JOURNAL OF VIROLOGY, Feb. 1992, p. 706–714
0022-538X/92/020706-09$02.00/0

Vol. 66, No. 2

Transcription of the Epstein-Barr Virus Gene EBNA-1 from Different Promoters in Nasopharyngeal Carcinoma and B-Lymphoblastoid Cells

P. R. SMITH* AND BEVERLY E. GRIFFIN

Department of Virology, Royal Postgraduate Medical School, University of London, Du Cane Road, London W12 0NN, United Kingdom

Received 30 July 1991/Accepted 24 October 1991

Transcriptional expression of the Epstein-Barr virus (EBV) genome has been shown to differ markedly between nasopharyngeal carcinoma (NPC) cells and latent B-cell lines, with a more limited pattern of gene expression seen in NPC. EBNA-1 is the only nuclear antigen so far detected in both NPC and Burkitt's lymphoma cells. We found previously that in a human NPC tumor passaged in nude mice, designated C15, the EBNA-1 mRNA contained a novel splice site in the *Bam*HI Q region of EBV which had not previously been described for B-cell lines. This lies within a region of the EBV genome to which EBNA-1 binds. Here, we further characterize the 5′ region of EBNA-1 transcripts and identify two splicing patterns in C15 cells; we show that they are derived from a common promoter region in the *Bam*HI F region of the viral genome. We also demonstrate that this region can function to initiate transcription of the chloramphenicol acetyltransferase gene in epithelial cells and that the promoter region is only partially methylated at CpG sites in the tumor. In contrast, a B-lymphoblastoid cell line derived from C15 uses a conventional promoter in *Bam*HI-C/W for expression of EBNA-1.

Epstein-Barr virus (EBV) is known to infect both mature B cells and squamous epithelial cells. This tissue tropism is reflected in the association of the virus with Burkitt's lymphoma (BL) (15, 21), a B-cell tumor, and nasopharyngeal carcinoma (NPC), a tumor derived from epithelial cells of the nasopharynx (27). EBV can also immortalize B cells in vitro to establish continuously proliferating B-lymphoblastoid cell lines. Analysis of the latter has produced a description of the pattern of latent gene expression associated with the establishment and maintenance of B-cell lines (6, 7, 14). Viral gene expression in such cells is apparently limited to that of six nuclear proteins, or EBNAs, and three membrane proteins, latent membrane protein and terminal proteins 1 and 2. Two small RNA species, EBERs, are also abundantly transcribed. Although little is known as yet of the function of most of these proteins, EBNA-1 has been shown to be involved in the maintenance of the EBV genome as an extrachromosomal episome (34). It has been found to bind to three regions of the EBV genome (13, 17), two in *Bam*HI-C, in the region (ori P) responsible for the maintenance of the EBV episome (19, 34) and for transactivation from a *Bam*HI C promoter (18, 32), and a third (of lower affinity) in the *Bam*HI Q region, for which no function has been identified (13).

In contrast to B-lymphoblastoid cell lines, in both of the EBV-associated tumors an even more limited pattern of EBNA expression is observed. To date, only EBNA-1, of the six EBNAs, has been observed in BL cells (20, 22). In NPC, in addition to EBNA-1, latent membrane protein can be found in up to two-thirds or more of the tumors examined (8, 16, 35), and recently we (11) and others (10) reported the occurrence in NPC cells of large amounts of a family of hitherto unidentified polyadenylated transcripts, although no protein product has yet been associated with the latter.

Transcription of the EBNA mRNAs has been shown earlier to be initiated from one of two promoter regions in the *Bam*HI W (24–26, 29, 30) or C (3, 4) region of the EBV genome. The selective use of these promoters depends on the cell line and also the differentiation state of the cell. It appears that, on infection of a B cell, the *Bam*HI W promoter is used first to initiate EBNA synthesis and that products of this promoter may then transactivate the *Bam*HI C promoter (33). Alternative splicing of an initial large primary transcript results in the various EBNA mRNAs (reviewed in reference 31). The splicing patterns of the EBNA messages in lymphoblastoid cell lines are complex, most EBNA-1 transcripts described to date originating in either the *Bam*HI W or C region and splicing into the *Bam*HI W repeat region to produce identical exons from this region, the precise number dependent on the copy number of W repeats in the viral genome. Although several alternate splicing patterns have so far been described for EBNA-1 transcripts, all seem to contain sequences in *Bam*HI-U that are then spliced onto the EBNA-1 open reading frame (ORF) in *Bam*HI-K.

Recently we presented data on a cDNA isolated from an NPC cDNA library which predicted a different splicing pattern in the transcript for EBNA-1 in the tumor. In this case, an exon from *Bam*HI-Q was identified, as well as those from *Bam*HI-U and -K, the latter being identical to those described previously for B-cell EBNA-1 transcripts (11). The position of the donor splice site in *Bam*HI-Q was within the reported EBNA-1 DNA binding region III (17). This observation raised the possibility that in NPC cells, EBNA-1 transcription arises from a different promoter and that EBNA-1 itself could play a role in controlling transcription or splicing of other EBNA species. In this study, we have further characterized the 5′ structure of the EBNA-1 transcript in the epithelial tumor, showing that two alternative splice sites exist in *Bam*HI-Q and that the EBNA-1 mRNA in C15 cells originate from a promoter region in the *Bam*HI

* Corresponding author.

F fragment of the EBV genome. We have also shown that the upstream region in *Bam*HI-F can act as a promoter to initiate chloramphenicol acetyltransferase (CAT) gene transcription in epithelial cells. This promoter does not, however, appear to be active in a B-lymphoblastoid lines generated with virus derived from the C15 tumor.

MATERIALS AND METHODS

Cells. C15 is a human NPC tumor of North African origin which can be passaged in nude mice (5). C17 and C18 are also human NPC tumors that are propagated in nude mice; they were originally derived from metastatic sites rather than primary lesions (5). NAD-C15-STO-B (referred to subsequently as NAD-C15) is a B-lymphoblastoid cell line derived from cocultivation of normal adult B cells with the C15 tumor. M-ABA is a B-lymphoblastoid cell line derived from an NPC patient, and B95-8 is an EBV-infected marmoset cell line which is permissive for viral replication. IB-4 is a latent lymphoblastoid cell line. Ramos is an EBV-negative BL cell line. All cell lines were propagated in RPMI 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine. The HEp-2 cell line, derived from a human carcinoma of the larynx, was maintained in Dulbecco modified Eagle medium supplemented with 5% fetal calf serum and 2 mM glutamine.

DNA clones. The initial EBNA-1 cDNA clone (designated K2) was isolated from a λgt10 cDNA library as described previously (11). Subclones of EBV sequences were generated by standard techniques (23) from an EBV *Bam*HI library derived from B95-8 cells or by polymerase chain reaction (PCR) techniques (see below).

