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The Visna Virus Long Terminal Repeat Directs Expression of a Reporter Gene in Activated Macrophages, Lymphocytes, and the Central Nervous Systems of Transgenic Mice

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Visna virus is a lentivirus which causes a slow progressive disease involving the immune system and the central nervous system. To determine the role of the viral long terminal repeat (LTR) in targeting the virus to specific host cells and tissues, transgenic mice were constructed which contained the visna virus LTR directing expression of the bacterial gene encoding chloramphenicol acetyltransferase (CAT). Analysis of the transgenic mouse tissues for CAT activity revealed that the viral LTR was responsible, in part, for the tropism of visna virus for macrophages and the central nervous system. Expression of the LTR required the macrophage to be in an activated state both in vivo and in vitro. Thioglycolate activation of peritoneal macrophages in vivo and 12-*O*-tetradecanoylphorbol 13-acetate treatment in vitro induced expression of the visna virus LTR. Lymphocytes from the spleens of the transgenic mice expressed CAT activity, suggesting that visna virus was able to replicate in lymphocytes, as did human immunodeficiency virus and simian immunodeficiency virus. These studies demonstrated that the lentivirus LTR was responsible, in part, for cell and tissue tropism in vivo.

Visna virus is a lentivirus of sheep which causes a chronic, progressive disease months to years after the initial infection (8, 9). Lentiviruses are an expanding group of host-specific viruses causing chronic diseases; human immunodeficiency virus, simian immunodeficiency virus, caprine arthritis-encephalitis virus, and equine infectious anemia virus are lentiviruses (1, 6, 13). A common feature in the pathogenesis of the diseases caused by lentiviruses is infection of cells of the immune system. This infection of immune cells causes a lifelong persistent infection and dysregulation of the immune system. In acquired immunodeficiency syndrome, which is the most striking example of this, the helper T lymphocytes are depleted, leaving the host immunodeficient and open to opportunistic infections (27). In visna virus, the dysregulation of the immune system is more subtle, causing a lymphoproliferative disorder which leads to the inflammatory reaction in target organs and clinical disease. The lifelong persistent infections in vivo (10, 21) are characterized by restricted viral replication. This contrasts with the highly permissive replication observed in tissue culture, in which the virus rapidly replicates to high titers, is cytolytic, and causes cell fusion (30). The differences between in vivo and in vitro viral replication have been considered indicative of the complex regulatory processes of the virus as well as the host cells.

Visna virus infects cells of the monocyte/macrophage lineage (4, 5, 22). In affected tissues, virus replication is almost exclusively associated with macrophages (4). It is also known that a subpopulation of monocyte/macrophage precursor cells in the bone marrow harbors the viral genome (4). Thus, experimental evidence supports a hypothesis that these cells act as a reservoir of infected stem cells. The stem cells appear to be silent carriers of the virus, making little viral RNA and no viral protein. As such, they probably are not targets for the host immune surveillance system. When these stem cells mature into macrophages, viral gene expression is induced (4, 5, 22). Only a subset of macrophages in an infected animal activates viral transcription. These include macrophages in the lungs, lymph nodes, spleen, brain, and synovia (joints). Since activation of visna virus gene expression occurs only in mature macrophages, factors which regulate macrophage-specific genes may be necessary for activation of the viral transcriptional control elements.

An in vivo system has been lacking to study the role of individual viral elements in lentivirus pathogenesis. To determine the contribution of the visna virus long terminal repeat (LTR) to cell-specific expression and tissue tropism in vivo, transgenic mice containing LTR sequences were produced. The LTR of visna virus contains both enhancer and promoter elements responsible for control of viral transcription (11). The visna virus LTR was linked to a reporter gene, bacterial chloramphenicol acetyltransferase (CAT). In vitro, this construct directs high levels of CAT activity in cells in which visna virus replicates; in addition, it is active in mouse L cells (11, 12). Since the viral LTR is present in every cell of the transgenic mouse, virus-receptor interactions are bypassed and these animals allow one to assess the role of the viral LTR in vivo in tissue and cell tropism, independent of other viral genes.

