RNA polymerase III-transcribed EBER 1 and 2 transcription units are expressed and hypomethylated in the major Epstein–Barr virus-carrying cell types

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The genome of Epstein–Barr virus (EBV) codes for two non-translated small RNA molecules, EBER 1 and 2. We found that both EBERs are expressed in the major EBV-carrying cell types, group I and III Burkitt's lymphoma (BL) cell lines, lymphoblastoid cell lines (LCLs) and in two nude mouse-passaged nasopharyngeal carcinoma (NPC) tumours. The relative amount of EBER 1 and EBER 2 varied in different host cells but did not correlate with the cellular phenotype. The EBER coding and flanking sequences were predominantly hypomethylated at HpaII sites not only in LCLs which usually carry hypomethylated EBV genomes but also in BL and NPC cell lines harbouring EBV episomes that are highly methylated in other regions. Thus, the EBER transcription units, actively transcribed by RNA polymerase III in the major EBV-carrying cell types, represent a methylation-free region in the EBV genome similarly to regulatory sequences of the latent membrane protein gene when the latter is transcribed by RNA polymerase II.

Introduction

The expression of latent (i.e. non-lytic) viral genes is differentially regulated in the three main Epstein–Barr virus (EBV)-carrying cell types, Burkitt's lymphoma (BL) cells, nasopharyngeal carcinoma (NPC) cells and lymphoblastoid cell lines (LCLs). BL cells in vivo as well as group I BL cell lines growing in vitro that maintain the BL biopsy phenotype express only EBNA 1 (a nuclear antigen involved in the maintenance of EBV episomes). In 65% of NPC biopsies the latent membrane protein (LMP) is coexpressed with EBNA 1. The control of latent EBV gene expression is even less stringent in LCLs and in group III BL cell lines of immunoblast phenotype: they synthesize EBNA 1 to 6 and LMP (Rowe et al., 1987; Fähreus et al., 1988). In addition to the growth transformation-associated proteins, BLs and LCLs also express two non-polyadenylated small RNA molecules, EBER 1 and EBER 2 (166 and 172 nucleotides, respectively), encoded by the BamHI C fragment of the EBV genome (Rymo, 1979; Lerner et al., 1981; Arrand & Rymo, 1982; Jat & Arrand, 1982; Howe & Shu, 1989). In addition to intragenic RNA polymerase III control regions, however, upstream elements associated with typical class II promoters also influence the transcription of EBER genes (Howe & Shu, 1989). As far as we know there has been no comparative analysis of EBER 1 and EBER 2 expression in BL cell lines of group I versus group III phenotype and in LCLs to date.

DNA methylation has been implicated in the control of viral gene expression (Doerfler, 1983). We have found previously that the methylation pattern of EBV DNA depends on the phenotype of the host cell (Minarovits et al., 1991). It is predominantly unmethylated in LCLs but is highly methylated at CCGG sequences in BL biopsy samples and in Rael cells, a stable group I BL cell line. In Rael cells, however, we also found an unmethylated region in the BamHI C fragment, within oriP, the latent origin of EBV replication (Ernberg et al., 1989). Another methylation-free region was detected in EBV genomes carried by NPC cells. Although the BamHI N fragment encoding LMP have been found to be highly methylated, the LMP regulatory sequences have been found to be unmethylated in LMP-expressing, but not in LMP-negative, NPC biopsies (Hu et al., 1991).
The present study had two main purposes. We wished to estimate the expression of EBER 1 and EBER 2 in group I and III BL cell lines, LCLs and NPCs using separately cloned EBER 1 and 2 sequences as probes (in earlier studies on BLs, LCLs and NPCCs the BamHI C and EcoRI J fragments of the EBV genome used did not distinguish between EBER 1 and 2 RNAs or only EBER 1 expression was analysed (Weigel et al., 1985; Rowe et al., 1987; Gilligan et al., 1990).

As sequence-specific methylation of promoter and adjacent regions of RNA polymerase III-transcribed genes has not been extensively investigated, we also wished to analyse the DNA methylation patterns of the EBER-encoding region in the major EBV-carrying cell types and determine whether its methylation status correlated with EBER 1 and 2 expression.

We found that both EBER genes were expressed in all of the BLs, LCLs and NPC cells analysed. No methylated HpaII sites could be detected within the EBER 1 and 2 coding sequences and their 5' and 3' flanking sequences with the exception of an HpaII site localized upstream of EBER 1 class II promoter elements. This site was completely or partially methylated in four different BL cell lines but its methylation did not inhibit expression of EBER 1.

