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The bovine leukemia virus p34 is a transactivator protein

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Recombinant Moloney murine retroviruses containing the BLV post-envelope long open reading frame were constructed and transfected into the ψ2 packaging cell line. They were shown to encode and to express a 34-kd protein able to transactivate the BLV long terminal repeat-directed gene expression in the respective transfected cells. These data demonstrate that the BLV X-LOR gene encodes a p34 transactivator product. Furthermore, the different cell lines produced infectious recombinant retroviruses capable of transferring X-LOR genes into recipient cells. The availability of the BLV transactivator protein should allow us to understand the role of the transactivator protein in BLV-induced leukemogenesis.
Key words: bovine leukemia virus/transactivator protein/Mo-MuLV recombinant retrovirus/gene expression

Introduction

Enzootic bovine leukosis (EBL) is a chronic lymphoproliferative disease of cattle (Burny *et al.*, 1980). The agent, bovine leukemia virus (BLV), is a retrovirus distantly related to the human T lymphotropic viruses (HTLV-I and II) (Poiesz *et al.*, 1980). The pathologies of BLV- and HTLV-I-induced diseases are notably similar, namely absence of chronic viremia, a long latency period and lack of preferred integration sites in tumors (Burny *et al.*, 1984).

Sequence comparisons between the BLV, HTLV-I and HTLV-II genomes show that the three viruses contain several overlapping open reading frames located between the *env* gene and the 3' long terminal repeat (LTR) (Seiki *et al.*, 1983; Rice *et al.*, 1984; Sagata *et al.*, 1985; Shimotohno *et al.*, 1985). In the three cases, that region of the genome has been shown to encode proteins (Lee *et al.*, 1984; Slamon *et al.*, 1984) that transactivate the LTR of the provirus (Sodroski *et al.*, 1984; Rosen *et al.*, 1986).

We recently reported the cloning, sequencing and *in vitro* expression of a cDNA (named BL1) corresponding to the X long (X-LOR) and short (X-SOR) open reading frames of the BLV genome (Willems *et al.*, 1987). The BL1 cDNA encodes a transactivator protein (Rosen *et al.*, 1986). However, this cDNA is able to encode two proteins (Sagata *et al.*, 1985): (i) p34, the translation product of the X long open reading frame, which is initiated at an AUG codon located 44 bases downstream from that of the *env* gene (Willems *et al.*, 1987); and (ii) p18 which shares the translation initiation codon with the *env* gp51 protein. The p18 amino terminus is common with that of the *env* protein and the carboxylic part is translated from the X short open reading frame.

We report here that fibroblastic cells infected with recombinant murine retroviruses expressing exclusively p34 specifically transactivate the BLV LTR sequences.

Results

The BL1 cDNA corresponding to the BLV post-envelope region is represented in Figure 1A. Its nucleotide sequence revealed that a splice event occurred between the end of the *pol* gene (GATGG/GTAAG) and the 5' end of the X long open reading frame (TTAAG/CAAGT). A *Sau*3A–*Pvu*II fragment containing all the X-LOR coding sequences was blunt-ended using the Klenow fragment of DNA polymerase and introduced into the Moloney murine leukemia virus-based vector pLS as illustrated in Figure 1B. In this construct, X-LOR should be translated from a viral genome-length mRNA whereas the *neo* gene, conferring resistance to G418 (Colbère-Garapin *et al.*, 1981), should be

