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Identification of human endogenous retroviruses with complex mRNA expression and particle formation

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ABSTRACT **Retroviruses comprise strains with considerable disease potential in animals and humans. In addition to exogenous strains transmitted horizontally, endogenous proviruses are transmitted through the germ line. Some of these endogenous retroviruses can be pathogenic in mice and possibly in other animal species. They may also be considered as mobile genetic elements with the potential to produce mutations. In humans, genomic DNA contains numerous endogenous retroviral sequences detected by their partial relatedness to animal retroviruses. However, all proviruses sequenced so far have been found to be defective. In this communication, we describe the expression of a family of human endogenous retrovirus sequences (HERV-K) in GH cells, a teratocarcinoma cell line producing the human teratocarcinoma-derived retrovirus (HTDV) particles previously described by us. Four viral mRNA species could be identified, including a full-length mRNA. The other three subgenomic mRNAs are generated by single or double splicing events. This expression pattern is reminiscent of the more complex control of virus gene regulation observed, for example, with lenti- or spumavirus strains, although HERV-K shows no sequence homology to human T-lymphotropic virus or human immunodeficiency virus. Sequence analysis of expressed HERV-K genomes revealed nondefective *gag* genes, a prerequisite for particle formation. Open reading frames were also observed in *pol* and *env*. Antisera raised against recombinant gag proteins of HERV-K stained HTDV particles in immunoelectron microscopy, linking them to the HERV-K family.**

A characteristic feature of retroviruses is their ability to persist as integrated proviruses in cellular genomes. While exogenous retroviruses are spread horizontally in their natural hosts, endogenous retroviruses are transmitted genetically and can therefore be detected in the genome of all cells of all members of the host species. A pathogenic potential of nondefective endogenous retroviruses has so far only been demonstrated in mice, in which they may induce tumors and immunological disorders (1, 2). In human DNA, endogenous retrovirus sequences (HERVs) are well known genetic elements. They all seem to be defective due to multiple termination codons, deletions, or the lack of a 5′ long terminal repeat (3, 4). Replication-competent human endogenous retrovirus genomes have not yet been isolated. HERVs are grouped into single and multiple copy number families and are classified according to the tRNA they may use as primer for reverse transcription. Thus, a retroviral RNA that carries a primer binding site homologous to the 3′ end of a lysine tRNA is called HERV-K (3, 4).

Several recent reports indicate that HERVs can be transcribed (5–11). Their expression pattern mirrors their genomic organization: full-length transcripts of multiple copy number families vary in size according to their extent of defectiveness. Subgenomic transcripts with aberrant splice patterns revealing cryptic splice donor or acceptor sequences have been described for the defective high copy number family HERV-H (12). Endogenous retroviral transcripts may also extend into adjacent cellular sequences joining retroviral to cellular genes (13).

Detailed electron microscopic surveys have revealed the existence of retrovirus-like particles in human placentas (14) and teratocarcinoma cell lines (15, 16). Human teratocarcinoma-derived viruses (HTDVs) have been shown to exhibit morphological features indistinguishable from placenta particles (17) and to contain an endogenous RNA associated with reverse transcriptase (RT) activity (18). However, due to the low rate of mature virus synthesis in teratocarcinoma cell cultures and their inability to grow in other host cells (19), their genome could not yet be characterized in detail (18).

Due to the fact that the human genome hosts numerous defective proviruses as evolutionary fossils, a search at the DNA level to identify nondefective human endogenous retroviruses, if they exist at all, appeared futile. Therefore, we resorted to an alternative experimental approach designed to detect retroviral transcripts in cellular RNA (20). Using mRNA from the particle producing teratocarcinoma cell line GH (19) and primers homologous to lysine tRNA, the generated cDNA turned out to encompass the 5′ U5 region of an endogenous retrovirus family called HERV-K (20). These sequences were originally identified by their homology to murine mammary tumor virus (21–23) and to Syrian hamster intracisternal A-type particles (24). The copy number of HERV-K genes was estimated to be 50 per haploid human genome (24). The sequence of one defective provirus was published (ref. 25; Fig. 1 *A* and *B*).§

In this communication, we are able to show that HERV-K proviruses are expressed in teratocarcinoma cells in a complex pattern of mRNA species and that these mRNAs contain long open reading frames with the potential to code for a complete set of viral structural proteins as well as for a putative regulatory protein. Furthermore, HERV-K appears to code for the retroviral particles previously designated HTDV (16).

