector pGEM1 (Promega, Madison, WI). Similarly, non-overlapping HERV-K *env* sequences were subcloned into

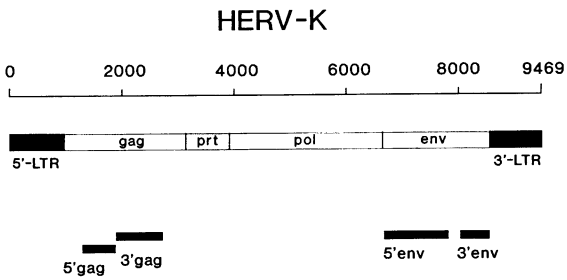

Figure 1. *Schematic representation of the HERV-K provirus gene structure indicating the location of the four gene probes used in this study. Sequences encoding* gag, prt, pol, *and* env *polyproteins are derived from four major open reading frames that are flanked by long terminal repeats (LTRs). Bars represent the specific probes as described in Materials and Methods.*

pGEM1, the 941-bp *Sma*I/*Sal*I fragment corresponding to nucleotide positions 6611 to 7552 (5′-*env*) and the 583-bp *Bam*HI/*Pst*I fragment corresponding to nucleotide positions 7832 to 8415 (3′-*env*; Figure 1). Serving as a control for the demonstration of sufficient levels of cellular RNA in tissue sections, immunoglobulin light chain-κ-type (IgLCκ) RNA probes were prepared by subcloning of the 550-bp *Sst*I fragment containing the human immunoglobulin light chain-κ segment[16] into pGEM1. A phage harboring an immunoglobulin light chain-κ genomic fragment was kindly provided by Dr. P. Leder, Cambridge, MA.

After linearization of the plasmids with either *Hin*dIII or *Eco*RI restriction endonucleases, T7 or SP6 RNA polymerases (BRL Gibco, Eggenstein, Germany), respectively, were employed to obtain run-off transcripts of either the antisense (complementary to mRNA) or sense (anticomplementary, negative control) strands. Transcription and labeling of RNA probes were performed as described in detail previously.[17,18]

In Situ *Hybridization*

Prehybridization, hybridization, removal of nonspecifically bound probe by RNAse A digestion, and additional washing procedures, autoradiography, and development were performed for both positive- and negative-strand RNA probes as described elsewhere.[17,18] Additionally, simultaneous control hybridizations with sense probes were carried out for all tissue blocks. Treatment of slides with *Micrococcus* nuclease (Boehringer-Mannheim, Mannheim, Germany) verified that RNA and not genomic DNA was the target of the hybridization procedure.

Immunoblotting

Tissue samples stored at −70°C were homogenized on ice in sample buffer. After incubation at 95°C for 10 minutes and centrifugation for 10 minutes at 15,000 × *g*, the supernatant was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon membranes (Millipore Corp., Bedford MA). The blots were incubated with a HERV-K Gag-specific rabbit antiserum (serum 6897) at a dilution of 1:200 and then stained indirectly using peroxidase-conjugated goat anti-rabbit antibodies.[6,19] The specificity of the reaction was controlled by competition experiments using a full-length recombinant HERV-K Gag protein expressed from plasmid pKG.[6]

Results

In Situ *Hybridization*

HERV-K-specific transcripts were detectable in all testicular and ovarian GCTs with the exception of teratomas and spermatocytic seminomas (Table 1). Two choriocarcinomas of gestational origin also expressed HERV-K-specific RNA. Tera 1 cells expressed HERV-K genes at high transcript levels. Concordant labeling patterns were found with all of the four HERV-K-specific probes. Little variation of signal intensity was observed among the four probes. The 3′-*gag*-specific probe, as the smallest one, required slightly longer autoradiographic exposure times. Within HERV-K-positive neoplasias, however, there was considerable variation in signal intensity from one cell to the other, with some tumor cells displaying signals close to background labeling whereas others were concealed by silver grains (Figure 2).

