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Redefining the Epstein–Barr virus-encoded nuclear antigen EBNA-1 gene promoter and transcription initiation site in group I Burkitt lymphoma cell lines

(Epstein–Barr virus/viral latency/TATA-less promoter)

BRIAN C. SCHAEFER*, JACK L. STROMINGER*, AND SAMUEL H. SPECK†

*Division of Tumor Virology, Dana–Farber Cancer Institute, Boston, MA 02115; and †Departments of Pathology and Molecular Microbiology, Center for Immunology and Division of Molecular Oncology, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110

Contributed by Jack L. Strominger, July 28, 1995

ABSTRACT **The Epstein–Barr virus-encoded nuclear antigen EBNA-1 gene promoter for the restricted Epstein–Barr virus (EBV) latency program operating in group I Burkitt lymphoma (BL) cell lines was previously identified incorrectly. Here we present evidence from RACE (rapid amplification of cDNA ends) cloning, reverse transcription-PCR, and S1 nuclease analyses, which demonstrates that the EBNA-1 gene promoter in group I BL cell lines is located in the viral *Bam*HI Q fragment, immediately upstream of two low-affinity EBNA-1 binding sites. Transcripts initiated from this promoter, referred to as Qp, have the previously reported Q/U/K exon splicing pattern. Qp is active in group I BL cell lines but not in group III BL cell lines or in EBV immortalized B-lymphoblastoid cell lines. In addition, transient transfection of Qp-driven reporter constructs into both an EBV-negative BL cell line and a group I BL cell line gave rise to correctly initiated transcripts. Inspection of Qp revealed that it is a TATA-less promoter whose architecture is similar to the promoters of housekeeping genes, suggesting that Qp may be a default promoter which ensures EBNA-1 expression in cells that cannot run the full viral latency program. Elucidation of the genetic mechanism responsible for the EBNA-1-restricted program of EBV latency is an essential step in understanding control of viral latency in EBV-associated tumors.**

Burkitt lymphoma (BL) is an Epstein–Barr virus (EBV)-associated neoplasm that occurs with high incidence in the malaria belt of equatorial Africa and sporadically elsewhere (1). Previously, BL tumor cells were believed to phenotypically resemble activated B-blasts and to contain EBV genomes that express six EBV-encoded nuclear antigens (EBNA-1, -2, -3a, -3b, -3c, and -4) and three membrane proteins (LMP-1, -2a, and -2b). This model was based on the study of cell lines established from BL biopsies and *in vitro* established lymphoblastoid cell lines (LCLs). However, analysis of fresh BL biopsies demonstrated that they do not phenotypically resemble LCLs and most BL cell lines (1, 2), but rather have a poorly differentiated resting cell phenotype (3). In addition, in these tumors only a single viral gene product (EBNA-1) is expressed (2, 4), demonstrating the existence of a form of latency that is restricted with respect to the well characterized LCL latency program. Characterization of other EBV-associated neoplasms, nasopharyngeal carcinoma and certain subtypes of Hodgkin disease, has also revealed restricted EBNA gene expression (5–7).

Previously, we (8) and others (9) identified a putative EBNA-1 gene promoter, Fp, located near the *Bam*HI F/Q junction in the viral genome in BL cell lines that retain the restricted pattern of EBNA gene expression (group I BL). However, there is now strong evidence that Fp is not the EBNA-1 gene promoter in group I BL (10). In this report, the group I BL EBNA-1 gene promoter is identified and is shown to be nested within the previously described FQ exon (8, 9, 11), immediately upstream of the two low-affinity EBNA-1 binding sites in *Bam*HI Q.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The LCLs X50-7, JY, and JC5 and the group III BL cell line clone 13 have been described and characterized (12, 13). DG75 is an EBV-negative BL cell line. Akata (14) and Rael (15) are group I BL cell lines. Mutu I and Mutu III are group I BL and group III BL cell lines, respectively, which were established from the same BL tumor (16). All cell lines were propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum.

