Epstein–Barr virus EBER1 and murine gammaherpesvirus TMER4 share conserved in vivo function to promote B cell egress and dissemination

Brett A. Hoffman[a], Yiping Wang[a], Emily R. Feldman[a], and Scott A. Tibbetts[a,1]

[a]Department of Molecular Genetics & Microbiology, University of Florida Health Cancer Center, University of Florida, Gainesville, FL 32610

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The oncogenic gammaherpesviruses, including human Epstein–Barr virus (EBV), human Kaposi's sarcoma-associated herpesvirus (KSHV), and murine gammaherpesvirus 68 (MHV68, γHV68, MuHV-4) establish life-long latency in circulating B cells. The precise determinants that mediate in vivo gammaherpesvirus latency and tumorigenesis remain unclear. The EBV-encoded RNAs (EBERs) are among the first noncoding RNAs ever identified and have been the subject of decades of studies; however, their biological roles during in vivo infection remain unknown. Herein, we use a series of refined virus mutants to define the active isoform of MHV68 noncoding RNA TMER4 and demonstrate that EBV EBER1 functionally conserves this activity in vivo to promote egress of infected B cells from lymph nodes into peripheral circulation.

herpesvirus | noncoding RNA | EBER | EBV | murine

Human gammaherpesviruses Epstein–Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), and murine gammaherpesvirus 68 (MHV68, γHV68, MuHV-4) are etiologic agents of a wide range of malignancies including B cell lymphomas. The ability of these viruses to establish life-long latency in circulating B cells is crucial for chronic infection and tumorigenesis; however, the precise determinants that mediate in vivo latency remain unclear (1). Gammaherpesviruses express several types of noncoding RNAs (ncRNAs) including microRNAs (miRNAs), small ncRNAs, long ncRNAs, and circular RNAs (reviewed in refs. 2–5). These include the EBV-encoded RNAs 1 and 2 (EBER1 and EBER2), which are among the first ncRNAs ever identified (6, 7). Despite decades of elegant molecular studies (reviewed in ref. 4), the in vivo functions of the EBERs remain poorly understood.

MHV68 expresses 8 distinct transfer RNA (tRNA)–miRNA encoding RNA (TMER) molecules which exhibit similarity to the EBV EBERs, including comparable size and secondary structure, polymerase III (pol III) transcription, and expression during latency (8–10). Individual TMERs lack significant sequence homology but display similar structure (Fig. 1*A*): Each 200- to 250-nt molecule is composed of a tRNA-like element (vtRNA) followed by 1 or 2 pre-miRNA stem–loops (SL1 and SL2) (Fig. 1*B*) (10–12). The vtRNAs do not function as charged tRNAs (10, 13) but do carry pol III promoters that drive TMER transcription (10, 11). Transcription and processing of individual TMER molecules results in the production of full-length TMER species, intermediately sized small ncRNAs, mature miRNAs, and free vtRNAs (11, 14, 15).

We previously demonstrated that 1) TMER4 is critical for hematogenous dissemination of infected cells to peripheral latency sites, 2) TMER4 vtRNA alone is not sufficient for function, and 3) TMER4 miRNAs are dispensable for function (15, 16). Here, we sought to define the critical TMER4 species. Using Northern blot on RNA from MHV68-infected cells (Fig. 1*C*), we detected distinct TMER4 species corresponding to 250-nt full-length, 70-nt vtRNA, and 2 intermediate species. Notably, the predominant form was 155 nt, consistent with vtRNA plus a single stem–loop. In contrast, in cells infected with a TMER4 mutant unable to undergo in vivo dissemination (15), only the vtRNA species was detected, suggesting that TMER4 function may be conveyed through the predominant 155-nt species. In parallel Northern blots using shorter probes, the 155-nt species was not detected by probe complementary to SL2 (Fig. 1*D*), demonstrating that the predominant TMER4 species was comprised of vtRNA plus the first pre-miRNA stem–loop (vtRNA-SL1).

