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Transcription Start Sites Downstream of the Epstein-Barr Virus (EBV) Fp Promoter in Early-Passage Burkitt Lymphoma Cells Define a Fourth Promoter for Expression of the EBV EBNA-1 Protein

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In Epstein-Barr virus (EBV)-transformed B lymphoblastoid and many Burkitt lymphoma cell lines, the EBV EBNA-1 protein is one of six viral nuclear antigens expressed from a common transcription unit under the control of one of two promoters, Wp or Cp. In contrast, EBNA-1 is the only EBV nuclear antigen expressed in Burkitt and other EBV-positive tumors. We previously identified a promoter of EBNA-1 transcription, designated Fp, in early-passage Mutu Burkitt tumor cells, and this promoter is also active in long-term Mutu and Akata Burkitt cell lines which maintain the exclusive expression of EBNA-1 characteristic of the tumor. However, transcription initiation within Fp reporter gene plasmids in EBV-negative cells occurs at positions 100 to 200 bases downstream of the Fp start site in the *Bam*HI-Q restriction fragment. Here we demonstrate that transcription initiation within newly established Burkitt lymphoma cell lines is consistent with the transcription initiation we observed in reporter plasmids. Furthermore, previous observations of transcription from Fp to generate EBNA-1 transcripts can be attributed to lytic-cycle gene expression. These data, in conjunction with our previous characterization of promoter regulatory elements, define a fourth EBNA-1 promoter, Qp, that is active in latently infected Burkitt tumor cells.

Primary infection by Epstein-Barr virus (EBV) results in the establishment of a lifelong latent infection within host B lymphocytes. In vitro, these cells will proliferate indefinitely as lymphoblastoid cell lines (LCLs) that express a subset of nine viral genes encoding the EBV latency-associated proteins (10). Six of these proteins constitute a family of EBV-encoded nuclear antigens (EBNAs) derived from a common transcription unit under the control of two alternatively used promoters, Wp and Cp (1, 19, 20, 29). The three remaining proteins, the so-called latent membrane proteins, are expressed in the plasma membranes of latently infected cells. With the exception of EBNA-1, each of the latency-associated proteins is known to contain epitopes recognized by cytotoxic T lymphocytes in EBV-seropositive individuals (9, 12). This apparent paradox of EBV persistence in a host that possesses a competent immunosurveillance against the latency-associated proteins is an issue of central importance in defining EBV pathogenesis and has been a primary focus of studies in our laboratories.

In contrast to LCLs, EBV gene expression in several EBV-associated tumors is restricted either to one known viral protein, EBNA-1, or to EBNA-1 and the latent membrane proteins. The expression of EBNA-1 alone, known as type I latency, is characteristic of group I Burkitt lymphoma (BL) cells, whereas expression of latent membrane proteins in addition to EBNA-1 (type II latency) is characteristic of undifferentiated nasopharyngeal carcinoma and EBV-positive Hodgkin's and T-cell lymphomas (2, 4–6, 14, 16, 25, 30). The realization that EBV gene expression is restricted during latency in tumors such as BL, which is not recognized by the virus-specific T-cell surveillance (15), has suggested that this may be a mechanism by which latently infected B cells persist in normal EBV-immune individuals.

We previously reported that exclusive expression of EBNA-1 in early-passage group I Mutu BL cells is associated with the expression of a novel EBNA-1 mRNA in the absence of detectable Wp or Cp activity (17). Employing an anchored-PCR protocol to generate EBNA-1 cDNAs from these cells, we amplified numerous cDNAs with 5′ ends mapping to different positions within a novel exon derived from the *Bam*HI-Q restriction fragment (17). In addition to containing a unique exon from *Bam*HI-Q, these cDNAs each contained a central 172-base exon from the *Bam*HI-U fragment (also present in EBNA-1 mRNAs expressed in LCLs), followed by a 3′ exon encoding EBNA-1. Longer cDNAs extending through *Bam*HI-Q into the adjacent *Bam*HI-F fragment could be obtained either by size selection of the cDNA prior to amplification, or by reverse transcription PCR (RT-PCR) with a 3′ primer specific for the EBNA-1 encoding exon K and 5′ primers with annealing sites beyond the ends of cDNAs beginning within the *Bam*HI-Q fragment. S1 nuclease mapping of these transcripts indicated that they initiate within the *Bam*HI-F fragment 24 bases downstream of a potential TATA box (17). Thus, in the absence of EBNA mRNAs containing structures characteristic of Wp- and Cp-specific transcripts, the unique FQ/U/K exon structure of the EBNA-1 mRNAs present within these group I BL cells suggested that a novel promoter, des-

