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Complex transcription of the Epstein–Barr virus *Bam*HI fragment H rightward open reading frame 1 (BHRF1) in latently and lytically infected B lymphocytes

(cDNA cloning/RNA blot analysis/*in vitro* transcription and translation/immunoprecipitation)

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ABSTRACT **Several cDNA clones containing the Epstein–Barr virus *Bam*HI fragment H rightward open reading frame 1 (BHRF1) have been recovered from the tightly latent lymphoblastoid cell line IB4. These clones contain the 5′ leader exons encoded in the major internal repeat 1 and the viral *Bam*HI fragment Y, identified in the rightwardly transcribed viral mRNAs associated with the latent viral life cycle. In addition, a cDNA clone containing BHRF1 from the Burkitt lymphoma cell line Jijoye was also recovered and exhibits a distinctive splicing pattern. *In vitro* transcription and translation of BHRF1, followed by immunoprecipitation with Epstein–Barr virus-positive human sera, indicates that this viral antigen is expressed during infection. RNA blot analyses with a wide panel of lymphoblastoid and Burkitt lymphoma cell lines revealed a complex pattern of transcription. Hybridization data obtained with several probes is presented.**

Epstein–Barr virus (EBV) is a human lymphotrophic herpesvirus carried by latently infected B lymphocytes in the majority of adult individuals. Infection is associated with two human neoplasias, nasopharyngeal carcinoma and Burkitt lymphoma. *In vitro*, the oncogenic potential of the virus is suggested by its ability to immortalize B lymphocytes. Cell lines containing EBV can be obtained from Burkitt lymphoma biopsies or by the *in vitro* infection of B lymphocytes. The majority of infected B cells maintain the virus in a latent state in the form of multiple nuclear episomes, but in some cell lines a fraction of the cells are spontaneous producers. The study of viral gene expression and function has focused on the latent viral life cycle, since these are the gene products likely to be responsible for the observed growth transformation of the host cell (for review, see ref. 1).

The EBV *Bam*HI fragment H rightward open reading frame 1 (BHRF1) encodes a putative transmembrane protein (2, 3) that shows partial sequence homology to a potential human oncogene activated in human follicular lymphomas, *BCL2* (4). The BHRF1 polypeptide has not yet been characterized or conclusively identified in EBV-infected cells. However, the observation that BHRF1 is transcribed in the tightly latent lymphoblastoid line IB4 (3) suggests that it may, along with other latent EBV antigens, play a role in the virus-mediated growth transformation of human B lymphocytes. The majority of the EBV proteins associated with the latent cycle of the virus are nuclear antigens. They are expressed from highly spliced rightward transcripts that appear to initiate from one of two promoters at the lefthand end of the genome (1, 5). The hallmark of these latent transcripts is the 5′ major internal repeat (IR) 1 exons (*Bam*HI fragment W repeat exons) they share (Fig. 1). To date the only described mRNA associated with the latent cycle that does not share these features is transcribed in the leftward direction from the *Bam*HI fragment Nhet at the righthand end of the viral genome. This is the most abundant viral mRNA found in the IB4 cell line and encodes a membrane protein (12, 13).

cDNA clones that contain BHRF1 have been isolated from the human lymphoblastoid cell lines JY and IB4 (3), but, in contrast to the partial cDNA clone obtained from the marmoset line B95-8 (11), they did not have a spliced structure. Here we report the isolation of spliced BHRF1 cDNA clones from the tightly latent human lymphoblastoid cell line IB4. These clones, which were obtained by screening bacteriophage λ cDNA libraries with a probe containing the IR1-encoded exons, show the 5′ splicing pattern typical of the rightward latent transcripts. We also report the structure of a BHRF1 transcript with a distinctive splicing pattern from the Burkitt lymphoma cell line Jijoye. The complexity of transcription suggested by the diversity of cDNA clones found is investigated further by RNA blot analyses with a wider panel of EBV-infected cell lines. Synthesis of the BHRF1-encoded protein *in vitro* with a coupled transcription–translation system followed by immunoprecipitation shows that this polypeptide is recognized by EBV-positive human sera.

