

Evolution of a cytoplasmic tripartite motif (TRIM) protein in cows that restricts retroviral infection

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Primate tripartite motif 5 α (TRIM5 α) proteins mediate innate intracellular resistance to retroviruses. In humans, TRIM5 is located in a paralogous cluster that includes TRIM6, TRIM34, and TRIM22. Although TRIM6 and TRIM34 orthologs are found in other mammals, TRIM5 has to date been identified only in primates. Cow cells exhibit early blocks to infection by several retroviruses. We identify a cytoplasmic TRIM protein encoded by LOC505265 that is responsible for the restriction of infection by several lentiviruses and N-tropic murine leukemia virus in cow cells. Susceptibility of N-tropic murine leukemia virus to 505265-mediated restriction is determined primarily by residue 110 of the viral capsid protein. Phylogenetically, cow LOC505265 segregates with the TRIM5/TRIM6/TRIM34 group, but is not an ortholog of known TRIM genes. The B30.2/SPRY domain of 505265 exhibits long variable regions, a characteristic of the proteins encoded by this paralogous group, and shows evidence of positive selection. Apparently, cows have independently evolved a retroviral restriction factor from the same TRIM family that spawned TRIM5 in primates. Particular features of this subset of cytoplasmic TRIM proteins may be conducive to the convergent evolution of virus-restricting factors.

capsid | convergent evolution | restriction factor | retrovirus | ungulates

Retroviruses initiate infection by fusion of the viral and target cell membranes and entry of the viral capsid into the cytosol (1, 2). Uncoating of the capsid into smaller subunits occurs before or concomitantly with reverse transcription. At this early phase of infection, certain retroviruses encounter blocks in the cells of particular mammalian species. These blocks are mediated by dominant host factors that can be competed by virus-like particles containing capsids related to that of the restricted virus (3–14). The study of recombinant retroviruses indicates that the viral capsid protein is the major determinant of susceptibility to these early restrictions (3–14). For example, the identity of residue 110 on the N-tropic murine leukemia virus (N-MLV) capsid protein strongly influences the ability of this virus to negotiate the early events of infection in human cells (7, 9, 15). HIV-1 and simian immunodeficiency virus of macaques (SIV_{mac}) encounter postentry blocks in the cells of Old World monkeys and New World monkeys, respectively (16–18). A genetic screen identified a tripartite motif (TRIM) protein, TRIM5 α , as the major factor restricting HIV-1 during the early phase of infection in Old World monkey cells (19). Differences among TRIM5 α orthologs in primate species account for the patterns of restriction observed for particular retroviruses (15, 20–25).

TRIM proteins contain RING, B-box, and coiled coil domains and constitute a large family of proteins with poorly understood functions (26, 27). Many cytoplasmic TRIM proteins, like TRIM5 α , contain a C-terminal B30.2(SPRY) domain that is thought to mediate binding to specific ligands (26, 27). Indeed, studies of recombinants of TRIM5 α from different primate species indicate that sequences in the B30.2 domain dictate the potency and specificity of the restriction of particular retroviruses (24, 28–37). Comparison of TRIM5 sequences among primate species indicates

that the TRIM5 α B30.2 domain, but not the RING and B-box 2 domains, has been subjected to strong positive selection during primate evolution (30, 34). The source of such selection may have been ancient retroviral epidemics, which studies of endogenous retroviral sequences indicate have plagued mammals repeatedly over millions of years of evolution (38–42).

Genetic lability characterizes the subset of TRIM genes related to TRIM5. In humans, TRIM5 is located in a paralogous cluster at 11p15.4 that includes TRIM6, TRIM34, and TRIM22 (30). Unlike most cytoplasmic TRIM proteins, the proteins encoded by these genes exhibit longer B30.2 domain variable regions than those of the putative ancestral TRIM protein (30). Presumably, these expansions were driven by requirements for binding particular ligands. Equally striking is the labile nature of TRIM5-related genes in the genomes of different mammalian species. Rodents and dogs have no TRIM5 ortholog, yet both groups retain TRIM6 and TRIM34 orthologs (30). Thus, TRIM5 appears to have arisen relatively recently in mammalian evolution (perhaps only in primates), probably by duplication of an ancestor of TRIM6 or TRIM34.

