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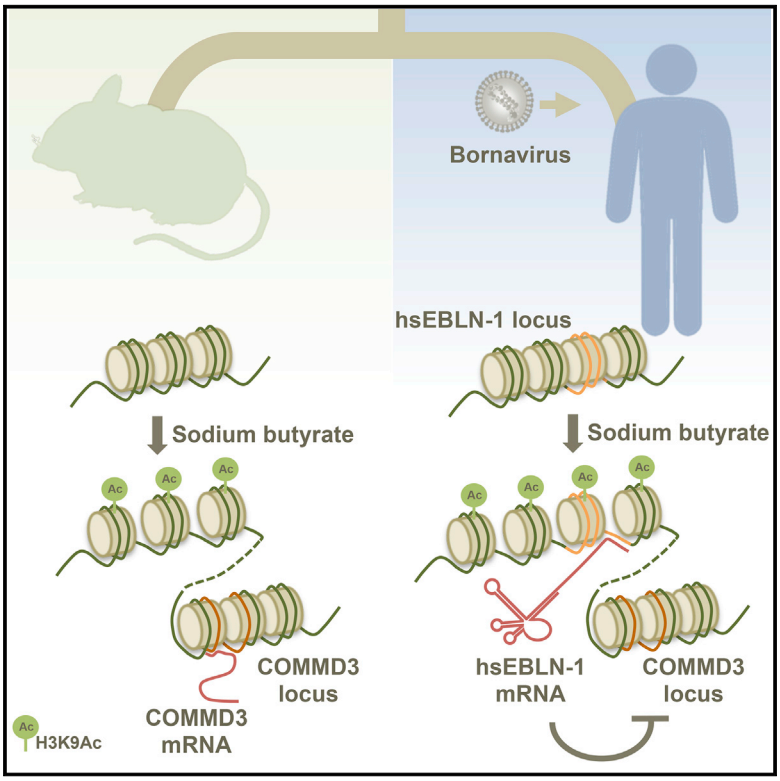
Transcription Profiling Demonstrates Epigenetic Control of Non-retroviral RNA Virus-Derived Elements in the Human Genome

Graphical Abstract



Highlights

- Seven *H. sapiens* endogenous bornavirus-like nucleoprotein elements are expressed as RNA in at least one tissue
- Expression of some hsEBLNs, for example, hsEBLN-1, is tissue-specific
- Expression from the hsEBLN-1 locus is epigenetically silenced in most tissues
- Integration of hsEBLN-1 in the genome affects the expression of a neighboring gene

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In Brief

DNA sequences derived from ancient bornaviruses (endogenous bornavirus-like nucleoprotein elements, EBLNs) are present in vertebrate genomes. Sofuku et al. demonstrate that *Homo sapiens* EBLN-1 (hsEBLN-1) is repressed epigenetically in many tissues. Expression of hsEBLN-1 in a limited, tissue-specific manner suggests a role in regulating neighboring gene expression and genome function.

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Transcription Profiling Demonstrates Epigenetic Control of Non-retroviral RNA Virus-Derived Elements in the Human Genome

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SUMMARY

Endogenous bornavirus-like nucleoprotein elements (EBLNs) are DNA sequences in vertebrate genomes formed by the retrotransposon-mediated integration of ancient bornavirus sequence. Thus, EBLNs evidence a mechanism of retrotransposon-mediated RNA-to-DNA information flow from environment to animals. Although EBLNs are non-transposable, they share some features with retrotransposons. Here, to test whether hosts control the expression of EBLNs similarly to retrotransposons, we profiled the transcription of all *Homo sapiens* EBLNs (hsEBLN-1 to hsEBLN-7). We could detect transcription of all hsEBLNs in at least one tissue. Among them, hsEBLN-1 is transcribed almost exclusively in the testis. In most tissues, expression from the hsEBLN-1 locus is silenced epigenetically. Finally, we showed the possibility that hsEBLN-1 integration at this locus affects the expression of a neighboring gene. Our results suggest that hosts regulate the expression of endogenous non-retroviral virus elements similarly to how they regulate the expression of retrotransposons, possibly contributing to new transcripts and regulatory complexity to the human genome.

