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Identification and Characterization of the Bovine Immunodeficiency-Like Virus *tat* Gene

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A cDNA clone of the bovine immunodeficiency-like virus (BIV) *trans*-activator gene (*tat*) was identified and characterized. The *tat* cDNA clone was generated by splicing, and on the basis of sequence analysis, the Tat protein was found to be encoded entirely by the first exon. It is 103 amino acids in size and shares sequence homology with the human immunodeficiency virus (HIV) Tat. The BIV *tat* clone can *trans* activate the BIV promoter effectively, as measured by the expression of the bacterial chloramphenicol acetyltransferase gene, when transfected into bovine cells. Besides activating the BIV promoter, the BIV Tat can also *trans* activate the HIV promoter effectively. It is possible that BIV Tat and HIV Tat employ similar mechanisms in *trans* activation of the viral long terminal repeat-directed gene expression.

Bovine immunodeficiency-like virus (BIV) is a recently characterized bovine lentivirus which causes lymphadenopathy, lymphocytosis, and central nervous system disorders in infected cattle (3, 22). The virus has been cloned, and the nucleotide sequence of the BIV genome has been determined (4, 10). The genome is approximately 9.0 kb in size and contains the structural genes *gag*, *pol*, and *env*, as well as other open reading frames analogous to the *vif*, *tat*, and *rev* genes of human immunodeficiency virus (HIV) (6, 7, 10). The HIV *tat* gene is an important regulatory gene (6, 8). Several studies have shown that HIV Tat increases the steady-state level of HIV mRNA in part by increasing the rate of transcription initiation (14, 15) and the efficiency of transcription elongation (12). It has been demonstrated recently that BIV-infected cells also express a *trans*-acting protein, similar to the HIV Tat (18). However, the *tat* gene of BIV has not been identified, and its *trans*-activation function has not been demonstrated. Analysis of the BIV transcriptional pattern by Northern (RNA) blot indicated that multiple transcripts were expressed, and several subgenomic viral RNAs that can potentially encode the BIV Rev and Tat proteins were detected (17). In order to specifically identify and characterize the BIV *tat* gene, we used polymerase chain reaction to amplify viral mRNAs that can potentially encode the Tat protein.

Cloning of BIV *tat* by polymerase chain reaction. On the basis of the BIV genomic sequence analyses (10), it was predicted that BIV *tat* is encoded by two exons (Fig. 1A). The first is encoded by an exon 5′ of the envelope gene from nucleotides (nt) 5228 to 5536, and the second is encoded by an exon located within the envelope gene from nt 7657 to 7782. In order to clone both *tat* exons, three different primers were used to amplify BIV-infected embryonic bovine lung (EBL) cell mRNA. The first primer, PT1, should prime 5′ to the first predicted *tat* exon, and the second primer, PT2, should prime 3′ of the predicted second exon (Fig. 1B). The third primer, PT3, was also used in place of PT2 to prime at the poly(A) tail located at the 3′ end of *tat* mRNA. Several clones were generated by either the first (PT1 and PT2) or the second (PT1 and PT3) set of primers. One clone generated from the first primer set (TATC-1) and one clone from the second primer set (TATC-2) were further mapped and characterized. They were found to be very similar. Both carry exons 1 and 2 of the putative *tat* gene (Fig. 1C). The TATC-1 insert is smaller and terminates at the end of the second *tat* exon at nt 7787, where the PT2 primer binds. The TATC-2 clone is larger, and its second exon extends to the 3′ end of the BIV genome, all the way to the polyadenylation site at nt 8485. The nucleotide sequence of TATC-1, its deduced amino acid sequence, and a comparison with a previously published BIV genomic sequence (10) are shown in Fig. 2. The sequence of our cDNA clone TATC-1 is very similar to the published genomic clone sequence: there are only three nucleotide differences between the two sequences, and two of these changes led to amino acid substitutions. Our cDNA sequence also indicates that the splice junction between *tat* exons 1 and 2 is different from the one predicted earlier (10). Our sequence indicates that the splice donor and acceptor sites for *tat* exon 2 are located at nt 5541 and 7638, respectively, whereas the previously predicted locations were at nt 5536 and 7657 (Fig. 1 and 2). Interestingly, the splice junction for *tat* is identical to that previously mapped for the BIV *rev* gene (17). In addition, our cDNA clone has three in-frame stop codons: one can be found before the splice junction, and the other two are in the second exon immediately after the splice junction. This suggests that the second exon may not encode any Tat protein sequence and that the 103-amino-acid (103-aa) Tat protein is encoded entirely within the first exon.

