Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 6550–6554, August 1991
Biochemistry

Exclusive expression of Epstein–Barr virus nuclear antigen 1 in Burkitt lymphoma arises from a third promoter, distinct from the promoters used in latently infected lymphocytes

(anchored-PCR cloning/selective promoter usage/5-azacytidine)

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ABSTRACT **Epstein–Barr virus transformation of human B lymphocytes *in vitro* results in the expression of six viral nuclear antigens (EBNAs) and three viral membrane proteins. However, examination of viral gene expression in fresh Burkitt lymphoma isolates has revealed expression of only one of the nuclear antigens, EBNA-1. Previous transcriptional analyses of the EBNA-encoding genes demonstrated that all these genes are driven from one of two distal promoters located near the left end of the viral genome, raising the question of how exclusive expression of EBNA-1 occurs in Burkitt lymphoma tumors. Although most established Burkitt lymphoma cell lines (group 3) exhibit the full-expression pattern of viral antigens seen in lymphoblastoid cell lines, a few cell lines have been established that retain the restricted pattern of viral gene expression (group 1). In this paper we characterize transcription of the EBNA-1 gene in a group 1 Burkitt lymphoma cell line and show that (*i*) neither Cp nor Wp, the promoters involved in driving EBNA gene expression in lymphoblastoid cell lines, are active in this cell line; (*ii*) treatment of this cell line with 5-azacytidine, previously shown to induce expression of all EBNA genes, induced Cp and Wp activity; (*iii*) sizes of the EBNA-1 transcripts detected in two group 1 Burkitt lymphoma cell lines correlated with each other and were distinct from the size of the EBNA-1 transcript seen in lymphoblastoid cell lines; (*iv*) the EBNA-1 transcripts in the group 1 Burkitt lymphoma cell lines do not hybridize to a probe containing the common 5′ exons present in all the EBNA transcripts from lymphoblastoid cell lines; and (*v*) anchored-PCR cloning the 5′ region of the EBNA-1 transcript from one of the group 1 cell lines identified two exons, FQ and U, upstream of the EBNA-1 coding exon. The FQ exon lies just downstream of a TATAA box, which may represent the promoter for transcription of EBNA-1 in these cells. It is particularly noteworthy that an incomplete EBNA-1 cDNA clone from a nasopharyngeal carcinoma tumor line that expresses EBNA-1, but not the other EBNAs, has been characterized; this EBNA-1 transcript also contains the FQ/U splice junction, suggesting that the organization of exons upstream of the EBNA-1 coding exon is the same and that this organization may reflect a viral program for exclusive EBNA-1 expression.**

Epstein–Barr virus (EBV) is a lymphotropic human herpesvirus that is the etiologic agent of infectious mononucleosis, a self-limiting lymphoproliferative disorder. In addition, EBV is closely associated with two human cancers, African Burkitt lymphoma (BL) and nasopharyngeal carcinoma (NPC). The role of EBV in the development of these tumors has, however, remained enigmatic. Two features of EBV infection of B lymphocytes in tissue culture are probably relevant to the issue of EBV-associated oncogenesis. (*i*) EBV predominantly establishes a latent infection in B lymphocytes with little or no virus production; thus, the virus persists for life in the infected individual. (*ii*) There is a concomitant growth transformation of the infected lymphocytes that gives rise to lymphoblastoid cell lines (LCL) that proliferate indefinitely in culture. However, these cells are not oncogenically transformed, as assessed by their inability to form tumors in nude mice (for review, see refs. 1 and 2).

In latently infected-growth-transformed B lymphocytes, six viral nuclear antigens (EBNAs) and three viral membrane proteins (latent membrane proteins, LMPs) are expressed (for review, see ref. 2). EBNA-1 is required for replication from the latency origin of replication, *oriP*, which also functions as an EBNA-1-dependent enhancer (3, 4). EBNA-2 is essential for growth transformation of B lymphocytes and has been shown to modulate the activity of several viral and cellular promoters (5–12). LMP-1 oncogenically transforms some established rodent cell lines (13, 14) and appears to be expressed at high levels in NPC tumors (15–18). The functions of the other viral genes expressed during latency is unknown. To date no one has successfully transformed B lymphocytes with individual EBV genes, and therefore the requirements for transformation of these cells remains undefined.