INTRODUCTION

Recently, we and others discovered that numerous vertebrate genomes contain endogenous sequences related to non-reverse-transcribing RNA and DNA viruses, not only retroviruses. Bornavirus, a nonsegmented negative-strand RNA virus, is unique because it is the only non-retroviral RNA virus with endogenous elements in the human genome (Horie et al., 2010). The majority of endogenous fragments of bornavirus in human genomes appear to have originated from reverse transcription and integration of nucleoprotein (N) mRNA of ancient bornavirus through long interspersed nuclear element-1 (LINE-1 or L1) activity. They are thus a unique form of processed pseudogenes and evidence a mechanism of retrotransposon-mediated RNA-to-DNA information flow from virus to host. We call these sequences endogenous bornavirus-like nucleoproteins (EBLNs) (Horie et al., 2010). A number of EBLNs are identified in the genomes of various mammalian species, including primates, rodents, and afrotherians (Horie et al., 2010). Whereas we have recently shown that an EBLN in the ground squirrel genome can inhibit Borna disease virus (BDV) replication (Fujino et al., 2014), it is unknown whether EBLNs have any biological significance in humans. At least seven independently integrated EBLNs are present in the human genome (Table S1). The motifs resembling the start signal motifs (SSMs) of BDV transcription were well conserved in flanking sequences of *Homo sapiens* EBLNs (hsEBLNs) except for hsEBLN-7, and hsEBLNs contain the target site duplications (TSDs) and poly A tracts, hallmarks of L1 retrotransposition process (Figure 1A). In one case the N mRNA appears to have been integrated as a chimera with a short interspersed nuclear element (SINE) (Horie et al., 2010). While EBLNs are not thought to be transposable themselves, they may share features with transposing elements on account of their origin via retrotransposition.

Transposable elements, such as L1 and endogenous retroviruses (ERVs), induce genomic changes through several mechanisms. Retrotransposition of LINEs themselves occurs in as many as one in eight human births and has been implicated in human disease (Kazazian, 1999). In addition, retrotransposons provide the molecular machinery for retroduplication of host genes, generating processed pseudogenes and new fusion genes (Esnault et al., 2000; Sayah et al., 2004). ERVs create new transcriptional landscapes by promoting transcription of protein-coding genes proximal to their insertion site (Whitelaw and Martin, 2001).

Retrotransposition mediated by these elements can have deleterious effects on the hosts, which have evolved redundant strategies that constrain retrotransposition. For example, in response to retrotransposon overexpression, host cells activate programmed cell death pathways (Chuma, 2014; Noutsopoulos et al., 2010). Should retrotransposon-encoded proteins become translated, many proteins can interact with them to inhibit their function (Goodier et al., 2013). However, at the front line of defense, epigenetic modification of transposon loci limits their transcription.

DNA methylation plays a major role in ERV silencing (Bourc'his and Bestor, 2004; Walsh et al., 1998; Yoder et al., 1997). Methylation of histone H3 lysine 9 (H3K9) is also required for ERV

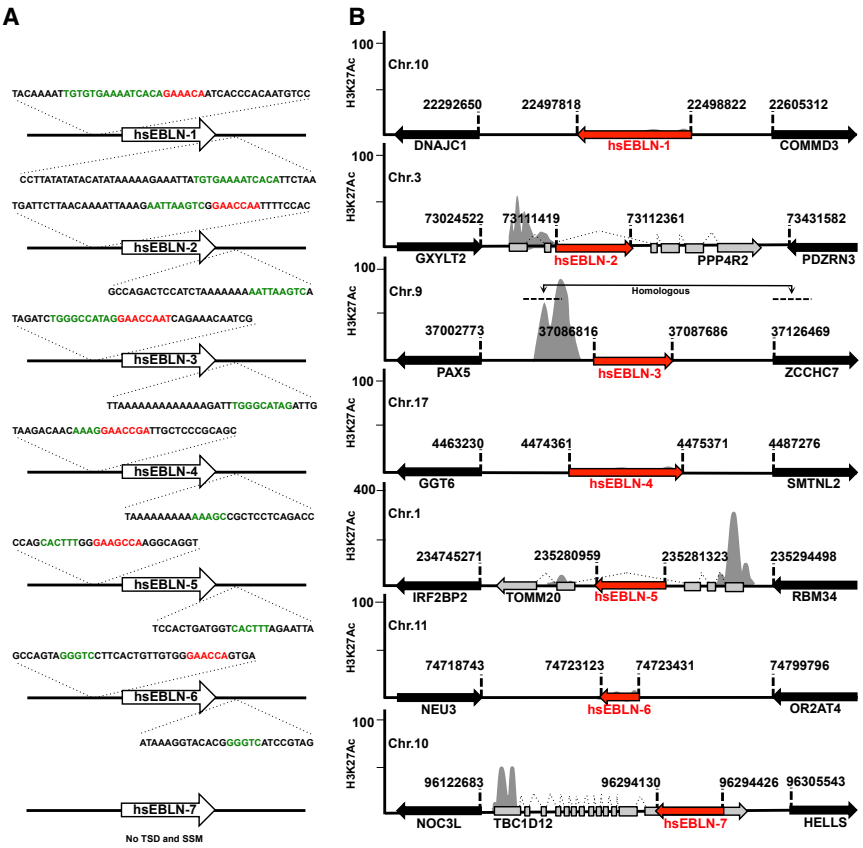

Figure 1. hsEBLN Loci in the Human Genome

(A) The SSM-like motif (red) and the TSD (green) around hsEBLN loci.

