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Restricted Epstein–Barr virus protein expression in Burkitt lymphoma is due to a different Epstein–Barr nuclear antigen 1 transcriptional initiation site

(herpesvirus/polymerase chain reaction/latency)

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ABSTRACT **Epstein–Barr virus (EBV) expresses six nuclear antigens (EBNAs) and three integral latent membrane proteins (LMPs) in latently infected growth-transformed B lymphoblastoid cell lines (LCLs). In contrast, EBV protein expression in Burkitt lymphoma tissue or in newly established Burkitt lymphoma cell lines is frequently restricted to the EBV genome maintenance protein, EBNA-1. EBNA-1 expression in the absence of other EBNAs and LMP-1 has been an enigma since, in LCLs, all EBNA mRNAs are processed from a single transcript. We now show that the basis for restricted EBV expression in Burkitt lymphoma cells is selective EBNA-1 mRNA transcription from a hitherto unrecognized promoter that is 50 kb closer to the EBNA-1-encoding exon than previously described EBNA-1 promoters. Infected cells with EBNA-1-restricted expression could preferentially persist *in vivo* in the face of EBV-immune T-cell responses, which are frequently directed against other EBNAs and are also dependent on LMP-1 expression.**

Epstein–Barr virus (EBV) is carried as an asymptomatic, largely latent infection in the B lymphocytes of most adult humans. Latently infected lymphocytes have the potential to proliferate indefinitely *in vitro* or to produce lymphoproliferative disease in immune-deficient patients, in children with X chromosome-linked susceptibility, in marmosets, or in severe combined immunodeficient (SCID) mice (ref. 1; for review, see ref. 2). The proliferation of EBV-infected B lymphoblastoid cell lines (LCLs) is associated with the expression of six EBV-encoded nuclear antigens [EBNAs 1, 2, 3A, 3B, 3C, and leader protein (LP)], three virus encoded integral latent membrane proteins (LMPs 1, 2A, and 2B), and B-lymphocyte activation and adhesion molecules (ref. 3; for review, see ref. 4). All EBNA mRNAs are differentially spliced from RNAs initiated at the same site; in some cells from the W_P promoter and in other cells from the adjacent C_P promoter (4–11; Fig. 1). LMP mRNAs are transcriptionally controlled by promoters near the opposite end of the genome, which are transactivated by EBNA-2 (4, 13, 14). The EBNAs and LMPs effect lymphocyte growth transformation and are sources of target epitopes for immune T-cell cytotoxicity (4, 15–19).

Endemic Burkitt lymphoma (BL) or early-passage BL cell cultures frequently exhibit a more restricted type of latent EBV infection, characterized by only EBNA-1 expression (20–22). These cells grow without clumping. They express high levels of CD10 on their surface but relatively low levels of activation or adhesion molecules—a phenotype termed group 1 (g1). With continued passage, many BL cell lines become EBNA-2- and LMP-1-positive and develop a "group 3" (g3) phenotype, similar to LCLs, that is characterized by growth in clumps, down-regulation of CD10, and up-regulation of B-lymphocyte activation and adhesion molecules (22). CD10 down-regulation and the up-regulation of activation and adhesion molecules are mediated by LMP1 and EBNA-2 (4, 15).

To determine the basis for the two types of latent infection, an early passage g1 BL cell line, Mutu, was cloned. Some clones exhibited a g1 phenotype and selectively expressed EBNA-1, while others exhibited a g3 phenotype and expressed all EBNAs and LMPs (22). The uncloned Mutu cell line and some g1 clones converted to a g3 phenotype with continued passage.

Since BL is a uniclonal malignancy that can give rise to clones of either phenotype, and since early-passage clones of g1 phenotype can switch to g3 over several further passages *in vitro*, the extent of EBV latent gene expression and the cell phenotype are likely to be determined by nongenetic factors. We now investigate the molecular basis for restricted EBNA-1 expression in cells of g1 phenotype.

MATERIALS AND METHODS

Cells. The Mutu cell line was established from a EBV (type 1 strain) infected African BL biopsy. The cloning at passage 10 and maintenance of g1 and g3 clones has been described (22). IB4 cells are a latently infected LCL established by infection with B95-8 strain EBV *in vitro* (11).