Among testicular neoplasias, HERV-K-specific labeling was observed in all cases of pure or mixed classical seminoma (Figure 2), embryonal carcinoma (Figure 3), yolk sac tumor (Figure 4), and choriocarcinoma. Syncytiotrophoblastic giant cells occurring in the setting of seminoma, embryonal carcinoma, and choriocarcinoma were found to express HERV-K transcripts at only minimal levels, often close to background signal. At variance, absence of HERV-K-specific signals was noted in all mature and immature teratomas and teratomatous elements in mixed testicular GCTs. Derivatives of all three embryonic layers, ie, ecto-, meso-, and endoderm, were represented in our teratoma cases. CIS/TIN cells occurring in the neighborhood of seminomas (Figure 5) and nonseminomatous GCTs, including teratomas

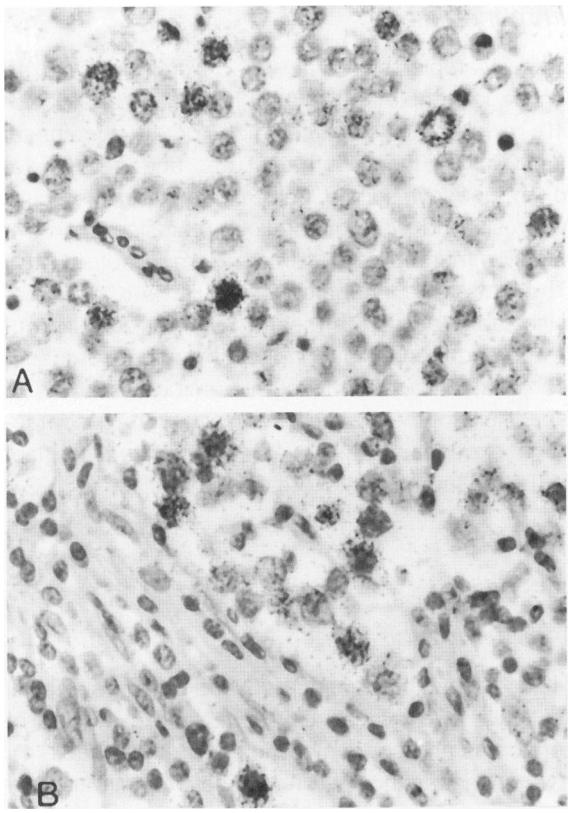

Figure 2. *Expression of HERV-K* gag *sequences in two cases of classical seminoma* (**A** *and* **B**). *Tumor cells display variably intense autoradiographic signal, whereas cells of the inflammatory infiltrate and blood vessels show background labeling. 5′-*gag *probe, 8 days of autoradiographic exposure; original magnification,* ×*100.*

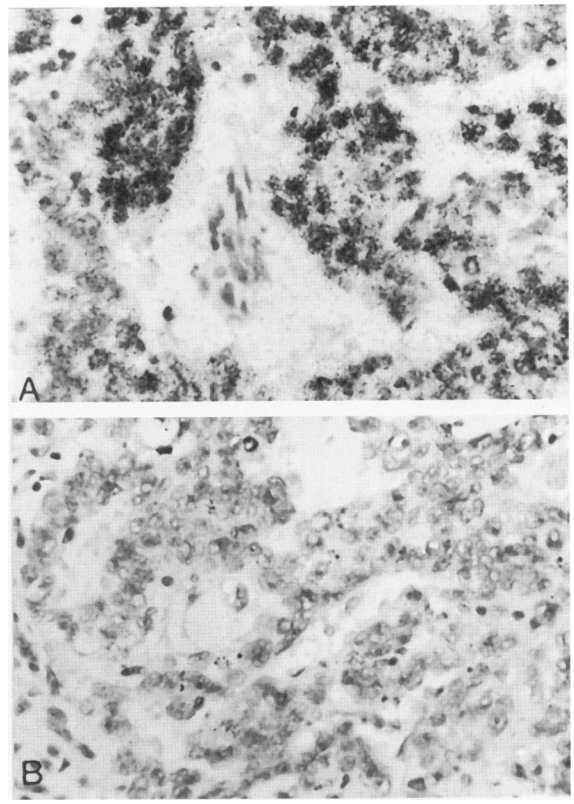

Figure 3. *Expression of HERV-K* gag *sequences in an embryonal carcinoma. Intense labeling is found using HERV-K probes in antisense orientation* (**A**), *whereas hybridization with RNA probes in sense orientation results in background signal* (**B**). *5′-*gag *probe, 8 days of autoradiographic exposure; original magnification,* ×*100.*

