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Two Species of Rev Proteins, with Distinct N Termini, Are Expressed by Caprine Arthritis Encephalitis Virus

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Several cDNA clones representing alternatively spliced Rev-specific transcripts were isolated from a cDNA library prepared from Himalayan tahr cells infected with caprine arthritis encephalitis virus (CAEV). We previously characterized two *rev*-like cDNA species, d1 and d2, and a *tat* e1 cDNA containing the *rev* coding sequence downstream to the *tat*. In these cDNAs, the *rev* coding domain derives its amino terminus from the N terminus of *env*, which is spliced to the 3′ open reading frame encoding the putative Rev protein. In this study, we report the genetic structure of a fourth *rev*-like cDNA (designated g1), which lacks the 5′ *env*-derived sequences. All of these *rev* transcripts, including cDNA g1, increased the level of chloramphenicol acetyltransferase expression when cotransfected with a reporter plasmid containing the CAEV Rev-responsive element-spanning region downstream of the *cat* coding sequences. Western blot (immunoblot) analysis showed that each transfected cDNA species gave rise to a 16-kDa protein lacking *env*-encoded amino-terminal epitopes. In contrast, CAEV-infected Himalayan tahr cells expressed only a 20-kDa protein, whose N terminus, in contrast, is derived from the *env*. Moreover, only the 20-kDa protein was also detected in the mature CAEV virions. These observations suggest that the transcripts d1, d2, and e1 can potentially, in appropriate cellular context, encode two Rev isoforms differing in their N termini, whereas the g1 transcript encodes only the 16-kDa species. Elucidation of the significance of the 16-kDa Rev protein in CAEV biology must await further studies.

Caprine arthritis encephalitis virus (CAEV), a lentivirus, is the etiologic agent of an inflammatory disease of domestic goats, characterized primarily by chronic arthritis and mastitis in adults and leukoencephalomyelitis in young animals (for a review, see reference 11). The genome of CAEV is somewhat less complex than the genomes of the primate lentiviruses (references 17 and 26 and our unpublished results). Thus, only three small open reading frames (ORFs), designated *orf1* (or Q), *orf2* (or S), and *3′orf*, are present. We and others have recently shown that *orf2* encodes the Tat protein (5, 18), while the *3′orf* was suggested to encode the Rev (6, 17, 19). Northern (RNA) hybridization and cDNA cloning experiments revealed the presence of a multiplicity of alternatively spliced transcripts, a characteristic of lentiviral genomes (5, 6, 17). In our previous analysis of cDNA libraries of CAEV-infected tahr cells, we identified two alternatively spliced species, designated pCEV/d1 and pCEV/d2, predicted to encode a putative Rev protein whose 38 N-terminal amino acids are encoded by the N terminus of *env*, joined in frame to 95 residues from the *3′orf* (6). A third cDNA, designated pCEV/e1, shown to encode Tat (5), contains a similar Rev-coding region downstream of the *tat* ORF and thus represents a polycistronic message.

We now report the isolation of an additional transcript, designated g1. For sequence analysis, the cDNA insert was subcloned into pBluescript SK vector (Stratagene) and sequenced by using a Sequenase DNA sequencing kit (United States Biochemical Corp.) as previously described (6). The 965-bp-long g1 cDNA (Fig. 1A) starts 27 nucleotides (nt) downstream of the transcription start site and ends at the polyadenylation site located at position 9181 of the genomic sequence, similar to the previously described CAEV cDNAs (5, 6). Comparison of the nucleotide sequence of this cDNA with the genomic sequence revealed that it contained two exons (Fig. 1). The first exon is identical to that of cDNAs d1, d2, and e1, whose donor splice sites are all located at nt 336 of the genomic sequence. However, in g1 the first exon is spliced directly to position 8514, within the *3′orf*. This transcript is similar to one detected previously in cells infected by CAEV (CO) (17).

