The Mechanism of Actinomycin D-Mediated Increase of Borna Disease Virus (BDV) RNA in Cells Persistently Infected by BDV

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Abstract: The transcriptional mechanism of Borna disease virus (BDV) has been poorly understood. We have analyzed transcription of the virus upon various stimuli in Madin-Darby canine kidney cells which were persistently infected by BDV (MDCK/BDV). Treatment with actinomycin D (ActD) increased the level of BDV RNA, shifting the size of RNA from 1.9 kb to 2.3 kb beginning 5 hr after the treatment. To understand the mechanism of this unique modulation of BDV RNA, we conducted several experiments. The RNA increase occurred at the stage in which synthesis of cellular intrinsic mRNA was intact, suggesting BDV does not compete with cellular transcriptional machinery for intrinsic RNA polymerase II. The BDV transcription was also enhanced by cycloheximide treatment, indicating that newly synthesized viral or cellular proteins are not necessary for viral transcription. However, a shift in the RNA size was not observed for cycloheximide-induced BDV RNA. The increase in viral transcription persisted during the cellular apoptotic process consequent to p53 gene accumulation beginning 1 hr after ActD treatment. Caspase inhibitors Z-VAD and DEVD-CHO repressed the apoptotic process but failed to block the increase in BDV transcription. In addition, adenovirus-mediated transduction of wild-type p53 did not alter the BDV transcription, indicating that the increase in BDV transcription was independent of the p53-mediated apoptotic process. Other various stimuli that evoke cellular signal transductions failed to alter BDV transcription. Agents inhibitory to topoisomerase except adriamycin failed to enhance BDV transcription, indicating that the increase in BDV transcription is not mediated by an inhibitory action to the topoisomerase II of ActD. Adriamycin showed an increase and size-shift of BDV RNA similar to ActD. These results suggest that intercalation of the viral genome itself with ActD is related to the stabilization of viral RNA and alteration of RNA size rather than secondary host cell changes.

Key words: Borna disease virus, Actinomycin D

Borna disease virus (BDV) contains a nonsegmented, negative-sense, 8.9 kilobase (kb), single-stranded RNA genome having at least six open reading frames encoding proteins of 40 (p40), 23 (p24), 10 (p10), 16 (gpl8), 57 (G) and 190 kDa (pol) (3, 5, 6, 26, 29, 30, 38, 39). Borna disease horses has been known as a chronic encephalomyelitis endemic to Germany and several other European countries (12). Natural infection of BDV has been reported in other vertebrates such as sheep, cattle, cats, rabbits and ostriches (26). Furthermore, accumulated epidemiological data suggest the association of BDV with certain psychiatric disorders in humans (2, 7, 27), raising concerns that it is a potential zoonosis. BDV multiplies at very low titers and hence causes no cytopathic effects in vitro (19, 20), which is the main reason for the chronic

Abbreviations: ActD, actinomycin D; BDV, Borna disease virus; CPT, camptothecin; DIG, digoxigenin; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; HIV, human immunodeficiency virus; hnRNP, heterogeneous nuclear ribonucleoprotein; MDCK, Madin-Darby canine kidney; MDCK/BDV, MDCK cell line persistently infected by BDV; TNF-α, tumor necrosis factor-alpha.
disease courses. However, precise mechanisms, through which viral transcription is maintained at a low level, remain unknown. We have recently demonstrated that the BDV RNA increases in virus-infected cells which are treated with actinomycin D (ActD) (22). ActD is a widely used anti-neoplastic agent that specifically inhibits RNA synthesis from DNA (24, 25). It is known to interact with single- and double-stranded DNA (25, 37) and RNA-DNA hybrids (33). ActD is known to inhibit the replication of some but not all RNA viruses; e.g., the growth of feline enteric coronavirus is inhibited by ActD, while the antigenically related feline infectious peritonitis virus is unaffected (18). ActD inhibits the reverse transcription of human immunodeficiency virus type 1 (HIV) through binding to the single-stranded DNA (16). Furthermore, ActD inhibits influenza virus replication not only by inhibition of transport of viral RNA from the cytoplasm to the nucleus (36), but most importantly through the inhibition of cellular transcription by RNA polymerase II, with the resultant depletion of the cap structure (17). To better understand the mechanism relevant to this unique up-modulation of BDV transcription, we undertook the present investigation.

