Cooperative Regulation of Bovine Leukaemia Virus Gene Expression by Two Overlapping Open Reading Frames in the XBL Region

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SUMMARY

Bovine leukaemia virus (BLV) induces syncytia in productively infected ovine and bovine monolayer cells. Expression of the env gene directly determines the syncytium-forming activity, since the expression of a cloned env gene directed by the simian virus 40 (SV40) early promoter efficiently induced syncytia in transfected ovine embryonic (OE) cells. However a BLV long terminal repeat (LTR)-directed expression plasmid (pLTRenv) failed to induce syncytia in transfected OE cells, suggesting insufficient promoter activity of the LTR sequences. To assess the role of the XBL genes, which are located in the 3' distal region of the genome, in viral gene expression we constructed SV40 early promoter-directed expression plasmids. These contained open reading frames (ORFs) in the XBL region, and were examined for syncytium-inducing activity by cotransfection with pLTRenv. The results suggest that both XBL-I (the longest ORF: x- lor) and XBL-II (a shorter overlapping ORF: x-sor) are trans-acting genes which cooperatively activate LTR-directed viral gene expression.

INTRODUCTION

Bovine leukaemia virus (BLV) is the causal agent of enzootic lymphoproliferative diseases in cattle (Burny et al., 1980). The proviral genome structure of BLV is similar to that of human T cell leukaemia virus-I (HTLV-I), the causal agent of adult T cell leukaemia/lymphoma, and HTLV-II (Wong-Staal & Gallo, 1985). These viruses are characterized by the presence of novel overlapping open reading frames (ORFs) between the env gene and the Y long terminal repeat (LTR) region designated X (Seiki et al., 1983; Haseltine et al., 1984; Shimotohno et al., 1984; Sagata et al., 1985).

The X region of these viruses is transcribed into subgenomic transcripts (XmRNA) by double-splicing mechanisms (Seiki et al., 1985; Sagata et al., 1985b; Wachsman et al., 1985; Rice et al., 1987). The XmRNA encodes at least two distinct polypeptides using overlapping ORFs (x- lor: XBL-I and x-sor: XBL-II) (Sagata et al., 1985a; Nagashima et al., 1987; Rice et al., 1987). The X region contains transcriptional activator genes which are essential for sufficient expression from specific viral LTRs (Cann et al., 1985; Felber et al., 1985; Sodroski et al., 1985; Rosen et al., 1986). The x-lor products of HTLV-I and BLV are sufficient for the LTR-directed expression of the bacterial chloramphenicol acetyltransferase (CAT) gene (Seiki et al., 1986; Katoh et al., 1987). However, a recent study by Inoue et al. (1987) suggested that the gene products of x-sor (p27xIII) and x-lor (p40x) of HTLV-I are both required for detectable expression of the viral gag gene. The XBL ORF-mediated regulation of BLV expression has not been fully characterized.

Ovine embryonic (OE) monolayer cells form syncytia on transfection with infectious molecular clones of BLV (Itohara & Sekikawa, 1987). By using the transfection-syncytium formation system, we have investigated the XBL ORF-dependent regulatory mechanisms.
Fig. 1. Schematic representation of expression plasmids for the env gene and XBL ORFs (a). BLV sequences are indicated as open boxes with nucleotide numbers at each end and translation initiation and termination codons are marked, as well as Sd and Sa sites. Shaded boxes indicate SV40-derived sequences. Unique EcoRI and CiaI sites of pSVX-I and pSVX-I-II were used to generate frameshift mutations within XBL-I and XBL-II. (b) Nucleotide sequences of the 5' end of ORFs in pSVX-I and pSVX-I-II. Deleted sequences are shown in parentheses.
**METHODS**

**Construction of expression plasmids of the env gene and ORFs in the XBL region.** To construct the env gene and XBL ORF expression plasmids, plasmid pSV2neo (Southern & Berg, 1982) was used as a vector. The BLV sequences were prepared from an infectious molecular clone (pB6490; Itohara & Sekikawa, 1987). All plasmid construction procedures were as described by Maniatis et al. (1982), and the structures were confirmed by extensive restriction endonuclease analysis and partial nucleotide sequencing. The enzymes used were purchased from Toyobo (Osaka, Japan).