MATERIALS AND METHODS

Production and screening of transgenic mice. A 3.2-kilobase-pair *Xho*I-*Pvu*I fragment of pVISLTRCAT (Fig. 1A) containing the visna virus LTR and the bacterial CAT gene was purified by sucrose gradient centrifugation. Approximately 100 to 1,000 copies of the DNA fragment were

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injected into nuclei of one-cell mouse embryos as described previously (7). Tail DNA was purified, digested with *Sst*I and *Hinc*II, electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled VISLTRCAT probe as previously described (29). Intact copies of the VISLTRCAT transgene were indicated by the presence of the 1.8-kilobase-pair band.

Cell and tissue extracts. Macrophages were isolated from mouse peritoneal cavities by lavage with 7 to 10 ml of normal saline. Cells were pelleted by centrifugation at 1,000 rpm in an RT6000 centrifuge (Ivan Sorvall, Inc.). Cells were suspended in 0.25 M Tris (pH 7.8) at a final concentration of 5 $\times 10^4$ cells per μl. Samples were freeze-thawed three times and heated at 65°C for 10 min to inactivate CAT enzyme inhibitors. Protein concentrations were determined by using a modified Lowry protocol (15). In most cases, protein concentrations were 1 μg/μl.

Tissues were flash frozen in liquid nitrogen, ground finely with a mortar and pestle, and suspended in 0.25 M Tris (pH 7.8). Extracts were then freeze-thawed three times as described above for cells and heated at 65°C for 10 min. Extracts were centrifuged for 15 min in a microcentrifuge, and the supernatants were flash frozen and stored at −70°C. Protein concentrations were determined as described for macrophage extracts.

CAT assays. CAT assays were performed essentially as described previously (11). Protein (50 μg) was used for each reaction, and incubations were for 16 h at 37°C. Spots were cut and counted. Specific activity was measured as percent conversion of chloramphenicol to its acetylated forms per microgram of protein.

Cell culture. Macrophages were isolated by peritoneal lavage and plated in RPMI medium plus 10% fetal bovine serum in 35-mm-diameter wells for 48 h. Activating factors were added in 1/10 the volume of medium to give conditions as described previously (25, 32). Briefly, these conditions were 160 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA); 0.5 ng of *Escherichia coli* lipopolysaccharide (LPS) per ml; 140 μM L-α-oleoyl-2-acetoyl-*sn*-3-glycerol (OAG); 500 μM dibutyrl cyclic AMP (dbcAMP), 1 μM muramyl dipeptide (MDP). Cells were harvested 24 h later, and CAT assays were performed. Cells were removed from the spleens by teasing tissue in Hanks buffered saline solution, drawing the cells once through a 25-gauge needle, and then plating at 10^6 cells per ml in RPMI medium containing 10% fetal bovine serum. Cells were cultured for 48 h and then treated with activators as described above. Additionally, in some cases phytohemagglutinin (PHA) was added at plating at a final concentration of 2.5 μg/ml and cells were harvested 72 h later. Adherent cells were assayed separately from nonadherent cells in each case.

RESULTS

Production of mice. A 3.2-kilobase-pair *Xho*I-*Pvu*I fragment of pVISLTRCAT (11) (Fig. 1A) containing the visna virus LTR and the CAT gene with minimal plasmid sequences was purified and injected into one-cell mouse embryos (7). Four founder mice, designated VC5, VC16, CAT5, and CAT6, containing VISLTRCAT sequences were obtained (Fig. 1B). VC16 contained approximately 10 copies, CAT5 contained >100 copies, and CAT6 contained approximately 20 copies of the intact VISLTRCAT sequences, as determined by Southern analysis (Fig. 1B) and dot blot quantitation (data not shown). VC5 contained approximately 50 partial copies of VISLTRCAT. VC16 mice

