

# Expression of Human Endogenous Retrovirus K in Melanomas and Melanoma Cell Lines

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## Abstract

**The human endogenous retrovirus K family (HERV-K) comprises 30 to 50 closely related proviruses, most of which are defective. In contrast to all other human endogenous retroviruses, some HERV-K proviruses have maintained open reading frames for all viral proteins. In addition to the structural proteins Gag and Env and the reverse transcriptase, two regulatory proteins (Rec and Np9) have been described. Malignant melanoma has the highest mortality among skin cancers and is particularly aggressive. To study the expression of HERV-K, a set of seven primers was developed that allows discrimination between full-length and spliced mRNA and mRNA from deleted and undeleted proviruses. Expression of full-length mRNA from deleted and undeleted proviruses was detected in all human cells investigated. Expression of spliced *env* and *rec* was detected in a teratocarcinoma cell line, in 45% of the metastatic melanoma biopsies, and in 44% of the melanoma cell lines. In normal neonatal melanocytes, spliced *rec* was detected but not spliced *env*. Viral proteins were shown to be expressed in primary melanomas, metastases, and melanoma cell lines by immunohistochemistry, immunofluorescence, and Western blot analyses using specific antisera. For the first time, antibodies against HERV-K were found in melanoma patients. Melanomas are, in addition to teratocarcinomas and human breast cancer, the third tumor type with enhanced expression of HERV-K. (Cancer Res 2005; 65(10): 4172-80)**

## Introduction

The human genome project revealed that the human genome comprises ~8% retroviral sequences and, therefore, represents a reservoir of potentially pathogenic retroviral genes (1). The human endogenous retrovirus K family (HERV-K) consists of 30 to 50 proviruses and is to date the only known human endogenous provirus that has retained open reading frames for all viral proteins (2, 3). Most of these proviral sequences are defective due to multiple mutations or deletions. Two major types of HERV-K proviruses are known. HERV-K type I is characterized by a 292 bp deletion that leads to the fusion of the *pol* and the *env* gene and the absence of the *rec* gene. The type II, which is the HERV-K prototype, has open reading frames for the structural proteins Gag and Env, the reverse transcriptase (4), and the regulatory protein Rec (5). Rec is a nuclear export factor and a functional homologue of the HIV-1 Rev protein (6, 7). In general, the expression of HERVs is repressed, but exogenous factors like UV radiation or endogenous

factors, such as cytokines or hormones, can activate their expression (8, 9). Although the full-length mRNA of HERV-K is expressed in many tissues (10), expression of viral proteins and particle production had, until recently, only been shown for teratocarcinomas (11, 12) when enhanced expression was also shown for melanomas (13). Although particle production was observed in some tumor cell lines, to date no infectious HERV-K has been described. In addition, expression of a spliced *env* mRNA was found in human breast tumors but no data are available on virus protein expression (14).

Malignant melanomas arise from the pigment-producing cells in the epidermis and UV radiation is thought to play a role in its development. It is the leading cause of death attributable to skin lesions and is highly aggressive. A sequence related to the envelope gene of a HERV-K provirus with a short open reading frame for an antigenic peptide termed HERV-K-MEL was found to be expressed in the majority of cutaneous and ocular melanomas as well as in normal testis and skin (15). This antigen is targeted by CTLs in melanoma patients. Here, we confirm and extend data by Muster et al. (13), showing enhanced expression of HERV-K in melanomas. For the first time, we show expression of viral proteins (including the transmembrane envelope protein) in melanomas using immunohistochemistry and a newly developed HERV-K-specific antiserum and the presence of HERV-K-specific antibodies in patients with melanomas.

## Materials and Methods

**Cell lines and clinical samples.** Melanoma biopsies obtained from patients were verified by immunohistochemistry using melanoma markers and by PCR using primers specific for melanoma markers. Excised melanoma biopsies were partitioned and one part was immediately snap frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until RNA extraction, whereas the other part was used for immunohistochemistry and for the establishment of melanoma cell lines. Normal neonatal male human epidermal melanocytes were obtained from Tebu-Bio (Offenbach, Germany). The human malignant melanoma cell lines SK-MEL-28 and SK-MEL-1 and a number of other cells including Jurkat (T lymphoblast); K37 (T lymphocyte); HL60 (promyeloblast); A27/80, 7774, and SK70V3 (ovarian carcinoma); U87 (astrocytoma, grade 3); SW620 and SW480 (colorectal adenocarcinoma); Hacat (keratinocyte); I20/87 (mamma carcinoma); 257/85 (gastric carcinoma); K562 (bone marrow, chronic myelogenous leukemia); Namalwa (B lymphocyte); D181/85 (pancreas); EA14 (glioma); and A431 NS (epidermoid carcinoma) cells were obtained from American Type Culture Collection (Manassas, VA). All other cell lines including the human malignant melanoma cell lines MEWO, GR-M, and G-361, the human embryonic kidney cell line 293, the feline astrocyte cell line PG4, and the Madin-Darby bovine kidney (MDBK) cell line were obtained from European Collection of Animal Cell Cultures. The human teratocarcinoma cell line GH has been described previously (11).

**Reverse transcription-PCR and PCR.** For the isolation of total RNA, a maximum of  $10^6$  cells were lysed in 1 mL TRI-Reagent (Sigma, Taufkirchen, Germany) and total RNA was purified from the aqueous phase using the

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RNeasy kit from Qiagen (Hilden, Germany), including on-column DNase digestion. RNA from viral pellets after centrifugation was also purified using the RNeasy kit from Qiagen, including on-column DNase digestion. RNA was stored at  $-80^{\circ}\text{C}$  until use.

Expression of HERV-K was investigated by one-step reverse transcription (Invitrogen, Karlsruhe, Germany). For each reaction, 100 ng total RNA were used. PCR cycles were as follows: 30 minutes at  $50^{\circ}\text{C}$ , 5 minutes at  $94^{\circ}\text{C}$ , 35 cycles of  $94^{\circ}\text{C}$  for 30 seconds, annealing temperature for 30 seconds and  $72^{\circ}\text{C}$  for 2 minutes, and  $72^{\circ}\text{C}$  for 10 minutes. Reverse transcription (RT-PCR) products were separated on 1% agarose gels and visualized by ethidium bromide staining. False-positive results due to residual DNA were excluded by parallel experiments omitting the reverse transcriptase. A set of seven primers was designed to allow investigation of HERV-K expression (Table 1; ref. 10). To verify melanoma characteristics, additional primers for the melanoma-specific genes *MIA* (melanoma inhibitory activity) and *MART-1* (melanoma antigen recognized by T cells 1; refs. 16, 17) were designed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as positive control.

