Epstein-Barr Virus Induction of Recombinase-Activating Genes RAG1 and RAG2

SHAMALA K. SRINIVAS AND JOHN W. SIXBEY*

*Departments of Infectious Diseases and Virology & Molecular Biology, St. Jude's Children's Research Hospital,
and University of Tennessee College of Medicine, Memphis, Tennessee*

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In experimental B-cell infections, Epstein-Barr virus induced sustained expression of V(D)J recombinase-activating genes RAG1 and RAG2, whose aberrant activity has been implicated in chromosomal translocations in B-cell neoplasms. In cell lines in which RAG1 and RAG2 were detected, virus integrated into cellular DNA rather than assumed the configuration of extrachromosomal episomes. Expression of the Epstein-Barr virus nuclear antigen 1 in transient transfection assays was sufficient to induce both recombinase-activating genes.

Epstein-Barr virus (EBV), a common human herpesvirus, infects 95% of the world's population by adolescence. Infection may be silent, manifest acutely as infectious mononucleosis, or be associated with tumors of lymphoid, epithelial, and myocytic origin during the lifelong virus carrier state. In the infected cell, EBV is maintained extrachromosomally in a circular (latent) or linear (replicating) molecular configuration (1, 12, 29). Recent detection in human lesions of EBV DNA rearrangement (31, 39, 43) and chromosomal integration (20, 35) suggests that recombination events are a central feature of EBV biology and pathogenesis (41, 46). Because internal rearrangements in the viral genome exhibit general sequence specificity (9, 16, 31, 41), we questioned whether site-specific recombinases involved in diversification of the host immune response mediate DNA rearrangements in this lymphotropic virus. V(D)J recombinase activity is restricted to immature lymphoid cells, whereas EBV normally targets mature B lymphocytes. Thus, in order to become substrate, EBV would have to activate RAG1 and RAG2, whose concerted expression has been shown sufficient for V(D)J recombination (30).

To determine if EBV infection stimulates RAG expression in mature B lymphocytes, we first examined established cell lines derived from sporadic (EBV-negative) Burkitt's lymphomas (sBL) paired with their in vitro-infected, EBV-positive counterpart (provided by C. Rooney, St. Jude Children's Research Hospital) (7). Sporadic Burkitt's tumors do not express RAG1 or RAG2 (5), and biopsy-derived cell lines have a relatively mature, surface immunoglobulin-positive cell phenotype (7, 19). Cytoplasmic RNA was extracted from sBL cell lines and the human pre-B-cell line Reh (American Type Culture Collection, Rockville, Md.) as previously described (4). Poly(A)⁺ RNA was isolated by using an oligo(dT)-cellulose microcentrifuge pack (Collaborative Biomedical Products, Bedford, Mass.) according to the manufacturer's protocol. RNA was electrophoresed in a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane (Micron Separations, Westborough, Mass.), and hybridized to a ³²P-labeled probe generated by random-primed labeling (11). Probe was derived from an *Xho*I-*Hind*III digestion fragment of a cloned 6.6-kb human RAG1 cDNA, H36 (gift of D. G. Schatz, Yale University).

Northern (RNA) blot analysis of poly(A)⁺ RNA from uninfected and EBV-converted sBL cell lines that had been maintained in long-term culture revealed RAG1 RNA in four of five EBV-infected sBL lines but not in their uninfected counterpart (representative blot in Fig. 1; Table 1). To control for RNA loading, blots were reprobed for the generally expressed glyceraldehyde 3-phosphate dehydrogenase gene, and the integrated optical density of bands was quantitated with a Visage 110 image analysis system (BioImage Products, Ann Arbor, Mich.). RAG1 RNA was approximately 50-fold less abundant in EBV-converted sBL lines than in the pre-B-cell control, Reh.

