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The endogenous retroviral insertion in the human complement *C4* gene modulates the expression of homologous genes by antisense inhibition

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Abstract Intron 9 contains the complete endogenous retrovirus HERV-K(C4) as a 6.4-kb insertion in 60% of human *C4* genes. The retroviral insertion is in reverse orientation to the *C4* coding sequence. Therefore, expression of *C4* could lead to the transcription of an antisense RNA, which might protect against exogenous retroviral infections. To test this hypothesis, open reading frames from the HERV sequence were subcloned in sense orientiation into a vector allowing expression of a β-galactosidase fusion protein. Mouse L cells which had been stably transfected with either the human *C4A* or *C4B* gene both carrying the HERV insertion (LC4 cells), and L(Tk$^-$) cells without the *C4* gene were transiently transfected either with a retroviral construct or with the wild-type vector. Expression was monitored using an enzymatic assay. We demonstrated that (1) HERV-K(C4) antisense mRNA transcripts are present in cells constitutively expressing *C4*, (2) expression of retroviral-like constructs is significantly downregulated in cells expressing *C4*, and (3) this downregulation is further modulated in a dose-dependent fashion following interferon-γ stimulation of *C4* expression. These results support the hypothesis of a genomic antisense strategy mediated by the HERV-K(C4) insertion as a possible defense mechanism against exogenous retroviral infections.

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Introduction

The human genome contains a great variety of endogenous retroelements, which have spread within the genome by retrotransposition. This abundant family of transposable sequences comprises short and long interspersed elements, like Alu repeats and L1 retroposons, respectively, which lack promotor-like long terminal repeats (LTRs), as well as LTR-containing retrotransposons and human endogenous retroviruses (HERVs). HERVs are characterized by complete genomic proviral sequences including *gag*, *pol*, and *env* genes, and are flanked by two LTRs. They can potentially spread horizontally to other cells by forming infectious virus particles. Thus HERVs share extensive homology with their exogenous counterparts, the simple type B, C, and D retroviruses, e.g., the mouse mammary tumor virus (MMTV) (for reviews, see Leib-Mösch and Seifarth 1996; Löwer et al. 1996; Urnovitz and Murphy 1996). With more than 30,000 copies, solitary LTRs are quite abundant, comprising approximately 1% of the human genome (Leib-Mösch et al. 1993). They are thought to represent former sites of retroviral integration which have been subsequently deleted, possibly due to homologous recombination (Mager and Goodchild 1989). HERV open reading frames are located on many chromosomes with the potential to express retroviral proteins (Mayer et al. 1997; Tönjes et al. 1999) and an almost complete 9.5-kb HERV-K insertion with intact sequence motifs has been recently identified (Mayer et al. 1999).

The HERV-K family is characterized by the presence of a lysine tRNA primer-binding site required for transcription initiation and by its sequence homology to MMTV (Larsson et al. 1989; Ono et al.

1986). A full-length proviral insertion with partial homology to HERV-K10 has been identified in intron 9 of the tandemly duplicated genes of the fourth component of human complement (*C4A* and *C4B*), and has been termed HERV-K(C4) (Chu et al. 1995; Dangel et al. 1994; Tassabejih et al. 1994). The *C4* genes are located in the class III region of the major histocompatibility complex (MHC) on Chromosome 6p21.3. Only approximately 60% of the *C4* genes carry the intron 9 insertion, giving rise to a gene length polymorphism of 22.5 and 16 kb, respectively (Prentice et al. 1986; Schneider et al. 1986; Yu 1998). As both long *C4A* and *C4B* genes have nearly identical insertion sequences, in particular, identical CAGACA target site repeats, retroviral insertion presumably either preceded the initial *C4* gene duplication event, or an insertion was transferred from one *C4* locus to the other by a secondary recombination event. Furthermore, Southern blots of DNA from Old World primates all exhibit multiple species-specific restriction fragment patterns when hybridized with a HERV-K(C4)-LTR probe, suggesting that approximately 30–50 copies of this LTR are present in these genomes (Dangel et al. 1994, 1995).

Interestingly, the transcriptional orientation of the HERV-K(C4) proviral insertion is opposite to the coding sequence of the *C4* genes. A proviral insertion in the same orientation as the *C4* coding sequence could have led to retroviral expression whenever the host *C4* gene was transcribed, thus leading to deleterious results for *C4*-expressing cells. Dangel and coworkers (1994) have therefore speculated that a retroviral insertion in opposite orientation to the *C4* coding sequence could very well confer a selective advantage to the host. If a retroviral antisense transcript is generated either in the course of *C4* transcription or by self-regulated transcription, it could provide protection in a case of acute exogenous retroviral infection by blocking the translation of retroviral mRNA. The authors supported their hypothesis by demonstrating in CAT promoter activity assays that the 3′-HERV-K(C4)-LTR exhibits *C4*-independent promoter activity only in antisense configuration, whereas the sense orientation and the 5′-LTR both appear to be inactive.