RNA extraction and Northern (RNA) blotting. Poly(A)$^+$ RNA was isolated either by the guanidinium-cesium chloride method followed by oligo(dT) chromatography or by using a Quik-prep (Pharmacia) mRNA extraction kit according to the manufacturer's instructions. For Northern blotting analysis, approximately 5 μg of poly(A)$^+$ RNA was loaded onto a 1% agarose gel containing 0.7% formaldehyde and 5 mM iodoacetamide and separated by electrophoresis. RNA was transferred to Bio-Dyne membranes (Pall) by capillary transfer. The Northern blots were probed with oligonucleotides, labelled at the 5′ termini with T4 polynucleotide kinase, that react with the glycine/alanine repeat region of the EBNA-1 ORF. Oligonucleotides used to probe for EBNA-1 sequences were 5′-TCCTCCTGCTCCTGCCCCTC-3′ and 5′-CTGCTCCTGCCCCTCCTGCT-3′. Blots were probed according to the method described by Hitt et al. (11) except that following hybridization, the membranes were washed at 60°C.

5′ RACE analysis of EBNA-1 transcripts. The 5′ regions of EBNA-1 transcripts were analyzed using the RACE (rapid amplification of cDNA ends) protocol of Frohman et al. (9). Briefly, 1 μg of poly(A)$^+$ RNA in a volume of 20 μl was digested for 10 min at 37°C with DNase; the RNA was then heated to 65°C for 10 min and cooled on ice. Next, 5 μl of 5× reverse transcription buffer (1× transcription buffer contains 50 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, and each deoxynucleoside triphosphate at a concentration of 1.5 mM) and 12 U of RNasin (Promega) were added, and the mixture was incubated for 60 min at 37°C and for 30 min at 52°C. Excess priming oligonucleotides were removed by centrifugation through a spun column (Pharmacia); the cDNA mixture was then dried under vacuum and resuspended in 23 μl of water. Then 1 μl of 6 mM dATP, 6 μl of 5× tailing buffer (Bethesda Research Laboratories), and 15 U of terminal deoxynucleotidyltransferase were added; the solution was incubated for 10 min at 37°C and heated at 65°C for 10 min before being diluted to 200 μl. A 10-μl sample of this solution was then routinely used in PCR amplification.

Amplification of cDNA products was performed by using a specific internal oligonucleotide, from either the 5′ region of the EBNA-1 ORF (oligonucleotide EBL; 5′-TGTTCCAC CGTGGGTCCCTTT-3′; position 108216 on the B95-8 EBV genome [2]) or the *Bam*HI U splice region (oligonucleotide EBU; 5′-GCGAAGCTTGGATGCCCTGAGACTACTCT-3′; position 67565 on the EBV genome), together with a dT_{17} adaptor oligonucleotide, 5′-GACTCGAGTCGACATCGAT TTTTTTTTTTTTTTT-3′, and a corresponding adaptor oligonucleotide lacking the T_{17} sequence. The reactions were amplified in a volume of 50 μl according to the manufacturer's conditions supplied with the *Taq* polymerase (Amplitaq; Cetus). The reaction mixtures were incubated at 94°C for 2 min and then at 72°C for 2 min prior to addition of the enzyme; the samples were incubated at 52°C for 5 min and at 72°C for 40 min before being subjected to 40 cycles of 94°C for 40 s, 52°C for 1 min, and 72°C for 3 min. The reactions were then analyzed for specific EBNA-1 amplification by Southern blotting and hybridization with a *Bam*HI-Q probe. Positive fractions were eluted from the gel and either cloned into Bluescript vectors at *Hin*dIII-*Cla*I sites or reamplified and then cloned. The latter were recloned into M13 vectors and sequenced according to standard protocols.

RNase protection experiments. The method used was essentially that described by Sambrook et al. (23). Briefly, a single-stranded ^{32}P-radiolabelled RNA probe containing the QU splice sequence, obtained from a RACE clone, was hybridized to 1 μg of C15 mRNA overnight at 45°C in a volume of 30 μl. The hybrid was then digested at 37°C with 300 μl of RNase digestion mixture containing 300 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 2 μg of RNase T_1 per ml, and 40 μg of RNase A per ml. The samples were then digested with proteinase K, extracted with phenol-chloroform, and precipitated with ethanol. The pellet was resuspended in sample buffer, separated on a 7.5% polyacrylamide sequencing gel, dried, and subjected to autoradiography.

Mapping of the 5′ region of EBNA-1 by PCR. A number of oligonucleotides upstream of the most 5′ sequences identified by the RACE protocol were synthesized and used in conjunction with both the *Bam*HI-U-specific oligomer described above and oligonucleotide EBQ1 (5′-GGCAAGCT TGAAGCACCCCCAT-3′; position 62511) to map the 5′ end of the EBNA-1 transcript. The oligonucleotides used, from left to right on the conventional EBV genome, were as follows: EBA, 5′-GGTTCGACTTGAAAAGGCGCGGG AT-3′ (position 62420); EBB, 5′-GGGTCGACTGTCTGGTC GCTAGAT-3′ (position 62367); EBC, 5′-AGGTCGACCC TCCGT-3′ (position 62278); and EBF5, 5′-CGGGATCCGT CTCTCGAGGTCT-3′ (position 62098).

For each reaction, 10 μl of the RACE product, obtained from poly(A)$^+$ RNA which had been digested with DNase and primed with oligonucleotides from *Bam*HI-K or *Bam*HI-U, was used as the starting material. The reaction conditions were those described above except that 30 cycles only were used and the extension time was decreased to 1 min. Following PCR, the samples were analyzed by Southern blotting for the presence of specific products.

Primer extension. An oligonucleotide covering the previously observed QU splice sequence was labelled with [γ-^{32}P]ATP and hybridized overnight at 45°C to poly(A)$^+$

RNA (1 μg). The hybrid was precipitated with ethanol on dry ice for 10 min and resuspended in 10 μl of 1× reverse transcriptase buffer (see above); 10 U of reverse transcriptase was added, and the reaction mixture was incubated for 2 h at 45°C. The extended products were precipitated at −70°C for 1 h and separated on a 7.5% sequencing gel.

CAT promoter analysis. The upstream region of the putative EBNA-1 promoter was cloned into CAT vectors to assess the ability of this region to function as a promoter. For this analysis, *Bam*HI-F EBV DNA was removed from plasmid DNA by digestion with *Bam*HI and the single-stranded ends were filled in with the Klenow fragment of DNA polymerase according to standard protocols. The fragment so obtained was digested with *Sph*I and cloned into blunt-ended *Sph*I basic CAT vectors (Promega Biotec) to generate plasmid E1 956. Alternatively, a fragment of 135 bp (coordinates 621002 to 62249) was generated by PCR and cloned into an *Xba*I-*Sal*I basic CAT vector (E1 135).

