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An Epstein–Barr virus transcript from a latently infected, growth-transformed B-cell line encodes a highly repetitive polypeptide

(alternative splicing/cDNA cloning/*in vitro* transcription and translation/immunoprecipitation)

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ABSTRACT **By screening a cDNA library prepared from poly(A)$^+$ RNA isolated from an Epstein–Barr virus latently infected, growth-transformed human B-lymphoblastoid cell line, we have recovered a clone corresponding to a highly spliced viral transcript encoded largely by the major internal repeat (IR1). The 5′ region contains one copy of a 26-base-pair (bp) exon (W0) [which is 28 bp downstream from a CAATT-$(N)_{34}$TATAAA sequence (N, unspecified base)] and seven copies of two small exons (W1, 66 bp; W2, 132 bp). In addition, there are three exons from the "unique" region of the *Bam*HI Y fragment of the viral genome. Two other cDNA clones that have been described, corresponding to latent viral transcripts, share homology in their 5′ regions with this clone and are clearly divergent at their 3′ ends. The cDNA clone described in this paper contains one long open reading frame that extends through the repeat element. *In vitro* transcription and translation of this open reading frame yielded a 62-kDa polypeptide that could be immunoprecipitated by an Epstein–Barr virus-positive human serum.**

Infection of cord blood lymphocytes with Epstein–Barr virus (EBV), a human lymphotropic herpesvirus, leads to the establishment of a latent carrier state with little or no virus production and a concomitant growth transformation of the infected B lymphocytes (reviewed in refs. 1–3). Viral transcription in latently infected cells is poorly understood. However, several transcriptionally active regions of the viral genome have been identified (4). The most abundant transcript is a 2.8-kilobase (kb) message encoded in the *Bam*HI Nhet fragment at the righthand end of the viral genome, which is transcribed in the leftward direction (4, 5). The other viral transcripts that have been identified are all transcribed in the rightward direction (4). These have been mapped, at least in part, to the *Bam*HI K (3.7-kb transcript); *Bam*HI W, Y, and H region (several transcripts); and to the *Bam*HI E fragment (4.5-kb transcript) (4, 6, 7) (Fig. 1).

In addition to mapping the viral transcripts present in latently infected cells, several viral antigens have been identified, and the sequences encoding three of these proteins have been mapped on the viral genome. The relatively abundant viral transcript from the *Bam*HI Nhet fragment encodes a membrane antigen (latent membrane protein) (5), while the 3.7-kb transcript from *Bam*HI K and the 3.0-kb transcript from the *Bam*HI W, Y, and H region encode nuclear antigens (EBNA1 and EBNA2, respectively) (11, 12). At least two other nuclear antigens have been identified (7, 13, 14, *), however, the regions of the viral genome encoding these antigens has not been determined.

A striking feature of the viral transcripts present in latently infected cells is their relatively large size. This was particularly intriguing in the case of the 3.7-kb transcript encoding EBNA1. Based on the apparent size of the viral antigen (11), only about 2 kb of coding information is required, leaving ≈1.7 kb unaccounted for. Furthermore, S1-nuclease analysis of this transcript revealed that only a single 2-kb exon can be mapped to the region of the *Bam*HI K fragment (15). This led us to clone a portion of the 5′ region of this transcript. Sequence analysis of this 5′ clone revealed that it is composed of a number of small exons that are spread over at least 70 kb of the viral genome (9). Specifically, the nucleotide sequence showed that it contains two exons from the major internal repeat (*IR1*; *Bam*HI W fragments) and two exons from the "unique" region of the *Bam*HI Y fragment, as well as exons from the *Bam*HI U, E, and K viral genomic fragments (9). This clone, however, did not contain the complete 5′ end of the transcript. Surprisingly, the exons from the *Bam*HI W and Y fragments were also present in another viral transcript that is divergent in its 3′ region from the transcript encoding EBNA1 (6), raising the possibility of a common 5′ terminus for several of the viral transcripts in latently infected cells (9).

In this paper we characterize a third viral transcript from a latently infected lymphoblastoid cell line, IB4. This message also contains the exons from the *Bam*HI W and Y viral genomic fragments and is clearly distinct in its 3′ region from the other two characterized viral transcripts.

