JOURNAL OF VIROLOGY, Mar. 1987, p. 743–747
0022-538X/87/030743-05$02.00/0

Vol. 61, No. 3

Characterization of Equine Infectious Anemia Virus Long Terminal Repeat

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Received 22 August 1986/Accepted 23 November 1986

The long terminal repeats (LTRs) of equine infectious anemia virus (EIAV) were examined with respect to their ability to function as transcriptional promoters in various cellular environments. Nucleotide sequence analyses of the LTRs derived from two unique proviral clones revealed the requisite consensus transcription and processing signals. One of the proviruses possessed a duplication of a 16-base-pair sequence in the CCAAT box region of the LTR which was absent in the other provirus. To assess its functional activity, each LTR was coupled to the bacterial chloramphenicol acetyltransferase gene and transfected onto various cell lines, including matched cultures of EIAV-infected and uninfected cells. The levels of chloramphenicol acetyltransferase activity directed by the EIAV LTRs were between 250 and 900 times greater in EIAV-infected cells compared with their uninfected counterparts. Thus, EIAV expression appears to be activated by a virus-induced *trans*-activation phenomenon analogous to that recently shown to amplify expression of certain other lentiviruses.

Equine infectious anemia virus (EIAV) is the etiologic agent of equine infectious anemia, a relapsing and remitting disease of horses (4). EIAV is an exogenous retrovirus of the subfamily *Lentivirinae* whose other family members include the visna-maedi caprine arthritis encephalitis virus, and the virus associated with the human acquired immune deficiency syndrome (HIV), formerly called human T-cell lymphotropic virus type III (HTLV-III) and lymphadenopathy-associated virus. The lentiviruses are characterized by similarities in morphology and genomic structure, limited sequence homology, and persistence in the host animal (6, 10, 29). The lifetime persistence of EIAV may, in part, be attributed to antigenic variation in the envelope glycoprotein (15, 22). However, transcriptional and posttranscriptional regulation of virus expression are also likely to be key determinants of the disease process (3).

Retroviral gene expression is governed by sequences located in the proviral long terminal repeats (LTRs) and by the cellular environment. The LTRs contain a transcriptional promoter whose utilization is controlled by upstream, *cis*-acting sequence elements (30, 31). Additionally, *cis*-acting sequences located downstream of the RNA start site have also been identified that may influence gene expression at transcriptional and posttranscriptional levels (8, 20). The interaction of virus-encoded *trans*-acting factors with *cis*-acting elements in the LTR is suggested by the observation that the promoters (LTRs) from the related lentiviruses, visnavirus and HIV, direct higher levels of gene expression in infected cells compared with uninfected cells (14, 25). This condition-specific activation is also seen in HTLV-I and HTLV-II and the bovine leukemia virus (BLV) (5, 7, 24). Expression of heterologous genes controlled by the LTRs from HTLV-I, HTLV-II, and BLV as well as HIV have recently been shown to be greatly increased in response to a product of the respective virus genome (1, 9, 23; D. Derse, unpublished observation). At present, it is unknown whether EIAV also encodes a gene product that specifically augments its own expression.

As a first step toward understanding the molecular mechanisms controlling EIAV expression, we have characterized the EIAV LTRs derived from two unique proviral clones with respect to structure and biological activity. Nucleotide sequence analysis revealed that the two LTRs differed solely in a region between the CCAAT and TATA boxes, suggesting potential differences in functional activity. We constructed plasmids in which the reporter gene, the chloramphenicol acetyltransferase (CAT) gene, was placed under the control of the EIAV LTRs. Transfection of these constructs onto EIAV-infected and uninfected cell lines resulted in a high level of CAT expression only in EIAV-infected cells. This condition-specific activation of the EIAV LTR resembles that of the other lentiviruses, visna virus, and HIV as well as HTLV-I, HTLV-II, and BLV.

MATERIALS AND METHODS

Plasmids. The EIAV proviral DNA from cells persistently infected with the Wyoming strain was cloned into the lambda vector EMBL4, and recombinants were characterized by restriction mapping (L. Levy, R. L. Talbott, E. G. Vivit, S. B. Payne, R. C. Montelaro, C. J. Issel, and J. W. Casey, submitted for publication). The LTRs from two unique lambda clones, lambda 12 and lambda 6, were subcloned into plasmid vectors for further analysis of nucleotide sequence and promoter activity.