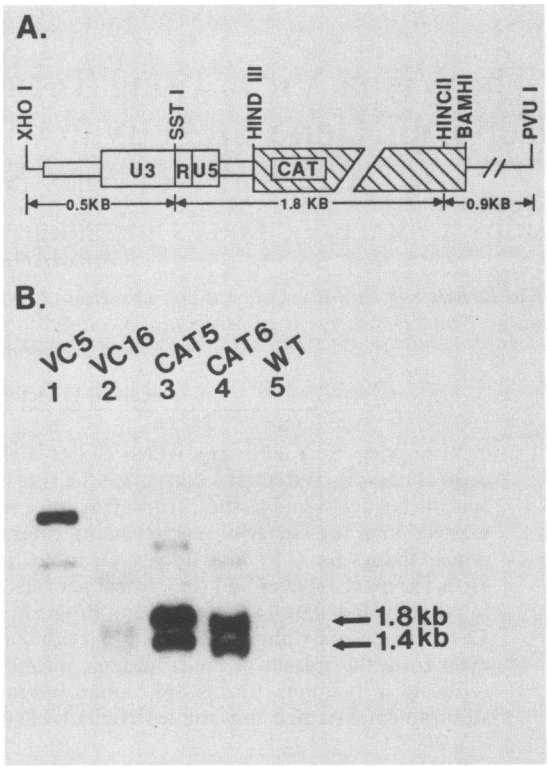

FIG. 1. Production of transgenic mice. (A) Map of the pVISL TRCAT *Xho*I-*Pvu*I fragment. The *Xho*I-*Pvu*I DNA fragment was purified on sucrose gradients and microinjected into one-cell mouse embryos as described previously (15). The *Sst*I and *Hinc*II sites used for analysis are indicated. ———, Plasmid DNA; ▭, visna virus DNA other than LTR; ☐, VISLTR (U3, R, and U5 regions); ▨, CAT gene and processing signals. (B) Southern analysis of four VISLTRCAT transgenic lines. Tail DNA was digested with *Sst*I and *Hinc*II, electrophoresed in 1% agarose gels, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled VISLTRCAT probe as described previously (31). Lanes 1 and 3 contain 2 μg of DNA and lanes 2, 4, and 5 contain 10 μg of DNA. VC16, CAT5, and CAT6 contained intact copies of VISLTRCAT sequences, indicated by the presence of the internal 1.8-kilobase (kb) band. The 1.4-kb band probably results from formation of head-to-tail concatamers of DNA, prior to integration, resulting in fusion of the 0.5- and 0.9-kb fragments. VC5 does not contain either of these fragments and therefore does not have intact copies of VISLTRCAT sequences. Other bands observed correspond to junction fragments with mouse sequences. These transgenic lines contain one integration site per transgenic mouse line, based on the transmission frequency to offspring (~50%). WT refers to DNA from a nontransgenic mouse.

were made homozygous by mating, and these mice were used for analyses. In the other three transgenic lines, hemizygous mice (containing one chromosomal integration per cell) were used for analyses.

Expression in macrophages. Since monocytes/macrophages are the target cells for visna virus expression in sheep (4, 5), expression of the VISLTRCAT transgene was first investigated in macrophages. Macrophages in the peritoneal cavity are mature but are not in an activated state. Activated macrophages were induced by injecting thioglycolate medium into the peritoneal cavity (31) 72 h before the cells were harvested. Activated macrophages or resident peritoneal macrophages were harvested by lavage, and CAT assays were performed. Equal numbers (2.5×10^6) of cells were used for each assay, which was equivalent to 50 μg of

