cis- and *trans*-Acting Regulation of Gene Expression of Equine Infectious Anemia Virus

PATRICIA L. DORN¹ AND DAVID DERSE^{2*}

Biological Carcinogenesis Development Program, Program Resources, Inc., National Cancer Institute Frederick Cancer Research Facility, and Department of Zoology, University of Maryland, College Park, Maryland 20742,¹ and Genetics Section, Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, Maryland 21701-1013²

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Deletion analysis of the equine infectious anemia virus long terminal repeat revealed that sequences responsive to virus-specific transactivation are located within the region spanning the transcriptional start site (−31 to +22). In addition, an active exon of a *trans*-acting factor (*tat*) was identified downstream of *pol* and overlapping *env* (nucleotides 5264 to 5461). Activation by *tat* is accompanied by an increase in the steady-state levels of mRNA directed by the equine infectious anemia virus long terminal repeat.

Equine infectious anemia virus (EIAV) is a member of the *Lentivirinae*, a subfamily of nononcogenic retroviruses. Retroviral gene expression is controlled by *cis*-acting regulatory sequences located within the long terminal repeats (LTRs) which flank the provirus and by factors which interact in *trans* with these sequences. In addition to core promoter and mRNA processing signals, LTRs have been shown to contain combinations of sequences, such as hormone response elements, enhancers, or regions which respond to virus-encoded *trans*-acting factors, which determine condition-specific activation. Certain retroviruses have also evolved or acquired genes which regulate virus gene expression in *trans*. These viral products directly or indirectly interact with the *cis*-acting LTR sequences to control expression at the transcriptional or posttranscriptional level, or both (2, 6, 7, 17).

Localization of *cis*-acting response region. Recent studies have shown that the EIAV LTR is activated in *trans* in cells infected with the same virus (3, 17). Furthermore, Sherman et al. have shown transactivation of the EIAV LTR upon cotransfection of cells with the EIAV provirus (17). To investigate the *cis*-acting regulatory elements, including the region in the LTR which is responsive to this virus-specific activation, we examined the effects of progressive deletions on promoter activity (Fig. 1). The LTR was deleted from either the 5' or the 3' end by using exonuclease III followed by exonuclease VII; the ends were repaired with T4 DNA polymerase, and the fragments were cloned into the poly-linker of the plasmid pUXcat (7). Constructs were named with respect to the transcriptional start site (+1), with deletion endpoints determined by dideoxynucleotide sequencing (15). Ten micrograms of these plasmids was then transfected onto EIAV-infected and uninfected adherent feline (E-FEA and FEA, respectively) cell lines, as described elsewhere (3). At 44 h after transfection, the cells were harvested and cell lysates were assayed for chloramphenicol acetyltransferase (CAT) activity by the thin-layer chromatography method of Gorman et al. (4). CAT activities are expressed relative to the amount of CAT activity obtained with pUXSV2cat, in which the simian virus 40 (SV40) early promoter is cloned upstream of the CAT gene in pUXcat. The levels obtained with pUXcat (background) were subtracted from all values. Plasmid pE(−111) showed

no difference in CAT activity compared with the whole LTR in either the E-FEA (induced-level) or the FEA (basal-level) cells (Fig. 1). Removal of an additional 35 base pairs (bp) [pE(−76)] actually increased CAT activity approximately twofold in E-FEA and approximately 30-fold in FEA cells. This increase in activity is also observed in certain cell types upon removal of a negative regulatory element from the U3 region of the human immunodeficiency virus type 1 (HIV-1) LTR (13). As this increase is not evident in later studies using D17 cells, the EIAV LTR may also contain a cell type-specific negative regulatory element. Further 5' deletions extending to −41 and −31, which retain the TATA box and CAP site, reduced promoter activity to approximately half the level observed with the entire LTR but still showed a >200-fold differential between uninfected and infected cells. Thus, upstream sequences, although important for a portion of the promoter activity, do not appear to be required for activation in E-FEA cells. In contrast to the other lentivirus LTRs, such as HIV-1 and HIV-2, the EIAV LTR examined in our study lacks Sp-1 or NF-κB consensus binding sites (5, 8, 11) or the long direct repeats that are present in visna virus and caprine arthritis-encephalitis virus LTRs (7). EIAV does, however, contain a 13-bp imperfect overlapping direct repeat encompassing the CAAT box which may be responsible for the activity we observed in this region. Compared with the LTR under study in this work, the LTRs of another clone we sequenced (3) and of a third isolate (19) do contain a 16-bp repeat separated by a 4-bp insertion. This duplication may have a slight effect on basal promoter activity but does not appear to influence transactivation (3).