RNA Analysis. For RNA blots, 5 μg of poly(A)$^+$ RNA per lane was denatured, fractionated in a 1.2% agarose/2.2 M formaldehyde gel, blotted onto a GeneScreen*Plus* membrane (DuPont/NEN), and probed with ^{32}P-labeled, nick-translated EBV DNA fragments (11, 23, 24).

S1 nuclease mapping of the 5′ end of the EBNA-1 mRNA used a ^{32}P-labeled oligonucleotide, 5′-CTCCGGCGACCTA-GTGGTCCCCTCCGGATCCCCCCTCCTCTCTATCCACC-GCCGCCCCGG-3′. After hybridization, digestion with 100 units of S1 nuclease was for 30 min at 37°C. The products were analyzed on an 8% polyacrylamide/7 M urea sequencing gel (25).

Characterization of EBNA-1 cDNAs. cDNAs from the 5′ leader of the EBNA-1 mRNA were derived by the RACE

Abbreviations: EBV, Epstein–Barr virus; EBNA-1, EBV nuclear antigen 1; BL, Burkitt lymphoma; LMP, latent membrane protein; LCL, lymphoblastoid cell line; g1 and g3, phenotypes termed groups 1 and 3; IR, internal repeat(s); PCR, polymerase chain reaction; nt, nucleotide(s).

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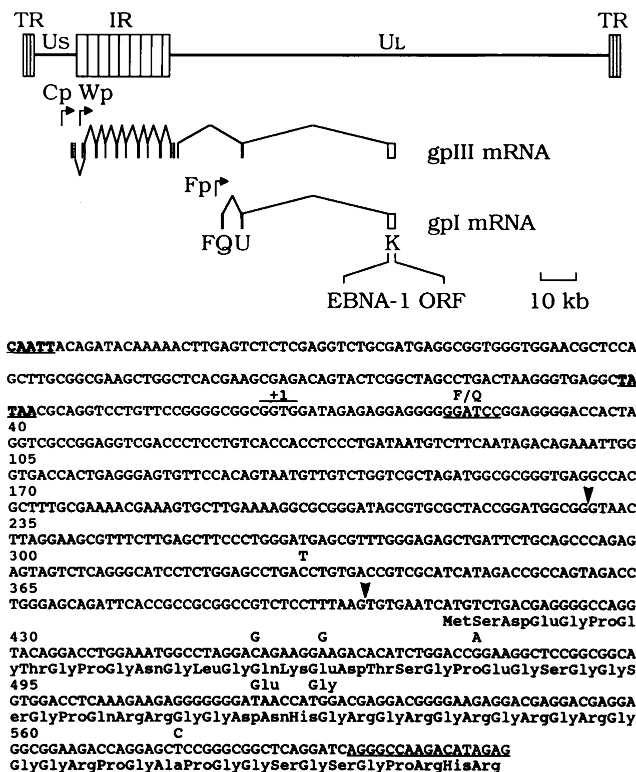

FIG. 1. Structure of the gI or g3 EBNA-1 mRNAs. (*Upper*) The EBV genome (4) is depicted with largely unique sequence domains (U_S and U_L) and long internal repeats (IR) or terminal repeats (TR). The gI and g3 EBNA-1 mRNAs are depicted below the genome. Exons (*FQ*, *U*, and *K*) are named according to the *Bam*HI restriction fragment from which they are derived, as are the different promoters (bent arrows). (*Lower*) The upstream sequence (derived from the B95-8 EBV sequence; ref. 12), and the cDNA sequences are shown. Potential promoter elements (CAAT and TATA boxes) are indicated in underlined boldface type. The putative transcription start site (+1) is overscored, and the *Bam*HI restriction site separating the *Bam*HI F and Q fragments is underlined, as is the 3′ primer annealing site. Exon junctions are indicated with arrowheads. The N-terminal portion of the EBNA-1 open reading frame is shown translated. Nucleotide and amino acid differences between the Mutu and B95-8 EBV isolates are indicated above or below the Mutu cDNA sequence, respectively. The differences shown were present in several independent cDNA clones and, therefore, were not due to misincorporation artifacts that arose during amplification.