Although these findings implicated vtRNA-SL1 as the critical species, it was plausible that a processed form of vtRNA alone instead conveyed activity and that the mutation in MHV68.ΔT4 altered vtRNA processing. To distinguish these possibilities we generated mutants lacking expression of either free vtRNA or the vtRNA-SL1 species. As expected (10, 14, 17), insertion of CCA (but not control AGT) at the tRNase Z discriminator nucleotide (Fig. 1*E*) prevented vtRNA processing but did not alter expression of other TMER4 species (Fig. 1*F*). Likewise, mutation of the pol III alternate stop sequence (Fig. 1*E*) resulted in complete loss of vtRNA-SL1 and a reciprocal increase in full-length TMER4 (Fig. 1*F*).

To define whether vtRNA-SL1 or free vtRNA was specifically required for in vivo function, we infected wild-type (WT) mice then quantified peripheral latency (Fig. 1*G*) using limiting dilution nested PCR (LDPCR) for viral genome (15). As expected, the frequency of splenocytes harboring viral genome was significantly reduced in mice infected with a control TMER4 promoter mutant lacking expression of any TMER4 species (WT, 1 in 470; Δpro, 1 in 3,800). While CCA mutant lacking free vtRNA established latency at a frequency nearly identical to that of WT virus (1 in 495), virus lacking vtRNA-SL1 was attenuated to a level (1 in 3,400) nearly equivalent to the promoter mutant, demonstrating that vtRNA-SL1 was the species critical for in vivo function.

Although TMER4 vtRNA-SL1 and EBV EBER molecules do not share predicted secondary structure (Fig. 2*A*), they exhibit similar size, pol III transcription, and expression during in vivo latency. We thus tested the ability of EBER1 to rescue the in vivo defect of TMER4 mutant viruses using a recombinant virus in which TMER4 was fully replaced by EBER1 (Fig. 2*B*). A virus containing mutations within EBER1 pol III promoter A and B boxes was generated as a control. Because adenovirus VA RNA I and II (VAI and VAII) are pol III-driven small RNAs (Fig. 2*A*) that have some overlapping activity with the EBERs (18), we also generated recombinants in which TMER4 was fully replaced by VAI (Fig. 2*B*). Northern blot confirmed EBER1 and VAI expression during virus infection (Fig. 2 *C* and *D*).

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The authors declare no competing interest.

[1]To whom correspondence may be addressed. Email: stibbe@ufl.edu.

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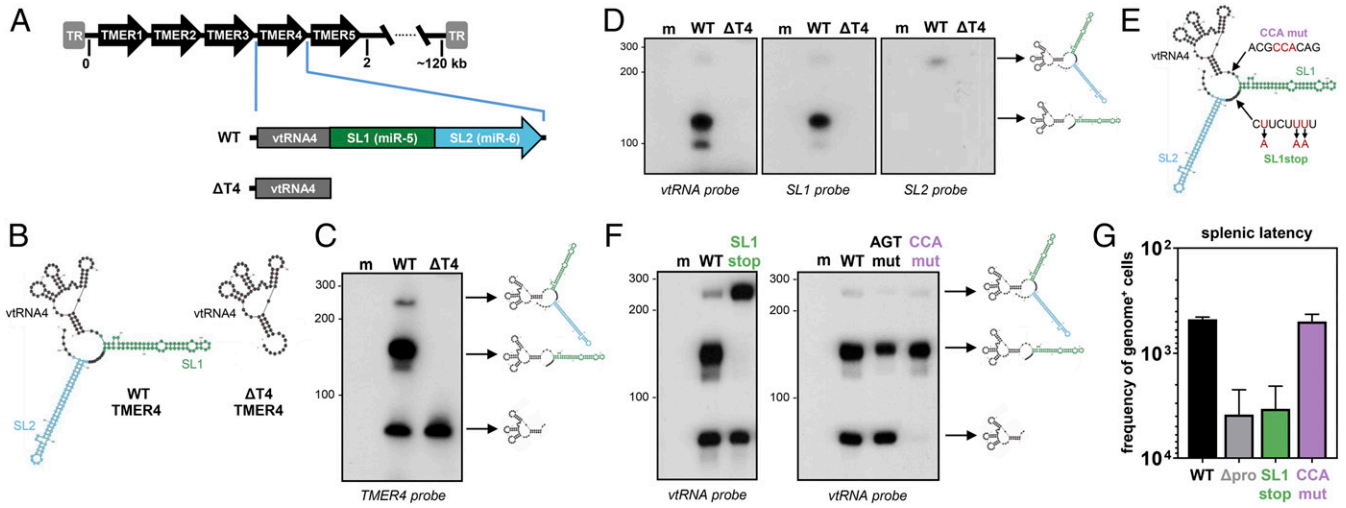