Materials and Methods

Cells, virus and reagents. Madin-Darby canine kidney (MDCK) cell line and a cell line persistently infected by BDV (MDCK/BDV) (14) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). The reagents used in this study are described in the figure legends.

RNA extraction. In most experiments, MDCK/BDV cells (1 X 10^5) were cultured in 25 cm² flasks (Corning, U.S.A.). After treatment of MDCK/BDV cells with ActD or other reagents, total RNAs derived from MDCK and MDCK/BDV cells were prepared using ISOGEN (Nippon Gene, Japan) as described previously (22).

Probes for Northern blot analysis. For detecting RNAs containing the p40 and p24 region of BDV, the RNA probes were synthesized using T3 and T7 RNA transcriptase systems with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) as described previously (21, 22). In this paper, we synthesized probes for detecting both genomic strand and anti-genomic strand RNA of the p24 region, and for detecting the anti-genomic strand RNA of the p40 region.

Expression of p53 RNA and protein using adenovirus. To simulate p53 accumulation in the absence of ActD, adenoviral-mediated p53 gene transfer into the cells was used (11). MDCK/BDV cells were infected with Ad-p53 at a 100 multiplicity of infection (m.o.i.) which was measured using 293 cells. The Ad-p53 induced cell death after 48 hr post-infection (p.i.), while the control vector expressing luciferase gene (Ad-Luc) at the same m.o.i did not.

Northern blot analysis. Aliquots of 5 µg of total RNA were fractionated in 1% agarose gel containing formaldehyde and transferred to nylon membranes, Hybond N+ (Amersham, U.K.). The 28S and 18S ribosomal RNAs indicated standardization of the amount of total RNA among lanes. The prehybridized membranes were then hybridized with RNA probes. Signals were detected by the DIG luminescent detection kit (Boehringer Mannheim, Germany) according to the manufacturer's instructions.

Western blot analysis. SDS-PAGE was performed under reducing conditions with 5% stacking gels and 12% separating gels. After electrophoresis, proteins were electrophoretically transferred to PVDF membrane (Millipore, U.S.A.). The membrane was blocked with 3% BSA in PBS for 60 min and then incubated with MAb for 60 min (35). After washing with PBS containing Tween 20, the membrane was incubated with horseradish peroxidase-conjugated goat-anti mouse IgG for 60 min and washed again. The membrane was incubated with a substrate solution (0.25 mg/ml 4-chloro-l-naphthol, 17% methanol, 0.2% H₂O₂ in PBS).

Results and Discussion

Increase and Band Shifting of BDV RNA by ActD
We used Madin-Darby canine kidney cells persistently infected with BDV (MDCK/BDV) (14) to analyze the effect of ActD treatment (2 µg/ml) on BDV transcription. RNA was extracted from cells and used for Northern blot analyses using digoxigenin (DIG)-labelled probes for p24 BDV mRNA and genomic RNA (Fig. 1). In association with the increase of BDV transcript, the

![Fig. 1. Genomic organization of BDV. Transcription map indicating relation among RNA probes and their target RNA for Northern blot analysis. The S and T indicate start site and termination site of transcription, respectively.](image-url)
band shifted from the size of 1.9 kb, which is capped neither at the 5' end nor polyadenylated at the 3' end (28), to 2.3 kb 5 hr after the ActD treatment (Fig. 2C). BDV-specific mRNAs also seemed to be synthesized at the region between 0.8 to 3.5 kb as smear bands, while genomic RNA slightly increased from 5 to 15 hr (Fig. 2B). Since transcription of BDV virus takes place in the nucleolus (23), at least five different mechanisms of ActD action on BDV transcription were conceivable: (1) recruitment of cellular RNA polymerases and/or their co-factors for

![Fig. 2](image1.png)