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ignated Fp, is responsible for EBNA-1 expression in type I latency. EBNA-1 transcripts with the Q/U/K splice structure, furthermore, were also detected in long-term BL cell lines maintaining a type I latency and in tumor cells from nasopharyngeal carcinoma and EBV-positive Hodgkin's disease exhibiting a type II latency (4, 7, 17, 23, 26).

Because Fp-specific EBNA-1 transcripts have been detected in type I and II latency, regulation of EBNA-1 expression by this promoter is potentially a key event in the maintenance of EBV infection in vivo and has therefore been the subject of intense interest. However, two reports from our laboratories have raised the possibility that Fp may not be the predominant promoter of EBNA-1 expression during these states of EBV latency (11, 13). First, on the basis of our initial observation that EBNA-1 mRNAs containing the Q/U/K splice structure could infrequently be detected by RT-PCR in cell lines maintaining an LCL-like pattern of EBV gene expression (type III latency), specifically those that spontaneously enter the EBV lytic cycle (17), it was demonstrated by nuclease mapping that transcripts initiating at the Fp start site are more easily detectable in these cells than in the latently infected group I BL cells (11). Furthermore, the levels of these transcripts, including those splicing to the EBNA-1 coding exon, increase dramatically upon induction of the EBV lytic cycle (11, 13). This indicated that Fp can function as a lytic-cycle promoter. Second, upon fine mapping of *cis*-regulatory elements, we found that transcription start sites within reporter plasmids in EBV-negative BL cells are clustered around positions +100, +165, and +195 relative to the Fp start site. However, little if any initiation of transcription at these downstream sites could be detected within the EBV genome in late-passage group I Mutu cells and the long-established group I BL cell line Akata, both of which predominantly utilize the Fp start site (13). The fact that Fp can function as a lytic-cycle promoter, combined with the discrepancy in transcription start sites, suggested that the Fp-specific cDNAs that we and others generated from group I BL cells (17, 23) may represent lytic-cycle transcripts and not EBNA-1 mRNAs expressed during latency.

Although our original analysis of EBNA-1 mRNAs was carried out with RNA extracted from early-passage group I Mutu cell clones that were predominantly latently infected, the level of lytic-cycle gene expression in these cells was not rigorously evaluated. If Fp is active exclusively during lytic infection, even a low level of lytic-cycle gene expression would have enabled us to amplify Fp-specific EBNA-1 cDNAs by PCR. Therefore, we retrospectively evaluated the level of lytic-cycle gene expression in these early-passage Mutu cells. To obtain an indication of the level of lytic-gene expression in these cells, RNA blots from our previous study (17) were probed for expression of the 2.65-kb EBV BMRF1 mRNA, which is actively expressed during the EBV replication cycle (21). As illustrated in Fig. 1A, the original group I Mutu BL cells (clone 179), from which Fp-specific cDNAs were obtained, expressed low but detectable levels of the BMRF1 mRNA. In comparison, BMRF1 mRNA was much more abundant in early-passage group III Mutu cells (clone 157) known to contain a relatively high proportion of lytically infected cells (typically 5% positive for BZLF1 expression) but was not detectable in the IB4 LCL which maintains a strict latent infection. These data indicate that our original early-passage group I BL cell line did contain a low percentage of lytically infected cells, consistent with our ability to amplify Fp-specific EBNA-1 cDNAs.

