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Expression of Tissue Factor Is Increased in Astrocytes within the Central Nervous System during Persistent Infection with Borna Disease Virus

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Persistent tolerant infection of rats with borna disease virus (BDV) results in a central nervous system (CNS) disease characterized by behavioral abnormalities. These disorders occur without inflammation and widespread cytolysis in the CNS. Therefore, mechanisms other than virally induced destruction of brain cells may explain the CNS disturbance caused by BDV. Previously, we have shown that astrocytes in the CNS express tissue factor (TF). TF functions as the primary cellular initiator of the coagulation protease cascades, resulting in the generation of the protease thrombin. Proteases and their inhibitors play important roles in the development and physiology of the CNS, and altered protease activity has been implicated in the pathophysiology of various neurological diseases. Here, we present evidence that TF expression in the brain is markedly increased during persistent infection with BDV. Persistent infection of cultured astrocytes with BDV also increased TF expression as a result of both increased transcription of the TF gene and stabilization of TF mRNA. We speculate that increased TF expression within the brain parenchyma may lead to increased protease activity in the CNS and contribute to virus-mediated CNS functional impairment by affecting neural cell interactions.

Borna disease virus (BDV) causes a central nervous system (CNS) disease characterized by behavioral abnormalities, accumulation of disease-specific antigens in limbic system neurons and frequently, but not always, the presence of cell infiltrates within the brain parenchyma (30, 34, 43). Naturally occurring infections with BDV have been documented in several vertebrate species including horses, cattle, cats, and ostriches (43). Moreover, the disease can be experimentally transmitted to a wide range of species from birds and rodents to nonhuman primates (43). The outcome of the infection varies depending on the species, age, and immune status of the host as well as the route of inoculation and viral strain used (43). Increasing evidence from a number of serological and molecular biological studies indicates that BDV can infect humans and is possibly associated with certain neuropsychiatric disorders (3, 4, 22, 33, 53).

BDV has been characterized recently as a negative nonsegmented single-stranded RNA virus (7, 15, 21, 47). Furthermore, BDV has a nuclear site for replication and transcription of its genome (14, 21, 47) and uses the host nuclear splicing machinery to generate some of its mRNAs (16, 48). These unique features for a negative nonsegmented single-stranded RNA virus indicate that BDV is the prototype of a new group of animal viruses (21, 47).

Experimental inoculation of BDV in immunocompetent rats leads to an immunologically mediated biphasic behavioral disease (51). In contrast, neonatally infected rats develop a persistent tolerant infection (PTI) associated with neurobehavioral abnormalities (1, 23). Despite a high viral load in neurons and astrocytes, rats with PTI do not contain inflammatory cell infiltrates in the CNS and there is no overt structural damage in the brain parenchyma (11, 30). Therefore, BDV-mediated impairment of CNS functions in rats with PTI is probably due to virus interference with specialized functions of brain cells.

Tissue factor (TF) is a transmembrane receptor that, on the basis of its structural features, has been classified as a member of the class II cytokine receptor family (2, 25). In addition to its role as the primary initiator of the blood coagulation (26), TF has been proposed to play a role in cellular signal transduction, angiogenesis, metastasis, and brain function (8, 25, 44, 55). Importantly, activation of the coagulation protease cascades by TF results in the generation of the protease thrombin (25). Thrombin plays an important role in neural development and plasticity, as well as in the regulation of neurite outgrowth and astrocyte morphology. These activities are important in the regeneration of synapses and cellular connections after brain injury (20). In addition, a dynamic balance between the levels of proteases and their inhibitors is important not only for the development and physiology of the CNS (28) but also for the repair of neural cell connections (18) and during neuropathological conditions (19, 20, 49).

We have previously shown that astrocytes are the primary source of TF in the CNS (24). Here, we demonstrate that TF expression is markedly increased in the CNS of rats during persistent BDV infection. We show that this upregulation is directly due to BDV infection of astrocytes, which leads to both increased TF gene transcription and stabilization of TF mRNA. Increased expression of TF due to BDV infection occurs in the absence of hemorrhage or blood-brain barrier

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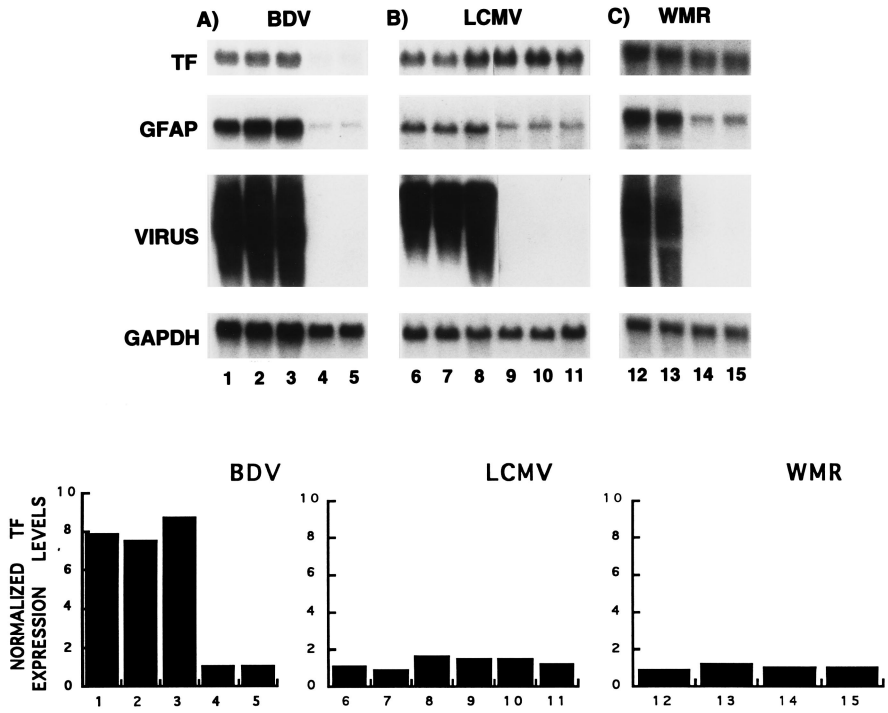