Fig. 1. MHV68 TMER4 intermediate species vtRNA-SL1 is essential for in vivo function. (*A*) Virus with TMER4 WT or carrying stem–loop deletions (ΔT4). (*B*) mFold RNA structure predictions. (*C*) Northern blot (full-length TMER4 probe) on RNA from fibroblasts: mock (m), MHV68 WT or with TMER4 SL1 and SL2 deletions (ΔT4). (*D*) Parallel Northern blots using probes to TMER4 vtRNA, SL1, or SL2. (*E*) TMER4 SL1stop and CCA mutations. (*F*) Northern blot on RNA from fibroblasts: mock, MHV68 WT or with TMER4 SL1 stop, CCA, or AGT mutation. (*G*) LDPCR for viral genome (15) to determine frequency of latently infected splenocytes in mice infected with MHV68 WT or with TMER4 promoter (Δpro), SL1stop, or CCA mutation (16 d postinfection).

We then tested the ability of mutant and control viruses to establish splenic latency in vivo. Notably, virus expressing full-length EBER1 in place of TMER4 established latency nearly equivalent to WT virus (WT, 1 in 490; E1, 1 in 510) (Fig. 2*E*). In contrast, insertion of EBER1 with mutated promoter (E1.mutAB, 1 in 3,500) or adenovirus VAI (1 in 2,690) in place of TMER4 had no impact on latency establishment (Fig. 2*F*). These findings strongly suggested that EBER1 shares a conserved in vivo function with TMER4.

The attenuation in peripheral latency exhibited by TMER4-deficient virus results from a severe defect in dissemination from the initial lung-draining mediastinal lymph node (MLN), resulting in a striking reduction in the number of infected cells that enter circulation (15, 16). Consistent with a shared function, replacement of TMER4 with EBER1 resulted in nearly complete restoration of the number of infected cells in circulation (WT, 1 in 11,400; ΔT4, 1 in 159,200; E1, 1 in 15,800) (Fig. 2*G*). Further, while virus deficient in TMER4 displayed an accumulation of infected cells in the MLN (WT, 1 in 12,400; ΔT4, 1 in 3,330), replacement of TMER4 with EBER1 resulted in restoration to a level nearly equivalent to WT virus (E1, 1 in 14,000) (Fig. 2*H*). Although it is formally possible that the EBER1 function for EBV