Plasmid DNAs were transfected into HEp-2 cells by using calcium phosphate, and CAT activity (analyzed by xylene extraction followed by liquid scintillation counting) was assessed after 24 or 48 h in culture following transfection. Results were calculated from duplicate measurements of four separate transfection experiments and expressed either as units of CAT activity per microliter of extract, as measured against a standard curve using purified CAT enzyme (Promega), or as a percentage of the activity of a control CAT plasmid containing the simian virus 40 promoter and enhancer elements (Promega).

Promoter usage in the NAD-C15 cell line. A PCR protocol was established and used to assess promoter usage in the NAD-C15 B-cell line, which was derived from the C15 tumor. This involved probing RACE products from either C15 or NAD-C15 RNA primed with an oligonucleotide from the *Bam*HI U exon (EBU) with either *Bam*HI-Q or *Bam*HI-W. Alternatively, the same oligonucleotides used to prime and reverse transcribe from either C15 or NAD-C15 mRNA, as described above, were used in conjunction with oligonucleotides specific for either the *Bam*HI Q or *Bam*HI W exon in a PCR reaction. Samples were separated on agarose gels and analyzed as described above.

DNA methylation. Total cellular DNA was digested with either *Msp*I or its methylation-sensitive isoschizomer, *Hpa*II. In each case, cellular DNA (20 μg) was digested overnight with 10 U of enzyme per μg of DNA. The digested DNA was analyzed by Southern blotting, using probes specific for the EBNA-1 DNA binding region in *Bam*HI-Q, a 313-bp fragment generated by *Bam*HI-*Rsa*I digestion of *Bam*HI-Q (EBV coordinates 62249 to 62562), or a 490-bp fragment covering the putative EBNA-1 promoter region, generated by *Rsa*I digestion of *Bam*HI-F (coordinates 61684 to 62174).

RESULTS

Northern blotting. Northern blot analyses of several cell lines, using specific ^{32}P-labelled oligonucleotides, were used to identify EBNA-1 transcripts (Fig. 1). The sizes of transcripts, which were up to 5 kb, varied among the cell lines examined, probably because of a combination of (i) differences in the number of *Bam*HI W repetitive elements in the virion, and hence in the number of corresponding *Bam*HI W exons in the mRNA, and (ii) the number of repetitive sequences (specifying glycine/alanine) found in the EBNA-1 ORF. Data on the variation in sizes of EBNA-1 antigens in different cell lines have already been correlated with variations in the numbers of glycine/alanine repeats. The EBNA-1 transcript in C15 (Fig. 1, lane A) was much smaller (ca. 2.5 kb) than the transcript (ca. 4.8 kb) identified in NAD-C15 cells (Fig. 1, lane B). This difference could not easily be ascribed to variations in the numbers of repeats, however, as the cells contain the same virus. The alternative, and most likely, explanation for the observed size differences between these two transcripts was that the C15 mRNA differed in its 5′ structure. Another B-lymphoblastoid cell line, M-ABA (Fig. 1, lane C), also derived from virus from an NPC, shows a similarly large EBNA-1 transcript (approximately 5.0 kb). These findings, together with our previous observation of a novel splice in *Bam*HI-Q in EBNA-1 transcripts from C15 (11), suggested that the promoter for EBNA-1 could be different in the NPC and lymphoblastoid cells. (Several minor bands seen in Northern blots in all cell lines analyzed were also identified in Ramos cells [Fig. 1, lane D, upon longer exposure of the film] and are thus presumably due to cross-hybridization with cellular sequences that correspond to the G/C-rich [glycine/alanine] repetitive sequence.)

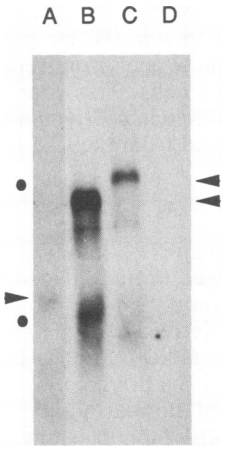

FIG. 1. Northern blots probed with an oligonucleotide from the sequence encoding the glycine/alanine repeats of the EBNA-1 ORF showing different-sized EBNA-1 mRNAs: C15 (lane A), NAD-C15 (lane B), C, M-ABA (lane C), and Ramos (lane D). The arrowheads indicate the positions of the specific EBNA-1 bands in three of the lanes; dots show the positions of the 28S and 18S rRNA bands at approximately 5.0 and 2.0 kb.

Mapping of the 5′ promoter region and splice sites in EBNA-1 transcripts. (i) By RACE analysis. Using the RACE protocol, we isolated a number of cDNA clones from C15 and from another NPC tumor, C17. However, no clones appeared to extend to the 5′ promoter region. That is, the clones isolated appeared to be too small to correlate with the Northern data (Fig. 1), and although not a strict requirement for transcription, there did not appear to be a TATA consensus sequence in the region immediately upstream of the 5′ end of any cDNA clones analyzed. However, sequencing a number of RACE clones did identify, in addition to the previously described *Bam*HI QU splice (11), a second (different) splice site in *Bam*HI-Q (subsequently called the Q′U splice). This splices from *Bam*HI-Q at position 62536 (2) into the identical site previously observed with regard to *Bam*HI-U (Fig. 2). Of seven clones sequenced, five were of the QU type and two were of the Q′U type. The results obtained with the RACE protocol are shown schematically in Fig. 2. A number of clones with the Q′U splice sequence, isolated from both the C15 and C17 tumors, all finished at an