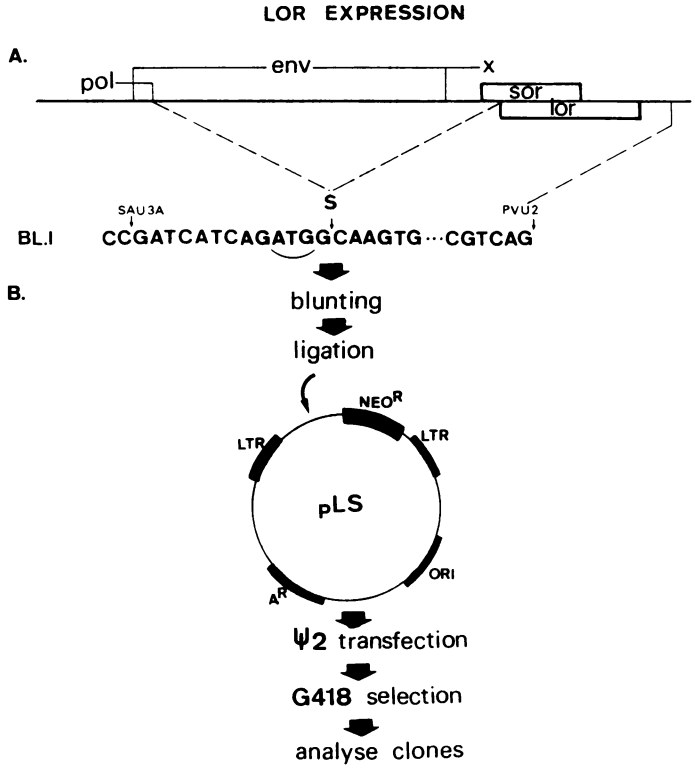

Fig. 1. Construction of X-LOR recombinant retroviruses and establishment of cells expressing p34. (**A**) Schematic representation of the BL1 cDNA corresponding to the BLV X region. S represents a splice event between the end of the *pol* gene and the X-LOR frame (X long open reading frame). (**B**) Construction of recombinant pLS/X-LOR plasmid and establishment of G418-resistant clones. The *Sau*3A–*Pvu*II BL1 insert was blunt-ended and cloned into the pLS plasmid (LTR, long terminal repeat; *Neo*^{R}, neomycin resistance gene; A^R, ampicillin resistance gene; ORI, bacterial origin of replication). The recombinant pLS/X-LOR plasmid was transfected into ψ2 cells. Individual cells were selected with G418 and used for further analysis.

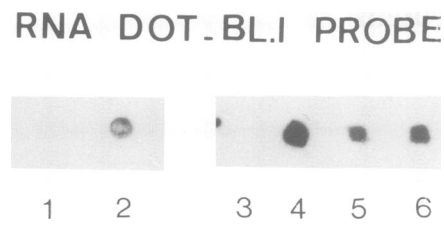

Fig. 2. RNA dot blot analysis. Total RNA was prepared (Maniatis *et al.*, 1982) from uninfected OVK cells (**lane 1**) and $\psi 2$ cells (**lane 3**). RNA was also prepared from three individual clones of transfected $\psi 2$ cells (clones 1, 2 and 3, **lanes 4–6** respectively). As positive control, FLK RNA was used (**lane 2**). The RNA (5 μg) was spotted onto nitrocellulose and hybridized to a ^{32}P-labelled BLV BL-1 probe.

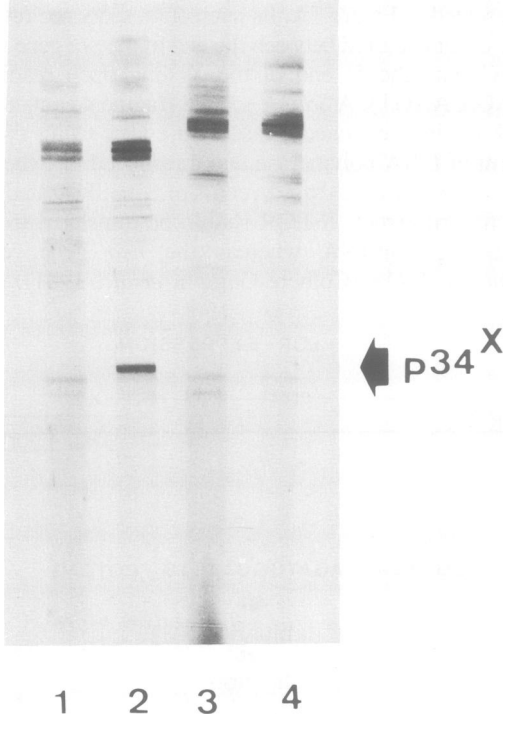

Fig. 3. Western blot analysis of Mo-MuLV/X-LOR-transfected $\psi 2$ lysate (clone 1). Antisera used are: non-infected bovine serum (**lanes 1** and **3**); BLV-infected bovine serum (**lanes 2** and **4**). Sera used in lanes 3 and 4 were preincubated with extracts of BLV-infected FLK cells as described by Goh *et al.* (1985).