(rapid amplification of cDNA ends) protocol (26). Prior to cDNA synthesis, RNA was treated with RQ1 DNase (Promega). First-strand cDNA was synthesized from 1 μg of poly(A)$^+$ RNA by using Moloney murine leukemia virus (Mo-MLV) reverse transcriptase under conditions specified by the manufacturer (BRL) for 1 hr at 45°C. cDNA synthesis was primed with 10 pmol of the synthetic oligonucleotide RT-2 (5′-CTTTGCAGCCAATGCAA-3′) complementary to the EBNA-1 mRNA beginning 250 nucleotides (nt) downstream of the EBNA-1 translation initiation site. After removal of excess primers, poly(dA) was added with terminal deoxynucleotidyltransferase (BRL) and dATP (26). The tailing reaction was terminated at 65°C for 5 min, and the cDNA was diluted to 500 μl with 10 mM Tris·HCl, pH 7.6/1 mM EDTA. A 10-μl aliquot of cDNA was amplified by using the polymerase chain reaction (PCR) in a 50-μl reaction volume containing 67 mM Tris·HCl (pH 8.8); 6.7 mM $MgCl_2$; 170 μg of bovine serum albumin per ml; 16.6 mM $(NH_4)_2SO_4$; 10% dimethyl sulfoxide; 1.5 mM each of dATP, dCTP, dGTP, and dTTP; 10 pmol of dT_{17}-adapter primer [5′-GACTCGAGTCGACATCGA(T)$_{17}$-3′]; 25 pmol of adapter primer (5′-GACTCGAGTCGACATCG-3′); 25 pmol of the gene-specific primer GSP-1 (5′-GTCTCCGGACACCATCT-3′) complementary to the EBNA-1 mRNA 217 nt downstream of the EBNA-1 initiation codon; and 2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus). The cDNA was amplified for 40 cycles, one cycle being 95°C for 40 sec/55°C for 2 min/72°C for 3 min. After the 40th cycle, the mixture was held at 72°C for 15 min to ensure complete polymerization. Five microliters of a 1:1000 dilution of the amplified cDNA was then reamplified for 30 cycles (as above) in a 100-μl reaction volume.

Primers (25 pmol of each) used for the second round of amplification were the adapter primer and the EBNA-1-specific primer GSP-3 (5′-CTCTATGTCTTGGCCCT-3′), which is complementary to the EBNA-1 mRNA 202 nt downstream of the EBNA-1 initiation codon. The specificity of the amplification reactions was monitored by Southern blot analysis. After electrophoresis through a 3% NuSieve GTG agarose gel (FMC), the cDNA was excised from the gel and ligated directly into the *Sma* I restriction site of pBluescript (Stratagene) for DNA sequence analysis.

RESULTS

Restricted EBV Protein and mRNA Expression in gI BL Cells. gI Mutu clones contained very low or undetectable levels of EBNAs 2, 3A, 3B, 3C, and LP and LMP-1, while EBNA-1 was expressed at levels comparable to the reference LCL IB4 (Fig. 2). In contrast, late-passage g3-phenotype Mutu cells and two representative Mutu g3 clones expressed all six EBNAs and LMP-1 (Fig. 2). Expression of LMP-2A and LMP-2B also appeared to be down-regulated in gI Mutu clones (data not shown), although with currently available antisera (3) we could barely detect these proteins in g3 clones or LCLs. By immunofluorescence microscopy, the gI clones contained 1–2% EBNA-2, LMP-1, or CD23$^+$ cells, indicative of partial switching to a g3 phenotype (22). This subpopulation accounts for the trace levels of EBNA-2 and LMP-1 in the gI immunoblots (Fig. 2).