FIG. 1. TF mRNA levels are markedly increased in the CNS during BDV infection but not during persistent LCMV infection or after WMR infection. Total RNA was isolated from the brains of persistently BDV-infected rats (lanes 1 to 3) (A), the brains of LCMV-infected mice (lanes 6 to 8) (B), and the brain stems of mice in the late stages of WMR infection (lanes 12 and 13) (C). Control animals (lanes 4 and 5, 9 to 11, and 14 and 15, respectively) were sham inoculated and treated identically. Northern blot analysis was performed with rat (lanes 1 to 5) and mouse TF cDNAs; BDV-, LCMV-, and WMR-specific cDNAs to illustrate viral infection; and GFAP and GAPDH cDNAs to indicate levels of reactive astrocytosis and relative loading of the different lanes, respectively. The exposure times for the Northern blot analysis of the different viral infections were not standardized; therefore, comparisons between them cannot be made. Levels of TF mRNA were normalized to GAPDH mRNA levels and are shown at the bottom of the figure.

disruption. This upregulation of TF in the CNS suggests that it may function in processes other than hemostasis during brain insults such as viral infection. Altered TF expression may contribute to the dysregulation of normal brain functions as seen in rats with PTI.

MATERIALS AND METHODS

Viral infection of animals and cells. Inbred Lewis rats younger than 24 h old were inoculated intracranially (i.c.) with a 20% (wt/vol) stock of BDV-infected rat brain homogenate as previously described (1). The rats used in this study were sacrificed 30 days postinoculation. Sham-infected Lewis rats of the same age served as controls. BALB/c WEHI mice were inoculated i.c. with 100 PFU of lymphocytic choriomeningitis virus (LCMV) Arm strain within the first 18 h of life. This results in the establishment of a lifelong persistent LCMV infection of the CNS, without signs of CNS inflammation, necrosis, or astrocytic infection (9, 27). Neonatal SWR/j mice were inoculated i.c. with the wild mouse retrovirus (WMR) clone WM 1504-E (cl N) as previously reported (40) and sacrificed after 8 to 12 weeks, when hindlimb paralysis and whole body tremor were apparent. Control mice for each infection were sham inoculated i.c. with 30 μl of medium.

Infection of C6 cells (ATCC CCL107) with BDV (C6BV) or LCMV was conducted as described previously (12, 14). The cells were kept at 37°C in Dulbecco's minimal essential high-glucose medium supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin, streptomycin, and 1% glutamine. Infected cells were used at early passages after infection.

Northern blot analysis. The mice and rats were sacrificed under deep anesthesia by cervical dislocation, and their organs were quickly removed and snap-frozen in liquid nitrogen. Total RNA was isolated from brains or tissue culture cells with TRI-Reagent (Molecular Research Center, Cincinnati, Ohio) as recommended by the manufacturer. The total RNA (10 μg) was size fractionated by 2.2 M formaldehyde–agarose gel electrophoresis (45), transferred by capillarity with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to MagnaGraph nylon membranes (MSI, Westboro, Mass.) and UV cross-linked. The blots were prehybridized for 30 min in 3 ml of Quikhyb (Stratagene, La Jolla, Calif.). Heat-denatured DNA probe, labeled with [α-^{32}P]dCTP by using random hexamers (Pharmacia LKB), and salmon sperm DNA were added to the Quikhyb buffer to concentrations of 5 ng/ml, and 30 μg/ml, respectively. Hybridization proceeded for 2.5 h, and the blots were washed twice at high stringency (0.2× SSC, 0.2% sodium dodecyl sulfate [SDS]) for 15 min. The temperature for prehybridization, hybridization, and washes ranged from 55 to 68°C depending on the probe. The blots were exposed to Biomax MR films (Kodak, Rochester, N.Y.) with intensifying screens at −70°C. mRNA levels were quantitated directly on the blots with a Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.). The following probes were used for Northern (RNA) blot analysis: 772- and 821-bp fragments of rat and mouse TF cDNA, respectively; a cDNA fragment of the BDV p40 gene (15); a 1-kb cDNA fragment of LCMV nucleoprotein (NP); a WMR Cas-Br-E cDNA (gift of J. Portis, Rocky Mountain Laboratories, Mont.); and a rat glial fibrillary acidic protein (GFAP) cDNA fragment (gift of R. Milner, Hershey Medical School). Hybridization signals were normalized to the signal obtained with a cDNA probe corresponding to the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Preparation of tissue and cells for histological testing. The rats were deeply anesthetized and perfused with 35 ml of phosphate-buffered saline (PBS) followed by 35 ml of 4% paraformaldehyde. The brains were removed and postfixed in 4% paraformaldehyde before being dehydrated and embedded in paraffin by standard histological procedures. Sections (7 μm thick) were cut with a rotary microtome, mounted onto Superfrost plus slides (Fisher Scientific, Pittsburgh, Pa.), and stored at 4°C before use.

Cells grown on Labtek slides (Nunc, Naperville, Ill.) were washed twice with PBS and then fixed in formalin-ethanol-acetic acid for 20 min at 4°C. After being quenched in 0.15 M ethanolamine for 20 min at 4°C, the slides were washed in PBS and stored in 70% ethanol at 4°C until used.