(B) The numbering corresponds to nucleotide positions in human genome (UCSC as of November 15, 2013). Red arrows indicate the region of homology with BDV N protein as annotated in Table S1. Black and gray arrows indicate neighboring genes around hsEBLNs. The enrichment of H3K27ac histone marks around hsEBLNs is plotted on the y axis in a.u.

silencing when DNA methylation levels are insufficient for ERV repression (Dong et al., 2008; Leung and Lorincz, 2012; Matsui et al., 2010). As for ERVs, DNA methylation and H3K9 methylation are critical for L1 silencing (Castro-Diaz et al., 2014; Di Giacomo et al., 2013; Ishizu et al., 2012; Tachibana et al., 2007). Furthermore, L1 retrotransposition is controlled by P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) in concert with the DNA methylation machinery (Bourc'his and Bestor, 2004; Kuramochi-Miyagawa et al., 2008) and endogenous RNAi in mouse (Tam et al., 2008; Watanabe et al., 2008) and human cells (Yang and Kazazian, 2006).

We hypothesized that expression of hsEBLNs might be epigenetically controlled by the host similarly to retrotransposons, even though they are not autonomously transposable. Here, to test this possibility, we profiled the transcription pattern of EBLNs in human tissues. We detected transcription of all seven hsEBLNs in at least one tissue examined. hsEBLN-1 was expressed in a testis-specific manner, and its expression was restricted by epigenetic modifications, reminiscent of retrotransposons.

RESULTS

Expression Profiles of hsEBLN RNAs

Figure 1B shows each hsEBLN within its genomic context, including nearest protein-coding genes. Although transcription from some hsEBLN loci has been predicted bioinformatically (Belyi et al., 2010), transcription of hsEBLN loci has never been systematically investigated experimentally. To do so, we first determined the expression level of hsEBLN RNAs in various human tissues (Figure 2). From these expression profiles, we noticed that hsEBLN loci could be categorized into two groups: the loci whose transcripts are expressed ubiquitously (UE loci; hsEBLN-2, hsEBLN-3, hsEBLN-5, and hsEBLN-7) and the loci whose transcripts are expressed mainly in testis (TS loci; hsEBLN-1, hsEBLN-4, and hsEBLN-6). Since hsEBLNs are a unique form of processed pseudogenes produced by L1, it is hypothesized that hsEBLNs themselves do not encode promoter sequences. We therefore searched putative nearby promoters that might drive hsEBLN transcription. UE loci were accompanied by upstream enrichment of histone H3 lysine 27 acetylation (H3K27ac), an active histone mark, and transcription factor binding in various cells at University of California, Santa Cruz (UCSC) Genome Browser (Figure 1B). The hsEBLN-2 and hsEBLN-5 loci are located in an intron of the *PPP4R2* and *TOMM20* genes, respectively. The hsEBLN-7 locus is located in an exon of the *TBC1D12* gene. The upstream sequence (chr9: 37079001–37086816) of the hsEBLN-3 locus, where H3K27ac was identified within various cell types, was highly homologous (92.2% identity) to the upstream region (chr9: 37119617–37129512) of the *ZCCHC7* gene, a neighboring gene of hsEBLN-3, where H3K27ac enrichment was also identified (Figure 1B). On the other hand, we detected putative promoter activity upstream of the TS hsEBLN-1 locus, where transcription factor binding, but not H3K27ac enrichment, was detected in UCSC Genome Browser, suggesting hsEBLN-1 may contain a putative promoter sequence with a suboptimal activity (Figure S1). Transcripts from all seven hsEBLN loci were detected in human testis, consistent with the report of elevated expression of processed pseudogenes in testis (Figure 2H) (Marques et al., 2005). Taken together, all hsEBLN loci have the potential to be transcribed because of a "borrowed" promoter sequence, as is the case with some other processed pseudogenes (Bradley et al., 2004; McCarrey and Thomas, 1987; Mighell et al., 2000).