The plasmid pSV2cat contains the origin of replication of pBR322 and the simian virus 40 early promoter linked to the coding sequences of the bacterial CAT gene (12). In pSV0cat, the entire simian virus 40 promoter region was deleted and replaced with a unique *Hin*dIII restriction site (12).

Plasmid pEI-1cat consists of a 478-base-pair (bp) *Bst*NI to *Pvu*II fragment containing the 5′ LTR derived from lambda

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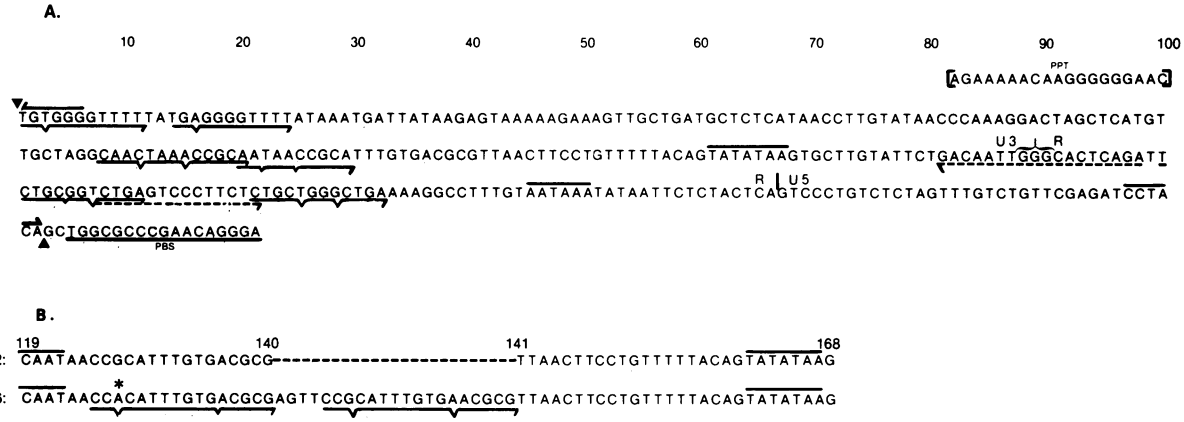

FIG. 1. (A) Complete nucleotide sequence of the 5′ LTR of lambda clone 12 and the polypurine tract adjacent to the 3′ LTR of lambda clone 6 (in brackets). LTR fragments were subcloned into pUC19 and sequenced by the method of Maxam and Gilbert (17). The inverted repeats marking the LTR boundaries are overlined with arrows, and the TATA box and polyadenylation signal are overlined. The three direct repeats are underlined with arrows, and the one region of dyad symmetry is underlined with dashes. The probable U3-R and R-U5 boundaries are indicated. (B) Nucleotide sequence comparison of the region between the CAAT box and the TATA box of the 5′ LTR of lambda clone 12 and the 3′ LTR of lambda clone 6. This region is located between nucleotides 119 and 168 of lambda clone 12 as shown in (A). The duplication in lambda clone 6 is underlined, and the single base change between the two clones is marked with an asterisk. A gap was introduced in lambda clone 12 to accommodate the duplication and insertion in lambda clone 6 in this region marked with dashes.

clone 12. This includes 175 bp of 5′ cellular flanking sequences, the entire 5′ LTR, and one additional base on the 3′ end. pEI-6cat is a 610-bp *TaqI* fragment encompassing the 3′ LTR of lambda clone 6 and includes 291 bp of viral flanking sequences and the LTR minus 11 bp at the 3′ end. After digestion and gel purification, the ends of both fragments were repaired with T4 DNA polymerase and modified by the addition of *Hin*dIII linkers. After a *Hin*dIII digest to remove excess linkers, the LTRs were inserted into the unique *Hin*dIII site of pSV0cat. Recombinants were analyzed by restriction enzyme mapping. Plasmids were grown in *Escherichia coli* JM-83, and plasmid DNA was extracted as described previously (16) by two cesium chloride banding isolations.

Sequence analysis. The 5′ and 3′ LTRs from proviral lambda clone 12 and the 3′ LTR from proviral lambda clone 6 were appropriately subcloned into pUC19 and sequenced by the Maxam and Gilbert method (17).