Computer analysis of pCEV/g1 revealed the existence of three ORFs (Fig. 1A). The first ORF, designated *rev* (nt 316 to 603), lacks an AUG codon. Assuming that initiation occurs at a non-AUG codon (e.g., isoleucine at nt 319) within the *3′orf*, the product would potentially represent a 95-residue-long Rev species which possesses an amino terminus different from that of the Rev protein encoded by d1, d2, and e1 cDNAs (Fig. 1B). The second ORF, designated ΔTM (nt 308 to 646), contains an AUG codon at nt 365 and, similar to cDNAs d1, d2, and e1, could thus encode a truncated transmembrane (TM) protein of 94 amino acids with a calculated molecular mass of 10 kDa (Fig. 1A). The third ORF, designated X ORF (nt 576 to 755), is identical to those of cDNAs d1, d2, and e1, contains an AUG at nt 576, and thus could potentially encode a 73-residue (8-kDa) protein (Fig. 1A).

Since the CAEV cDNA libraries were constructed in the pCEV/27 vector, in which transcription is driven by the Moloney murine leukemia virus long terminal repeat (LTR) (6), the coding capacity of each cDNA could be directly assessed in a eukaryotic system. However, tahr cells, which are highly permissive for CAEV replication (27), cannot be established as drug (G418)-resistant lines (our unpublished observations). Therefore, we used a canine cell line, Cf2Th, which was shown to enable efficient transcriptional activity of the Moloney mu-

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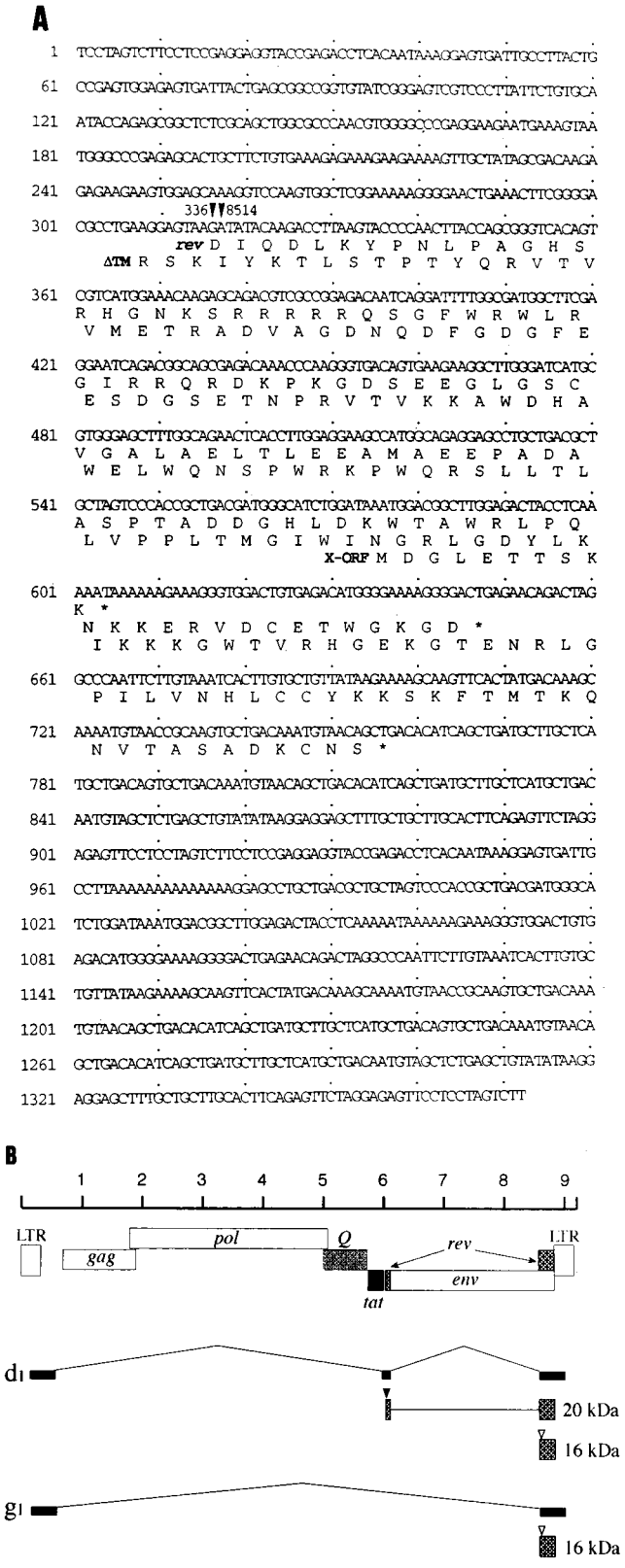