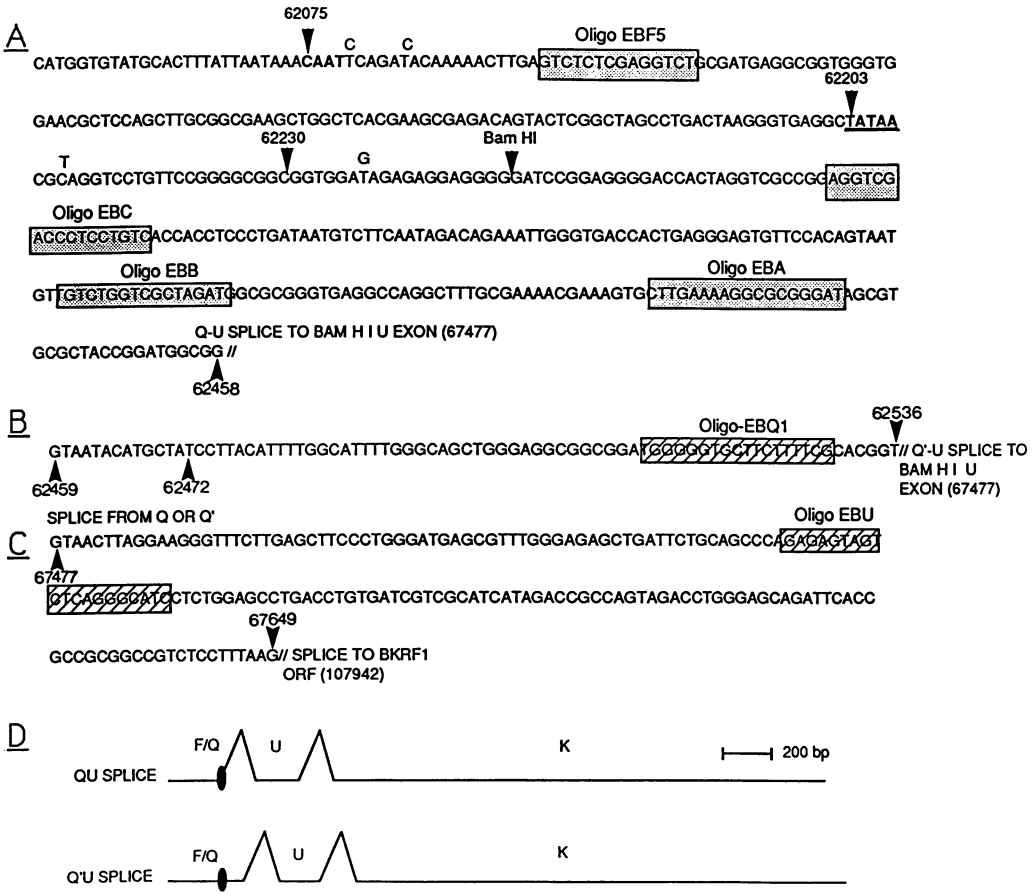

FIG. 2. Sequences of the promoter and other regions associated with EBNA-1 transcripts. (A) Sequence upstream from the QU splice at position 62459, putative TATA box at position 62203 (underlined), and possible CAAT box sequence at position 62075 in the EBV genome; (B) additional sequence from the Q'U splice at position 62536; (C) sequence of the *Bam*HI U exon; (D) schematic diagram of the EBNA-1 mRNAs in C15. The location of the DNA binding site III ( ) is indicated. Boxed regions identify the positions of oligonucleotides used to map the promoter and initiation sites of EBNA-1 transcripts. Stippled boxes show oligomers 5'-3' (left-to-right orientation), and hatched boxes show oligomers 5'-3' (right to left). Base changes identified in the C15 sequence are indicated by letters placed above the DNA sequence of the B95-8 genome (2).

identical base (position 62472), suggesting that this could represent the 5' end for this RNA species, or alternatively that there is some secondary structure in the RNA which blocks the progression of the enzyme beyond this point. In support of the latter suggestion, initial RNase protection studies showed this region to be extremely insensitive to RNase digestion. (Examination of the sequence between positions 62430 and 62476 shows a remarkable degree of potential intrastrand secondary structure.)

(ii) By RNase protection analysis. The RACE analysis, followed by product cloning and DNA sequence analysis, showed that mRNAs with alternative splices exist for EBNA-1 in the C15 and C17 tumors (Fig. 2D). Numerically, the recombinant DNA clones obtained suggested that one of the two (the QU spliced form) was the more abundant of the two mRNAs. To investigate this question further, we carried out RNase protection experiments using a segment of radiolabelled RNA that encompassed both splices and conditions that resulted in digestion of single-stranded RNA (23). The data obtained were consistent with those from the RACE analysis, showing the Q'U RNA (and possibly a third unidentified RNA) to be present at lower levels than the QU spliced form (Fig. 3).

(iii) By PCR. As the RACE procedure did not appear to extend completely to the 5' end of the EBNA-1 messages, PCR was used to amplify regions around putative TATA sequences upstream of the 5' end of the RACE products generated in the analysis described above. The results obtained (Fig. 4) with use of an oligonucleotide (EBQ1; Fig. 2B) located in the Q'U spliced form of EBNA-1 mRNA, in conjunction with specific 5' oligonucleotides (for positions, see Fig. 2A), showed that it was possible to amplify sequences up to position 62279 (oligomer EBC). No amplification was seen with oligomer EBF5 (position 62098), indicating that the EBNA-1 mRNA in C15 could be initiated from a sequence between these coordinates. As a control, C15 DNA used as a substrate for PCR showed that the oligonucleotide pair EBF5/EBQ1 could amplify specific sequences over this region, indicating that the lack of amplification was not due to structures in the EBV sequence which were difficult to copy (data not given). The only TATA box in this region of the genome is found at position 62203, suggesting that this sequence might be used to initiate transcription of EBNA-1 messages. Use of oligonucleotides around this sequence, in particular oligonucleotides designated EBD (62238 to 62254) and EBE (62180 to 62197), in

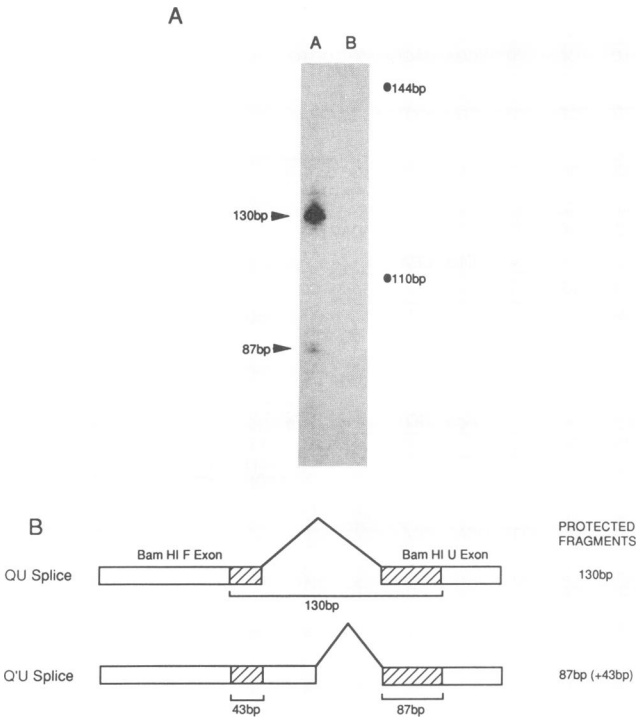

FIG. 3. RNase protection experiment showing relative amounts of two alternate spliced EBNA-1 mRNAs. (A) Pattern obtained when a 130-base fragment containing the QU spliced form of EBNA-1 mRNA was used to protect C15 poly(A)$^+$ mRNA. Lane A shows a fully protected 130-bp fragment and a smaller band at approximately 87 bp, which corresponds to the protected U sequence found in the Q'U spliced form of EBNA-1 mRNA; lane B shows the results obtained when C15 mRNA was omitted from the digest and complete digestion of the probe was obtained. The other (43-bp) fragment which should also be protected could not be observed on this gel. Reciprocal experiments using the Q'U spliced form were not possible, as an RNA probe containing this sequence could not be obtained. (B) Schematic view of the two spliced forms of EBNA-1 transcripts showing the protected regions (hatched).