Deletions from the 3' end of the LTR, pE(+40) or pE(+22), retained essentially full promoter activity when transfected onto FEA or E-FEA cells. However, deletion of an additional 18 nucleotides, pE(+4), abolished CAT activity in both EIAV-infected cells and uninfected cells. Thus, the sequences required for activation in infected cells are contained within the region −31 to +22.

Identification of a *tat* gene. Several retroviruses have been shown to encode a *trans*-acting factor or *tat* gene in open reading frames (ORFs) located between the *pol* and *env* genes (18) or in the 3' end of the viral genome (1, 2). The lentiviruses studied to date produce *tat* from at least a three-exon message in which the exon derived from the central region, between *pol* and *env*, is sufficient for transac-

* Corresponding author.

FIG. 1. Schematic diagram and CAT activity of plasmids that express the CAT gene under the control of an intact EIAV LTR or truncated LTRs. The structure of the parental plasmid, pUXcat, is shown at the top of the figure and contains the polylinker from pUC18 (hatched box) followed by the bacterial CAT gene and the polyadenylation signals from SV40. The entire LTR (pE1cat) or LTRs deleted of 5' or 3' sequences (solid lines indicate EIAV sequences retained) were cloned into the polylinker of pUXcat. The endpoints of the truncated LTRs were determined by dideoxy sequencing (15) and are numbered with respect to the transcriptional start site (*1). Ten micrograms of these plasmid constructs was transfected onto E-FEA and FEA cells by the calcium phosphate coprecipitation method (12). Approximately 44 h later, the cells were harvested and cell lysates were assayed for CAT activity (12). Levels of CAT activity are expressed relative to levels obtained with pUXSV2cat. Background levels obtained with pUXcat were subtracted from all values.

tivation (16). The EIAV genome contains a much smaller central region than do the other lentiviruses (HIV-1, HIV-2, or visna virus). Two ORFs are present in this region in EIAV: S1 is downstream of *pol* (nucleotides 5104 to 5253) and has a coding potential of 50 amino acids; S2 is downstream of S1, overlaps the *env* gene (nucleotides 5264 to 5461), and has a coding potential of 66 amino acids (14). A larger ORF completely within the 3' end of the *env* gene (S3, nucleotides 7215 to 7619) has a coding potential of 135 amino acids (14). The second codon of S2 is an ATG (Fig. 2, asterisk); however, neither S1 nor S3 contains a translational start site. To test these subgenomic fragments for the transactivating function, we cotransfected cells with the EIAV LTR-CAT plasmid (pE1cat) and constructs designed to express these ORFs. The expression vector, pS, is shown in Fig. 2 and contains the promoter-enhancer and the polyadenylation sequences of SV40 cloned into extreme ends of the polylinker in pBluescribe (pBS+; Stratagene, San Diego). Fragments encompassing S1 plus S2 (0.8-kilobase *NcoI*-to-*SmaI* fragment [nucleotides 4870 to 5670]) or S3 (1.3-kilobase *HpaI* fragment [nucleotides 6715 to 8029]) were cloned into pS, creating plasmids pS1,2 and pS3, respectively. pS1 was constructed by removing all viral sequences between the *Bam*HI and *SmaI* sites (nucleotides 5318 to 5670) in pS1,2, which left ORF S1 intact but truncated S2 after the first 18 codons. pS1 and pS1,2 also contain a 234-bp section of 5' sequence which overlaps the 3' end of the *pol* gene and contains a few short ORFs (the longest is 38 codons), several splice donor and acceptor sites, and translational start sites. From these expression plasmids, 5 μ g was cotransfected with 5 μ g of the LTR-CAT constructs onto D17 cells