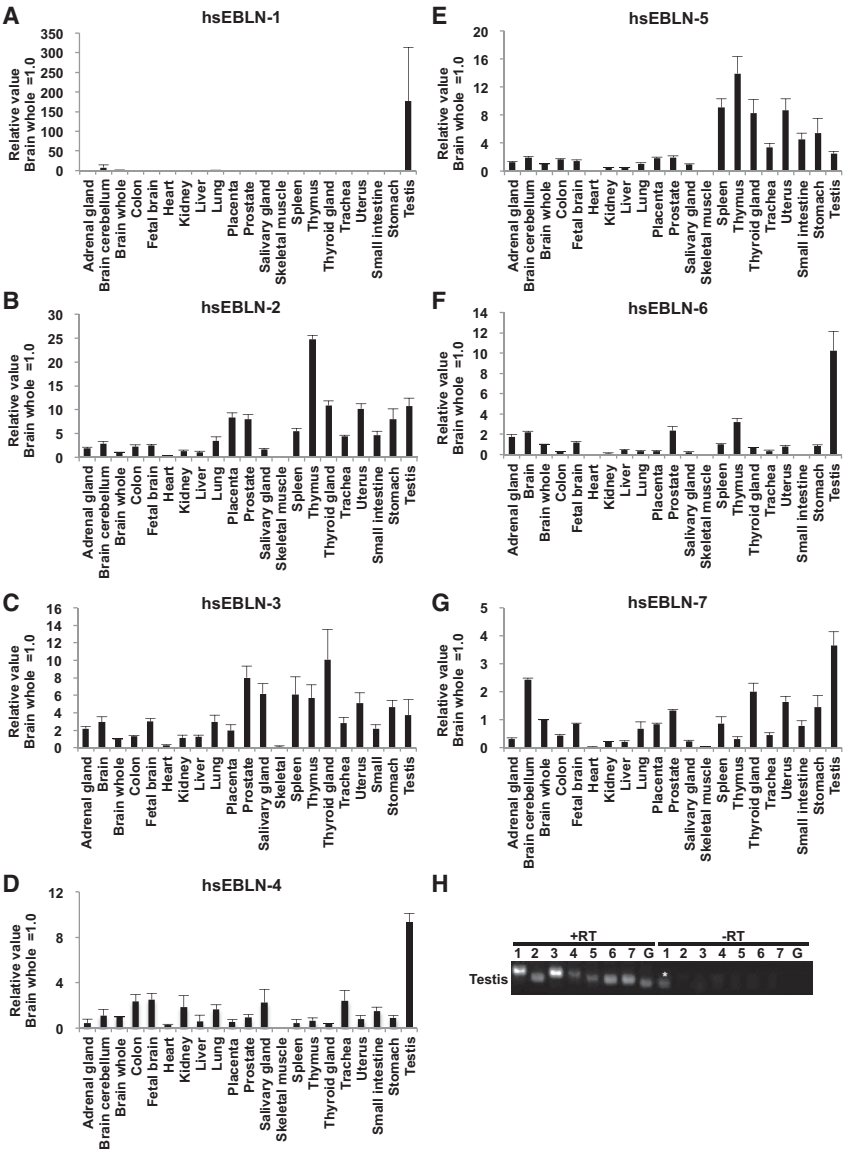

Figure 2. Expression Profiles of hsEBLN RNAs

(A–G) Quantitative analysis of hsEBLN RNAs in various human tissues. (A) hsEBLN-1. (B) hsEBLN-2. (C) hsEBLN-3. (D) hsEBLN-4. (E) hsEBLN-5. (F) hsEBLN-6. (G) hsEBLN-7. The expression level of hsEBLN RNA was normalized to that of GAPDH mRNA. The relative levels of hsEBLN RNAs in various tissues were calculated with that in brain whole. Values are expressed as the mean + SD.

(H) RT-PCR analysis of hsEBLN RNA expression in human testis. Lane 1, hsEBLN-1; lane 2, hsEBLN-2; lane 3, hsEBLN-3; lane 4, hsEBLN-4; lane 5, hsEBLN-5; lane 6, hsEBLN-6; lane 7, hsEBLN-7; lane G, *GAPDH*. * Non-specific band.

Regulation of hsEBLN-1 Expression by Histone Deacetylation and DNA Methylation

The testis-enriched expression pattern of TS loci raised the possibility of host silencing of these sequences in somatic tissues, similar to retrotransposons. Such silencing would be of potential interest, especially because these sequences are homologous to an exogenous virus. The TS hsEBLN-1 locus has a 1,004-nt sequence of continuous homology to the N gene of BDV. Because of this homology and its striking testis-specific expression, we focused on the hsEBLN-1 locus for additional research.

We examined whether transcription from the hsEBLN-1 locus is also restricted in human cultured cell lines derived from two somatic tissues. As shown in Figure 3A, transcription from the hsEBLN-1 locus was limited in OL (human oligodendroglioma cells) and 293T (human embryonic kidney cells) cells, although it was detected by increasing amplification cycles as reported previously (Horie et al., 2010). On the basis of these observations, we used OL cells to investigate the silencing mechanisms of the hsEBLN-1 locus.

First, we evaluated the contribution of histone deacetylation to silencing of the hsEBLN-1 locus. Treatment of OL cells with a histone deacetylase (HDAC) inhibitor, sodium butyrate (SB) or trichostatin A (TSA), led to dose-dependent induction of hsEBLN-1 RNA and histone H3 acetylation (H3ac) (Figures 3B, 3C, S2A, and S2B). Chromatin immunoprecipitation (ChIP) revealed that SB treatment increased the level of histone H3 lysine 9 acetylation (H3K9ac) on the hsEBLN-1 promoter (Figure S2C). These results demonstrate that histone deacetylation contributes to silencing of the hsEBLN-1 locus in OL cells. We next evaluated the contribution of DNA methylation to silencing of the locus. A DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5-Aza), enhanced transcription from the hsEBLN-1 locus to a similar level as SB, suggesting DNA methylation also contributes to silencing of the hsEBLN-1 locus (Figures S2D and S2E).

