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Expression and Packaging of a Human Endogenous Retrovirus-K Genomic DNA Clone

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Human contains large number of human endogenous retroviruses (HERVs) in its genome. One of the HERV families, HERV-K, entered human genome most recently and includes many members with full-length intact proviruses. Normally, these proviruses do not express but infrequently they seem to express in cancers or autoimmune disease patients. To investigate expression mechanisms of these endogenous retroviruses, a DNA copy of HERV-K was cloned and its expression was studied. The transfection of the full-length clone into human cell lines did not produce any detectable viral capsid protein, Gag, and the transcription from its own promoter in LTR was extremely poor. The transcription was less than 10 percent compare to the exogenous retrovirus. However, when the Gag coding region was cloned under CMV promoter, Gag could be expressed efficiently and secreted as particles, probably virus like particles. The efficient expression also required a nuclear export signal. The expressed Gag could also package its own genomic RNA. These results indicate that the LTR of HERV-K is normally not active but its genes have a potential to express and possibly produce infectious particles.

Key Words: HERV-K, Genome, Gag, Expression, Packaging

INTRODUCTION

HERV sequences make up about 8% of the human genomic DNA (11). Evolutionarily they have entered the genome millions of years ago by germline infections of former exogenous retroviruses (14). Over the last two decades, there have been numerous reports linking human endogenous retroviruses (HERV's) with diseases such as cancer, diabetes, multiple sclerosis and others [see review (1)].

Most HERVs are defective because they have accumulated numerous nonsense mutations over time (14). However, a few exceptions are known. In particular, several proviruses belong to HERV-K (HML-2) family still display intact retroviral genes for Gag, Prt, Pol, or Env (2,7,21,23) making them potentially the most recent of the ancient proviruses. HERV-K also encodes a regulatory protein, Rec (formally known as cORF). Rec is a functional homologue of Rev (15). The Rec protein binds to an RNA element, the Rec-responsive element (RcRE), in the 3' LTR of HERV-K and mediates nuclear export through the RcRE (16). HERV-K is closely related to types A and D retroviruses, such as mouse mammary tumor virus MMTV and MPMV (20). One of the HERV-K family, HERV-K108, has complete uninterrupted ORF including REC. HERV-K108 has close sequence similarity to HERV-K113 and 115 which are known to be most recent entered to the human genome accounted by the mutation in the LTR and the presence of polymorphism in the population (3,25).

HERV-K family attracted some attention partly due to its possible linkage to cancers, autoimmune diseases and the

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presence of its virus like particles (VLPs) in cancer cell lines such as teratocarninoma (13) or melanoma (19). Some members also encode active enzymes and full-length Gag protein (18,22), and seem to direct the synthesis of intact (but noninfectious) virions seen in placenta and in some malignancies (5). Recently, it has been reported HERV-K has functional envelope gene (7). The expressed envelope gene could be pseudotyped with other retroviruses and was able to infect a certain cell type, though not a human cell type tested.

Despite frequent detection of HERV-K mRNAs in many different tissues, more in cancer tissues, the presence of full-length ORFs, as well as production of VLPs in a few carcinoma cell lines, the activity of HERV-K as infectious agent is not yet observed. A teratocarcinoma cell line, GB, produces HERV proteins as well as VLPs into the medium. The VLPs from GB cell supernatant showed reasonable RT activity (21), however the VLPs were not infectious. It is possible that the RT activity observed in VLPs could be derived from some other sources. It is also possible that the Gag protein might not be able to package corresponding viral genomes. The full-length clone of HERV-K/HDTV generated from cDNA library of teratocarcinoma cell line could produce VLPs in baculovirus expression system, but was not infectious (21). So far, most studies were based on the cDNA clones expressed in tumor cells since they were unable to find the corresponding genomic copy to those cDNA. It is possible that the genomic copy of HERV expressing tumor cell lines contain multiple mutations different from the ones in normal cellular genome (19).

In this study, we have tested if the genomic copy of HERV-K from normal human DNA has functional HERV-K Gag protein and if it can package the genome. To study if normal HERV genomic copy has the ability to express functional proteins, one of the most recently entered endogenous retrovirus, HERV-K 108, was cloned from normal human DNA and analyzed for its expression.