Fig. 2. Effect of ActD on viral transcription in MDCK cells persistently infected by BDV. MDCK/BDV cells were exposed to 2 µg/ml of ActD (Sigma, U.S.A.) and cellular RNA was extracted at 0, 5, 10 and 15 hr after treatment. Aliquots of 5 µg (A, B) of total RNA were fractionated on 1% agarose gel and transferred to a nylon membrane (Hybond N+; Amersham, U.K.). The prehybridized membrane was then hybridized with an RNA probe for detection of BDV p24 sense RNA (A, C) and with an RNA probe for detecting BDV-genomic RNA (B). An X-ray film was exposed to the membrane filter for approximately 30 min. (C) Total RNA (0.5 µg) derived from ActD-treated (5 hr) MDCK/BDV cells was fractionated. Lane N, ActD non-treated MDCK/BDV cells; Lane M, total RNA from MDCK cells as a negative control. The 28S and 18S ribosomal RNAs of cells are indicated.

![Fig. 3](image2.png)

Fig. 3. Host cell factors or newly synthesized viral protein are not necessary for the enhancement of BDV transcription. (A) MDCK/BDV cells were treated with ActD (2 µg/ml) and cellular RNA was extracted at 0, 5, 10 and 15 hr after treatment. Northern blot analysis was performed using a β-actin RNA detecting probe which was transcribed and labelled with DIG-11-dUTP from a β-actin-mouse antisense control template (Nippon Gene, Japan) using T7 RNA polymerase. An X-ray film was exposed to the membrane filter for about 1 hr. (B) Northern blot analysis using a BDV p40 RNA detecting probe. Lane N, ActD non-treated MDCK/BDV cells; Lane M, total RNA from MDCK cells as a negative control. The 28S and 18S ribosomal RNAs of cells are indicated.
viral RNA synthesis through the inhibition of cellular RNA synthesis, (2) contribution of the redox mechanism and caspase cascade by ActD-induced cellular apoptotic process to BDV transcription, (3) direct effect of p53 protein on viral enhancer or indirect effect of secondary cellular proteins induced by p53-mediated transcriptional activation, (4) inhibition of cellular topoisomerase activity by ActD which may suppress BDV transcription, and (5) direct action of ActD on viral ribonucleoprotein complex or RNA. We tested these hypothetical mechanisms by conducting the following experiments.

**Effect of Cellular RNA Polymerase II on Up-Regulation of BDV Transcription by ActD Treatment**

First, we examined the effect of ActD on cellular mRNA synthesis. As shown in Fig. 3A, the amount of β-actin mRNA in ActD-treated cells did not change until 10 hr after treatment and thereby decreased only slightly at 15 hr, whereas the increase in BDV transcription began at least 5 hr (Fig. 2A), denying the possibility of the recruitment of cellular RNA polymerases. Pyper et al reported that cellular RNA polymerase I did not affect BDV transcription (23), and the present results suggest that cellular RNA polymerase II does not either.

**Effect of Newly Synthesized BDV Protein on Up-Regulation of BDV Transcription**

To examine whether newly synthesized viral proteins are needed for viral mRNA synthesis, p40 mRNA and protein were analyzed by Northern and Western blot analyses. As shown in Fig. 3B, p40 mRNA synthesis increased from 5 to 15 hr after ActD treatment, while the amount of p40 protein remained in a steady state. Furthermore, a 5 hr-treatment with cycloheximide increased BDV transcription (Fig. 3C). Taken together, these results strongly indicate that ActD-induced BDV transcription does not depend on a newly synthesized viral or cellular protein. It should be noted that the band shift from 1.9 kb to 2.3 kb was not observed in the BDV transcript enhanced by cycloheximide treatment (Fig. 3C), indicating the presence of distinct mechanisms for ActD- and cycloheximide-induced BDV transcription.

**Effect of Apoptotic Events on Up-Regulation of BDV Transcription**

Both ActD and cycloheximide caused DNA fragmentation in MDCK/BDV cells as assessed by DNA-ladder formation (data not shown). We thus examined the possibility of an enhancement in BDV transcription in association with the cellular apoptotic process. A number of cellular proteins are known to migrate into the nucleus during apoptosis, including poly (ADP-ribose) polymerase, DNA-dependent protein kinase, lamin, nucleolin, actin, heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and C1/C2 (4). The cells were treated with tumor necrosis factor (TNF-α) to induce apoptosis. In spite of the apoptosis induced by TNF-α as assessed by microscopical examination, DNA laddering (data not shown) and degradation of 28S ribosomal RNA, BDV transcription was not affected (Fig. 4A). Moreover, viral RNA synthesis was not inhibited by either cas-