Given the long history of exposure of many vertebrate species to retroviruses (38–42) and the potential benefit of expressing virus-restricting elements, the evolution of proteins with TRIM5-like functions in nonprimate species might be expected. Although TRIM6 and TRIM34 genes are found in the genomes of a number of mammalian species, neither has been shown to encode a protein with antiretroviral activity (ref. 30 and X.L., unpublished work). Although rodents lack TRIM5, their genomes contain TRIM6/TRIM34 paralogs not found in the human genome (e.g., TRIM12, TRIM30, 9230105E10Rik in the mouse). However, there is currently no evidence that the proteins encoded by these genes restrict retrovirus infection (refs. 26 and 30 and B.S., unpublished observations).

Surveys of the susceptibility of cells from different mammalian species to retroviral vectors have suggested that early blocks to N-MLV or feline immunodeficiency virus (FIV) might exist in nonprimate lineages (9, 25). The sporadic distribution pattern of restriction on the phylogenetic tree of vertebrate species hints that the ability to encode retroviral restriction factors may have been convergently acquired multiple times during mammalian evolution (9). Here, we investigate retroviral restriction in cow cells, which have been reported to be poor hosts for N-MLV and FIV vectors

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Abbreviations: MLV, murine leukemia virus; N-MLV, N-tropic MLV; B-MLV, B-tropic MLV; TRIM, tripartite motif; SIV_{mac}, simian immunodeficiency virus of macaques; SIV_{agm}, SIV derived from African green monkeys; FIV, feline immunodeficiency virus; BIV, bovine immunodeficiency virus; EIAV, equine infectious anemia virus; siRNA, short interfering RNA; MDBK, Madin-Darby bovine kidney; Ka/Ks, nonsynonymous/synonymous.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ381150 and DQ381151).

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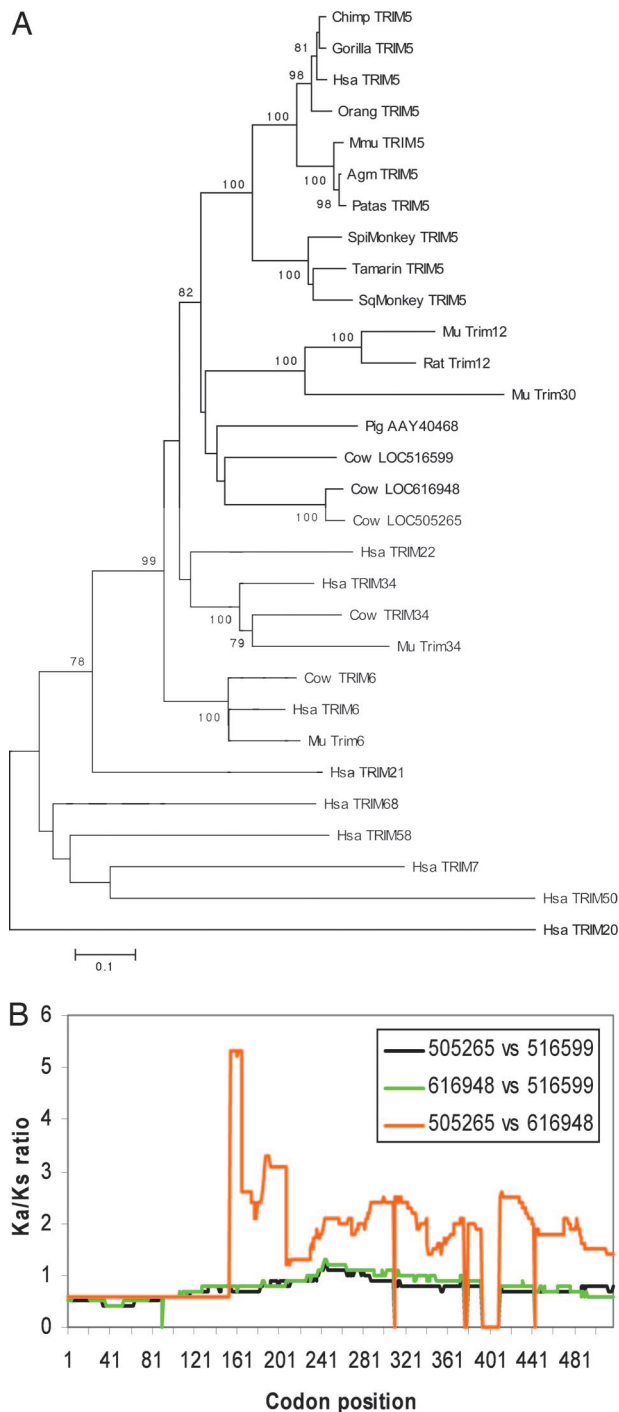