(Figure 6) as well as CIS/TIN in each one case of dysgenetic gonads (Figure 7) and maldescended testes, displayed HERV-K transcript levels similar to seminomas. The presence of HERV-K RNA in CIS/TIN cells correlated with placental alkaline phosphatase positivity as assessed by immunohistology on adjacent sections. Cells of intact spermatogenesis and placental-alkaline-phosphatase-negative spermatogonia detectable in 12 testicular specimens and present distant to the tumor areas in GCTs and tubules with CIS/TIN cells were in all instances free of HERV-K-specific signals.

Two cases of spermatocytic seminoma were available for this study. HERV-K transcripts were detectable neither in tumor cells nor in neighboring intratubular spermatogonia (Figure 8A). Positive labeling of a proportion of plasma cells and small lymphocytes within the scant inflammatory infiltrate verified that lack of HERV-K-specific signals was not due to absence of RNA in the tissue sections (Figure 8B).

Ovarian GCTs showed the same pattern of HERV-K transcript expression as testicular GCTs. Normal oocytes in primordial and Graafian follicles occurring adjacent to GCTs did not display HERV-K-specific signals. The two choriocarcinomas of gestational origin displayed intense HERV-K-specific signals in most of the cytotrophoblastic cells, whereas syncytiotrophoblastic tumor cells rarely expressed HERV-K transcripts (Figure 9). Twelve

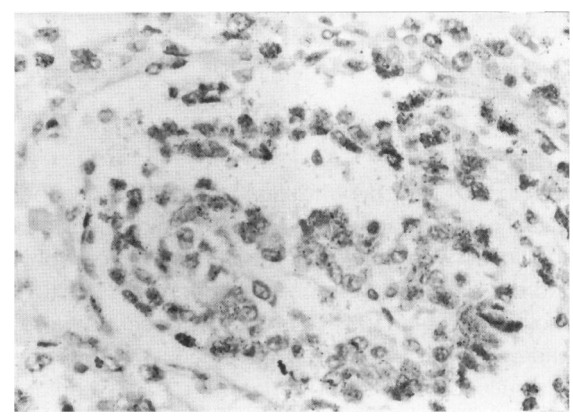

Figure 4. *Expression of HERV-K* gag *sequences in a yolk sac tumor. Tumor cells display autoradiographic labeling, whereas endothelial cells of the blood vessel forming the core of the Schiller-Duval body show background signal. 5′-*gag *probe, 8 days of autoradiographic exposure; original magnification,* ×*100.*