Rapid Amplification of cDNA Ends (RACE) Cloning, Reverse Transcription (RT)-PCR, and Southern Hybridization Analyses. RACE cloning (17, 18) was performed as described (8). RT-PCR was performed according to the method of Kawasaki (19). One microgram of poly(A)-selected RNA from the indicated cell lines was reverse transcribed using Superscript reverse transcriptase (GIBCO/BRL) and the indicated RT primer; 1/50th of the RT product (equivalent to 0.05 μg of RNA) was then PCR amplified for 25 cycles using the indicated primers, and 1/5th of the resulting PCR product was separated by electrophoresis on a 1.5% agarose gel Southern blotted by established protocols (20). The blot was probed with a random-primed ^{32}P-labeled *Bam*HI U exon probe derived from bases 1250–1731 (*Xho* I/*Cla* I) of the EBV *Bam*HI U fragment, which includes the entire U exon. The sequences of the oligonucleotide primers used for priming cDNA syntheses were 5′-CATTTCCAGGTCCTGTACCT-3′ (K primer) and 5′-CTTAAAGGAGACGGCCGCGG-3′ (U primer). The following primers were used for PCR amplification: Q1, 5′-ATATGGATCCGGAGGGGACCACTA-3′; Q2, 5′-ATATGAGCTCGGGTGACCACTGAGGGT-3′; Q3, 5′-GTGCGCTACCGGATGGCG-3′; Y3, 5′-TGGCGTGTGACGTGGTGTAA-3′; K, 5′-TATAGGTACCTGGCCCCTCGTCA-3′; U, 5′-CGGTGAATCTCGTCCCAGGT-3′.

Generation of Plasmids. The FQUGlobin plasmid was generated by first ligating the *Sac* I/*Eag* I region of the *Bam*HI U fragment (1221–1513 bp) to the *Sac* I/*Eag* I sites of the Bluescript KS + (Stratagene) polylinker. This fragment was then subcloned (through several intermediate constructs) directly upstream of the chloramphenicol acetyltransferase reporter gene in the pGL2CAT construct (21) (UpGL2CAT). *Bam*HI F sequences 5840–7396 bp (*Kpn* I/*Bam*HI) and

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Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV-encoded nuclear antigen; BL, Burkitt lymphoma; RACE, rapid amplification of cDNA ends; LCL, lymphoblastoid cell line; RT, reverse transcription.

*Bam*HI Q sequences 1–2206 bp (*Bam*HI/*Xho* I) were joined to the UpGL2CAT construct *Kpn* I site (within the vector polylinker) and *Xho* I site (*Bam*HI U fragment coordinate 1250) in a three-part ligation. Finally, the rabbit β-globin gene was cloned in place of the chloramphenicol acetyltransferase gene. The plasmid CW1Globin has been described (13).

Electroporation, RNA Preparation, and S1 Nuclease Protection Analysis. Reporter constructs were transiently transfected into cell lines by electroporation as described (10). Cytoplasmic RNA was prepared by the method of Favaloro *et al.* (22). Polyadenylylated RNA was purified on an oligo(dT)-cellulose column as described (23). Total RNA was isolated from transfected cells via the single-step method using guanidium isothiocyanate/phenol prepared according to Chomczynski and Sacchi (24), followed by treatment with RQ1 DNase (Promega) according to the manufacturer's instructions. Synthetic oligonucleotides were labeled with [γ-^{32}P]ATP by established protocols (20). Labeled oligonucleotides were hybridized overnight to RNA samples, digested with S1 nuclease, and analyzed by electrophoresis on denaturing polyacrylamide gels as described (12). All hybridizations and digestions were performed at 37°C, except for experiments with the Qp S1 oligonucleotide in which hybridization and digestion were both performed at 45°C. The sequences of the oligonucleotides used to assess transcription initiation and exon usage were either previously described (10) or are as follows: Qp, 5′-CCGCCATCCGGTAGCGCACGCTATCCCGCGCCTTTTCAAGCACTTTCGTTTTCGCAAAGC-3′; U/K splice, 5′-CTCGTCAGACATGATTCACACTTAAAGGAGACGGCCGCGGTCAAGCGTAC-3′; Q/U splice, 5′-AGAAACGCTTCCTAAGTTACCCGCCATCCGGTAGCGCACGATTAAAATAT-3′; β-actin, 5′-ACATAGGAATCCTTCTGACCCATGCCCACCATCACGCCCTGGGAAGGAAAGGACAAGA-3′.