Methods

Cell lines and tissue culture. LCLs and BL cell lines were grown in RPMI 1640 tissue culture medium (Gibco) supplemented with 5% foetal calf serum. The cells were fed every 3 to 4 days and cultured at 37 °C in incubators with a 5% CO2 atmosphere and high humidity. The following cell lines were analysed: Rael, Akata and Mutu BL I C1216 which are group I BL cell lines maintaining the BL biopsy phenotype; Jijoye p79, Mutu BL III CI 99 and Namalwa which are group III BL cell lines with an LCL-like phenotype; CB-M1-Ral-STO, IARC 176B, Cherry, NAD-20-E95-STO, IARC 171 and CBC-Seb-CHI-20 which are LCLs and B95-8 which is an EBV-producing marmoset cell line. The NPC tumours C15 (Busson et al., 1988) and CAO (Cao, 1987) were maintained in vivo in nude mice.

DNA isolation and analysis of DNA methylation. High M, DNA was isolated from cell lines and biopsy samples and digested with HpaII (CpG methylation-sensitive) or MspI (CpG methylation-insensitive), both recognizing CCGG (Waalwijk & Flavell, 1978), as described in a previous study (Ermberg et al., 1989). Separation of the digested DNA fragments by agarose gel electrophoresis, transfer to Hybond N membranes (Amersham) and hybridization with [32p]dCTP-labelled EBV fragments was done according to standard procedures (Maniatis et al., 1982). The probes were removed from the filters by repeated washings (10 min each) in boiling 0.1 x SSC 0.1% SDS. Complete removal of the label was monitored by autoradiography before hybridization with a new probe.

Probes. A cloned 0.5 kb SstI- Sau3AI subfragment of the EcoRI J fragment of EBV DNA containing EBER 1 coding sequences (pJJJ 1) and a cloned 0.5 kb Sau3AI–EcoRI subfragment of the EcoRI J fragment, containing EBER 2 coding sequences (pJJJ 2, Jat & Arrand, 1982; Fig. 1) was a gift from Dr Nigel Sharp, St George's Hospital Medical School, London, U.K. These DNA fragments show little or no cross-reaction when hybridized to EBER 1 or EBER 2 RNAs, respectively (Jat & Arrand, 1982). The cloned EcoRI J fragment of the EBV genome was a gift from Dr Lars Rymo, Gothenburg, Sweden.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated from cell cultures and homogenates of nude mouse-passaged NPC tumours by centrifugation of a guanidinium isothiocyanate lysate through a CsCl cushion (Maniatis et al., 1982). RNA (10 µg per lane) was subjected to electrophoresis through a 1% agarose gel containing formaldehyde and transferred to a Hybond N membrane (Amersham). Filters were then hybridized to probes pJJJ 1, pJJJ 2 and a plasmid containing cDNA corresponding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tao et al., 1985). The conditions for hybridization and washing were the same as those for the Southern blots.

Results

Expression of EBERs in BL cell lines and LCLs

Fig. 2 shows the expression of EBER 1 and EBER 2 in group I and III BL cell lines, LCLs and B95-8, a virus-producing marmoset cell line. All of these cell lines expressed both EBERs. The amount of EBER 1 relative to EBER 2 RNA in various host cells was calculated from densitometric readings of the autoradiographs and the results are summarized in Table 1.

Methylation patterns of the EBER coding and flanking DNA sequences in LCLs

The methylation analysis of EBER 1 and EBER 2 coding region in LCLs is shown in Fig. 3. In all of the LCLs analysed the HpaII sites localized within the EBER
Expression and methylation of EBER genes

Fig. 2. Northern blot analysis of EBER 1 and 2 expression in BLs, LCLs and B95-8 cells. Total cellular RNAs were hybridized to an (a) EBER 1- or (b) EBER 2-specific probe. Lane 1, Rael; lane 2, Akata; lane 3, Jijoye p79; lane 4, Mutu BL I CI 216; lane 5, Mutu BL III CI 199; lane 6, Namalwa; lane 7, CB-M1-Ral-STO; lane 8, IARC 176B; lane 9, Cherry; lane 10, NAD-20-E95-STO; lane 11, IARC 171; lane 12, CBC-Seb-CH1-20; lane 13, B95-8. Positions of 28S and 18S ribosomal RNAs are indicated.

Genes and in their flanking sequences were unmethylated, resulting in HpaII and MspI fragments of identical size. It cannot be excluded, however, that one of the two HpaII sites, only 13 bp apart from each other in the EBER 1 gene, is methylated, since the resulting HpaII fragment cannot be distinguished from the unmethylated cleavage product by electrophoresis through 1.5% agarose gels. Owing to restriction fragment length polymorphism, the MspI cleavage pattern of Cherry and CB-M1-Ral-STO differs from that of the B95-8 transformed IARC 171 and NAD20-E95-STO.