Our more recent work on Fp usage has been performed with late-passage group I and III Mutu cell lines and the long-established group I BL line Akata. Although each of these lines retains the pattern of EBV latent-gene expression that defines either type I or III latency, it was important to assess the level of lytic-cycle gene expression in these cells as well. This was particularly critical since we have shown by RNase protection analysis that the late-passage group I Mutu BL line (clone 59) maintains a high level of Fp usage (13), unlike the early-passage group I clones in which Fp-specific transcripts were difficult to detect (17). As demonstrated in Fig. 1B, BMRF1 mRNA levels were highest in the late-passage group I Mutu cells (clone 59) and in Akata cells that had been treated with anti-immunoglobulin G to induce EBV replication. This indicates that the clone 59 Mutu cells maintain a high level of lytic-gene expression. Much lower levels of BMRF1 mRNA were detected in late-passage group III Mutu (clone 176) and uninduced Akata cells, indicating that these are relatively latent. Most importantly, the relative levels of BMRF1 mRNA in these cells corresponded precisely with the relative levels of Fp- and EBNA-1-specific transcripts previously detected in the same RNA preparations (Fig. 1C; reprinted with permission [13]). Of particular note is the finding that BMRF1 mRNA levels, like those of Fp-specific transcripts detected by RNase protection assay and EBNA-1 mRNAs detected by Northern (RNA) analysis, were at least as high in the group I Mutu cells as in the lytically infected Akata cells (compare the first and last lanes of Fig. 1B and C, respectively). These data, as well as similar data published by Schaefer et al. (22) after the present manuscript was submitted, strongly suggest that previously detected EBNA-1 transcripts initiating at the Fp start site represent lytic- instead of latent-cycle transcripts.

Although EBNA-1 cDNAs extending to the Fp start site could be amplified from the RNA of early-passage group I Mutu cells, the 5′ ends of the majority of the cDNAs that we obtained by anchored PCR were located further downstream within the same exon. Because EBV infection of these cells was

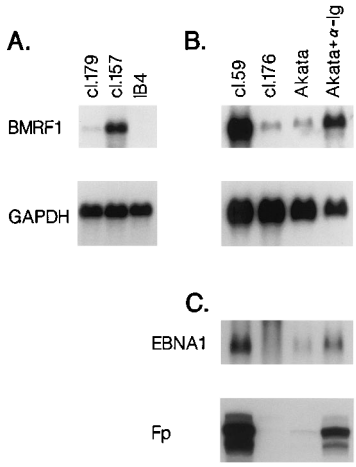

FIG. 1. Fp activity correlates with EBV lytic-gene expression. Northern blots containing 5 μg of poly(A)$^+$ RNA per lane were probed for the mRNAs encoding BMRF1 as an indication of lytic-cycle gene expression, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control for RNA loading, or EBNA-1. (A) RNA from early-passage group I (clone 179 [cl. 179]) and group III (cl.157) Mutu BL cell clones (6, 16) and the latently infected IB4 LCL. (B) RNA from late-passage group I (cl.59) and group III (cl.176) Mutu cell lines and the group I BL cell line Akata; the EBV lytic cycle in Akata cells was induced by cross-linking of surface immunoglobulin G (α-Ig) with 0.1 mg of goat anti-human immunoglobulin G per ml for 3 days. (C) Detection of the 2.5-kb EBNA-1 mRNA by Northern blot analysis and Fp-specific transcripts by RNase protection assay in the same RNA preparations as shown in panel B. The RNase protection probe extended across the Fp start site from −18 to +232. Data shown in panel C were published previously (13) and are presented here with permission of Academic Press, Inc., Orlando, Fla.