RESULTS

The U Exon Is Present in the EBNA-1 Transcripts Isolated from Group I BL Cell Lines. To determine whether the initial RACE analyses (8, 9), which identified transcripts containing the U exon spliced to the EBNA-1 coding exon (K exon), were correct, quantitative S1 nuclease protection analyses were used to compare the levels of K exon transcripts and U/K spliced transcripts (Fig. 1). RNA from three group I BL cell lines (Akata, Rael, and Mutu I) and three *in vitro* immortalized lymphoblastoid cell lines (JC5, JY, and X50-7) was examined. The K exon was readily detected in all cell lines examined, although the abundance of EBNA-1 transcripts in group I cell lines was significantly lower than in the LCLs. Utilization of an oligonucleotide probe diagnostic for the U/K splice junction indicated that most, if not all, of the EBNA-1 transcripts present in the cell lines examined contain the U exon directly upstream of the K exon. However, when Q/U splicing was analyzed, only the Akata, Rael, Mutu I, and JY cell lines exhibited a detectable S1 nuclease signal. Our previous analysis of Fp-initiated transcripts (10) demonstrated that lytic transcripts initiated from Fp contain the U exon, although these transcripts splice to the K exon only at a very low frequency. Thus, the detection of Q/U spliced transcripts in the Akata, Mutu I, and JY cell lines, at least in part, reflects lytic transcripts initiated from Fp. However, the presence of Q/U spliced mRNAs in the Rael cell line (which is tightly latent and does not exhibit any detectable Fp-initiated transcription) at a level consistent with K exon-containing transcripts indicates that the EBNA-1 transcripts in group I BL cell lines most likely also contain the Q/U splice junction. An S1 nuclease probe for β-actin exon 3 was used to show that all poly(A) RNAs were of similar quality. The LCLs exhibited an ≈4-fold higher β-actin signal than the group I BL cell lines, indicating a lower abundance of β-actin mRNA in the group I BL cell lines compared to LCL.

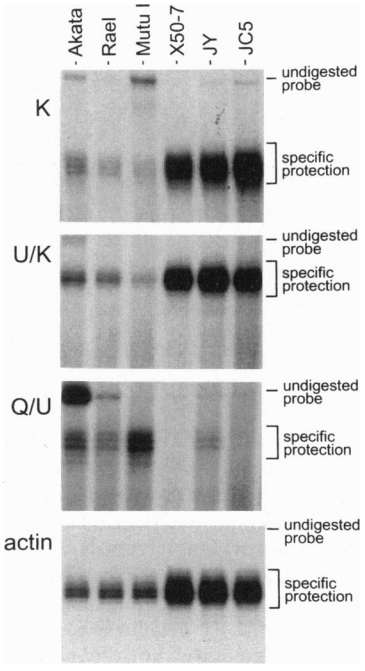

FIG. 1. Quantitative S1 nuclease protection analysis of the abundance of EBNA-1 transcripts in comparison to the abundance of transcripts containing the U exon/EBNA-1 coding exon and Q exon/U exon splice junctions. Akata, Mutu I, and Rael are group I BL cell lines, while JC5, JY, and X50-7 are *in vitro* immortalized LCLs. The oligonucleotide probes used are indicated on the left. K probe spans the EBNA-1 coding exon splice acceptor site. The U/K and Q/U probes span the U exon/EBNA-1 coding exon and Q exon/U exon splice junctions, respectively. The splice junction oligonucleotide probes contain 10 nucleotides of nonhomologous sequence at their 3′ ends, which allows specific protection to be distinguished from undigested probe. The actin probe spans the β-actin exon 3 splice acceptor site. Either 10 μg (K, U/K, Q/U) or 2 μg (β-actin) of polyadenylylated RNA was used for each protection reaction.