(uninfected adherent canine cells, plated the day before at 5×10^6 cells per 60-mm dish), and cell lysates were assayed for CAT activity by the method of Neumann et al. (12). Briefly, 5% (5 μ l) of the cell extract was added to chloramphenicol (1.0 mM), Tris hydrochloride (pH 7.8, 100 mM), and [14 C]acetyl coenzyme A (0.1 μ Ci) in a microscintillation vial. The 250- μ l reaction was overlaid with Econofluor (New England Nuclear Corp., Boston, Mass.), and the production of the labeled enzymatic reaction product, acetylchloramphenicol, was monitored by counting in a liquid scintillation counter. Results of these experiments are shown in Table 1. Plasmid pS3 was inactive in the cotransfection experiments. In contrast, pS1,2 was able to transactivate the EIAV LTR: approximately 10-fold-higher levels of CAT activity were observed in cells cotransfected with pS1,2 compared with those of the control plasmid, pUXSV2 β globin (Table 1). pUXSV2 β globin was used to control for the amount of SV40 promoter sequences present in the transfection. To more precisely localize the transactivator gene, pS1,2 was truncated at the *Bam*HI site, which removed all but the first 18 amino acids of S2 (pS1). Since this deletion, which left S1 intact, was no longer able to transactivate the LTR (Table 1), we conclude that S2 is required for transactivation and encodes an active part of the *tat* protein. However, since the active construct, pS1,2, also contains sequences from the 3' end of *pol* which include small ORFs in addition to S1, these studies do not rule out a possible contribution of sequences upstream of S2 in a multiply spliced *tat* message. pS1,2 appears to specifically activate the EIAV LTR: neither the HIV-1 LTR (pC15cat [16]) nor the SV40 promoter (pUXSV2cat) were affected by pS1,2 (Table 1). Similar

FIG. 2. (A) Genetic organization of the 3' end of EIAV. The ORFs including *pol*, *env*, S1, S2, and S3 are indicated as boxes. The positions of the first ATG codons are marked by asterisks. Restriction sites used in cloning are as follows: N, *Nco*I; B, *Bam*HI; S, *Sma*I; H, *Hpa*I. Distances are given in nucleotide bases. (B) Schematic diagram of the expression vector, pS, and regions of the 3' end of the viral genome inserted into pS. pS was derived from pBS+ (see text) by adding the promoter-enhancer and the polyadenylation sequences from SV40 to the extreme ends of the polylinker (hatched box). For the expression constructs, a 0.8-kilobase *Nco*I-to-*Sma*I fragment (encompassing ORFs S1 and S2) or a 1.3-kilobase *Hpa*I fragment (including S3) was cloned into the *Hinc*II site in the polylinker, producing plasmids pS1,2 and pS3, respectively. pS1 was constructed by removing the *Bam*HI-to-*Sma*I region of pS1,2.

results were observed in FEA cells (data not shown). The data presented here are in agreement with those of Sherman et al., who identified the region between nucleotides 4473 and 5775 (which corresponds to nucleotides 4455 to 5757 in

TABLE 1. CAT activity directed by the EIAV or HIV-1 LTRs in cotransfected cells^a

Plasmid source of LTR	Cotransfecting plasmid ^b	Relative CAT activity (SEM) ^c
pElcat	Control ^d	0.30 (± 0.04)
pElcat	pS1	0.55 (± 0.16)
pElcat	pS3	0.27 (± 0.13)
pElcat	pS1,2	3.05 (± 0.14)
pC15cat	Control ^d	0.80 (± 0.16)
pC15cat	pS1,2	0.65 (± 0.04)

^a EIAV LTR from pElcat; HIV-1 LTR from pC15cat.