![Fig. 4. Apoptotic process does not affect BDV transcription. (A) MDCK/BDV cells were exposed to a recombinant human TNF-α (Pepro Technology Inc., U.S.A.) for 5 (100 ng/ml) and 24 (10 ng/ml) hr, and Northern blot analysis was performed using a BDV p24 RNA detecting probe. An X-ray film was exposed to the membrane filter for approximately 30 min (24 hr treatment with TNF-α) and 1 hr (5 hr treatment). (B) Z-VAD-CH2-DCB (Phoenix Pharmaceuticals, Inc., U.S.A.) and DEVD-CHO (Biomol Research Laboratories, Inc., U.S.A.), 100 mm each, was added to MDCK/BDV cells for 1 hr before treatment with ActD (2 μg/ml) for 5 hr. Northern blot analysis was performed using a BDV p24 RNA detecting probe. Lane N, ActD non-treated MDCK/BDV cells. The 28S and 18S ribosomal RNAs of cells are indicated.](https://example.com/fig4.png)
pase inhibitor, DEVD-CHO or Z-VDAD-CH2-DCB (Fig. 4B), indicating that at least the final common pathway of cellular apoptosis is not related to ActD-induced BDV transcription.

Effect of p53 Accumulation on BDV Transcription

The production of a 2.3-kb band of BDV RNA in place of a 1.9-kb band occurred at an early stage after ActD treatment; it began at 1 hr and increased at 2 hr after treatment (data not shown). Therefore, early cellular events might be important for viral transcription. Because the accumulation of tumor suppressor protein p53, which does not need de novo protein synthesis but post-translational stabilization by phosphorylation (1, 31, 32), was detected at 1 hr following ActD treatment (Fig. 5A), we next tested whether the p53 protein has an enhancing effect on viral transcription. It is known that viral promoters of human papillomavirus (8) and HIV (10) are modulated by p53. To simulate p53 accumulation in the absence of ActD, we used adenoviral-mediated p53 gene transfer into the cells (11). As shown in Fig. 5B, although there was high expression of p53 protein by Ad-p53, there was no obvious change in the amount and size of the BDV transcript. Further, to examine whether p53 facilitates the ActD- or cycloheximide-induced transcriptional enhancement of BDV, 24 hr p.i. Ad-p53 cells were treated with ActD or cycloheximide for 3 or 5 hr, respectively. However, there was no surplus increase in viral transcription observed for either ActD or cycloheximide. These results indicate that the p53 is not an important factor for the enhancement of viral transcription.

Effect of Topoisomerase Inhibitors on BDV Transcription

ActD is known to have another activity as an inhibitor of topoisomerase II. Topoisomerase IIa increases during DNA synthesis (S to G2 phases) and returns to the basal level at the G1 phase of the cell cycle (13). Because BDV mRNA increases in growth-arrested MDCK/BDV cells as compared to growing cells (22), the activity of topoisomerase IIa might have an inhibitory effect on BDV transcription. We therefore examined the effects of topoisomerase inhibition on viral transcription. MDCK/BDV cells were treated with the topoisomerase II inhibitors adriamycin, etoposide (VP16) and ICRF-193 as well as the topoisomerase I inhibitor camptothecin (CPT) for 24 hr. As shown in Fig. 6A and B, adriamycin strongly enhanced BDV transcription and caused a band shift to 2.3 kb, while VP16, ICRF-193 and CPT

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**Fig. 5.** Tumor suppressor protein p53 does not affect BDV transcription. (A) MDCK/BDV cells were treated with ActD (2 µg/ml) and Western blot analysis was performed using an anti-human p53 antibody (Transduction Lab., U.S.A.). (B) MDCK/BDV cells were infected with 100 m.o.i. of each Ad-p53 and Ad-Luc. After 24 hr p.i., Northern and Western blot analyses were performed using a p24 RNA detecting probe and anti-p53 antibody, respectively. (C) After transduction of Ad-p53 and Ad-Luc at 100 m.o.i. to MDCK/BDV cells for 24 hr, ActD (2 µg/ml) and cycloheximide (100 µg/ml) were treated for 3 and 5 hr, respectively. Northern blot analysis was performed using a p24 RNA detecting probe. Lane N, ActD non-treated MDCK/BDV cells. The 28S and 18S ribosomal RNAs of cells are indicated.