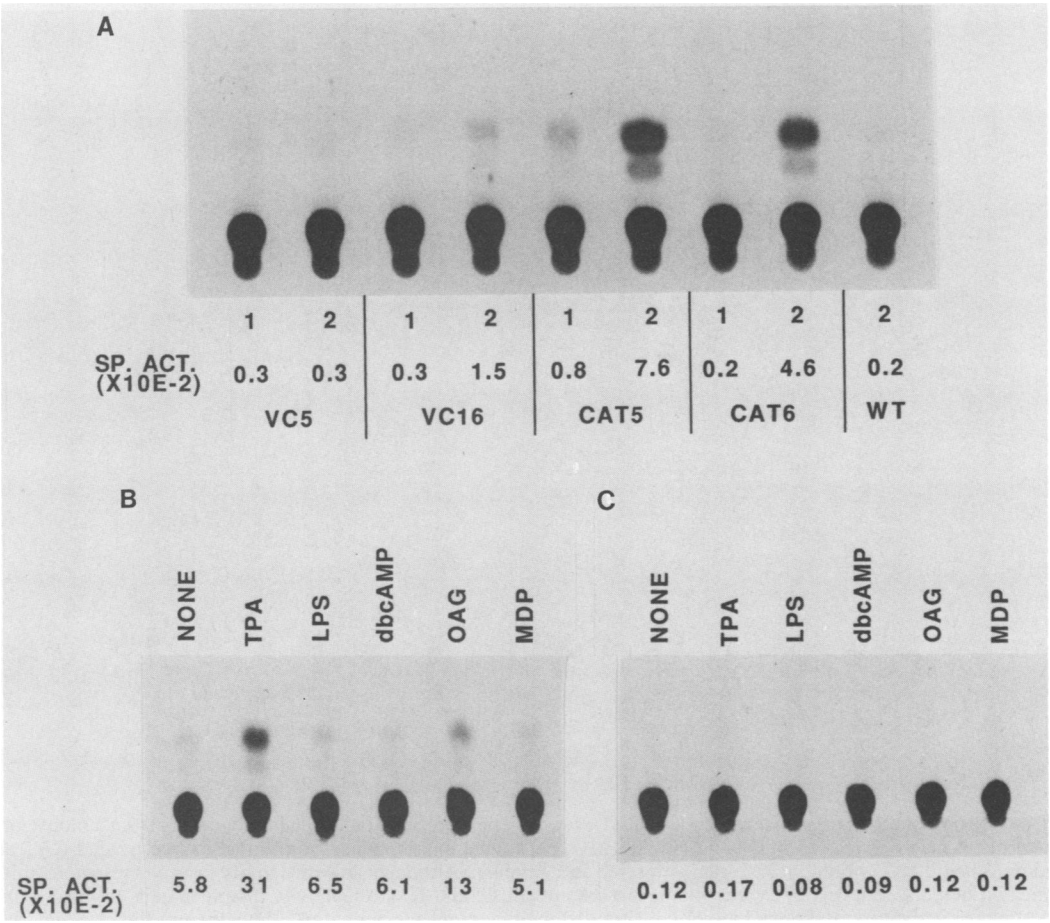

FIG. 2. CAT assays of transgenic mouse macrophages. CAT specific activity (SP. ACT. X10E-2) is given as percent conversion of chloramphenicol per microgram of protein. (A) CAT assays of mouse peritoneal macrophages. Macrophages were isolated by lavage from either control (lane 1) or thioglycolate-stimulated (lane 2) transgenic mice. CAT assays were performed as described previously (13), except that 50 μg of protein was used per assay, and incubations were done for 16 h. VC5, VC16, CAT5, and CAT6 are mice from transgenic lines; WT was a nontransgenic littermate of the CAT6 mouse. (B and C) In vitro activation of peritoneal macrophages of CAT6 transgenic mouse (B) and WT littermate (C). Macrophages were plated in RPMI medium plus 10% fetal bovine serum for 48 h and then treated for 24 h with activating factors as indicated. No activating factor (NONE) was used in the first lanes. The activating factors and their concentrations were 160 nM TPA, 0.5 ng of LPS per ml, 500 μM dbcAMP, 140 μm OAG, and 1 μM MDP.

protein. Assays were incubated at 37°C for 16 h. Low or undetectable levels of activity were found in resident macrophages from all four lines. There was a higher level of expression of CAT in the thioglycolate-stimulated activated macrophages from lines VC16, CAT5, and CAT6, but no induction of CAT activity occurred in VC5 (Fig. 2A; Table 1). Stimulation of activity was most evident in CAT5 and CAT6 mice, in which up to 20-fold induction of CAT activity was observed when the macrophages were in an activated state (Fig. 2A; Table 1). In VC16 homozygous animals, a 2- to 10-fold stimulation was observed. Lack of expression in the VC5 animals was attributed to the loss of cell-specific regulatory elements in the LTR during integration. Thus, the cell-specific expression of visna virus in sheep macrophages is mirrored in the transgenic mice containing only the viral LTR.