For provirus detection, DNA from  $10^6$  cells was purified using the DNA Blood kit from Qiagen and stored at  $-20^{\circ}\text{C}$ . The primers P2 and P3 (Table 1) and 100 ng total template DNA were used for each reaction. PCR cycles were as follows: 10 minutes at  $94^{\circ}\text{C}$ , 35 cycles at  $94^{\circ}\text{C}$  for 30 seconds,  $60^{\circ}\text{C}$  for 30 seconds and  $72^{\circ}\text{C}$  for 2 minutes, and  $72^{\circ}\text{C}$  for 10 minutes.

**Recombinant transmembrane envelope protein of human endogenous retrovirus.** The major part of the sequence encoding for the ectodomain of the HERV-K transmembrane envelope (TM) protein (amino acids 488-586) was amplified using DNA from the human teratocarcinoma cell line GH, the forward primer 5'-CTGGATCCGGAGTTGCATTCAC-TCTTCTG and the reverse primer 5'-GAGAAGCTTTACGTAGTACTTCAATGGTC. The amplified DNA was sequenced, compared with other HERV-K TM genes, cloned into the pMAL-Vector (New England Biolabs, Frankfurt am Main, Germany), and transformed into *Escherichia coli*. HERV-K TM fused  $\text{NH}_2$ -terminally to a 40 kDa maltose-binding protein was produced, purified by amylose resin affinity chromatography, and characterized by PAGE.

**Antiserum specific for the transmembrane envelope protein of human endogenous retrovirus K.** Goat 26 was immunized i.m. with 500  $\mu\text{g}$  of the purified recombinant fusion protein emulsified in Freund's adjuvant and boosted twice (3 and 6 weeks after immunization). The HERV-K TM-specific antibodies were purified from whole serum using a Sepharose-coupled recombinant HERV-K TM column.

**Immunofluorescence.** Cells were grown in chamber slides and fixed with 4% paraformaldehyde for 30 minutes at room temperature, washed with PBS, and permeabilized with 0.5% Triton X in PBS. The cells were then washed again and incubated with 3% skimmed milk in PBS for 30 minutes at room temperature to block unspecific binding. Immunofluorescence staining was done by incubating the cells with either anti-HERV-K TM

protein serum (or the corresponding preimmune serum) diluted 1:750 or with undiluted monoclonal anti-HERV-K Gag for 1 hour at  $37^{\circ}\text{C}$  in a humidified box. Monoclonal anti HERV-K Gag antibodies were kindly provided by Dr. Boller (Paul Ehrlich Institute, Langen, Germany; ref. 18). After washing, the cells were incubated with the corresponding secondary antibody labeled with FITC at a dilution of 1:2,000 for 1 hour at  $37^{\circ}\text{C}$  in a humidified box. After a last wash, the cells were mounted in Prolong antifade reagent (Molecular Probes, Göttingen, Germany) and examined using a Zeiss microscope with appropriate filters.

**Immunohistochemistry.** Melanoma biopsies were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into sections of 4 to 6  $\mu\text{m}$  with a microtome. The melanoma biopsies were characterized using the monoclonal antibodies HMB45 (DAKO, Hamburg, Germany) and antityrosinase antibodies (Novocastra, Newcastle upon Tyne, United Kingdom) both at a dilution of 1:50. HERV-K proteins were detected using the undiluted HERV-K Gag-specific monoclonal antibody and the HERV-K TM protein-specific goat serum 26 at a dilution of 1:100. The appropriate peroxidase-labeled secondary antibodies were used at a dilution at 1:500 (LSAB kit, DAKO). Hematoxylin was used for counterstaining.

**Protein isolation.** Total protein was isolated from GH, SK-MEL-28, and 293 cells using TRI-Reagent according to the manufacturer's instructions.

**Western blot analysis.** SDS-PAGE and Western blotting were done as described (19). Four micrograms of purified recombinant HERV-K TM protein were used as antigen. Test sera were incubated for 12 hours at  $4^{\circ}\text{C}$  at a 1:10 or higher dilution, followed by a 1:2,000 dilution of peroxidase-coupled species-specific antiserum (Sigma, Deisenhofen, Germany) for 2 hours at room temperature. Antibody binding was visualized using metal-enhanced diaminobenzidine and peroxide (Pierce, Bonn, Germany). Twenty micrograms of total protein isolated from cells were used as antigen to investigate HERV-K protein expression. Anti-HERV-K TM protein-specific goat serum 26 was used at a dilution of 1:500. Additionally, antibodies against  $\beta$ -actin (Sigma) at a dilution of 1:5,000 and GAPDH (Abcam, Cambridge, United Kingdom) at a dilution of 1:1,000 were used to show equal amounts of protein were loaded. The sera were incubated for 2 hours at room temperature followed by a 1:2,000 dilution of antigoat or a mixture of antimouse and antirabbit peroxidase-coupled species-specific secondary antibodies for 2 hours at room temperature. Antibody binding was visualized as described above.