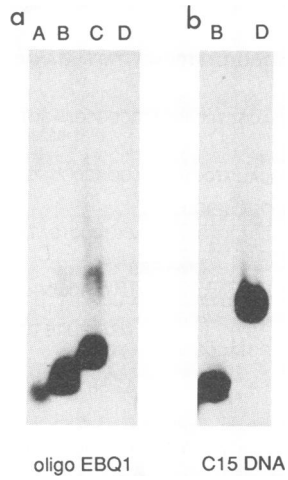

FIG. 4. PCR mapping of the 5' region of the EBNA-1 promoter in C15 cells. The initial oligonucleotide used to prime from RNA (a) was EBQ1 (position shown in Fig. 2). Panel b contains PCR products from C15 DNA, as noted. The Southern blots were probed with a 313-bp *Bam*HI-*Rsa*I fragment from the 5' end of the *Bam*HI Q fragment of the EBV genome (coordinates 62249 to 62562). The 5' oligonucleotides used in conjunction with EBQ (positions shown in Fig. 2) are oligomers EBA (lane A), EBB (lanes B), EBC (lane C), and EBF5 (lanes D).

PCR with EBQ1 (Fig. 2) produced results consistent with this hypothesis, the former being positive and the latter being negative, in preliminary experiments (data not shown). Other experiments using the EBU oligonucleotide (Fig. 2C) in the *Bam*HI U exon as a primer gave similar findings. Results from the PCR analysis (Fig. 4) cannot exclude the possibility that all sequences amplified are derived from a QU-type message and that the Q'U message identified by the RACE protocol contains a different 5' region. We have no data to support this notion, however, and at present all results are consistent with the conclusion that both types of EBNA-1 mRNAs in C15 (QU and Q'U spliced forms) initiate from the same promoter sequence. The sizes of the transcripts initiating from the TATAA sequence at position 62203 would be consistent with the Northern data (Fig. 1).

(iv) By primer extension. To confirm the location of the 5' initiation site of EBNA-1 in C15, primer extension analysis was used. Priming of C15 mRNA with an oligonucleotide covering the QU splice site (5'-TACCGGATGGCGGGT AATACAT; eight bases in the U exon and 13 bases in the Q exon) yielded extended products, the largest of which was about 230 bp (Fig. 5). This would locate the end of the message at approximately nucleotide 62235, that is, 32 bp 3' to the putative TATA site. This procedure provides evidence consistent with the TATAA box at nucleotide 62203 being the sequence used to initiate transcription of EBNA-1 in C15 cells. No bands of similar sizes were seen in IB-4 cells, a B-lymphoblastoid cell line, implying that this promoter is not used in this latent B-cell line. In similar experiments, priming from sequences in the *Bam*HI-U exon identified bands of higher molecular weight, consistent with sizes predicted for

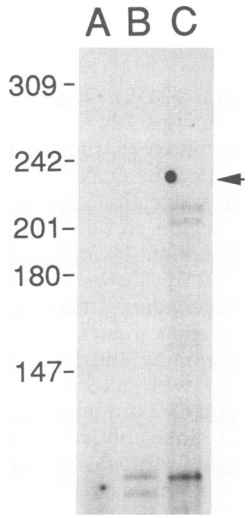

FIG. 5. Primer extension analysis of the 5' region of the EBNA-1 promoter in C15. Lanes: A, *Escherichia coli* tRNA; B, IB-4 mRNA; C, C15 mRNA. The oligonucleotide used to prime from the RNA was positioned across the QU splice. The size of the largest extended product was approximately 230 bp, indicating that the 5' end of the transcript is located at approximately nucleotide 62235 in the EBV B95-8 genome.

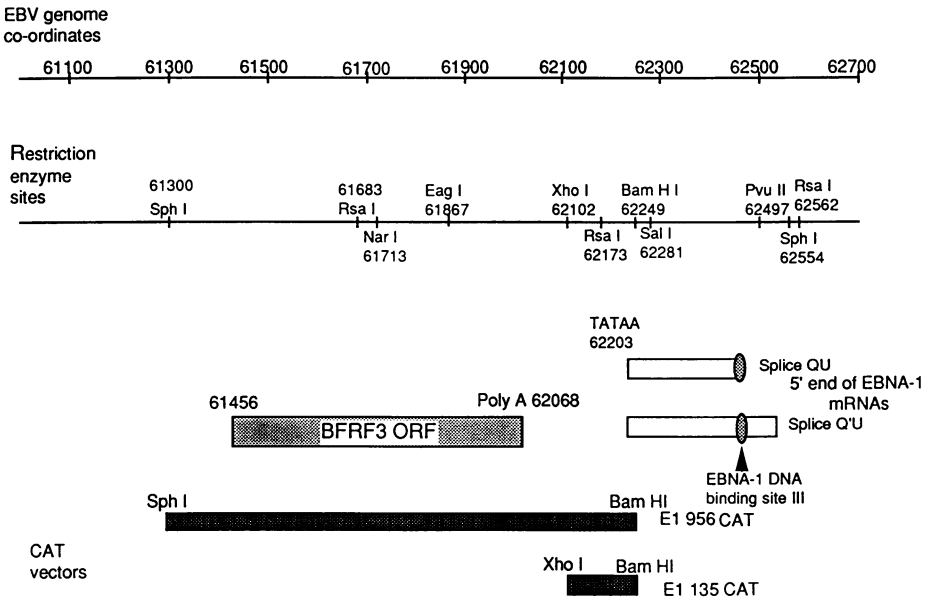

FIG. 6. Positions of clones E1 956 CAT and E1 135 CAT used in CAT assays for functional analysis of the C15 promoter relative to the EBV genome, the EBNA-1 transcripts, and the BFRF3 ORF.

the alternate spliced mRNAs (QU and Q'U forms) if they both originated from the same region in C15. Figure 2D illustrates schematically the structures of the EBNA-1 mRNAs in C15, as derived from data obtained with the experiments described above.

CAT assays. To confirm that the region upstream from nucleotide 62203 (Fig. 2) can function to initiate transcription, a series of constructions 5′ to the putative promoter site were cloned into CAT vectors (Fig. 6). These constructs were assessed for their ability to initiate transcription of the CAT gene in epithelial cells in standard assays. The results (Table 1) show that in the cells (HEp-2) examined, both constructions E1 956 and E1 135, containing upstream regions of the putative EBNA-1 promoter, could function in this assay. The levels of CAT synthesis in cells transfected with the larger construct (E1 956) were, however, consistently lower than those obtained with the small E1 135 CAT construction. This result is probably due to the presence of an ORF in the E1 956 plasmid (BFRF3) which may interfere with translation from the EBNA-1 promoter region and therefore lower the efficiency of the CAT translation in the cells used. BFRF3 is not transcribed in C15 cells.