Immunohistochemistry and in situ hybridization. Immunohistochemistry and in situ hybridization were performed essentially as described previously (5). For immunohistochemistry, deparaffinized sections or cells were incubated for 1 h at room temperature with rabbit polyclonal antibodies against GFAP (Dakopatts, Carpinteria, Calif.) at 1:1,000 or against the BDV p40 protein at 1:450. Binding of primary antibodies was detected with a species-matched biotinylated secondary antibody and revealed with an avidin-biotin peroxidase kit (ABC; Vector Laboratories, Burlingame, Calif.) and diaminobenzidine as a substrate. For double-labeling experiments, in situ hybridization was performed after immunohistochemistry. In situ hybridization was performed as described previously (5). ^{35}S-UTP-labeled riboprobes complementary (antisense) to murine TF and BDV p40 mRNA were prepared as described previously (46). Adjacent sections were processed in parallel with a murine TF sense riboprobe to provide a control for

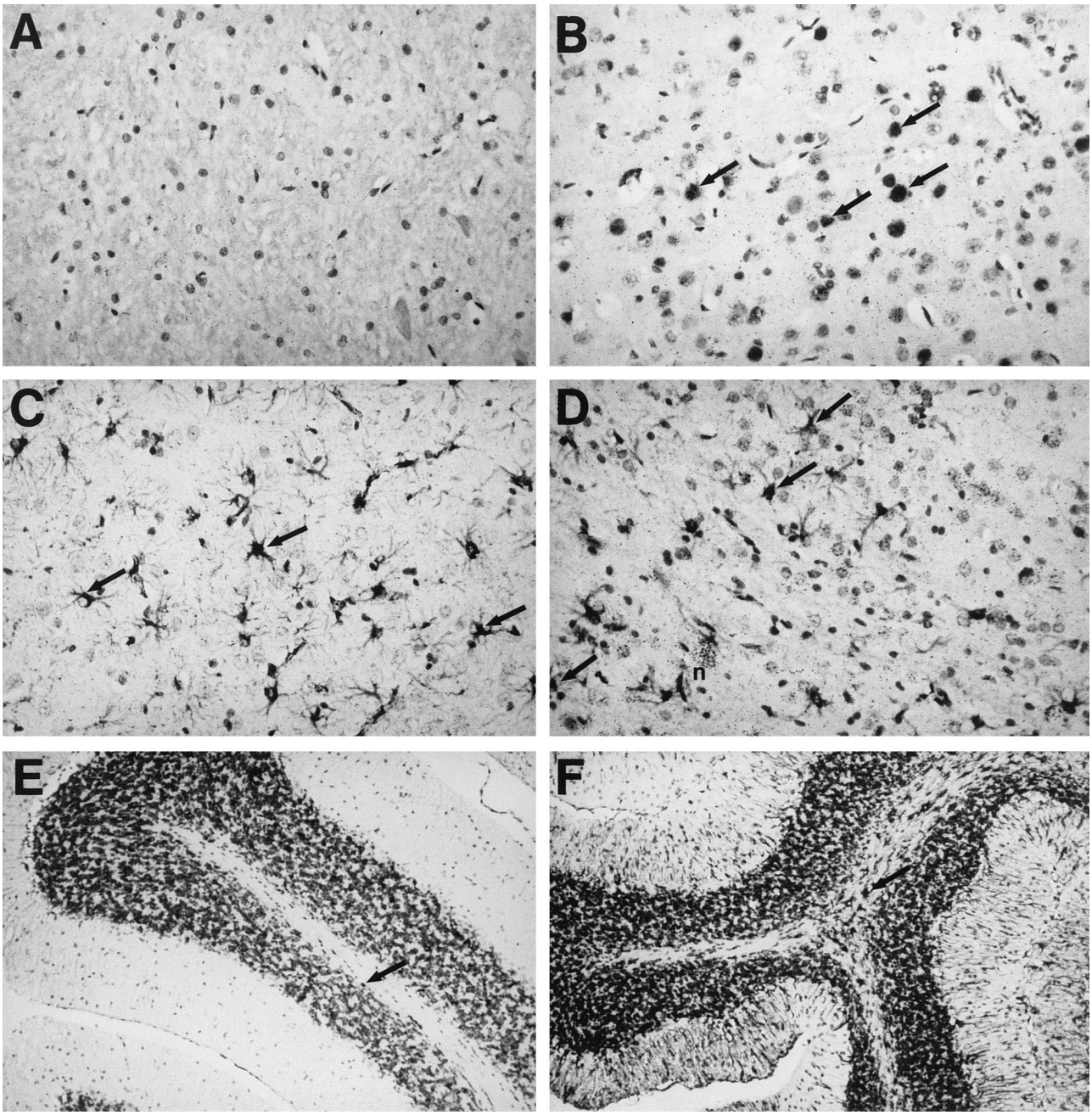

FIG. 2. In situ analysis of TF mRNA activation in BDV-infected rat brains. (A and B) Simultaneous detection of BDV p40 antigen and TF mRNA in control (A) and infected (B) rat brains. In situ hybridization grains, corresponding to TF mRNA levels, are below the threshold level of detection in control rat brains (A) but are markedly upregulated in BDV-infected animals (B) and colocalize with the DAB staining corresponding to BDV p40 antigen, which is mainly nuclear. Arrows indicate representative examples of double-labeled cells. Similar fields of the hippocampal region in infected and control animals are shown here. Signals for TF were stronger in this area. The autoradiography exposure time was 30 days at 4°C. Magnification, ×225. (C) Simultaneous detection of GFAP antigen and TF mRNA in infected rat brain sections. TF mRNA-positive cells expressed GFAP (arrows), indicating that they were astrocytes. Signals for TF are lower in the brain area shown here, but the choice of this field was dictated by the fact that the prominent astrocytosis, and hence the intense GFAP staining, complicates the visualization of autoradiography grains in areas of the brain containing a higher proportion of astrocytes. The autoradiography exposure time was 30 days at 4°C. Magnification, ×225; (D) Simultaneous detection of GFAP antigen and BDV mRNA in brain sections of rats with PTI. A fraction of cells expressing BDV mRNA are also stained with the GFAP antibody (arrows). However, the majority of cells infected throughout the brain appear, from morphological criteria, to be neurons (n). The autoradiography exposure time was 30 h at 4°C. Magnification, ×225. (E and F) BDV-induced cerebellar hypoplasia and astrocytosis. Sections from control (E) and infected (F) rats were stained with the GFAP antibody. The two fields presented here were taken at the same magnification to show the important cerebellar hypoplasia present in rats with PTI. The marked increase of GFAP staining is clearly visible in this area. Consistent with this activation, astrocytes are significantly hypertrophied in infected brains (compare the arrowed cells in panels E and F). Magnification, ×90.