FIG. 1. Genetic structure of cDNA g1. (A) Nucleotide sequence and deduced amino acid sequence. Numbers at the left refer to the cDNA sequence. Filled-in arrowheads indicate exon borders according to the nucleotide positions of the CAEV genomic sequence (17). The predicted amino acid sequences of the putative ORFs are given in standard single-letter code. The *rev* ORF (nt 316 to 603), the ΔTM ORF (nt 308 to 646), and the X ORF (nt 576 to 755) are indicated. (B) Schematic representations of cDNA g1 and cDNA d1. The exons (black boxes) are shown with the positions relative to the proviral genome. The AUG codons which presumably serve for translation initiation of the 20-kDa Rev protein are indicated by a filled-in arrowhead. The putative non-AUG codon for translation initiation of the 16-kDa Rev protein is indicated by an empty arrowhead.

rine leukemia virus LTR (16). An antiserum raised against a *3′orf*-derived oligopeptide, ALAELTLEEAMAEEP (designated anti-*3′orf* oligopeptide serum), detected a 16-kDa band in each transfectant (Fig. 2A). This reactivity could be competed for by the peptide immunogen (not shown). The band was not observed in cells transfected with vector DNA.

Next, experiments were performed to analyze the *3′orf*-encoded proteins in CAEV-infected tahr cells. Data showed that, in contrast to cDNA d1-transfected Cf2Th cells which expressed the 16-kDa protein, CAEV-infected tahr cells expressed only a 20-kDa *3′orf*-encoded protein (Fig. 2B). This protein was not detected in uninfected cells or in infected cell extracts incubated with preimmune serum or with anti-*3′orf* oligopeptide serum that had been preadsorbed with the immunizing oligopeptides (not shown).

It was reported that the Rev protein of visna virus is present in mature virions (9). We likewise detected the Rev protein in mature equine infectious anemia virus (EIAV) virions (16). It was thus of interest to search for the presence of the *3′orf*-encoded protein in CAEV virions. Therefore, virions collected from supernatants of CAEV-infected tahr cells harvested at 24-h intervals were subjected to sequential immunoprecipitation and Western blotting (immunoblotting) by using the anti-*3′orf* oligopeptide serum. A protein of 20 kDa, similar to that expressed in CAEV-infected cells, was specifically detected in CAEV virions (Fig. 2B).

To analyze the genetic structures of the 20- and 16-kDa *3′orf*-encoded proteins, the putative N-terminal domain (nt 6015 to 6122 in the genomic sequence) of the 20-kDa protein was amplified by PCR, using sense (GATGCCGGGGCCAG ACATATTCGC) and antisense (TCATTGCTGCCTCTCAA TGCAGCCTTT) oligonucleotide primers (6), and inserted in frame into pET17xb (Novagen). The bacterially synthesized protein was used to raise an antibody (designated anti-N-terminus serum) in rabbits. Western analysis of cell lysates prepared from pCEV/d1-transfected Cf2Th cells or from CAEV-infected tahr cells showed that while the 16-kDa protein reacted only with the anti-*3′orf* serum, the 20-kDa protein reacted with both the anti-*3′orf* and anti-N-terminus sera (Fig. 2C). These data suggest that in contrast to the 20-kDa protein expressed in CAEV-infected tahr cells, the 16-kDa protein expressed in Cf2Th cells transfected with various cDNA lacks the *env*-encoded N terminus and thus would initiate at the non-AUG codon within the *3′orf*.