MATERIALS AND METHODS

Source and Preparation of RNA. RNA was prepared from the EBV latently infected cell line IB4, established by transforming human placental lymphocytes with the B95-8 EBV strain (16). Cells were harvested and stored frozen at −70°C until ready for use. Cytoplasmic RNA was prepared by the method of Favaloro *et al.* (17). Poly(A)$^+$ RNA was isolated by fractionation on oligo(dT)-cellulose (18).

cDNA Synthesis. Double-stranded cDNA was prepared by a modification of the method of Gubler and Hoffman (19), as described (9) except that after ligation of synthetic *Eco*RI linkers and digestion with *Eco*RI, the cDNA was size-fractionated on a 1% low-melting agarose gel, and the fraction ≥2.0 kb was cut out and recovered from the gel (20). This fraction was then ligated into the cDNA cloning vector λgt10 (9, 21).

DNA Sequencing. Appropriate restriction endonuclease-digested DNA fragments were subcloned into phage M13mp18 and M13mp19 directly from low-melting point agarose as described by Crouse *et al.* (20). The number of W1-W2 exons was determined by partial digestion of ^{32}P-end-labeled restriction fragments of the IB4WY-1 cDNA clone with *Sma* I. Several 198-bp *Sma* I fragments (*Sma* I cuts once in the W1 exon) were sequenced and found to be

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Abbreviations: EBV, Epstein–Barr virus; bp, base pair(s); kb, kilobase(s).

*Rowe, D., Heston, L., Metlay, J. & Miller, G., Tenth International Herpesvirus Workshop, August 11–16, 1985, Ann Arbor, MI, abstr. 308.

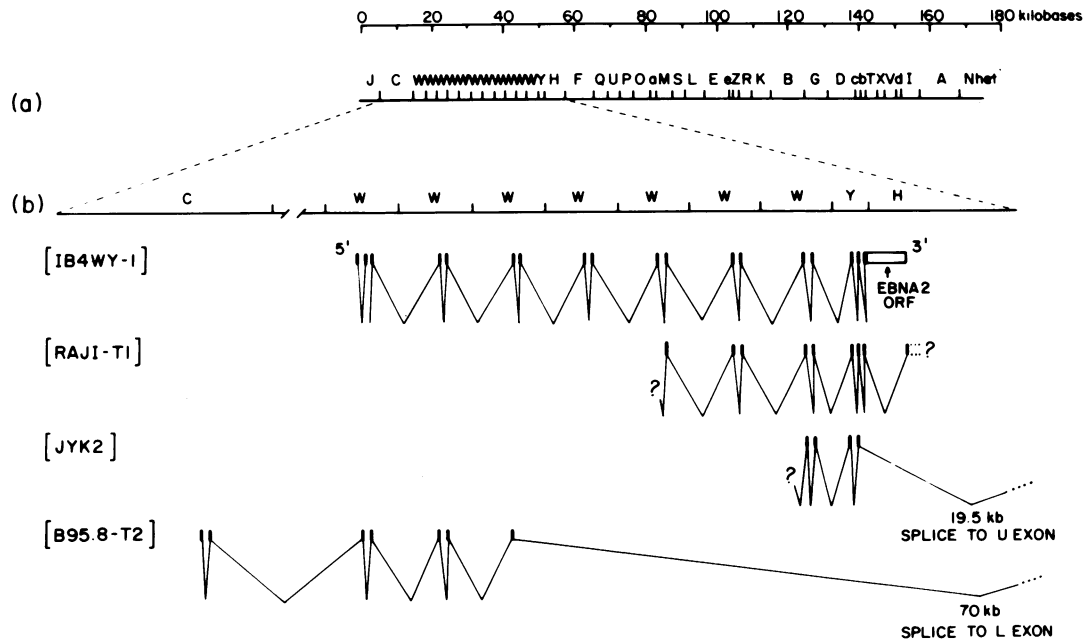

FIG. 1. (*a*) *Bam*HI restriction endonuclease map (8) of the B95-8 strain of EBV. (*b*) Schematic exon maps for the IB4WY-1, Raji-T1 (6), JYK2 (9) and B95.8-T2 (10) cDNA clones. Filled in boxes represent sequences present in each clone, and open boxes represent proposed structures.

identical. DNA sequencing was carried out by the dideoxynucleotide chain termination method of Sanger *et al.* (23) using dATP[^{35}S].