Analysis of EBV mRNAs in early-passage gI and g3 clones and in an LCL (11) revealed a direct correlation with the protein levels and a surprisingly smaller size EBNA-1 mRNA in the gI cells. The EBNA-1 mRNAs in the LCL and g3 clone were 3.4 and 3.6 kilobases (kb), respectively (Fig. 3 *B* and *C*), as expected for typical EBNA-1 mRNAs transcribed from the W_P or C_P promoters, and contained multiple exons derived from the long internal repeat IR1 (ref. 27; Fig. 1). EBNA-2,

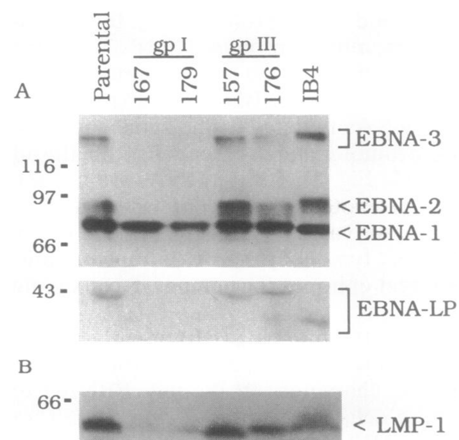

FIG. 2. EBV protein expression in gI or g3 Mutu BL clones. EBNA proteins were detected (10, 21, 22) with an EBV-immune human serum (*A*) and LMP-1 with the monoclonal antibody S12 (*B*). Protein extracts were from the parental Mutu BL cell line, two early-passage gI or g3 subclones or the IB4 LCL. Molecular mass standards in kDa are indicated to the left of the blots.

LMP-1, EBNA-3B, EBNA-3C, LMP-2A, and LMP-2B mRNAs were also similar in size and abundance in the LCL and g3 clone (ref. 11; Fig. 3 *B* and *C*). In contrast, the EBNA-1 mRNA in two representative g1 clones was smaller, 2.3–2.5 kb (Fig. 3 *A* and *C*; also, data not shown). The IR1 exon probe, which detects the leader sequence common to all previously characterized EBNA mRNAs (4), hybridized to the 3.0-kb EBNA-2 mRNA in the g1 clones but not to the 2.5-kb EBNA-1 mRNA (see IR1 in Fig. 3*A*). This indicates that the 2.5-kb g1 EBNA-1 mRNA does not have the usual EBNA leader sequence. The g3 clone also had a low level of the 2.5-kb EBNA-1 RNA (Fig. 3 *B* and *C*). The relative EBNA-1, EBNA-2, or LMP-1 mRNA abundances in g1, g3, or LCL RNAs were directly compared in the same Northern blots, indicating that the g1 RNA was deficient in EBNA-1, EBNA-2, and LMP-1 mRNAs (Fig. 3*C*). Curiously, EBER RNAs were at least as abundant in the g1 RNA as in the g3 or LCL RNA (data not shown).

The g3 EBNA-1 and -2 mRNAs were 0.2 kb larger than the LCL mRNAs (Fig. 3*C*) probably because of a larger number of IR1-derived exons in the leader of the g3 mRNAs (4). EBNA-LP, which these exons encode, is larger in the g3 Mutu cells than in the LCL (ref. 27; Fig. 2*A*). This was confirmed by immunoblotting with an LP-specific antibody (data not shown).