Fig. 1. Identification of candidate bovine restriction factors. (A) The predicted amino acid sequences of the proteins encoded by bovine *LOC516599*, *LOC616948*, *LOC505265*, *LOC514492* (cow TRIM6) and *LOC539820* (cow TRIM34) were aligned with those of other TRIM proteins by using CLUSTAL X (50). The alignment was used to build trees in MEGA3.1 by using neighbor joining, maximum parsimony, the Unweighted Pair Group Method with Authentic Mean (UPGMA), and maximum evolution methods with 1,000 bootstrap iterations. Values of 100 represent 99–100% concordance in the bootstrap analysis. The scale bar represents evolutionary distance in substitutions/amino acid residue. Hsa, *Homo sapiens*; Mmu, *Macaca mulatta*; Spi, spider monkey; Sq, squirrel monkey; Mu, mouse. (B) The plot shows the Ka/Ks ratios at various codon positions for pairwise comparisons of *LOC516599*, *LOC616948*, and *LOC505265*. The alignment is shown in Fig. 5. The Ka/Ks ratio across gaps in the alignment was arbitrarily set to 0. The Ka/Ks ratios, calculated as described (45), were estimated as rolling averages for a window of 200 codons.

Table 2. Lengths of the B30.2 domain variable regions in TRIM proteins

TRIM protein	B30.2 variable region length, aa			
	v1	v2	v3	v4
505265 (cow)	20	43	28	5
616948 (cow)	20	29	29	5
516599 (cow)	20	26	29	5
AAY40468 (pig)	20	20	29	5
TRIM6 (human, mouse)	19	23	25	5
514492/TRIM6 (cow)	18	23	25	5
TRIM12 (rat)	19	43	30	5
TRIM12 (mouse)	19	30	30	5
TRIM22 (human)	19	29	29	5
TRIM34 (human, mouse, rat, dog)	13	23–25	29	5
539820/TRIM34 (cow)	13	25	31	5
TRIM5 (Old World primates)	26–46	17	32	5
TRIM5 (New World primates)	17	15–17	41–96	5
TRIM20 (human, chimpanzee)	20	12	23	10
TRIM50 (human, mouse, rat, pig)	20	12	23	9
Consensus	19–21	11–13	20–23	4–10

The boundaries of the TRIM B30.2 domain variable regions are defined as in Song *et al.* (30). The variable region lengths in amino acid residues are reported, along with those expected of the putative ancestral TRIM protein (Consensus). Variable region lengths greater than those of the consensus are in bold.

Transfection of siRNA 4, but not the control *TRIM21* siRNA, also resulted in an increase in BNBB-MLV and FIV infection of MDBK cells. (Fig. 2 and Fig. 6, which is published as supporting information on the PNAS web site). Transfection of siRNA 4 had less of an effect on the infectivity of NBNN-MLV, and had no significant effect on the susceptibility of MDBK cells to B-MLV, EIAV, or BIV vectors. These results suggest that the proteins encoded by *LOC616948* and *LOC505265*, either alone or together, contribute to restriction of N-MLV and FIV infection in MDBK cells.

To test whether N-MLV restriction could be rescued in the siRNA 4-transfected MDBK cells, we established MDBK cells stably expressing mutated *LOC616948* or *LOC505265* cDNAs. These cDNAs were modified with identical silent nucleotide changes that are predicted to render them resistant to siRNA 4. The encoded TRIM proteins contain a C-terminal epitope tag from influenza hemagglutinin. In MDBK cells stably expressing the mutated *LOC505265* cDNA, transfection of siRNA 4 did not relieve the restriction to N-MLV infection (Fig. 2E). By contrast, stable expression of the mutated *LOC616948* cDNA did not attenuate the ability of siRNA 4 to increase the susceptibility of MDBK cells to N-MLV infection. Differences in the steady-state levels of expression of the protein products of the mutant *LOC505265* and *LOC616948* cDNAs did not explain the observed differences in the rescue of restriction (Fig. 2F). These results indicate that *LOC505265* encodes a protein that restricts N-MLV infection in MDBK cells.