***In Vitro* Transcription and Translation.** The IB4WY-1 clone was digested with *Bst*X1 (New England Biolabs) (which cuts at nucleotide 24 of the EBV sequence; see Fig. 2), blunted with T4 DNA polymerase (New England Biolabs) (leaving the ATG initiation codon intact), followed by digestion with *Eco*RI (Bethesda Research Laboratories). This fragment was inserted into the *Sma* I–*Eco*RI sites of 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 as described employing SP6 RNA polymerase (24, 25). RNA was translated in the rabbit reticulocyte system (New England Nuclear) as described by Paterson *et al.* (26).

Immunoprecipitations, Gel Electrophoresis, and Fluorography. Immunoprecipitations were performed essentially as described by Kessler (27) and modified by Edson *et al.* (28). Both immunoprecipitations and protein fractions were analyzed on $NaDodSO_4$/polyacrylamide gels as described by Laemmli and Favre (29). Fluorography was carried out as described by Bonner and Laskey (30).

RESULTS

Sequence of IB4WY-1 cDNA Clone and Comparison to the Viral Genomic Sequence. A size-selected cDNA library (≥2.0-kb inserts), prepared from cytoplasmic poly(A)$^+$ RNA from the latently infected, growth-transformed lymphoblastoid cell line IB4 was screened with the characterized JYK2 clone [containing about 200 base pairs (bp) of the EBNA1 exon and 1 kb of 5′ sequences] (9) in an attempt to recover clones that contain the 5′ end of the viral transcript encoding EBNA1. From this screening a 2.0-kb clone was recovered that is encoded almost entirely by the major internal repeat of EBV (IR1; *Bam*HI W fragments) (Fig. 1). The sequence of this clone, IB4WY-1, is shown in Fig. 2, and a comparison of the exons present in this clone with the viral genomic sequence (31) from which they derive is shown in Fig. 3.

Surprisingly, the 5′ ≈400 bp of this clone are from non-EBV sequences. These sequences are almost certainly an artifact of cloning, most likely representing a cDNA dimer, since: (*i*) such dimers have frequently been observed in this and other laboratories; (*ii*) selection for large inserts will select for dimers (which probably occur during ligation of synthetic linkers onto blunt cDNA); (*iii*) the junction between the non-EBV and EBV sequences does not constitute a splice junction based on the EBV genomic sequence; and (*iv*) the 5′ end of the viral sequences in this clone are consistent with their representing the 5′ terminus of a transcript, as is discussed below.

With respect to the EBV sequences in this clone, the 5′ end contains one copy of a small 26-bp exon (W0). W0 is spliced to a 61-bp exon (W1′) 149 bp downstream, which in turn is spliced to a 132-bp exon (W2) separated by an 85-bp exon. The W2 exon splices to a 66-bp exon (W1) (2793-bp intron) that differs from the W1′ exon only in that the splice acceptor utilized for the W2-W1 splice is 5 bp upstream from the splice acceptor employed by the W0-W1′ splice junction (see Fig. 3). The W1-W2 exons are repeated seven times in this clone, with the ultimate W2 exon splicing to a 31-bp exon (Y1) encoded in the U2 region of the *Bam*HI Y fragment. The W2 and Y1 exons are separated by a 2208-bp intron (assuming that the 3′ W2 exon is encoded by the righthand most *Bam*HI W fragment). This clone also contains two other exons from the U2 region of the *Bam*HI Y fragment. The Y2 exon is 121 bp long and is 85 bp downstream from the Y1 exon, while the Y3′ exon is at least 88 bp long and is separated from the Y2 exon by a 386-bp intron. The appropriate splice donor and acceptor sequences (32) are present in the viral genomic sequence (31).