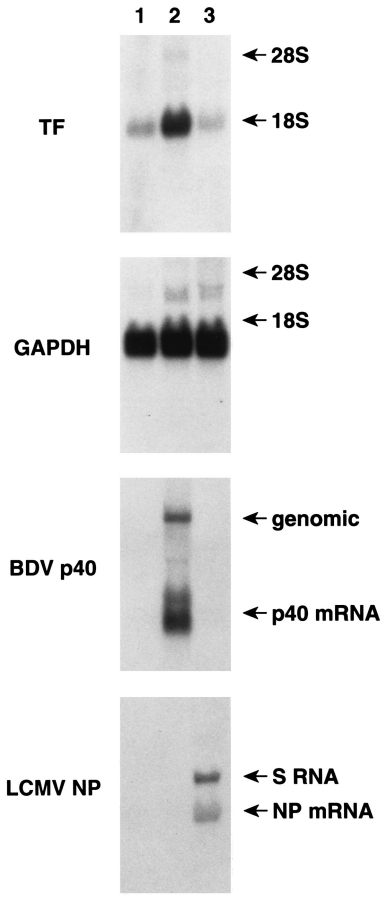

FIG. 3. Steady-state TF mRNA levels are increased in C6 cells persistently infected with BDV. RNA extracted from the C6 astrocyte cell line or C6 cells persistently infected with BDV or LCMV (lanes 1, 2, and 3, respectively) was analyzed by Northern blot hybridization with probes for mouse TF, GAPDH, BDV p40, and LCMV NP (probes indicated on the left). Arrows on the right indicate the expected sizes of 18S and 28S RNAs and BDV and LCMV transcripts. BDV infection of the C6 cells resulted in a clear increase in steady-state TF mRNA levels, whereas persistent LCMV infection did not alter TF expression. Phosphorimager quantitation of the signals and normalization to GAPDH revealed a sixfold increase in TF mRNA levels in C6BV cells.

nonspecific hybridization. No signal was observed with this probe (data not shown). Comparisons of hybridization signals between infected and noninfected sections were made with the same regions of the brain which had been processed in parallel.

Coagulation assay. TF activity was assayed by a single-stage plasma coagulation assay as described previously (31). Cell lysates from confluent monolayers of C6 and C6BV cells were prepared by resuspending the cell pellets in 15 mM octyl β-D-glucopyranoside (500 μl for 10^6 cells) and incubating the mixture for 15 min at 37°C. Two volumes of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) were added, and the samples were vortexed and kept on ice before being assayed. Aliquots of the samples (100 μl) were mixed with 100 μl of prewarmed human citrated plasma in 15- by 75-mm borosilicate glass tubes. After 30 s, 100 μl of 20 mM $CaCl_2$ was added, the tubes were rocked manually, and the time required for a visible fibrin gel to form was noted. Functional TF was quantitated by using a standard curve established with phospholipid-reconstituted affinity-purified TF from human brain. The use of heterologous human plasma in these studies provides only a qualitative measurement of rat TF activity but does allow comparison between infected and noninfected cell lines.

Study of mRNA stability. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a specific RNA polymerase II inhibitor (42), was added at 65 μM (21 μg/ml) to monolayers of C6 and C6BV cells. The cells were maintained at 37°C for up to 18 h without any further modification of culture conditions and harvested 0, 2, 4, and 18 h after addition of DRB. The decay of TF mRNA was analyzed by Northern blotting. After Phosphorimager quantitation of signals and autoradiography, the blots were stripped by being boiled twice in stripping buffer (2 mM EDTA, 5 mM Tris [pH 7.5], 0.1% SDS) and rehybridized with a GAPDH probe.

Transfections. Functional analysis of the human TF promoter was performed with plasmids containing the wild-type TF promoter and mutated derivatives cloned upstream of the luciferase reporter gene (35). We also used a plasmid containing mutated EGR-1 sites described previously (17). As a control, we used a plasmid containing the cytomegalovirus promoter cloned upstream of the luciferase gene. Qiagen-prepared (Qiagen, Chatsworth, Calif.) plasmid DNAs were used for the transfections. C6 and C6BV cells were seeded in 24-well plates (10^5 cells per well) 16 h prior to transfection and transfected with Lipofectamine (GibcoBRL, Grand Island, N.Y.). We used 2 μl of Lipofectamine and 0.5 μg of DNA per well. For each transfection experiment, every DNA was transfected in four separate wells, and this was repeated five times (i.e., each plasmid was tested 20 times) before processing of the data. Luciferase activity was determined 16 h posttransfection by using the luciferase assay system (Promega, Madison, Wis.) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, Calif.). In parallel, 10-μl aliquots of the samples were used to determine protein concentrations in a commercial protein assay system (Bio-Rad Laboratories, Hercules, Calif.). Results were expressed as luciferase activity divided by protein concentration.

RESULTS

Increased TF mRNA expression in brains of rats with PTI. Quantitation of mRNA steady-state levels by Northern blot analysis revealed a substantial increase in the amount of TF mRNA in brains of rats with PTI compared with the amount found in sham-inoculated rats (Fig. 1A). The exposure time for Fig. 1A, lanes 1 to 5, was chosen to demonstrate upregulation of TF mRNA expression. However, a longer exposure of the autoradiogram revealed constitutive expression of TF mRNA in noninfected brains. Quantitation of the signals shown in Fig. 1A and normalization with GAPDH revealed that mRNA levels were increased 8.0-fold ($n = 3$; standard error of the mean = 0.3; $P < 0.001$ by Student's t test) in infected brains. Hybridization with a GFAP cDNA probe showed upregulation of GFAP mRNA, indicating the presence of reactive astrocytosis in BDV-infected brains.