MATERIALS AND METHODS

1. Cell lines and transfection

A human embryonic kidney cell line, 293T, the African green monkey cell line, COS-1, a dog osteosarcoma cell line, D17, and a mouse fibroblast cell line, NIH3T3, were maintained in DMEM with 10% fetal calf serum (FCS). Tera-1, a human teratocarcinoma cell line, was purchased from ATCC and cultured in McCoy's 5A (Invitrogen) with 15% FCS. Transfection was carried out by lipofectamin plus reagent (Invitrogen) as the manufacture's instruction.

2. Cloning of full length HERV-K 108 genome

The human genomic DNA was purchased from Roche. The genomic clone of HERV-K108 was cloned in two PCR steps. The primers were designed based on the sequence in GenBank (AC074054). PCRs were performed with *Taq* Platinum High Fidelity (Invitrogen) in 50-μl volumes. In general, reactions were set up with the following mixture: 1 μg human genomic DNA 5 pmol each primer, 1 unit polymerase, 2 mM deoxynucleoside triphosphates, 1 x manufacturer's buffer and distilled water to a final volume of 50 μl. The 5' portion including 5' LTR and *gag*, *pol* and 5' region of *env* was PCR amplified using the forward primer in 5' flanking sequence (TTTTTATTTTAGTAGAGATGGGTTTC) and the reverse primer in *env* region (AGGAGGATAATGATACCCAATG). The 3' portion of HERV containing *env* and 3' LTR was PCR amplified using the forward primer in *pol* region (GAAATAGGGAAGGTGATAACGT) and the reverse primer in 3' flanking sequence (TAATTTGGGGGATTTTAGATATTAG). Two PCR products were cut with AatII (New England Biolab), gel purified and ligated. The ligated PCR product was then cloned into pCR-BluntII-Topo vector (Invtrogen). Two independent clones, pF9 and pF12, were picked and a full-length clone (pF9) was confirmed by restriction mapping and sequencing.

3. LacZ reporter construction

The lacZ expressing packaging reporter, pK-lac, was cloned by replacing the gag-env region of pF9 with *lacZ* open reading frame (ORF). The *lacZ* ORF was derived from pSV-β galactosidase (Promega, nucleotide number 686~3793). The *lacZ* ORF was PCR amplified with 5' forward primer (CCCGGGAGATCTAACTAGTGCGCTGTATGGCGAGAT) and 3' reverse primer (CCCGGGACTAGTAGGCCTGCGAAATACGGGCAGAC). The pF9 was cut with BglII and SpeI (nucleotide number 1276~7780 based on AF164614) and replaced with PCR amplified *lacZ* ORF fragment. The pMP-lac reporter was cloned by replacing the *gag-env* region (nt 1386~6680 of M12349) of MPMV

with *lacZ* ORF. For this, the full length MPMV clone, pSHRM15 (generous gift from Dr. S. Rhee), was cut with SpeI and replaced with the SpeI digested lacZ PCR product.

4. LacZ in-situ assay

Two days after transfection, the cells were washed once with PBS, fixed with 0.2% paraglutaldehyde in PBS for 15 min and then stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) solution [0.1 M $NaPO_4$, pH 7.3, 1.3 mM $MgCl_2$, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6$, 1 mg/ml X-Gal].

5. The Gag expression plasmids

The HERV-K *gag* ORF was PCR amplified from pF9 using the primer pair (5' primer-CACCATGGGGCAAAC-TAAAAGTAAAAT; 3' primer-GTCGACCTGCTGCACT-GCCGC) and cloned into pcDNA3.1D/V5-His-Topo vector (Invitrogen), pKgag13A-His. MPMV *gag* ORF was PCR amplified from pSHRMl5 using the primers (5' primer-ACCATGGGGCAAGAATTAAGCC; 3' primer-GTCGA-CATACTGTGTGGGAGGTGGAAC) and cloned into pcDNA3.1D/V5-His-Topo, pMPgag3A-His. The pKgag13A -His and pMPgag3A-His were also modified to have HA tag by replacing His tag with HA tag (pKgag13A-HA and pMPgag3A-HA, respectively). The Gag expression constructs with constitutive transport element (CTE) were cloned as following. The CTE fragment (pSHRMl5) was PCR amplified with the primers (5' primer-GGGTCT-AGAACCTCCCCTGTGAGCTAGACGGGTCTAGA; 3' primer-CCAAGACATCATCCGGG) from pSHRMl5 and cloned into XbaI site of pKgag (pKgag13A-CTE) and pMP-gag (pMPgag3A-CTE).