This clone exhibits a number of features that are worth noting. First, the W0 exon is 28 bp downstream from a consensus eukaryotic promoter [CAATT(N)$_{34}$TATAAA sequence (N, nonspecified base)], strongly suggesting that this represents the 5′ terminus of the transcript. Second, the W1, W2, Y1, and Y2 exons are identical to exons described for two other latent viral transcripts (6, 9). In addition, the Y3′ exon is the same as that described for the Raji-T1 cDNA clone (6), except that it continues through the splice donor site used in the Raji-T1 clone (the GT underlined in the Y3′ exon, Fig. 3*b*). Furthermore, the putative EBNA2 initiation codon is 40 bp downstream of the 3′ end of the IB4WY-1 clone, indicating that the corresponding transcript may also contain the EBNA2 open reading frame. The latter point has

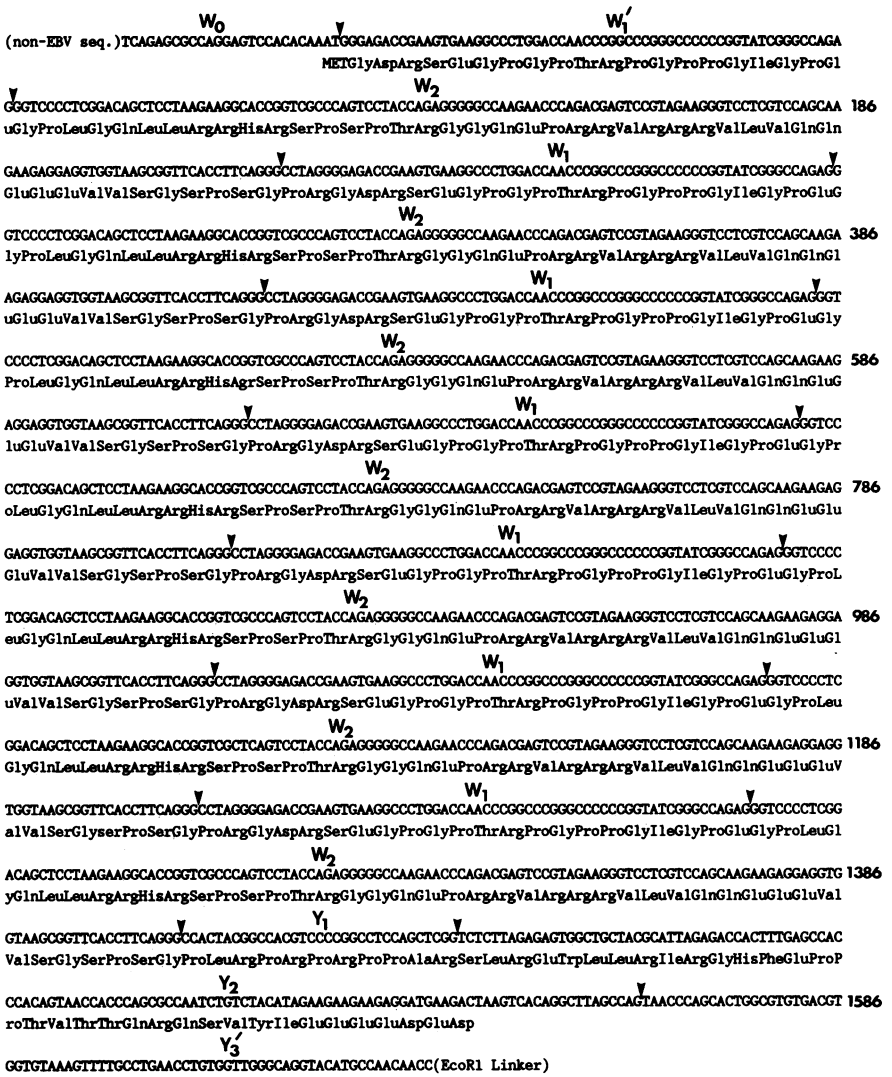

FIG. 2. Nucleotide sequence of the IB4WY-1 cDNA clone and translation of the long open reading frame. The exon borders are indicated by arrowheads, and the exons are labeled corresponding to the appropriate *Bam*HI genomic restriction endonuclease fragment in which they are encoded.

been supported by the partial characterization of a putative EBNA2 cDNA clone (33).