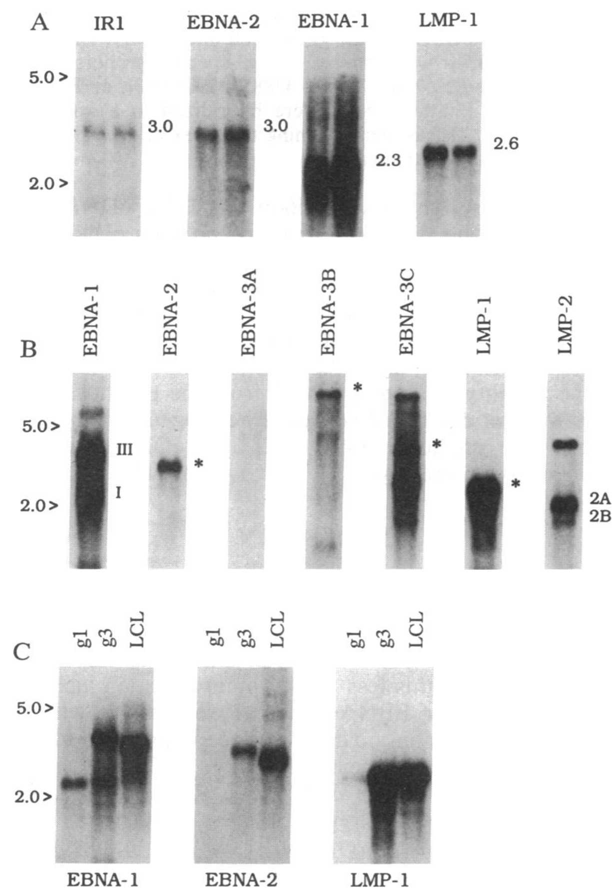

FIG. 3. Northern blot analysis of EBV latent gene mRNAs in two early-passage Mutu BL g1 clones (clones 167 or 179) (*A*) or an early-passage Mutu BL g3 clone (clone 157) (*B*). (*C*) Direct comparison of EBNA-1, EBNA-2, and LMP-1 mRNA abundances in early-passage g1 or g3 clones (clones 179 or 157, respectively) or in the IB4 LCL. The probes used to detect these transcripts have been described elsewhere (11). Asterisks designate the latent gene transcripts. The g1 and g3 EBNA-1 transcripts are so indicated. Positions of the 5.0-kb (28S) and 2.0-kb (18S) rRNA markers are shown to the left of the blots. The g1 EBNA-1 mRNA size was 2.3–2.5 kb in various Northern blots (*A*, *C*, and data not shown).

g1 EBNA-1 mRNA Is Transcribed from a Different Promoter. The finding of a much smaller EBNA-1 mRNA in g1 cells and the relative absence of other EBNA mRNAs is consistent with the hypothesis that a promoter that transcribes only EBNA-1 mRNA is activated in g1 cells. This putative promoter would likely be downstream of IR1 (Fig. 1), since the g1 EBNA-1 RNA has a shorter leader and lacks IR1-derived exons (Fig. 3*A*). The g1 EBNA-1 mRNA leader is predicted to be 0.3 kb because the RNA is 2.5 kb, of which the EBNA-1 open reading frame is 1.9 kb, the open reading frame exon is 2.0 kb, and the polyadenylate sequence is likely to be 0.2 kb. Because g1 and g3 cells have the same size EBNA-1 (Fig. 2), they are likely to have the same EBNA-1 open reading frame and the same 3′ EBNA-1-encoding exon.

To define the putative g1 EBNA-1 mRNA 5′ exons and thereby locate the promoter, we characterized cDNAs derived from g1 poly(A)$^+$ RNA. First-strand cDNA synthesis used a synthetic oligonucleotide that would prime 250 nt 3′ to the EBNA-1 initiation codon. Oligo(dA) was then added to the 3′ end of the cDNA. EBNA-1 cDNAs were then specifically amplified by PCR by using an EBNA-1 open reading frame-specific oligonucleotide primer and the RACE protocol (26). The most abundant cDNAs had the "normal," LCL-like, EBNA-1 mRNA splice from a *Bam*HI *U* exon to the EBNA-1 open-reading-frame *K* exon (Fig. 1). These cDNAs began 40 nt downstream of the *U* exon splice acceptor site. S1 nuclease protection analysis with g1 or LCL control RNA confirmed that the *U* exon in g1 cell poly(A)$^+$ RNA was identical to the previously characterized EBNA-1 *U* exon in LCLs (data not shown). An EBV DNA *Bam*HI *U* DNA fragment probe hybridized to the 2.5-kb gI transcript on Northern blots (data not shown), further confirming that the 2.5-kb RNA contains the *U* exon.

To obtain the 5′ end of the 2.5-kb mRNA, first-strand cDNA synthesis was primed with an oligonucleotide whose annealing site at bases 67613–67629 in the EBV genome (12) is near the 3′ end of the *U* exon. After amplification, cDNAs of the expected size were cloned and sequenced. The two longest cDNA clones begin at either of two guanosines within 3 nt of each other—24 and 27 nt downstream of a TATAA element at base 62202 (12) in the EBV DNA *Bam*HI F fragment (Fig. 1). These guanosines are likely to be the beginning of the RNAs because each cDNA has an extra nongenomic guanosine added before the oligo(dA) sequence, as reverse transcriptase often does when it reaches the end of a template. The putative first exon was designated *FQ* because it extends from the EBV DNA *Bam*HI F fragment to *Bam*HI Q DNA fragment and corresponds to the 229 nt from 62230 to 62458 in the EBV DNA sequence (12). The *FQ* splice donor site at 62458 is linked to the *U* exon acceptor at 67478 (7, 12). The structure of the mRNA predicted from the cDNAs is shown in Fig. 1.