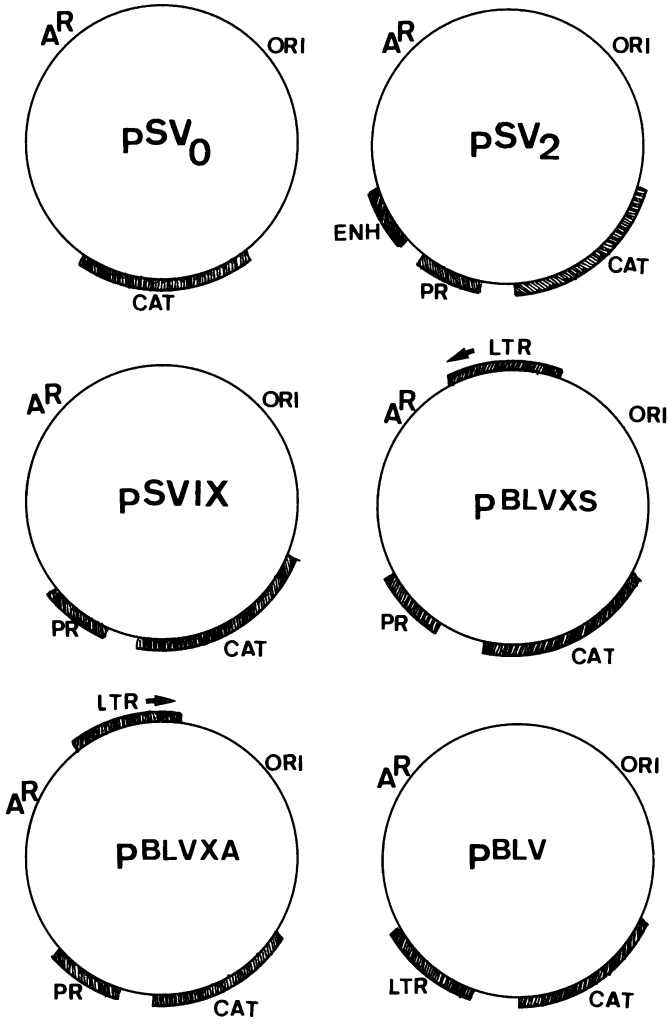

Fig. 4. Schematic representation of plasmids used in transfection experiments. pSV_0CAT contains the chloramphenicol acetyl transferase (CAT) gene; pBLVCAT contains the BLV LTR cloned upstream of the CAT gene; pSV_2CAT is composed of the SV40 early region transcription enhancer (ENH) and promoter (PR) cloned upstream to the CAT gene; plasmids pBLVXS and pBLVXA derive from pSVIXCAT and contain the BLV LTR sequences ~2.8 kb 5′ to an enhancerless SV40 promoter (PR) (Celander and Haseltine, 1984).

expressed from a spliced subgenomic mRNA. Infectious helper-free X-LOR virus was obtained by transfection of the pLS/X-LOR construct into the $\psi 2$ packaging cell line (Mann *et al.*, 1983).

To test for the presence of X-LOR gene transcripts in individual G418-resistant colonies, total cellular RNA was extracted from several clonal $\psi 2$ isolates. Figure 2 demonstrates that X-LOR gene transcripts were present in three independent G418-resistant colonies (Figure 2, lanes 4–6) at levels similar to that found in the BLV-producing FLK cell line (lane 2). No viral transcripts were detected in control cell lines (untransfected $\psi 2$ cells: lane 3; uninfected OVK cells: lane 1). These data proved that the X-LOR gene can be efficiently transcribed from the recombinant murine retroviral vector.

In order to verify the efficient translation of these transcripts, Western blot analyses were performed on one transfected $\psi 2$ isolate (clone 1). Serum of BLV-infected cattle specifically detected high levels of a 34 000 M_r product in these cells (Figure 3, lane 2). This protein is absent from non-transfected control $\psi 2$ cells (data not shown) and is not detected by the serum of a non-infected animal (Figure 3, lanes 1, 3). The reactivity of the BLV-infected cattle serum towards this 34-kd protein is specifically blocked upon adsorption with extracts of BLV-producing FLK cells (Figure 3, lane 4).