**Infection experiments.** Cell culture supernatants from 293, SK-MEL-28, and GH cells were pelleted over a 20% sucrose cushion by ultracentrifugation at  $113,000 \times g$  for 3 hours. For this, the cells were seeded with  $1 \times 10^4$  cells/ $\text{cm}^2$  and cultured until 95% confluence. Thirty milliliters of each cell culture supernatant were pelleted. The pellets were resuspended in PBS and stored at  $-20^{\circ}\text{C}$ . The presence of reverse transcriptase activity in the cell supernatants and viral pellets was determined using the reverse transcriptase assay CAVIDI HS kit  $\text{Mg}^{2+}$  RT (Cavidi, Uppsala, Sweden). Supernatants of HERV-K-producing SK-MEL-28 and GH cells were sterile

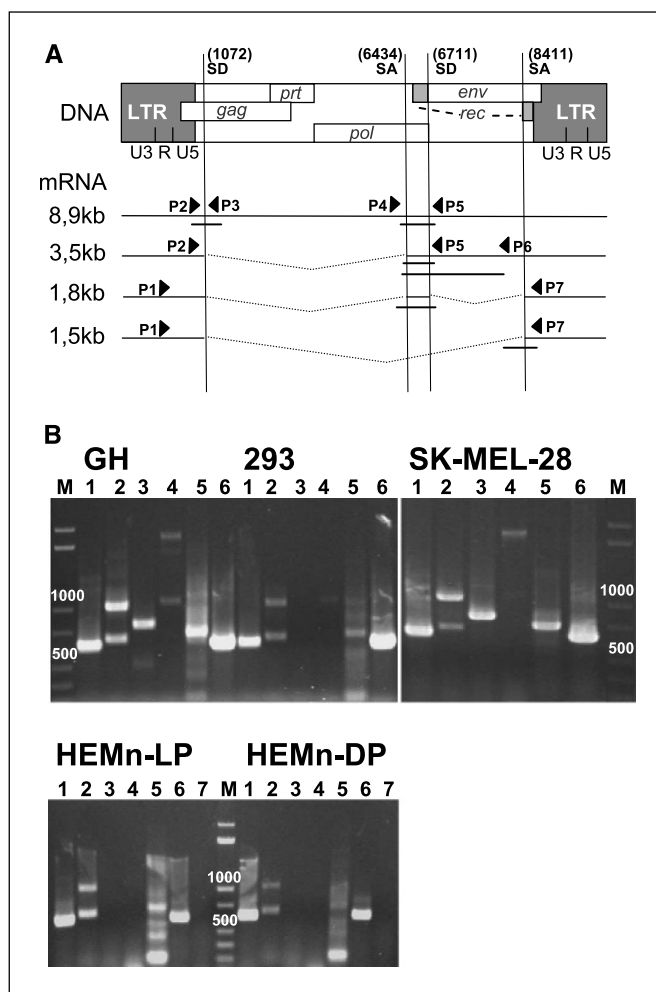
**Table 1.** Primers used for RT-PCR and PCR investigations

Primer	Localization	Sequence
<i>GAPDH</i> forward	Glyceraldehyde-3-phosphate dehydrogenase	5'-CTCAGTGTAGCCCAGGATGC
<i>GAPDH</i> reverse		5'-ACCACCATGGAGAAGGCTGG
P1	HERV-K LTR (U5)	5'-GAGGCTGGCGGGATCCTC
P2	HERV-K LTR (leader)	5'-GAAGGTACGCTCGAGCGTAATCATTGAG
P3	HERV-K <i>gag</i>	5'-GAGCCATTACCGGCTCTGTACATATTGC
P4	HERV-K <i>pol</i>	5'-GTACCACTCCTCAGATGCAACTTAATCTAGC
P5	HERV-K <i>env</i> (SU)	5'-GTGACATCCCGCTTACCATGTGATAAGTG
P6	HERV-K <i>env</i> (TM)	5'-CGTCTAACCATGTCCCAGTGATGC
P7		5'-ACAAGCTTCCTACGTCATCATGGCCCG
<i>MIA</i> forward	Melanoma inhibiting activity	5'-CTGGTGTGCCTTGGTGTGCATCATCT
<i>MIA</i> reverse		5'-TCTTCACATCGACTTTGCCAGGTTT
<i>MART-1</i> forward	Melanoma antigen recognized by T-cell 1	5'-ACTGCTCATCGGCTGTTGGTATTG
<i>MART-1</i> reverse		5'-TTTCAGCATGTCTCAGGTGTCTCG

filtered and added to the target cells together with 6 µg/mL polybrene and incubated overnight. Additionally, different initial ratios of SK-MEL-28 cells and hygromycin-resistant MDBK cells (3:1, 2:1, 1:1, and 1:2) were cocultivated for 2 weeks, routinely splitting the cultures 1:5. Then, the cocultivation was subjected to 500 µg/mL hygromycin, which killed the SK-MEL-28 cells and left the hygromycin-resistant MDBK cells. Infection was measured as provirus integration by PCR.

## Results

**Expression of human endogenous retrovirus K messenger RNA in melanoma cell lines.** A set of seven previously designed primers (10) was used to analyze expression of HERV-K (Fig. 1A). The primers were located before and behind the splice donor and the splice acceptor and allow the full-length and spliced mRNA of *env* as well as of doubly spliced *rec* mRNA to be discriminated. To discriminate between proviruses with and without a 292 bp deletion between *pol* and *env*, primers flanking the deletion were used. The human teratocarcinoma cell line GH, known to produce full-length and spliced HERV-K mRNA as well as virus-like particles (11, 12), was used as positive control (Fig. 1B). In contrast, in 293 human embryonic kidney cells, the expression of full-length mRNA of HERV-K type I and type II, but not of spliced mRNA, was detected. The expression of HERV-K full-length and spliced mRNA was observed in the human malignant melanoma cell lines SK-MEL-28 (Fig. 1B), MEWO, and G361 (not shown). In contrast, the human malignant melanoma cell lines SK-MEL-1 and GR-M showed expression of full-length HERV-K but not of spliced *env* or *rec* mRNA. Expression of full-length mRNA of HERV-K type I and type II was detected in all 19 melanoma cell lines developed in our laboratory, but spliced *env* and *rec* were only detected in 44% (7 of 19; Table 2). All melanoma cell lines showed at least a weak expression of the melanoma markers *MIA* and *MART-1*. In addition, 16 cell lines (Jurkat, K37, HL60, A27/80, 7774, SK70V3, U87, SW620, SW480, Hacat, 120/87, 257/85, K562, Namalwa, D181/85, EA14, and A431 NS) established from various other tumors, such as T-cell and B-cell carcinomas, ovary or epidermoid carcinomas, and several other tumors were investigated. All these cell lines were tested negative for the expression of the melanoma marker *MART-1*. Expression of full-length mRNA of HERV-K type I and type II, but no expression of spliced *env* or *rec*, was observed in 15 of 17 cell lines. Only the cell line 120/87 derived from a mamma carcinoma and SK70V3 derived from an ovary carcinoma showed a weak expression of spliced *env*. These data are in agreement with previous data in which the same RT-PCR method was used to study expression of HERV-K in different other tissues. The tissues studied included kidney carcinoma, testicular tumor, yolk sac tumor, testis, epididymis, ovary carcinoma, cervix uteri carcinoma, placenta, thyroid gland carcinoma, normal thyroid gland, mammary carcinoma, brain, skeletal muscle, spleen, lymphoma, bone marrow, peripheral blood mononuclear cells (either unstimulated or stimulated with 12-*O*-tetradecanoylphorbol-13-acetate, concanavalin A, phytohemagglutinin, or lipopolysaccharide or in mixed leukocyte reaction), teratocarcinoma cell lines GH and Tera-1, hepatocyte cell line HepG2, myeloid cell line K562, and different T cell lines, such as Molt4, MT4, and C8166. Spliced *env* mRNA and *rec* RNA were found in kidney carcinomas, in some testicular tumors, in ovary carcinoma, and in teratocarcinoma cell lines GH and Tera-1 (10). However, normal ovaries and kidneys had not been tested. In addition, expression of full-length mRNA has been analyzed in more than 30 normal tissues using dot blot hybridization with