Our data showing that the 20-kDa protein is expressed in CAEV-infected tahr cells, whereas the 16-kDa species is expressed in canine cells, might suggest a translational regulation. In addition, the absence of the 16-kDa isoform in CAEV-infected tahr cells might result from the lower frequency of g1 cDNA than of the d1 species in the cDNA library prepared from CAEV-infected tahr cells (reference 6 and our unpublished results). Nevertheless, the presence of the 20-kDa Rev protein in CAEV-infected tahr cells strongly suggested that the Rev cDNAs d1, d2, and e1 would express the 20-kDa protein in an appropriate cellular context. Likewise, the 16-kDa protein, which was not expressed during CAEV infection in tahr cells, might be expressed and function during CAEV infection under certain conditions in vivo. However, elucidation of the

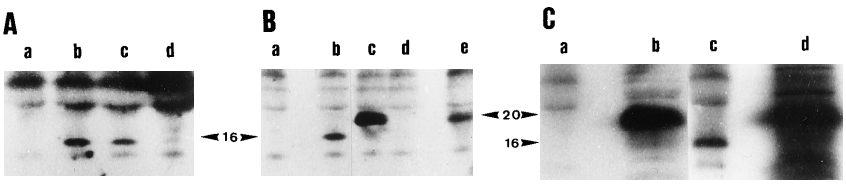

FIG. 2. Expression of the *3′orf*-encoded proteins. (A) Analysis of cDNA encoding capacity. Semiconfluent canine thymus cells (Cf2Th [ATCC CRL 1430]) were stably transfected with 5 μg of pCEV27 (lane a), pCEV/g1 (lane b), pCEV/d1 (lane c), or pCEV/d2 (lane d) as previously described (5). Following selection in the presence of G418 (400 μg/ml), total lysates of 6×10^6 stable transfectants were preadsorbed with preimmune rabbit serum (for 1 h at 0°C) followed by protein A-Sepharose (for 30 min at 0°C) and then immunoprecipitated with anti-*3′orf* oligopeptide serum. Proteins were fractionated on sodium dodecyl sulfate–15% polyacrylamide gels and blotted onto nitrocellulose filters. Detection was performed with anti-*3′orf* oligopeptide serum followed by ^{125}I-protein A as previously described (16). The arrowheads indicate the positions of 16-kDa *3′orf*-encoded proteins. (B) Analysis of CAEV-infected cells and CAEV virions for the presence of *3′orf*-encoded proteins. Total cell lysates of 5×10^6 stably pCEV27 (lane a)- or pCEV/d1 (lane b)-transfected Cf2Th cells, CAEV-infected (lane c) or noninfected (lane d) tahr cells, or CAEV purified virions harvested from 160-ml supernatants of CAEV-infected tahr cells (lane e) were preadsorbed with a preimmune serum, immunoprecipitated with anti-*3′orf* oligopeptide serum, and fractionated on an SDS–15% polyacrylamide gel. The gel was subjected to immunoblot analysis using anti-*3′orf* oligopeptide serum. Detection was performed by enhanced chemiluminescence (Amersham) as previously described (5). The arrowheads indicate the positions of the 20- and 16-kDa *3′orf*-encoded proteins. (C) Genetic structures of the *3′orf*-encoded proteins. Lysates prepared from stably pCEV/d1-transfected Cf2Th cells (lanes a and c) or from CAEV-infected tahr cells (lanes b and d) were subjected to Western analysis using anti-N-terminus (lanes a and b) or anti-*3′orf* (lanes c and d) serum. Detection was performed by enhanced chemiluminescence. The arrowheads indicate the 20- and 16-kDa *3′orf*-encoded proteins.

relevance of the 16-kDa protein to CAEV infection must await further studies.