EBNA-1 promoter usage in different cells. Northern blotting studies showed that there was a large difference in size between the EBNA-1 mRNAs from C15 and the B-cell line derived from the tumor NAD-C15, implying that the messages are initiated from different promoters. To confirm this finding, appropriate PCR analyses were performed. The results (Fig. 7) confirm that in the C15 tumor (lanes A and B), EBNA-1 mRNA is not apparently transcribed from the promoter regions in *Bam*HI-W or -C. In contrast, the EBNA-1 mRNA in NAD-C15 was not identified with oligomers derived from *Bam*HI-Q but was identified with sequences from *Bam*HI-W (lane D), implying that in the B cells, transcription initiates from previously described EBNA promoter regions in *Bam*HI-W (or -C).

DNA methylation. Methylation of CpG sequences is thought to be one means by which promoter regions are maintained in an "off" position, although whether the methylation is the initial switch or merely a consequence of the process is still unclear. Use of methylation sensitive/insensitive restriction enzyme isoschizomers, such as *Hpa*II/*Msp*I, can show the extent to which regions are methylated

TABLE 1. CAT activity induced by the C15 EBNA-1 promoter in HEp-2 cells

Vector	CAT activity[a]	
	U (10^3)/μl of extract[b]	As % of control CAT plasmid activity
Basic CAT vector	0	0
Control CAT vector	8.5	100
E1 956	0.7	8.2
E1 135	1.14	13.4

[a] Results are means of four separate transfection experiments; each assay was performed in duplicate.
[b] Calculated from a standard curve using purified CAT enzyme.

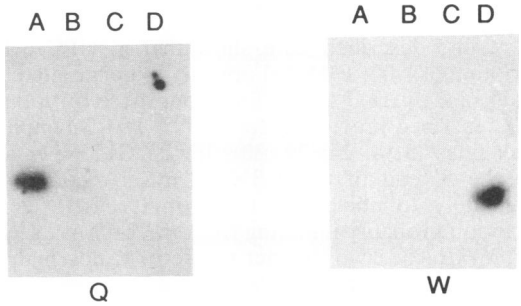

FIG. 7. Use of different EBNA-1 promoter regions in C15 and NAD-C15 cells as assessed by RACE. Lanes: A, products primed from the QU splice of C15 mRNA; B, products primed from the U exon of C15 mRNA; C, products primed from QU splice of NAD-C15 mRNA; D, products primed from the U exon of NAD-C15 mRNA. The Southern blots were probed with the ^{32}P-labelled EBV *Bam*HI fragments indicated below the panels.

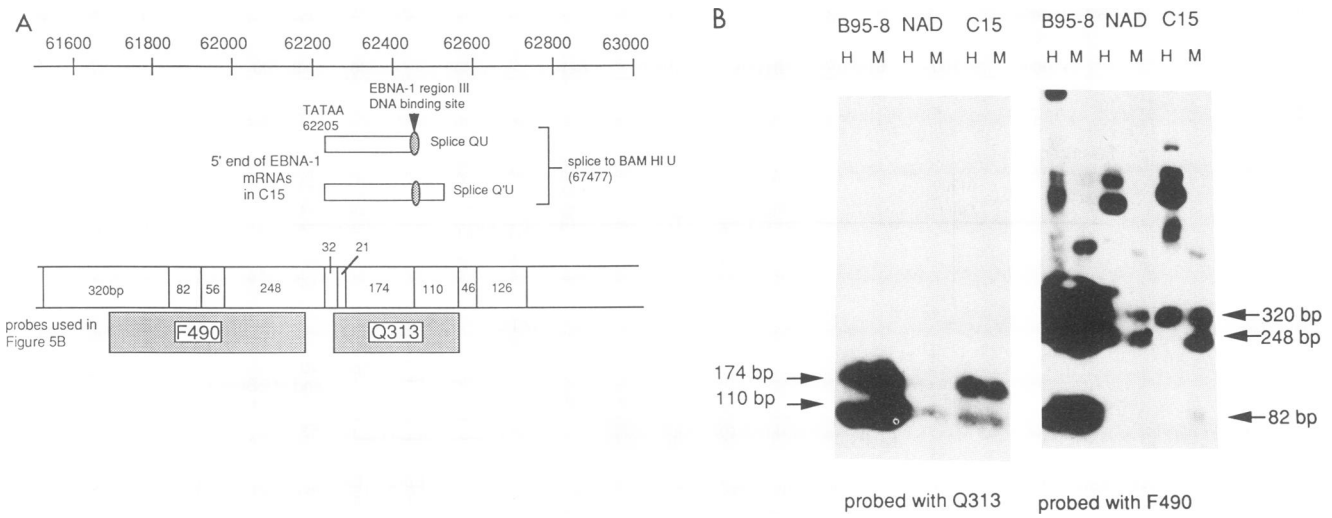

FIG. 8. (A) Positions of *Msp*I sites around the EBNA-1 promoter region in C15 and positions of probes F490 and Q313, used for Fig. 5, lane B. (B) Patterns of DNA methylation as assessed by *Hpa*II or *Msp*I digestion of cellular DNA. H, *Hpa*II-digested DNA; M, *Msp*I-digested DNA isolated from cell lines, as indicated. Sizes of expected restriction enzyme fragments are noted by arrows.

and hence provide some notion of the likelihood for transcription being obtained over any prescribed region. Figure 8A shows the predicted *Msp*I restriction pattern around the EBNA-1 promoter region and the region III EBNA-1 DNA binding site in C15. Figure 8B shows the patterns obtained following hybridization with probes shown in Fig. 8A. The results with the Q313 probe (Fig. 8A) show that the region containing the EBNA-1 DNA binding site is unmethylated, in striking contrast to the results obtained with the F490 probe, which covers the transcription initiation site and upstream sequences. The results obtained with the latter, in which a 320-bp fragment is demonstrated to be present, suggest that the sites around a potential 56-bp cleavage fragment are methylated. The data on sequences upstream of this are ambiguous, however. The methylation status in this region is obviously complex and is being investigated in greater detail by using DNase protection and methylation interference assays. These experiments imply, however, that the EBNA-1 DNA binding region III could be important in the control of transcription not only in epithelial cells but also in B cells.