Surprisingly, analysis of early-passage EBV-positive BL cell lines revealed that they exhibit a very restricted pattern of viral gene expression (19). These cell lines were observed to express EBNA-1 but not to express the other EBNAs or LMP genes. Immortalizing virus could be recovered from these cell lines, and the resulting LCL established with these viruses expressed the full array of viral antigens classically seen in LCL. The latter result indicated that the viral genomes in these BL tumors are intact and that the restricted gene expression did not result from mutation of the viral genome. Most established BL cell lines drift from this restricted viral gene expression (group 1 BL phenotype) to a pattern of viral gene expression similar to that seen in LCL (group 3 BL phenotype) (20). Downregulation of EBNA and LMP gene expression in BL may be an important mechanism of evading cytotoxic T-lymphocyte immune surveillance (21–23). Notably, these results indicate that functions of EBV required for immortalization of B lymphocytes (except for EBNA-1) are not necessary in the maintenance and propagation of BL tumors, although these functions may be important during BL genesis.

In LCL and group 3 BL, transcription of the six EBNA genes initiates from one of two promoters, Cp or Wp, located near the left end of the viral genome (see Fig. 1) (for review, see ref. 24). The coding exons are not clustered, however, but rather are spread out over the left-hand half of the viral

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Abbreviations: BL, Burkitt lymphoma; LCL, lymphoblastoid cell line; NPC, nasopharyngeal carcinoma; EBNA, Epstein–Barr virus nuclear antigen; LMP, latent membrane protein; nt, nucleotide(s).

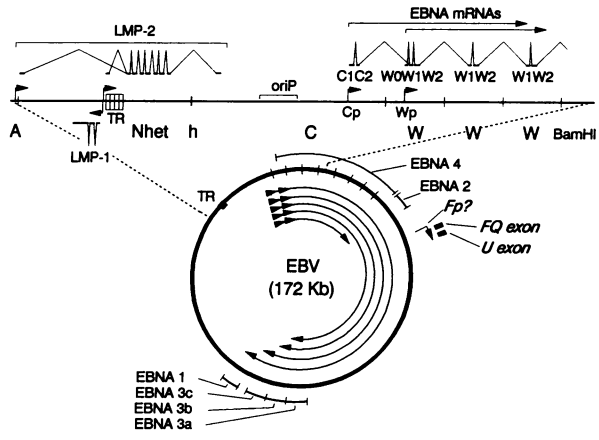

FIG. 1. Transcriptional organization of the EBV genes expressed during latent viral infection of B lymphocytes. Regions encoding the various antigens are indicated. The inner concentric arcs represent the various primary transcripts encoding the EBNAs, whereas the two arrowheads at the 5′ ends represent transcription initiation from either Cp or Wp. The exploded portion of the diagram illustrates the organization of exons present at the 5′ end of all EBNA mRNAs, as well as the exon organization of the LMP genes with respect to the *Bam*HI restriction endonuclease map of that region. Also shown are the two exons, FQ and U, found upstream of the EBNA-1 coding exon in group 1 BL cell lines. TR, terminal repeats; Fp?, putative EBNA1 promoter in group 1 BL.

genome. The activities of Cp and Wp are mutually exclusive in clonal cell lines (25), and transcription initiating from these promoters gives rise to long primary transcripts that are alternatively spliced to yield the individual EBNA mRNAs. All the EBNA mRNAs contain common 5′ exons encoded within the major internal repeat of the virus, which are spliced to the 3′ coding exons. Wp is exclusively used during the initial stages of infection, followed by a switch to Cp usage (26).

In this paper, the issue of exclusive expression of EBNA-1 in group 1 BL cell lines is addressed. The results of these studies indicate that transcription of EBNA-1 in these cells is driven by a previously unrecognized promoter located downstream of the major internal repeat of the virus, putatively in the viral *Bam*HI F fragment. We propose that this putative promoter is an integral component of an EBV program, which limits viral gene expression to EBNA-1 in lymphocytes.