**Fig. 6.** Topoisomerase inhibitors, with the exception of adriamycin, do not affect BDV transcription. (A) MDCK/BDV cells were treated with adriamycin (5 µM; Sigma, U.S.A.), VP16 (1 µM; Topo Gen, Inc., U.S.A.), CPT (1 µM; Topo Gen, Inc., U.S.A.) and ICRF-193 (1 µM; Funakoshi, Japan) for 24 hr. Northern blot analysis was performed using a BDV p24 RNA detecting probe. (B) An X-ray film was exposed to the membrane filter used (A) for half time. Lane N, Ad-p53 + ActD; Lane M, total RNA from MDCK cells was the negative control. The 28S and 18S ribosomal RNAs of cells are indicated.
had no effect. The known difference of adriamycin and ActD from CPT, VP16 and CPT is that adriamycin and ActD form an intercalation of nucleic acids (9, 15, 34). Taken together, it is suggested that the increase in BDV RNA is attributable to the intercalating activity of the two agents rather than the inhibitory activity to topoisomerase II itself.

Effect of Other Reagents on BDV Transcription

To rule out other cellular events for BDV transcriptional activation, we tested the effects of various bioactive molecules that evoke cellular signal transductions, including hepatocyte growth factor, epidermal growth factor, transforming growth factor-β, 5-azacytidine, herbimycin A, genistein, pentoxifyline, retinoic acid, lipopolysaccharide, 12-O-tetradecanoyl-phorbol-13-acetate, dexamethasone, urea, caffeine, cytohalasin B and calcium ionophore. The molecules were acted on the cells for 5 or 24 hr and viral RNA was analyzed. However, we could not detect any effect on BDV transcription (data not shown).

In this paper, we found that the amount of BDV mRNAs including viral genome are increased early after ActD treatment. This finding could not be explained by the indirect effects of ActD, such as the inhibition of RNA polymerase II activity, induction of apoptosis, accumulation of p53 and topoisomerase activity. Therefore, we think the unique effect of ActD and adriamycin appears to be attributable to the specific effect on intercalation of nucleic acids. Vogel and Scholtissek speculated from their experiments using influenza virus that ActD stabilizes a bulky secondary structure of single-stranded RNA by intercalating, and this secondary structure of RNA might be retained in the nucleus (36). Since our result indicates that BDV protein is kept at a steady level regardless of the increasing amount of viral mRNAs in the presence of ActD, the newly synthesized BDV mRNA associated with ActD might not be able to cross the pores of the nuclear membrane and translated. The explanation may be that BDV mRNAs accumulate by a stabilizing effect of ActD.

We also found that the 1.9-kb RNA changed to 2.3 kb by ActD and adriamycin treatment. We previously reported that the transcription of 1.9-kb RNA was more abundant in growth-arrested cells in confluent culture than those growing rapidly (22), suggesting that molecules which are related to the G1 checkpoint have an enhancing activity on BDV transcription or that molecules which propel the cell cycle have inhibitory activity. However, because the size of the 1.9-kb RNA never changes in the confluent MDCK/BDV cells (22), this effect is distinct to that of ActD and adriamycin but might be similar to that of cycloheximide. Since the 2.3-kb RNA signals were obtained from Northern analysis using both p24 and p40 probes, the 2.3-kb RNA is thought to contain the same sequence as 1.9-kb RNA except for the additional 0.4 kb. Although we used different procedures that would identify the 3' end of the 2.3-kb RNA, these procedures failed to work in our lab (data not shown). An alternative approach is needed to identify the exact structure of the 2.3-kb RNA. Though merely speculative, it is considered that a specifically targeted intercalation on the viral genome might cause a read-through of transcript termination at the T1 site (Fig. 1) and stop the transcription at a novel termination site, and/or the 2.3-kb RNA might receive additional polyadenylation, resulting in the production of a larger RNA species.

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