RACE Cloning of cDNA Specifically Primed from the EBNA-1 Coding Exon Reveals the Existence of a Promoter in *Bam*HI Q. Previously, we and others (8, 9) attempted to locate the 5′ end of the group I BL EBNA-1 message by RACE cloning. Using cDNA primed within the K exon (EBNA-1 coding exon), it was determined that the U exon [previously described as an exon present in EBNA-1 and EBNA-3c cDNAs isolated from libraries prepared from LCLs (25)] lies immediately upstream of the K exon. Four clones were isolated in which the U exon was spliced to an upstream exon encoded within the viral *Bam*HI Q fragment (Q exon). In our analysis, clones initiating at Fp were identified only when a second round of RACE was carried out employing a RT primer within the U exon. Because the U exon was subsequently shown to be present in lytic transcripts initiated from Fp (10, 26), the results of the initial RACE were ambiguous. To reassess the structure of the EBNA-1 transcript, new cDNA was synthesized by using RNA prepared from the Rael cell line and a primer from the *Bam*HI K exon (EBNA-1 coding exon) to ensure that only clones representing bonafide EBNA-1 transcripts would be obtained. The RACE clones generated by this approach all terminated within the same cluster of bases in *Bam*HI Q defined by the four original Q/U-spliced clones described above (Fig. 2). A total of 18 independent clones with this structure were isolated. It should be noted that a number of published reports, which used PCR to detect EBNA-1 transcripts in group I BL cell lines, employed a Q exon primer that hybridizes to a region of the transcript downstream of the 5′

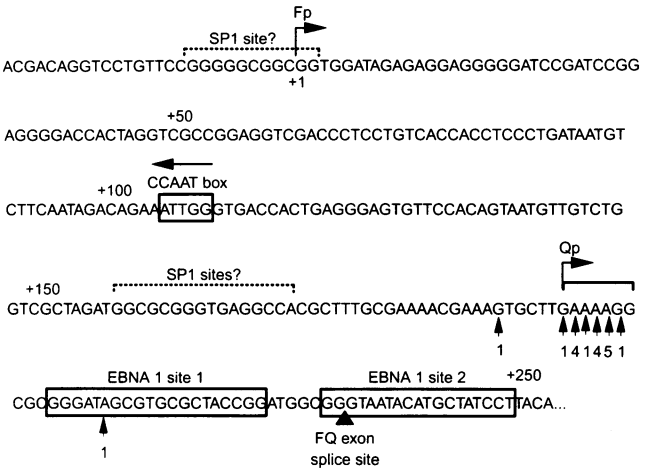

FIG. 2. Identification of the 5′ end of the EBNA-1 transcript in group I BL cell lines by RACE. The 5′ ends of 18 independent clones obtained by RACE PCR are indicated by vertical arrows. Numbers under vertical arrows indicate the number of clones whose 5′ end mapped to that position. Location of Fp is indicated and genomic sequence is numbered relative to the Fp transcription initiation site. In addition, locations of an inverted CCAAT box, potential Sp1 binding sites, the low-affinity EBNA-1 binding sites, and the FQ exon splice donor site are indicated.

ends described here (9, 27, 28). Thus, the results obtained in those reports would not distinguish between transcripts initiating from Fp and those initiating within the *Bam*HI Q region. Based on the RACE analysis presented here, it appears likely that a promoter within the EBV *Bam*HI Q fragment is the group I BL EBNA-1 gene promoter. In addition, our previous deletion mapping of the region required for reporter gene activity identified this region of *Bam*HI Q as essential and sufficient for activity (10). This putative promoter is hereafter referred to as Qp.