MATERIALS AND METHODS

Cells, 5-Azacytidine, and Antiimmunoglobulin Treatment. Rael (27) and Akata (28) are group 1 BL cell lines, and X50-7 (29) is a human LCL transformed with EBV. All cell lines were grown at 37°C in RPMI 1640 medium/10% (vol/vol) fetal bovine serum. Akata and Rael cells were treated either with 5-azacytidine or with anti-human IgG whole or Fab_2 antibodies (Cappel Laboratories), as described (26, 28, 30).

RNA Preparation, Blotting, and S1 Nuclease Analysis. Total cellular RNA was prepared by the method of Auffray and Rougeon (31). Cytoplasmic RNA was prepared as described (32), and poly(A)$^+$ RNA was isolated by fractionation on oligo(dT)-cellulose (33). Poly(A)$^+$ RNA (10 μg) was fractionated by electrophoresis on a formaldehyde/agarose gel (34) and subsequently transferred to a nylon membrane (Amersham). RNA was crosslinked to the hybridization membrane by exposure to UV light for 5 min. Blots were probed either with a 2.9-kilobase (kb) *Bam*HI–*Hin*dIII fragment of the EBV genome containing the EBNA-1 coding exon or with the IB4-WY1 cDNA, which contains seven copies of the W1/W2 repeat exons as well as the Y1, Y2, and Y3 exons (35). Conditions for hybridization and washing were as previously described.

Twenty five to fifty micrograms of total cellular RNA was hybridized with specific oligonucleotide probes diagnostic for Cp or Wp activity, EBNA-1 coding exon use, or transcription of the early lytic gene *BHLF1*. The C1 58-mer is specific for Cp activity, whereas the W0W1 60-mer is diagnostic for Wp activity, both of which have been described (25, 26). The EBNA-1 60-mer oligonucleotide spans the EBNA-1 exon splice-acceptor site (5′-CCAGGTCCTG-TACCTGGCCCCTCGTCAGACATGATTCACAC-TAAAAGAGATCAATAGACA-3′), whereas the BHLF1 60-mer spans the 5′ end of the *BHLF1* gene (5′-CTCAGTCT-TGGCTTCTCAGTTGCTCGCTGCAGATGCGGTTGGAG-TACGAGGCGCCGCAGC-3′). Hybridization and S1 nuclease digestions were done as described (25, 26).

Primer Extension and Anchored-PCR Cloning. An 18-nucleotide (nt) primer homologous to a region 235 base pairs (bp) downstream of the EBNA-1 coding exon splice-acceptor site was used to prime first-strand cDNA synthesis with poly(A)$^+$ RNA prepared from the Rael cell line (36). The first-strand cDNA was tailed with poly(dG) by using terminal deoxynucleotide transferase to allow hybridization of an anchor oligonucleotide (37, 38). Second-strand synthesis was subsequently primed, using an oligonucleotide containing a polylinker with four restriction endonuclease sites coupled to a 15-nt poly(dC) tail. Forty cycles of PCR amplification were done on the double-stranded cDNA by using a primer homologous to the polylinker region upstream of the homopolymer tail as the 5′ primer and a 3′ 33-nt primer containing a *Not* I restriction endonuclease site linked to a 21-nt region homologous to the EBNA-1 coding exon 81 nt downstream of the splice-acceptor site. The resulting material was digested with *Apa* I and *Not* I, cloned into a Bluescript plasmid (Stratagene), and positive clones were identified by colony hybridization with an EBNA-1 exon probe. A 240-bp clone (103K/U) was recovered that contained the described (36) U exon spliced to the EBNA-1 coding exon. This clone apparently did not reflect the entire 5′ region because the 5′ end of the clone did not contain the poly(dC) tract (the *Pst* I region of the PCR anchor appeared to crosshybridize to the region of the U exon containing a *Pst* I site).