^b Plasmids express subregions of the EIAV genome. Five micrograms of an LTR-CAT plasmid was combined with 5 μ g of an ORF expression plasmid and transfected onto D17 cells. CAT assays were performed as described elsewhere (12).

^c Values are means and are expressed relative to the values obtained with pUXSV2 cat.

^d Plasmid pUXSV2 β globin was used to control for the amount of SV40 promoter sequences present in the transfection.

TABLE 2. CAT activity directed by the EIAV and truncated LTRs in cotransfected cells^a

Plasmid source of LTR	Relative CAT activity (SEM) with cotransfecting plasmid ^b	
	Control ^c	pS1,2
pElcat	0.30 (± 0.04)	3.05 (± 0.14)
pE(-111)cat	0.22 (± 0.02)	2.02 (± 0.21)
pE(-76)cat	0.27 (± 0.02)	2.03 (± 0.21)
pE(-41)cat	0.04 (± 0.01)	0.69 (± 0.08)
pE(-31)cat	0.04 (± 0.01)	0.92 (± 0.16)
pE(+40)cat	0.23 (± 0.03)	2.21 (± 0.49)
pE(+22)cat	0.25 (± 0.03)	1.23 (± 0.12)
pE(+4)cat	0.004 (± 0.002)	0.01 (± 0.005)

^a EIAV LTR in pElcat and truncated LTRs in other plasmids.

^b Five micrograms of an LTR-CAT plasmid was combined with 5 μ g of an ORF expression plasmid and transfected onto D17 cells. CAT assays were performed as described elsewhere (12). Values are means and are expressed relative to the values obtained with pUXSV2 cat.

^c Plasmid pUXSV2 β globin was used to control for the amount of SV40 promoter sequences present in the transfection.

this EIAV clone) as necessary for transactivation (17). Thus, EIAV looks similar to HIV-1 in that *tat* is likely derived from a multiply spliced message containing essential coding sequences from the central region. In EIAV the central region is much smaller than that in HIV, and so a portion of the *tat* gene overlaps the *env* ORF. Cloning of cDNA sequences expressing the *tat* gene will unambiguously identify the splice sites and *tat* coding sequences.

To define the *cis*-acting sequences in the EIAV LTR which are necessary for response to *tat*, we cotransfected pS1,2 with the truncated EIAV LTRs linked to CAT. This allowed analysis of the response sequences in cellular environments differing only in the presence or absence of the transactivator. Results shown in Table 2 demonstrate that removal of upstream sequences, although reducing CAT activity by four- to eightfold with or without *tat*, did not impair the activation of the LTR by *tat*. Further delineation of 5'-minimal *tat*-responsive sequences will require point mutational analysis, since further deletions would remove the TATA box.

Deletion analysis in which sequences from the 3' end of the LTR were removed revealed that sequences distal to +22 were not required for transactivation (Table 2). Since the deletion to +4 abolished all promoter activity, basal as well as activated, it is likely that this deletion interferes with the transcriptional start site or disrupts some requisite promoter element. Therefore, the same region required for activation in virus-infected cells is required for response to pS1,2. Our data confirm and extend the results of Sherman et al., who showed that a deleted LTR construct that contains only three bases of the R region was not responsive to transactivation (17). A large inverted repeat present in the *trans*-acting-factor response region of HIV-1 was postulated to be involved in transactivation because removal of half of this repeat abolishes transactivation (10). The EIAV LTR contains a similar inverted repeat largely contained in the R region (3). However, the LTR deleted to +22 was still fully responsive to *trans*-acting factors but was missing most of one half of the inverted repeat. This result suggests that formation of this secondary structure is not essential for transactivation in EIAV. In summary, our results localize the *trans*-acting-factor response sequences to a 53-bp region (-31 to +22) spanning the RNA start site.