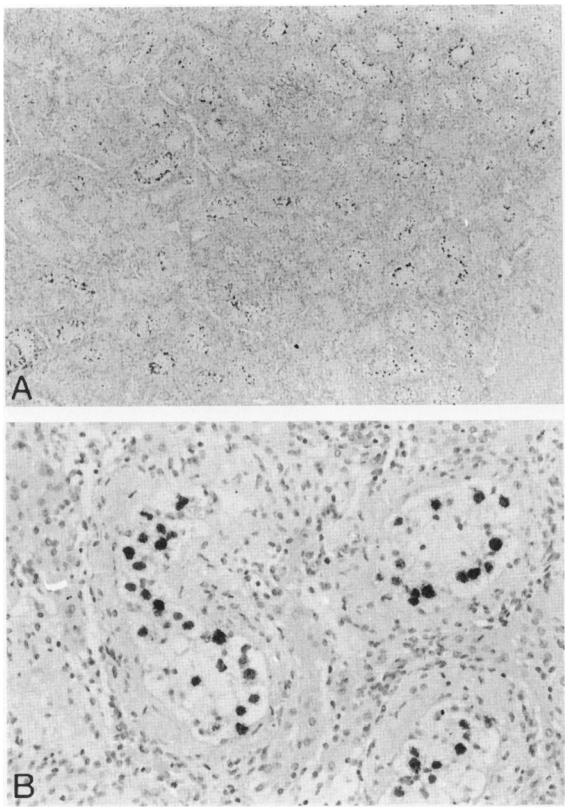

Figure 5. *Expression of HERV-K* env *sequences restricted to atypical spermatogonia (so-called testicular carcinoma* in situ*) within tubules adjacent to seminoma. 5′-*env *probe, 8 days of autoradiographic exposure; original magnification,* ×*40* (**A**) *and* ×*100* (**B**).

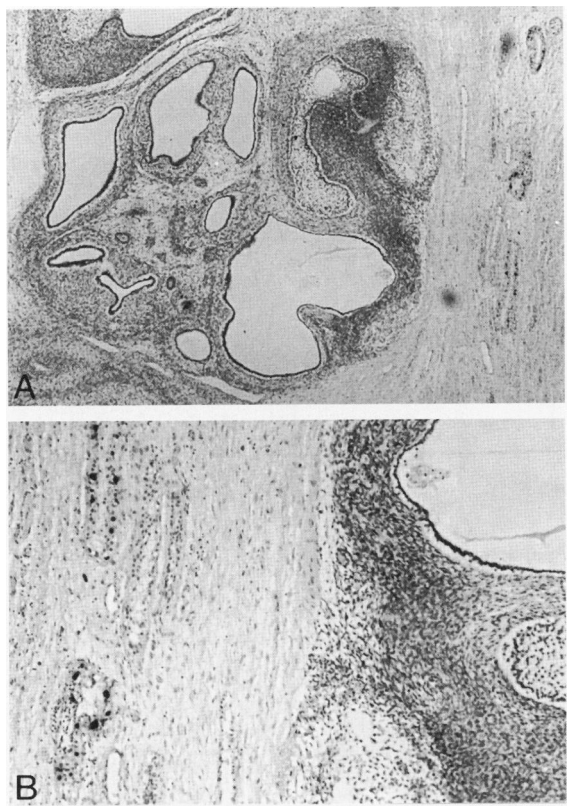

Figure 6. *Expression of HERV-K* env *sequences in atypical spermatogonia (so-called testicular carcinoma* in situ*) within a tubule adjacent to a teratoma mass. Note the absence of specific autoradiographic signal from the teratoma. 3′-*gag *probe, 8 days of autoradiographic exposure; original magnification,* ×*40* (**A**) *and* ×*100* (**B**).

cases of partial molar pregnancy representing benign GTD did not display HERV-K transcripts at levels detectable by our technique.

HERV-K transcripts were also absent from normal placental and embryonal tissues representing different gestational stages, cases of breast and prostate carcinoma, Hodgkin's disease, non-Hodgkin's lymphoma, and various other malignancies tested as controls.

Immunoblots

Snap-frozen GCT tissues were processed and analyzed by immunoblotting with rabbit anti-HERV-K Gag serum. Representative results are shown in Figure 10. The rabbit anti-Gag serum specifically recognizes a protein band of 80 kd that represents the Gag polyprotein.[6] This band is shared by the cell line Tera 1 and the GCT samples but is absent from the Epstein-Barr virus producer cell line B95-8 and splenic tissue used for control. The binding of the specific antibodies to the 80-kd protein could be abolished by preincubation of the serum with recombinant full-length HERV-K Gag fusion protein (pKG[6]; as demonstrated in Figure 10). Several background bands with an apparent molecular weight of approximately 50 kd were found, the nature and derivation of which, however, is presently not known.