A portion of the cDNA was reamplified for 40 cycles by using the same anchor primer and a 3′ 39-mer primer containing a *Not* I restriction site linked to 27 nt homologous to the U exon. From this amplification a 126-bp clone (D2U/Q) was recovered that contained the 5′ region of the U exon, which was spliced to an upstream exon encoded in the viral *Bam*HI Q fragment. In addition to D2U/Q, three other positive clones exhibiting the same exon structure were obtained and are thought to be independent isolates because they contained 8, 10, and 12 fewer nucleotides from the Q exon at their 5′ ends. All these clones ended in a poly(dC) tract, suggesting that they either represented amplication full-length clones or partial cDNAs generated by a strong reverse transcriptase stop site.

To attempt to extend the cloning further upstream, a 31-nt Q exon primer was used in a third round of PCR amplification by using the original cDNA primed with the EBNA-1 exon primer. Southern blotting of the PCR-amplified material revealed hybridization of a 240- to 280-bp fragment to a Q exon probe. However, the quantities of this material were small, and we were not successful in cloning it. To generate more cDNA that extended to the 5′ end of the transcript, cDNA synthesis was primed with the U exon primer described above (previously used in the PCR amplification of

the original cDNA). PCR amplification (40 cycles) of this cDNA using the Q exon and anchor primers yielded high molar quantities of a single product that appeared the same size as the amplified product obtained with the original cDNA. This material was cloned, and nine positive clones were isolated, five of which were sequenced. One of the clones (S1Q) was a short clone that contained only an additional 80 bp upstream of the Q primer. The remaining four clones (XPQF2-5) extended from the Q primer upstream, past the *Bam*HI Q/F junction, 21 nt into the *Bam*HI F fragment. All four clones ended at the same place but contained poly(dC) of different lengths, suggesting that they may have arisen from different cDNA clones.

RESULTS

The Rael Cell Line Does Not Exhibit Cp or Wp Activity. To begin characterizing EBNA-1 transcription in the group 1 BL cell line Rael, S1 nuclease protection assays were carried out employing probes diagnostic for transcription initiating from Cp or Wp. In addition, a probe homologous to the region spanning the EBNA-1-coding exon splice-acceptor junction was used as a positive control. By using these probes, transcription of the EBNA-1 exon was readily apparent (Fig. 2, lane 1). However, no transcription initiation from either Cp or Wp could be detected (lanes 2 and 3). This result indicates that transcription of EBNA-1 in this group 1 BL cell line initiates from a promoter other than Cp or Wp.

Examination of another group 1 BL cell line, Akata, using the same probes revealed very low-level transcription initiation from Cp and no detectable Wp activity (data not shown). This observation was confirmed by probing a blot of Rael and Akata RNAs with a probe containing the C1 and C2 exons, which revealed low-level Cp expression in Akata but *not* in Rael (data not shown). However, the level of Cp activity detected in Akata cells could only account for a small fraction of EBNA-1 transcripts in these cells. Because many group 1 BL cell lines have been shown to drift toward the group 3 phenotype, the low-level Cp activity detected in this cell line probably represents a population of cells that has drifted to the group 3 phenotype.

Treatment of the Rael Cell Line with 5-Azacytidine Induces Cp and Wp Activity. Treatment of the Rael cell line with 5-azacytidine has been demonstrated (30) to upregulate expression of all EBNA proteins, as well as of LMP-1. To determine whether this induction of EBNA proteins is associated with induction of Cp and Wp activity, total cellular RNA was prepared from Rael cells at various times after addition of 5-azacytidine. These RNAs were assayed by S1 nuclease protection with the described probes. As expected,