6. Isolation of VLP

Two days after transfection, the supernatant was collected and filtered through HA filter (Milipore) to remove cell debris. The filtered supernatant was centrifugated through 20% sucrose cushion for 20 minutes, 300,000 x *g*.

7. Western blot analysis

Two days after transfection, the VLP pellet was resuspended directly by 1 x protein loading dye. The protein was analyzed through SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked in 5% skim milk and 0.1% Tween 20 in PBS for 1 h, and incubated sequentially with the primary antibody for 1.5 h, and the horse radish peroxide (HRP)-conjugated secondary antibody for 1 h. The protein band of interest was detected using the Western Blot ECL Reagent (Amersham). The anti-HERV-K Gag antibody was kindly provided by Dr. Sauter (Universitat Skliniken Des Saarlandes, Germany) and anti-MPMV Gag antibody was provided by Dr. Rhee (Public Health Research Institute, USA).

8. RT-PCR and realtime PCR

After 2 days of transfection, cells were washed twice with PBS and total RNAs was prepared by Qiagen total RNA prep kit. For VLP associated RNA preparation, the transfected cell supernatants were filtered through HA filter (*Milipore*) and centrifuged 300,000 x *g* for 20 minutes. The RNA associated with VLPs was purified by Qiagen total RNA prep kit. For quantitative RT-PCR, The RNAs were treated 3 times with Rnase-free DNaseI to remove any residual plamid DNA contamination. DNA-free RNA was resuspended in 50 μl and 5 μl was used for cDNA synthesis. 2.5 μl of cDNA was used RT-PCR or realtime PCR. cDNA was synthesized by reverse transcription with Moloney murine leukemia virus (MMLV) reverse transcriptase and an oligo dT primer in 50 μl. For RT-PCR, cDNA was 1'st amplified with the MPMP specific 5' forward primer (CACCATGGGGCAAGAATTAAGCC) and 3' reverse LacZ primer (described above) or HERV-K108 specific 5' forward primer (TGATAATGGGGCAAACTAAAA) and 3' reverse LacZ primer. 2 μl of 1'st amplified PCR reaction was re-amplified with nested LacZ primer set (5' primer-TGGCAGGGTGAAACGCAGGTC; 3' primer-CGTTCA-GCAGCAGCAGACCAT). Real time PCR was performed in a SYBR Green PCR Master Mix (Applied Biosystems) containing SYBR Green 1 Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, lacZ primer set, and optimized buffer components. Initial activation at 95℃ for 10 min and 40 cycles at 95℃ for 15 sec / 60℃ for 1 min were carried out using the GeneAmp 7000 Sequence Detection System (Applied Biosystems). The amount of mRNA of the samples was normalized to that of human GAPDH using the ΔCt method, as described in manufacture's protocol.

RESULTS

1. Cloning and expression of full length HERV-K108

To study if the genomic HERV-K sequence can be expressed and packaged, the full length genomic fragment of HERV-K108 was cloned as described in Materials and Methods. A full-length construct, pF9, was chosen and confirmed the sequences to have complete proviral structures and sequences including LTR and ORF of *gag*, *pol*, *env* and *rev* (Fig. 1A). pF9 showed a few sequence differences different from the reported ones in GenBank. It could be due to the PCR cloning errors. However, considering the several GenBank sequences were slightly different each other, it was also possible that this represented differences in samples.

To test if these full-length clones could express Gag proteins, several cell lines, 293T, COS-1 and D17, were transfected with two independent full-length constructs, pF9 and pF12. After 2 days transfection, the cell supernatant was collected and VLPs were isolated through 20% sucrose cushion and analyzed by the western blot. For a comparison, a replication competent D-type retrovirus MPMV DNA clone, pSHRM15, was used as a control. As shown in Fig. 1, MPMV full-length genome could efficiently express and produced infectious particles in human cell lines as known before. MPMV Gag cross-reacted with HERV-K Gag antibody weakly and p27 and a few MPMV Gag proteins could be detected. The expression of MPMV Gag was also confirmed by anti-MPMV Gag antibody. MPMV expressed best in a human cell line, 293T cells, but not in dog cell line, D17. However, pF9 and pF12 did not produce any visible amount of Gag in any of the cell lines either in the supernatant or within the cell (data not shown).