Hierarchical Regulation of hsEBLN-1 by Multiple Epigenetic Modifications

We then evaluated the contribution of histone methylation to hsEBLN-1 silencing using siRNA against various histone lysine methyltransferases (HKMTs) (siG9a, siEZH2, or siESET). All three siRNAs efficiently downregulated the target mRNA (Figures S2F–S2H). However, there was no significant change in the expression of hsEBLN-1 RNA by these siRNA treatments (Figure 3D). Since hsEBLN-1 silencing is enforced by at least two other mechanisms, we considered that reductions in HKMTs may be insufficient to induce transcription from the hsEBLN-1 locus. We therefore knocked down HKMTs in the presence of SB or 5-Aza. In the presence of SB, siESET led

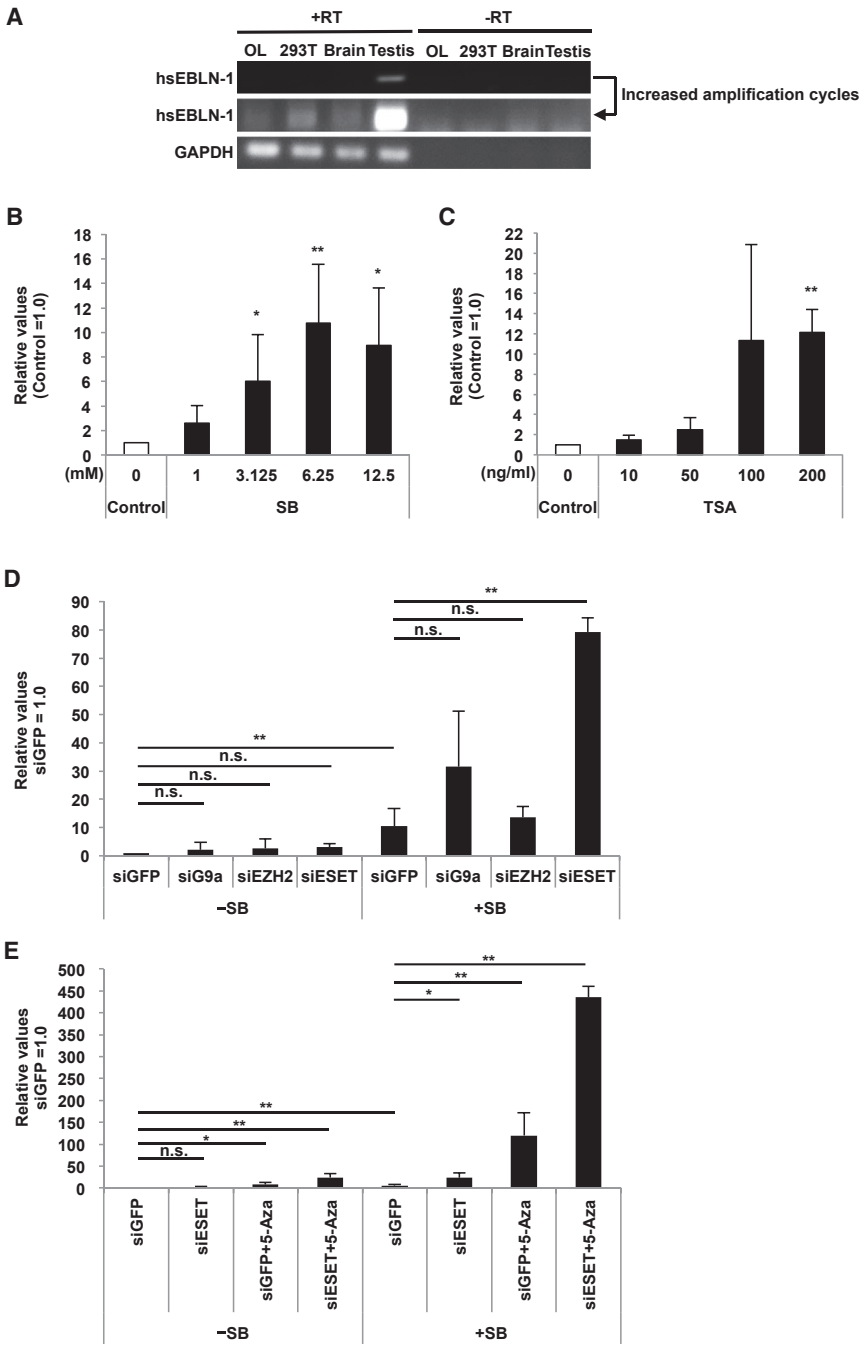

Figure 3. Epigenetic Regulation of hsEBLN-1 RNA Expression

(A) RT-PCR analysis of hsEBLN-1 RNA expression in human brain, testis, OL, and 293T cells. GAPDH mRNA expression is indicated as a loading control.

(B and C) Quantitative analysis of hsEBLN-1 RNA after treatment of HDAC inhibitors. OL cells were incubated for 18 hr after addition of 12.5 mM SB (B) or 200 ng/ml TSA (C). The expression level of hsEBLN-1 RNA was normalized to that of GAPDH mRNA. The relative values were calculated using untreated group (Control).