We developed a simple experimental approach to test their hypothesis. As a model system for exogenous retroviral expression, we used HERV-K(C4) open reading frames from the *gag*, *pol*, or *env* genes subcloned into a β-galactosidase (β-gal) fusion protein expression vector. Thus the level of β-gal activity served as a marker for expression of the "infecting" retroviral construct when transiently transfected into mammalian host cells. As target cells, we used mouse fibroblast L cells stably transfected with either the human *C4A* (LC4A) or *C4B* (LC4B) gene (Miura et al. 1987), because we wanted to ensure that there is no undefined genomic background of HERV-homologous sequences. Thus wild-type L(Tk–) cells could be used as negative controls not expressing HERV-specific antisense transcripts. In these experiments, evidence was obtained supporting the hypothesis of genomic antisense protection mediated by the HERV-K(C4) insertion as a possible defense mechanism against retroviral infections.

Materials and methods

Reverse transcription-polymerase chain reaction

Total RNA of LC4A cells, LC4B cells, and human HepG2 cells was isolated using the high pure RNA extraction kit (Roche Diagnostics, Mannheim, Germany). The extraction procedure included a digestion with DNAseI to prevent carryover of genomic DNA. RNA was reverse transcribed into first-strand cDNA using MuLV reverse transcriptase (GeneAmp RNA PCR Kit; Applied Biosystems, Weiterstadt, Germany) with env-, pol-, and gag-5′ primers. As a control, the same amount of RNA was reverse transcribed into *C4* exon-specific cDNA (exons 4–9) with the *C4* exon 9/3′ primer. The reverse transcription products were amplified by *Taq* polymerase (Applied Biosystems) using *env*-, *pol*-, *gag*-, and *C4*-specific primer pairs (C4 exon 4/5′: 5′-GGGTCTTTGCTCTGGATCAGA-3′; C4 exon 9/3′: 5′-CCT GGAACTCTGCCT TTGAGA-3′). Aliquots of the PCR products were separated in a 1.5% agarose minigel and visualized under UV light after ethidium bromide staining.

Plasmid construction

The plasmid vector CMV-βFUSb for the expression of β-gal fusion proteins in eukaryotic cells was generously provided by Dr. Z. Ivics (Ivics and Izsvák 1997). Three open reading frames from the *gag/pol*, *pol*, and *env* regions of HERV-K(C4) were PCR-amplified from a genomic *C4A* clone containing the complete HERV-K(C4) insertion (Prentice et al. 1986). To all primers, a 5′ flanking *Hin*dIII recognition site (underlined) was added to facilitate subcloning of the resulting PCR products, and to the respective 5′ primers, a translation signal ATG start codon (bold face) was also added. PCR primer sequences were as follows (sequences listed according to GenBank accession no. U07856; Dangel et al. 1994; the actual PCR products have an additional 23 bp due to the 5′ primer modifications):

1. *gag/pol* fragment (516 bp, pos. 876–1391)
   - gag-5′: 5′-GGATAAGCTT**ATG**GAAGGAGTTGGAAAG GACCTTA-3′
   - gag-3′: 5′-GGATAAGCTTATAAAGCACCCCCAACTTT TC-3′
2. *pol* fragment (346 bp, pos. 1846–2190)
   - pol-5′: 5′-GGATAAGCTT**ATG**GCTGAATTACTTATAG CACCTG-3′
   - pol-3′: 5′-GGATAAGCTTTAAAGGCAAGTCTGAGTC AATC-3′
3. *env* fragment (267 bp, pos. 4506–4772)
   - env-5′: 5′-GGATAAGCTT**ATG**TACATCTCTGATCACA CTATGG-3′
   - env-3′: 5′-GGATAAGCTTTAATTTGGAGTGTTTAGGG CCTA-3′

The resulting PCR products were inserted in frame into the *Hin*dIII site upstream of the *β-gal* gene of CMV-βFUSb as shown in Fig. 1. The constructs were transfected into competent *Escherichia coli* JM109 or DH5α cells, and the bacterial colonies were screened for the presence of recombinant plasmid constructs by PCR using the same primers. Both the sense and antisense orientations of the *gag/pol*, *pol*, and *env* fragments relative to