**Figure 1.** A, organization of HERV-K proviruses, expression pattern of mRNA, and primers used for RT-PCR analysis. The localization of primers P1 to P7 regarding the splice acceptors (SA) and splice donors (SD) and the corresponding RT-PCR products are shown. B, HERV-K expression in GH cells, 293 cells, SK-MEL-28 cells, and in normal melanocytes (HEMn-LP and HEMn-DP). HERV-K full-length mRNA: P2 + P3 (1) and P4 + P5 (2); spliced *env* mRNA: P2 + P5 (3) and P2+P6 (4); spliced *rec* mRNA: P1 + P7 (5) and GAPDH (6); reverse transcriptase (7) was omitted to show absence of cellular DNA; M, marker in bp.

a Gag-specific probe. High expression was observed in the brain, testis, ovary, kidney, and liver (10).

When normal human epidermal melanocytes were tested, expression of the full-length mRNA and of the doubly spliced *rec* mRNA, but not of the spliced *env* mRNA, was observed in both the darkly pigmented (–DP) and the lightly pigmented (–LP) cell lines (Fig. 1B). *MIA* and *MART-1* were not expressed in these normal melanocytes.

**Expression of human endogenous retrovirus K messenger RNA in melanoma biopsies.** Using the same RT-PCR method, HERV-K expression was investigated in 34 biopsies of human melanoma metastases. Full-length mRNA of HERV-K type I and type II were detected in all biopsies tested but spliced *env* and *rec* could only be detected in 45% (11 of 34; Table 2). The availability of cell lines and biopsy samples from the same metastases allowed HERV-K expression in both to be investigated in parallel and, therefore, allowed changes caused by cultivation to be identified. From all biopsies that were negative

for spliced *env* and *rec* mRNA, the corresponding cell line was also negative. In one biopsy (AC) positive for spliced *env* and *rec* mRNA, the corresponding cell line was also positive, but in another case (AB) a relatively weak expression was observed in the biopsy without a corresponding expression in the cell line, suggesting the selection and growth of a cell type with a very low HERV-K expression.

In general, the biopsies showed a higher expression of melanoma markers compared with the cell lines. Although LH is not a melanoma but a Merkel cell carcinoma, it shows expression of *MIA* but at a lower level compared with the melanoma biopsies.

**Generation of antibodies specific for the transmembrane envelope protein of human endogenous retrovirus K.** To generate a HERV-K TM-specific antiserum, the major part of the sequence encoding for the ectodomain of HERV-K TM (amino acids 488-586) was cloned using an amplified sequence from human teratocarcinoma GH cells. Sequence comparison with several HERV-K Env sequences showed that the cloned sequence C6-5 most closely resembled the TM protein encoded by HERV-K

101 (Fig. 2A). The sequence differed in 2 bp from the HETM2 sequence cloned previously by Phelps et al. (10) that had been used for the generation of another antiserum specific for the HERV-K TM protein (goat serum 7) and also used in a recent publication by Muster et al. (13). Whereas the HETM2 clone is identical to HERV-K 108, clone C6-5 differs from HERV-K 108 in two positions, leading to amino acid changes at position 466 (Arg to His) and position 481 (Lys to Asn) and from HERV-K 101 by an amino acid exchange in position 481 (Lys to Asn). After expressing the protein as a maltose-binding fusion protein in *E. coli* and purification by maltose-binding protein affinity chromatography, an antiserum was generated in goat 26.

**Human endogenous retrovirus K-specific antibodies in sera from melanoma patients.** Sixty sera from melanoma patients, 20 sera from normal blood donors, and 20 sera from patients with alopecia were tested for antibodies specific for the HERV-K transmembrane envelope protein. Twenty-two percent (13 from 60) of the melanoma patients' sera reacted strongly with the recombinant HERV-K TM by Western blot (Fig. 2B). None of the