The structures of the expression plasmids are schematically presented in Fig. 1. In plasmid pSVenv nucleotides 4789 to 6446 were inserted between the HindIII and SmaI sites of pSV2neo in the sense orientation by blunt end ligation. The nucleotide numbering corresponded to that of a completely sequenced BLV (Sagata et al., 1985c). This plasmid uses simian virus 40 (SV40)-derived sequences of the early promoter, the splicing junction and the transcription terminator. In plasmid pLTRenv, nucleotides 7925 to 1152 (EcoRI to SalI) containing LTR sequences and nucleotides 4118 to 6823 (HindIII to XhoI) containing the env gene were joined together with a SalI–HindIII linker and inserted in the sense orientation between the PvuI and HpaI sites of pSVneo-d by blunt end ligation. Plasmid pSV2neo-d is a derivative of pSV2neo in which the sequence from the BamHI site to the EcoRI site has been deleted. Plasmid pLTRenv uses an SV40-derived terminator. In plasmid pSVX-I-II nucleotides 4789 to 5087 (BglII), containing initiation codons for the XBL-I and XBL-II reading frames and the splice acceptor site (Sa), were joined at the BglII and BamHI ends, and inserted in the sense orientation between the HindIII and HpaI sites of plasmid pSV2neo-d by blunt end ligation. The resultant plasmid uses the SV40-derived early promoter and terminator, and has intact sequences of both XBL-I (x-lor) and XBL-II (x-sor). The structure of plasmid pSVX-I was essentially the same as that of pSVX-I-II, but nucleotides 4789 to 4827, containing an initiation codon for the XBL-II frame, have been deleted (Fig. 1 b). Hence, pSVX-I has intact XBL-I sequences but lacks the initiation codon for XBL-II (Sagata et al., 1985b; Rice et al., 1987).

To create an XBL-I-specific mutation, the EcoRI site (nucleotide position 7294) of pSVX-I-II and pSVX-I was cleaved, a blunt end was formed by Klenow fragment treatment and the plasmids were religated. The resultant plasmids pSVX-II-sX-I and pSVfsX-I have frameshift mutations in XBL-I caused by insertion of four base pairs. To mutagenize both XBL-I and XBL-II, the ClaI site (nucleotide position 7319) of pSVX-I-II was cleaved, a blunt end was formed by Klenow fragment treatment, and the plasmid was religated. The resultant plasmid pSVfsX-I-II has frameshift mutations of both XBL-I and XBL-II caused by the insertion of two base pairs.

Closed circular DNA of the plasmids was purified twice by caesium chloride–ethidium bromide centrifugation and stored at 4 °C until it was used for transfection assays.

**DNA transfection assay.** Ovine embryonic kidney cells (3.5 x 10^5) (Itohara & Sekikawa, 1987) were seeded in a 60 mm culture dish with growth medium (Dulbecco's modified Eagle's medium pH 7.2, 10% foetal calf serum). On the next day, semi-confluent monolayers were transfected with 5 to 10 μg of DNA by the calcium phosphate coprecipitation method (Graham & Van der Eb, 1973) and incubated for 4 h in a 5% CO2 incubator at 37 °C. Then the monolayers were washed once with serum-free medium, treated for 3 min with 25% DMSO (Stow & Wilkie, 1976) at room temperature, washed three times with a medium containing 5% foetal calf serum, and incubated with the growth medium at 37 °C in a 5% CO2 incubator. On the next day, the monolayers were trypsinized and distributed into two 60 mm dishes containing an assay medium (Dulbecco's modified Eagle's medium pH 7.4, 10% foetal calf serum, 1% DMSO, 4 μg of polybrene per ml) and then cultured for an additional 3 to 5 days. The monolayers were then stained with Giemsa solution, and examined under the microscope for the appearance of syncytia containing five or more nuclei.

**RESULTS**

**The env gene directly determines syncytium-forming activity**

In order to identify the gene(s) responsible for the syncytium-forming activity of BLV, we initially constructed a series of defective proviruses from plasmid pB6490 which carries an infectious circular proviral genome, and examined them for syncytium-forming activity by DNA transfection assay using OE cells. Although a gag–pol deletion mutant showed syncytium-forming activity with an efficiency equivalent to that of the wild-type genome, mutants having a deletion or insertion within the env gene or XBL region completely lost the activity (data not shown). The results suggest that the transfection-syncytium formation system is suitable as a sensitive assay to determine the mechanisms regulating expression of viral genes involved in syncytium formation.