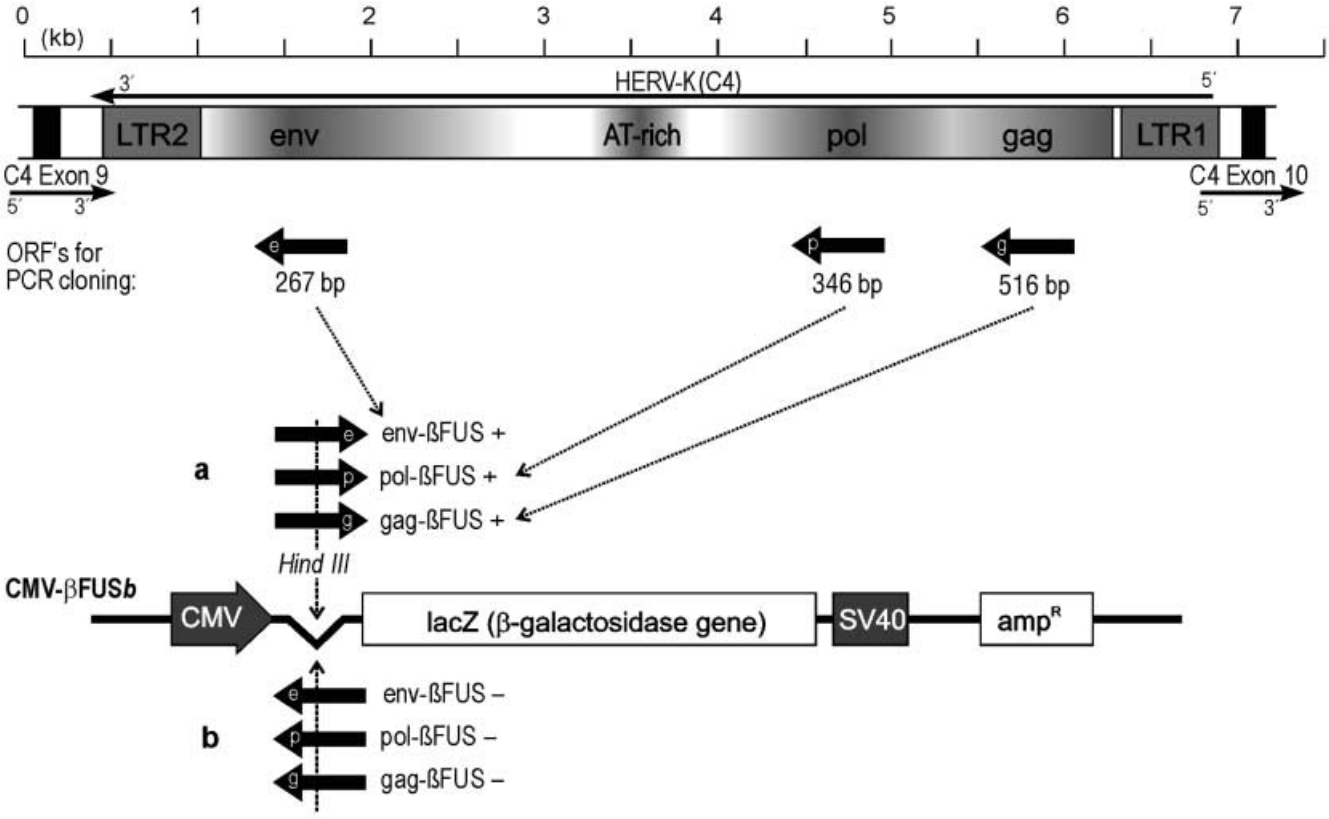

Fig. 1 Cloning strategy for the retroviral constructs used as a model system for retroviral infections in transient transfection experiments. The complete intron 9 retroviral insertion in the human *C4* gene is shown at the top with the transcriptional orientiation of the *C4* and HERV coding sequences (Chu et al. 1995). Three open reading frames (ORFs) were generated by PCR from three retroviral gene regions, and cloned into the CMV-*β*FUSb plasmid vector suitable for expression of a *β*-gal fusion protein (Ivics and Iszvák 1997). The three fragments were inserted independently in sense (*a*) as well as antisense (*b*) orientation to generate six separate constructs as indicated, which were then used for the transfection experiments

the *β-gal* gene were isolated and termed gag/pol/env-*β*FUS+ for the sense, and gag/pol/env-*β*FUS– for the antisense constructs (Fig. 1). To ensure the fidelity of the *β-gal* fusion gene coding sequence, all constructs were analyzed by cycle sequencing using fluorescent dideoxynucleotides followed by capillary electrophoresis in an ABI Prism 310 Genetic Analyzer according to the manufacturer's instructions (Applied Biosystems).