RT-PCR Analysis Confirms That Qp, and Not Fp, Is Responsible for Generation of Q/U/K-Spliced Transcripts in Group I BL Cell Lines. To clearly distinguish between the partially overlapping transcriptional units initiated from Qp and Fp, an extensive RT-PCR analysis was carried out with RNA derived from three group I BL cell lines—one LCL and two group III BL cell lines (Fig. 3). Two separate cDNA syntheses were carried out, one with a primer near the 5′ end of the *Bam*HI K exon and the other with a primer near the 3′ end of the *Bam*HI U exon. The K-primed cDNA was first amplified with three sets of primers: a single 3′ primer in the K exon combined with one of three 5′ primers in the Q or FQ exon. The Q1 and Q2 primers are upstream of the putative Qp start site, but downstream of the Fp start site, and are therefore specific for Fp-initiated transcripts. The other 5′ primer, Q3, is complementary to a sequence downstream of the Qp initiation site and can thus amplify messages initiated from either Qp or Fp (Fig. 3*A*).

Results of this RT-PCR experiment (Fig. 3*B*) clearly demonstrated that only the group I BL cell lines Akata, Rael, and Mutu I contain significant quantities of Q/U/K-spliced transcripts, and these transcripts are amplified only by the Q3/K primer pair. Since little or no product was amplified by the Q1/K and Q2/K primer pairs, which are specific for Fp-initiated transcripts, the Q/U/K-spliced messages detected in the group I BL cell lines by the Q3/K primer pair must be initiated from Qp and not from Fp. Very faint signals were detected with the Q1/K and Q2/K primer pairs in the producer cell lines Akata, Mutu I, and JY (as well as with the Q3/K pair in JY), indicating that in these cell lines a very small population of Fp-initiated transcripts are spliced from the FQ exon to the U exon to the EBNA-1 coding exon in *Bam*HI K.