The translated TATC-1 sequence displays several common features that are found in other lentiviral Tat proteins (5). There is a cysteine-rich domain (aa 38 to 46) containing a cluster of five cysteines. Mutations of these analogous cysteine residues in HIV can abolish Tat function completely (13). There is also a core region of BIV Tat, HCQLCF LQKNLGINYG (aa 49 to 64), which bears striking homology to the HIV core region, HCQVCFITKALGISYG (aa 33 to 48). Alignment of the HIV-1 and BIV Tat sequences indeed shows a 54% amino acid homology within their cysteine-rich and core regions; both regions are essential for HIV-1 Tat protein to function in *trans* activation (5). In the BIV Tat protein, there also exists a basic domain, RGKGRRIRR (aa 73 to 81), which bears some homology to the domain (RKKRRQRRR) found in HIV-1 (19). This domain was predicted to be the nuclear localization motif for HIV. Further experiments are required to

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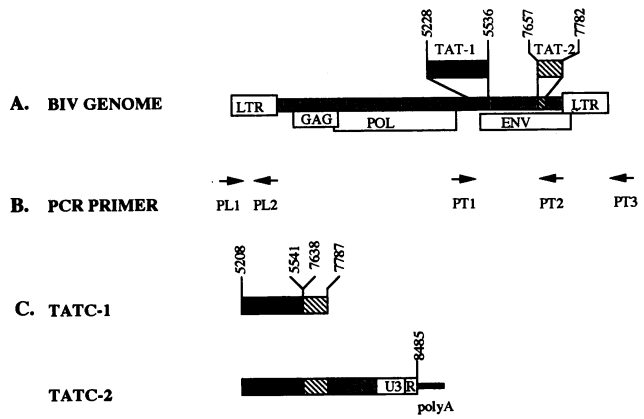

FIG. 1. (A) Schematic representation of organization of the BIV genome. The predicted BIV *tat* exon 1 is indicated by the solid box, and exon 2 is indicated by the slashed box. Numbers indicate the predicted nucleotide positions of the *tat* exons (10). (B) Locations of primers used for cloning *tat* cDNA and the BIV LTR. The primers are as follows: PL1, 5'-CGCCACGCTCCTATTTTA-3' (nt 7953 to 7970), which is located in the 5' end of the U3 region of the LTR; PL2, 5'-CCGAGCCAGTGCCTCTC-3' (nt 8435 to 8419, an antisense primer), which is located in the R region of the LTR; PT1, 5'-CATTCATAGATTGTGGCA-3' (nt 5208 to 5225), which is located before the predicted *tat* exon 1; PT2, 5'-GTTCAACCAAT TCTCGTAGA-3' (nt 7787 to 7768, an antisense primer), which spans the predicted stop codon of *tat* exon 2; and PT3, 5'-TTGTC GACTTTTTTTTTTTTTTTT-3', which is an oligo(dT) primer containing a *SalI* recognition site at the 5' end. PCR, polymerase chain reaction. (C) Schematic representation of the two *tat* cDNA clones obtained with primers PT1 and PT2 (TATC-1) and with primers PT1 and PT3 (TATC-2). Numbers indicate the nucleotide positions and the location of the splice junction between *tat* exons 1 and 2. R, R region.