**Table 2.** HERV-K expression in melanoma cell lines and in melanoma biopsies

Patient	HERV-K expression			Melanoma marker expression		Clinical data						
	FL*	<i>env</i>	<i>rec</i>	MIA	MART-1	Age <sup>†</sup>	Sex	Tumor thickness (mm)	Stage <sup>‡</sup>	Type (c/l)		
1	BA	+	+	+	++	++	41	f	4	III	c	
	LAU	+	+	+	++	++	52	m	n.d.	III	c	
	SCHM	+	+	+	++	++	55	f	2	IV	c	
	SCHW	+	+	+	++	++	42	f	4	IV	c	
	TR	+	+	+	++	+	69	f	n.d.	IV	l	
	ZO	+	+	+	++	++	64	f	2.55	III	c	
2	AC	++	+	++	n.d.	++	69	f	n.d.	IV	c	
	AD	+	+/-	-	n.d.	+	55	f	n.d.	III	l	
3	KU	+	-	-	++	++	61	m	5	IV	c	
	RA	+	-	-	++	++	84	f	3.7	IV	c	
	SO	+	-	-	++	++	59	m	5	IV	l	
	ZD	+	-	-	++	++	55	m	5	IV	c	
	AB	+	-	-	n.d.	++	68	m	1.1	IV	c	
	AF	+	-	-	n.d.	+/-	39	m	1	IV	n.d.	
	AG	+	-	-	n.d.	++	80	f	0.64	IV	c	
	AI	+	-	-	n.d.	+/-	47	m	n.d.	IV	l	
	AJ	+	-	-	n.d.	+	46	m	10	IV	c	
	AK	+	-	-	n.d.	+/-	63	m	5	IV	l	
	MA <sup>§</sup>	+	-	-	-	-	59	f	n.d.	n.d.	n.d.	
	5	FE	+	+	+	++	++	80	f	n.d.	III	l
		FM	+	+	+	++	++	28	f	n.d.	IV	Lung
KLM		++	++	++	++	++	50	m	n.d.	III	l	
MA		+	+	+	++	++	58	f	n.d.	IV	l	
SC		+	+	+	++	++	64	m	n.d.	III	l	
TB		+	+	+	++	++	58	f	n.d.	IV	l	
HI		+	+	+	++	++	67	f	n.d.	IV	Brain	
AA		++	+	+	n.d.	++	62	m	n.d.	IV	l	
AB		++	+/-	+/-	n.d.	++	68	m	n.d.	IV	c	
AC		++	+	+/-	n.d.	++	69	w	n.d.	IV	c	
AH		++	++	+	n.d.	++	44	w	n.d.	IV	c	
6		LU	+	+/-	-	++	++	69	f	n.d.	III	c
		SE	+	+	-	+	++	86	f	n.d.	IV	c
	AF	++	-	+	n.d.	++	39	m	n.d.	IV	n.d.	
	AG	+	-	+	n.d.	++	80	w	n.d.	IV	c	

(Continued on the following page)

**Table 2.** HERV-K expression in melanoma cell lines and in melanoma biopsies (Cont'd)

Patient	HERV-K expression			Melanoma marker expression		Clinical data					
	FL*	<i>env</i>	<i>rec</i>	MIA	MART-1	Age <sup>†</sup>	Sex	Tumor thickness (mm)	Stage <sup>‡</sup>	Type (c/l)	
7	BN	+	—	—	++	+	44	m	n.d.	III	l
	GB	+	—	—	++	+/-	42	f	n.d.	IV	Lung
	HK	+	—	—	++	++	42	f	n.d.	IV	c
	KH	+	—	—	++	++	64	f	n.d.	III	c
	LA	+	—	—	++	++	44	m	n.d.	IV	c
	LAN	+	—	—	++	++	65	m	n.d.	III	l
	LG	+	—	—	++	++	75	m	n.d.	III	l
	OL	+	—	—	++	+	41	m	n.d.	n.d.	n.d.
	PV	+	—	—	++	++	81	f	n.d.	III	c
	SR	+	—	—	++	++	60	m	n.d.	n.d.	l
	VH	+	—	—	++	++	81	m	n.d.	n.d.	l
	WD	+	—	—	+	++	59	m	n.d.	IV	l
	WR	+	—	—	++	+	72	m	n.d.	IV	c
	AD	++	—	—	n.d.	++	55	w	n.d.	III	l
	AE	++	—	—	n.d.	++	56	w	n.d.	IV	c
	AI	++	—	—	n.d.	++	47	m	n.d.	IV	l
AJ	++	—	—	n.d.	++	46	m	n.d.	IV	c	
AK	++	—	—	n.d.	++	63	m	n.d.	IV	l	
8	LH <sup>  </sup>	+	—	—	+	—	71	f	n.d.	n.d.	n.d.

NOTE: Patient groups 1, 2, and 3: melanoma cell lines; patient groups 5, 6, and 7: melanoma biopsies; ++, strong expression; +, expression; +/-, weak expression; —, no expression.

Abbreviations: n.d., not determined; c, cutaneous; l, lymph node metastases.

\*Full-length mRNA.

<sup>†</sup>Age at first tumor diagnosis.

<sup>‡</sup>American Joint Committee on Cancer 2001: stage III—lymph node or cutaneous metastases between primary tumor and regional lymph node; stage IV—distant metastases.

<sup>§</sup>Breast carcinoma.

<sup>||</sup>Merkel cell carcinoma.

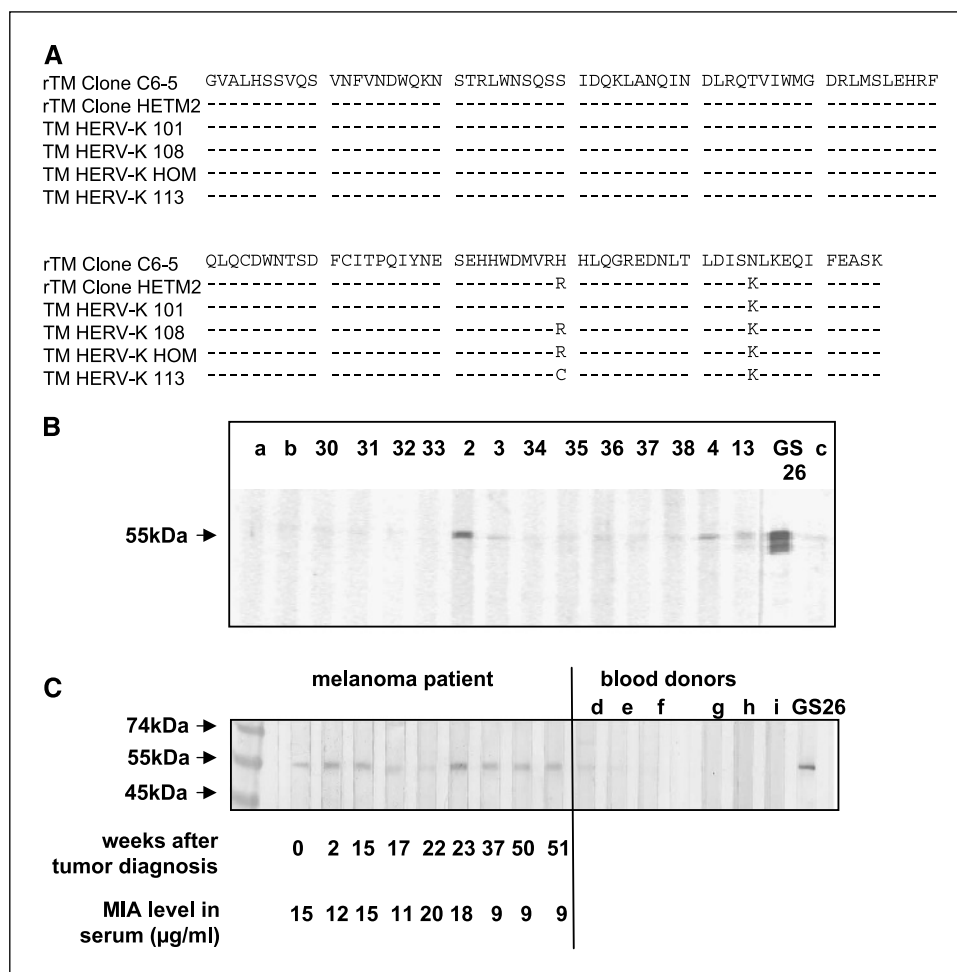
sera from normal blood donors and alopecia patients were positive in this assay, confirming previous results showing 1 positive serum from 30 normal blood donors (2). One melanoma patient was studied in more detail. Over a period of 1 year, sera were collected nine times and tested for the melanoma marker MIA and for antibodies specific for HERV-K TM (Fig. 2C). The time points at which enhanced levels of MIA were detected in the serum (0, 17, and 22 weeks after the initial tumor diagnosis and excision) correspond to tumor reappearance in the patient. At these time points, the levels of HERV-K-specific antibodies as measured by Western blot remained low.

