Sequence analysis and transcriptional activity of the LTR of OLV-CU1, a North American ovine lentivirus

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Although ovine lentiviruses have been described in the United States since the early part of this century, North American strains of sheep lentiviruses remain relatively uncharacterized at the molecular level. The LTR of a North American ovine lentivirus, OLV-CU1, was found to be closely related at the molecular and functional levels to visna virus, the Icelandic ovine lentivirus. Sequence analysis of the LTR revealed high identity to other ovine and caprine lentiviruses in key regulatory elements of the upstream promoter region (−25 to −115). However, the R region of the LTR was much less homologous. Transcriptional control of OLV-CU1 in transient transcriptional assays required a conserved putative AP-4 region and possibly an AP-1 like element in the upstream promoter region for moderate to high levels of transcription, much like visna virus. In contrast to visna virus, the downstream region beyond the transcriptional start site was required for virus-specific transactivation.

Ovine lentiviruses comprise a small group of the lentivirus subfamily of retroviruses and cause slowly progressive multi-organ diseases with incubation periods of months to years (Narayan & Clements, 1989). We have isolated and biologically characterized a New York ovine lentivirus (OLV-CU1) that causes lesions typical of classic ovine progressive pneumonia (OPP) when inoculated into newborn lambs. The OLV-CU1 provirus is related to visna virus by hybridization, restriction analysis and sequence relatedness (84% and 78% at the amino acid level in the pol and env regions, respectively) (Campbell et al., 1993).

Our initial goal was to determine the sequence relatedness of the OLV-CU1 LTR to other ovine and caprine lentivirus LTRs. In order to clone the OLV-CU1 LTR, two 22-mer primers corresponding to the LTR ends were made after sequencing the partial OLV-CU1 clones, pCU15.5 and pCU10.5 (generated from a SacI λgt11 library of OLV-CU1 infected fibroblasts) (Campbell et al., 1993). The U3 and U5 primers, with HindIII restriction sites at their 5′ ends, were AGAAGCTTTGGACTGTCAGGAC and AGAAGCTTCGAGATCCGCTCG, respectively. PCR was performed on DNA extracted from infected fibroblasts. A subclone, pCU1LTR#3, that matched the sequences of the initial OLV-CU1 clones derived from a SacI λgt11 library of infected cells (data not shown), was used for subsequent studies (GenBank accession no. U39826).

Sequence alignments of the LTRs of the following ovine and caprine lentiviruses were generated using the Clustal V program (Higgins & Sharp, 1989) and further aligned manually (Fig. 1): OLV-CU1; visna virus (GenBank accession no. M18039) (Sonigo et al., 1985); SA-OMVV, derived from an epithelial cell line isolated from a South African sheep (M31646) (Querat et al., 1990); EV-1, cloned from the peripheral blood mononuclear cells of a British sheep (S51392) (Sargan et al., 1991); and caprine arthritis-encephalitis virus (CAEV) (M33677) (Saltarelli et al., 1990). The OLV-CU1 LTR had significant identity with the other LTRs (visna virus 82%, SA-OMVV 75% and CAEV 60%). The most striking difference between the visna virus and OLV-CU1 LTRs was the lack of a 43 bp repeat in the OLV-CU1 LTR. This lack of a duplication may not be functionally relevant for two reasons. First, deletion of one of the repeats in the visna virus LTR does not significantly change the levels of CAT activity in transient assays (Hess et al., 1985). Second, the OLV-CU1 LTR was even more active as a promoter than was the visna virus LTR in the transcriptional experiments outlined later in this paper. There were several areas of identity in the U3 region with all of the aligned LTRs, especially between 25 and 115 bp upstream of the TATA box. The putative AP-4 site was highly conserved among the viruses, while the putative AP-1 protein binding site immediately downstream had a 2 bp inversion in both the SA-OMVV and OLV-CU1 LTRs as compared to the consensus site found in the visna virus LTR. A third region important for...
visna virus promoter activity, TCCGCTTAA. 18 bp upstream of the putative AP-4 site, was also different between the OLV-CU1 and visna virus LTRs. The R region was the most divergent among the ovine LTRs, whereas the CAEV LTR differed from the others throughout the entire sequence.

Primer extension was performed on oligo(dT)-selected RNA from infected cells and OLV-CU1 virion RNA using the OLV-CU1 U5 primer previously described. The transcriptional start site and not that of the visna virus site.