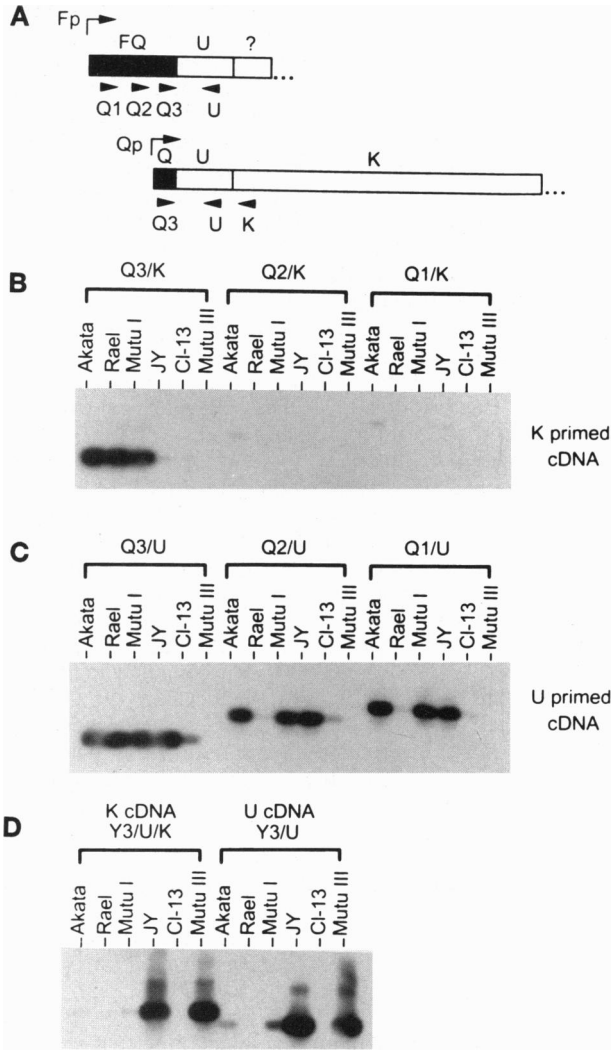

FIG. 3. Semiquantitative PCR analysis with several upstream primers within the FQ exon confirms RACE identification of a transcription initiation site near the 3′ end of the FQ exon. All PCR amplifications were carried out for 25 cycles, in all cases employing the indicated primers. The reactions were all within the linear range, as assessed by varying the number of cycles (data not shown). PCR mixtures were fractionated on agarose gels, blotted, and probed with a random-primed ^{32}P-labeled U exon probe. Akata, Mutu I, and Rael are group I BL cell lines; JY is an *in vitro* established LCL; and clone 13 and Mutu III are group III BL cell lines. (*A*) Model of the structures of Fp- and Qp-initiated transcripts. Approximate locations of PCR primers are indicated below the transcripts. (*B*) PCR amplification of cDNA specifically primed with an EBNA-1 coding exon primer (K cDNA primer). The K PCR primer is homologous to a region immediately upstream of the region primed for cDNA synthesis. (*C*) PCR amplification of cDNA specifically primed with a U exon primer (U cDNA primer). The U PCR primer was homologous to a region immediately upstream of the region primed for cDNA synthesis. (*D*) PCR amplification of cDNA specifically primed with either an EBNA-1 coding exon primer (same cDNA used in *B*) or a U exon-specific primer (same cDNA used in *C*) employing an upstream primer homologous to a region within the Y3 exon and either the K or U PCR primer described above.

However, Q/U/K splicing occurs primarily in group I BL cell lines, and Qp-initiated EBNA-1 transcripts represent the vast majority of EBNA-1 transcripts in the group I BL cell lines.

To rule out the possibility that mRNA secondary structure or some other artifact prevents efficient RT or amplification of Fp-initiated transcripts, cDNA generated by priming from the *Bam*HI U exon was PCR amplified using the same 5′ Q1, Q2, and Q3 primers and a 3′ primer near the 3′ end of the U exon

(Fig. 3*C*). In contrast to the results obtained with K-primed cDNA, all three sets of PCR primers yielded amplification products hybridizing with similar intensities when U-primed cDNA derived from producer cell lines (Akata, Mutu I, JY, and to a lesser extent clone 13) was the starting template. In addition, a strong positive signal was generated when U-primed Rael cDNA was amplified with the Q3/U primer pair, but not with the Q2/U or Q1/U primer pairs, indicating that Qp-initiated transcripts were present in the Q3/U amplified products. These data demonstrate that Fp-initiated transcripts are efficiently reverse transcribed, as well as efficiently PCR amplified by the Q1 and Q2 5′ primers. The observation that U-primed cDNA from producer cell lines yields positive PCR signals with all three primer pairs is consistent with our previous data (10) demonstrating that Fp is a lytic promoter that drives transcription of a message which is frequently spliced from the FQ exon to the U exon.

As an additional control, aliquots of the K- and U-primed cDNAs described above were amplified with Y3/K and Y3/U primer pairs, respectively (Fig. 3*D*). The Y3 5′ primer hybridizes to the Y3 exon common to EBV transcripts initiated from either Cp or Wp (25). As anticipated, amplification of JY and Mutu III cDNAs gave rise to strong positive signals, since both of these cell lines use Cp to drive transcription of EBNA transcripts. The Wp using cell line clone 13 has a deletion that includes the Y3 exon, and there is thus no signal from the clone 13 PCR. Little or no signal was detected from the Qp using group I BL cell lines. The weak signals generated when Akata and Mutu I cDNAs were amplified are consistent with previously published data (26) that group I cell lines can pass through a group III intermediate phenotype as the lytic cycle is activated.