Cell lines and culture conditions

Mouse fibroblast L cells [L(Tk–)] were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS; Life Technologies, Karlsruhe, Germany), and used as HERV-K(C4)-negative controls in all experiments. Mouse LC4A and LC4B cell lines, which had been stably transfected either with human complement *C4A* or *C4B* (Miura et al. 1987), were maintained in HAT medium (DMEM with 10% FCS and 1.36 µg/ml hypoxanthine, 17.4 µg/ml aminopterin, 388 µg/ml thymidine). All cell lines were incubated in a humidified atmosphere with 5% CO_2 at 37 °C.

Transfection of mouse L cells

The day before transient transfection, 1.5–2×105 cells were plated per 30-mm dish in 2 ml of the appropriate growth medium. Two micrograms of plasmid DNA was transfected using the SuperFect polycationic transfection reagent as recommended by the supplier (Qiagen, Hilden, Germany). For the co-transfection experiments of sense and antisense plasmid constructs, a constant amount of *β*FUS+ sense plasmid DNA (2 µg) was transfected together with increasing doses of *β*FUS– antisense constructs (0.5, 1, 1.5, and 2 µg). To monitor transfection efficiencies, the pSV-*β*-Galactosidase control vector (Promega, Mannheim, Germany) was used in independent transfection experiments using the same protocol. For interferon (IFN)-*γ* stimulation, recombinant mouse IFN-*γ* (Life Technologies) was applied directly after transfection in three increasing doses (10, 100, 200 units/ml medium). All transfection experiments were carried out in three separate assays to allow calculation of mean values and standard deviations.

β-Gal assay

Total cell lysates were prepared 48 h after transfection using the Reporter lysis buffer system (Promega). The expression of *β*-gal fusion protein was measured as *β*-gal enzymatic activity using the *β*-Galactosidase Enzyme Assay System performed in a 96-well plate assay according to the manufacturer's instructions (Promega). Absorption was read at 405 nm using a Titertek Multiscan Plus ELISA Reader (ICN Biomedicals, Eschwege, Germany). A standard curve was established and all *β*-gal activities were normalized for the total protein content of the cell extracts (µunits/µg protein), which were determined by a protein quantification assay (BIO-RAD, Munich, Germany) after removal of precipitates by centrifugation.

Results

Detection of HERV-specific antisense mRNA

To confirm the existence of antisense mRNA specific for HERV-K(C4), RT-PCR was carried out using total RNA extractions from the mouse fibroblast cell lines transfected with the human *C4* genes, and from the human hepatoma cell line HepG2 constitutively expressing *C4* (Miura et al. 1987). The primers for reverse transcription were selected such that only mRNA in coding orientation with respect to *C4* was suitable for cDNA synthesis. The unspliced *C4* mRNA should also include the intron 9-derived transcripts in the antisense orientation to the endogenous retroviral genes. Subsequently, specific PCR products could be detected with sizes of 290 bp for *env*, 368 bp for *pol*, 539 bp for *gag*. As a control, a transcript of 397 bp containing *C4* exons 4–9 could be amplified from the spliced *C4* mRNA (Fig. 2). These fragments were not derived from genomic DNA carried over in the RNA purification process, because the extracted RNA was subjected to a DNAse digestion, and the size of the *C4*-specific fragment indicated the absence of genomic intron sequences. Furthermore, we performed a control experiment using extracted cell line RNA directly as template for PCR amplification. No amplification could be achieved using HERV- and *C4*-specific primers, providing evidence for the absence of genomic DNA in our RNA preparations (data not shown). Thus, two types of mRNA are present in HepG2 and *C4*-transfected L cells, comprising either the *C4* coding sequence or the intron 9-derived HERV antisense transcripts. The *C4*-specific PCR products clearly have a stronger intensity in the gel, providing evidence that more mRNA template was available for reverse transcription compared to the weaker HERV-specific fragments. These could have been transcribed either from unspliced *C4* mRNA, from excised intronic mRNA segments, or from 3′ LTR-driven HERV transcription, as suggested by Dangel and co-workers (1994) based on functional LTR promotor studies using CAT assays.