DISCUSSION

The results described here show that in NPC cells the transcription of EBV EBNA-1 appears to be initiated from a promoter in *Bam*HI-F that differs from those (in *Bam*HI-W and -C) described previously for EBNA-1 in B-lymphoblastoid cell lines (3, 4, 24, 29, 30). In the C15 NPC-derived tumor, the 5′ end of the EBNA-1 mRNA corresponded approximately to nucleotide 62235 in the EBV genome, transcription probably initiating from the TATAA sequence at position 62203. Two distinct transcripts, designated QU and Q′U, may initiate from this promoter, the QU spliced form of the mRNA appearing to be the more abundant of the two forms in the C15 and C17 NPC-derived tumors. The significance of two different splice sites in *Bam*HI-Q, both encompassing an EBNA-1 DNA III binding region, are difficult to explain at this time, although not unusual since multiple spliced forms of EBNA-1 transcripts have been described in B cells (reviewed in reference 3). In another group of mRNAs found in NPC and transcribed across the *Bam*HI A region, different spliced patterns for related mRNAs have also been described (11).

Analysis of the presumed C15 EBNA-1 promoter region showed that it was functionally active in CAT assays in epithelial cells. The lower activity observed with a large plasmid, E1 956, than with a smaller plasmid CAT construction, E1 135, is probably due to the presence of a second ORF (BFRF3) in the former, which interferes with transcription/translation from the EBNA-1 promoter region in HEp-2 cells. This ORF is not transcribed in C15 cells, but its deletion (plasmid E1 135) increased the CAT activity observed following transfection. We are at present investigating the activity of the *Bam*HI F EBNA-1 promoter in a range of cell types. Our evidence suggests that it is not a major promoter in the B-lymphoblastoid cell line NAD-C15.

EBNA-1 has been shown to be a DNA-binding protein that binds to three specific sites in the EBV genome, two (I and II) designated high affinity and one (III, in *Bam*HI-Q) designated low affinity (13, 17). Recent reports show that EBNA-1 contains both a modified leucine zipper sequence and a possible helix-turn-helix region, characteristic of DNA-binding proteins (1, 12). EBNA-1 has also been shown to bind DNA as a homodimer in vitro (1). However, from our methylation data, the possibility must be raised that EBNA-1 may also form a dimer with a cellular protein(s), to control transcription from the F promoter in NPC cells. This hypothesis could account for the fact that in C15, as well as in B95-8 cells, the low-affinity DNA binding site III in *Bam*HI-Q is unmethylated, and as such could bind a protein (or be protected from methylases by a bound protein. It is of interest that the sequence around the *Bam*HI FQ splice site permits formation of a fairly stable hairpin loop). In cells that use the *Bam*HI W promoter, lack of specific EBNA-1-binding proteins or, alternatively, a specific EBNA-1 inhibitory protein may be present, which would then direct transcription from the *Bam*HI W (or *Bam*HI C) promoter rather than the F promoter used in epithelial cells.

The significance of the existence of an exon in the EBNA-1 message which maps to the region of the genome hitherto identified as a low-affinity EBNA-1 DNA binding

region cannot at present be explained. It is possible that EBNA-1 itself is controlling transcription from the F promoter or, alternatively, that it may be controlling the splicing of the pre-mRNA. It is noteworthy that none of the other EBNAs (EBNA-2 to -6) are expressed in the C15 tumor (8, 11, 35). These species may be transcribed from the W or C promoter only, and the latter be inactive in epithelial cells. Alternatively, EBNA-3, -4, and -6, at least, which could be initiated from the F promoter (as the reading frames for these proteins are found downstream of this promoter), may be dependent on cell differentiation for expression and therefore not observed in the undifferentiated C15 tumor.

EBV is known to be expressed in at least two main cell types, in squamous epithelial cells and mature B cells. There is mounting evidence to suggest that in epithelial cells, the control of viral transcription is markedly different from that previously described for B-lymphoblastoid lines. For example, in addition to the results for EBNA-1 presented here, we have recently shown that in C15 cells transcription of terminal proteins is restricted to the expression of terminal protein 2 only (28). Also, latent membrane protein appears to initiate from an alternative promoter in C15, in addition to that previously described in B cells (10). It is apparent that transcription of EBV is complex and that in different cell types, and even in different stages of differentiation of the same cells, the control of EBV transcription may differ. Further investigation of the role of EBNA-1 in the regulation of transcription in NPC, and the factors that determine which promoter is used in the different cell types, may serve as a model for understanding the modulation of EBV gene expression in diverse cellular environments.

ACKNOWLEDGMENTS

This work was supported by the Cancer Research Campaign of Great Britain.

We thank Y. Gao for assistance with some of the work and M. Jones and D. Snudden for helpful discussions. We thank P. Busson and T. Turz for initial gifts of tumor materials and I. Ernberg for NAD-C15 cells.

ADDENDUM

After this report was submitted, two reports appeared in the literature on the use of an alternative promoter for EBNA-1 in group I Burkitt's lymphoma cells corresponding to the promoter identified here (22a, 26a).