The IB4WY-1 cDNA Clone Contains a Long Open Reading Frame. An analysis of potential open reading frames in the IB4WY-1 sequence revealed a single long open reading frame (ORFwy) that extends through the W1-W2 repeat exons and the Y1 exon, and terminates in the Y2 exon (see Fig. 2). Interestingly, the ATG initiation codon is generated at the splice junction of the W0-W1′ exons and is the only possible translation initiation signal in the entire open reading frame. However, it should be noted that the nucleotide sequence around the initiation codon does not predict to a favorable start sequence, since there is a pyrimidine at position −3 (34). ORFwy potentially encodes a proline-(17.8%) and arginine-(18%) rich polypeptide, and since the W1-W2 repeat exons remain in frame the protein has a 66-amino acid sequence repeated seven times. The size of the putative viral protein (VPwy) is 506 amino acids with a predicted molecular size of ≈61 kDa, and an overall charge of about +32.

***In Vitro* Transcription and Translation of ORFwy.** The IB4WY-1 cDNA was cloned into a plasmid containing the bacteriophage SP6 RNA polymerase promoter, thereby readily allowing *in vitro* generation of capped transcripts, to determine: (*i*) whether ORFwy can be translated *in vitro* by the rabbit reticulocyte lysate system; (*ii*) the apparent size of VPwy; and (*iii*) whether the *in vitro* translation product can be recognized by a human serum from an individual infected with EBV. *In vitro* transcription of this construction was carried out using SP6 RNA polymerase, employing diguanosyl triphosphate in the transcription reaction to generate 5′-capped transcripts (25). These *in vitro* transcripts were translated in the rabbit reticulocyte lysate system (26) and analyzed on $NaDodSO_4$/polyacrylamide gels. As shown in Fig. 4, a single predominant *in vitro* translation product of ≈62 kDa in size was visible, in good agreement with the predicted size of VPwy. Immunoprecipitation with an EBV-positive human serum clearly recognizes the *in vitro* translation product, although the EBV-negative control human serum also very weakly immunoprecipitated this polypeptide. This result was not very surprising since the deduced amino acid sequence for VPwy predicts that it is a very basic protein and as a result may bind nonspecifically to added IgG or to the *Staphylococcus aureus CI* employed in the immunoprecipitation.

DISCUSSION

The IB4WY-1 clone appears to represent the 5′ end of an EBV transcript present in latently infected, growth-transformed B lymphocytes. Indeed, the fact that the W0 exon is 28 bp downstream from a consensus eukaryotic promoter is consistent with the location of other eukaryotic transcription initiation sites (35). This raises the question as to which of the 11 *Bam*HI W fragments, present in the B95-8 genome (31) of the IB4 cell line, contains the transcription initiation site since there are only 7 W1-W2 exon repeats in the IB4WY-1 cDNA clone. It is possible that several of the *Bam*HI W promoters are active in latently infected cells leading to the