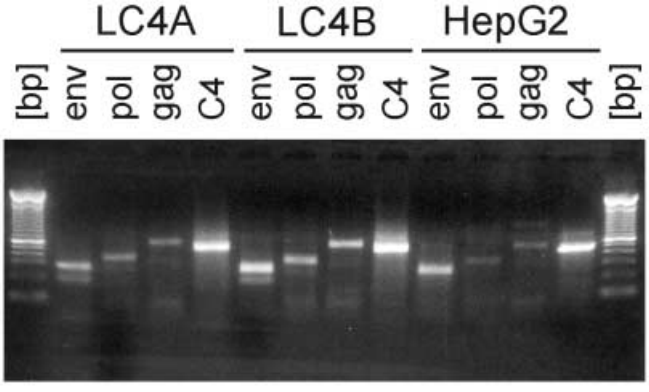

Fig. 2 RT-PCR analysis of retroviral antisense transcripts in transfected mouse fibroblasts and human hepatoma cells. Amplification from contaminating genomic DNA carried over from the RNA extraction procedure was prevented by DNAseI treatment [LC4A, LC4B: mouse L cells stably transfected with a human *C4A* and *C4B* gene, respectively, containing the intron 9 HERV insertion; HepG2: human hepatoma cell line constitutively expressing *C4*; env, pol, gag: RT-PCR fragments of antisense open reading frames from the HERV *env* (290 bp), *pol* (368 bp), and *gag*/*pol* (590 bp) gene regions (each including 23 bp of 5′ PCR primer modification); C4: RT-PCR fragment from *C4* exon 4–9 coding sequence (397 bp); bp: 100-bp ladder as size marker]

Antisense inhibition of βFUS+ expression in C4-transfected L cells

Wild-type L(Tk–) cells and the *C4*-expressing LC4A and LC4B cell lines were transiently transfected in separate assays with 2 μg of the env-, pol-, and gag-*β*FUS+ constructs, and *β*-gal activity was determined in the cell lysates after 2 days. The results demonstrate a significant decrease in pol- and gag-*β*-gal fusion protein expression in the *C4*-transfected cell lines compared to the wild-type cells (Table 1). Using the two-tailed *t*-test, this reduction was statistically significant (*P*-values between 0.0008 and 0.034 for the individual comparisons; Table 1). The strongest reduction of *β*-gal activity, 64.2% compared to the L(Tk–) cells, was observed for the gag-*β*FUS+ construct in LC4B cells. The transfection experiments with the env-*β*FUS+ construct failed to generate functionally active *β*-gal fusion protein (data not shown), although the DNA sequence analysis did not reveal any artifacts causing mutations or frameshifts in the coding sequence of the construct. We therefore assume that the enzyme activity could have been inhibited by specific structural features of the env-*β*-gal fusion protein. The env-*β*FUS+ construct was therefore not included in subsequent experiments. The gag-*β*FUS+ construct always exhibited a higher baseline *β*-gal activity compared to the pol-*β*FUS+ construct (Table 1), supporting the assumption that the retroviral gene-derived portion of the fusion protein might influence enzyme activity.

Control inhibition of βFUS+ expression by antisense βFUS– constructs

To test our in vitro model system for retroviral expression and to demonstrate that the antisense inhibition is specifically directed at the retroviral sequence contained in the *β*FUS+ constructs, control experiments were carried out in wild-type L(Tk–) cells alone. These were co-transfected with constant amounts of pol- and gag-*β*FUS+ constructs (2 μg) as well as increasing amounts of the corresponding *β*FUS– constructs (0, 0.5, 1, 1.5, and 2 μg) containing the same *pol* and *gag* inserts in reverse orientation. A dose-dependent decrease in *β*-gal activity down to 46.5

Table 1 Antisense inhibition of pol- and gag-βFUS+ expression in *C4*-transfected L cells and untransfected controls

Cell line	pol-βFUS+			gag-βFUS+		
	μunits/μg protein	%	*P* (*t*-test)	μunits/μg protein	%	*P* (*t*-test)
L(Tk–)	152.7±9.5	100.0±6.2		251.7±4.9	100.0±2.0	
LC4A	125.3±0.6	82.1±0.4	0.034	176.0±3.6	69.9±1.4	0.0025
LC4B	113.0±6.1	74.0±4.0	0.024	161.7±4.7	64.2±1.9	0.0008

Table 2 Control inhibition of pol- and gag-βFUS+ expression by antisense pol-, gag-, and env-βFUS– constructs