REFERENCES

1. **Ambinder, R. F., M. Mullen, Y.-N. Chang, G. S. Hayward, and S. D. Hayward.** 1991. Functional domain of Epstein-Barr virus nuclear antigen EBNA-1. J. Virol. **65**:1466–1478.
2. **Baer, R., R. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tufnell, and G. B. Barrell.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310**:207–211.
3. **Bodescot, M., and M. Perricaudet.** 1986. Epstein-Barr virus mRNAs produced by alternative splicing. Nucleic Acids Res. **14**:7103–7114.
4. **Bodescot, M., M. Perricaudet, and P. J. Farrell.** 1987. A promoter for the highly spliced EBNA family of RNAs of Epstein-Barr virus. J. Virol. **61**:3424–3430.
5. **Busson, P., G. Ganem, P. Flores, F. Mugneret, B. Clauss, B. Caillou, K. Braham, H. Wakasugi, M. Lipinski, and T. Tursz.** 1988. Establishment and characterization of three transplantable EBV-containing nasopharyngeal carcinomas. Int. J. Cancer **42**:599–606.
6. **Dambaugh, T., K. Hennessy, S. Fennewald, and E. Kieff.** 1986. The EBV genome and its expression in latent infection, p. 13–45. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus: recent advances. John Wiley & Sons, New York.
7. **Dillner, J., and B. Kallin.** 1988. The Epstein-Barr virus proteins. Adv. Cancer Res. **50**:95–158.
8. **Fåhreus, R., L.-F. Hu, I. Ernberg, J. Finke, M. Rowe, G. Klein, K. Falk, E. Nilsson, M. Yadav, P. Busson, T. Tursz, and B. Kallin.** 1988. Expression of Epstein-Barr virus encoded proteins in nasopharyngeal carcinoma. Int. J. Cancer **42**:329–338.
9. **Frohman, M. A., M. K. Dush, and G. R. Martin.** 1988. Rapid production of full length cDNAs from rare transcripts. Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85**:8998–9002.
10. **Gilligan, K., H. Sato, P. Rajadurai, P. Busson, L. S. Young, A. B. Rickinson, T. Tursz, and N. Raab-Traub.** 1990. Novel transcription from the Epstein-Barr virus terminal *Eco*RI fragment, DI Jhet, in a nasopharyngeal carcinoma. J. Virol. **64**: 4948–4956.
11. **Hitt, M. M., M. J. Allday, T. Hara, L. Karran, M. D. Jones, P. Busson, T. Tursz, I. Ernberg, and B. E. Griffin.** 1989. EBV gene expression in an NPC-related tumour. EMBO J. **8**:2639–2651.
12. **Inoue, N., S. Harada, T. Honma, T. Kitamura, and K. Yanagi.** 1991. The domain of the Epstein Barr virus nuclear antigen 1 essential for binding to *Ori*P region has a sequence fitted for the hypothetical basic-helix-loop-helix structure. Virology **182**:84–93.
13. **Jones, C. H., S. D. Hayward, and D. R. Rawlins.** 1989. Interaction of the lymphocyte derived Epstein-Barr virus nuclear antigen EBNA-1 with its DNA binding sites. J. Virol. **63**:101–110.
14. **Kieff, E., and D. Liebowitz.** 1990. Epstein-Barr virus and its replication, p. 1889–1920. *In* B. Fields, D. Knipe, R. Chanock, M. Hirsch, J. Melnick, T. Monath, and B. Roizman (ed.). Virology, 2nd ed. Raven Press Ltd., New York.
15. **Lenoir, G., G. O'Conor, and C. L. M. Olweny (ed.).** 1985. IARC scientific publication no. 60. International Agency for Research on Cancer, Lyon, France.
16. **Raab-Traub, N., R. Hood, C. Yang, B. Henry, and J. Pagano.** 1983. Epstein-Barr virus transcription in nasopharyngeal carcinoma. J. Virol. **48**:580–590.
17. **Rawlins, D. R., G. Milman, S. D. Hayward, and G. S. Hayward.** 1985. Sequence specific DNA binding of the Epstein Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region. Cell **42**:659–668.
18. **Reisman, D., and B. Sugden.** 1986. *trans*-activation of an Epstein-Barr viral (EBV) transcriptional enhancer by the EBV nuclear antigen-1. Mol. Cell. Biol. **6**:3838–3846.
19. **Reisman, D., J. Yates, and B. Sugden.** 1985. A putative origin of replication of plasmids derived from Epstein Barr virus is composed of two *cis*-acting components. Mol. Cell. Biol. **5**:410–413.
20. **Rowe, D. T., M. Rowe, G. Evan, L. E. Wallace, P. J. Farrell, and A. B. Rickinson.** 1986. Restricted expression of EBV latent genes and T-lymphocyte detected membrane antigen in Burkitt's lymphoma cells. EMBO J. **5**:2599–2607.
21. **Rowe, M., and C. Gregory.** 1989. Epstein-Barr virus and Burkitt's lymphoma, p. 237–259. *In* G. Klein (ed.), Advances in viral oncology, vol. 8. Raven Press Ltd., New York.
22. **Rowe, M., D. T. Rowe, C. D. Gregory, L. S. Young, P. J. Farrell, H. Rupani, and A. B. Rickinson.** 1987. Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells. EMBO J. **6**:2743–2751.

22a. **Sample, J., L. Brooks, C. Sample, L. Young, M. Rowe, C. Gregory, A. Rickinson, and E. Kieff.** 1991. Restricted Epstein-Barr virus protein expression in Burkitt lymphoma is due to a different Epstein-Barr nuclear antigen 1 transcriptional initiation site. Proc. Natl. Acad. Sci. USA **88**:6343–6347.

23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Sample, J., M. Hummel, D. Braun, M. Birkenbach, and E. Kieff.** 1986. Nucleotide sequences of mRNAs encoding Epstein-Barr

virus nuclear proteins: a probable transcriptional initiation site. Proc. Natl. Acad. Sci. USA **83**:5096–5100.

25. **Sample, J., and E. Kieff.** 1990. Transcription of the Epstein-Barr virus genome during latency in growth transformed lymphocytes. J. Virol. **64**:1667–1674.
26. **Sawada, K., M. Yamamoto, T. Tabata, M. Smith, A. Tanaka, and M. Nonoyama.** 1989. Expression of the EBNA-3 family in fresh B lymphocytes infected with Epstein-Barr virus. Virology **168**:22–30.

26a. **Schaeffer, B., M. Woisetschlaeger, S. Strominger, and S. Speck.** 1991. Exclusive expression of Epstein-Barr virus nuclear antigen 1 in Burkitt lymphoma comes from a third promoter, distinct from the promoters used in latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **88**:6550–6554.

27. **Simons, M. J., and K. Shanmugaratnam.** 1982. UICC technical report series 71, report 16. UICC, Geneva, Switzerland.
28. **Smith, P. R., and B. E. Griffin.** 1991. Differential expression of Epstein Barr viral transcripts for two proteins (TP1 and LMP) in lymphocyte and epithelial cells. Nucleic Acids Res. **19**:2435–2440.
29. **Speck, S. H., A. Pfitzner, and J. L. Strominger.** 1986. An Epstein Barr virus transcript from a latently infected, growth transformed B-cell line encodes a highly repetitive polypeptide. Proc. Natl. Acad. Sci. USA **83**:9298–9302.
30. **Speck, S. H., and J. L. Strominger.** 1985. Analysis of the transcript encoding the latent Epstein Barr virus nuclear antigen 1: a potentially polycistronic message generated by long range splicing of several exons. Proc. Natl. Acad. Sci. USA **82**:8305–8309.
31. **Speck, S. H., and J. L. Strominger.** 1989. Transcription of Epstein Barr virus in latently infected, growth transformed lymphocytes, p. 133–150. *In* G. Klein (ed.), Advances in viral oncology, vol. 8. Raven Press Ltd., New York.
32. **Sugden, B., and N. Warren.** 1989. A promoter of Epstein Barr virus that can function during latent infection can be transactivated by EBNA-1, a viral protein required for viral DNA replication during latent infection. J. Virol. **63**:2644–2649.
33. **Woisetschlager, M., C. Yandava, L. Furmanski, J. L. Strominger, and S. H. Speck.** 1990. Promoter switching in Epstein Barr virus during the initial stages of infection of B lymphocytes. Proc. Natl. Acad. Sci. USA **87**:1725–1729.
34. **Yates, J., N. Warren, D. Reisman, and B. Sugden.** 1984. A *cis*-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81**:3806–3810.
35. **Young, L. S., C. Dawson, D. Clark, H. Rupani, P. Busson, T. Tursz, A. Johnson, and A. Rickinson.** 1988. Epstein-Barr virus gene expression in nasopharyngeal carcinoma. J. Gen. Virol. **69**:1051–1065.