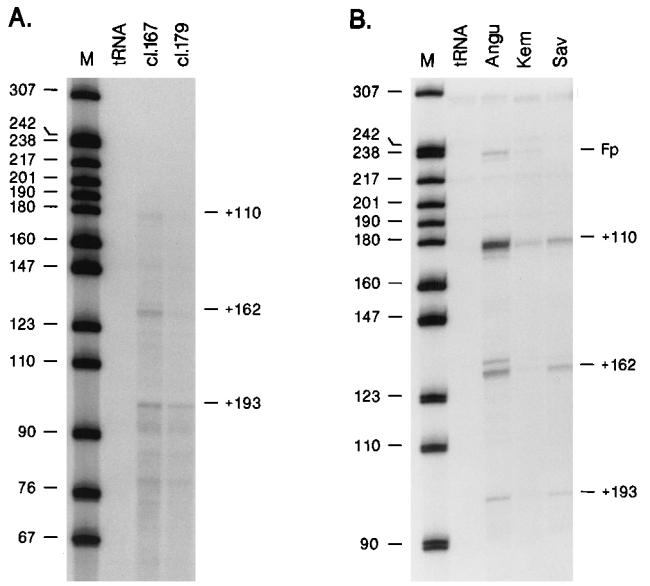

FIG. 2. Identification of transcription start sites within early-passage group I Mutu cells (A) and three newly-established group I BL cell lines (B). Twenty micrograms of total cytoplasmic RNA or yeast tRNA was subjected to RNase protection analysis as described previously (13) with a ^{32}P-labeled cRNA probe spanning bases 50 to 291 of the Fp-specific EBNA-1 mRNA. Numbers to the right indicate the positions of start sites relative to the Fp start site (+1). Bands indicative of initiation at the Fp start are so labeled on the left. Size markers (lanes M) were labeled *Msp*I fragments of pBR322. cl.167 and cl.179, clones 167 and 179, respectively.

predominantly latent, as indicated by the Northern blotting data in Fig. 1A, it is possible that some of these original cDNAs did represent full-length copies of mRNAs initiating from a latent-cycle promoter downstream of Fp. This would be consistent with our finding that transcription initiates within reporter plasmids in EBV-negative BL cells exclusively at sites 100 to 200 bases downstream of the Fp start site (13). Since our long-term group I Mutu BL cells (clone 59) are not representative of the early-passage cells because of their high level of lytic-cycle gene expression and since transcripts within latently infected Akata cells initiating at either Fp or sites downstream are difficult to detect at best, we extended our analysis to other group I BL cell populations whose lytic-cycle statuses are known.

We first analyzed the total RNA from two of the original early-passage group I Mutu clones by RNase protection assay. As illustrated in Fig. 2A, major protected probe fragments indicative of transcripts initiating at approximately positions +110, +162, and +193 were detected in both clones. Moreover, a signal indicative of initiation at the Fp start site was not detected, at least in a 5-day exposure of the film, consistent with the low level of lytic-gene expression in at least one of these cell lines (clone 179) as demonstrated in Fig. 1A. Our recent prospective analysis of early-passage BL lines retaining the group I phenotype indicates that spontaneous entry of a small proportion of cells into the lytic cycle always occurs, although absolute values vary between individual tumors (unpublished observations). We therefore selected three newly-established BL lines (Angu, Kem, and Sav) for which immunofluorescence staining indicated 100% of the cells to be positive for EBNA-1 with no detectable EBNA-2 or EBNA-LP. The levels of BZLF1 expression in these cells, a sensitive marker of EBV lytic-gene expression, were 1.5 (Angu), 0.5 (Kem), and <0.1% (Sav). As illustrated in Fig. 2B, these cells also contained transcripts with potential 5′ termini mapping to positions +110, +162, and +193. Thus, within five early-passage group I BL lines we observed transcription start sites similar (+110) or identical (+162 and +193) to those observed in reporter plasmids following transfection into EBV-negative BL cells. Furthermore, the relative levels of Fp-specific transcripts detected in the Angu, Kem, and Sav lines (Fig. 2B), were proportional to the percentage of cells expressing the BZLF1 lytic-cycle protein.