MATERIALS AND METHODS

Cell Lines. Cells were grown at 37°C as described (7). B95-8 is a marmoset line carrying IM-derived EBV (14). IB4 (15), JY (16), X50-7 (17), and JC5 (18) are human lymphoblastoid cell lines transformed with EBV. Jijoye (19) and clone-13, a subclone of P3HR-1 (20), Daudi (21), and Raji (22) are Burkitt lymphoma cell lines. Clone-13 and Daudi carry nontransforming strains of EBV, and Raji is a nonproducer. BJAB is an EBV-negative Burkitt lymphoma (23), and BJABc is derived from BJAB by infection with the B95-8 strain of EBV.

RNA Preparation. Cells were harvested in the exponential phase of growth and stored, after washing in cold isotonic phosphate-buffered saline, as pellets at −70°C. Total cellular RNA was prepared (24) and poly(A)$^+$ RNA was selected on oligo(dT)-cellulose (25).

cDNA Synthesis and DNA Sequencing. cDNA was prepared from poly(A)$^+$ RNA by a modified version of the method described by Gubler and Hoffman (26). Libraries were plated on the C600 hfl strain of *Escherichia coli* and screened with nick-translated probes. The cDNA clones were sequenced after subcloning appropriate restriction endonuclease-digested DNA fragments into phage M13 by the dideoxynucleotide chain-termination method (27).

***In Vitro* Transcription, Translation, and Immunoprecipitation.** The IB4.WYH/F3 clone was digested with *Bgl* II, which cuts 40 nucleotides upstream of the initiation codon of

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Abbreviations: EBV, Epstein–Barr virus; BHLF1, *Bam*HI fragment H leftward open reading frame 1; BHRF1, *Bam*HI fragment H rightward open reading frame 1; IR1, major internal repeat 1.