The functional activity of the X-LOR protein was tested by transfection of cell lines with plasmid constructs in which the CAT gene activity is under control of various regulatory elements (Figure 4). The level of CAT activity was measured in cell extracts prepared at 48 h post-transfection. The results are shown in Figure 5 and summarized in Table I. Transfection with plasmid pBLVCAT resulted in a marked increase in the level of LTR-directed gene expression in FLK (BLV) cells (used as positive control, Figure 5, lane 2) and in the Mo-MuLV/X-LOR transfected cell lines (Figure 5, lanes 4–6). No measurable CAT activity was observed in uninfected OVK and $\psi 2$ cells (Figure

Table I. Relative enzyme activity of the CAT gene product

Plasmids	Enhancer	Promoter	Cell lines					
			OVK	FLK	ψ2	Clone 1	Clone 2	Clone 3
pSVIXCAT	–	SV40	1	1	1	1	1	1
pSV_2CT	SV40	SV40	0.80	10.18	1.35	10.70	1.62	1.14
pBLVCAT	BLV-LTR	BLV-LTR	0.87	16.50	1.91	105.30	27.46	20.40

The numbers represent the fold stimulation of CAT gene expression in infected compared with uninfected cells, all normalized to the CAT activity obtained with plasmid pSVIXCAT. Results represent the average of at least two independent transfections.

5, lanes 1 and 3 respectively). Lane 7 represents the level of acetylation obtained with 50 mU of CAT enzyme. To establish a control for differences in transfection efficiencies that might be associated with the individual clones, the level of CAT activity was normalized to the activity obtained following transfection of the same cells with plasmid pSVIXCAT (Figure 4). In this plasmid, CAT expression is under the control of the SV40 promoter. These data are presented in Table I. It appeared that the levels of transactivation measured in three transfected ψ2 isolates (clones 1, 2 and 3) were up to six times higher than the activity observed in FLK cells (105.30 versus 16.50).

Previous studies have shown that factors present in BLV-infected cells activate enhancer sequences present within the BLV LTR (Derse *et al.*, 1985; Derse and Casey, 1986; Rosen *et al.*, 1986). To examine the ability of the product of the X-LOR gene to effect BLV enhancer activity, plasmids pBLVXS (Figure 6, lanes 2) and pBLVXA (Figure 6, lanes 3) (Rosen *et al.*, 1986), that contain both orientations of the BLV enhancer sequences 5′ to the early region SV40 promoter (plamid pSVIXCAT, Figure 6, lanes 1), were transfected into recipient cells. The results of these experiments are shown in Figure 6 and summarized in Table II. No stimulation of BLV enhancer activity was observed in OVK or ψ2 cells (Figure 6A and C respectively). In contrast, as expected, in FLK cells a marked increase of CAT activity was observed with plasmids pBLVXA and pBLVXS (Figure 6B, lanes 2 and 3) while absent with control plasmid PSVIXCAT (Figure 6B, lane 1). The three Mo-MuLV/X-LOR-transfected ψ2 cell lines also showed an enhancer activity (Figure 6D, E and F, lanes 2 and 3).

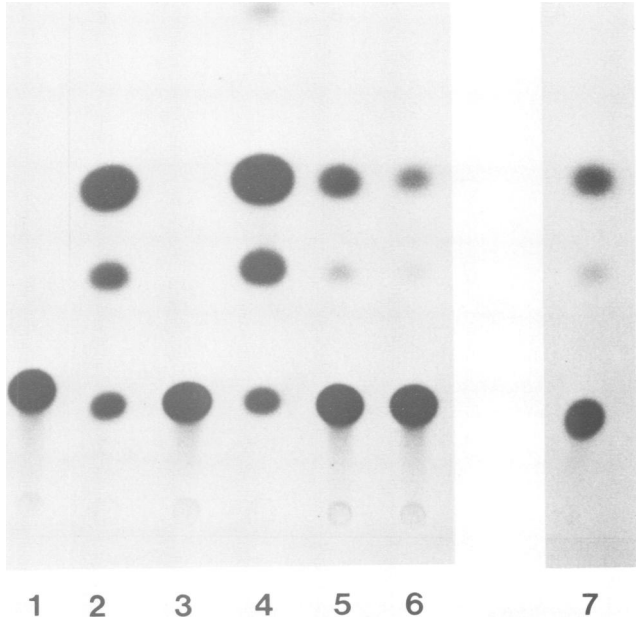

Fig. 5. CAT assays after transfection of pBLVCAT plasmid into various cell lines. **Lane 1**, uninfected OVK cells; **lane 2**, BLV-infected FLK cells; **lane 3**, uninfected ψ2 cells; **lanes 4, 5** and **6**, Mo-MuLV/X-LOR-transfected ψ2 cells (clones 1, 2 and 3 respectively); **lane 7**, CAT activity corresponding to 50 mU of CAT enzyme. See Figure 4 for plasmids used and Materials and methods for CAT assays.