To determine whether transcripts initiating at sites downstream of the Fp initiation site actually splice to the EBNA-1 encoding exon K, we performed RT-PCR analysis on EBNA-1 cDNAs prepared from total cytoplasmic RNA from the early-passage Angu and Sav BL cells. cDNA synthesis was primed under previously described conditions (17) with a 17-mer oligonucleotide complementary to the EBNA-1 mRNA 250 bases downstream of the start site for EBNA-1 translation in the K exon. EBNA-1-specific cDNAs were then amplified for 35 cycles as described previously (17) with a K-specific 17-mer primer which anneals downstream of the annealing site for the primer used to prime cDNA synthesis and a 17-mer primer that had an annealing site within the FQ exon beginning at position +195, +165, +134, +27, or −24 relative to the Fp start site (+1). Following electrophoresis through an agarose gel, the PCR products were analyzed by Southern blot hybridization with a probe specific for the U exon, which is present within all known EBNA-1 mRNAs. As illustrated in Fig. 3, EBNA-1-specific cDNAs were amplified from Angu BL RNA with each 5′ primer except the −24 primer, which would anneal only to a cDNA of a transcript extending beyond the Fp start site. These data, therefore, are consistent with our RNase mapping data (Fig. 2B) which indicated that there is detectable Fp activity in these cells. Furthermore, these data clearly confirm that transcripts initiating at the Fp start site do splice to the EBNA-1 coding exon, in contrast with a recent report which argues otherwise (22). Within Sav BL cells, which did not exhibit detectable Fp activity, EBNA-1 cDNAs were amplified with the +195 and +165 primers only, indicating that Qp transcripts initiating at +193 and +162 do result in EBNA-1 mRNAs. Endogenous EBNA-1 transcripts initiating at the +195 site have also been observed by Schaefer and Speck, as reported recently (21a). Since no Sav BL cDNA was amplified with the +134 primer, transcripts putatively initiating at +110 would not appear to splice to the EBNA-1 coding exon. Although our RNase mapping data implicating a transcript initiating at +110 could also result from a transcript initiating upstream of Fp, we have previously detected this

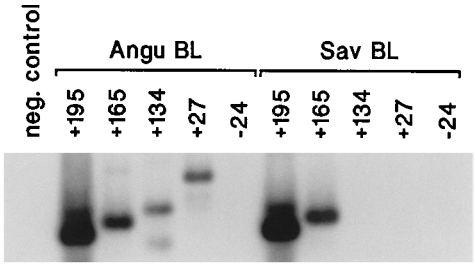

FIG. 3. RT-PCR analysis of EBNA-1 mRNAs. A Southern blot of the PCR products following hybridization to a ^{32}P-labeled U exon probe is shown. EBNA-1 cDNAs generated from early-passage Angu and Sav group I BL cell RNA were amplified with a 3′ primer specific for the EBNA-1 coding exon and 5′ primers with annealing sites successively closer to the Fp start site (+1) within the FQ exon. The negative control amplification lane (neg. control) contained the 3′ primer and the +195 5′ primer but no cDNA template.

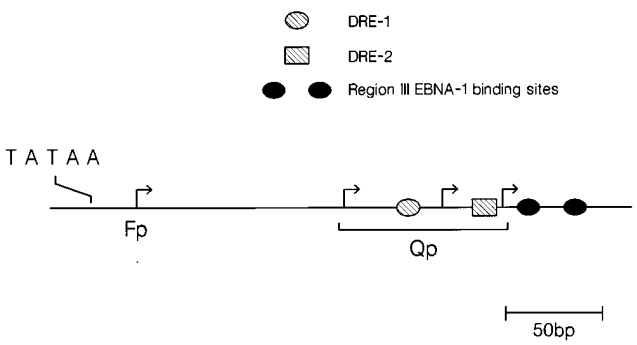

FIG. 4. Organization of the Fp/Qp promoter complex. The positions of major transcription start sites (bent arrows) and previously identified regulatory elements are indicated. Downstream regulatory elements (DRE-1 and DRE-2) are positive *cis* elements (13); the region III EBNA-1 binding sites are negative autoregulatory elements (18, 27). The Fp TATA box is depicted; no recognizable TATA element is present near the Qp start sites.

putative start site in long-term BL cells with a probe extending upstream of the Fp start site (13), therefore arguing against this possibility. Since we detect very little initiation at the +110 site in reporter plasmids (13) and EBNA-1 mRNAs do not appear to be generated from such a transcript (Fig. 3), the identity and significance of this RNA are unknown.