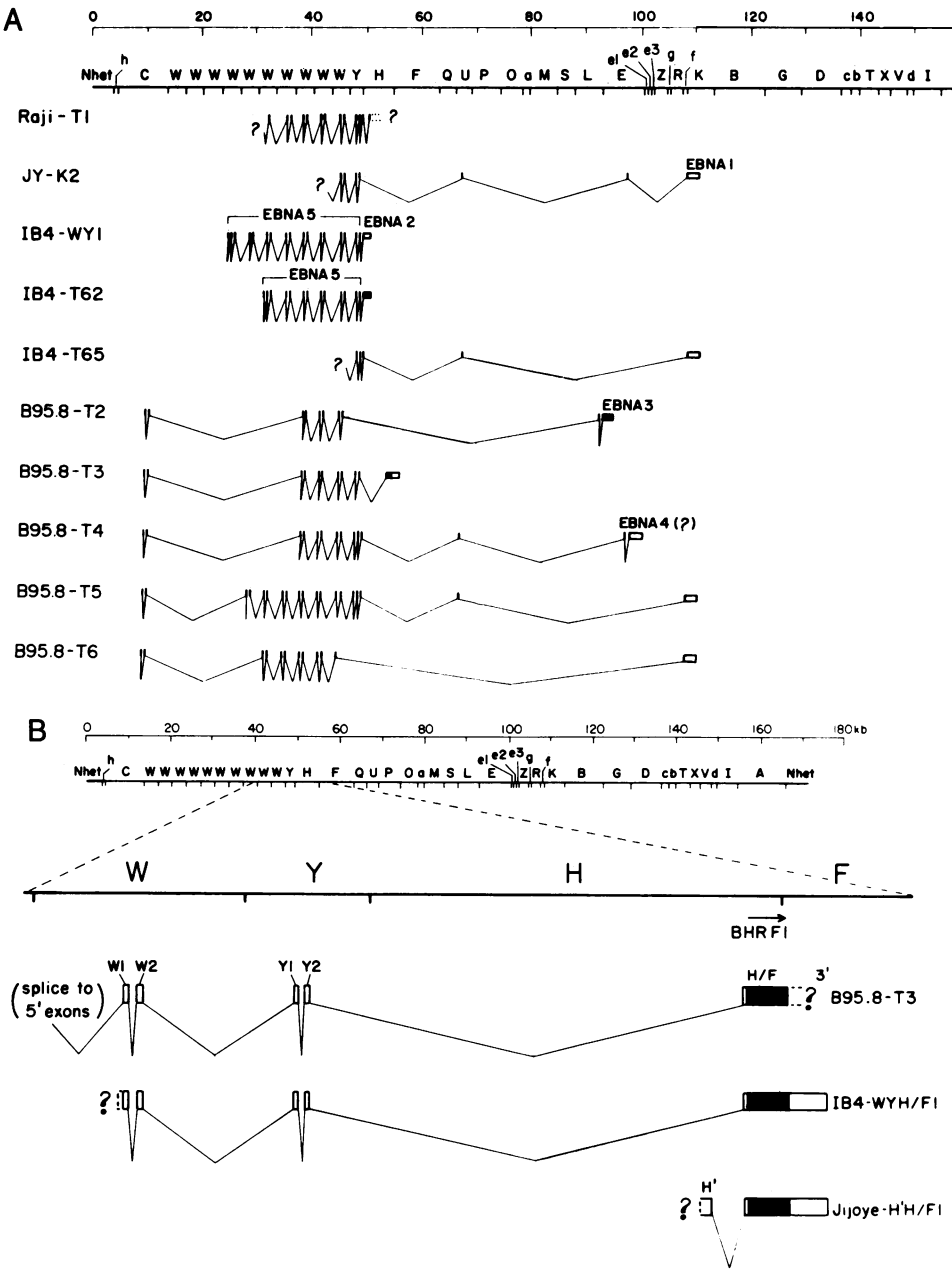

FIG. 1. (*A*) Schematic exon maps. Summary of cDNA clones corresponding to the rightwardly transcribed viral mRNAs associated with the latent life cycle of the virus. Also shown is a *Bam*HI restriction endonuclease map of the EBV genome. Solid boxes represent exons present in cDNA clones; open boxes represent proposed structures. The cDNA clones are from the following: Raji-T1 (6); JY-K2 (7); IB4-WY1 (8); IB4-T62-T65 (9); B95.8-T2 (10); and B95.8-T3, -T4, -T5, and -T6 (11). (*B*) Schematic exon maps of the B95.8-T3, IB4-WYH/F1, and Jijoye-H'H/F1 cDNA clones. Solid box represents BHRF1, and open boxes represent flanking sequences.

BHRF1 (Fig. 2). The 5′ protruding ends were filled in using *E. coli* polymerase I, and *Hin*dIII linkers were ligated to the blunt ends. After digestion with *Hin*dIII and *Eco*RI, the 1150-base-pair fragment, which contained the complete BHRF1 plus 3′-untranslated sequences, was cloned into the pGEM3 plasmid (Promega Biotec, Madison, WI). Prior to *in vitro* transcription, the template was linearized by digestion with *Eco*RI. Capped runoff transcripts were generated (8, 28, 29), and the RNA was translated in the rabbit reticulocyte system (New England Nuclear) (30). Immunoprecipitations were performed essentially as described by Kessler (31) and modified by Edson *et al.* (32). Immunoprecipitations and protein fractions were analyzed by $NaDodSO_4$/polyacrylamide gels as described by Laemmli and Favre (33). Fluorography was carried out as described by Bonner and Laskey (34).

RNA Blot Analysis. Poly(A)$^+$ RNA (5 μg per lane) was fractionated on formaldehyde/agarose gels (35), transferred to a nylon membrane (Hybond, Amersham) by capillary blotting in 20× SSC (3 M NaCl/0.3 M sodium citrate, pH 7.0), and subsequently cross-linked to the membrane by UV irradiation. The blots were prehybridized for at least 8 hr before the addition of labeled probe in fresh hybridization solution. Hybridization with nick-translated probes was performed in 50% (vol/vol) formamide/5× SSC/50 mM Hepes, pH 7.0/5× Denhardt's solution/denatured salmon sperm at 100 μg/ml at 42°C (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). Hybridization with labeled RNA probes, generated by SP6 RNA polymerase from appropriate pGEM constructs (36), was performed in the above buffer with the addition of yeast RNA at 100 μg/ml and 0.1% $NaDodSO_4$, at 50°C. Hybridization with kinase-treated oligonucleotide probes (37) was performed in 6× SSC/50 mM Hepes, pH 7.0/5× Denhardt's solution/0.1% $NaDodSO_4$/denatured salmon sperm at 100 μg/ml at 42°C. Blots probed with oligonucleotide probes A (50% G+C rich) and B (60% G+C rich) were washed in 6× SSC/0.1% $NaDodSO_4$ at 55°C and 60°C, respectively. Blots probed with the nick-translated or RNA probes were washed in 0.1× SSC/0.1% $NaDodSO_4$ at 68°C.