2. Cloning and expression of Gag proteins

For expression HERV-K108 Gag proteins in human cell lines, HERV-K108 or MPMV *gag* coding regions were cloned into pcDNA vector to have His tag (pKgag13A-His and pMPgag3A-His) or with HA tag (pKgag13A-HA and

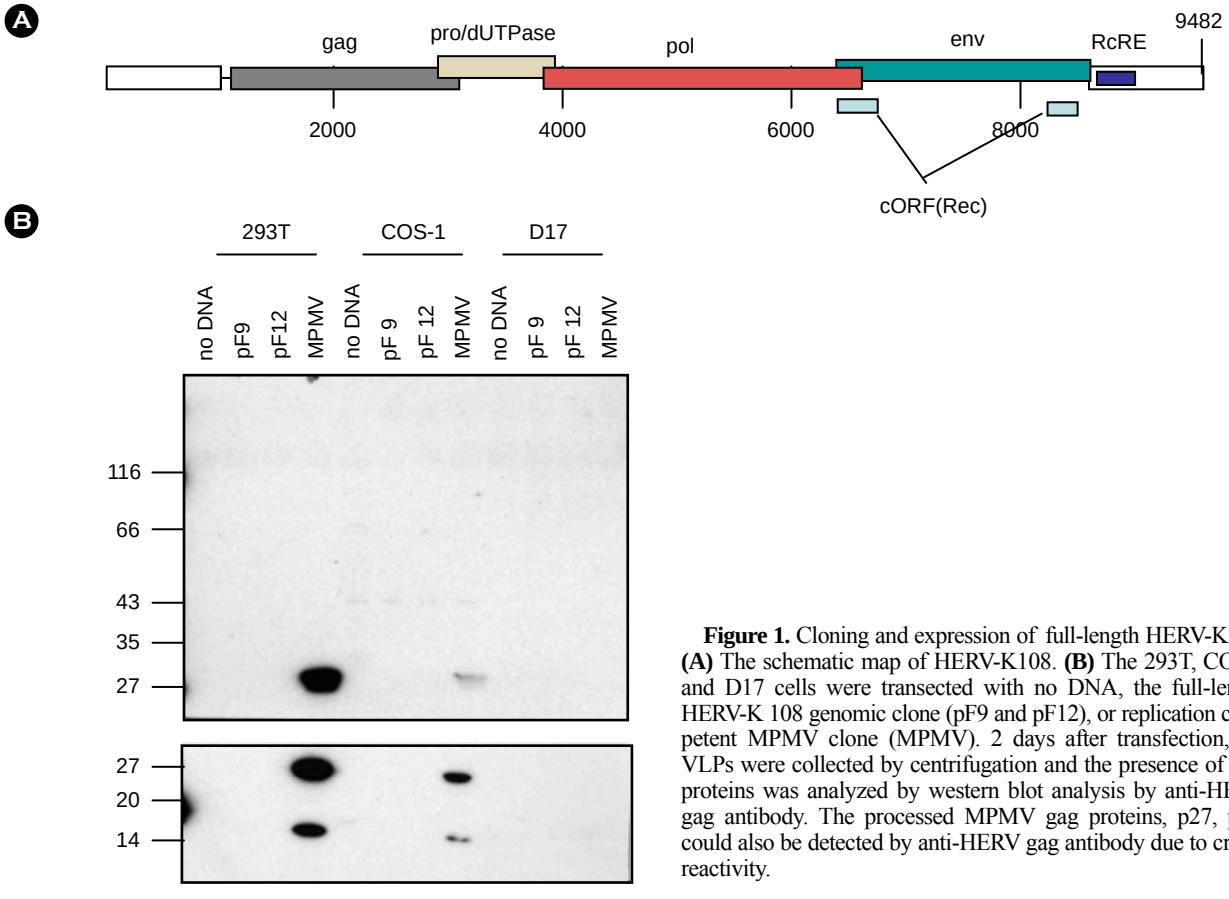

Figure 1. Cloning and expression of full-length HERV-K108. **(A)** The schematic map of HERV-K108. **(B)** The 293T, COS-1 and D17 cells were transected with no DNA, the full-length HERV-K 108 genomic clone (pF9 and pF12), or replication competent MPMV clone (MPMV). 2 days after transfection, the VLPs were collected by centrifugation and the presence of gag proteins was analyzed by western blot analysis by anti-HERV gag antibody. The processed MPMV gag proteins, p27, p17, could also be detected by anti-HERV gag antibody due to cross-reactivity.