Antisense construct (μg/2× 105 cells)	pol-βFUS+/pol-βFUS–		pol-βFUS+/env-βFUS–		gag-βFUS+/gag-βFUS–		gag-βFUS+/env-βFUS–	
	μunits/μg protein	%	μunits/μg protein	%	μunits/μg protein	%	μunits/μg protein	%
0	152.7±9.5	100.0±6.2	157.7±8.1	100.0±5.2	251.7±4.9	100.0±2.0	282.3±2.1	100.0±0.7
0.5	116.3±5.8	76.2±3.8	150.3±1.5	95.3±1.0	188.7±8.7	75.0±3.5	284.3±10.8	100.7±3.8
1.0	100.0±4.4	65.5±2.9	160.0±7.8	101.5±5.0	153.0±14.9	60.8±5.9	274.0±10.4	97.0±3.7
1.5	89.3±1.5	58.5±1.0	151.3±13.7	96.0±8.7	128.0±12.1	50.9±4.8	272.7±7.6	96.6±2.7
2.0	71.0±2.0	46.5±1.3	154.7±3.1	98.1±1.9	110.7±5.1	44.0±2.0	270.3±6.7	95.7±2.4

and 44% for *pol* and *gag*, respectively, of uninhibited control expression was observed (Table 2). The specificity of this antisense inhibition was further tested by co-transfection experiments using either pol- or gag-βFUS+ constructs with increasing amounts of the unrelated env-βFUS– construct. As expected, no inhibition of β-gal activity could be observed (Table 2). The three antisense constructs, pol-, gag-, and env-βFUS–, do not exhibit any β-gal activity on their own due to numerous reading frame mutations, and thus cannot influence the observed β-gal levels at the protein level.

Dose-dependent antisense inhibition of βFUS+ expression by IFN-γ-induced C4 expression

L(Tk–) controls as well as LC4A and LC4B cells were transiently transfected with either pol- or gag-βFUS+ constructs and stimulated with IFN-γ in three increasing doses of 10, 100, and 200 units. *C4* gene expression had earlier been shown to be specifically upregulated by this cytokine (Kulics et al. 1990; Miura et al. 1987). Unstimulated and stimulated cells L(Tk–) cells exhibited almost identical levels of β-gal activity, thus excluding the possibility that other genes or a *C4*-independent IFN-γ response element influences the expression of the β-gal fusion proteins. In contrast, the enzyme activity was clearly reduced in a dose-dependent fashion down to levels between 63.3 and 47.2% for gag-βFUS+ in LC4A and pol-βFUS+ in LC4B, respectively, compared to the unstimulated pol/gag-βFUS+-transfected LC4A and LC4B cells (Table 3). To assess total antisense activity of constitutive and IFN-γ-enhanced expression in comparison to the situation without any endogenous antisense activity, the results were also compared to those from the L(Tk–) control cells without inhibition of fusion protein expression. Using this approach, a more pronounced decrease was observed in each experiment, down to a minimum of 33.7% for pol-βFUS+ expression in LC4B cells (Fig. 3, Table 4). Table 4 summarizes all the antisense inhibition experiments indicating the cell lines and constructs used for each assay. The results are shown based on the maximal observed reduction of relative β-gal activity, i.e., 0% in L(Tk–) indicates no inhibition.

Discussion

Substantial evidence already exists for an important role of antisense transcripts in the regulation of sense gene expression in prokaryotes as well as eukaryotes including humans (Vanhée-Brossolet and Vaquero 1998; Wagner and Simons 1994). These antisense nucleic acids are usually generated by transcription of the noncoding strand of a normally expressed gene (Adelman et al. 1987). There are few examples, however, for an interaction of sense and antisense transcripts derived from different loci. In the case of myelin-deficient mice, reduction in myelin basic protein has been observed to result from the existence of an antisense transcript expressed from a second *MBP* gene, which originated from a tandem duplication and contains an inversion of exons 3–7. Thus, a partial antisense mRNA is created which interacts with the regular *MBP* message by heteroduplex formation (Tosic et al. 1990).