**Expression of human endogenous retrovirus K proteins in melanoma cell lines.** To determine whether HERV-K-specific proteins were expressed in human melanoma cells, immunofluorescence was done using the HERV-K TM protein-specific goat serum 26. Both GH and SK-MEL-28 cells were shown to express the HERV-K TM protein (Fig. 3A-a and c), whereas human 293 kidney cells were negative (Fig. 3A-e). This correlates with the differences in expression of HERV-K-specific mRNA in all three cell lines. The preimmune serum showed no reaction (Fig. 3A-b, d, and f). The staining pattern of SK-MEL-28 is similar to that of GH cells, indicating the presence of virus clusters on the cell surface. Expression of the TM protein of HERV-K was also shown by Western blot analysis using specific antisera (Fig. 3B).

**Expression of human endogenous retrovirus K proteins in primary tumors and metastatic lesions.** Expression of HERV-K proteins was analyzed in primary tumors and metastatic lesions by immunohistochemistry. To enable the tumor to be located and characterized, metastases were analyzed using antibodies specific for tyrosinase and the antibody HMB-45 (Fig. 3C-a). Expression of HERV-K TM and Gag proteins was detected in melanoma cells, whereas the surrounding lymphoid tissue in a lymph node metastases was negative (Fig. 3C-b and d). The preimmune serum did not react (Fig. 3C-c). Not every tumor cell expressed HERV-K; 3 of 6 (50%) primary tumors were positive for staining with HERV-K specific serum and 4 of 5 (80%) were positive for staining with anti-HERV-K Gag. Of the melanoma metastases investigated, 7 of 15 (47%) were positive for HERV-K TM expression and 16 of 23 (70%) were positive for Gag expression (Fig. 3D).

**Virus particles released from the melanoma cell line SK-MEL-28 are not infectious.** GH cells are known to produce virus-like particles; therefore, the melanoma cell line SK-MEL-28 was also tested for release of infectious particles. Supernatants from cultured SK-MEL-28 and GH cells were tested positive for reverse transcriptase activity. Additionally, pellets were obtained by ultracentrifugation of cell culture supernatants of SK-MEL-28, GH, and 293 cells and were tested by RT-PCR for HERV-K viral RNA

**Figure 2.** A, comparison of the recombinant HERV-K TM sequence (Clone C6-5) with that of HETM2 used previously (10), HERV-K101, HERV-K 108, HERV-K HML-2.HOM, and HERV-K113 sequences (Genbank accession nos. P61566, Q69384, and Q902F9). B, Western blot analysis of sera from melanoma patients (Arabic numbers) and normal blood donors (letters) using the transmembrane envelope protein of HERV-K as antigen. a to c, sera from normal blood donors; GS26, positive control, goat serum. C, sera from one melanoma patient collected at different time points; d to i, sera from normal blood donors; GS26, positive control, goat serum 26.



as well as for reverse transcriptase activity. Supernatants and pellets from SK-MEL-28 and GH cells were found positive, whereas 293 cells did not release HERV-K particles (Fig. 4A). These data suggest the release of viral particles from SK-MEL-28 and GH cells. Preliminary electron microscopy analyses showed the presence of retroviral particles in the pellets from SK-MEL-28 cells; however, most of them seemed defective.<sup>3</sup> To investigate whether the released particles are infectious, nonhuman PG4 and MDBK cells and human 293 cells were used for the infection experiments. Although HERV-K-specific sequences were detected by PCR in PG4 cells after exposure to supernatants from GH and SK-MEL-28 cells, this signal was lost after four rounds of passage in each of which cells were split 1:5. This suggests that the positive result was due to the presence of residual cellular DNA. No provirus was detected after exposure of MDBK and 293 cells either by cell-free viruses or during cocultivation (see Materials and Methods), indicating that the released particles were not infectious.