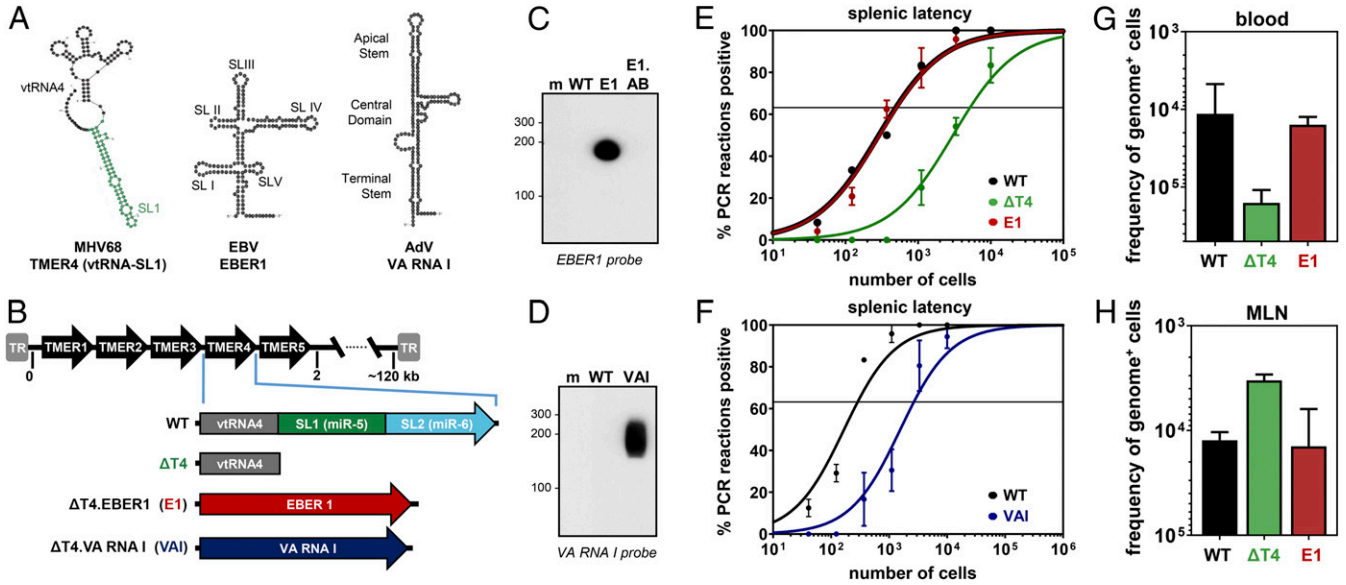

Fig. 2. EBV EBER1 fully restores the in vivo attenuation of TMER4-deficient MHV68. (*A*) RNA structure predictions for TMER4 vtRNA-SL1, EBV EBER1 (4), and adenovirus VA RNA I (20). (*B*) Virus with TMER4 WT, deletions of stem–loops (ΔT4), or replaced by EBV EBER1 (E1) or adenovirus VA RNA I (VAI). (*C* and *D*) Northern blots on RNA from fibroblasts: mock (m), MHV68 WT, or MHV68 carrying EBV EBER1 (E1), EBV EBER1 with mutated promoter (E1.AB), or adenovirus VA RNA I (VAI) in place of TMER4. (*E*) LDPCR on splenocytes carrying viral genome during in vivo latency. (*F*) LDPCR on splenocytes carrying viral genome during in vivo latency. (*G*) LDPCR on blood cells carrying viral genome during dissemination, 6 d postinfection. (*H*) LDPCR on lung-draining lymph node cells carrying viral genome during dissemination, 8 d postinfection.

may be distinct from the rescue function that EBER1 provides for TMER4-deficient MHV68, the findings presented here strongly suggest that EBV EBER1 shares a conserved in vivo function with TMER4 to promote hematogenous dissemination of infected B cells.

Methods

Viruses. Parental WT marker virus MHV68.ORF73βla, MHV68.ΔTMER4, and virus generation were previously described (15, 19). Sequences of EBER1 plus 5′ 140 nt and 3′ 30 nt, or VA I plus 5′ 81 nt and 3′ 50 nt, replaced TMER4 plus 5′ 41 nt and 3′ 9 nt. EBER1 A/B mut: CGCTGCCCTAGAGGTT/GGGTACAAGTCCC to TAGCCTAGGCTTCAGC/ATCCGGTCTGAGT.

Mice and Infections. C57BL/6J mice were housed at a University of Florida (UF) biosafety level 2+ laboratory in accordance with federal and Institutional Animal Care and Use Committee (IACUC) guidelines, and all procedures were approved by UF IACUC. Mice were infected intranasally (10^4 plaque-forming units) as described (15). Latency, MLN, and blood assays used 3, 4 to 12, and 4 mice per group per experiment, respectively.

Virus Assays. Latency levels were determined by LDPCR (15). The number of infected MLN B cells was quantified by flow cytometry (15).

Northern Blots. NIH 3T12 murine fibroblasts were infected at a multiplicity of infection of 5 (18 h). Northern blots were performed as described (15). VAI RNA probe primers: CCCATCACCCCGTCATAAGAG, CATCGCAAGCGTTTCCACC. EBER1 RNA probe primers: CACCAACTATAGCAAACCCCG, AGACTTGGGACATCTGGG. End-labeled TMER4 probes: vtRNA (TGAACCGAGAACCTGCAGGGGATGA), SL1 (GGGGAGACGACCCGATCTCAACTCT), SL2 (GGCTAAGACTCTGAAATTGTGGGAG GTGGTTG).

Statistical Analysis. Frequencies of genome-positive cells were determined by Poisson distribution from nonlinear regression (indicated by the line at 63.2% in Fig. 2 *E* and *H*).

Data Availability. Data have been deposited in the National Center for Biotechnology Information GenBank database with accession numbers AJ507799.2 for EBER1 and AC_000008.1 for VAI (21, 22).

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