Discussion

HERV-K expression was a consistent finding in most entities of testicular and ovarian GCT, GCT precursor lesions, and choriocarcinomas of gestational origin. Despite the obvious uncertainties relating to the exact role of endogenous retroviral gene products, the findings have implications for the pathobiology, classification, and clinical management of these tumors as well as for the understanding of the function of retroviral elements in the human genome.

Preliminary studies have shown that, at variance with non-GCT, seminoma patients display increased titers of HERV-K Gag-specific antibodies.[6] These studies were extended to the analysis of HERV-K Env products with similar results.[20] Serological demonstration of Gag and Env polypeptides and corresponding antibodies in seminoma patient sera was

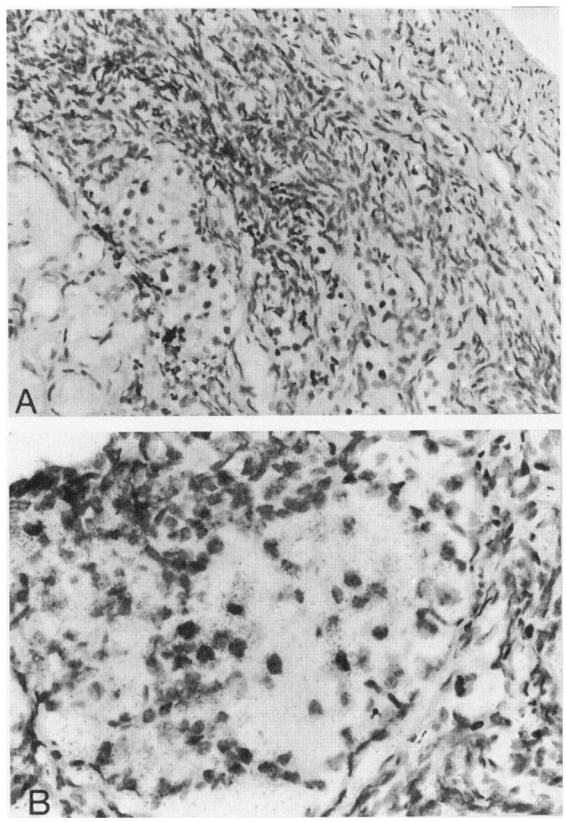

Figure 7. *Expression of HERV-K* env *sequences in atypical gonocytes within tubular structures placed in a case of mixed gonadal dysgenesia. Note the absence of specific autoradiographic signal from the stromal cells. 3′-*gag *probe, 8 days of autoradiographic exposure; original magnification,* ×*40* (**A**) *and* ×*100* (**B**).

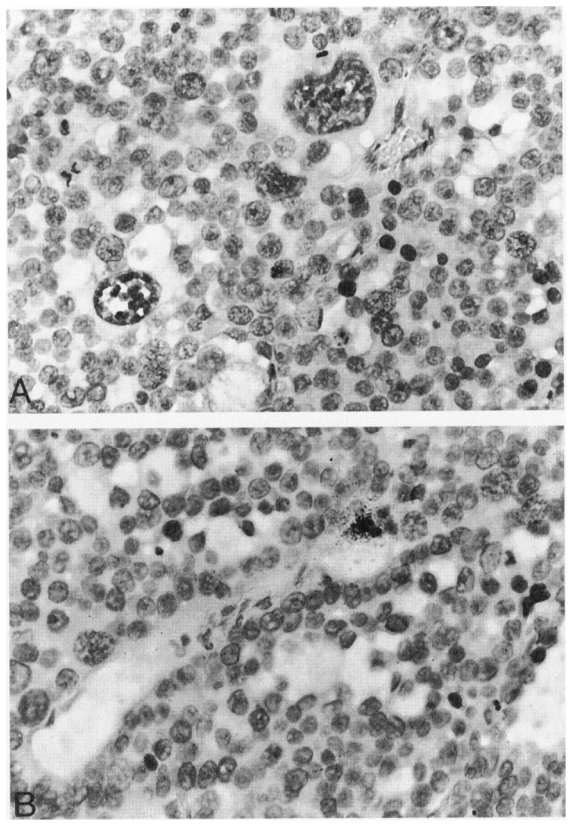

Figure 8. In situ *hybridization of a spermatocytic seminoma with the 3′-*gag *probe displays absence of specific autoradiographic signals from all tumor cells including giant cells and tumor cells with hyperchromatic nuclei* (**A**). *After hybridization with an immunoglobulin type k light chain probe, plasma cells and occasional B lymphocytes display autoradiographic signals indicating the presence of sufficient levels of cellular RNA in the section* (**B**). *Eight days of autoradiographic exposure; original magnification,* ×*100.*