Methylation patterns of the EBER coding and flanking DNA sequences in BL cell lines

In the BL cell lines (Fig. 4), similarly to the LCLs, at least four HpaII sites were unmethylated in the EBER 1 and 2 coding region. In the BL cell lines Rael and Akata, and in the group I and III Mutu clones there was an additional MspI site upstream from the EBER 1 gene and this site is absent from the B95-8 genome. This extra CCGG sequence (indicated in Fig. 1a) is completely methylated in Rael and Mutu BL I CI 216 cells and partially methylated in Akata and Mutu BL III CI 199 cells. All of the HpaII sites assayed were unmethylated in B95-8 cells.

Expression and methylation of the EBER coding region in nude mouse-passaged NPCs

Both EBERs were expressed in the nude mouse-passaged NPC lines C15 and CAO (Fig. 5a). Hybridization of MspI-digested DNAs with the EBV EcoRI J fragment resulted in an altered cleavage pattern in both NPCs compared to B95-8 (Fig. 5b). Comparison of the HpaII and MspI fragments, however, indicated that the 326 and approximately 436 bp fragments are flanked by unmethylated CCGG sequences in the EBER coding region, whereas a 371 bp fragment lying outside this region is flanked by methylated HpaII sites.

Discussion

The transcription units of small RNA (EBER) genes of EBV combine RNA polymerase II and III promoter elements (Howe & Shu, 1989). The RNA polymerase II promoter elements include an Sp1 protein-binding site, an activating transcription factor-binding site and a TATA box-like sequence. All of these elements are

<table>
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<tr>
<th>Cell line</th>
<th>EBER 1/EBER 2*</th>
<th>EBER 1/GAPDH</th>
<th>Eber 2/GAPDH</th>
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<tr>
<td>Group I BLs</td>
<td></td>
<td></td>
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<tr>
<td>Rael</td>
<td>1.18</td>
<td>0.547</td>
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<td>Akata</td>
<td>1.41</td>
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<td>Group II BLs</td>
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<td></td>
</tr>
<tr>
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<td>2.26</td>
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<td>0.019</td>
</tr>
<tr>
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<td>0.66</td>
<td>0.083</td>
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<td>CB-M1-Ral-STO</td>
<td>0.72</td>
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<td>IARC 171</td>
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<td>CBC-Seb-CHI-20</td>
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<td>Marmoset cell line B95-8</td>
<td>1.97</td>
<td>0.900</td>
<td>0.458</td>
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</table>

* Relative amounts of RNA were calculated on the basis of densitometric measurements using an LKB Ultrascan XL Laser Densitometer.
localized within 77 nucleotides upstream of the transcriptional initiation site of EBER 1 or EBER 2. The intragenic RNA polymerase III control regions consist of boxes A and B which determine the initiation site and the level of transcription, respectively. Although both EBER genes require upstream sequences for maximum expression in transient transfection assays, their transcription by RNA polymerase III in vitro is independent of the upstream sequences (Howe & Shu, 1989; Jat & Arrand, 1982). The combination of RNA polymerase II and III promoter elements may contribute to the ubiquitous expression of EBERs in all of the major EBV-carrying cell types, BLs, LCLs and NPCs.

It has been consistently observed that the EBER 2 gene is transcribed more efficiently in vitro than is the EBER 1 gene. In contrast, in the BL line Raji, the abundance of EBER 1 RNA greatly exceeds that of EBER 2 RNA (Jat & Arrand, 1982; Howe & Shu, 1989). We found that the relative amount of EBER 1 compared to EBER 2 RNA was variable in group I and III BLs and in LCLs (Table 1). The EBER 1/EBER 2 RNA ratio did not correlate with the phenotype of the cells.
of functional importance, however, are exempt from transcription; however, it may reflect the higher overall genomes like the BL and NPC biopsies. Certain regions group I BL cell line, carries highly methylated EBV are indicated. (b) HpaII- (odd lanes) or MspI- (even lanes) digested DNAs were hybridized to an EBER 1- (lanes 1 and 2) or EBER 2- (lanes 3 and 4) specific probe. Lanes 1 and 3, NPC C15 RNA; lanes 2 and 4, CAO RNA. Sizes of 32P-labelled fragments of bacteriophage lambda DNA are indicated. (b) HpaII- (odd lanes) or MspI- (even lanes) digested DNAs were hybridized to the EcoRIJ fragment of EBV DNA. Lanes 1 and 2, NPC C15; lanes 3 and 4, CAO; lanes 5 and 6, B95-8. The sizes of B95-8 MspI fragments are indicated. The 436 bp fragment comigrated with a 428 bp fragment.