Blockade of HIV-1 Infection in MDBK Cells Before Reverse Transcription. We wanted to examine whether the poor infectivity of HIV-1 vectors in MDBK cells resulted from a block before reverse transcription and whether the 505265/616948 proteins were involved. MDBK cells were mock-transfected or transfected with siRNA 4 or *TRIM21* siRNA. A real-time PCR assay was used to monitor viral cDNA synthesis at various times after incubating these MDBK cells with the vesicular stomatitis virus G-pseudotyped HIV-1-Luc vector. Two sets of PCR primers and Taqman probes were used. One set measures minus-strand strong-stop DNA, the initial DNA product of reverse transcription. The second set detects later DNA products that rely on the second

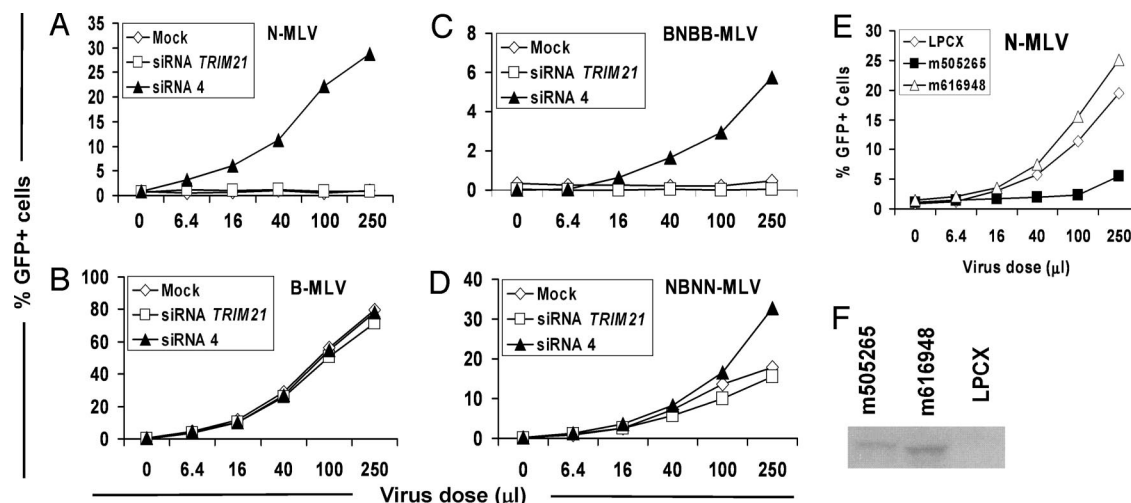


Fig. 2. Implication of bovine 505265 in retroviral restriction in MDBK cells. (A–D) MDBK cells were mock-transfected or transfected with 100 pmol of siRNA directed against *TRIM21* or siRNA 4. The cells were then incubated with increasing amounts of the indicated GFP-expressing viruses. Forty-eight hours later GFP-positive cells were counted by fluorescence-activated cell sorting. The results shown are typical of those obtained in three independent experiments. (E) MDBK cells were transduced with the empty LPCX vector or LPCX vectors expressing cow *LOC505265* or *LOC616948* cDNAs with mutations that render them resistant to siRNA 4. Cells were then transfected with siRNA 4 and incubated with increasing amounts of the GFP-expressing N-MLV vector. GFP-positive cells were counted by fluorescence-activated cell sorting. The experiment was performed twice with similar results. (F) MDBK cells transduced with the empty LPCX vector or LPCX vectors expressing the mutant cDNAs (m505265 or m616948) described in *E* were lysed. Cell lysates were Western-blotted with an antibody directed against the hemagglutinin epitope tag at the C terminus of the protein.