The 5′ end of the *FQ* exon of the g1 EBNA-1 mRNA was confirmed by S1 analysis with an oligonucleotide probe that extended from −10 to +50 relative to the more 5′ of the two putative transcriptional initiation sites. g1 cell RNA protected fragments of 44–47 and 50 nt, whereas no similar size fragments were protected by RNA from noninfected BL2 cells (Fig. 4). A full-length probe was also protected by the g1 cell RNA, possibly because of non-EBNA-1 RNAs (see below).

Since there are no potential splice acceptor sites within 100 nt of the 5′ end of the *FQ* exon, the TATAA and CAAT sequences beginning at −28 and −156 relative to the *FQ* start site, are likely to be components of a g1 EBNA-1 mRNA promoter designated F_P (Fig. 1). To further confirm the 5′ g1 EBNA-1 mRNA site, we tested five different g1 BL cell lines, including Mutu, for *FQ*/*U*/*K*-spliced RNA. cDNAs were made from the RNA of each cell line, and PCR amplifications were attempted with a 3′ primer, GSP-1, complementary to

a site near the beginning of the EBNA-1 open reading frame in the *K* exon and 5′ primers corresponding to sites beginning at −264, −192, −135, −67, −24, or +28, relative to the F_P start site. All primers could amplify the expected size fragments from Mutu DNA by using an appropriate nearby primer for the complementary DNA strand. However, for all five cell lines, only the +28 primer could amplify a cDNA when used with a 3′ primer within the *K* exon EBNA-1 open reading frame (data not shown). When a 3′ primer complementary to a site in the *U* exon, corresponding to 67480-67500 in the EBV DNA sequence (12), was used with the cDNAs that it had been primed within *U* and the same set of 5′ primers, *FQ*/*U*-spliced cDNAs were detected with 5′ primers up to position −135 (data not shown). This confirms the finding of some fully protected probes in the S1 nuclease analyses (Fig. 4). This cDNA does not arise from a EBNA-1 mRNA because EBNA-1 open reading frame-primed cDNA did not amplify with any primers 5′ to the putative F_P start site. Since *Bam*HI F fragment is extensively transcribed in productive infection (23) and a small fraction of the g1 cells are permissive for virus replication (22), this latter *FQ*/*U*-spliced RNA could be a virus replication cycle RNA.

Use of the F_P Promoter in Other g1 BL Cells. At passage 100, the g1 and g3 Mutu clones were phenotypically stable, and the parental Mutu cell line was largely g3. PCR amplification of cDNAs from these cells, from passage 100 g3 clones, from Mutu-BL parental cells, or from three other EBV-infected largely g1 BL cell lines (WAN, ELI, or BL37) confirmed the presence of FQ/U/K RNAs in the g1 cells (Fig. 5). The first amplifications used a 3′ primer complementary to the EBNA-1 open reading frame and a 5′ primer from the *FQ* exon or from the *Y3* exon that is characteristic of LCL EBNA-1 mRNAs (4, 7). A Southern blot of the amplified cDNAs was hybridized to an oligonucleotide probe specific for the *U* exon, which is common to both messages (Fig. 5*A*). The predicted 239-nt FQ/U/K cDNA was amplified from the Mutu g1 clones; while the predicted 268-nt Y3/U/K cDNAs were amplified from the Mutu g3 clones, from LCLs, or from the passage 100 g3 Mutu parental BL cell line (Fig. 5*A*).