We constructed a series of independent env and XBL ORF expression plasmids as shown in Fig. 1. When OE cells were transfected with these plasmids, significant syncytium formation...
Table 1. Trans-activation by the XBL ORF expression plasmids*

<table>
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<tr>
<th>Plasmids</th>
<th>Donor</th>
<th>Test</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
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<td>ND</td>
</tr>
<tr>
<td>None</td>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
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* OE cells were cotransfected with 5 μg each of donor and test plasmids per 60 mm dish. The transfected cells were cultured for 4 days in experiments (expt.) 1 and 2, and for 6 days in expt. 3.
† Average number of syncytia in two to four dishes.
‡ ND, No data were obtained.

was observed in cells transfected with pSVenv (Table 1, Fig. 2a), but not with any ORF expression plasmids (Table 1, Fig. 2c). The results clearly demonstrate that the env gene directly determines the syncytium-forming activity of BLV. However, pLTRenv, which is an LTR-directed env gene expression plasmid, failed to induce syncytia (Table 1, Fig. 2b); this failure could be due to insufficient activity of the LTR sequence as a promoter.

Both XBL-I and XBL-II encode trans-acting genes that activate LTR-directed expression of the env gene

To determine the role of XBL ORFs in env gene expression directed by the LTR, OE cells were cotransfected with the plasmid pLTRenv (5 μg/dish) and equal amounts of various ORF expression plasmids. As shown in Table 1, pSVX-I, which has only an intact XBL-I reading frame, and pSVX-I-II, which has intact XBL-I and XBL-II reading frames, induced efficient syncytium-forming activity of pLTRenv. pSVfsX-I, which has a frameshift mutation in XBL-I, and pSVfsX-I-II, which has frameshift mutations in XBL-I and XBL-II, failed to induce syncytia. However pSVX-IIfsX-I, which has an intact XBL-II sequence and a frameshift mutation in XBL-I, induced a small but significant number of syncytia. These results suggest that both XBL-I and XBL-II reading frames encode trans-acting genes which induce and/or enhance LTR-detected env gene expression. The number of syncytia produced by 5 μg of pSVenv was slightly increased by the addition of pUC18 as a carrier DNA in the precipitation mixture (Table 1, expt. 1). If XBL-I, or XBL-I and XBL-II were expressed by the SV40 promoter, the syncytium-forming activity of pSVenv was not influenced by cotransfection (Table 1), suggesting specificity of the trans-activation by these genes for the LTR.

Kinetics of trans-activation by XBL-I and XBL-II

To determine the modes of action of the trans-acting genes, we determined the dose–responses of the XBL ORF expression plasmids. Fig. 3(a) shows the results of cotransfection with a constant amount of pLTRenv (5 μg/dish) and various amounts of XBL ORF expression plasmids (50 pg to 500 ng/dish). The dose–response of pSVenv (50 ng to 5 μg/dish) was also determined as a control. Plasmid pSVenv showed significant activity at 50 ng per dish and the
activity increased linearly up to 5 μg per dish. On the other hand, XBL ORF expression plasmids showed significant activity at very low concentrations. The number of syncytia induced increased linearly with increasing amounts of pSVX-I, pSVX-IIfsX-I and pSVX-I-II up to approximately 50 ng per dish, and reached a plateau. Plasmid pSVfsX-I-II did not show significant activity at any concentration.