Fig. 1. Alignments of the OLV-CU1 (CU1), EV-1 (EV1), SA-OMV (SA), visna virus (Visna) and CAEV LTRs. Identical sequences are indicated by a (-), and gaps are noted by a (.). Visna virus and CAEV transcriptional start sites are indicated by up (visna virus) or down (CAEV) arrows and a (○) symbol. The transcriptional start site of OLV-CU1 is indicated by the bold G that also corresponds to the down arrow. Putative transcription factor binding sites of the OLV-CU1 LTR are underlined, with the name above the sequence [AP1, activator protein 1; AP4, activator protein 4; TATA, TFIID binding site; and putative poly(A) signal sequence].
virus or Tat expression plasmid, range from 3- to 20-fold, whereas CAEV or a CAEV Tat expression plasmid transactivates its own LTR much less efficiently, only between 2- and 6-fold (Hess et al., 1985, 1986; Jackson et al., 1991). Interestingly, there is one report that the CAEV Tat is not required for virus replication either in vitro or in vivo (Harmache et al., 1995).

In order to determine if other ovine lentiviruses function similarly at the transcriptional level, we measured the basal and transactivated activity of the OLV-CU1 LTR in transient CAT assays. An OLV-CU1 LTR–CAT reporter plasmid was constructed by standard molecular cloning techniques using the HindIII site of pUC-CAT (Janice Clements, Johns Hopkins University, Baltimore, Md., USA). Cultured primary fetal ovine bone marrow (FOBM) cells were infected with OLV-CU1 or visna virus (m.o.i. values of 5-0 TCID₅₀ (Reed & Muench, 1938; Campbell et al., 1993). Reporter constructs were transfected into uninfected or infected FOBM cells 24 h post-infection by calcium phosphate precipitation (Sambrook et al., 1989) using reporter plasmid and carrier plasmid (pUC19) for a final concentration of 40 μg/ml. Cell extracts were prepared and an ELISA for CAT detection was performed according to the manufacturer's (5 prime-3 prime) instructions. The transcriptional activity of the OLV-CU1 LTR was examined in uninfected and OLV-CU1 infected FOBM cells (Fig. 2a). Activity of the OLV-CU1 promoter was increased at least 9-fold when 0-1 μg of input pCU1LTRCAT was used as the template for stimulation (values between 0-1 μg and 5 μg of transfected reporter plasmid were within the linear range of the CAT assay; data not shown). The level of transactivation decreased to between 3- and 4-fold when higher levels (either 0-5 or 1-0 μg) of input pCU1LTRCAT were used to transfect the cells. For comparison, plasmids containing the SVO, RSV or visna virus promoters placed in front of the CAT reporter gene were also tested in OLV-CU1 or visna virus infected and uninfected FOBM cells (Fig. 2a). Moderate CAT levels were obtained after transfection with the visna virus LTR construct. Even higher basal levels were found after transfection with the OLV-CU1 LTR (2- to 3-fold increases above the visna virus LTR CAT levels). Both the visna virus and OLV-CU1 LTRs gave between a 2- and 5-fold increase in CAT protein levels in OLV-CU1 or visna virus infected cells in at least three separate transfection experiments. No differences were observed in transfection efficiencies between uninfected and infected cells when a β-actin LacZ expression plasmid was used in parallel experiments (data not shown).

To determine the effect of heterologous tat gene products on the expression of the OLV-CU1 LTR, transient CAT assays were performed with the visna virus or CAEV Tat expression plasmids (pVLDXS, pRSV1.9; kindly supplied by J. Clements, Johns Hopkins University, and W. Cheevers, Washington State University, Pullman, Wash., USA, respectively) co-transfected with either the pRSVCAT, pVISLTRCAT or pCU1LTRCAT reporter plasmids (Fig. 2b). Plasmids used as negative controls were pVLflglobin and pRSV1.9R (CAEV tat in reverse orientation). The only consistent transactivation
Fig. 3. OLV-CU1 deletion constructs and CAT protein levels. A linear map of the OLV-CU1 LTR in front of the CAT reporter gene (p17E, WT), and constructs 1–8 and 10–11. Deletion fragments were generated using the indicated restriction enzymes and cloned into pUC-CAT. Constructs 10 and 11 were generated using PCR and primers A (10) or B (11) and primer 1; see text. a, 50 ng of plasmid used for transfection; b, 1 μg; c, 2.5 μg; d, 1 μg.
found with the visna virus LTR was with the visna Tat expression plasmid. The transactivation observed with the Tat expression plasmid on either the pVISLTRCAT or pCU1LTRCAT constructs ranged from 3- to 4-fold above cotransfections with the β-globin construct. However, a 2- to 2.5-fold transactivation was also observed with the CAEV tat expression plasmid, pRSV1.9, cotransfected with the CU1LTRCAT expression plasmid, which is less than reported elsewhere (Jackson et al., 1991). The cotransfection experiments were repeated at least twice with triplicate transfections each time. The difference in the response of the visna virus and OLV-CU1 LTRs to the CAEV transactivator, although small, may indicate that other regions regulate the activity of the transactivator with cis-acting regions within the promoter, such as the putative AP-4 site.