positively correlated with clinical parameters such as failure to respond to therapy and development of metastases.[6] If confirmed by immunohistology with antibodies yet to be developed, the finding raises the possibility that serological detection of HERV-K Gag and Env polypeptides may be useful to complement serological monitoring with markers such as β-HCG and α-fetoprotein. Except in pediatric patients, eg, yolk sac tumors rarely occur in pure form but usually as a component of mixed GCTs, the metastases of which may not contain yolk sac tumor elements at all. The same is true for β-HCG and choriocarcinoma as a component of GCT or β-HCG-secreting syncytiotrophoblastic giant cells occurring in a proportion of seminomas. In case of GTD, β-HCG forms a reliable serological marker that, however, cannot be used to distinguish potentially malignant from nonmalignant forms of GTD. Our analysis of GTD lesions suggests that HERV-K expression in trophoblast cells may be associated with an increased risk to develop overtly malignant GTD such as choriocarcinoma. It is reasonable to anticipate a role for assessment of HERV-K expression in diagnosis and clinical monitoring of GCT and GTD. We have good evidence that expression of HERV-K *gag* is not restricted to the RNA level as HERV-K Gag can be detected by immunoblotting in tumor biopsies[6] (Figure 10). Because at the present time no qualified HERV-K Env antibodies are available, the detection of Env protein has to be a subject of future studies. On the other hand, the prevalence of Env antibodies in patients with seminoma suggest the expression of this protein in tumor tissues.[20]

HERV-K expression patterns suggest a common molecular pathogenesis of GCT and malignant GTD. HERV-K expression patterns support the classification of testicular GCT as originally devised by Friedman and Moore[21] and adopted by the World Health Organization.[11] These classifications distinguish seminoma, GCT with extraembryonic differentiation, and teratoma. At variance, the classifications proposed by the British Testicular Tumor Panel[22] did not accept the concept of embryonal carcinoma and classified testicular tumors into seminoma and tera-

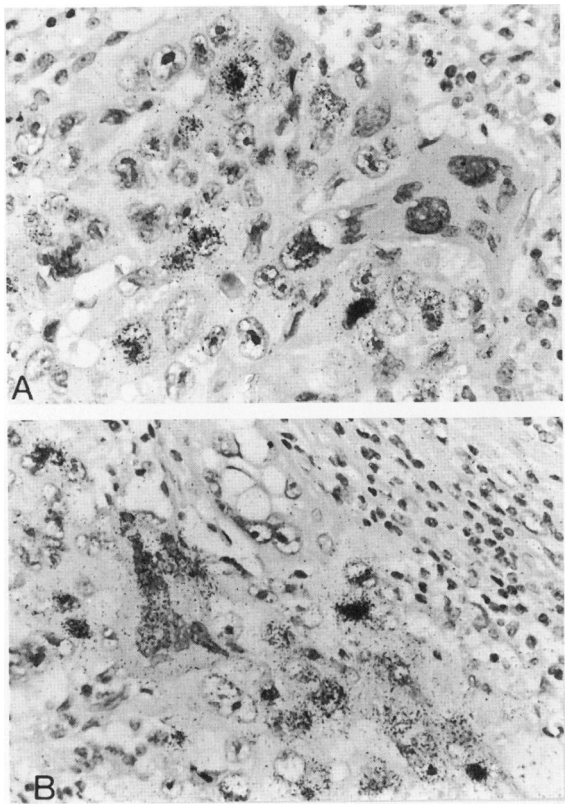

Figure 9. *Expression of HERV-K* env *sequences in a uterine choriocarcinoma. Note the reduced autoradiographic signal in syncytiotrophoblastic giant cells, whereas cytotrophoblastic parts of the tumor display variable, but usually strong signal* (**A**). *Occasional giant cells, however, display clearly positive signals with HERV-K probes* (**B**). *5′-*env *probe, 8 days of autoradiographic exposure; original magnification, ×100.*