TF mRNA levels were also examined following two other persistent CNS infections. Northern blot studies with total brain RNA indicated that TF mRNA expression was not significantly altered in persistently LCMV-infected mice (Fig. 1B). Nevertheless, a distinct increase in GFAP mRNA levels was apparent in those brains, suggesting a low-scale astrocytosis. Likewise, analysis of RNA extracted from the brain stem of WMR-infected mice, which is the major region of pathological changes, revealed no significant increase in TF mRNA levels (Fig. 1C). Again, GFAP mRNA levels were increased, indicating an ongoing reactive astrocytosis.

To analyze the cellular pattern of BDV-induced TF mRNA upregulation, we performed coupled immunohistochemistry and in situ hybridization on 7-μm-thick rat brain sections. We used a rat TF or BDV p40 riboprobe combined with an anti-GFAP or an anti-p40 antibody. After autoradiography, the slides were counterstained with Harris hematoxylin and eosin and examined by light microscopy (Fig. 2). The histological appearance of the brain was indistinguishable between control and infected rats. In particular, no inflammatory infiltrate or disruption of the blood-brain barrier was apparent in those animals. However, brains of infected rats exhibited a prominent and widespread increase in the number of astrocytes expressing GFAP, consistent with the Northern blot data shown in Fig. 1. In addition, astrocytes in infected rats displayed a hypertrophied morphology (compare the arrowed cells in Fig. 2E and F). As previously reported (11), the cerebellar area of BDV-infected animals was markedly hypoplastic, with a cerebellum measuring approximately half of the size of that in age-matched control rats (compare Fig. 2E and F). The astrocytosis was remarkably widespread in this area (Fig. 2F).

Analysis of TF mRNA expression by in situ hybridization

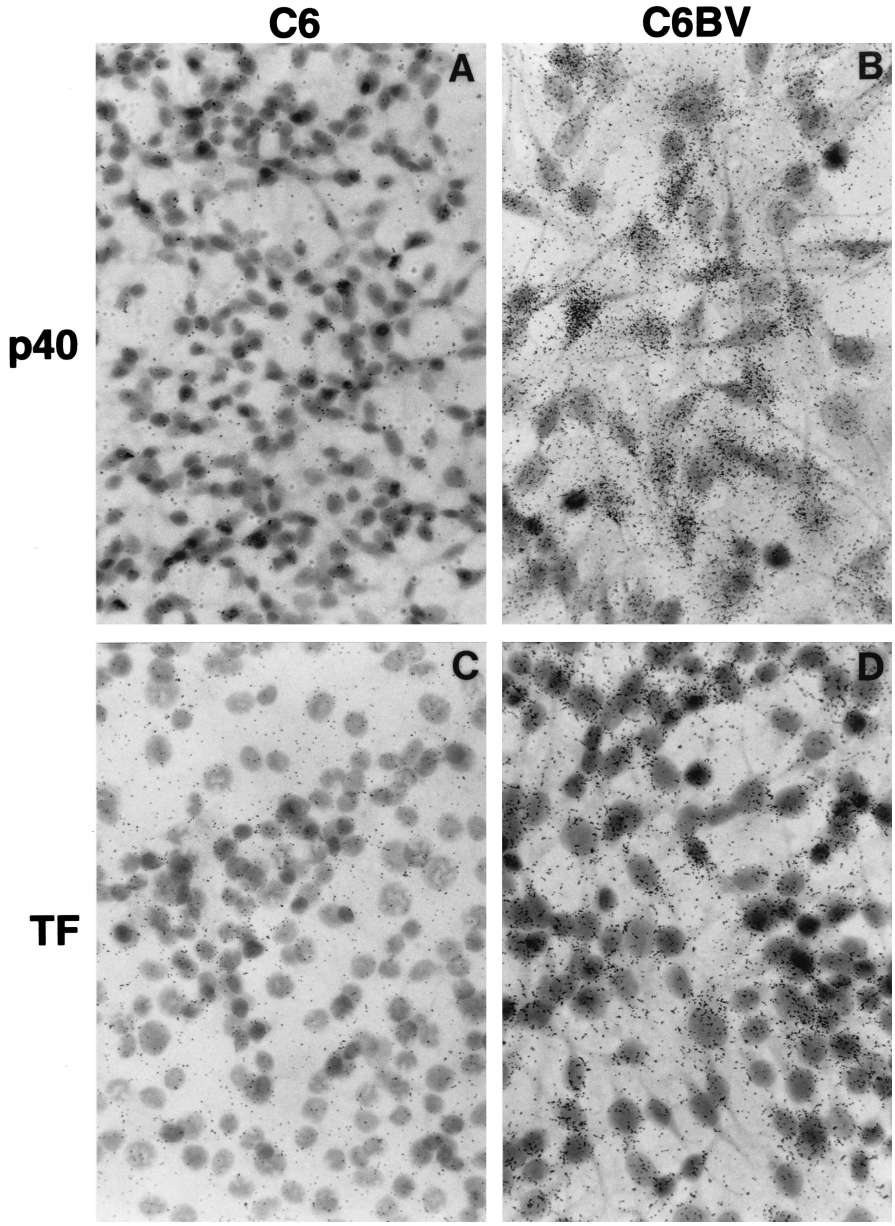

FIG. 4. Analysis of TF and BDV mRNA expression in C6 and C6BV cells by in situ hybridization. BDV p40 mRNA expression was studied by in situ hybridization in C6 (A) and C6BV (B) cells. The signal varies from cell to cell, reflecting asynchronous viral replication in the cell population. Likewise, detection of TF mRNA in C6 (C) and C6BV (D) cells revealed the same heterogeneous pattern in C6BV cells. The exposure time was 24 h (A and B) or 9 days (C and D) at 4°C. Magnification, ×250.

was performed after an autoradiography exposure time of 30 days. TF mRNA hybridization signals were detected in the brain parenchyma of rats with PTI, whereas the endogenous level of TF mRNA in control rats could not be detected at this time of exposure. The TF hybridization signal was not evenly observed in the brain parenchyma of infected rats and seemed to correlate with the regions having the highest viral load. For example, the strongest signals were observed in the hippocampal region, known to be one of the main areas for BDV replication (Fig. 2A and B) (10), whereas no signal was detected in the cerebellum of rats with PTI, despite infection of some of the cerebellar astrocytes (data not shown). The fact that the cerebellum was also the area with the most prominent astrocytosis suggests that increased TF mRNA expression is not a simple consequence of astrocyte activation.