RESULTS

Structure and Sequence of the Spliced cDNA Clones from IB4 and Jijoye. The IB4 cDNA libraries were screened with the characterized JY-K2 cDNA clone (7) that contains W1,

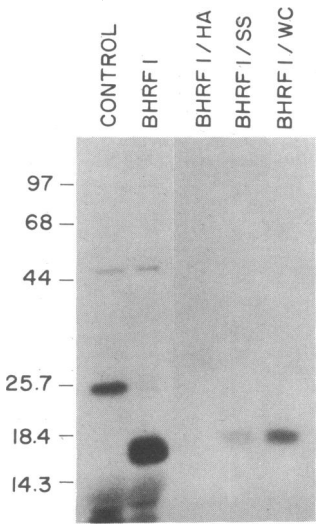

FIG. 2. Analysis of cell-free translation products synthesized from capped mRNA generated by an *in vitro* transcription reaction with SP6 bacteriophage RNA polymerase. Immunoprecipitations with EBV-negative (lanes BHRF1/HA) and EBV-positive (lanes BHRF1/SS and BHRF1/WC) human sera are shown.

W2, Y1, Y2, U, E, and K exons (Fig. 1). Subsequent screening of the clones obtained with the EBV genomic *Bam*HI fragments H and F led to the isolation of three clones, IB4-WYH/F1, -2, and -3. The cDNA clone from Jijoye was isolated by screening with the viral *Bam*HI fragment H (Fig. 1*B*).

All four clones contain the complete BHRF1 open reading frame (Fig. 1*B*; only the longest IB4 clone IB4-WYH/F1 is shown). They utilize the splice acceptor site found 40 nucleotides upstream of the initiation codon and employ the polyadenylylation site 575 nucleotides downstream of the stop codon (3). The three clones from IB4 are incomplete at their 5′ ends but contain the W2, Y1, and Y2 exons seen in other rightward latent transcripts. The Y2–HF splice junction is identical to that observed in the B95.8-T3 cDNA clone isolated by Bodescot and Perricaudet (11), although a common 3′ structure cannot be assumed since B95-8-T3 terminates prematurely, just downstream from BHRF1. The W2, Y1, and Y2 exons, as shown, may encode the carboxyl terminus of EBV-encoded nuclear antigen 5 (8, 9). If this transcript utilizes the consensus promoter present in IR1 (8, 9), the W0–W1′ splice junction would generate the initiation codon for the EBV-encoded nuclear antigen 5 open reading frame, and thus the spliced IB4 transcripts would be bicistronic with just 51 nucleotides between the two reading frames.

The cDNA clone from Jijoye uses a splice donor within the *Bam*HI fragment H [located at nucleotide 53,917 of the B95-8 genome (2)], representing a pattern of splicing not previously described. The splice acceptor is the same as for the IB4 and B95.8 cDNA clones (Fig. 1*B*). The 5′ end of this clone is incomplete, leaving open the possibility that further upstream splicing may occur (5′ end of clone at nucleotide 53,787).

***In Vitro* Transcription and Translation of BHRF1.** *In vitro* transcription of BHRF1 employing the bacteriophage SP6 RNA polymerase and translation of the capped transcripts in the rabbit reticulocyte system generated a 17-kDa product (Fig. 2). This is smaller than the predicted size of the polypeptide (22 kDa), most likely reflecting anomalous migration on $NaDodSO_4$/polyacrylamide gels rather than any post-translational cleavage. The translation product was immunoprecipitated by EBV-positive human sera from two individuals, but not by an EBV-negative human serum. These results demonstrate that the BHRF1 protein is expressed during the course of EBV infection *in vivo*, but they do not determine at what stages of the viral life cycle this occurs.

RNA Blot Analyses of BHRF1 Transcription. The isolation of both unspliced (3) and spliced BHRF1 cDNA clones from IB4, and the distinctive spliced BHRF1 clone from Jijoye, suggested that several alternatives exist for the transcription of BHRF1. To explore this further, mRNA from 10 EBV-infected B cell lines was analyzed by RNA blotting (Fig. 3).

Before examining the transcription of BHRF1, the mRNAs were probed first with an HLA class I gene to check their integrity and subsequently probed with a probe for the most abundant early mRNA, a 2.5-kilobase (kb) transcript that spans the *Bam*HI fragment H leftward open reading frame 1 (BHLF1) (see Fig. 3*B*).