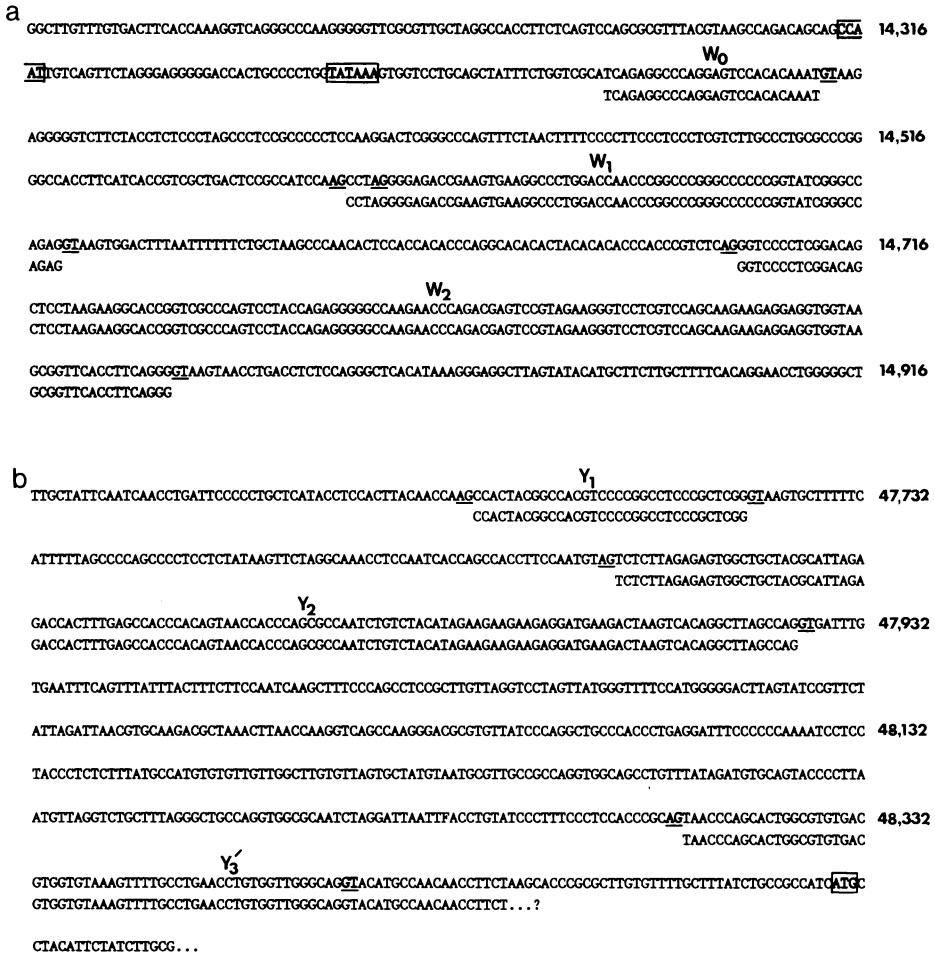

FIG. 3. (*a*) Comparison of the EBV genomic sequence (31) from the region of the *Bam*HI W fragment and the exons present in the IB4WY-1 cDNA clone (the genomic sequences from nucleotides 14,217–14,916, representing the first *Bam*HI W repeat, are shown). The CCAAT and TATAA sequences are boxed, and the splice donor and acceptor sequences are underlined. (*b*) Sequence of the EBV genome (31) from the region of the *Bam*HI Y fragment (nucleotides 47,633–48,449) encoding three exons present in the IB4WY-1 cDNA clone. The splice donor and acceptor sequences are underlined [the splice donor sequence employed in the Raji-T1 cDNA clone (6) is underlined in the Y3′ exon], and the putative ATG initiation codon for the EBNA2 open reading frame is boxed.

production of a family of polypeptides of various sizes based on the number of W1-W2 exon repeats, as has been suggested by others (14, *).

The occurrence of alternative splicing in EBV transcripts produced during latent infection was apparent from comparisons of the Raji-T1 (6) and JYK2 (9) cDNA clones and is further reinforced by the IB4WY-1 cDNA clone. As is schematically shown in the compiled exon maps for the latent viral transcripts that have been characterized (Fig. 1*b*), (*i*) the W2 exon has been shown to splice to both the W1 and Y1 exons, (*ii*) the Y2 exon can splice to either the Y3 or U exon, and (*iii*) the Y3′ exon in the IB4WY-1 clone fails to splice at a splice site utilized in the Raji-T1 clone (6, 9). Furthermore, comparison of the W0-W1′ and W2-W1 splice junctions supports the concept of specific splice donor–acceptor pairs in splice junction formation. In addition, the splicing of the W0 exon to a splice acceptor 5 bp downstream from the splice acceptor utilized by the W2-W1 splice is most likely not due to defective splice junction in the first *Bam*HI W repeat (or any of the other *Bam*HI W fragments) since the sequence of these repeats in the B95-8 genome is identical (31). The putative EBNA2 cDNA clones described (33) are not included in the compilation of latent transcripts since they were only partially characterized and as a result have ambiguous structures.

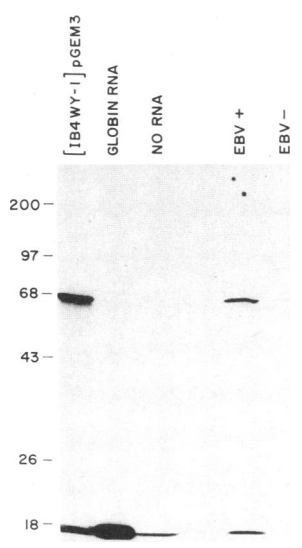

FIG. 4. Analysis of cell-free translation products synthesized from either capped RNA generated by an *in vitro* transcription reaction using SP6 bacteriophage RNA polymerase ([IB4WY-1] pGEM3) or globin mRNA. SP6 transcripts generated employing the [IB4WY-1]pGEM3 plasmid as a template were used to direct the rabbit reticulocyte cell-free translation system, and products were analyzed on a $NaDodSO_4$/10% polyacrylamide gel. Immunoprecipitations with an EBV-positive (EBV+) human serum, named RWM, and with an EBV-negative (EBV−) human serum are shown.