The great majority of cells expressing TF mRNA were also positive for the BDV p40 antigen (Fig. 2B), suggesting that infection of the cell with BDV is required for TF mRNA upregulation. Consistent with our previous results showing that astrocytes are the primary source of TF in the CNS (24), the cells expressing TF mRNA were also found to be GFAP positive (Fig. 2C). Finally, we also confirmed that a subpopulation of BDV-infected cells consisted of astrocytes (10) on the basis of their positive staining for GFAP (Fig. 2D). However, on the basis of morphological criteria, neurons represented the majority of cells infected by BDV.

Astrocyte TF expression in vitro following infection by BDV. To further dissect the mechanisms that underlie the BDV-induced increase in TF mRNA levels, we analyzed an astroglial tissue culture model. BDV persistent infection was established in the rat astrocytoma cell line C6. RNA was isolated from both infected and uninfected cell monolayers, and the levels of TF mRNA were analyzed by Northern blot hybridization (Fig. 3). TF mRNA levels were markedly increased in BDV-infected (C6BV) cells, whereas persistent LCMV infection did not result in any increase in TF mRNA expression despite significant levels of LCMV replication (Fig. 3). Phosphorimager quantitation of the TF mRNA signal and normalization to GAPDH revealed that the level of TF mRNA was increased 6.0-fold ($n = 4$, standard error of the mean = 0.7; $P < 0.001$ by Student's t test) in C6BV cells. In contrast, GFAP levels, which are known to be low in C6 cells (54), were not significantly modified after infection with either BDV or LCMV (data not shown).

Having shown that BDV-infected cells contained enhanced levels of TF mRNA, we sought to compare the levels of procoagulant activity between C6 and C6BV cells. Using a single-stage clotting assay, we assayed the total cellular level of functional TF protein. C6BV cells expressed a markedly increased level of procoagulant activity (3,189 ± 435, $n = 4$) compared with normal C6 cells (425 ± 20, $n = 4$). This 7.5-fold difference was highly significant ($P < 0.001$ by Student's t test). Therefore, BDV infection of cultured astrocytes induces high levels of TF mRNA and functional procoagulant activity.

To study the single-cell pattern of TF mRNA expression, C6 and C6BV cells were examined by in situ hybridization (Fig. 4). We used both p40 and TF riboprobes in separate experiments. More than 90% of C6BV cells were positive for p40 mRNA (Fig. 4B). As can be seen in Fig. 4B, the intensity of the signal varied from cell to cell, reflecting asynchronous viral replication in the cell population. Interestingly, the signal obtained in infected cells hybridized with the TF riboprobe also followed a similar pattern (Fig. 4D), suggesting that the activation of TF mRNA expression is related to the level of BDV expression in the same cell.

Mechanisms of BDV-induced TF mRNA upregulation. The BDV-induced increase in TF steady-state mRNA levels could result from increased TF gene transcription and/or mRNA stabilization. Therefore, we determined mRNA decay rates following blockade of RNA polymerase II transcription and assessed transcription from the TF promoter independently in transient transfection experiments.

(i) TF mRNA stability. TF mRNA has a short half-life (45 to 90 minutes) (6) and has several AU-rich motifs in its 3′ untranslated region. These motifs have been involved in the regulation of the stability of several transiently expressed mRNAs (50), including TF mRNA, following treatment of monocytic cells with lipopolysaccharide (LPS) (6).

RNA was isolated from C6 and C6BV cells at different time points after addition of the RNA polymerase II inhibitor DRB, and the levels of TF and GAPDH mRNAs were determined by Northern blot analysis (Fig. 5). TF mRNA was more stable in BDV-infected cells than uninfected cells, whereas GAPDH mRNA levels showed similar rates of decay in both infected and uninfected cells.

(ii) TF gene transcription. To determine whether BDV infection also increases TF gene expression, we performed transient transfection experiments. We used plasmids containing either the wild-type or mutant TF promoter cloned upstream of the luciferase gene (Fig. 6A). Luciferase activity was assayed 16 h posttransfection and was normalized to the concentration of protein in each sample. Means and standard deviations were