template switch of reverse transcription. Synthesis of strong-stop DNA was inefficient in the mock- or *TRIM21* siRNA-transfected MDBK cells at all time points examined. By contrast, significant increases in the amount of strong-stop DNA were observed in cells transfected with siRNA 4 (Fig. 3A). Similar results were obtained when late reverse transcripts were studied (Fig. 3B). The luciferase

activities in these cells, which reflect the efficiency of HIV-1-Luc infection, paralleled the increases in viral cDNA synthesis (Fig. 3C). The block to HIV-1-Luc infection in MDBK cells transfected with siRNA 4 was rescued by expression of an siRNA 4-resistant *LOC505265* cDNA, but not by an *LOC616948* cDNA (Fig. 3D). The protein products of both of these mutated cDNAs were

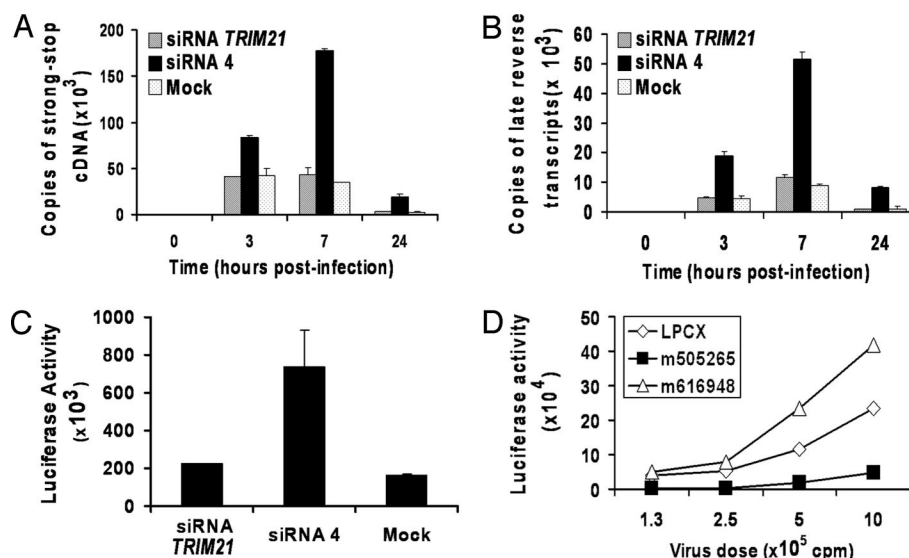


Fig. 3. The block to HIV-1 infection in MDBK cells occurs before reverse transcription. (A and B) MDBK cells were mock-transfected or transfected with siRNA 4 or siRNA directed against *TRIM21*. Cells were then incubated with vesicular stomatitis virus G-pseudotyped, DNase-treated HIV-1-Luc virus. Extrachromosomal DNA was extracted at the indicated times and used to detect early, strong-stop (A) and late (B) reverse transcripts by quantitative PCR. Incubation of cells with control HIV-1-Luc viruses lacking envelope glycoproteins resulted in very low signals; these background signals were subtracted from those obtained with entry-competent HIV-1-Luc viruses. Error bars indicate the variation observed between two parallel infections assayed in duplicate. (C) A portion of the MDBK cells transfected as described above was incubated with 2×10^6 reverse transcriptase-cpm of HIV-1-Luc viruses. Forty-eight hours later, luciferase activity was measured in the cell extracts. Error bars indicate the variation in luciferase activity between duplicate infections. Similar results were obtained in two independent experiments. (D) MDBK cells were transduced with the empty LPCX vector or LPCX vectors expressing LOC505265 or LOC616948 cDNAs with mutations that render them resistant to siRNA 4. The level of expression of the 505265 and 616948 proteins in these cells is shown in Fig. 2F. Cells were transfected with siRNA 4 and incubated with increasing amounts of vesicular stomatitis virus G-pseudotyped, HIV-1-Luc virus. Forty-eight hours later, luciferase was measured in the cell extracts. Similar results were obtained in two independent experiments.