Cells and transfection procedures. EIAV-infected and uninfected feline (E-FEA and FEA, respectively) and canine (E-Cf2th and Cf2th, respectively) cell lines were obtained from Larry Arthur (Program Resources, Inc., Frederick Cancer Research Facility, Frederick, Md.) (23). The EIAV-infected cells are continuous cell lines (>100 passages) in which virus production does not result in cytopathic effects (2). Other cell lines used included a human rhabdomyosarcoma line (RD-4), murine fibroblasts (NIH 3T3), African green monkey kidney cells (CV-1), and a human B-lymphocyte line (Raji). With the exception of Raji, all cells were grown in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum. The lymphocytes (Raji) were grown in RPMI 1640 (GIBCO) with 10% fetal calf serum. Fibroblasts were plated the day before transfection at 3×10^5 cells per 60- by 15-mm culture dish (Falcon, Oxnard, Calif.). Plasmid DNA (10 μg) was transfected onto fibroblasts as a calcium phosphate coprecipitate followed by a 15% glycerol shock as described previously (11, 13). A DEAE-dextran method (18, 26), modified as follows, was employed for transfection of lymphocytes. Cells were suspended in fresh medium before transfection; four hours later, the cells were pelleted and suspended in 250 μg of DEAE-dextran (Pharmacia, Uppsala, Sweden) per ml–50 mM Tris hydrochloride (pH 7.3) in RPMI 1640 at a concentration of 10^7 cells per 5 ml. This cell suspension was added to tubes containing 10 μg of the plasmid DNA and was gently shaken for 45 to 60 min at 37°C. The cells were then spun down, washed two times with complete medium, and finally suspended in 10 ml of complete medium.

CAT assays. The harvesting of cells and the CAT assays were performed as previously described (11,12), except that the reactions were done in 100-μl volumes containing 5.2 nmol of [^{14}C]chloramphenicol (specific activity, 50 mCi/mmole; New England Nuclear Corp., Boston, Mass.), 1 mM acetyl coenzyme A, 0.15 M Tris hydrochloride (pH 7.8), and 5 to 40 μl of cell extract. The reaction times and amounts of extract used were varied to insure that the rate of product formation was in the linear range. The reactions were extracted with ethyl acetate and resolved by thin-layer chromatography. Appropriate sections were cut out, and substrates and products were quantified by liquid scintillation counting.

RESULTS AND DISCUSSION

The molecular cloning of proviruses from fetal equine kidney cells infected with the Wyoming strain of EIAV will be described elsewhere (Levy et al., submitted). Two unique proviral clones, designated lambda clone 6 and lambda clone 12, were used as the sources of EIAV LTRs in the following studies. Proviral fragments containing LTR sequences were subcloned into plasmid pUC19 for sequence analyses or pSV0cat for examination of promoter activity.

The nucleotide sequences of both the 5′ and the 3′ LTRs and flanking sequences of lambda clone 12 were determined, thus permitting an unambiguous assignment of the LTR termini. In addition, the sequence of the 3′ LTR of lambda clone 6 was analyzed. Figure 1A shows the nucleotide sequence of the 302-bp LTR of lambda clone 12. The provirus is flanked by 5-bp direct repeats of cellular DNA

(CAAAG in the case of lambda clone 12), and the LTRs are bounded by imperfect 6-bp inverted repeats (5'-TGTGGG and 3'-CCTACA). An 18-nucleotide polypurine tract, probably used in the initiation of plus-strand synthesis during replication, is located upstream of the 3' LTR. Immediately following the 5' LTR, the provirus contains the tRNA primer-binding site for initiation of minus-strand synthesis (Fig. 1A); this $tRNA_3^{Lys}$ primer site is shared with HIV and mouse mammary tumor virus (28). Moreover, visna virus and simian retrovirus type 1 contain the related $tRNA_{1,2}^{Lys}$ primer-binding site, which is identical to that of EIAV in the first eight nucleotides (19).

The nucleotide sequence data permitted the identification of the signals and sites utilized for transcription initiation and mRNA processing. A consensus Goldberg-Hogness or "TATA" box is present between nucleotides 160 and 170 (Fig. 1A). Approximately 25 bp downstream of this TATA box are three G residues (positions 188 to 190, Fig. 1A), any of which could serve as the initiation site for RNA synthesis. Indeed, S1 nuclease mapping places the mRNA CAP site within this 3-bp region and therefore establishes the U3-R boundary (data not shown). A consensus poly(A) addition signal, AATAAA, is located between nucleotides 245 and 250 (Fig. 1A). The site which forms the 3' terminus of mature mRNA and onto which the poly(A) tail is attached is generally found 20 to 30 bp downstream of the AATAAA sequence and ends in the dinucleotide CA. In addition, this site, which defines the R-U5 border, is often followed at a distance of about 10 to 25 bp by the sequence TTGT (30). The CA dinucleotide at position 265 fits all of these criteria and is likely to define the 3' end of viral RNA. In this case, the EIAV LTR would possess a U3 region of approximately 189 bp, an R region of 77 bp, and a U5 region of 36 bp; the U3 and U5 regions are notably small among retroviruses.