(D) Quantitative analysis of hsEBLN-1 RNA after treatment with siG9a, siEZH2, or siESET with or without SB.

(E) Quantitative analysis of hsEBLN-1 RNA after treatment with siESET and 5-Aza with or without SB. OL cells were incubated for 3 days after treatment with siESET and 10 μM 5-Aza with or without SB. The relative values were calculated using siGFP-treated group without SB (siGFP). Values are expressed as the mean + SD. *$p < 0.05$, **$p < 0.01$, and n.s., no significance (Student's t test). At least three independent experiments were performed.

to efficient transcription from the hsEBLN-1 locus (Figure 3D). On the other hand, siEZH2 and siG9a had no significant effect on the level of hsEBLN-1 RNA even in the presence of SB (Figure 3D). In the presence of 5-Aza, siESET also led to transcription from the locus (Figure 3E). Furthermore, combination treatment with SB, 5-Aza, and siESET showed a synergistic effect on the locus (Figure 3E). Taken together, these results indicate that silencing of the hsEBLN-1 locus is enforced by several epigenetic blocks, dominantly histone deacetylation and DNA methylation.

The Expression of Neighboring Genes around the hsEBLN-1 Locus

Our results above suggest that the host may significantly regulate the expression of the hsEBLN-1 locus by the epigenetic machineries similarly to the regulation of retrotransposon expression. These observations raised the possibility that hsEBLN loci could influence the expression of neighboring genes similarly to retrotransposons (McClintock, 1956). We therefore examined whether transcription from the hsEBLN-1 locus affects the expression level of neighboring protein-coding genes, human *DNAJC1* and *COMMD3* genes. Treatment with SB or TSA led to transcription from the hsEBLN-1 locus (Figures 3B and 3C) as well as upregulation of *DNAJC1* expression (Figure 4A). On the other hand, the expression of human *COMMD3* gene was downregulated (Figure 4B). Given that treatment of SB or TSA either enhances or does not affect gene transcription in general, this downregulation might be due to the influence of hsEBLN-1 expression. To evaluate this, we suppressed hsEBLN-1 RNA induction after SB treatment using siRNA against hsEBLN-1 RNA (si-hsEBLN-1) (Figure 4C). Suppression of the hsEBLN-1 RNA induction eliminated the SB-induced downregulation of the *COMMD3* gene expression (Figure 4D). Taken together, hsEBLN-1 RNA is likely to affect expression of the neighboring *COMMD3* gene.