pMPgag3A-HA), respectively (Fig. 2A). However, the expression of these clones was extremely poor (Fig. 2B and C, lanes 2 and 3). It was possible that the export or the stability of HERV mRNA required the nuclear export signal, such as Rec (17). We therefore cloned the cis-acting constitutive transport element (CTE) sequence of MPMV, which should eliminate the necessity of Rec expression (6), into the *gag* constructs (Fig. 2A). This strategy indeed increased the expression to a detectable level, indicating the importance of nuclear export signal in HERV-K and MPMV Gag expression (Fig. 2B and C, lanes 4). As in Fig. 2B, the *gag* gene constructs with CTE, pKgag13A-CTE and pMPgag3A-CTE, efficiently produced unprocessed gag polyproteins, respectively. Furthermore, the unprocessed Gag protein was secreted into the medium probably forming particles that could pellet through 20% sucrose cushion, implying the formation of VLPs.

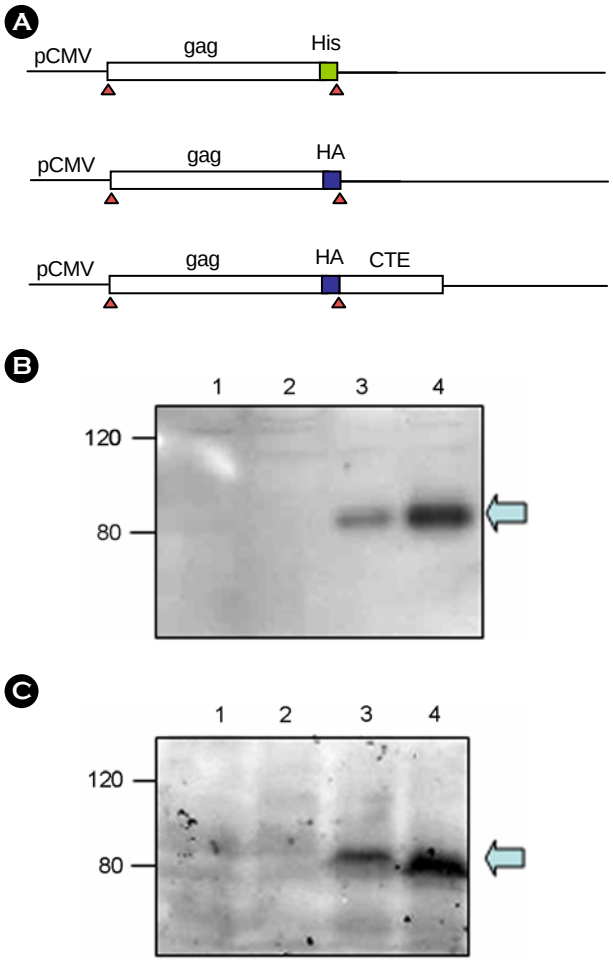

Figure 2. Gag expression requires nuclear export signal. **(A)** the schematic drawing of gag expression clones. The small triangles indicate the location of start and stop codons. His, histidine tag; HA, hemagglutinin tag; CTE, constitutive transport element. **(B and C)** 293T cells were transfected with various constructs including gag expression clones. 2 days after transfection the supernatants were collected and analyzed by western analysis with anti-HERV gag antibody **(B)**. 1. no DNA; 2. pSV-lac (negative plasmid control); 3. pKgag13A-HA; 4. pKgag13AHA-CTE. **(C)** Western analysis with anti-MPMV gag antibody. 1. no DNA; 2. pMPgag3A-His; 3. pMPgag13A-HA; 4. pMPgag13A-CTE. The arrows on the right indicate gag proteins.