In contrast to the early-passage g1 or g3 cells, which were not entirely of one phenotype (Fig. 3), by passage 100 all or virtually all g1 EBNA-1 mRNAs had Q/U/K splices, and g3 EBNA-1 mRNAs had Y3/U/K splices (Fig. 5*A*). Similar experiments were done with RNA from the g1 BL cell lines WAN, ELI, and BL37 by using a different *K* exon 3′ primer and either a *FQ* or *Y2* 5′ primer (Fig. 5*B*). The predicted 584-nt FQ/U/K cDNA was amplified from the WAN, ELI, and BL37 cells, whereas a 556-nt Y2/U/K cDNA was amplified from the WAN cell line, and no such cDNA was amplified from the ELI or BL37 cell lines (Fig. 5*B*). However, we could amplify the expected Y2/U/K cDNAs, using the same primers and cDNAs from RNAs derived from LCLs established by transforming B lymphocytes with EBV from the ELI or BL37 cell lines (data not shown). This indicates that the inability to amplify cDNA from the parent BL cell RNAs was not due to Y2 sequence heterogeneity (data not shown). The predicted 556-nt Y2/U/K cDNA was also amplified from a prototype EBV LCL (Fig. 5*B*).

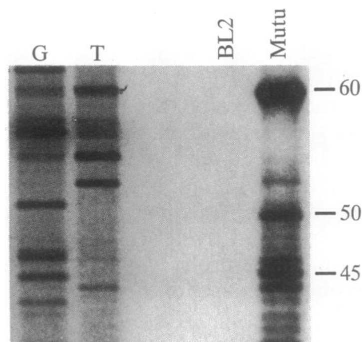

FIG. 4. S1 nuclease mapping of the g1 EBNA-1 mRNA initiation site. Poly(A)$^+$ RNA (15 μg) from g1 Mutu BL cells (lane Mutu) or total RNA or from an EBV$^-$ BL cell line, BL2, were hybridized to a 60-mer spanning the putative cap site and digested with S1 nuclease before analysis on a sequencing gel. The sizes of protected fragments in this and other experiments and in experiments with another g1 cell line RNA are indicated. Products of sequencing reactions (G, T) served as markers for size.

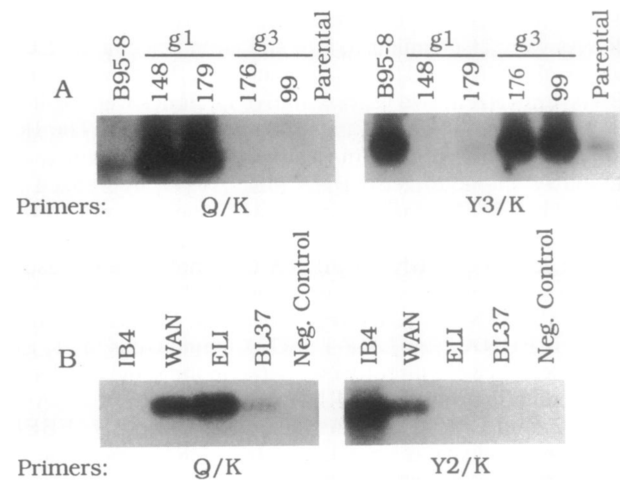

FIG. 5. PCR analysis of F_P usage in various cell lines. (*A*) cDNA derived from late-passage g1 or g3 subclones of the Mutu BL cell line, the parental Mutu BL cell line, or the B95-8 LCL were amplified with primers specific for the g1 (*Q*/*K* exons) or g3 (*Y3*/*K* exons) EBNA-1 mRNAs. The *Q* primer comprises bases 62440–62457 of the EBV genome (12). The *Y3* primer comprises bases 48397–48416. The *K* primer comprises bases 107967–107987. Southern blots of amplified cDNAs were hybridized to a ^{32}P-labeled oligonucleotide (5′-AGAGAGTAGTCTCAGGGCAT-3′) specific for the *U* exon. (*B*) cDNAs of the IB4 LCL and the g1 BL cell lines WAN, ELI, and BL37 were amplified with other g1- or g3-specific primers corresponding to bases 62257–62273 (*Q*), 47885–47901 (*Y2*), or 108150–108166 (*K*) (12). Southern blots were hybridized to a randomly labeled *Xho* I-*Sst* II fragment spanning the *U* exon. The negative control did not contain template.