Discussion

Recent studies have demonstrated that the 3′ region of BLV provirus encodes a transactivator protein able to activate *in trans* BLV LTR-directed gene expression in the absence of other viral proteins (Rosen *et al.*, 1986). The experiments reported here establish that p34, the protein product of BLV X-LOR, is a transactivator.

Using a Mo-MuLV-based retroviral vector we were able to express a functional X-LOR product in ψ2 cells. In fact transactivation BLV-directed CAT gene expression appears higher in some cells transfected with the recombinant retroviral vector than the level observed in BLV-infected FLK cells. Since the levels of transactivation represent the average of at least two independent transfections normalized to a control plasmid (pSVIXCAT), the differences observed are most probably due to p34 expression efficiencies or to the presence of cellular factors in individual clones.

Enhancer activity of the X-LOR gene product is limited to certain cell lines (Rosen *et al.*, 1986). Here we show that BLV enhancer activation is observed in ψ2 cells expressing the X-LOR product (p34). Consequently additional cellular factors probably required for enhancer activity are present in these cells.

It has been proposed that expression of the X-LOR gene product may exert an effect on transcription, not only of the BLV LTR, but also of cellular genes (Rosen *et al.*, 1986). In the HTLV-I system, the transactivator proteins induce expression of the IL2 and IL2-receptor genes (Greene *et al.*, 1986). Functional similarities between BLV and HTLV transactivator proteins suggest that p34 may induce cellular genes involved in B cell proliferation, immortalization or transformation.

To study the physiological consequences of the X-LOR gene expression, a high-efficiency vector capable of transferring these genes into cells is required. Disadvantages associated with the standard transfection methods include low efficiency of gene transfer and inability to transfect certain cell types efficiently. In this context, retroviral vectors offer several advantages, including efficient infection and predictable integration configuration (Varmus and Swanstrom, 1982).

The different Mo-MuLV/X-LOR-transfected ψ2 isolates produce infectious recombinant retroviruses ($\sim 10^3$ particles/ml of culture fluid) able to infect NIH-3T3 cells and to express a functional p34 (data not shown). Recombinant X-LOR retroviruses that express a functional transacting product permit efficient

Table II. BLV enhancer activity in transfection experiments

Plasmids	Enhancer origin	Enhancer orientation	Promoter	Cell lines					
				OVK	FLK	ψ2	Clone 1	Clone 2	Clone 3
pSVIXCAT	–	–	SV40	1	1	1	1	1	1
pBLVXS	BLV-LTR	sense	SV40	1.03	49.60	1.09	110.44	6.58	4.95
pBLVXA	BLV-LTR	anti-sense	SV40	0.93	37.20	0.82	82.83	4.93	2.21

Numbers indicate the fold stimulation of CAT gene expression as in Table I.

