Borna disease virus (BDV) is a non-segmented, negative-strand RNA virus that belongs to the order \textit{Mononegavirales} (Schneemann \textit{et al.}, 1995; Tomonaga \textit{et al.}, 2002). The BDV genome harbours six genes that encode the nucleoprotein (N), phosphoprotein (P), non-structural protein (X), matrix protein (M), glycoprotein (G) and polymerase protein (L) (Briese \textit{et al.}, 1994; Cubitt \textit{et al.}, 1994a). Among these, N, P and L form ribonucleoproteins (RNPs) with viral genome RNA and initiate viral transcription and replication (Schneider, 2005). Uniquely among animal-derived RNA viruses, BDV transcribes and replicates in the nucleus and establishes persistent infection without overt cellular damage (Briese \textit{et al.}, 1992; Cubitt \\& de la Torre, 1994; Herzog \\& Rott, 1980). In the infected nucleus, BDV forms inclusion bodies, termed viral speckles of transcripts (vSPOTs), which are associated with chromatin in the nucleus, the host factors involved in the maintenance of vSPOTs remain largely unknown. In this study, we identified X-linked RNA-binding motif protein (RBMX) as a nuclear factor interacting with BDV nucleoprotein. Interestingly, knockdown of RBMX led to disruption of the formation of vSPOTs and reduced both transcription and replication of BDV. Our results indicate that RBMX is involved in the maintenance of the structure of the virus factory in the nucleus.

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At present, only a host factor, high mobility group box protein 1 (HMGB1), has been identified as being localized at vSPOTs (Kamitani \textit{et al.}, 2001). HMGB1 is known to be a multifunctional nuclear protein widely involved in the chromosomal maintenance of host cells and has been shown to be required for the stabilization of BDV RNPs on chromosomes, as well as efficient BDV RNA transcription in the nucleus (Matsumoto \textit{et al.}, 2012). HMGB1 is not, however, required for the generation of vSPOTs. Therefore, it is still unknown how the structure of vSPOTs is maintained in the nucleus and whether or not BDV replication is involved. In a previous study (Matsumoto \textit{et al.}, 2012), we demonstrated that N is an essential component of RNPs and is more stable on the chromatin than P, suggesting that N-bound host factors may be involved in the association of vSPOTs with chromosomes. In this study, therefore, we tried to identify the BDV N-bound factors affecting vSPOT formation, using the nuclear insoluble fraction of infected cells. We found that the X-linked RNA-binding motif protein (RBMX/hnRNP-G), which is known to be a cohesion regulator...
maintaining the proper cohesion of sister chromatids, is associated with the structural maintenance of vSPOTs. Our results demonstrate a host factor-dependent maintenance of the virus factory of BDV in the nucleus.

Proteins in the nucleus have different resistances to biochemical treatments (Hirai et al., 2013). When BDV-infected OL cells (Nakamura et al., 2000) were treated with CSK buffer [10 mM PIPES-NaOH (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 1:100 Protease Inhibitor Cocktail (Nacalai tesque) and 0.5 % Triton X-100], BDV N and P were still present in vSPOTs in the nucleus (Fig. 1a). Because CSK buffer removes cytosolic and loosely bound nuclear proteins, this suggested that vSPOTs exist as nuclear structures tightly bound to the nucleus. To determine the host nuclear factors that affect the structure of vSPOTs, we prepared the detergent-resistant nuclear fraction of BDV-infected OL cells, and immunoprecipitated with antibody–bead complexes prepared by mixing anti-N antibody and Dynabead Protein G (Thermo Fisher Scientific). The N-bound proteins were identified by mass spectrometry as described by Hirai et al. (2013).

In this study, we focused on the host factors that associate with host chromosomes, because our previous study demonstrated that the formation of vSPOTs, as well as the dynamics of viral RNPs, appeared to be associated with the structural dynamics of chromosomes, such as mitosis (Heinrich et al., 2009; Matsumoto et al., 2012). Among the candidate host factors (Table S1, available in the online Supplementary Material), we focused on a nuclear protein, RBMX, because only this protein has been shown to be associated with chromosomal structure and to form nuclear foci (Heinrich et al., 2009). RBMX is an RNA-binding protein that participates in the splicing process and plays a role in alternative splicing (Heinrich et al., 2009). Furthermore, this protein is known to be essential for the maintenance of proper sister chromatid cohesion (Matsunaga et al., 2012). N was co-immunoprecipitated with FLAG-tagged RBMX in BDV-infected cells using an anti-FLAG antibody (M2; Sigma-Aldrich) (Fig. 1b). We could not show a direct interaction between RBMX and BDV N, but we verified that RBMX associates with BDV RNPs in the infected cells.

We observed subcellular localization of endogenous RBMX in uninfected and BDV-infected cells. Uninfected or BDV-infected OL cells cultured on coverslips were fixed with 4 % paraformaldehyde for 10 min and then incubated with anti-RBMX (Abcam)/anti-N (Kobayashi et al., 2003; Watanabe et al., 2000) antibodies as primary antibodies. After washing, the cells were incubated with secondary antibodies (Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 555-conjugated goat anti-mouse IgG) and DAPI. Microscopic observation was carried out at room temperature using a confocal laser-scanning microscope (A1; Nikon). As shown in Fig. 1(c), BDV infection did not affect the subcellular localization of RBMX. RBMX seemed to exist adjacent to vSPOTs in the infected cells but did not co-localize with them (Fig. 1c, d). This suggested that, despite the interaction with N, RBMX does not enter the viral factories.