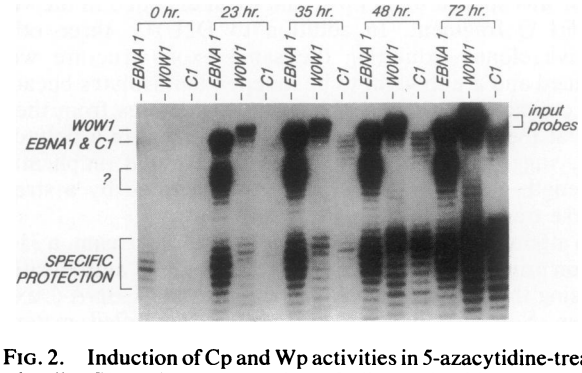

FIG. 2. Induction of Cp and Wp activities in 5-azacytidine-treated Rael cells. S1 nuclease protection of oligonucleotide probes diagnostic of EBNA-1 exon usage (EBNA-1), and Wp (WOW1) and Cp (C1) activity. RNA was prepared from Rael cell at the times indicated after induction with 5-azacytidine. Protected fragments were resolved on a 10% Tris/borate/EDTA/urea-acrylamide gel.

conversion of Rael from group 1 to group 3 phenotype was associated with induction of Wp and Cp activity (Fig. 2; Wp, lanes 5, 8, 11, and 14; Cp, lanes 6, 9, 12, and 15).

Treatment of Rael with 5-azacytidine also significantly increased the level of EBNA-1 transcription (Fig. 2; lanes 4, 7, 10, and 13). In addition, however, after 5-azacytidine treatment strong total protection of the probe was also seen as well as an additional protection starting 12–13 nt upstream of the EBNA-1 coding exon splice-acceptor site. The latter protection does *not* correlate with the presence of a splice-acceptor site (based on the sequence of the B95.8 genome) and, therefore, may represent transcription initiation from a previously unrecognized promoter immediately upstream of the EBNA-1 coding exon. Alternatively, this protection may result from sequence differences between the Rael EBV genome and the standard B95.8 genome. Thus, protection of the entire probe by transcripts initiating further upstream might result in partial cleavage by S1 nuclease at the site(s) of sequence difference to yield both full protection and protection 12–13 nt shorter than the full-length probe.

The complex transcription pattern of the EBNA-1-coding exon seen in Rael cells treated with 5-azacytidine may result from induction of the viral lytic cycle, as well as a shift from the group 1 to group 3 phenotype. To determine whether 5-azacytidine treatment of the Rael cell line induces the lytic cycle, S1 nuclease protection with a probe diagnostic for transcription of BHLF1 (an abundant early lytic transcript) was done. As control for level of lytic-cycle induction, Akata cells were treated with goat anti-human IgG, shown to efficiently trigger the viral lytic cycle in this cell line (28). Indeed, as assessed by induction of BHLF1 transcription, treatment of Rael cells with 5-azacytidine induces the viral lytic cycle (Fig. 3). Notably, treatment of the Akata cell line with 5-azacytidine did not induce the lytic cycle; nor did treatment of the Rael cell line with goat anti-human IgG induce the lytic cycle to any significant extent. Thus, these two group 1 BL are induced into the viral-lytic cycle by distinct stimuli, and at least for Rael induction is accompanied by activation of the latent promoters, Wp and Cp, with concomitant expression of all EBNAs.

EBNA-1 Transcripts in Group 1 BL Cell Lines Are Distinct in Size from the EBNA-1 Transcript in LCL and Do Not Contain the 5′ Common Exons. To further characterize EBNA-1 transcription in group 1 BL cell lines, cytoplasmic polyadenylylated RNA was prepared from the Rael and Akata cell lines. In addition, RNA was prepared from a representative LCL, X50-7. RNA blots were hybridized with

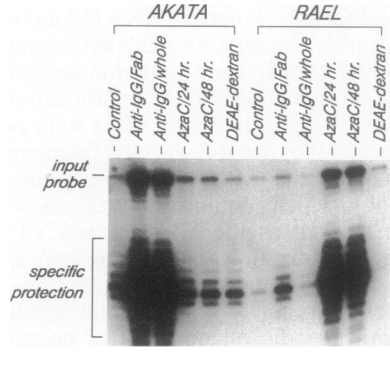

FIG. 3. Induction of EBV early-lytic gene *BHLF1* in the Akata and Rael cell lines by various reagents. Cells were treated with indicated reagents, and RNA was prepared either 24 hr after induction (anti-human IgG whole or Fab_2 antibodies, 5-azacytidine (AzaC), and DEAE-dextran) or 48 hr after addition of 5-azacytidine. S1 nuclease protection was done by using an oligonucleotide probe spanning the *BHLF1* gene transcription initiation site. Protected fragments were resolved on a 10% Tris/borate/EDTA/urea-acrylamide gel.

either a probe containing the EBNA-1 coding exon or a probe prepared from the IB4-WY1 cDNA clone (35), which contains seven copies of the W1/W2 exons found at the 5′ end of all EBNA transcripts in LCL.