To further investigate the induction of the visna virus LTR in activated macrophages, a number of different activating factors were used. Phorbol esters and diacylglycerol induce activation of macrophages via protein kinase C pathways (27, 32), while cyclic AMP works via the cyclic AMP signal transduction cascade (32). Bacterial endotoxins (LPS) (14, 32, 33) or other bacterial products (MDP) (14, 32) cause activation of macrophages via a non-TPA-stimulated protein kinase C pathway. Peritoneal macrophages from the transgenic mouse lines VC16, CAT5, and CAT6 were collected by lavage and cultured in vitro for 48 h. Activating factor TPA,

TABLE 1. CAT expression in macrophages

Line	Avg % conversion[a]		Induction[b] (fold)
	Control	Thioglycolate	
VC5	0.12	0.12	1
VC16	0.13	0.72	5.5
CAT5	0.31	3.4	11.0
CAT6	0.17	2.2	12.9
WT[c]	0.06	0.06	1

[a] For VC16, CAT5, and CAT6, these results are the average of at least three animals. For VC5 and WT, the results are from two animals. Percent conversion is defined as the number of counts in the acetylated forms of chloramphenicol divided by the total counts, then multiplied by 100%.

[b] Induction refers to the increase of expression in thioglycolate-treated macrophages over control, noninduced macrophages.

[c] WT refers to a nontransgenic mouse.