To confirm these findings and to detect less abundant RAG2 whose levels fluctuate throughout the cell cycle (23), we performed reverse transcription (RT)-PCR to detect RAG RNAs (Fig. 1B). For RT-PCR, total RNA was isolated by using RNAzol B (Cinna/BiotecX Laboratories International Inc., Friendswood, Tex.). RNA was incubated for 20 min at 37°C in DNase I (RNase free; Promega, Madison, Wis.) to remove contaminating DNA. cDNA was reverse transcribed from 1 µg of total cellular RNA, using the 3' primers specified below with avian myeloblastosis virus reverse transcriptase as specified by the manufacturer (Life Sciences, St. Petersburg, Fla.). After unincorporated nucleotides were removed by centrifugation over a Centricon-100 (Amicon, Beverly, Mass.), cDNA was amplified for 35 cycles (1.5 min at 94°C, 2 min at 52°C, and 2 min at 70°C) with *Taq* polymerase (Stratagene, La Jolla, Calif.). RAG1-specific primers were 5'-CCATCATGCAGGGAAAGG-3' (5' primer; bp 1479 to 1496) and 5'-GCTTCTCACTCACGTC TC-3' (3' primer, bp 1928 to 1946) (38); RAG2 primers were 5'-TGAGATGGAGACCCAGATT-3' (5' primer, bp 2101 to 2120) and 5'-TCACAAGTAGGGCAGCATGT-3' (3' primer, bp 2453 to 2472) (15). The PCR products were separated on a 3% NuSieve-1% SeaKem agarose gel (FMC Bio-products, Rockland, Maine), transferred onto a nylon membrane, and hybridized to ³²P-end-labeled oligonucleotide probes specific to RAG sequences internal to the primers. Results achieved by RT-PCR for RAG1 were identical to those of Northern blot hybridizations (Fig. 1). In all infected sBL cell lines except one (BL30/P3HR-1), RAG-1 and RAG-2 were coordinately transcribed (Table 1). These results concur with recent findings derived independently in a second laboratory by Kuhn-Hallek et al. (19) showing RAG expression in EBV-infected BL cell lines. Detection of RAG2 in the absence of RAG1, seen in BL30/P3HR-1 cells, has been described previously in B-lineage acute lymphocytic leukemia cells (5).

* Corresponding author. Mailing address: Departments of Infectious Diseases and Virology & Molecular Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis TN 38101-0318.

FIG. 1. RAG1 and RAG2 expression in EBV-infected sBL cells. (A) Northern blot analysis for the approximate 6.6-kb RAG1 mRNA in BL41 cells infected with two EBV strains. BL41 lanes contained 10 µg of poly(A)⁺ RNA; the REH lane contained 0.2 µg of mRNA from control pre-B-cell line Reh, which expresses abundant RAG. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe controlled for RNA loading. (B) Ethidium bromide gel of RT-PCR amplification products for RAG1 and RAG2 in infected BL41 cells. Reaction mixtures contained 50 ng of positive control Reh RNA; 1 µg of RNA was used for all other assays. Amplifications without reverse transcriptase (RT -) excluded contaminating DNA as a reaction template. Data are representative of four separate assays.

and chicken B cells undergoing immunoglobulin gene conversion (8).

To relate RAG induction temporally to virus infection, one sBL line (BL41) was acutely infected with the B95-8 strain of EBV as described elsewhere (7). After exposure to virus, approximately 2% of the total cell population expressed EBV nuclear antigen 2 (EBNA2), as determined by indirect immunofluorescence staining with monoclonal antibody PE-2 (gift of L. S. Young, University of Birmingham) (45). The bulk culture

TABLE 1. EBV-associated RAG1 and RAG2 transcription in sBL cells

Cell line ^a	Transcription	
	RAG1 ^b	RAG2 ^c
BL2	—	—
BL2/B95-8	+	+
BL30	—	—
BL30/B95-8	+	+
BL30/P3HR-1	—	+
BL41	—	—
BL41/B95-8 (5D3)	+	+
BL41/P3HR-1	+	+

^a P3HR-1 and B95-8 are nontransforming and transforming laboratory strains of EBV.