3. Cloning LacZ expression packaging reporter

The packaging reporter clones were cloned such that *β-galactosidase* gene was expressed under normal LTR promoters. These clones retained intact proviral sequences including LTR and about 100 nt of 5' *gag* known to be required for MPMV packaging (Fig. 3A). The HERV K reporter, pK-lac, and MPMV reporter, pMP-lac, were then transfected to human cell lines, 293T and Tera-1, and a mouse cell line, NIH3T3, and compared for *β-galactosidase* gene expression. As can be seen in Fig. 3B, pK-lac reporter showed extremely weak β-galactosidase activity compared to pMP-lac in those human cell lines. However, in NIH 3T3 cells pK-lac expressed large amounts of β-galactosidase, indicating that the constructs were capable of producing active protein. The poor expression of β-galactosidase in 293T cells was probably due to the weak promoter activity of HERV LTR in human cells. Several other human cell lines also revealed similar results as in 293T (data not shown). The realtime PCR confirmed that the transcription from pK-lac reporter indeed was 10 times less than pMP-lac (Table 1).

4. Packaging of lacZ reporter RNA into Gag particles

To evaluate the packagibiligy of HERV-K RNA by its

Table 1. 293T cells transfected with pK-lac and pMP-lac and the cytoplasmic and nuclear RNA were prepared from 2 days after transfection. The RNA samples were normalized with GPDH and relative amount of RNA was calculated with realtime PCR with the appropriately diluted control DNA (pMP-lac)

	DNA transfected to 293-T	Relative amount of RNA
Cytoplasmic RNA	pK-lac	37.8
	pMP-lac	357.0
Nuclear RNA	pK-lac	32.4
	pMP-lac	417.0

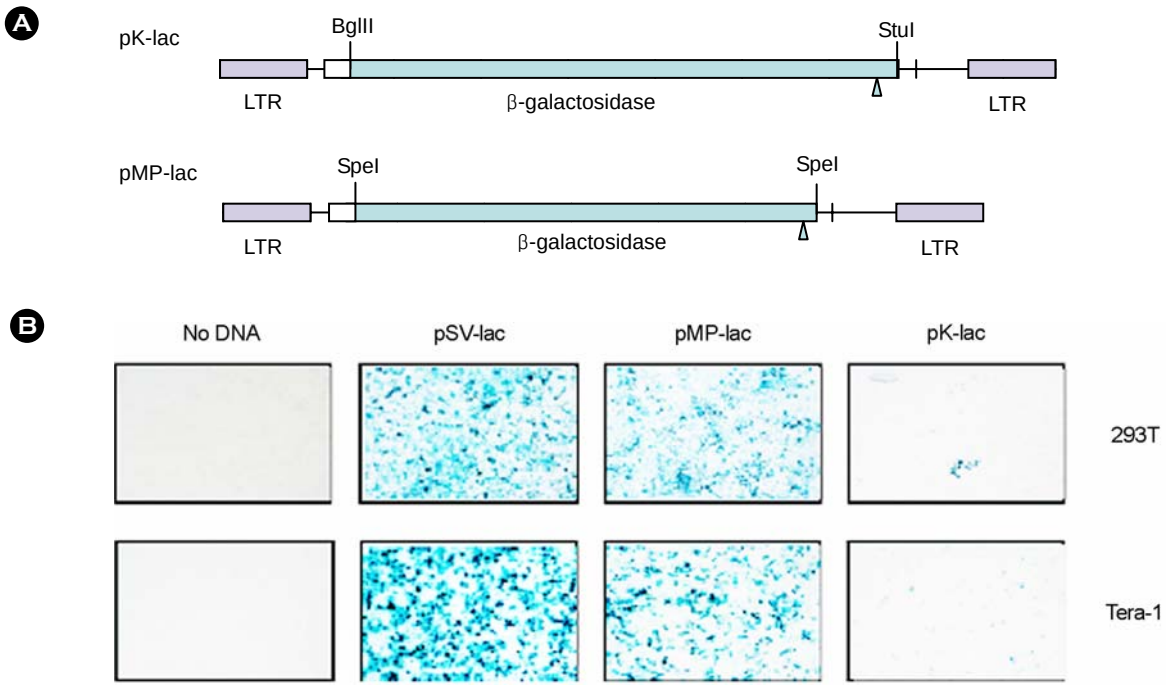

Figure 3. Construction and expression of LacZ reporter constructs. **(A)** the schematic drawing of LacZ expression reporter. The triangles indicate the location of translation stop site. **(B)** LacZ reporters were transfected into 293T and Tera-1 cells and the LacZ expression was analyzed *in-situ* X-gal staining as described in Materials and Methods.