Lastly, a series of OLV-CU1 LTR deletion CAT reporter constructs was prepared and assayed to identify the regions of the OLV-CU1 LTR necessary for promoter activity and virus-specific transactivation. As shown in Fig. 3, deletion constructs 1 to 4 were generated from pLTR#3 by double endonuclease digestions and blunt-end cloned into Smal digested pUC-CAT. Deletion constructs of the 5’ portion of the LTR (5 and 6) were constructed as follows: pLTR#3 was digested with HindIII and either NheI (5) or PvuII (6), and then ligated to restriction cleaved pUC-CAT (XbaI–HindIII for construct 5 or Smal–HindIII for construct 6). 3’ deletion construct 8 was generated by cleavage of pLTR#3 with SmalA and blunt-end cloned into Smal digested pUC-CAT. Two additional 3’ deletion fragments, 10 and 11, were generated by the use of PCR. PCR primers 1 (start of LTR), A (5’ AGAAGCTTAGGCGAGCCAGGAG3’) and B (5’ AGAAGCTTACGAATCCCGATA3’), complementary to the positive strand 58 and 115 bp downstream of the transcriptional start site of OLV-CU1, were synthesized with HindIII restriction sites at their ends to facilitate subcloning into the pUC-CAT HindIII site. All transformants were checked by DNA sequence analysis.

The basal level of CAT in deletion construct 1, which was composed of only the U3 region and 15 bp of the R region, was approximately 25% of wild type (Fig. 3). No transactivation was observed with this construct, nor with the other three constructs that had the same 3’ end (2, 3 and 4). Deletion constructs 2, 3 and 4 were consecutive 5’ deletions of the upstream region, and had sequentially less transcriptional activity based on the levels of CAT protein detected. Neither the basal level of activity nor the transactivation of the LTR was restored after a 17 bp addition to the 3’ end of construct 1 to generate construct 8 (3’ end at +32). However, when the full R region and parts of the U5 region of the virus were rejuvenated in constructs 5, 10 and 11, the basal level of CAT protein was increased to greater than wild-type levels. The extent of transactivation of these constructs and of the wild-type LTR were low, presumably because the system was oversaturated from the high basal levels of promoter activity. In fact, when 1/100th of wild-type OLV-CU1 LTR CAT plasmid was used in parallel transfections (50 ng), a greater than 9-fold increase was observed in OLV-CU1 infected FOBM cells. Alternately, this phenomenon could be explained by a titration out of repressors by the increasing amounts of promoter present. For example, the viral transactivator may act by removing repressors from the DNA elements within the LTR or transactivators may be modulated by repressor elements located at distal sites within the LTR. When the entire R and U5 regions were added to construct 4 (where no CAT was detected) to generate construct 6, a very low basal level of activity was observed in uninfected cells. More interestingly, this addition of the R and U5 regions in construct 6 restored the ability of the LTR to respond to transactivation by OLV-CU1. The transactivated response was greater than 4-fold, and was highly reproducible (three additional experiments, data not shown).

Several key regulatory regions were delineated by CAT deletion analysis. Clearly, sequences spanning the putative AP4, AP1 and TATA regions are required for basal activity of the OLV-CU1 LTR. Deletion constructs of the OLV-CU1 LTR which disrupt the putative AP4 site result in a dramatic decrease in CAT levels. Sequences outside the region between the putative AP-4 site and the transcriptional start site are also necessary for full basal activity of the OLV-CU1 LTR. There may be a potential negative regulatory element (NRE) located farther than 115 bp downstream of the beginning of R or upstream of the Nhel site, because constructs 5, 10 and 11 have greater than wild-type basal activity. Alternatively, deletion of either of these regions may increase the translatability or stability of the mRNA.

OLV-CU1 and visna viruses are functionally similar in their transactivation capabilities according to the experiments described in this paper. These results are not surprising, given the similarities in the biology and, at the molecular level, of the OLV-CU1 and visna virus genomes (Campbell et al., 1993; Hess et al., 1989). In contrast to the visna virus LTR, sequences downstream of the OLV-CU1 transcriptional start site seem to be necessary for virus-specific transactivation to occur. The deletion experiments described here do not, however, delineate between transactivation at the transcriptional or post-transcriptional levels.

References


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