We observed earlier that the overall methylation of the EBV genome follows cell type-specific patterns. In LCLs the EBV DNA is hypomethylated whereas Rael, a stable group I BL cell line, carries highly methylated EBV genomes like the BL and NPC biopsies. Certain regions of functional importance, however, are exempt from methylation even if the overall level of EBV DNA methylation is high, for instance the two HpaII sites within the oriP region in Rael cells and the regulatory sequences of the LMP gene in NPCs expressing LMP (Ernberg et al., 1989; Hu et al., 1991; Minarovits et al., 1991).

The HpaII sites in the EBER 1 and 2 transcription units are unmethylated in all of the cell lines analysed, with the exception of a single HpaII site upstream of the EBER 1 transcription unit. This site (indicated in Fig. 1a) is completely methylated in the group I BL cell lines Rael and Mutu BL I CI 216 and is partially methylated in Akata (a group I BL cell line) and Mutu BL III CI 99 (a group III BL cell line) (Fig. 4). As this site is far upstream of the RNA polymerase II-like promoter elements of the EBER 1 gene, its methylation may not influence EBER 1 transcription; however, it may reflect the higher overall level of EBV DNA methylation in BLs compared to LCLs. Methylation at this site did not result in a decrease of the relative amount of EBER 1 transcripts compared to that of EBER 2 since the EBER 1/EBER 2 ratio is higher in the group I Mutu clone (where this site is completely methylated) than in the group III clone (harbouring the same virus), where its methylation is partial. In addition, the relative amount of EBER 1 RNA is higher in Rael cells (where this site is completely methylated) than in CB-M1-Ral-STO, an LCL harbouring the virus rescued from Rael cells, where it is unmethylated.

Methylation of CCGG sequences in the internal regulatory region of the RNA polymerase III-transcribed VAI gene of adenovirus type 2 inhibits its expression in an in vitro cell-free transcription system (Jutterman et al., 1991). Box B in the intragenic RNA polymerase III control region of the EBER 1 gene is flanked by two HpaII sites on its 5' and 3' ends (Jat & Arrand, 1982). At least one of these sites remains unmethylated in BL cells, LCLs and in the NPCs C15 and CAO. The 3' end of box B in the EBER 2 internal control region is also flanked by a HpaII recognition sequence that is also consistently unmethylated in the EBER 2-expressing cells included in our study.

We concluded that both EBERs are expressed in the major EBV-carrying cell types. CCGG sequences in the region of the RNA polymerase III-transcribed EBER 1 and 2 transcription units are hypomethylated even if other regions of the EBV genome are highly methylated in the host cell (e.g. in Rael cells). This phenomenon is similar to the hypomethylation of regulatory (but not coding) sequences of the LMP gene, transcribed by RNA polymerase II, in LMP-expressing NPC biopsies (Hu et al., 1991).

We are grateful to Dr Nigel Sharp and Dr Lars Rymo for providing EBV DNA clones and to Dr Alan Rickinson and Dr Kenzo Takada for providing BL cell lines. This study was supported by the Swedish Cancer Society and PHS grant 5 RO1 CA28380-03 from the National Cancer Institute. J. M. is on leave from the Microbiological Research Group of the National Institute of Hygiene, Budapest, Hungary, as a recipient of a fellowship from CRI/Concern. L.-F. H. is supported by CRI/Concern and by the National Science Foundation and National Oncogene Research Centre, China.

References


Fig. 5. Expression and methylation analysis of EBER 1 and 2 transcription units in NPC lines C15 and CAO. (a) Total cellular RNA hybridized to an EBER 1- (lanes 1 and 2) or EBER 2- (lanes 3 and 4) specific probe. Lanes 1 and 3, NPC C15 RNA; lanes 2 and 4, CAO RNA. Sizes of 32P-labelled fragments of bacteriophage lambda DNA are indicated. (b) HpaII- (odd lanes) or MspI- (even lanes) digested DNAs were hybridized to the EcoRIJ fragment of EBV DNA. Lanes 1 and 2, NPC C15; lanes 3 and 4, CAO; lanes 5 and 6, B95-8. The sizes of B95-8 MspI fragments are indicated. The 436 bp fragment comigrated with a 428 bp fragment.


(Received 22 August 1991; Accepted 24 February 1992)