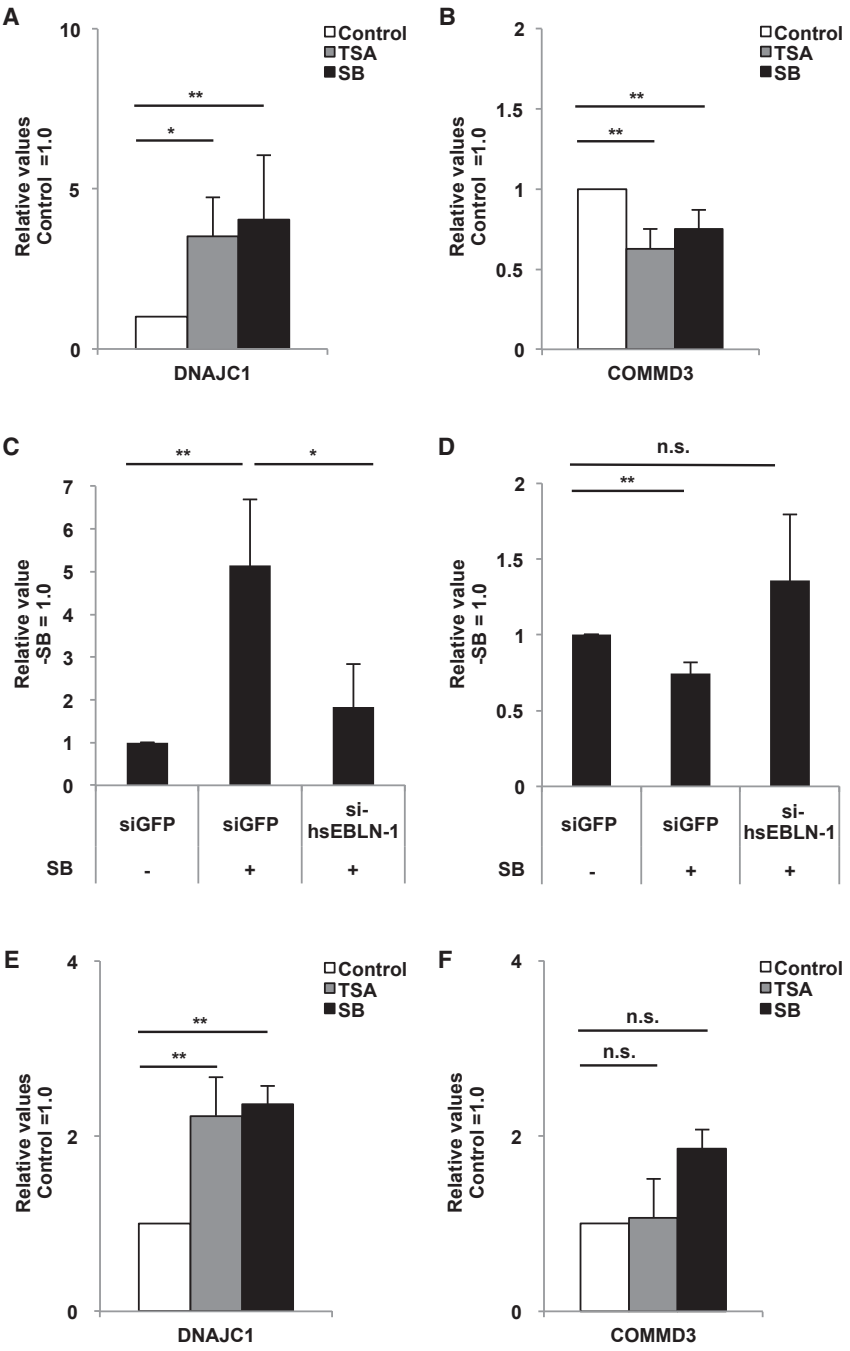

Figure 4. The Expression of Neighboring Genes around the hsEBLN-1 Locus

(A and E) Quantitative analysis of the expression of the *DNAJC1* gene after treatment of SB or TSA. Human OL (A) or mouse B7 (E) cells were incubated for 18 hr after treatment of 12.5 mM SB or 200 ng/ml TSA.

(B and F) Quantitative analysis of the expression of the *COMMD3* gene after treatment of SB or TSA. OL (B) or B7 (F) cells were incubated for 18 hr after treatment of SB or TSA.

(C and D) Quantitative analysis of the expression of hsEBLN-1 RNA (C) and the *COMMD3* gene (D) after treatment of the hsEBLN-1-specific siRNA and SB. OL cells were treated with the hsEBLN-1-specific siRNA and were incubated for 18 hr after treatment of SB. The expression levels of the indicated genes were normalized to that of GAPDH mRNA. The relative values were calculated using untreated group (Control). Values are expressed as the mean + SD. *$p < 0.05$, **$p < 0.01$, and n.s., no significance (Student's t test). At least three independent experiments were performed.

We then investigated whether EBLN integration indeed influenced epigenetic regulation around the hsEBLN-1 locus. EBLN-1 through EBLN-4 integrated into the genome of an ancestor of all haplorhini primates (Horie et al., 2010). Except for the EBLN, this region is syntenic between rodents and primates. Therefore we tested the effect of HDAC inhibitors on expression of *DNAJC1* and *COMMD3* orthologs in cells derived from the African green monkey (Vero cells), which has an hsEBLN-1 ortholog in this locus, as well as cells from mouse (B7 pulmonary epithelial cells) and rat (C6 neuroblastoma cells), which do not (Figure S3A). As in human cells, the expression of monkey, mouse, and rat *DNAJC1* orthologs was upregulated by treatment with HDAC inhibitors (Figures 4E, S3B, and S3D). In contrast, HDAC inhibitor treatment led to decreased transcription of *COMMD3* orthologs in human and monkey cells, whereas transcription of mouse and rat *COMMD3* orthologs was not affected (Figures 4F, S3C, and S3E). These results suggest that EBLN integration may affect the epigenetic status of its neighboring gene, further supporting the idea that the expression of human *COMMD3* gene is influenced by the hsEBLN-1 locus.

DISCUSSION

This is systematic evaluation of endogenous non-retroviral virus elements in the human genome. In this study, we demonstrated that all hsEBLNs have the potential to be transcribed in some tissues. hsEBLN loci can be categorized into two groups based on expression profile: UE and TS loci. hsEBLN loci were located in an existing gene or downstream of a potent promoter region. This is consistent with the proposed nature of EBLNs as pseudogenes generated from mRNA in an L1-dependent manner; processed pseudogenes are generally expressed only when they acquire new promoter and regulatory sequences or happen to integrate within an existing transcription unit (Bradley et al., 2004; McCarrey and Thomas, 1987; Mighell et al., 2000). Our observation that four of seven hsEBLNs are currently transcribed fairly ubiquitously is unexpected based on

transcription of pseudogenes processed from host mRNA, of which only 4%–6% are transcribed (Harrison et al., 2005). The unexpected extent of transcription from hsEBLNs might suggest a possible function of some of these elements.