Although the 16-kDa Rev isoform lacks the *env*-encoded N-terminal domain of the 20-kDa isoform (6), it is predicted to contain the other two conserved regions encoded by the *3′orf*. The first is KSRRRRR, which is analogous to the arginine-rich motif of the Rev proteins of primate lentiviruses thought to be involved in binding to Rev-responsive elements and in nuclear localization. The second is the leucine-rich domain, LAELTLEE, at the carboxy terminus, which corresponds to the putative acidic activation domain of the primate lentiviral Rev that would function by binding to cellular factors involved in RNA transport (for a review, see reference 2). It was thus important to assess Rev activity of the 16-kDa *3′orf*-encoded isoform.

The Rev protein of CAEV (19, 21) likely functions similarly to those of other lentiviruses (reviewed in reference 2), by increasing the expression of the unspliced or singly spliced viral messages in the cytoplasm. To examine the function of Rev, we used a reporter chloramphenicol acetyltransferase (CAT) plasmid (designated pCAEV.LTR-CAT-RRE$_{CAEV}$) that contained the fragment designated envΔrev region (nt 4076 to 9181 in the genomic sequence), which spanned the CAEV RRE domain (5, 17, 19) but lacked the *rev* ORF (nt 8516 to 8569 in the genomic sequence). The expression of *cat* was driven by the CAEV LTR. Each construct stimulated CAT activity by two- to fourfold (Fig. 3A), suggesting that the Rev protein expressed by the various Rev cDNAs species acts similarly to other lentiviral Rev proteins (reviewed by Antoni et al. [2]), by facilitating the cytoplasmic expression of Rev-responsive element-containing viral transcripts.

We previously characterized the CAEV *tat* transcript (pCEV/e1) in which the *rev* ORF is downstream of *tat* (5). To determine whether pCEV/e1 also expresses Rev, we constructed an additional CAT reporter vector (pCMV-CAT-RRE$_{CAEV}$) in which the CAEV envΔrev region was inserted downstream of the cytomegalovirus enhancer-promoter region in the cytomegalovirus-CAT construct described previously (16). The absence of the CAEV LTR in this reporter vector did not enable Tat expressed by pCEV/e1 to exert its activity.

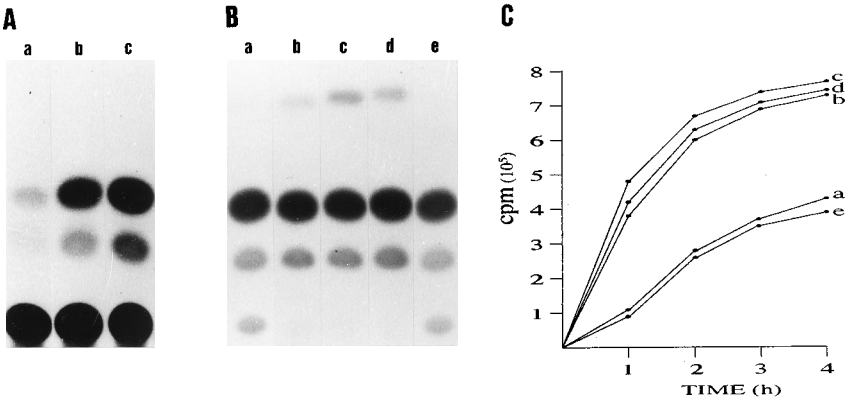

FIG. 3. Functional analysis of the Rev cDNAs in a Rev-dependent gene expression assay. The reporter CAT vectors pCAEV.LTR-CAT-RRE$_{CAEV}$ and pCMV-CAT-RRE$_{CAEV}$ were constructed as follows. To abolish the *rev* coding sequence, a deletion inside the *3′orf* (nt 8516 to 8569 of the genomic sequence [17]) was performed in the CAEV Env cDNA isolated from a cDNA library constructed in pCEV27 (reference 6 and our unpublished results). The Rev-deleted *env* coding sequence (4076-[Δ8516-8569]-9181), designated envΔrev, was inserted downstream of the *cat* gene, the expression of which was driven by the CAEV LTR or by a cytomegalovirus early transcriptional unit described previously (16). (A) Cf2Th cells were cotransfected with 2 μg of the CAT reporter plasmid pCAEV.LTR-CAT-RRE$_{CAEV}$ and 5 μg of either pCEV27 (lane a), pCEV/d1 (lane b), or pCEV/g1 (lane c). After 48 h, CAT levels were assessed by thin-layer chromatography of acetylated chloramphenicol derivatives as previously described (16). (B) Cf2Th cells were cotransfected with 2 μg of pCMV-CAT-RRE$_{CAEV}$ and either pCEV27 (a), pCEV/d1 (b), pCEV/g1 (c), pCEV/e1 (d), or pCEV/e1Δrev (e). CAT levels were determined 48 h after transfection by thin-layer chromatography (B) or by the method of Neumann et al. (12) (C) as previously described (16).