## Discussion

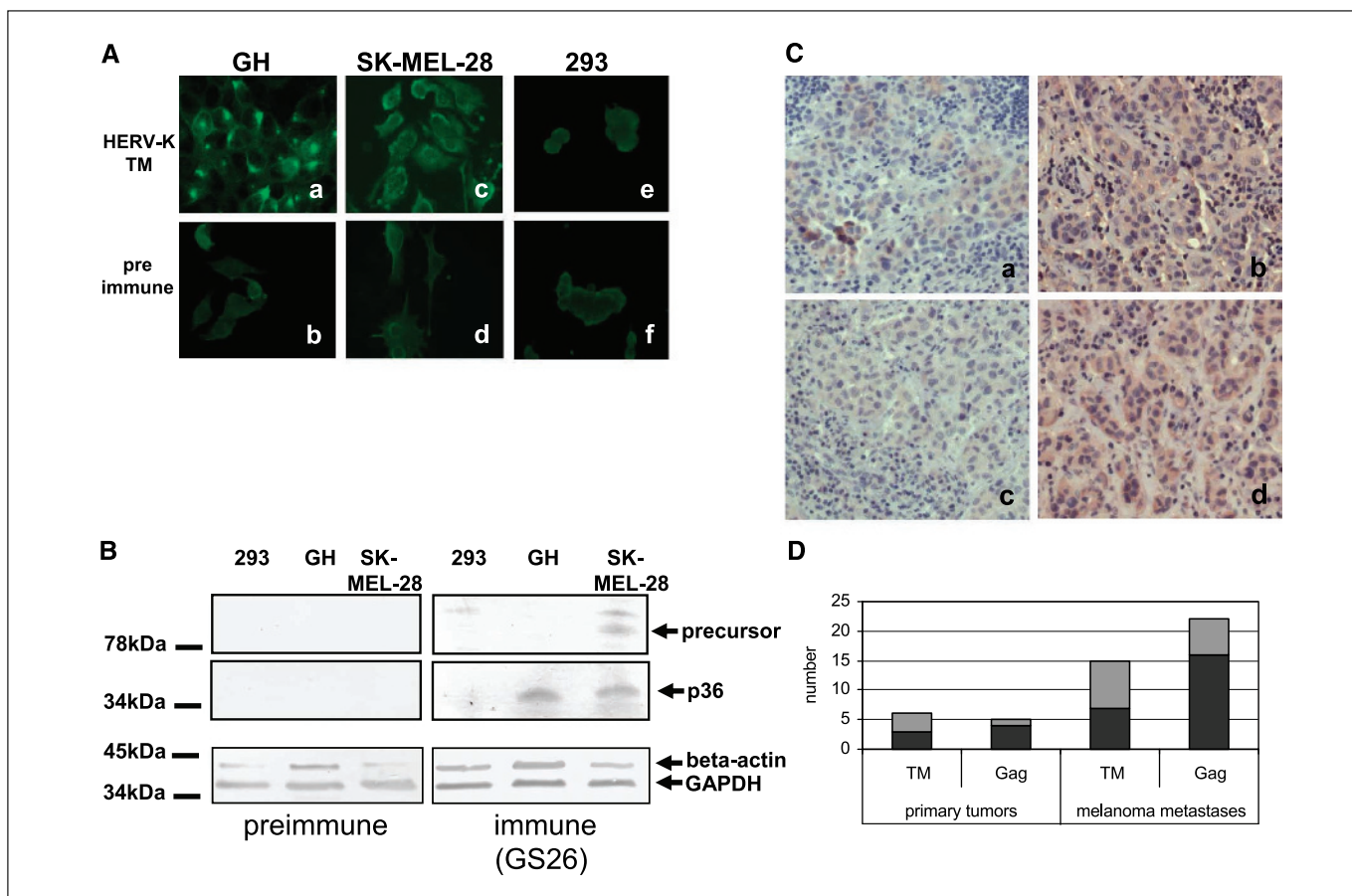
Although the expression of HERV-K full-length mRNA can be detected in most human tissues (10, 20), spliced *env* and *rec* mRNA has only thus far been found to be expressed in teratocarcinoma and melanoma tumors (13, 21). Transcripts of subgenomic HERV-K *env*,

but not of *rec*, were also detected in the breast cancer cell line T47D (22) and in some breast tissues (14). Here, we confirm and extend data on the enhanced HERV-K expression in human melanomas reported by Muster et al. (13). In contrast to their results, expression of HERV-K proteins was detected in only in about half of those primary tumors and metastases studied directly by immunohistochemistry (Fig. 3C) and spliced *env* and *rec* mRNA was found in only 45% of the metastases tested (Table 2). A similar frequency of expression was found in melanoma cell lines, with 44% of the newly established cell lines expressing spliced *env* and *rec* mRNA. When long-established cell lines used for many years in melanoma research were studied (SK-MEL-28, SK-MEL-1, MEWO, GR-M and G-361), expression of spliced *env* and *rec* mRNA was detected in only SK-MEL-28, MEWO, and G-361, but not in SK-MEL 1 and GR-M cells. The frequency of patients producing antibodies against the transmembrane envelope protein of HERV K is even less at 22%, suggesting that expression in the other tumors is too low for induction of a humoral response. In contrast, up to 85% of the patients with seminomas and other germ cell tumors produce antibodies directed against the Env protein of human endogenous retroviruses and all Env-positive germ cell tumor patients tested generate high titers of antibodies directed against the transmembrane domain (23). The generation of HERV-K-specific antibodies also indicates a lack of tolerance and suggests that expression during ontogenesis (when discrimination between self and non-self is made) does not occur. Such antibodies were detectable in none of

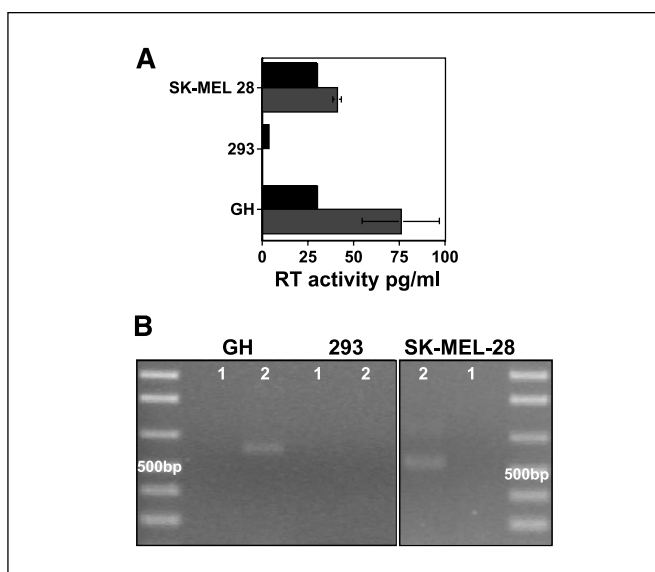
<sup>3</sup> M. Özel, personal communication.