METHODS

Cells. Cells were grown as described (20). The cell lines Vero (African green monkey kidney), MRC-5 (human fibroblast), A204 (human rhabdomyosarcoma), and AV-3 (human amnion) were obtained from the American Type Culture Collection. The origin of the cell lines JAR (human choriocarcinoma), Tera 2 (human teratocarcinoma), and GH (human teratocarcinoma) was described earlier (19, 20).

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Abbreviations: HERV, human endogenous retrovirus sequence; HTDV, human teratocarcinoma-derived retrovirus; RT, reverse transcriptase.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. X72790 and X72791).

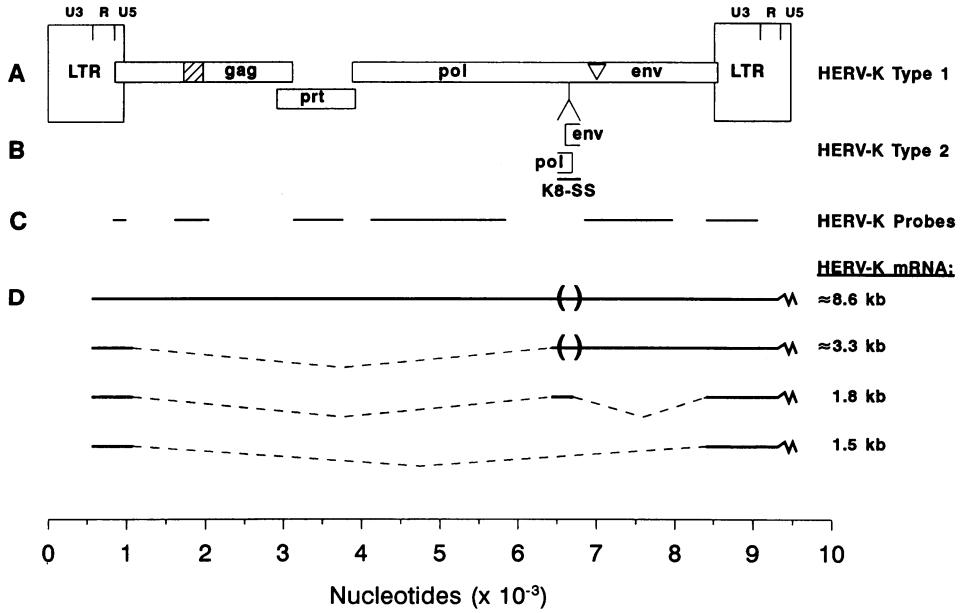

FIG. 1. (*A* and *B*) Schematic representation of genomic organization of HERV-K proviruses. Two types of full-length genomes were described (24), which differ in size by an ≈290-nt insert (designated K8-SS) located at the boundary between the polymerase (*pol*) and envelope (*env*) genes. The complete nucleotide sequence of a genomic clone representing type 1 has been published and named HERV-K10 (25). For type 2, sequence information is only available for an additional 290 nt found in a genomic clone called HERV-K8. A composed sequence had been generated by insertion of these additional nucleotides (K8-SS) into the HERV-K10 sequence at position 6551 and was designated HERV-K10(+) (25). This sequence is used for numbering nucleotides in this report. There is no stop codon between the *pol* and *env* genes in type 1, while in type 2 both genes are separated. The *gag* gene of the published genomic HERV-K10 sequence (25) is split into two reading frames (hatched box, overlapping section). A stop codon interrupts the *env* gene (▽). LTR, long terminal repeat. (*C*) Location of the probes used in Northern blot analyses (see Figs. 2 and 3). (*D*) Expression of HERV-K in GH cells. The overall pattern of mRNAs was derived from Northern blot analysis (see Figs. 2 and 3). The small transcripts as well as part of the *env* gene between nt 6072 and nt 7980 were amplified from GH mRNA and sequenced. Parentheses indicate mRNAs with and without K8-SS inserts.