Hybridization with the EBNA-1 probe revealed two transcripts in both Akata and Rael cell lines (Fig. 4). The smaller transcript is ≈2.5 kb in length, whereas the longer transcript is ≈5 kb. The overall EBNA-1 transcription pattern in these two group 1 BL is strikingly similar, while the size of the EBNA-1 transcript in X50-7 does not correspond to either of the transcripts seen in group 1 BL cell lines. In addition, the EBNA1 transcripts in group 1 BL do not hybridize to a probe containing W1/W2 repeat exons (Fig. 4). This result is consistent with the observed lack of Cp and Wp activity in these cell lines. Taken together, these results strongly indicate that EBNA-1 transcription in group 1 BL is driven by a completely different transcriptional unit than that in LCL and group 3 BL cell lines.

PCR Cloning the 5′ Region of the Group 1 EBNA-1 Transcript Identified a Putative Promoter Mapping to the Viral *Bam*HI F Fragment. Using anchored-PCR cloning, exons upstream of the EBNA-1 coding exon in the Rael EBNA-1 transcript were cloned. The compiled sequence from two rounds of primer extension coupled with PCR amplification revealed the presence of two upstream exons (Fig. 5). One is the U exon, which has previously been found in several EBNA-1 and EBNA-3c cDNA clones isolated from LCL (24, 36), and the other is an exon that spans the *Bam*HI F and Q junction of the viral genome (referred to as the FQ exon). The FQ exon has *not* previously been found in any of the EBNA-1, -3a, -3b, or -3c cDNA clones characterized. However, a portion of this exon was present in a partial EBNA-1 cDNA clone isolated from an NPC line that expresses EBNA-1 but *not* the other EBNAs (17). In addition, this NPC cDNA clone also contained the U exon, suggesting that the upstream exon structure of the EBNA-1 transcripts in these cells is the same as in the Rael cell line.

The 5′ end of the FQ exon lies 23 nt downstream of a putative TATAA box (see Fig. 5). There is no obvious CCAAT box, although there is a CAATT sequence at −157 bp. It is attractive to speculate that this region represents the promoter for EBNA-1 in group 1 BL cell lines. However, functional studies will be required to determine whether this is, indeed, the case.

DISCUSSION

EBV gene expression in newly established BL cell lines appears restricted to expression of EBNA-1 only (19). This downregulation in expression of the other viral gene products characteristic of latently infected LCL and group 3 BL may facilitate the evasion of group 1 BL tumor cells from immune surveillance (21–23). If this is indeed the case, then the question arises whether this represents a viral program or, alternatively, results from strong negative selection against expression of some EBV antigens (e.g., EBNA-2 and LMP-1). Negative selection might be expected to give rise to mutated viral genomes and would not necessarily be expected to yield a uniform solution. Conversely, if exclusive expression of EBNA-1 is part of a viral program, then transcription of EBNA-1 in all group 1 BL cell lines should be the same.

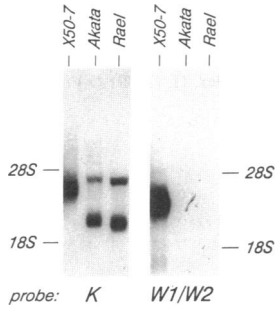

FIG. 4. RNA blot of RNAs prepared from an EBV-infected lymphoblastoid cell line (X50-7) and two group 1 BL cell lines (Akata and Rael). Blots were probed either with the region of the viral *Bam*HI K fragment containing the EBNA-1 coding exon (probe K) or with the cDNA clone IB4-WY1, containing multiple copies of W1/W2 repeat exons (probe W1/W2). 28S and 18S, migration positions of rRNAs.