In cells transfected with the pCEV/e1 construct, CAT expression reached levels similar to those induced by pCEV/d1 (Fig. 3B and C). Moreover, the increase in CAT levels was abolished when the CAT reporter vector was cotransfected with a mutated pCEV/e1, designated pCEV/e1(ΔRev), in which part of the *rev* ORF (nt 8516 to 8569) was deleted. Thus, we conclude that, like other lentiviral *tat-rev* transcripts (4, 10, 15, 16, 22, 24, 25), cDNA e1 is polycistronic, expressing the Rev protein from a downstream initiation codon. This suggestion is consistent with the leaky scanning mechanism (7, 8), by which the positions −3 and +4 relative to the A in the AUG are the most important nucleotides for efficient translation. Thus, the absence of G at position +4 of the Tat AUG (AAGAUGA) (5) may allow translation from a downstream initiation site, the AUG located at residue 1 of the *rev* ORF of cDNA e1, thus giving rise to the 20-kDa Rev protein. In addition, the AUG of the *rev* ORF of cDNAs d1, d2, and e1 occurs also in a suboptimal context, UAGAUGG (5, 6), in which the key purine at position −3 is absent, thus enabling translation from a non-AUG initiation codon further downstream within the 3′ *rev* ORF, giving rise to the 16-kDa Rev protein.

Our data suggest that the Rev protein of CAEV is expressed via alternatively spliced messages whose genetic structures and coding capacities resemble those of the *rev* transcripts of visna virus (4, 10), EIAV (16), bovine immunodeficiency virus (13, 14), feline immunodeficiency virus (15), and primate lentiviruses (2). However, as found for Rev transcripts of EIAV (16), our data strongly suggest that whereas the multiply spliced *rev* cDNAs of CAEV might give rise to two species of the Rev protein with distinct N termini, the double-exon cDNA might express only the smaller species of Rev. Interestingly, in contrast to the primate Rev proteins (reviewed in reference 2), the N terminus of the 20-kDa Rev is *env* encoded, similar to the N termini of the Rev proteins encoded by visna virus (4), bovine immunodeficiency virus (14), feline immunodeficiency virus (15), and EIAV (16). Although the 16-kDa Rev of CAEV lacks the *env*-encoded N terminus, our data suggest that it functions via a mechanism similar to that of the other lentiviral Rev proteins. In other systems, it has been shown that the use of alternative initiation codons from within the same message results in differential protein localization (1, 3, 23). It is noteworthy that the Rev protein of visna virus, in contrast to primate lentiviral Rev proteins, accumulated also in the cytoplasmic/membrane fraction of infected cells (9, 20) as well as in the nucleus. The Rev protein of CAEV is also localized both in the nucleus and in the cytoplasmic fraction of CAEV-infected cells (21). Hence, it will be of interest to determine whether the two Rev species of CAEV exhibit distinct intracellular localization. It is noteworthy that the larger species of the Rev proteins of visna virus (10) and EIAV (16) are packaged into mature viral particles, as we show here for the 20-kDa Rev of CAEV. Thus, the 20-kDa Rev protein might play a role in the assembly of viral particles or in the early steps of the new cycle of virus infection, in addition to regulating nuclear export of the viral structural transcripts.

Nucleotide sequence accession number. The nucleotide sequence of cDNA g1 has been entered into the EMBL database under accession number X64828.

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