Since pSVX-I-II showed higher activity than pSVX-I and pSVX-IIfsX-I, it is likely that XBL-I and XBL-II cooperatively activate the \textit{env} gene expression directed by the LTR. To confirm the cooperative nature of the XBL-I and XBL-II genes, we conducted a cotransfection experiment with a constant amount of pSVX-I or pSVX-IIfsX-I (150 ng/dish, a concentration that caused maximum activity), and various amounts of pSVX-IIfsX-I or pSVX-I (0 to 500 ng/dish). As shown in Fig. 3(b), the basal activities of pSVX-I and pSVX-IIfsX-I were significantly increased by the addition of pSVX-IIfsX-I and pSVX-I, respectively. The activity of 1.5 ng of pSVX-I in the presence of pSVX-IIfsX-I was equivalent to that of 150 ng of pSVX-I without XBL-II. The maximum numbers of syncytia obtained by using the respective combinations were equivalent to those obtained by using pSVX-I-II. These results strongly suggest that XBL-I and XBL-II cooperatively activate LTR-directed \textit{env} gene expression.
Fig. 3. Kinetic analysis of trans-activation by XBL-I and XBL-II. (a) OE cells were cotransfected with a constant amount of pLTRenv (5 μg/dish) and serially diluted pSVX-I (○), pSVX-II-fsX-I (●), pSVX-I-II (■) or pSVfsX-I-II (▲). Plasmid pSVenv (▲) was serially diluted (50 ng to 5 μg/dish) and used for transfection. (b) Plasmid pLTRenv (5 μg/dish) was used for cotransfection with pSVX-I (150 ng/dish) and serially diluted pSVX-II+fsX-I (0 to 500 ng/dish) (○). Plasmid pLTRenv (5 μg/dish) was used for cotransfection with pSVX-II+fsX-I (150 ng/dish) and serially diluted pSVX-I (0 to 500 ng/dish) (○). The final concentration of the precipitation mixture was adjusted by adding pUC18 DNA. Each point and small vertical bar represent the average number of syncytia from four dishes and the standard deviation.

**DISCUSSION**

An initial observation in this study was that transient expression of the *env* gene is sufficient to cause syncytium formation during BLV infection in the absence of viral spreading. This result clearly demonstrates that syncytium formation by BLV in OE cells is mediated by fusion from within. This syncytium-forming activity of the *env* gene used in the context of transfection experiments provides a sensitive, quantitative and simple assay, which does not require nucleic acid or protein extraction and secondary assay, to evaluate viral gene expression. Using the transfection–syncytium formation system, we investigated the mechanisms of regulation of viral gene expression mediated by ORFs in the XBL region. The reliable quantitative range of the assay system is zero to approximately 10000.

The expression of the *env* gene from pLTRenv is markedly enhanced by pSVX-I, which is expected to express only intact XBL-I products (38K protein; Sagata et al., 1985a), but not by pSVfsX-I, which has a +4 frameshift mutation in XBL-I. The results demonstrate that XBL-I (*x-lor*) is a trans-acting gene which is essential for the transient LTR-directed expression of the viral *env* gene and agree with results obtained for the CAT gene expression assay system in BLV (Katoh et al., 1987) and HTLV (Felber et al., 1985; Seiki et al., 1986). Furthermore, whereas pSVfsX-I and pSVfsX-I-II were completely unable to induce syncytium formation, pSVX-II+fsX-I, which has an intact XBL-II sequence and a frameshift mutation in the XBL-I sequence, showed a low but significant enhancing effect on syncytium formation (approximately one-tenth the activity of pSVX-I in Fig. 3a). This suggests that XBL-II encodes a second trans-acting gene which enhances viral gene expression directed by the LTR. The product of this gene (a 19K phosphoprotein) has been detected in BLV-infected cells (Rice et al., 1987).
The results of two series of kinetic analysis (Fig. 3a, b) of the trans-activation system suggest that XBL-I is a major trans-activator gene, and that its activity is significantly enhanced by cooperation of a minor trans-activator gene (XBL-II). The XBL-II products enhanced the activity of the XBL-I products approximately fivefold.

It has been suggested that p40\(^X\) (x-lor) and p27\(^X\)-III (x-sor) of HTLV are transcriptional and post-transcriptional activators respectively, and are both required for detectable expression of the gag gene of HTLV-I (Inoue et al., 1987). In their case, independent expression of p40\(^X\) or p27\(^X\)-III was not sufficient for detectable expression of the gag gene. In contrast, our results demonstrate that independent expression of XBL-I (x-lor) and XBL-II (x-sor) is able to induce or enhance env gene expression significantly. The discrepancy between the two experiments may be due to the difference in sensitivity of the assay systems used.

In any case, our results suggest that expression and replication of BLV is regulated by two trans-activator genes. Further analysis is necessary to elucidate the fine regulatory mechanisms of the BLV-coded trans-activator genes in the virus replication cycle.

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