toma, assuming a derivation of seminomas from germ cells and of nonseminomatous tumors from blastomeric remnants. Obviously, HERV-K expression patterns are not easily reconciled with the latter classification but rather point to a closer biological relationship between embryonal carcinoma and seminoma than between embryonal carcinoma and teratoma. Interestingly, our two cases of spermatocytic seminoma were not associated with detectable HERV-K expression. This supports previous notions that, by differing from classical seminoma in morphology, immunophenotype, incidence, and clinical behavior, spermatocytic seminoma forms an entity of testicular malignancy distinct from classical seminoma.[23]

More importantly, HERV-K expression patterns strongly support the concept of testicular CIS/TIN as a precursor lesion for all testicular GCTs.[8] Atypical spermatogonia occurring in the neighborhood of seminomas were already described in the last century.[24] The demonstration of such cells in testicular biopsies of patients who subsequently developed GCTs formed the basis for a histogenetic model proposing CIS/TIN as the precursor for all forms of GCT.[25] CIS/TIN cells are regularly found in the vicinity of up to 90% of GCTs, in 5% of the contralateral testis of GCT patients, and in up to 6% of patients with streak gonads or maldescended testes.[10] All of these conditions bear a well documented increased risk for development of GCT. Our finding of HERV-K expression in CIS/TIN cells in, at present, each one case of dysgenetic and maldescended gonads, respectively, and in the neighborhood of classical seminomas and nonseminomatous GCTs, including teratomas, supports this CIS/TIN concept and also suggests a role for HERV-K in the molecular etiology of all GCTs but spermatocytic seminoma.

The uniform expression of HERV-K in most forms of malignant GCT and malignant GTD raises questions as to the role of HERV-K gene products in the etiology of these lesions. Variable levels of HERV-K *gag* and *env* RNA within a tumor cell population suggest that expression of HERV-K genes may be cell cycle dependent, related to mitotic activity. Inappropriate expression of HERV-K may lead to malignant GTD when occurring during placental development. HERV-K derepression in trophoblast cells may produce precursor cells analogous to CIS/TIN that may explain the occasional occurrence of choriocarcinoma after normal pregnancy. During later stages of embryonal development, derepression of HERV-K in embryonal cells may be restricted to primitive germ cells and gonocytes, giving rise to the formation of CIS/TIN GCT precursor cells. The development of overtly malignant GCTs may then require additional stochastic genetic alterations occurring during adolescence and early adult life. Because of the consistent absence of HERV-K expression from teratomas, repression of HERV-K seems to go parallel to the induction of differentiation along ecto-, meso-, or endodermal lines of embryonal development.

The biological significance of HERV-K expression is currently not clear. HERV-K gene expression may be secondary to the activation of an as yet unknown oncogene. HERV-K gene products may merely represent another phenotypic feature or epiphenomenon of GCT and GTD. The relevance of HERV-K detection may then be limited to clinical applications as a surrogate marker for a molecular process governing the induction of most GCTs and malignant GTD. Conversely, HERV-K gene products themselves may have oncogenic potential when expressed in a specific cellular context. These questions remain to be elucidated