The nucleotide sequence of the EIAV LTR was examined for potential regulatory elements or structures present in other retroviral LTRs. A potential CCAAT box, whose function is poorly understood, appears at positions 119 to 123 (Fig. 1A); its location, which is approximately 70 bp upstream from the RNA start site, is typical of this sequence. The core enhancer sequence, GTGG T/A T/A T/A G, is absent from the LTR. Although there are no long direct repeats in the U3 region, several short direct and inverted repeats are present (Fig. 1A, underlined), which may function in the control of transcription. Two clustered regions of homology in the LTRs of EIAV and visna virus were identified: in U3, nucleotides −67 to −52 of EIAV with nucleotides −48 to −35 of visna virus (12 of 14 nucleotides matching); and in R, nucleotides +54 to +69 of EIAV with +34 to +49 of visna virus (13 of 16 nucleotides matching). The sequences are numbered with respect to the RNA start sites (27) and were aligned by using the nucleotide alignment program of Wilbur and Lipman (32). No regions of sequence homology with HIV were found, including the *trans*-acting factor response element identified in the HIV LTR (21, 28). Furthermore, EIAV lacks the long direct repeats present in visna virus or the numerous dyad symmetries seen in the HIV LTR.

An interesting and unexpected result of the sequence analyses was the finding that the LTR of lambda clone 6 was different from the LTR of lambda clone 12. The lambda clone 6 LTR possesses a duplication of a 16 bp sequence between the CCAAT and TATA boxes which is absent in lambda clone 12 (Fig. 1B). In addition, the duplicated elements are separated by a 4-bp insertion (AGTT; Fig. 1B). The sole base pair change between the LTRs of clones 6 and 12 is an A-to-G transition located within the duplication. It is unclear at present whether the sequence variation occurred in vivo or during the course of virus propagation in cell culture. The location of the sequence duplication within a known regulatory region suggested the possibility that the LTRs might differ with respect to their control of gene expression.

TABLE 1. CAT activity[a]

Cell line	CAT activity relative to pSV2cat	
	pEI-1cat	pEI-6cat
Raji	0.060	
CV-1	0.021	
NIH 3T3	0.026	
RD-4	0.080	
FEA	0.027	0.080
E-FEA	24.92	20.71
Cf2th	0.020	
E-Cf2th	7.97	

[a] Levels of CAT enzyme activity directed by EIAV LTRs in various cell lines. EIAV-infected and uninfected feline (E-FEA and FEA, respectively) and canine (E-Cf2th and Cf2th, respectively) cell lines were obtained from Larry Arthur (2). Other cell lines included a human rhabdomyosarcoma line (RD-4), murine fibroblasts (NIH 3T3), African green monkey kidney cells (CV-1), and a human B-lymphocyte line (Raji). Monolayer cells were plated the day before transfection at 3×10^5 cells per 60-mm culture dish and transfected by the calcium phosphate coprecipitation method with 10 μg of plasmid DNA (13). Approximately 10^7 lymphocytes were transfected with 10 μg of plasmid DNA by the DEAE-dextran method (18, 26). Cells were harvested 40 to 48 h after transfection, and extracts were prepared and assayed as previously described (11, 12). Levels of CAT activity are expressed relative to the level obtained with pSV2cat in each cell type. Each value represents the average of at least three transfections with the variation between experiments less than 30% of the average.

The nucleotide sequence data indicated that requisite promoter elements were present in the EIAV LTRs. To determine whether these LTRs were in fact competent to direct gene expression, they were examined in transient expression assays. DNA fragments containing each LTR were coupled to the bacterial CAT gene in the plasmid pSV0cat. The EIAV lambda clone 12 and lambda clone 6 LTRs were placed in the sense orientation with respect to CAT coding sequences to produce the plasmids pEI-1cat and pEI-6cat, respectively (Materials and Methods). The levels of CAT enzyme activity produced shortly after transfection has previously been shown to reflect promoter strength (11, 12). To control for differences in transfection efficiencies among the various cell types examined here, levels of CAT activity are expressed relative to the levels obtained with pSV2cat, which we have found to be an active promoter in all cells tested. Table 1 summarizes the results of the transfections of CAT plasmids onto a variety of cell types. The levels of CAT enzyme activity obtained with pEI-1cat were low in all of the uninfected cell lines examined. Activities directed by the EIAV LTR ranged from about 2% (in CV-1 cells) to 8% (in RD-4 cells) of the levels directed by pSV2cat (Table 1). Thus, the EIAV LTR is able to function, albeit at low levels, in uninfected cells. When the LTRs were inserted into pSV0cat in the antisense orientation, CAT activity was not detected above the background levels observed with pSV0cat (data not shown). Expression of CAT activity was next compared in two sets of cultures in which one partner of each set was productively infected with EIAV. The productively infected cell lines E-FEA and E-Cf2th were derived from the parental feline (FEA) and canine (Cf2th) cells, respectively. These are stable, continuous cell lines in which production of virus is not accompa-