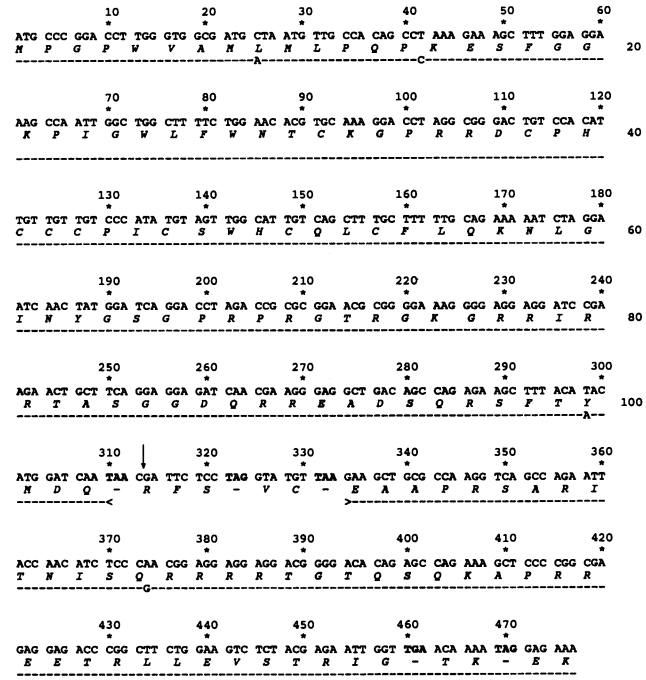

FIG. 2. Nucleotide sequence and translated amino acid sequence of BIV *tat* cDNA clone TATC-1. Nucleotide numbers are given at the top, and the amino acid numbers are given on the side. The TATC-1 sequence (top sequence) and the published BIV *tat* genomic sequence (10) (bottom sequence) are shown. The dashed line represents sequence identity between TATC-1 and the published genomic sequence, and the differences in the sequences are indicated. The arrow between nt 314 and 315 represents the splice junction between *tat* exons 1 and 2; < > represents the predicted splice donor and acceptor sites (10). Termination codons are also indicated (boldface).

demonstrate the functions of these domains in the BIV Tat protein.

The *tat* cDNA clone can *trans* activate the BIV promoter. To test whether our *tat* cDNA clone can *trans* activate BIV long terminal repeat (LTR) expression, a BIV-CAT construct which contains the chloramphenicol acetyltransferase (CAT) gene under the control of the BIV LTR was made. Our *tat* cDNA clone TATC-2 was also cloned into a plasmid under the control of the simian virus 40 promoter to generate the *tat* expression vector pBTATC. This clone was then cotransfected into EBL cells along with the BIV-CAT plasmid to check for *trans* activation of CAT expression. In the presence of pBTATC, BIV-CAT was activated and resulted in a 20-fold increase in CAT activity (Table 1). Since the putative Tat protein is encoded entirely by the first exon, our results suggest that exon 1 alone is sufficient for *trans* activation of the BIV LTR. To further confirm that a functional BIV Tat protein can be entirely encoded by only one exon, a second BIV-*tat* clone was generated from the genomic BIV clone (1). This plasmid, pBTATG, was constructed by inserting the BIV proviral DNA fragment containing an intact proviral 5' LTR and the predicted *tat* exon 1 into a pUC18 vector. This genomic clone thus lacks the second *tat* exon and the 3' end of the BIV genome. When pBTATG was cotransfected into EBL cells along with BIV-CAT, *trans* activation of the BIV LTR-directed CAT expression was observed. There was a 25-fold increase of CAT activity in the presence of pBTATG (Table 1). Interestingly, the genomic pBTATG clone consistently yields slightly higher levels of *trans*-acting activity than our *tat* cDNA clone, pBTATC. It is possible that pBTATG, which uses the BIV LTR as a promoter, is more effective in expressing *tat* mRNA, and the Tat protein produced may in turn provide positive feedback to further *trans* activate the BIV LTR.

BIV Tat is similar to HIV Tat and can *trans* activate the HIV LTR. Since the BIV Tat protein sequence shares some homology with the HIV-1 Tat core and cysteine-rich regions (19), it would be of interest to test whether BIV Tat could *trans* activate the HIV LTR and vice versa. EBL cells were either transfected with HIV-CAT (11) alone or cotransfected with the BIV *tat* cDNA clone pBTATC or genomic clone pBTATG. The results in Table 1 show that the HIV LTR can indeed be *trans* activated by BIV Tat and that the presence of pBTATC or pBTATG elevated HIV LTR-directed CAT gene expression by approximately 8- and 18-fold, respectively. Even though this level of *trans* activation is significant, it is still lower than the previously observed 50- to 100-fold activation of the HIV LTR by its homologous HIV *tat* gene product. In bovine cells, the lower level of activation of the HIV LTR by BIV Tat cannot be due to the absence of appropriate cellular factors, since the HIV LTR can be activated by HIV Tat by approximately 101-fold in these cells (Table 1). Thus, the lower level of *trans* activation of the HIV LTR by BIV Tat may simply reflect structural differences between HIV and BIV Tat proteins. The *trans* activation of HIV-CAT by BIV Tat is specific; other viral