Our current analysis of RNA from latently infected early-passage group I BL cells is in agreement with our previous reporter gene analyses, which indicated that in the absence of a stimulus to induce virus replication, transcription initiates downstream of the Fp start site. Since mutational analysis has indicated that these downstream start sites are associated with a promoter distinct from Fp and located in the adjacent *Bam*HI-Q fragment (13), this promoter is designated Qp. The organization of the Fp/Qp promoter complex with respect to major transcription start sites and known regulatory elements is presented in Fig. 4. Unlike Fp, Qp does not contain a recognizable TATA box, consistent with the multiple sites of transcription initiation associated with Qp. We have identified two *cis*-regulatory elements, downstream regulatory elements 1 and 2, that are required for Qp activity in reporter gene assays (13). The regulatory proteins that interact with these elements, however, have not been definitively identified. Additionally, EBNA-1 can negatively autoregulate expression within reporter plasmids containing both Fp and Qp through two binding sites downstream of the 3′-most Qp start site (18, 27). Since only the Qp start sites are used in these reporter constructs (13), it is currently unclear whether EBNA-1 can also autoregulate Fp-mediated expression. Despite the fact that Fp and Qp are active during distinct stages of the EBV life cycle, our preliminary data suggest that these closely spaced promoters are mutually exclusive and, therefore, not totally independent with regard to their regulation.

Recently, Schaefer et al. (22) reported that transcripts other than EBNA-1 mRNAs are expressed from Fp and that few if any Fp-specific transcripts contain the EBNA-1 coding exon as we and others had previously demonstrated (11, 17, 26). The latter conclusion by these authors was based primarily on two observations, the first of which was the lack of detection of a parallel increase in the levels of EBNA-1- and Fp-specific transcripts (determined by nuclease protection) upon induction of the EBV lytic cycle in P3HR-1 BL cells. This interpretation, however, does not take into consideration previous observations (8, 24) that induction of the lytic cycle in such cells also results in down-regulation of EBNA-1 transcription from the Cp/Wp promoters; since a probe specific for the EBNA-1 coding exon in this assay does not distinguish between EBNA-1 mRNAs originating from Cp, Wp, or Fp, one would expect little if any change in total EBNA-1 mRNA levels upon induction, as was observed. Second, little if any increase in EBNA-1 mRNA levels was detected by Northern blot analysis upon treatment of group I Rael BL cells with 5-azacytidine to induce EBV lytic-gene expression. The level of Fp activation as a result of this treatment, however, appeared to be low, and the inability to detect an increase in EBNA-1 mRNA levels in this instance may have been further complicated by production of more abundant Fp-specific transcripts not encoding EBNA-1. Additional experiments to detect Fp-specific EBNA-1 mRNAs also did not employ cells exhibiting a high level of Fp activity, such as the late-passage group I Mutu cells or induced Akata cells. The data presented in Fig. 1C, however, clearly demonstrate a good correlation between the levels of Fp- and EBNA-1-specific transcripts within Akata BL cells upon induction of the EBV lytic cycle; the fact that Fp-specific transcripts actually splice to the EBNA-1 coding exon was previously confirmed by RT-PCR analysis (11, 17) and is further supported by RT-PCR data presented in Fig. 3.

We have recently detected EBNA-1 transcripts with the Q/U/K splice structure in normal EBV-infected lymphocytes from peripheral blood by RT-PCR (28). Although the 5′ primer used in this study would not have distinguished Qp- from Fp-specific transcripts, the inability to detect transcripts of the BZLF1 gene in the same RNA preparations suggests that Qp will prove to be responsible for EBNA-1 expression in these cells. Chen et al. (3) have also reported detection by RT-PCR of EBNA-1 transcripts in peripheral blood B cells, but only with primers specific for the U/K exon structure and not with primers that would implicate Qp-, Wp-, or Cp-specific transcripts. Although the inability to amplify Q/U/K cDNAs may have been due to technical factors, it could also suggest the presence of an additional EBNA-1 promoter. If Qp is responsible for EBNA-1 expression during a type I or II latency in normal B lymphocytes, what then is the role of Fp? It is currently unknown what function, if any, EBNA-1 contributes to virus replication. Alternatively, the primary function of Fp may be to mediate the expression of other EBV proteins necessary for EBV replication (22).

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