the patients with non-germ cell tumors. Antibodies recognizing synthetic HERV-K peptides were detected at a very low frequency (1/30) in the sera of healthy blood donors, consistent with the observation that HERV-K is expressed at only a low level in normal tissues (2). There are at least three possible biological reasons for retroviral protein expression in tumors. First, they could be activated during transformation and tumor progression as a result of an increase in transcription activators or other factors able to enhance expression of endogenous retroviruses. Second, they may actively participate in malignant transformation. Normally, retroviruses do not contain oncogenes and transformation is usually induced by insertional mutagenesis that activates expression of cellular oncogenes or disrupts the open reading frames of tumor suppressor genes. Indeed, a murine retrovirus associated with melanomas in C57Bl/6 mice is inserted into the *c-maf* proto-oncogene in transformed cells (24). HERV-K is a more complex retrovirus than the murine leukemia viruses because it contains two additional genes, *rec* and *np9*. *Rec*, a regulatory protein located in the nucleus and with functions similar to the Rev protein of HIV-1 (5–7), was shown to support cell transformation and to induce tumors in nude mice. Nude mice that received cells expressing *Rec* developed tumors, as did three mice that received cells producing the *Rec* splice

variant instead of Env (25). A supportive role for both proteins in melanoma development may, therefore, be possible. The third possible biological reason for retroviral protein expression in tumors concerns the expression of the HERV-K transmembrane envelope protein in some of the tumors. Many retroviruses, including HIV-1, feline leukemia virus (FeLV), and murine leukemia virus (MLV), induce immunodeficiencies in the infected host (26). The transmembrane envelope protein gp41 of HIV-1 and p15E of FeLV as well as synthetic peptides corresponding to a highly conserved domain of the TM protein, the so-called immunosuppressive domain, inhibit lymphocyte proliferation and modulate cytokine production (27, 28). The TM proteins of MLV (29), of the Mason-Pfizer monkey virus (30), and of HERV-H (31) support tumor development when expressed on the surface of tumor cells unable to grow in immunocompetent mice. Tumor cells expressing a retroviral transmembrane envelope protein may, therefore, have a selective advantage due to protection from immunologic rejection. However, when comparing the clinical data from patients with melanomas that express HERV-K with data from patients with melanomas that do not express HERV-K, no obvious differences were observed. Neither age nor sex of the patient nor the tumor stage or thickness correlated with the expression of spliced HERV-K mRNA, and no correlation between cutaneous or



**Figure 3.** A, immunofluorescence analysis of GH, SK-MEL-28, and 293 cells stained with HERV-K TM-specific serum goat 26 (a, c, and e) and with preimmune serum (b, d, and f), both at a dilution of 1:500. B, Western blot analysis of 293, GH, and SK-MEL-28 cells using lysates of these cell lines and goat serum 26 specific for the HERV-K TM protein as well as the preimmune serum. The major precursor and the p36 are indicated. Additionally, antibodies against human  $\beta$ -actin and GAPDH were used to show that equal amounts of protein were loaded. C, immunohistochemistry of a melanoma metastases stained with antityrosinase, a tumor marker for malignant melanomas (a); a monoclonal antibody specific for HERV-K Gag (b); a preimmune serum from goat 26 (c); and with the HERV-K TM-specific goat 26 antiserum (d). The cells were counterstained with hematoxylin. D, number of primary melanoma tumors and metastases tested in immunohistochemistry for HERV-K TM and Gag protein expression. Positive tumors were depicted in black; negative tumors are in light gray.



**Figure 4.** A, determination of reverse transcriptase activity in cell culture supernatants (black) and in the viral pellets obtained by ultracentrifugation of supernatants from 293, GH, and SK-MEL-28 cells (gray). B, RT-PCR analysis of RNA from viral pellets obtained from 293, GH, and SK-MEL-28 cell supernatants. HERV-K-specific full-length primers P2 and P3 and RNA were used in lane 2; reverse transcriptase was omitted in lane 1 to show the absence of cellular DNA.

lymphoid metastases and expression of HERV-K *env* and *rec* transcripts was found.

The apparent loss of spliced HERV-K *env* and *rec* mRNA in a cell culture derived from a biopsy positive for spliced HERV-K expression confirmed the data presented by Muster et al. (13) showing that Rec was expressed in ~20% of the analyzed cells, whereas the expression of Env was detected in ~10%, suggesting that not all tumor cells express HERV-K at the same level. However, differences in the sensitivity of the methods used may also be responsible for such discrepancies.

The newly developed goat serum 26, specific for the HERV-K transmembrane envelope protein, is a powerful tool for investigating the expression of this putatively immunosuppressive protein on the surface of tumors, including melanomas. Although the epitopes recognized by this antiserum were not mapped, it is unlikely that the one amino acid difference between the proteins derived from clones C6-5 and HETM2 is associated with differences in the specificities of the corresponding antisera (goat sera 26 and 7, respectively). Goat serum 7 has also been used by Muster et al. (13) to show expression of HERV-K in melanomas.

Endogenous retroviruses of other species (e.g., the porcine endogenous retroviruses) are infectious (32–34). Infectious HERV-K has never been detected even when cell-to-cell contact was allowed (presumably a very efficient way of transmission) by cocultivation with target cells. Although positive PCR signals were briefly detected after exposure of different cell lines (including MDBK) to HERV-K-containing supernatants from GH or SK-MEL-28 cells, these were almost certainly due to contaminating cellular DNA; the proviral sequence detected by Muster et al. (13) in MDBK cells was also possibly due to DNA integration alone. Attempts to identify budding proviruses on the surface of SK-MEL-28 cells unfortunately failed due to the presence of numerous villi. However, in the viral pellet from the supernatant of SK-MEL-28 cells, retroviral particles were detected and most of them were defective.<sup>3</sup>

Further studies are required to determine whether the expression of HERV-K in certain forms of melanoma has a prognostic or diagnostic use. In addition, the retroviral proteins expressed may provide excellent targets for an antitumor vaccine.

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## Expression of Human Endogenous Retrovirus K in Melanomas and Melanoma Cell Lines

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