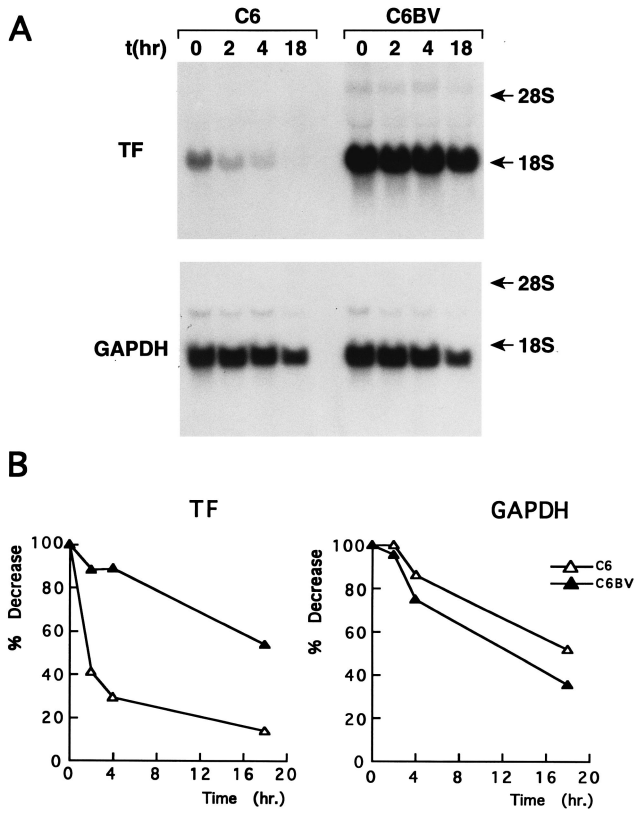

FIG. 5. Increased TF mRNA stability in C6BV cells. RNA was isolated from C6 and C6BV cells at different time points (0, 2, 4, and 18 h) after addition of the transcriptional inhibitor DRB, and levels of mRNA encoding TF or GAPDH were determined by Northern blot analysis. (A) Autoradiograph depicting representative hybridization signals (probes are indicated on the left). (B) Phosphorimager quantitation of mRNA decay rates for TF and GAPDH. Levels are indicated relative to mRNA levels at time zero, which were set as 100%.

calculated from five independent transfection experiments. Determination of normalized luciferase activities revealed that TF promoter activity was three to six times higher ($P < 0.001$) in C6BV cells than in uninfected cells whereas the activity of the cytomegalovirus promoter was indistinguishable in uninfected and infected cells (Fig. 6B). Increased activity of the TF promoter in infected cells was maintained in pTF(−111)Luc, which contains three overlapping binding sites for Sp1 and EGR-1 transcription factors (17). Luciferase activities in all cases were at least 20 times above the background of the assay, or above the levels of luciferase activity expressed by pTF(−21)Luc construct, which does not express any promoter activity (35). To evaluate the contribution of EGR-1 in BDV-induced activation of the TF promoter, we used two plasmids: pTF(−111 to +14)Luc, which contains the wild-type TF promoter, and pTF(EGR-1m)Luc, which contains mutations in all three EGR-1 sites without affecting Sp1 binding (Fig. 6C). Mutation of the three EGR-1 sites completely abolished the increased TF gene transcription in infected cells, suggesting that EGR-1 mediates the increased TF promoter activity observed in C6BV cells.

DISCUSSION

Persistent viral infections of the CNS can induce slowly progressive neurological disorders which are associated with diverse neurochemical abnormalities and pathological findings