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acagtagagggcacgaaacatggtgtatgcactttattaataaacaatta -151
cagatacaaaaacttgagtctctcgaggtctgcgatgaggcggtgggtgg -101
aacgctccagcttgcggcgaagctggctcacgaagcgagacagtactcgg  -51
ctagcctgactaagggtgaggctataacgcaggtcctgttccggggcggc   -1
GGTGGATAGAGAGGAGGGGGATCCGGAGGGGACCACTAGGTCGCCGGAGG   50

TCGACCCTCCTGTCACCACCTCCCTGATAATGTCTTCAATAGACAGAAAT  100

TGGGTGACCACTGAGGGAGTGTTCCACAGTAATGTTGTCTGGTCGCTAGA  150

                    FQ                  PCR Q oligo
TGGCGCGGGTGAGGCCACGCTTTGCGAAAACGAAAGTGCTTGAAAAGGCG  200
CGGGATAGCGTGCGCTACCGGATGGCGGGTAACTTAGGAAGCGTTTCTTG  250

                PCR U oligo/cDNA primer 2
AGCTTCCCTGGGATGAGCGTTTGGGAGAGCTGATTCTGCAGCCCAGAGAG  300
                    U
TAGTCTCAGGGCATCCTCTGGAGCCTGACCTGTGATCGTCGCATCATAGA  350
CCGCCAGTAGACCTGGGAGCAGATTCACCGCCGCGGCCGTCTCCTTTAAG  400
TGTGAATCATGTCTGACGAGGGGCCAGGTACAGGACCTGGAAATGGCCTA  450
                    K                   PCR K oligo
GGAGAGAAGGGAGACACATCTGGACCAGAAGGCTCCGGCGGCAGTGGACC  500
TCAAAGAAGAGGGGGTGATAACCATGGACGAGGACGGGGAAGAGGACGAG  550
GACGAGGAGGCGGAAGACCAGGAGCCCCGGGCGGCTCAGGATCAGGGCCA  600
                                        cDNA primer 1
AGACATAGAGATGGTGTCCGGAGACCCCAAAAACGTCCAAGTTGCATTGG  650
CTGC...
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FIG. 5. Composite nucleotide sequence of the 5′ region of the EBNA-1 transcript present in the Rael cell line. Cloning of the 5′ region of the EBNA-1 transcript was done by using specific cDNA primers followed by anchored-PCR cloning. Positions of the specific cDNA and PCR primers used are indicated by overbars. Exon boundaries are indicated by open triangles, and the respective exons are labeled according to the viral *Bam*HI restriction endonuclease fragment in which they are encoded. The EBNA-1 binding site within the FQ exon is shown in boldface, as is the putative TATAA box at −28 to −24 bp.

In this paper, transcription of the EBNA-1 gene in two group 1 BL cell lines, Akata and Rael, has been examined. In both cases, two EBNA-1 transcripts of ≈2.5 and 5.0 kb were detected, which were clearly distinct in size from the 3.7-kb EBNA-1 transcript present in LCL. In addition, neither of these EBNA-1 transcripts hybridized to a probe containing multiple copies of the W1/W2 exons found at the 5′ end of all EBNA transcripts characterized from LCL (for review see ref. 24). The latter result was further substantiated by the lack of demonstrable Cp or Wp activity in the Rael cell line. The Akata cell line did exhibit low-level Cp activity, but this activity could not account for the level of EBNA-1 transcripts in these cells and, therefore, most likely represents a small percentage of cells in the culture that have drifted to the group 3 phenotype. The striking similarity in the EBNA-1 expression pattern in these two group 1 BL cell lines is consistent with a viral program.

Anchored-PCR cloning the 5′ region of the EBNA-1 transcript produced in the Rael cell line revealed two upstream exons, FQ and U. Notably this upstream exon structure appears the same as that previously observed for an EBNA-1 transcript from an NPC tumor line that expresses EBNA-1 but not the other EBNAs (17). In contrast, the FQ exon has not been found in any of the LCL EBNA cDNA clones analyzed. This result further substantiates a viral program model for exclusive expression of EBNA-1. It should be noted that in the NPC tumor line, LMP-1 and several other viral genes are expressed. With regard to the latter point, in contrast to data obtained in B lymphocytes, LMP-1 expression in NPC appears independent of the expression of EBNA-2.