Northern Blots. The conditions for poly(A)$^+$ RNA preparation, electrophoresis, blotting, and hybridization were detailed recently (20). Probes were generated with poly(A)$^+$ RNA of GH cells by using either a RNA PCR kit (Perkin–Elmer) or cloned and purified simian immunodeficiency virus (African green monkey) RT (ref. 26; gift of H. König, Paul-Ehrlich-Institut, Langen, F.R.G.). Reverse transcription was typically carried out for 10 min at 37°C followed by 30 min at 42°C. Amplification reactions were optimized for each pair of primers. With reference to the HERV-K10(+) sequence (25), the following gene segments were amplified: core protein gene (*gag*), nt 1600–2037; protease gene (*prt*), nt 3124–3765; polymerase gene (*pol*), nt 4114–5843; envelope gene (*env*), nt 6848–7970; U3, nt 8403–9055 (Fig. 1*C*). The U5 probe has been prepared by R-U5 PCR (20).

Cloning and Sequencing of the Small mRNAs. The small transcripts were amplified by RT PCR using primers specific for U5 (GAGGCTGGCGGGATCCTC with an internal *Bam*HI site) and U3 (TGGAGCATACAATCGG with an added *Eco*RI site). After digestion with appropriate enzymes, the products were ligated into the *Eco*RI/*Bam*HI site of pGEM-3. Sequences were determined by the dideoxynucleotide chain-termination method. Critical sections with differences to the published sequence were verified by several runs and sequencing of both strands.

Cloning the *gag* Gene. The entire *gag* gene was cloned in a two-step procedure: nt 972–2424 and nt 2086–3159 were amplified, cloned separately, and linked afterward using an *Hpa* I site in the overlapping region.

Electron Microscopy. Ultrathin sections were prepared and stained according to standard procedures (27). For ultrathin frozen sections, cells were fixed with 2% formaldehyde/0.1% glutaraldehyde. The sections were immunolabeled and embedded in methylcellulose according to Tokuyasu and coworkers (28, 29). Rabbit anti-HERV-K gag antiserum was used at 1:500 dilution. Goat anti-rabbit IgG antibody, coupled to 10 nm colloidal gold (purchased from BioCell, Plano, Marburg, F.R.G.), was diluted 1:100. All pictures were taken with Zeiss electron microscope EM 902.

RESULTS

HERV-K Is Expressed in Teratocarcinoma Cell Lines. Using the cloned 5′ U5 HERV-K cDNA as a probe, a variety of cell lines including the teratocarcinoma line GH were analyzed in Northern blots (Fig. 2). In contrast to all other cells tested, only in GH cells were positive signals revealing four bands at 8.6, 3.3, 1.8, and 1.5 kb detected. To further analyze these mRNA species, probes from the gag, prt, pol, env, and U3 regions were generated by RNA PCR using primers synthesized according to the published HERV-K10(+) sequence (Fig. 1*C*). Probes from the *gag*, *prt*, and *pol* genes only hybridized to the ≈8.6-kb mRNA, which has the size of the predicted full-length transcript. The *env* probe detected both the full-length message as well as a broad band around 3.3 kb, which also hybridized to the U3 probe. Thus, this 3.3-kb transcript is composed of sequences from U5, *env*, and U3 regions as expected for a singly spliced subgenomic retroviral env mRNA. The small RNA species hybridized only to U5 and U3 probes, lacking detectable *gag*, *prt*, *pol*, and *env* sequences. These RNAs may have been transcribed from defective genomes or may be the consequence of alternative splice acceptor usage. In addition to these prominent signals, another 4.5-kb RNA hybridized strongly to *env* and U3 probes but only very faintly to the U5 probe. Whether these RNA transcripts result from unusual splicing events or indicate expression of defective HERV-K proviruses remains to be elucidated. In the teratocarcinoma cell line Tera 2 in which HTDV particles are seen extremely rarely, HERV-K transcripts can be detected only after very long exposure times