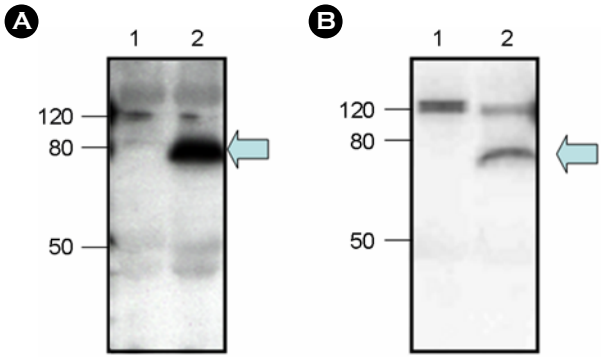

Figure 4. co-transfection of LacZ reporter with gag expression clones. **(A)** 293T cells were transfected either with (1) pK-lac or (2) pK-lac and pKgag13A-CTE. Two days later, the supernatant were assayed for the presence of gag VLP by western analysis using anti-HERV gag antibody, **(B)** 293T cells were transfected either with (1) pMP-lac, or (2) pMP-lac and pMPgag3A-CTE and analyzed with anti-MPMV gag antibody. The arrows on the left indicate gag proteins.

own Gag proteins, the Gag expression construct and packaging reporter were co-transfected into 293T cells. Two days after the transfection, supernatants were collected and VLPs were purified through 20% sucrose cushion. The resulting pellets were divided to test Gag proteins expression (Fig. 4 A and B, lanes 2) and packaging Gag particle-associated RNA. For Gag VLP associated RNA preparation, samples were treated extensively with RNase-free DNase to remove any residual plasmid DNA before further PCR analysis. The total *lacZ* RNA expressed in 293T cells are shown in Fig. 5A. Since the amount of VLP associated RNA was very small, the cDNA was first amplified with MPMV (Fig. 5B) or HERV-K108 (Fig. 5C) specific primer set and re-amplified by *lacZ* nested PCR (see Materials and Methods). Despite low expression of RNA expression from pK-lac in 293T, HERV reporter RNA was relatively efficiently packaged by HERV Gag protein (Fig. 5C, lane 4).

DISCUSSION

Several lines of evidences showed that the HERV-K gag coding sequences is at least able to make and/or secrete VLP under Baculovirus expression system (21,22). In this study, the complete provirus was cloned from normal human DNA and efficient expression system of gag was developed in a human cell line, 293T. The results showed that the expression of genomic RNA from its LTR promoter was very poor and expression of gag ORF under different pro-