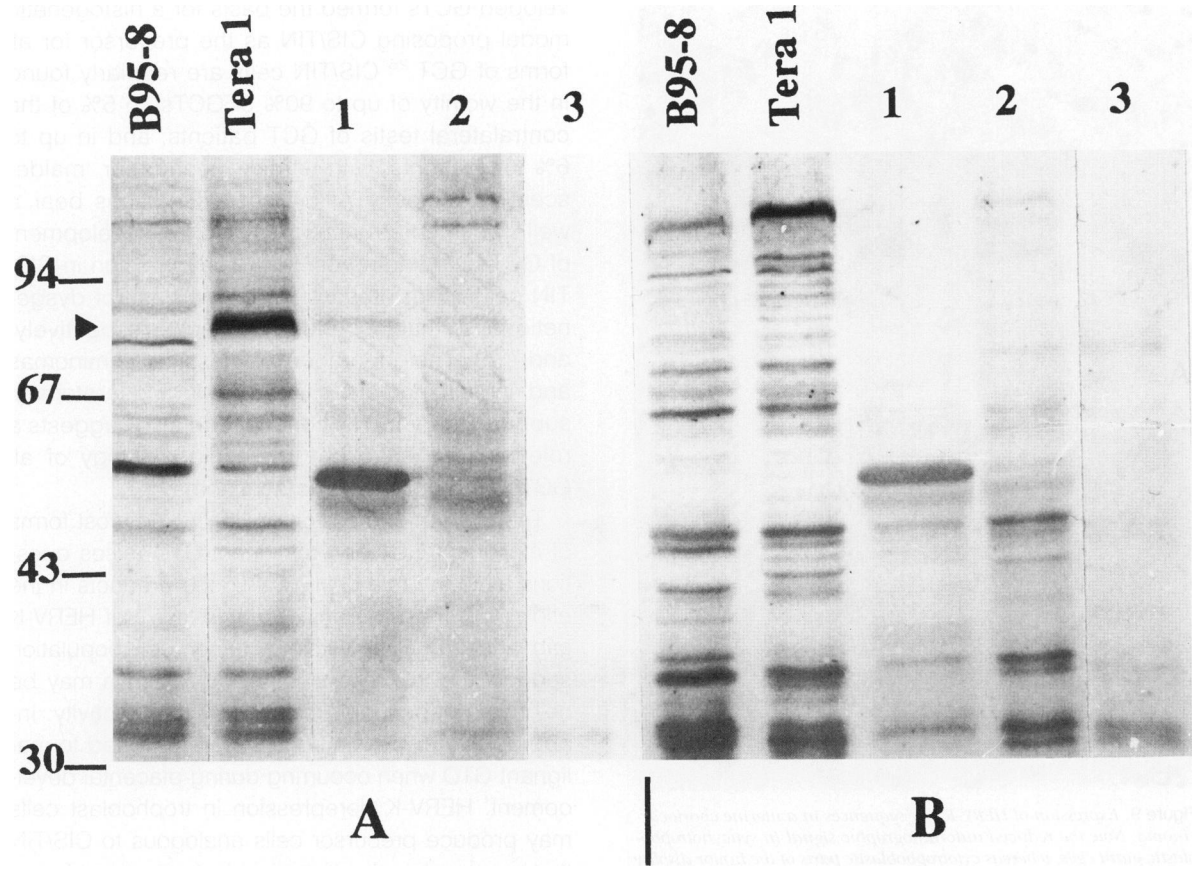

Figure 10. *Immunoblotting analysis of tumor tissues (testicular embryonal carcinoma and mixed testicular GCT composed of seminoma and yolk sac tumor;* lanes 1 *and* 2, *respectively), splenic tissue (*lane 3*), and B95-8 cells serving as negative and Tera 1 cells as positive controls. The position of the 80-kd Gag polyprotein is indicated by an* arrow. *The apparent molecular weight was calculated from co-migrating molecular mass standards and is expressed in kilodaltons. Filter membranes were probed with an anti-Gag polyclonal rabbit serum (*A*) or, for proving the specificity the antiserum, was preincubated with 2 μg of prokaryotic HERV-K Gag fusion protein per ml (*B*).*

in future studies. Future research will also have to address questions as to the role of a putative physiological expression of HERV-K in fetal development, which could not be studied at present due to the lack of fetal gonadal tissue. Such research may ultimately answer the question of why the human genome harbors intact and functional retroviruses, ie, whether such genomes may be beneficial for development.

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