DISCUSSION

The most significant aspect of this and previous work with g1 and g3 BL or BL cell lines (20–22) is the delineation of a less active EBV latent infection characterized by expression of EBNA-1 without expression of the other EBNAs or the LMPs. Therefore, this less active latent infection lacks the effects of the other EBNAs or LMPs on cell growth and activation or adhesion molecule surface expression (4, 15); G1 cells would be much less susceptible to T cell-mediated immune cytolysis because EBNA-2, EBNA-3s, and LMP-1 contain target epitopes for HLA class I-restricted T-cell cytolysis (16–19). Moreover, LMP-1 expression induces adhesion molecule surface expression and functionally activates B/T-lymphocyte interaction (15). This less active state of EBV latency has been best defined in BL cells and tissues (20–22) but also may be quite important in EBV's persistence in normal lymphocytes or epithelial cells *in vivo*. EBV infection of B lymphocytes or epithelial cells at particular stages of cell differentiation *in vivo* may be associated with use of F_P and hence with selective expression of the virus genome-maintenance protein EBNA-1. EBNA-1 expression would be necessary in a cell that is in a naturally proliferative compartment, such as germinal centers, to ensure EBV episome transmission to progeny cells (28). Similar phenom-

ena may be important in epithelial infection. An EBNA-1 cDNA that begins with 19 nt of the *FQ* exon spliced to the *U* exon was derived from a nude mouse passage of nasopharyngeal carcinoma (NPC) cells (29). Thus, EBNA-1 is likely to be expressed from the F_P promoter in NPC cells as it is in gl BL cells. This would correlate with EBNA-1 expression and the absence of other EBNAs in NPC (30, 31).

The molecular basis for the two types of EBV latent gene expression appears to be the activation of the W_P or C_P EBNA promoters in BL cells at one stage of differentiation or the mutually exclusive activation of the herein described F_P latent promoter at another stage. This working hypothesis is consistent with several lines of evidence. First, the gl state is associated with the exclusive expression of EBNA-1 mRNA initiated downstream of the F_P promoter and the exclusive expression of EBNA-1 protein, whereas the g3 state is characterized by the expression of all EBNAs and LMPs and by EBNA mRNAs similar to those transcribed by W_P or C_P in EBV growth-transformed LCLs. Second, the switch from gl to g3 phenotype almost certainly has an epigenetic basis. The gl and g3 Mutu clones have the same viral and cellular DNAs. Burkitt lymphoma is a uniclonal malignancy that frequently exhibits a gl phenotype (20–22). Clones of a single gl BL tumor have gl or g3 or mixed phenotypes, and gl clones frequently convert rapidly in culture to a g3 phenotype. The number of passages over which gl clones convert to g3 is dependent on specific culture conditions. Although the early-passage g3 Mutu clones still had some gl type EBNA-1 mRNAs, by passage 100, only the LCL type EBNA-1 mRNA was detectable in g3 clones, and only F_P-initiated EBNA-1 mRNAs were detected in the gl clones. Third, much of the EBV genome is methylated in gl BL cells, whereas EBV DNA in LCLs is relatively unmethylated. Treatment of gl BL cells with 5-azacytidine inhibits methylation and induces a shift to full latent EBV gene expression (32). This relationship between EBV DNA methylation and the shift to the g3 phenotype is indicative of transcriptional regulation.

The unique expression of the EBNA-1 mRNA from the F_P promoter in the gl cells is of interest from the perspective of transcriptional regulation, posttranscriptional regulation, and the role of EBNA-1 in these processes. The transition to the g3 phenotype and activation of the upstream C_P or W_P promoters may inactivate the F_P promoter by transcriptional interference or may be associated with an inactivating change in transcriptional regulatory factors. These hypotheses should be testable by analyzing the activity of these promoters cloned upstream of reporter genes and transfected into gl or g3 cell clones. Posttranscriptional splice site utilization must also be critical to the exclusive generation of the gl EBNA-1 mRNA. The 3′ coding exons of the EBNA-3 mRNAs are between the *U* and *K* exons (4) but are skipped in the gl cells. Perhaps related to this differential splicing over the EBNA-3 exons in the gl EBNA mRNA are the two low-affinity EBNA-1 binding sites GGGATAGCGT and CGGGTAATAC (33) at +203 and +227 in the F_P transcript (Fig. 1). Thus, EBNA-1 may play a different role in the regulation of latent gene expression in gl versus g3 cells.

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