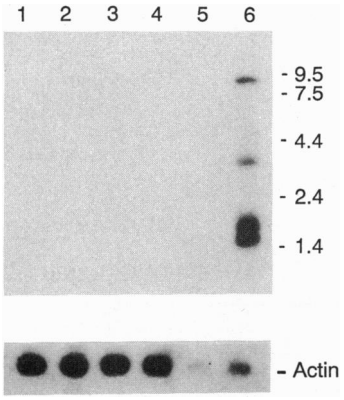

FIG. 2. Northern blot analysis of poly(A)$^+$ RNAs of the following cell lines with a probe specific for HERV-K U5: Vero (lane 1), MRC-5 (lane 2), A204 (lane 3), JAR (lane 4), AV-3 (lane 5), and GH (lane 6). The probe was synthesized by R-U5 PCR of putative endogenous retroviral mRNAs (see ref. 20) using a primer homologous to the 3′ end of lysine tRNA. To verify the presence of RNA, the blot was rehybridized with a chicken β-actin probe (gift of B. Paterson, National Institutes of Health, Bethesda, MD). In additional experiments, use of higher amounts of AV-3 mRNA gave the same negative result as illustrated here. Numbers on right are kb.

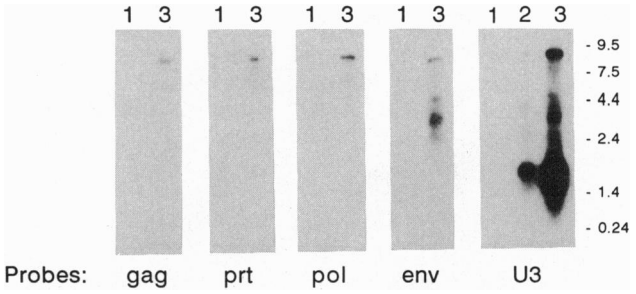

FIG. 3. Northern blot analysis of poly(A)$^+$ RNAs of the cell lines MRC-5 (lane 1) and GH (lane 3) with probes specific for selected parts of the HERV-K genome. In addition, the hybridization pattern of another teratocarcinoma cell line, Tera 2 (lane 2), with a U3-specific probe is shown. Numbers on right are kb.

(Fig. 3). Interestingly, a 1.5-kb message could not be detected and transcripts in the range of 3.3–4.5 kb are of slightly different sizes compared to GH transcripts. As HERV-K represents a multicopy number family, it is conceivable that in Tera 2 another subset of proviruses is expressed.

Unusual Small HERV-K Transcripts. Each retroviral transcript starts with the sequence R-U5 and ends with U3-R. To study the small transcripts in GH cells RNA PCR was initiated by using primers from the 5′ U5 and 3′ U3 sequence of HERV-K10(+), yielding two products of 0.9 and 1.2 kb. In experiments with genomic DNA, a product of different size was amplified with the same primers, which is most probably derived from a defective provirus (data not shown). These results suggest that the small HERV-K transcripts are generated by splicing. In all RNA PCRs, no PCR signal could be detected when RT was omitted, excluding the possibility of contaminating DNA in RNA preparations.

Cloning of the 0.9-kb PCR product and subsequent sequencing of several clones revealed a transcript encompassing the 5′ sequences of HERV-K up to a splice donor at nt 1076, the existence of which had already been postulated by Ono *et al.* (25). A splice acceptor could be defined in the 3′ *env* region at nt 8408 (see Fig. 1*D*). A total of three clones were sequenced, with each of them exhibiting far more point mutations with respect to each other and to HERV-K10 as can be expected from PCR or RT errors (30–32). Thus, it is likely that more than one of the different HERV-K proviruses are transcriptionally active in GH cells. Taking into account that at both ends of the observed 0.9-kb RNA sequence 253 nt of the R region as well as a 3′ poly(A) tail have to be added, the resulting viral mRNA has a predicted length of 1.4–1.5 kb, which corresponds well to the 1.5-kb Northern blot band (Fig. 3).