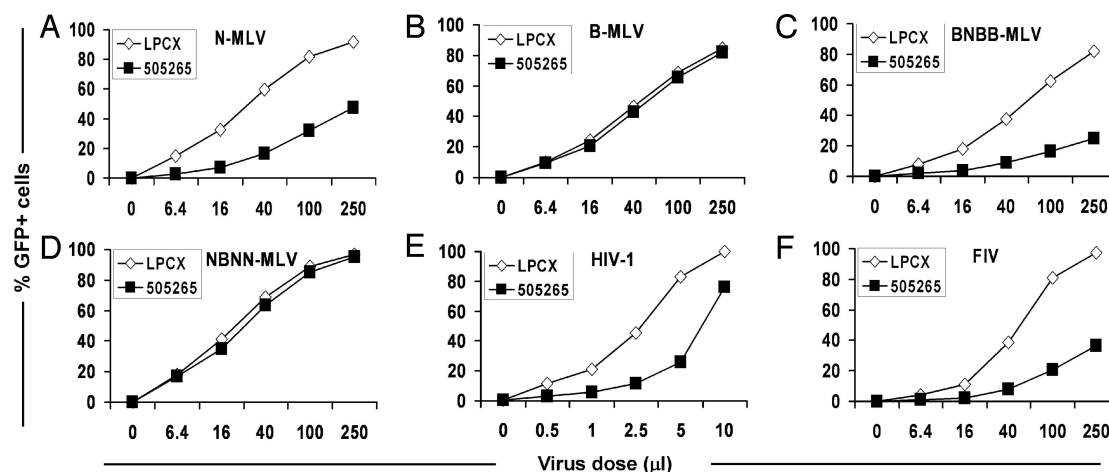


Fig. 4. Specific inhibition of retroviral infection by cow 505265. Cf2Th cells transduced with the empty LPCX vector or an LPCX vector expressing cow 505265 protein were incubated with various amounts of the indicated viruses expressing GFP. GFP-positive cells were counted by fluorescence-activated cell sorting. The data shown are representative of those obtained in three independent experiments.

expressed efficiently in the MDBK cells (see Fig. 2F). We conclude that at least part of the block to HIV-1 infection in MDBK cells is mediated by the 505265 protein and involves a decrease in the efficiency of early events before or concurrent with the initiation of reverse transcription.

Effects of 505265 Expression on Retroviral Infection. To examine directly the effects of the cow 505265 protein on the efficiency of retroviral infection in permissive cells, Cf2Th canine thymocytes stably expressing this protein were established by transduction with LPCX vectors containing the *LOC505265* cDNA. Control cells were transduced with the empty LPCX vector. Staining with an antibody directed against the C-terminal hemagglutinin epitope tag revealed that the 505265 protein was located in the cytoplasm and excluded from the nuclei of these cells (Fig. 7, which is published as supporting information on the PNAS web site). The 505265-expressing Cf2Th cells were incubated with different amounts of the retroviral vectors described above and the efficiency of infection was monitored by GFP fluorescence. Expression of 505265 significantly inhibited infection by N-MLV, BNBB-MLV, FIV, HIV-1, and *SIV_{mac}*, compared with the infection of cells transduced with the empty LPCX vector (Fig. 4 and Fig. 8, which is published as supporting information on the PNAS web site). By contrast, infections by B-MLV, NBNN-MLV, BIV, and *SIV_{agm}* were not significantly affected by 505265 expression. 505265 partially inhibited EIAV infection. We conclude that the expression of cow 505265 is sufficient to account for most of the specific restrictions to retroviral infection observed in MDBK cells.

Discussion

TRIM proteins appeared with the metazoans and dramatically expanded in number during vertebrate evolution (27, 30). Approximately 70 TRIM genes have been identified in mammals (26), but the function of most of the encoded TRIM proteins is unknown. Some TRIM family members have been reported to exhibit antiviral activity, but to date only primate TRIM5 α variants have demonstrated high potency and specificity for particular retroviruses (26). Here, we identify a second TRIM protein, cow 505265, that exhibits substantial ability to restrict infection by several lentiviruses and a gammaretrovirus. Like TRIM5 α (15, 19), 505265 blocks infection before the initiation of reverse transcription. As changes in the viral capsid protein influence susceptibility to 505265-mediated restriction, 505265 likely targets the incoming capsid complex. The pattern of retroviruses blocked by 505265 parallels the susceptibility of MDBK cells to infection; one excep-

tion is *SIV_{agm}*, which did not efficiently infect MDBK cells, yet was not blocked by 505265. Treatment of MDBK cells with siRNA 4 did not increase the efficiency of *SIV_{agm}* infection, and expression of 616948 in Cf2Th cells did not block *SIV_{agm}* infection (data not shown). Further studies will be required to dissect the blocks encountered in MDBK cells by *SIV_{agm}*. Of note, 505265 did not inhibit infection by BIV, which naturally infects cows. The effects of 505265 on infection by EIAV, another ungulate lentivirus, were minimal. A similar situation applies to primate TRIM5 α proteins, which at best partially restrict their cognate lentiviruses (26). Retroviruses have apparently evolved capsids that are only moderately restricted by the TRIM protein(s) expressed by the natural host.