FIG. 3. Northern blot analysis of the promoter activity of the EIAV LTR and deleted LTRs in the presence or absence of *tat*. Various promoter units directing expression of a β -globin cDNA (5 μ g of plasmid DNA) were transfected onto D17 cells in the presence of pS1,2 (lanes 3, 5, and 7) or a control plasmid, pUXSV2cat (lanes 1, 2, 4, 6, and 8). Promoters placed upstream of β globin are as follows: pUX, no promoter (lane 1); pUXSV2, the SV40 early promoter (lane 2); pEI, the entire EIAV LTR (lanes 3 and 4); pE(-47), an EIAV LTR deleted from the 5' end to -47 with respect to the RNA start site (+1) (lanes 5 and 6); and pE(+4), an LTR deleted from the 3' end to +4 (lanes 7 and 8). Poly(A)⁺ RNA isolated from cells 44 h after the transfection was analyzed by Northern blotting, hybridized with a radiolabeled β -globin probe, and visualized by autoradiography (A). The blot was then rehybridized with an actin probe (B).

Increase of steady-state levels of RNA in the presence of pS1,2. To address the mechanism of transactivation by EIAV, we analyzed the steady-state levels of RNA directed by the EIAV LTR in the presence and absence of pS1,2. An increase in the levels of LTR-directed RNA in the presence of the transactivator would indicate that transactivation functions, at least in part, by increasing the rate of transcription initiation or stability of the message, or both. The entire EIAV LTR (pEI) and selected truncated LTRs were cloned upstream of a β -globin cDNA. Ten micrograms of these plasmid DNAs was transfected onto D17 cells (plated the day before at 10^6 per 100-mm petri dish) with pS1,2 or control plasmid DNA. pUXSV2cat was used to control the amount of SV40 sequences present in the transfection. Forty-four hours later, the cells were harvested and total cellular RNA was isolated by the hot-acid phenol method (9). Poly(A)⁺ RNA was purified on oligo(dT)-cellulose as described for batch selection (9) and was subjected to Northern (RNA) blot analysis by using a radiolabeled β -globin probe (Fig. 3). After visualization by autoradiography, the blot was rehybridized with an actin probe to quantify the amount of RNA in each lane. The plasmid pUX β globin, which lacks a promoter, did not direct observable levels of β -globin RNA (Fig. 3, lane 1). Transfection of cells with pUXSV2 β globin resulted in moderate levels of β -globin RNA (lane 2). Cells transfected with the entire EIAV LTR- β -globin construct expressed 64-fold-higher levels of β -globin RNA with cotransfections of pS1,2 (lane 3)

compared with those of the control plasmid (lane 4) as quantified by densitometry of the autoradiogram. Plasmid pE(-47) β globin expressed diminished levels of β -globin RNA compared with levels expressed by the whole LTR in the presence of *tat* (lane 5), and it expressed undetectable levels of β -globin RNA in the absence of *tat* (lane 6). The plasmid with the LTR deleted to +4 which was inactive in CAT assays, pE(+4), was also inactive as pE(+4) β globin in directing β -globin RNA in either the presence or the absence of *tat* (lanes 7 and 8). The results shown in Fig. 3 support a transcriptional activation mechanism for stimulation by *tat*. The data do not rule out stabilization of LTR-directed mRNA by *tat* and do not directly address the effects on translational efficiency.

Results of these studies demonstrate that EIAV encodes a *trans*-acting factor with *cis*-acting response sequences present in the EIAV LTR. The presence of a positive regulator with the potential for a potent positive feedback loop does not explain the quiescent phase of the disease or the lifetime persistence of EIAV. However, the presence of *tat* does raise the possibility of activation of virus expression from a latent form of EIAV which is then amplified by *tat*. Engagement of such a positive feedback cycle could then contribute to the bursts of viremia and ultimately to the clinical episodes during the acute phase of equine infectious anemia.

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