^b Determined by Northern blot analysis and RT-PCR.

^c Determined by RT-PCR analysis only.

FIG. 2. EBNA1 induction of RAG1 and RAG2 in sBL cells. Shown is a Southern blot of RT-PCR products after BL41 cells were transiently transfected with EBV gene EBNA1, EBNA2 (type A), or EBNA3C or with pSG5 vector alone. BL41, mock-transfected cells. RT, reverse transcriptase.

was then cloned with a FACStar cell sorter (Becton Dickinson, Braintree, Mass.) at one cell per well into 96-well plates containing human foreskin fibroblast feeder layers. Of 300 clones screened, one (5D3) was EBNA2 positive. Within the first passage of this newly converted EBV-positive clone, we verified de novo induction of RAG1 and RAG2 (Fig. 1, clone 5D3). Five EBV-negative clones screened for RAG mRNA by RT-PCR did not contain transcripts (data not shown).

Latent infection by prototype EBV is associated with constitutive expression of six EBNA proteins and three latent membrane proteins. Whereas both B95-8 and P3HR-1 virus strains induced RAG expression in sBL cells (Fig. 1), infection with P3HR-1 results in a more restricted pattern of viral gene expression (28) due to deletions in that virus of DNA encoding EBNA-LP and the transactivating protein EBNA2 (6, 18, 33). On this basis, we selected three gene constructs for transfection into sBL to determine what viral genes might be responsible for RAG induction: EBNA1 (required for EBV plasmid maintenance in infected cells) (34, 36, 44), EBNA3C (a transcription factor essential for EBV transformation) (27, 42), and EBNA2 (deleted in P3HR-1 and not anticipated to stimulate RAG expression).

Fifteen micrograms of vector DNA (pSG5; Stratagene), pSG-EBNA1, pSG-EBNA2 (type A), or pSG-EBNA3C (provided by C. Sample, St. Jude) was transfected into 8×10^6 BL41 cells, using an electroporator (Gene Pulser; Bio-Rad, Hercules, Calif.) at 250 V and capacitance of 960 µF. After resuspension in 10 ml of RPMI 1640, cells were cultured for 48 h and then harvested. Comparable expression of introduced genes was confirmed by protein immunoblotting and immunofluorescence staining with monoclonal antibody PE-2 (anti-EBNA2) and monospecific human sera AM (anti-EBNA1) and JT (anti-EBNA3C) (from C. Sample). Total RNA was analyzed for RAG1 and RAG2 transcripts by RT-PCR. At 48 h, EBNA1-transfected sBL cells contained both RAG1 and RAG2 RNA by RT-PCR analysis. Cells mock transfected or electroporated with EBNA2, EBNA3C, or the pSG5 vector alone did not express RAG genes (Fig. 2).

Although characteristically episomal, EBV regularly integrates in converted sBL lines (14). To determine if integration events coincided with recombinase expression, total cellular DNA was digested with restriction endonuclease *Bam*HI, and the molecular configuration of EBV DNA was determined by analysis of terminal repeat sequences as described previously (32). After electrophoretic separation on 0.8% agarose gels, DNA was transferred to nylon membranes for hybridization with riboprobes to unique DNA at either side of EBV terminal

FIG. 3. EBV integration into genomic DNA of cells expressing RAG. (A) Schematic representation of EBV terminus analysis. Probes specific for unique viral DNA at the left (L) and right (R) of terminal repeats, but within *Bam*HI restriction sites (arrows), distinguish episomal from integrated EBV DNA on Southern blots. Detection of equal-size bands with both R and L probes suggests joined ends characteristic of episomal EBV; unequal fragments indicate integration by terminal sequences into cellular DNA (hatched boxes). (B) Southern blots of *Bam*HI-restricted total cellular DNA hybridized to ³²P-labeled L probe and then stripped and reprobed with the R probe. Virus in newly converted BL41/B95-8 (clone 5D3) cells integrated by sequences internal to the L probe (data not shown), so only right terminal sequences are detected. B95-8 control (rightmost lanes) is a virus-producing cell line with episomal bands at approximately 10.3 kb and smaller unjoined ends of linear (replicating) EBV DNA. Sizes at the left are indicated in kilobases.