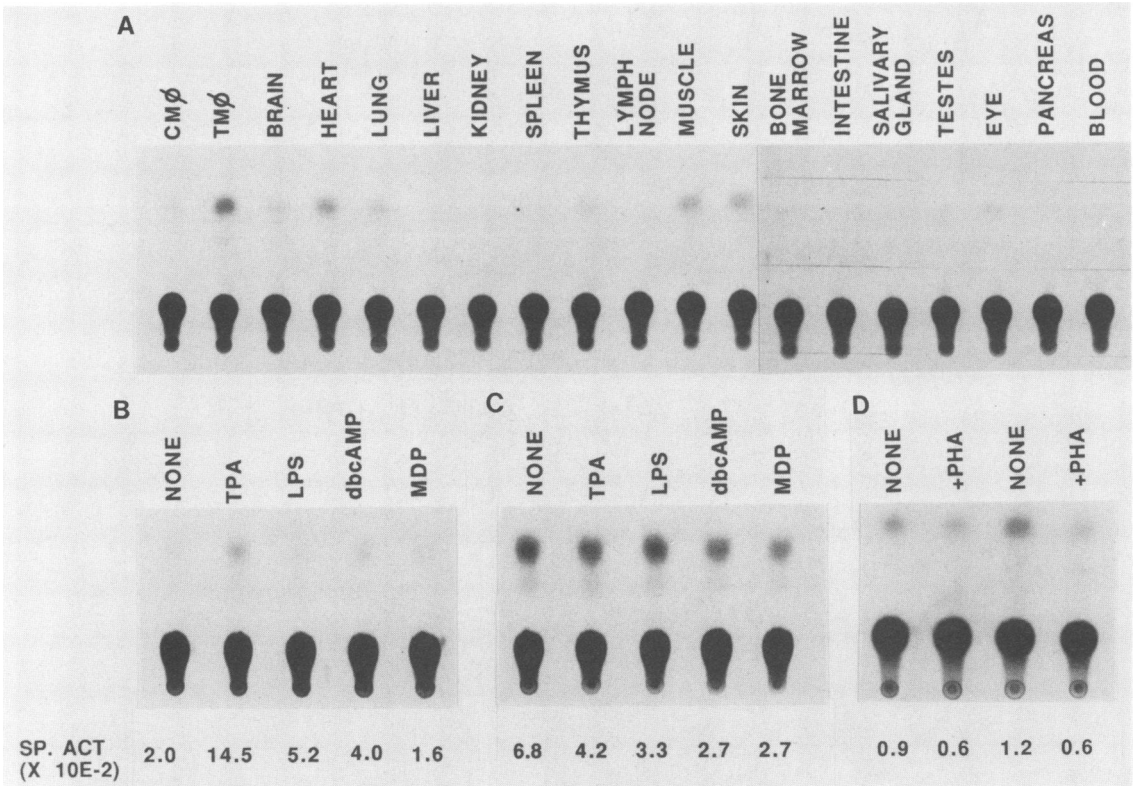

FIG. 3. CAT expression in tissue and spleen cells. (A) CAT assays were performed on tissue homogenates from a mouse of transgenic line CAT5. CMØ, peritoneal macrophages from CAT5 animal; TMØ, peritoneal macrophages from thioglycolate-treated CAT animal. Activity can be detected in lanes TMØ, brain, heart, lung, muscle, and thymus. Although spleen activity was very low in this animal, other experiments indicated detectable levels of expression in the spleen. (B, C, and D) CAT activity in spleen cells in vitro. (B) Adherent cells treated with macrophage activators; (C) nonadherent cells treated with macrophage activators; (D) adherent (lanes 1 and 2) or nonadherent (lanes 3 and 4) cells. Spleen cells were plated in culture for 48 h and treated for 24 h with activating factors as described in the legend to Fig. 2. Panel D, lanes 2 and 4, Cells were plated in culture medium containing 2.5 μg of PHA per ml. Cells were harvested and assayed after 72 h in culture.

OAG, dbcAMP, LPS, or MDP was then added to cells and cultured for 24 h (Fig. 2B).

The phorbol ester, TPA, and diacylglycerol analog, OAG, stimulated expression of the visna virus LTR six- and threefold, respectively, over untreated cultured peritoneal macrophages. Cyclic AMP analog dbcAMP, LPS, and MDP had no effect on CAT expression driven by the viral LTR. No CAT activity was observed by treating macrophages from wild-type mice with any activating factors (Fig. 2C). These data suggest that activation of the visna virus LTR can occur by cellular factors induced in activated macrophages via the TPA-stimulated protein kinase C pathway. These results demonstrate for the first time that specific activation of the macrophage is required for induction of the visna virus LTR.

Tissue-specific expression. To define the tissue specificity of the visna virus LTR-directed CAT activity, homogenates were made from the brain, heart, lung, liver, kidney, spleen, thymus, lymph node, muscle, skin, blood, bone marrow, intestine, salivary gland, pancreas, eye, and gonads of mice from VC16, CAT5, and CAT6 lines. In the transgenic line CAT5, CAT activity could be measured in the brain, heart, lung, muscle, and skin and at lower but detectable levels in the spleen and thymus (Fig. 3A). There was no detectable CAT activity in other tissues tested. A similar pattern of expression was observed for VC16 and CAT6 transgenic lines (data not shown). Tissues from the transgenic line VC5 were not analyzed.

Expression of visna virus LTR CAT in spleen was further investigated to determine if macrophage-activating factors or PHA stimulation of cells derived from this tissue would induce expression by the visna virus LTR. Spleens were removed from mice, and splenocytes were placed into culture. At 48 h after plating, cells were treated separately with activating factor TPA, MDP, LPS, or dbcAMP. Adherent and nonadherent cells were harvested 24 h later and assayed separately. Replicate cultures of splenocytes were treated with or without PHA, and cells were harvested 72 h later. The adherent cells were primarily macrophages, while the nonadherent cells were primarily lymphocytes (16). Splenic macrophages cultured for 72 h had low levels of CAT activity (Fig. 3B). However, similar to the peritoneal macrophages, expression of the visna virus LTR was induced (Fig. 3B) 2.5-fold by treatment with the phorbol ester TPA. MDP, LPS, and dbcAMP had no effect on expression of CAT in the splenic macrophages.