We next investigated the structural relationship between RBMX and vSPOTs. To this end, we knocked down RBMX in the infected cells using small interfering RNA (siRNA) (Qiagen) and observed the subcellular localization of N and P. Interestingly, when the expression level of RBMX was reduced (Fig. 2a), the formation of vSPOTs seemed to be disrupted and became indeterminate (Fig. 2b). Furthermore, interestingly, the signals of N and P appeared to be dispersed to heterochromatin regions stained by DAPI, including the regions surrounding the nucleolus (Fig. 2c, arrowheads). Consistent with this observation, N in the knockdown cells was observed in association with the heterochromatin stained by anti-H3K9me3 antibody (Fig. 2d arrowheads). These data suggested that RBMX affects the structural formation of vSPOTs, resulting in translocation of the viral RNPs to heterochromatin regions from the vSPOTs.

To investigate whether disruption of the formation of vSPOTs is linked to BDV replication, we measured the level of BDV RNA in BDV-infected, RBMX-knockdown cells. Total RNA was extracted from RBMX-knockdown or control siRNA-treated BDV-infected cells and reverse transcribed with a Verso cDNA Synthesis kit (Thermo Scientific) using a BDV-specific primer or an anchored oligo(dT) primer. Quantitative real-time PCR was performed using a TaqMan PCR Assay and a SYBR-Green PCR Assay (Toyobo), and products were detected with a Rotor-Gene Q System (Qiagen). The Taqman probe was labelled with 6-carboxyfluorescein (FAM) as the 5′ fluorescent reporter and tetramethylrhodamine (TAMRA) as the 3′ quencher. The primers and probe used in this study are shown in Table S2. When the expression level of RBMX was decreased, both transcription and replication of BDV were significantly decreased (Fig. 3), suggesting that RBMX positively regulates the transcription and replication of BDV in the nucleus.

Our results demonstrate that RBMX is a nuclear factor that is involved in the structural formation of vSPOTs in the nucleus. A recent study demonstrated that RBMX is a nuclear factor contributing to the maintenance of the proper cohesion of sister chromatids (Matsunaga et al., 2012). These authors also showed that RBMX associates with chromatin in the interphase cells. On the other hand, we reported that BDV uses the chromatin structure as a scaffold for assembly of vSPOTs in the interphase nuclei (Matsumoto et al., 2012). We also found that the viral RNPs were dispersed from vSPOTs and distributed to the surfaces of the condensed chromosomes in mitosis (Matsumoto et al., 2012). These observations suggested that a proper chromatin structure would be necessary for the maintenance of vSPOT formation in the nucleus. Therefore, it is conceivable that the change of chromosome structure by the depletion of RBMX leads to the collapse of vSPOTs, even in interphase nuclei.
Alternatively, RBMX may maintain the structure of vSPOTs through regulation of the mRNA splicing of BDV transcripts. RBMX is also known to be involved in the control of pre-mRNA splicing (Hofmann & Wirth, 2002; Nasim et al., 2003; Wang et al., 2011). Indeed, the transcription units encoding the M, G and L genes of BDV are spliced to produce mature mRNAs (Cubitt et al., 1994b; Schneider et al., 1994; Tomonaga et al., 1995).
Fig. 2. RBMX maintains the structure of vSPOTs. (a) BDV-infected OL cells were transfected with siRNA against RBMX and harvested transfected cells were subjected to SDS-PAGE, followed by Western blotting by using each specific antibody. (b–d) siRNA-transfected cells were subjected to immunofluorescence staining using anti-P and anti-N antibodies (b, c), or anti-H3K9me3 and anti-N antibodies (d). Specific signals were observed by confocal microscopy. The inset areas in the merged panel in (b) are enlarged in (c). Bars, 5 μm (b, d); 1 μm (c).
2000). If RBMX participates in the splicing of BDV mRNAs, accumulation of abnormal splicing products may suppress transcription and replication of BDV in the vSPOTs. This in turn would lead to disruption of the structure of vSPOTs.

Note that the knockdown RBMX did not entirely prevent vSPOT formation and still allowed virus replication. This indicates the possibility that other nuclear factors also contribute to the formation of vSPOTs and the replication of BDV in infected nuclei.

In this study, we found a host factor associated with viral factory formation in the nucleus. Further studies are needed to elucidate the role of RBMX for the maintenance of the structure of vSPOTs in the infected nuclei. Although the function of RBMX in the nucleus has not yet been fully elucidated, our study provides a probe for understanding the fundamental questions about replication of RNA viruses in the nucleus, such as persistent infection and viral factory formation.

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**Fig. 3.** RBMX positively regulates the transcription and replication of BDV. The amounts of transcripts (a) or complementary RNA of genomic RNA (b) of BDV were measured by quantitative real-time PCR using the comparative Ct method. The specific signal intensity was normalized by the amount of glyceraldehyde 3-phosphate dehydrogenase mRNA. For statistical analysis, Student’s t-test was used. *P<0.01. Error bars indicate SD (n=3) from three independent experiments.