repeats (1.9-kb *Xho*Ia and *Bam*HI J portion of *Eco*RI-I in pGEM2; gift of N. Raab-Traub, University of North Carolina). By analysis of EBV terminal sequences, we found complete concordance between expression of RAG genes and EBV integration. The infected cell line (BL30/P3HR-1) that expressed only RAG2 maintained EBV in its episomal form (Fig. 3). Virus in newly infected BL41 cell clone 5D3 also integrated, indicating that recombination detected in sBL cells is not a consequence of long-term cell culture.

To strengthen the correlation between integration and RAG expression, five additional EBV-converted sBL cell lines (BL30/B95-8, BL31/B95-8, BL31/P3HR-1, BL40/B95-8, and BL41/72; provided by D. Thorley-Lawson, Tufts University) previously described as containing viral episomes (14) were analyzed by RT-PCR for RAG1 expression. Ubiquitously expressed *c-abl* mRNA was amplified as a control to ensure RNA integrity. RAG1 mRNA was not detected in these sBL lines (data not shown). Also of note is the distinct correlation in two B95-8-converted clones of BL30 from separate laboratories: one contained a single copy of integrated EBV and expressed RAG (Fig. 3), whereas the second was RAG negative with multiple copies of episomal EBV (14). Although insufficient to implicate V(D)J recombinase specifically, viral integration does indicate the presence of recombinogenic activity in RAG-

positive cells. Whether increased RAG transcription correlates with a functional V(D)J recombinase or represents a more general stimulation of cellular recombination mechanisms (3, 41) is the focus of ongoing studies.

Our findings indicate inappropriate RAG expression after B-cell infection by EBV and identify EBNA1 as sufficient for induction of both RAG1 and RAG2 in some cells. Whereas all EBV-converted sBL lines express EBNA1, RAG induction was sporadic and presumably reflects cell context. For example, we detected only transient RAG expression on infection of peripheral blood B cells (40). Despite a mature B-cell phenotype, sBL may represent a subset of B lymphocytes, such as those in the pre-B-to-B-cell transition (26), capable of simultaneously expressing surface immunoglobulin as well as genes normally restricted to the pre-B developmental stage. Because DNA viruses induce a variety of cellular genes required for viral replication, it is likely that cellular recombinogenic machinery is activated for cleavage and packaging of EBV DNA concatamers or, alternatively, for circularization of incoming linear DNA (3, 10, 17, 41, 46). These findings may be useful in delineating potential regulatory factors affecting RAG transcription.

What makes activation of this host enzymatic machinery by EBNA1 of particular note is the well-documented role that aberrant V(D)J recombination plays in human tumorigenesis. At least one-fifth of childhood malignancies appear to involve translocations derived from site-directed lymphoid recombination (21). The one constant feature of EBV-positive African BL is chromosomal translocation of the *c-myc* proto-oncogene into an immunoglobulin locus (13). In this tumor, only EBNA1, a protein which activates the EBV plasmid origin of replication (34, 36, 44) but has no growth-transforming activity per se, is expressed (37). Rather than a mistake in V(D)J joining at the pre-B-cell stage as the developmentally regulated expression of V(D)J recombinase has presupposed, our findings raise the possibility that such translocations also occur at later maturational states as a result of EBV-initiated V(D)J recombinase activity. Given the widespread nature of EBV infection, such a mechanism would have far-reaching implications and could contribute to V(D)J recombinase-generated cytogenetic alterations reported in lymphoid tissue and peripheral blood lymphocytes of a large percentage of the healthy human population (2, 22, 24, 25).

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