Our results clearly demonstrate that antisense transcripts derived from the HERV-K(C4) insertion are present in total RNA extracts from cells expressing *C4* (Fig. 2), and that these antisense sequences can interact with primary transcripts of transiently expressed homologous genes not coming from the cellular genome. This interaction between sense and antisense mRNA leads to a significant reduction in the respective gene product, presumably due to inhibition

Table 3 Dose-dependent inhibition of pol- and gag-*β*FUS+ expression after IFN-*γ* stimulation of *C4* gene expression

IFN-*γ* (units/ml)	pol-*β*FUS+/L(Tk–)		pol-*β*FUS+/LC4A		pol-*β*FUS+/LC4B		gag-*β*FUS+/L(Tk–)		gag-*β*FUS+/LC4A		gag-*β*FUS+/LC4B	
	µunits/µg protein	%	µunits/µg protein	%	µunits/µg protein	%	µunits/µg protein	%	µunits/µg protein	%	µunits/µg protein	%
0	158.0±6.9	100.0±4.4	125.3±0.6	100.0±0.5	113.0±6.1	100.0±5.4	248.0±6.2	100.0±2.5	176.0±3.6	100.0±2.0	161.7±4.7	100.0±2.9
10	162.3±7.8	102.7±4.9	107.0±3.0	85.4±2.4	91.7±2.5	81.1±2.2	258.7±12.9	104.3±5.2	162.3±1.5	92.2±0.9	148.0±1.7	91.5±1.1
100	156.3±4.9	98.9±3.1	85.0±1.0	67.8±0.8	70.7±1.2	62.5±1.0	243.7±8.6	98.3±3.5	144.3±4.9	82.0±2.8	126.7±0.6	78.4±0.4
200	162.7±8.5	103.0±5.4	73.0±2.6	58.2±2.1	53.3±1.2	47.2±1.0	261.7±13.7	105.5±5.5	111.3±2.1	63.3±1.2	93.0±5.3	57.5±3.3

Fig. 3 Dose-dependent decrease in retroviral fusion protein expression after IFN-*γ* stimulation in transfected LC4A and B cells and in L(Tk–) control cells. To assess total antisense activity of constitutive and IFN-*γ*-enhanced *C4* expression in comparison to the situation without any endogenous antisense activity, the results were compared to those from the L(Tk–) control cells without inhibition of fusion protein expression. The results are shown as a percentage for all tested combinations of either pol- or gag-*β*FUS+ constructs in LC4A or LC4B cells relative to the expression of the two constructs in the wild type L(Tk–) without the *C4* gene [*β*-gal activity in L(Tk–) cells=100% expression quantified as µunits *β*-gal activity/µg protein]. LC4A, LC4B, and L(Tk–) cells were stimulated with 0, 10, 100, and 200 units IFN-*γ*/ml culture medium in three independent assays at each concentration

of mRNA translation by heteroduplex formation. The specificity of this interaction was shown by the absence of any inhibition in wild-type mouse L(Tk–) cells not carrying any HERV-related gene sequences (Table 1), and by the dose-dependent inhibition of fusion protein expression after stimulation of the LC4A and LC4B cells by IFN-*γ*, which is not observed in wild-type L(Tk–) cells (Table 3, Fig. 3). Additional control experiments based on co-transfection of sense and antisense constructs (Table 2) demonstrated that

Table 4 Results of the antisense inhibition experiments summarizing the maximal relative inhibition of β-gal activity

Experiment Transfected construct	*Target cell line for transfection* Inhibition of β-gal expression (%)			
Constitutive antisense activity gag-/pol-βFUS+:	*LC4A* 18–31%	*LC4B* 26–36%	*L(Tk⁻)* 0%	
IFN-γ-stimulated activity gag-/pol-βFUS+:	55–54%	62–66%	0%	
Antisense control βFUS+ (sense): with βFUS– (antisense):	*L(Tk⁻)* gag gag: 56%	env: 4%	pol gag: 54%	env: 2%

the inhibition of β-gal expression is specifically targeted at the *pol* and *gag* open reading frames in the fusion protein βFUS+ constructs, as no inhibition could be observed when the unrelated env-βFUS– construct was co-transfected.

The observed changes in expression of the retroviral constructs by antisense inhibition appear to be modest, and it might be argued that this limited effect may not be sufficient to support the hypothesis of significant inhibition of retroviral infection. Although the transfected plasmid constructs are controlled by the highly efficient CMV promotor, strongly enhancing transcription, when the experimental system was established, we found that using less than 1 µg of the constructs per 105 cells for transfection would have made it quite difficult to detect expression of the β-gal fusion proteins and their downregulation at measurable levels. Therefore, although numerically small, the observed reduction in fusion protein expression to one-third of the uninhibited controls (Fig. 3, Table 4) represents a highly efficient mechanism of antisense inhibition at the molecular level.

Antisense activity was consistently more prominent in LC4B than in LC4A cells (Tables 1, 3). This can be explained by the observation described in the original study that L(Tk–), LC4A, and LC4B cells contain 0, 4.8, and 12.6 copies, respectively, of the *C4* gene per haploid genome (Miura et al. 1987). The correlation of the quantititative effect with the number of *C4* gene copies may serve as additional proof for the specificity of the antisense inhibition.