Transcription from the hsEBLN-1 locus was repressed in most tissues except for testis by multiple epigenetic mechanisms, dominantly by histone deacetylation and DNA methylation. These results indicate that hsEBLN-1 is repressed by the host epigenetically, similar to retrotransposons, even though it has no transposition activity. However, there are some differences between mechanisms silencing hsEBLN-1 and those silencing retrotransposons. One such difference is that transcription from the hsEBLN-1 locus is rather reversible since an HDAC inhibitor or 5-Aza alone can reactivate the locus. This reversible control of transcription from the hsEBLN-1 locus is similar to silencing of exogenous retroviruses (Blazkova et al., 2009; Nabel and Baltimore, 1987). On the other hand, silencing of endogenous retrotransposons is mediated by redundant mechanisms, and transcription from these elements is not easily induced (Di Giacomo et al., 2013; Leung and Lorincz, 2012; Matsui et al., 2010). Another difference is that histone deacetylation plays a major role in silencing the hsEBLN-1 locus, again more similar to that of exogenous retroviruses (Williams et al., 2006) than to ERV or L1 where DNA methylation, but not histone deacetylation, plays a major role (Matsui et al., 2010). Collectively, silencing mechanisms of the hsEBLN-1 locus might be more similar to those of exogenous retroviruses than to those of endogenous retrotransposons.

Retrotransposons were first characterized as elements capable of controlling the expression of neighboring genes (McClintock, 1956). Indeed, ERVs, L1s, and processed pseudogenes can influence the expression of neighboring genes by various mechanisms. For example, retrotransposons or transposed genes can regulate the expression of homologous genes in other loci by competing for transcription factor(s) (Kalmykova et al., 1998; Livak, 1990). Additionally, retrotransposons may provide alternative promoters, exons, terminators, and splice junctions to protein-coding loci, facilitating genome evolution (Babushok et al., 2007; Häsler et al., 2007; Kazazian, 2004; Speek, 2001). hsEBLNs were formed via horizontal gene transfer from the environment to mammals, which is an unexplored mechanism of genome innovation in mammals. Here, using hsEBLN-1 as an example, we showed the possibility that endogenous non-retroviral virus elements could be a novel source of regulatory control of nearby protein-coding genes. In the rodent genome, lacking an orthologous EBLN at this locus, the expression of rodent *COMMD3* gene was not affected by SB or TSA. This suggests that the *COMMD3* locus itself is not inherently regulated by histone acetylation in the absence of an EBLN. When the expression from the EBLN locus in the human or monkey genome was induced, the expression of *COMMD3* orthologs was downregulated. si-hsEBLN-1 abrogated this regulation of the *COMMD3* gene. Perhaps hsEBLN-1 RNA may function as a long non-coding RNA that scaffolds transcriptional repressors for the *COMMD3* gene around the locus, downregulating its expression. EBLN-encoded proteins could potentially have biological functions as shown previously (Fujino et al., 2014). However, the involvement of the protein encoded by hsEBLN-1 in the *COMMD3* gene expression is unlikely, because the protein is located in the cytoplasm (Fujino et al., 2014). As piRNAs generated from rodent EBLN sequences are found in the NCBI database, it is possible that piRNAs are generated from hsEBLN-1 and are involved in this regulation. We cannot exclude the possibility that other SB- or TSA-responsive factors contribute to downregulation of the *COMMD3* gene in anthropoid cells, but our data highlight a previously underappreciated source of epigenetic regulation as a consequence of EBLN integration. Further studies are clearly required for the understanding of significance of EBLNs in this regard.

EXPERIMENTAL PROCEDURES

Cells

OL cells were cultured in DMEM (Gibco) containing 5% fetal bovine serum (FBS). B7 cells were cultured in DMEM containing 10% FBS. Cells were cultured at 37°C under 5% CO_2.

Chemicals and siRNAs

SB, TSA, and 5-Aza were purchased from Sigma-Aldrich. siESET was purchased from Santa Cruz Biotechnology. siGFP, siG9a, siEZH2, and si-hsEBLN-1 were purchased from Sigma-Aldrich. The sequences of siRNAs are listed in Table S2.

RT-PCR Analysis

Total RNAs were extracted using TRIzol reagent (Invitrogen). Total RNAs of human tissues were purchased from Clontech. Reverse transcription was performed using Verso cDNA Synthetic Kit (Thermo Scientific). Real-time RT-PCR assays were carried out using Thunderbird SYBR qPCR Mix (Toyobo). The primers used are listed in Table S2.

Knockdown by siRNA

OL cells were transfected with siG9a, siEZH2, siESET, si-hsEBLN-1, and siGFP using Lipofectamine 2000 (Invitrogen).

ACCESSION NUMBERS

Monkey COMMD3 and DNAJC1 partial mRNAs reported in this paper have been deposited to the GenBank and are available under accession numbers GenBank: AB934382.1 and GenBank: AB934381.1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.007.

AUTHOR CONTRIBUTIONS

K.S. and T.H. designed and performed experiments; K.S. and T.H. analyzed data; T.H. and K.T. conceived the project; and K.S., N.F.P., T.H., and K.T. wrote the manuscript.

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