S1 Nuclease Analysis of Promoter and Exon Usage Demonstrates Utilization of a Qp Transcription Initiation Site in Group I BL Cell Lines. In an S1 nuclease analysis of promoter usage (Fig. 4*A*), group I BL cell lines were shown to be negative for Cp activity, consistent with previous observations (8). The JY LCL was positive for Cp activity as reported (12), and the X50-7 LCL was positive for Wp activity (12, 29) (data not shown). Hybridization with an oligonucleotide that spans the putative Qp transcription initiation site gave a detectable signal only in the three group I BL cell lines Akata, Rael, and Mutu I (Fig. 4*A*). Thus, the Qp S1 nuclease protection data are in complete agreement with the RT-PCR analysis (Fig. 3), which demonstrated that significant levels of Q/U/K-spliced transcripts, which are initiated from Qp (Fig. 2), are observed only in group I BL cell lines and not in LCL or group III BL cell lines. As expected, Fp activity (Fig. 4*A*) segregated to cell lines exhibiting spontaneous lytic activity (Akata, Mutu I, and JY) rather than to group I BL cell lines (Rael exhibited no detectable Fp activity). Lytic activity was confirmed by S1 nuclease protection analysis of transcripts initiated from the BHLF1 early lytic promoter (Fig. 4*A*), which was most active in the Akata, Mutu I, and JY cell lines.

To examine whether transcription from exogenous reporter constructs initiated at the same Qp start site utilized by the virus, and to ascertain whether transcription initiates from Fp in the context of an exogenous reporter construct, we cloned an ≈5-kb region containing Fp, Qp, the FQ exon (also Q exon), a large proportion of the FQ/U (also Q/U) intron, and the first 159 bp of the U exon directly upstream of the rabbit β-globin gene. This reporter construct was transfected into both the EBV-negative BL cell lines DG75 and the group I BL cell line Mutu I. As a control, the same cell lines were transfected with the previously described CW1Globin construct (20), which contains a functional Cp promoter. S1 nuclease analysis of Cp usage demonstrated that Cp in the CW1Globin constructs was very active in both cell lines, while no Cp signal was detected in the FQUGlobin transfectants (Fig. 4*B*). The latter result underscores our previous observation that the transcription factors necessary to drive Cp are present in group I BL cell lines, and thus the lack of Cp activity from the endogenous viral genome is likely due to extensive methylation of the viral genome as has been postulated (30, 31).

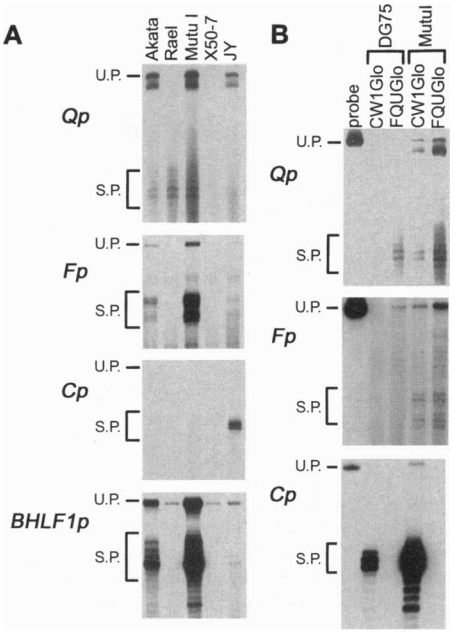

FIG. 4. (*A*) S1 nuclease protection analysis of endogenous Qp activity in a panel of group I BL cell lines and LCLs. Oligonucleotide probes spanning the transcription initiation sites for Qp, Fp, Cp, and the early lytic promoter BHLF1p were used to assess activity. Either 10 μg (Qp, Fp, Cp) or 5 μg (BHLF1p) of polyadenylylated RNA was used for each analysis. Akata, Mutu I, and Rael are group I BL cell lines, while JY and X50-7 are *in vitro* established LCLs. Positions of undigested probe (U.P.) and specific protection (S.P.) are indicated. (*B*) Detection of Qp activity from a transiently transfected reporter construct. A reporter construct driven by Cp (CW1Glo) or a reporter construct containing both Fp and Qp (FQUGlo) was transiently transfected into either the EBV-negative BL cell line DG75 or the Mutu group I BL cell line. The S1 nuclease probes used (indicated on the left) span the transcription initiation sites of the respective promoters. Either 25 μg (Qp, Fp) or 10 μg (Cp) of total RNA was used for each analysis. Positions of undigested probe (U.P.) and specific protection (S.P.) are indicated.