Cloning and sequencing of the 1.2-kb PCR product revealed a multiply spliced transcript. The same splice donor as for the 0.9-kb PCR product is used to excise the *gag*, *prt*, and *pol* genes, but sequencing revealed a different splice acceptor in the *pol*/*env* junction region (nt 6433). The sequence downstream of this splice acceptor is at the beginning homologous to the published HERV-K10(+) sequence but thereafter homologous to the HERV-K type 2-specific sequence (K8-SS in Fig. 4), uncovering another splice donor (nt 6708) that connects the sequence to the splice acceptor at nt 8408 described above (Fig. 4). Again, adding the size of two R regions and a poly(A) tail to the observed 1.2-kb PCR product results in a 1.8-kb sequence, which corresponds to the 1.8-kb polyadenylylated mRNA detected in Northern blots (Fig. 3). A product with identical structure and sequence (except for point mutations) was obtained with Tera 2 mRNA using the same U5 and U3 specific primers.

The sequence described here exhibits remarkable differences to the published genomic HERV-K10(+) sequence. First of all, a stop codon (TAA at nt 6495–6497) is changed to an arginine codon (AGA). Second, two insertions (A after nt 6612, C after nt 6687) are found. These alterations contribute to an open reading frame that has the potential to encode a 12-kDa protein. Analysis of the protein reveals a stretch of basic amino acids (RRRRHRNR, nt 6486–6509). This basic domain closely resembles the basic domain of regulatory proteins of lenti- and spumaviruses (Fig. 5). It is assumed that these stretches of basic amino acids are essential for RNA binding (36–38). In particular, recent data emphasize the role of a single arginine (position 6 in Fig. 5)

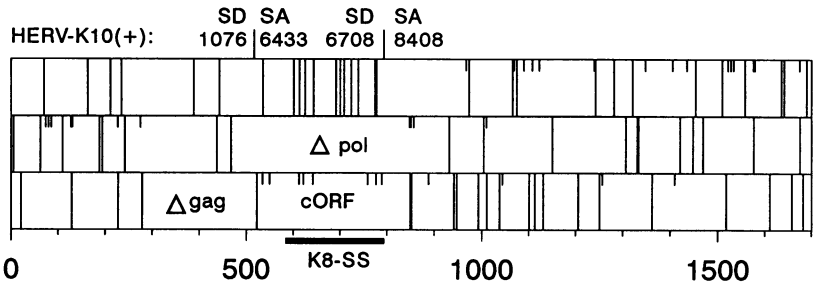

FIG. 4. Analysis of the 1.8-kb HERV-K mRNA for open reading frames (ORF). mRNA was amplified and sequenced between nt 254 and nt 1442. The remaining redundant sequences (5′ R and 3′ R) were taken from the published HERV-K10(+) sequence (25). In the three reading frames, long vertical lines represent stop codons; short vertical lines represent codons for methionine. Positions of splice donors (SD) and splice acceptors (SA) relative to the HERV-K10(+) sequence are indicated. Parts of the ORFs for *gag* and *pol* are marked Δgag and Δpol. Because of its position in the genome, the newly described ORF is tentatively designated central ORF (cORF).

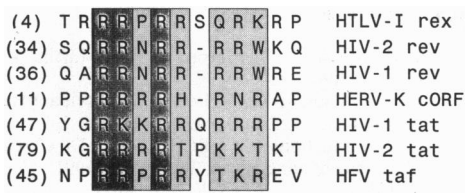

FIG. 5. Comparison of putative RNA binding domains of HIV-1 and HIV-2 tat and rev (EMBL nucleotide sequence data base accession numbers P04606, P04616, P04605, and P04615, respectively), HTLV-I rex (33), human foamy virus taf (bel1; refs. 34 and 35), and HERV-K cORF. Highly conserved amino acids are darkly shaded and less well conserved amino acid positions are lightly shaded. Numbers delineate position of the first amino acid in the corresponding protein.

for binding of tat to the bulge of the TAR stem–loop structure (39). This arginine embedded in a basic surrounding is conserved between all domains listed.

The complex splicing pattern of the 1.8-kb mRNA of HERV-K is similar to the splicing pattern of mRNAs coding for regulatory proteins of lenti- and other complex retroviruses. Furthermore, the existence of a putative RNA binding motif suggests that this HERV-K transcript might also code for a regulatory protein. The splice events are summarized in Fig. 1*D*. To our knowledge, there has been no previous report that a human endogenous retrovirus family exhibits the complex RNA expression pattern reminiscent of exogenous retroviruses.