The 1.7-kb HLA class I transcript was detected in all cell lines, whereas levels of the 2.5-kb BHLF1 transcript varied considerably (Fig. 3*B*). B95-8 is a spontaneous virus producer, and the strong signal obtained on hybridizing with BHLF1 is indicative of its active lytic cycle. The lymphoblastoid lines JC5 and JY also express BHLF1, whereas the signal seen in X50-7 and the nonproducer Burkitt line Raji is very weak. At this exposure the 2.5-kb BHLF1 transcript was not detected either in the EBV-converted BJAB line or in the tightly latent lymphoblastoid line IB4. The virus strains in clone-13 and Daudi carry deletions that span BHLF1 (Fig. 3*A*). However, they do contain sequences homologous to IR2 and the D_L promoter elsewhere in the genome (38) and the 2.7-kb and 2.5-kb mRNAs detected in these cell lines are presumably from this homologous region. Probing an RNA gel blot with a single-stranded RNA probe complementary to 200 nucleotides at the 3′ end of the BHLF1 mRNA (Fig. 3*B*; P180) confirms these results and demonstrates that the high molecular weight band detected by the nick-translated probe represents a rightward rather than a leftward transcript. No transcripts were detected in clone-13 and Daudi with this probe, since it did not extend into the region of homology (IR2 and D_L).

When the same RNAs were probed with a nick-translated plasmid that includes the BHRF1 open reading frame and 3′-untranslated sequences a complex pattern of transcription was revealed, as illustrated by the multiplicity of hybridizing bands (Fig. 3*B*; BHRF1). The only simple observation to emerge from these data is that transcription of these sequences occurs in all the EBV-infected lines examined.

To distinguish between transcripts that contained the Y2–HF splice junction and those with the unspliced structure of the JY and IB4 cDNA clones characterized (3), oligonucleotide probes were synthesized. Probe A was designed to detect the spliced transcripts and is complementary to 10 nucleotides on either side of the Y2–HF splice junction. Probe B is complementary to a stretch of 20 nucleotides found 170 bases upstream of the HF splice acceptor site and was designed to detect transcripts that do not splice in this region.

The results obtained on probing blots with oligonucleotide probe A (Fig. 3*B*; oligo A) show that the transcripts containing the Y2–HF splice are present at much lower levels than the apparently unspliced species (Fig. 3*B*; oligo B). The blot probed with oligo A was exposed for three times longer than the blot probed with oligo B, and labeled bands represent a small minority of the bands seen with the nick-translated probe. Expression of the spliced transcripts is highest in the lymphoblastoid cell lines JY and JC5 and faintly detectable in the more tightly latent lymphoblastoid cell lines X50-7 and IB4 and the Burkitt lymphoma cell line Jijoye. Surprisingly, given the isolation of the cDNA clone B95-8.T3 (11), no transcripts were detected with this oligonucleotide probe in the B95-8 cell line. The absence of any signal from clone-13 or Daudi was expected since the *Bam*HI fragment Y is deleted in these strains. Variation in the transcript sizes is