Still unresolved is the question of whether all the rightwardly transcribed messages present in latently infected cells share a common 5′ structure (promoter and transcription initiation site) and, as in the case of the IB4WY-1 clone, utilize one of the promoters present in IR1. Alternatively, one or more promoters for latent transcription (in addition to the IR1 promoters) that map near the lefthand end of the viral genome may exist. With regard to the latter possibility, as shown in Fig. 1*b*, an exon map of the 5′ region of a viral transcript present in B95-8 cells (B95.8-T2) (10) shows striking similarities to the general exon structure and splicing pattern present in the latent transcripts that have been characterized. However, transcription initiation of this message does not occur in IR1, and its 5′ terminus appears to lie within the *Bam*HI C fragment. This location is intriguing since a latent origin of replication (*orip*) has also been mapped to this region of the viral genome (36), suggesting an organization similar to simian virus 40 in which the origin of replication is tightly associated with viral promoters and transcriptional regulatory elements (37). However, it is unclear if the transcript from B95-8 cells is associated with

the latent or lytic cycle of EBV, since the B95-8 cell line actively produces virus (38).

The protein product encoded by ORFwy is interesting because of its predicted highly repetitive structure. *In vitro* transcription and translation of ORFwy, followed by immunoprecipitation with an EBV-positive human serum strongly suggests that VPwy is expressed in infected cells. Rowe *et al.** have identified by immunoblotting what appears to be a family of nuclear antigens that vary in size by about 6-kDa increments. Furthermore, a synthetic peptide homologous to a region of VPwy was used to generate heterosera in rabbits against VPwy (14), confirming the earlier speculation that this family of viral nuclear antigens is encoded in IR1. These data are consistent with the prediction that a number of the IR1 promoters may be active in latently infected cells, thus producing VPwy polypeptides of various lengths (14, *). While there is not an obvious nuclear targeting signal present in the deduced amino acid sequence of VPwy that is homologous to the simian virus 40 tumor antigen nuclear targeting signal (Pro-Lys-Lys-Lys-Arg-Lys-Val-) (39), there is a sequence encoded in the W2 exon that follows the general motif of proline followed by a number of basic residues (-Pro-Arg-Arg-Val-Arg-Arg-Arg-Val-). This general motif has been noted for the yeast ribosomal protein L3 and histone H2B (40); however, the VPwy sequences differs in that the basic residues are exclusively arginine while in the yeast and simian virus 40 signal sequences the basic residues are mostly lysine (39, 40).

A final interesting feature of VPwy is the presence of an -Arg-Gly-Asp- sequence that is repeated six times in the protein. This tripeptide has been shown to be crucial for the interaction of fibronectin (and several other proteins) with its cell surface receptor (41) and is thought to constitute a recognition system for cell surface signaling. As discussed above, VPwy appears to be a nuclear antigen (14, *) that is obviously not consistent with a role at the cell surface. However, as is the case with simian virus 40 large tumor antigen (42), it is possible that some small amount of VPwy may be associated with the cytoplasmic membrane.

The data presented in this paper underscore the complex viral transcription pattern present in EBV latently infected, growth-transformed B lymphocytes and the importance of precise molecular characterization of these transcripts. If indeed the viral transcript corresponding to the IB4WY-1 clone also contains the EBNA2 open reading frame, as suggested by our results and those of Sample *et al.* (33), this would again raise the question of whether the viral transcripts encoding EBNA1 and EBNA2 are really polycistronic (9). It is possible that the virus provides a mechanism for efficient translation of more than one open reading frame. Alternatively, given the complex splicing pattern exhibited in the latent viral transcripts, it is conceivable that the exons encoding EBNA1 and EBNA2 are also present in messages in which they are the only open reading frame. Resolution of this problem will obviously require further characterization of the viral transcripts present in latently infected, growth transformed B lymphocytes.

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