Analysis of the HERV-K *gag*, *pol*, and *env* Genes. In an initial attempt to analyze the region between the *pol* and *env* genes, a sequence was amplified by RNA PCR starting at nt 6078 in the putative integrase domain and ending at nt 7970 in the *env* gene. Sequencing of two resulting clones demonstrated that they belonged to HERV-K type 1 (Fig. 1*A*) and that they differed in an internal *Eco*RI restriction site. No stop codon could be determined in the putative *pol*/*env* junction. One of the clones had exactly the same sequence as HERV-K10, revealing that this provirus is also transcriptionally active. The other clone differed from HERV-K10 in several point mutations, one of which abolished the stop codon in *env* (▽ in Fig. 1*A*).

In the published HERV-K10(+) provirus sequence, a frameshift splits the *gag* region into two open reading frames preventing normal *gag* gene expression (Fig. 1*A*; ref. 25). As there are ≈50 copies of HERV-K in the human genome (24), it is conceivable that not all of the transcripts may have the same *gag* gene structure. Therefore, we studied *gag* genes expressed in GH cells by amplifying with the help of RNA PCRs a region from nt 1600 to nt 2037 surrounding the frameshift between the two *gag* open reading frames in the published HERV-K10(+) sequence. Four individual clones were sequenced. They exhibited a limited number of point mutations but differed from HERV-K10(+) in one significant feature—they all possessed a G insertion after nt 1749, leading to a long open reading frame as expected for a functional *gag* gene. To confirm these results, RNA PCR was performed with primers located at both ends of the *gag* region (nt 972–3159). The resulting fragments were cloned and three clones were sequenced. They all had the G insertion abolishing the stop described for HERV-K10(+). One clone had a stop codon 175 nt downstream from the G insertion, but the other two possessed long open reading frames spanning the whole *gag* region (data not shown).

HERV-K Codes for HTDV Particles. One of us (N.M.-L.) had recently cloned the HERV-K *gag* region from cellular DNA, expressed the gene in *Escherichia coli*, and raised antibodies to the purified product in rabbits (40). When we used this gag antiserum in immunoelectron microscopic studies with GH cells, it stained the HTDV particles (Fig. 6). As there is also a quantitative correlation between the increased synthesis of HERV-K mRNA and HTDV in GH compared to Tera 2 teratocarcinoma cells, it is very likely that one or more HERV-K proviruses code for HTDV.

DISCUSSION

HERVs were previously only known as defective genomes unable to code for virus particles. Since there are so many defective HERVs (0.6–1% of the haploid genome; refs. 3 and 4), direct cloning and sequencing of corresponding genomic DNA is unlikely to lead to the identification of putative full-length nondefective endogenous retroviral loci.

Approximately 50 copies of HERV-K are integrated in the human genome. As seven slightly different *gag* gene sequences could be identified, our studies suggest that a number of different HERV-K proviruses are transcriptionally active. The known prototypic HERV-K10(+) sequence contains only a few stop codons disrupting longer open reading frames (25), suggesting the probability that other copies of the proviruses may possess open reading frames for one or all genes. Indeed, our sequencing of PCR products of critical areas revealed that the stop codon described for the putative *env* region may be absent. Similarly, the published disruption of the *gag* gene by a frameshift (25) could not be demonstrated in the HERV-K mRNA in GH cells, suggesting that a complete set of gene products can be expressed.

HERV-K expression in the teratocarcinoma cell lines GH and Tera 1 (20) strongly resembles the pattern observed for exogenous retroviruses: full-length transcripts are accompanied by subgenomic env mRNAs. Singly and multiply spliced retroviral mRNA species coding for regulatory proteins were described for lenti- and spumaviruses as well as for murine mammary tumor virus and HTLV (41–43). In HERV-K, a singly spliced small mRNA reveals a splice acceptor site 93 nt upstream of the U3 region. It contains no large open reading frame. A similar small mRNA has recently been described for another family of human endogenous retroviral sequences (ERV-9; ref. 44). In contrast, the doubly spliced transcript of HERV-K was found to contain an open reading frame with coding capacity for a protein carrying a putative RNA binding motif.