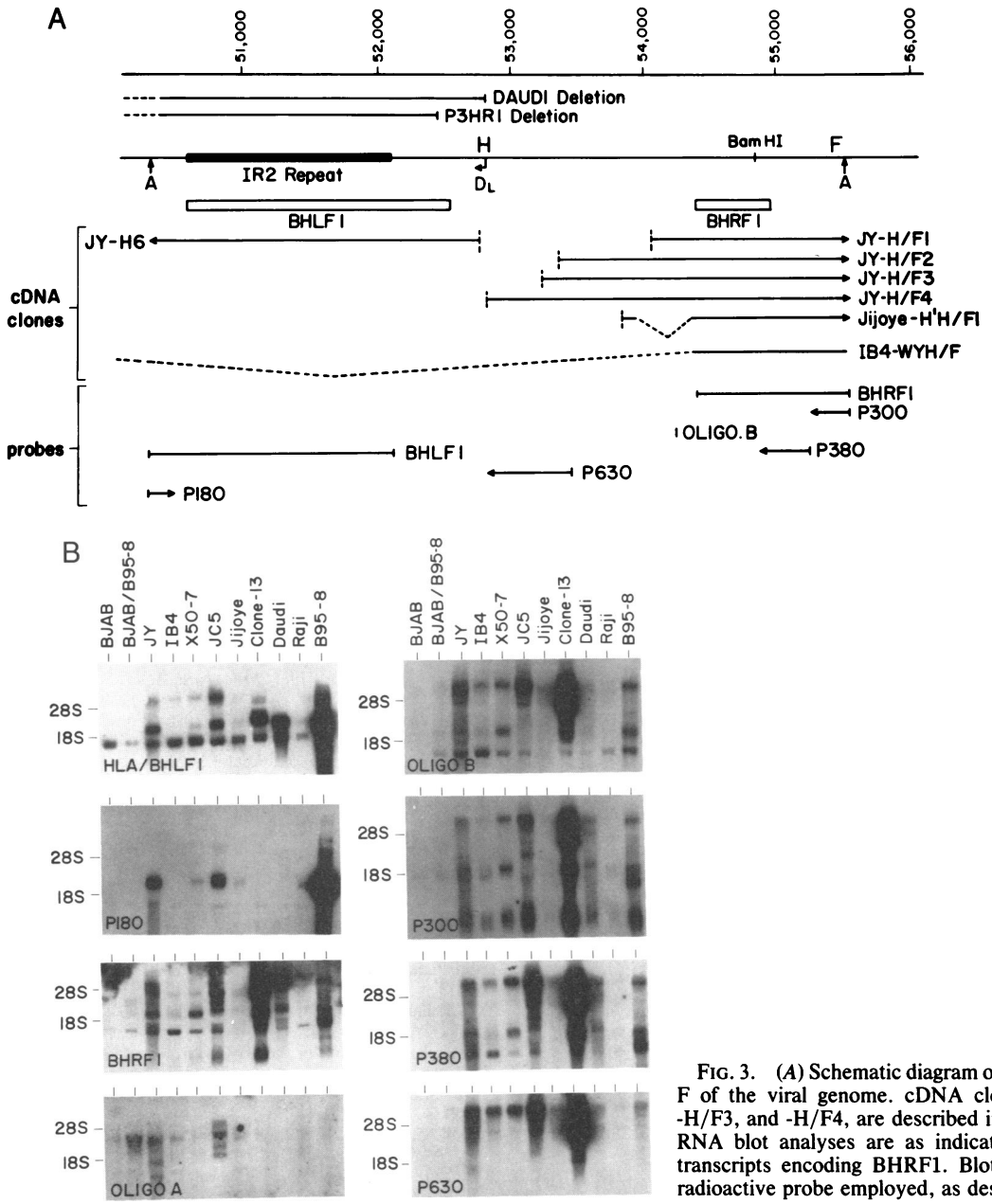

FIG. 3. (*A*) Schematic diagram of the EBV *Bam*HI regions H and F of the viral genome. cDNA clones JY-H6, JY-H/F1, -H/F2, -H/F3, and -H/F4, are described in ref. 3. The probes used in the RNA blot analyses are as indicated. (*B*) RNA blot analyses of transcripts encoding BHRF1. Blots are labeled according to the radioactive probe employed, as described in *A* and the text.

likely to reflect the incorporation of different numbers of the IR1 repeat exons. The distinctive pattern observed in JC5 may be due to initiation of rightward transcription from the promoter in the BamHI fragment C, a phenomenon that occurs in B95-8 and several Burkitt lymphoma lines but not in the other lymphoblastoid cell lines examined (5).

Oligonucleotide probe B detects most of the prominent transcripts detected by the nick-translated BHRF1 probe. These include a 1.6-kb transcript that appears to be present in most (possibly all) of the EBV-infected cell lines analyzed, a high molecular weight transcript(s) that migrates more slowly than the 28S RNA band (>5 kb) and some (but not all) of the intermediate-sized transcripts such as the 2.2-kb mRNA seen in X50-7. In the case of JC5 the reduced complexity seen with oligonucleotide probe B can be accounted for, at least in part, by spliced transcripts that are detected by oligonucleotide probe A. Alternative splicing patterns may explain the loss of other bands that are seen by the nick-translated probe such as those between 1.7 and 2.0 kb in JY, JC5, Daudi, and B95-8.

Fig. 3*B* also shows the results of probing with P630, a single-stranded RNA probe that extends (5′ to 3′) from a point 2.1 kb upstream of the BHRF1 polyadenylylation signal into the region of the D_L promoter. Only the high molecular weight transcripts, plus mRNAs of 3.2–4.0 kb in size from clone-13 and a low abundance 3.5-kb mRNA from JC5, are detected by this probe indicating that the sequences immediately downstream of the D_L promoter are not included in any of the smaller transcripts. The explanation for the failure to detect the 2.2-kb transcripts (prominent in X50-7 and B95-8) may be for quantitative reasons since the predicted overlap between P630 and a 2.2-kb unspliced transcript would be only 100 base pairs. An alternative explanation is that these transcripts contain a 5′ splicing event.