Heteroduplex or double-stranded (ds)RNA does not form stable complexes, possibly explaining why it is so difficult to isolate these duplexes from cells (Nellen and Liechtenstein 1993). dsRNA in the nuclear compartment is believed not to interact with cytoplasmatic pathways. Instead, it could become the target of nucleases specific for or activated by dsRNA, causing its rapid degradation. On the other hand, dsRNA in the cytoplasmatic compartment may trigger various metabolic pathways including IFN and protein kinase activation (Kumar and Carmichael 1998). This may represent an important cellular defense mechanism in cases of viral infection which are often characterized by the presence of dsRNA intermediates. Thus, the IFN-γ-dependent increase in *C4* expression might be further enhanced by the formation of heteroduplexes composed from infecting retroviral RNA and its antisense counterpart contained in the unspliced *C4* mRNA. However, it is not yet clear in which cellular compartment the specific interaction between the endogenous antisense transcript and the viral RNA might occur, and whether these complexes are sufficiently stable to induce activation of the IFN pathways. Furthermore, viral infections trigger the increased expression of cytokines as well as of complement components. It is well established that C3 and C4 may also directly neutralize infectious virus particles (Cooper 1998).

In the context of a hypothetical antisense protection mechanism, however, an important prerequisite for any interaction between an endogenous antisense RNA transcript with RNA of an infecting virus is sufficient sequence homology to allow the formation of a heteroduplex RNA. In our model system, a perfect homology was established by experimental design, as the genomic sequences already present in intron 9 of the *C4* gene were used for constructing the recombinant fusion protein. This was done on purpose to allow tight control of the assay conditions.

Nucleotide database searches had established that the HERV-K(C4) retroviral sequence exhibits significant sequence identity of >65% across several hundred base pairs, and 90% for short stretches of up to 30 bp with known human and animal viruses. This identity has been found predominantly in the conserved *pol* gene region in comparisons, e.g., with HIV, MMTV and Jaagsiekte sheep retrovirus (Chu et al. 1995). At the time of the initial retroviral integration, there must obviously have been an exogenous counterpart of the HERV-K(C4) provirus with homologies over a longer sequence range to allow for stronger sense-antisense interactions. Therefore, our results provide support for the hypothesis suggested previously that the continuous presence of endogenous retroviral sequences in opposite transcriptional orientation with respect to the "host" gene could be the result of natural selection (Dangel et al. 1994). LTRs have been shown to act as independent promoters in both orientations on neighboring genes (Domansky et al. 2000), and *C4*-independent promoter activity has been demonstrated in antisense orientation for the 3′ LTR of HERV-K(C4) (Dangel et al. 1994). There are other examples of both complete HERV as well as

LTR insertions in human genes in reverse orientation (Kambhu et al. 1990; Kjellmann et al. 1999), but additional systematic data are required to support the assumption that this could be a result of selection rather than by coincidence.

Some studies have focused on the potential role of HERVs regarding possible pathogenic effects of expressed retroviral gene products, e.g., in the case of autoimmune disease (Venables and Brooks 1992). These could be side-effects of a primarily beneficial role of endogenous retroviruses. In this context, it is interesting to note that the most common Caucasoid MHC haplotype *HLA-A1*,*Cw7*,*B8*,*DR3*,*C4AQ0*,*C4B1* does not carry the HERV-K(C4) insertion, as the *C4A* gene is deleted, and the *C4B* gene is always short (Schneider et al. 1986). At the same time, this haplotype is significantly associated with a number of autoimmune diseases of unknown etiology, such as insulin-dependent diabetes mellitus and systemic lupus erythematosus, as well as with rapid progression of HIV infection (Lechler and Warrens 2000). It is tempting to speculate that the lack of this protective HERV insertion might confer an additional risk factor for this particular haplotype. However, as yet there is no convincing evidence for any retroviral involvement in the pathogenesis of the MHC-associated autoimmune diseases. Furthermore, the HERV insertion is present in other MHC risk haplotypes, as well as in haplotypes considered to be protective (Atkinson and Schneider 1999). The proposed genomic antisense protection could provide another good reason why ERVs are so abundant in the genome of humans and other vertebrates, and have been maintained by positive selection mechanisms (Sverdlov 2000). However, further studies using a retroviral infection model will be required to demonstrate that this protective role could also be a relevant in vivo cellular defense strategy.

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