In Northern blots, expression of HERV-K could only be demonstrated in teratocarcinoma cell lines but not in other human lines. Preliminary RT PCR studies suggest, however, that HERV-K may be expressed in many if not all human cells at levels too low to be detectable in Northern blots (data not shown). The basis for the significant quantitative difference in expression between teratocarcinoma cells and other

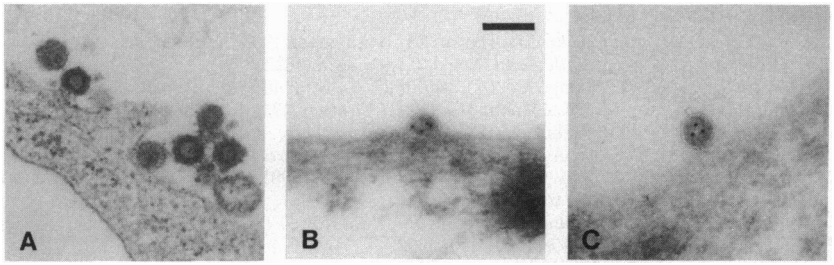

FIG. 6. HTDV particles from GH cells. (*A*) Ultrathin section of resin-embedded cells. (*B* and *C*) Ultrathin frozen sections immunolabeled with monospecific rabbit anti-HERV-K gag antiserum. (Bar = 200 nm.)

cell lines is not clear. It is intriguing to speculate that a cellular factor(s) may regulate the synthesis of HERV-K mRNA depending on the cell type or the state of differentiation. In this context, it should be remembered that other retroid elements [ERV-9 (9), RTVL-H (20, 45), LINE-1 (46; unpublished observation)] are also preferentially expressed in human teratocarcinoma cells.

The HTDV particles, which can be observed in human teratocarcinoma cells by electron microscopy (17), have not yet been shown to be infectious (18). They possess a distinct morphology: (*i*) they lack the electron lucent space normally seen between viral envelope and core, (*ii*) they seem to be predominantly arrested in budding stages, and (*iii*) collapsed cores, a consequence of a final proteolytic step in virus maturation, have never been observed. As long as the *gag* precursor remains uncleaved, resulting particles are not likely to be infectious. However, preliminary information indicates that HERV-K *prt* genes can code for an enzymatically active protein (40).

In some retroviruses, the viral RT proteins are translated by a double frameshift event (47). In HERV-K10(+) the *gag*, *prt*, and *pol* genes are located in different reading frames and synthesis of RT would also require a double frameshift. In the HERV-K10 genome, there is no stop codon at the *pol/env* boundary (Fig. 1*A*; ref. 25), an observation we could confirm in two clones. Synthesis of a large pol/env fusion protein would presumably result in a drastically impaired polymerase activity. HTDV particles contain a low amount of active reverse transcriptase, but the enzyme is unique in that it favors an endogenous reaction using the viral RNA as template over an exogenous reaction with synthetic templates (18).

It is often very difficult to demonstrate the assembly of viral envelope proteins in electron micrographs, as env proteins are easily sheared from the particle surface during preparation procedures. In some electron micrographs, HTDV spikes can be observed (e.g., Fig. 6). In any case, we have already found one HERV-K transcript with an open reading frame in *env*. The predicted features of the HERV-K proteins suggest that the HERV-K family encompasses a candidate genome for HTDV particles and include the possibility that several expressed copies might have to complement each other. The fact that the HTDV particles are stained by an anti-HERV-K gag antiserum in immunoelectron microscopy strongly supports this assumption.

Expression of endogenous retroviruses has been reported in many animal tumor cell lines (1), but they have only occasionally been shown to be causally responsible for tumor formation. Nevertheless, such potentially transposable genetic elements might participate in the development of human diseases by processes involving reinsertion of proviruses at new sites. It will be interesting to elucidate the role, if any, of HERV-K in tumorigenesis.

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