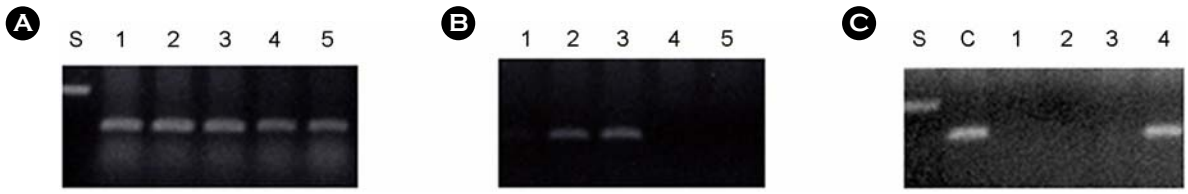

Figure 5. Packaging of reporter RNA by gag VLP. **(A)** Total reporter RNA in 293T cells. 293T cells were transfected with (1). pMP-lac, (2) pMP-lac and pMPgag3A-CTE, (3) pMP-lac and pMPgag3A-CTE, (4) pK-lac, (5) pK-lac and pKgag13A-CTE. 2 days after transfection, total cellular RNA was prepared and analyzed by RT-PCR with a LacZ primer set **(B)** MPMV reporter RNA in MPMV gag VLP. 293T cells were transfected with (1) pMP-lac, (2) pMP-lac and pMPgag3A-CTE, (3) pMP-lac and pMPgag3A-CTE, (4) pK-lac, (5) pK-lac and pKgag13A-CTE. Two days after transfection, supernatant VLP was prepared and analyzed by RT-PCR. First amplification was carried out using a MPMV specific primer set and second amplification was with lacZ nested primer set. **(C)** HERV-K reporter RNA in HERV gag VLP. 293T cells were transfected with (1) pK-lac and pKgag13A, (2) pMP-lac and pMPgag3A-CTE, (3) pK-lac and pKgag13A-HA, (4) pK-lac and pKgag13A-CTE. 2 days after transfection, supernatant VLP was analyzed by RT-PCR. First amplification was carried out using an HERV-K108 specific primer set and the second with LacZ nested primer set; C, control DNA (pK-lac).

moter, pCMV, requires nuclear export signal. The resulting expressed Gag protein was able to package its own genomic RNA.

It is not clear how the expression of HERV is regulated in human. In human genome, there are many full-length HERV-K proviruses with uninterrupted coding frames (14). However, HERV-K expression is only observed in cancer cells or in a few autoimmune disease patients. One of the probable possibilities is the regulation of promoter activity. Promoter activity can be controlled by methylation (12) or suppressed by mutations accumulated in LTR. The reporter expression was generally poor or non-existant in most human cell lines tested. Considering transfected plasmid DNA is not methylated, it is possible that methylation may not be the only regulation upon HERV LTR. Lavie et. al. (12) also have shown that HERV-K LTR were methylated in different levels but the methylation was not sole determinant for 5' LTR activity. The endogenized virus also could have accumulated many mutations to reduce harmful activities to the host. A human teratocarcinoma cell line, Tera-1, is known to express endogenous HERV-K mRNA as well as proteins, however the transfected exogenous full-length or lac reporter plasmid failed to express any viral proteins. It is possible that the endogenous HERV-K proviruses contained mutations accumulated which have reversed the suppressive mutations in LTR.

HERV-K genome encodes Rev like trans-acting factor, Rec, which facilitates nuclear export of unspliced and incompletely spliced RNAs (15). Other D-type retrovirus, MPMV, encodes a *cis*-acting RNA element, CTE. CTE promotes export of incompletely spliced mRNAs from the nucleus (6,8). Presence of nuclear export related protein, Rec, and ReRE in the HERV genome indicate that the export of genomic RNA requires proper export machinery. As in most retroviruses, HERV-K108 *gag* is expressed from full-length genomic RNA. However, the cloning of HERV-K108 *gag* coding fragment with no other viral sequences under control of the CMV promoter produced very little amount of Gag. Efficient expression of Gag was only possible in the presence of *cis*-acting nuclear transport signal, CTE. Such result indicates that nuclear export of most viral RNA is highly controlled by the nuclear export machinery regardless of splicing. It has been shown that cloning of CTE sequence improved efficient HIV Gag expression (26), indicating nuclear export of viral mRNA might be generally controlled by nuclear export machinery. It is also possible *gag* ORF could have accumulated cryptic splicing sequence due to mutations.

Human cells successfully suppress the activation of endogenous viruses only occasionally fail to do so as in carcinoma cells. However, the study presented here shows that the suppression is not at least due to the mutation in ORF or in translational block. The Gag proteins and genomic RNA still retain the ability to interact to form a particle, albeit it is not clear yet if this particle is infectious or not. Although many proviruses have previously been reported to encode functional RT, PR, and IN proteins (4,9,24) and Env (7), no infectious endogenous retrovirus particle was found. The failure could be partly due to the improper transcriptional initiation. Recently, there has been a report that HERV-K transcription started from the U5, downstream of R, indicating genomic LTR promoter has been modified

(10). Improper transcriptional initiation could interfere with cDNA synthesis after infection, thus aborting further replication steps. The activation of endogenous retroviruses can be harmful for it host. Once the proper promoter and nuclear export signal were supplied, the gag was efficiently expressed and packaged the genomic RNA, indicating that the capsid protein retains some of its functions but the transcription is highly regulated.

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