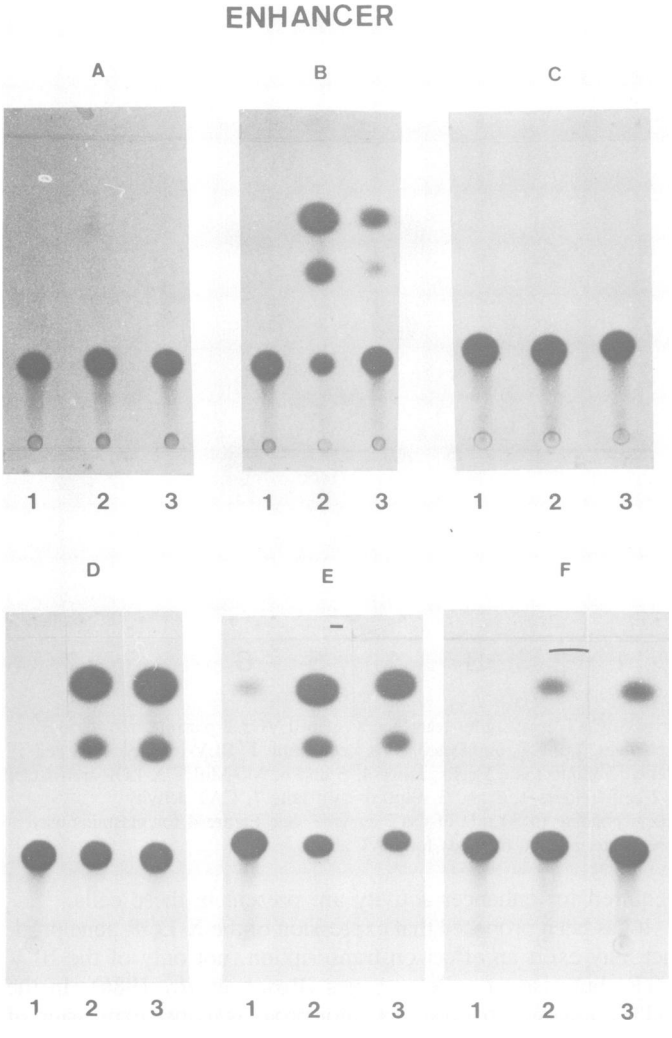

Fig. 6. CAT assays (see Materials and methods) after transfection with plasmids pSVIXCAT (**lanes 1**), pBLVXS (**lanes 2**) and pBLVXA (**lanes 3**) (see Figure 4). **A**, non-infected OVK cells; **B**, BLV-infected FLK cells; **C**, non-infected ψ2 cells; **D**, **E** and **F**, Mo-MuLV/X-LOR-transfected ψ2 cells (clones 1, 2 and 3 respectively).

transfer to ψAM (packaging cell line able to express an amphotropic virus) and consequently to a variety of cell types including bovine B lymphocytes. The availability of a BLV X-LOR protein active within these cells should allow the role of the transactivator protein in BLV-induced leukemogenesis to be understood.

Materials and methods

Retroviral vector

The Moloney murine leukemia virus-based retroviral vector (pLS) was obtained from Dr E.Wagner (EMBL). This vector contains the 3′ and 5′ LTR of Mo-MuLV, sequences required for efficient RNA encapsidation and a polylinker cloning sequence. This vector also includes the bacterial gene for neomycin resistance (*neo*^{R}) located downtream from a splice acceptor site. This allows selection of transfected cells on the basis of their resistance to the antibiotic G418 (Southern and Berg, 1982).

Cloning and sequencing of the BL1 cDNA corresponding to the BLV X region has been described previously (Willems *et al.*, 1987). The *Sau*3A–*Pvu*II insert containing the long open reading frame of the cDNA was subcloned at the blunt-ended *Bam*HI site in the polylinker region of the pLS plasmid (Figure 1). The resulting construct pLS/X-LOR plasmid was transfected into ψ2 cells using the calcium phosphate co-precipitation procedure (Gorman *et al.*, 1982). Forty-eight hours after transfection, cells were cultivated in DME medium containing 1 mg/ml of G418. After 2–3 weeks, neomycin-resistant clones were picked and amplified in the continuous presence of G418.

Cell lines

BLV-infected fetal lamb kidney cells (FLK) were established by Van der Maaten and Miller (1976). OVK cells are uninfected ovine kidney cells. ψ2 cell lines constitutively produce ecotropic murine leukemia virus proteins but cannot package the viral transcripts (Mann *et al.*, 1983). All cell lines were cultivated in DME medium supplemented with 10% fetal calf serum.

Plasmids

The construction of plasmids pBLVCAT (Rosen *et al.*, 1985), pBLVXA, pBLVXS (Rosen *et al.*, 1986), pSV$_2$CAT, pSV$_0$CAT, pSVIXCAT (Gorman *et al.*, 1982), was described elsewhere.

DNA transfections and CAT assays

All cell lines were transfected using the calcium phosphate co-precipitation method as modified by Wigler *et al.* (1979). In short, 10 μg of plasmid were transfected into 10^6 cells. Cytoplasmic extracts were prepared 48 h after transfection and CAT assays were performed as described by Gorman *et al.* (1982).

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Note added in proof

After submission of this communication, Derse reported that the BLV X-LOR encodes a transacting factor. Derse,D. (1987) *J. Virol.*, **61**, 2462–2471.