The *LOC516599*, *LOC616948*, and *LOC505265* genes are not orthologs of any known gene, but clearly belong to the TRIM5/6/12/22/30/34 group. These three TRIM genes are thus paralogs of cow TRIM6 and TRIM34 and probably arose as ungulates diverged from other mammals. In this respect, *LOC516599*, *LOC616948*, and *LOC505265* resemble the TRIM12 and TRIM30 genes, which have been found only in rodent species, and TRIM5 and TRIM22, found only in primates. Apparently, a number of novel genes have arisen within the TRIM5/6/12/22/30/34 group in different mammalian species. The acquisition of specific antiretroviral activity by *LOC505265* in cows and TRIM5 in primates represents an example of convergent evolution. It is of interest that this group of TRIM genes was used in at least two separate instances to create antiviral factors. Certain features of the TRIM proteins in this group, including intracellular association with particular host molecules, oligomerization state, or ligand-binding characteristics, may be conducive to the acquisition of antiviral activity.

Ancient retroviral epidemics probably imposed sporadic, but powerful, selective pressure on any TRIM proteins that could modulate the efficiency of infection. TRIM5 genes exhibit evidence of positive selection, particularly in the sequences encoding the C-terminal B30.2 domain (30, 34). Sequence divergence in the variable regions of the B30.2 domain, which is critical for capsid recognition (46), determines the potency and viral specificity of restriction by TRIM5 α proteins from different primate species. Similarly, the B30.2 domains of other TRIM proteins with antiviral activity might be expected to exhibit hallmarks of selection imposed by the requirement to coevolve with rapidly changing viruses. The *LOC516599*, *LOC616948*, and *LOC505265* genes are closely related to each other, supporting the idea that they arose by successive duplication events, first of a common ancestor and later of an *LOC505265* and *LOC616948* ancestor. The B30.2 variable regions

of the encoded proteins are expanded, compared with those of the putative TRIM ancestor. Our analysis of Ka/Ks ratios suggests that, after the divergence of *LOC505265* and *LOC616948*, positive selection has operated on the coiled coil and B30.2 domains of the encoded proteins, whereas purifying selection has preserved the RING and B-box 2 amino acid residues. This pattern is reminiscent of that seen for TRIM5 variants in primates (30, 34).

As seen for susceptibility to Fv1 and human TRIM5 α (7, 9, 15, 47), the identity of the amino acid at residue 110 in the MLV capsid protein strongly influences the sensitivity of MLV variants to the cow 505265 protein. Residue 110 is located at the outer tip of the spokes that compose the hexameric unit of retroviral capsids (48). The spokes of adjacent hexamers flank a trimeric depression on the capsid surface that has been hypothesized to represent a potential binding site for the trimeric TRIM5 α proteins (49). The charged side chain of residue 110 is well positioned to influence the interactions of restriction factors with this putative binding site. Future studies should test this hypothesis.

Materials and Methods

cDNA Cloning, Analysis, and Expression. Generation of cDNA clones for cow *LOC616948* and *LOC505265*, phylogenetic analysis, and expression of these cDNAs is described in *Supporting Materials and*

Methods, which is published as supporting information on the PNAS web site. The sequences of *LOC505265* and *LOC616948* cDNAs from MDBK cells have been deposited in GenBank under accession nos. DQ381150 and DQ381151, respectively.

Quantitative Real-Time PCR. Levels of strong-stop and late reverse transcription products were assessed as described (refs. 15 and 23 and *Supporting Materials and Methods*).

Single-Round Infections. Retroviral vectors expressing GFP or luciferase were made and used as described (refs. 15 and 23 and *Supporting Materials and Methods*).

RNA Interference. The sequences of the siRNAs and the transfection of these siRNAs into MDBK cells are described in detail in *Supporting Materials and Methods*.

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