The sizes of the FQ and U exons (399 nt), in addition to the EBNA-1 coding exon [≈2.2 kb, assuming a 200-nt poly(A) tail], reasonably account for the smaller EBNA-1 transcript

(2.5–2.6 kb) seen in Rael and Akata. At this time, the origin of the larger transcript is unclear. It may reflect a partially spliced heteronuclear contaminant, an alternatively spliced species, readthrough of the poly(A) signal at the end of the EBNA-1 coding exon, or it may be derived from a completely different transcriptional unit.

The 5′ end of the FQ exons lies just downstream of a putative TATAA box, and it is attractive to speculate that this region represents the promoter involved in driving transcription of the EBNA-1 gene in these cells. However, preliminary attempts to demonstrate promoter activity with genomic fragments containing this region have been negative. This result may possibly reflect the fact that we have not yet tested the reporter constructs in the appropriate cell lines (e.g., Rael and Akata, which are difficult to transfect). Alternatively, a distal enhancer may be required for promoter activity. Resolution of this issue would allow definitive identification of the third promoter involved in viral latency.

Interestingly, the region of the FQ exon contains two EBNA-1 binding sites (in addition to those present in *oriP*), one of which is completely contained within the exon and the other of which spans the FQ splice-donor site. Several possible functions for these sites can be envisaged. (*i*) They may be part of an EBNA-1-dependent enhancer that autoregulates Fp in the appropriate cells. Notably the EBNA-1-dependent enhancer in *oriP* is involved in both plasmid replication of EBV and in modulating the activity of Cp (3, 4, 25). (*ii*) They may serve to attenuate (i.e., downregulate) transcription of EBNA-1. This could apply equally to transcripts initiating for Cp or Wp, as well as those initiating from Fp because all these transcriptional units contain the EBNA-1 binding sites in the *Bam*HI Q fragment. (*iii*) They may be involved in regulating splicing. With respect to splicing, it is important to consider that the U exon has been found in EBNA-1 *and* EBNA-3c transcripts from LCL. Thus, in LCL the U exon is alternatively spliced to either the EBNA-1 or -3c coding exons. However, alternative splicing from the U exon does *not* appear to occur in group 1 BL cell lines. The 5′ structure of the primary transcript probably plays a prominent role in dictating the splicing pattern. Indirect evidence from the early events in EBV infection suggests that the switch from Wp to Cp usage alters the splicing pattern of the primary transcripts encoding the EBNA genes (26, 39).

In summary, transcription of the EBNA-1 gene in group 1 BL cells is driven by a transcriptional unit distinct from that used in group 3 BL or LCL. The observation that transcription of the EBNA-1 gene appears very similar in two group 1 BL cell lines and in an NPC line, all of which share the same common phenotype of expressing EBNA-1 but not the other EBNAs, suggests that this expression pattern is programmed. Thus, the full program for the genesis of EBV-associated BL may involve the sequential use of three distinct viral promoters. We propose that in the initial stages of EBV infection of B lymphocytes Wp is active and gives rise to expression of EBNAs-2 and -4. Expression of EBNA-2 facilitates a switch from Wp to Cp usage. Finally, in the appropriate cells via an unknown mechanism, transcription from Cp is downregulated with the concomitant upregulation of EBNA-1 transcription driven from Fp. The latter results in the attenuation of EBNA gene expression with the exception of EBNA-1, thereby providing a mechanism for escape from immune surveillance.

We thank Dr. G. Klein for providing the Rael cell line and Dr. K. Takada for providing the Akata cell line. This research was supported by Grants CA43143 (S.H.S.) and CA7554 (J.L.S.) from the National Institutes of Health and by a Postdoctoral Fellowship to M.W. from the Fonds zur Foerderung der Wissenschaftlichen, Vienna.

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