The level of CAT expression in the untreated splenic lymphocytes was similar to that in macrophages (Fig. 3C). However, CAT activity was not induced by any of the activators used for macrophages (Fig. 3C) nor with PHA (Fig. 3D), which causes lymphocyte proliferation (16). In fact, there was a twofold reduction in CAT activity in

PHA-stimulated splenic lymphocytes. It was surprising that CAT activity was detected in the splenic lymphocytes, since visna virus infection had not been detected previously in lymphocytes from infected sheep.

DISCUSSION

In this study, we have demonstrated that the visna virus LTR was responsible in part for the cellular tropism of the virus for cells of the monocyte/macrophage lineage. The regulation of the viral LTR in mouse macrophages mirrored events that occur during natural infection in sheep. Expression was restricted in transgenic mouse monocytes and lymphocytes, based on the lack of CAT activity in bone marrow, blood, and lymph nodes, where precursor or immunologically unstimulated cells are located. Also, only a low level of expression was detected in the spleen and in resident, unactivated peritoneal macrophages. When the mouse macrophages were activated, induction of the visna virus LTR occurred, resulting in expression of CAT activity. The studies demonstrate that a specific pathway in the activation of macrophages induce the viral LTR. This is analogous to the specific activation of the human immunodeficiency virus LTR by NF-κB factor in activated lymphocytes (20).

The activation of the visna virus LTR in lymphocytes is the first demonstration that transcription factors required for activity of visna virus gene expression are present in lymphocytes. Expression of visna virus LTR CAT was not detected in blood samples, although blood contains a substantial amount of lymphocytes. This may reflect the fact that in vitro culture of the spleen lymphocytes alters the state of the cell, i.e., allows activation and proliferation which may cause expression of the visna virus LTR. Further, this suggests that lymphocytes may be infected in the natural infection, albeit at a very low level. Finally, activation of the visna virus LTR in the brain provides an important link in the neuropathogenesis of the virus. Previously, brain lesions were thought to be mediated by an indirect immunopathological mechanism, rather than by infection of neural cells directly. Our data suggest that gene expression from the viral LTR may be supported by certain populations of cells in the brain. However, we cannot exclude the possibility that we are detecting expression of the visna virus LTR in macrophage populations in the brain.

The activation of the visna virus LTR in cultured macrophages and lymphocytes suggests that specific pathways induced when cells are activated are responsible for the positive regulation of visna virus gene expression. In macrophages, the protein kinase C pathway induced by TPA and diacylglycerol may be involved in the activation of the viral LTR. TPA also rapidly induces the expression of a nuclear protein encoded by c-*fos* which complexes with the cellular DNA-binding proteins AP-1 and c-*jun* (24). The viral homolog v-*fos* stimulates transcription from specific promoters in *trans* (28). Our laboratory has demonstrated, by genetic analysis, multiple AP-1 binding sites in the visna virus LTR which are important for transcriptional control of the virus (J. Hess et al., submitted for publication). These sites regulate gene expression by the binding of the cellular transcription factor AP-1 in concert with other factors, such as c-*fos* and c-*jun* (3). c-*fos* induction occurs when monocytes differentiate into macrophages (17, 19), as well as after electrical or chemical stimulation of the brain (2, 18, 26). Preliminary experiments in our laboratory (data not shown) indicate that cotransfection of rat c-*fos* and visna virus LTR CAT in mouse peritoneal macrophages result in a higher level of CAT expression than with visna virus LTR CAT alone. Additionally, production of seizures in mice, which induces c-*fos* in the brain (2, 26), results in a small increase in CAT expression in the brains of transgenic mice. These results are consistent with the hypothesis that c-*fos* expression results in activation of the visna virus LTR in the brain and in macrophages. Therefore, c-*fos* might provide the link to the common cellular pathway which activates visna virus gene expression in its multiple target organs.

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