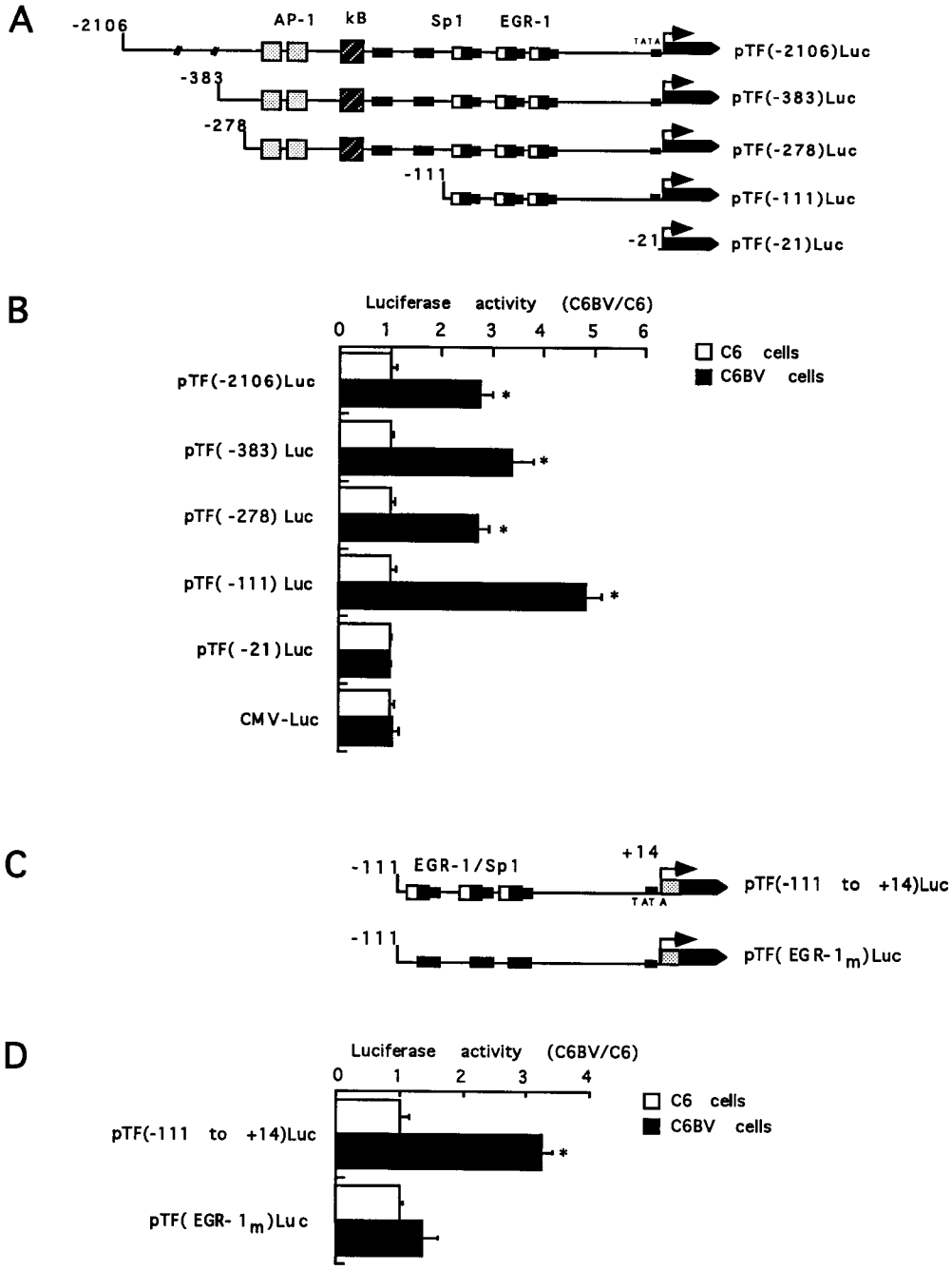

FIG. 6. Functional analysis of the TF promoter in C6 and C6BV cells. (A and C) Plasmids used in this study. The plasmids are named as previously described (17, 35). Sites for binding of transcription factors described in the TF promoter are shown. In particular, EGR-1 sites are indicated by the open boxes and Sp1 sites are indicated by the solid boxes. Results obtained after determination of luciferase activity were normalized to the protein concentration in each sample and are expressed as C6BV/C6 ratios. Differences between infected and uninfected cells were statistically significant ($P < 0.001$ by Student's t test) when indicated (*). (B) Results obtained with the promoter deletion series shown in panel A. Statistically significant differences were observed with deletions of the promoter up to position −111. In contrast, no difference was observed when the immediate-early cytomegalovirus promoter (CMV-Luc) was used. (D) Results obtained with the promoters described in panel C. Mutation of the EGR-1-binding sites completely abolished the statistically significant difference seen with the corresponding vector containing functional EGR-1 sites.

(52). These infections can take place in the absence of lymphoid cell infiltration and lysis of infected cells (52). However, they may alter differentiated functions of the infected brain cells. This, in turn, can disrupt the CNS homeostasis and lead to disease. Therefore, it is important to understand the mechanisms whereby viruses can interfere with the normal functions of the CNS in the absence of structural damage within the brain parenchyma. In this respect, BDV provides a very valuable model to study both the mechanisms and consequences of a persistent infection of the CNS.

In this study, we have demonstrated that TF expression in the CNS is markedly increased during persistent infection of

rats with BDV. Simultaneous in situ hybridization and immunohistochemistry strongly suggests that increased TF mRNA levels are due to BDV infection of parenchymal astrocytes. We could not detect expression of TF mRNA in other cells, such as endothelial cells, microglia, or neurons. All the cells expressing high levels of TF were positive for BDV p40 protein. Moreover, TF upregulation was not evenly observed in the brain parenchyma and seemed to correlate with the regions having the highest viral load. Taken together, these findings suggest that BDV infection of the astrocyte is required for increased expression of TF mRNA. Our results also argue against the hypothesis that TF upregulation in infected brains could be due to a soluble factor released by surrounding infected cells. Indeed, treatment of C6 cells with tissue culture supernatant from persistently infected C6BV cells for up to 2 days did not result in any increase in TF mRNA levels (not shown).

Rats with PTI are characterized by intense reactive astrocytosis as determined by increased GFAP expression. Our data also show that TF mRNA upregulation and astrocytosis appear to be independent phenomena, at least in this model. For example, TF expression was not increased in the cerebellum of infected rats despite a very prominent astrocytosis. In contrast, no difference in TF expression was observed following murine persistent CNS infection caused by LCMV or WMR, despite infection of endothelial cells in both cases (27, 39). Transient induction of TF in cultured human endothelial cells has been described following acute infection with herpes simplex virus type 1, avian hemorrhagic virus, and measles virus (32, 37, 41). In contrast, our studies with BDV provide the first evidence of a constitutive increase of TF mRNA levels during viral persistent infection, in the absence of inflammation.

To further dissect the mechanisms whereby BDV affects TF gene expression, we conducted tissue culture studies with C6 cells persistently infected with BDV. C6BV cells exhibited increased TF mRNA levels and functional TF protein. Measurement of TF mRNA decay rates showed that BDV infection caused significant stabilization of the TF mRNA. This suggests that the increase in TF mRNA levels in infected cells results, at least in part, from mRNA stabilization. Finally, functional studies revealed that the TF promoter was three to six times more active in C6 infected cells than in uninfected cells. This transcriptional activation of TF in C6BV cells appears to be mediated by the three EGR-1 motifs present in the TF promoter. EGR-1 is a nuclear phosphoprotein that binds specifically to the DNA sequence 5′-GCGGGGGCG-3′ in a zinc-dependent manner (13). Our recent studies have shown that EGR-1 mediates serum induction of the TF gene in human epithelial cells (17). Interestingly, EGR-1 is strongly activated following acute BDV infection (29). EGR-1 site-dependent enhancement of TF gene expression is currently under investigation.

The results presented here show that TF gene expression in the brain is activated following persistent BDV infection of the astrocyte. Since this phenomenon occurs in the absence of hemorrhage, inflammation, or disruption of the blood-brain barrier, it raises interesting questions about the consequences of TF upregulation and suggests that TF may fulfill functions other than hemostasis in the CNS. Indeed, the regulation of TF expression suggests a more pleiotropic role for this protein besides being an initiator of blood coagulation (25). Architectural features of the TF molecule have classified it as a member of the class II cytokine receptor family (25), and a recent report has shown that TF might actually work as a signaling receptor by inducing intracellular calcium levels upon stimulation (44). Moreover, it has been suggested that TF plays a role in angiogenesis and in metastatis (8, 55).

There is increasing evidence that proteins of the coagulation and fibrinolysis systems may function in the CNS independent from blood clotting, such as in regulating normal brain development and defending the brain against damage caused by stroke, trauma, and other injuries (36). Any imbalance in the protease activities may contribute to neuronal damage. TF is the primary initiator of the thrombin activation system, a protease which has been implicated in several neurodegenerative diseases, such as Alzheimer's disease (36, 38). Our results suggest that upregulation of TF expression due to BDV infection may play an important role in the CNS response to this viral assault and perhaps also in the BDV-mediated disturbances of CNS function.

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