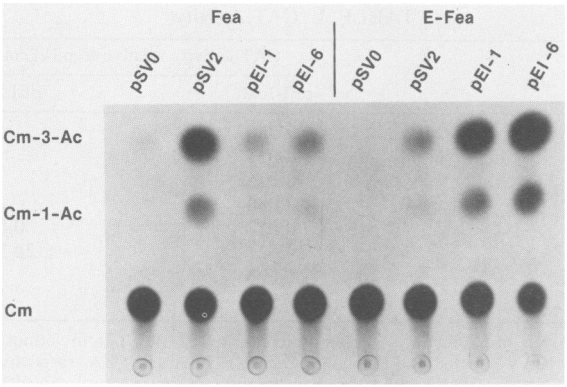

FIG. 2. Conversion of chloramphenicol (Cm) to its monoacetylated forms (Cm-1-Ac and Cm-3-Ac) in uninfected (FEA) and EIAV-infected (E-FEA) cells. Transfections and CAT assays were performed as described in Materials and Methods. The reactions were run in a 100-μl volume; the uninfected FEA cells with 40 μl of cell extract and 4 h of incubation and the infected E-FEA cells with 5 μl of cell extract and 30 min of incubation. The substrates and products were extracted with ethyl acetate, separated by thin-layer chromatography, and visualized by autoradiography.

nied by cytopathic effects. Figure 2 graphically illustrates the different responses of the EIAV LTR in uninfected and EIAV-infected FEA cells. Both pEI-1cat and pEI-6cat directed the expression of CAT to levels which ranged between 250 and 900 times higher in EIAV-infected cells compared with those of the uninfected parental cell lines (Table 1). This increase in promoter activity resulting from EIAV infection was observed in both FEA and Cf2th cells (Table 1). Such a condition-specific response, i.e., amplification of LTR-controlled gene expression as a result of infection with the same virus, has also been described for HIV and visna virus as well as the HTLV-I, HTLV-II, and BLV group of retroviruses. These data suggest that EIAV-encoded or -induced factors mediate this activation.

The sequence duplication that differentiates the LTRs of lambda clone 12 and lambda clone 6 apparently has no outstanding effect on promoter activity detectable in these experiments. In EIAV-infected FEA cells, the lambda clone 12 and lambda clone 6 LTRs were about equally active (Table 1). However, a slight but consistent difference in CAT expression, favoring the lambda clone 6 LTR, was seen in uninfected FEA cells. Thus, the presence of the extra 20 bp in the clone 6 LTR might influence basal promoter activity slightly, but this is overshadowed when the LTRs are compared under "activated" conditions. It is possible that these differences in the LTRs may influence their function in vivo.

Nucleotide sequence analysis revealed that the EIAV LTRs contain the hallmark signals required for retroviral RNA synthesis and processing. However, the regulatory elements which control the promoter must be unique to EIAV, since we were unable to identify a core enhancer sequence or elements homologous to other retroviral regulatory units (with the exception of the two small regions of homology with visnavirus). It is evident that the EIAV LTR is responsive to an activation phenomenon mediated by factors unique to EIAV-infected cells. It is likely that EIAV encodes the gene(s) responsible for the activation of its own promoter since similar genes have been identified in HIV, HTLV-I, HTLV-II, and BLV. At present it is not clear whether the activation of gene expression observed in these systems is at the level of transcription initiation, posttranscriptional events, or a combination of several levels of control. It will be of interest to determine whether the various lentiviruses share common strategies for the regulation of gene expression. We are presently examining the EIAV LTR to identify the regions responsive to the effects of virus-encoded factor(s) and to elucidate the mechanisms of activation.

ACKNOWLEDGMENTS

This research sponsored in part by Public Health Service contract NO1-CO-23910 from the National Cancer Institute with Program Resources, Incorporated.

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