The results of two additional RNA blots illustrate a surprising deviation from the expected 3′ structure of the BHRF1 transcripts. By probing with P300 that contains sequence complementary to the 3′ end of all the BHRF1 cDNA clones, we have found a pattern that differed from the pattern of the nick-translated BHRF1 probe in two respects. First, enhanced hybridization of the low molecular mRNA species was seen, suggesting that these short transcripts utilize the same poly(A) site as the cDNA clones. The second, and more significant, difference is the complete absence of the 1.6-kb transcript in

all cell lines, although a transcript of 1.7 kb was detected by this probe in B95-8. To support and extend these findings, the same blot was stripped and reprobed, first with oligonucleotide B, then with P380, which is complementary to 93 nucleotides at the 3′ end of BHRF1 (encoding the putative transmembrane and cytoplasmic domains) and to the first 290 nucleotides of 3′-untranslated sequence. Probing with oligonucleotide B reproduced the pattern seen with oligonucleotide B (Fig. 3*B*) and confirmed the distinction between the 1.6- and 1.7-kb transcripts in B95-8 (data not shown). P380 also detected the 1.6-kb transcript but not the low molecular weight transcripts. Premature termination seems unlikely to account for the absence of 3′ sequences in the 1.6-kb transcripts, since there are no apparent upstream poly(A) sites that could be employed. The results, therefore, suggest a 3′ splicing event, either to a point immediately preceding the polyadenylylation signal described here or to sequences further downstream.

DISCUSSION

A significant argument favoring the transcription of BHRF1 during latency is the isolation of cDNA clones from IB4 that contain 5′ exons typical of the rightward latent transcripts. The spliced transcripts are present at very low levels and may encode EBV-encoded nuclear antigen 5 in addition to BHRF1. Since the RNA blots show that BHRF1 transcripts containing the Y2–HF splice are in a minority, even in the tightly latent lymphoblastoid line IB4, the further characterization and classification of the unspliced species becomes a matter of priority. The 1.6-kb mRNA is of particular interest, since it is the predominant band detected in IB4. The level of this mRNA does not appear to show any correlation with the level of BHLF1 transcription, suggesting that it is not upregulated on lytic induction. It would thus seem to meet all the criteria for being a true latent transcript.

The significance of the high molecular weight transcripts remains unclear, especially with regard to protein expression. They appear to be detected with all the probes that detect rightward transcription, including the BHLF1 nick-translated probe, suggesting that they include sequences transcribed from the D_L promoter region. These transcripts are most abundant in JC5, but they are as abundant in IB4 as in the spontaneously lytic cell line B95-8 and as such could be classified as latent transcripts. With the exception of clone-13, they are more prominent in the lymphoblastoid cell lines than in the Burkitt lymphoma lines, and one might speculate that they fulfill a function that is no longer required in the fully transformed tumor cell lines. It is possible that antisense transcription of BHLF1 may serve to prevent translation of low level BHLF1 transcripts.

The D_R and D_L promoters are known to be highly responsive to induction of the lytic cycle, and studies have implicated the immediate early transacting factor, encoded by the open reading frame BZLF1, in their activation (39, *). In addition, a study suggests that the viral protein encoded by BRLF1 specifically activates transcription from the D_L promoter in a rightward (rather than a leftward) direction, leading to expression of an early cytoplasmic antigen that is likely to correspond to transcription of BHRF1 (*). In this paper we described a 1.7-kb transcript that is expressed exclusively in B95-8 and a number of other transcripts that are expressed (preferentially if not exclusively) in cell lines with evidence of D_L promoter activity (as assayed by BHLF1 expression). However, the sequences immediately downstream of the bidirectional D_L promoter are predominantly only present in mature transcripts that are >5 kb in size, emphasizing the question of where these transcripts are being initiated.

It is clear from the data presented here that there is a heterogeneity of BHRF1 transcripts. Alternative splicing appears to play a significant role in their generation. Furthermore, it seems probable that several transcripts that contain the spliced leader exons are expressed during latency.

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