TABLE 1. *trans* activation of CAT promoter constructs by different *tat* plasmids[a]

Construct and plasmid	CAT enzyme activity (cpm/h/10^6 cells)[b]	Fold activation
BIV-CAT		
None	1,820	
pBTATC	36,804	20
pBTATG	44,941	25
HIV TAT	5,366	3
HIV-CAT		
None	499	
pBTATC	4,198	8
pBTATG	9,071	18
HIV TAT	50,550	101
ΔBIV-CAT		
None	2,068	
pBTATC	5,559	3
pBTATG	16,121	8
pSV_2-CAT		
None	2,936	
pBTATC	2,916	
HTLV-I-CAT		
None	2,756	
pBTATC	2,945	

[a] EBL cells were transfected with 2 μg of CAT plasmid and 10 μg of *tat* plasmid by the standard calcium phosphate precipitation procedures (2). Transfected cells were harvested at 48 h for CAT analyses. All samples were corrected for protein concentration variations before they were assayed. Procedures for CAT assays with [^{3}H]acetyl coenzyme A were described previously (16).

[b] The amount of CAT enzyme activity per 10^6 transfected cells was measured over a 5-h period, and the values were calculated by taking the slope of the linear portion of the curve and expressed as counts per minute incorporated over a 1-h period.

promoters, such as the human T-cell leukemia virus type I promoter construct (HTLV-I-CAT) or the simian virus 40 viral promoter construct (pSV_2-CAT), cannot be *trans* activated by BIV Tat (Table 1). In addition, the BIV LTR can also be *trans* activated when cotransfected into EBL cells with the heterologous HIV *tat* expression plasmid (Table 1) (11). However, the activation of the BIV LTR by HIV-1 Tat is only about threefold and is much lower than the observed 20- to 25-fold activation by BIV Tat. Nevertheless, this suggests that *trans* activation by the BIV Tat protein may involve mechanisms similar to those of HIV Tat.

***Trans* activation of a deletion BIV LTR construct by BIV Tat.** The *trans*-activation target of HIV Tat has been well characterized and is located downstream of the transcription initiation site (9, 20). Similarly, the Tat responding element for equine infectious anemia virus is also located within the LTR (21). Since BIV and HIV Tat proteins may use similar mechanisms in activation of their respective LTRs, it was of interest to determine whether the target site of BIV Tat is also located within the LTR. In order to test for the presence of the BIV Tat responding element, a BIV LTR deletion clone was tested. This clone, ΔBIV-CAT, was generated by using polymerase chain reaction primers to specifically amplify the BIV LTR from the U3 region, up to position +64 in the R region, with respect to the mRNA start site. The amplified, deleted LTR was then cloned in front of the CAT gene to study LTR-directed CAT expression. When ΔBIV-CAT was cotransfected into EBL cells along with the pBTATC or pBTATG plasmid, even though activations of ΔBIV-CAT were still observed, the levels of *trans* activation were much lower (Table 1). Levels of activation of ΔBIV-CAT by pBTATC and pBTATG were about six- and threefold lower than those of intact BIV-CAT, respectively. Our results suggest that the deletion in ΔBIV-CAT has removed part of the *cis*-acting target element which may be involved in the Tat-directed *trans* activation.

Our study provides further evidence to support the idea that there are similarities between BIV and other lentiviruses, such as HIV. Not only are there similarities in genomic organization, nucleotide sequence (10), and antigenicities (1), but the BIV Tat is also very similar to that of HIV, as we have now demonstrated. These proteins appear to function through similar mechanisms. Further elucidation of the mechanism of Tat-mediated stimulation of BIV viral gene expression will require detailed mutagenesis studies of the functional domains of the BIV Tat protein and its *cis*-acting *trans*-activation target element, as well as studies to identify other cellular proteins that may be involved.

Nucleotide sequence accession number. The GenBank nucleotide sequence accession number for the BIV *tat* gene is M90681.

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