Analysis of Qp usage in the DG75 cell line demonstrated specifically initiated Qp transcription with the FQUGlobin transfectant and no activity in the CW1Globin transfectant (Fig. 4*B*). In the Mutu I cell line, high levels of Qp-initiated transcription were detected in the FQUGlobin transfectant. The lower level of Qp activity detected in the CW1Globin transfectant represents transcription from the endogenous viral Qp. S1 nuclease protection analysis of Fp transcripts (Fig. 4*B*) revealed no specific initiation in the DG75 cell line when either the FQUGlobin or CW1Globin reporter construct was transfected. In the Mutu I cell line, a low and nearly equivalent Fp signal was detected in both the FQUGlobin and CW1Globin transfectants, indicating that the majority of the signal corresponds to endogenous Mutu I Fp activity and that the transfected FQUGlobin Fp was largely inactive. Thus, constructs containing both Qp and Fp, as well as several kilobases of surrounding sequence, appear to initiate transcription exclusively from Qp and the site of initiation is the same as that utilized by the endogenous viral Qp.

DISCUSSION

Elucidation of the genetic mechanisms responsible for the EBNA-1-restricted program(s) of EBV latency is an essential

step in understanding control of viral latency in both EBV-associated tumors and persistently infected lymphoid cells of healthy seropositive individuals. We have shown that EBNA-1 transcripts in group I BL cell lines arise from a previously unidentified promoter, Qp, located near the junction of the viral *Bam*HI F and Q fragments and not from Fp as previously postulated (8, 9). Because the EBNA-1 transcript has been shown to have the Q/U/K-spliced structure in Hodgkin disease tumor biopsies (7, 32), nasopharyngeal carcinoma tissues (5, 6, 11, 33), and in B cells of persistently infected normal seropositive donors (27), we postulate that Qp is the EBNA-1 gene promoter in all cases where expression of the EBNA genes is restricted to EBNA-1.

An interesting feature of the architecture of Qp is that there is no TATAA sequence upstream of the initiation site. TATAA-less promoters are most typically found to direct the transcription of housekeeping genes. The primary positively acting elements found in TATAA-less promoters of housekeeping genes are the initiator element (Inr) and Sp1 binding sites. The initiator element includes bases immediately surrounding the initiation site that are required to bind a specific Inr protein. The Inr protein is responsible for recruiting the basal transcription complex and directing site-specific initiation (34). Sequences surrounding Qp + 1 do not correspond to any known initiator element, and thus transcription from Qp may be initiated by a previously unknown Inr protein.

Recent reports suggest that one role of Sp1 is to prevent methylation of housekeeping promoters during embryogenesis (35, 36). The methylation of cytosine at CpG residues of promoter sequences results in the promoter being packaged in nucleosomes, blocking access of transcription factors. Promoters that are not protected from methylation are thus inactivated. The EBV genomes in group I BL cell lines are known to be heavily methylated (30, 31). In this report (Fig. 4), it is clearly demonstrated that group I BL cell lines transfected with constructs containing Cp will efficiently initiate transcription from the exogenous Cp, even though the endogenous viral Cp is quiescent. However, Cp of the endogenous viral genome can be activated in group I BL cell lines by the demethylating agent 5-azacytidine (8, 16, 30). Thus, there is considerable evidence that the LCL/group III BL program of latency is blocked in group I BL cell lines by methylation of Cp, presumably resulting in inactivation of Cp due to its incorporation into nucleosomes. The presence of potential Sp1 binding sites in the G+C-rich islands close to the Qp initiation site (see Fig. 2) may indicate that Qp, like the *aprt* gene promoter (35, 36), is protected from methylation by the binding of Sp1.

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