NOTES

Two Related Epstein-Barr Virus Membrane Proteins Are Encoded by Separate Genes

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The structures of the 2.3- and 2.0-kilobase Epstein-Barr virus (EBV) mRNAs, partially encoded within the EcoRI J fragment DNA of the viral genome, were determined by analysis of their cDNAs. Both mRNAs are transcribed across the fused terminal repeats of the EBV episome and consist of nine exons. The mRNAs are transcribed from different promoters and have a unique 5' exon from the U5 region of the genome but eight common exons from the U1 region. One principal open reading frame is present in each mRNA and is predicted to encode 54,000- and 40,000-dalton integral membrane proteins. This result was confirmed by in vitro translation of RNAs in the presence of canine pancreatic microsomes. The 2.3-kilobase mRNA is not expressed in Raji cells, owing to the deletion of the 5' regulatory and coding region of this gene, whereas neither mRNA is expressed in Namalwa cells, owing to inactivation as a result of integration of the EBV genome via the terminal repeats. Since these mRNAs are readily detected in largely latently infected cells and do not increase in abundance with EBV replication, these putative latent-infection membrane proteins are tentatively designated LMP-2A and LMP-2B, respectively.

Epstein-Barr virus (EBV) is the etiologic agent of heterophile-positive infectious mononucleosis and is also associated with anaplastic nasopharyngeal carcinomas and B-cell lymphomas (for a review, see reference 5). Following a primary infection, EBV establishes a latent infection within some of the B lymphocytes of the host, causing growth transformation of the infected cells. The 180-kilobase-pair linear EBV DNA circularizes via a covalent fusion of its terminal repeats, and the genome is subsequently maintained within the cell as an episome but can integrate into cellular DNA (3, 19, 21, 23, 26). Seven virus-encoded proteins are known to be expressed during latent infections. Six of these proteins are the nuclear proteins EBNA-1, -2, -3A, -3B, -3C, and -LP (4, 27, 28, 37). The seventh protein is the latent-infection membrane protein (4, 9). The proteins expressed in latent infections are of particular interest because of their presumed role in maintaining EBV latent infections or their associated growth transformation of lymphocytes.

While the role of EBV latent-infection-associated proteins in latency and growth transformation is being ascertained through functional assays (31, 34, 35, 36, 38), the possibility persists that there may be other EBV proteins which are essential to latency or growth transformation. In fact, 2.3- and 2.0-kilobase (kb) poly(A)⁺ cytoplasmic RNAs have been known for some time to be encoded by the left end of the EBV genome and expressed in a latently infected growth-transformed lymphocyte cell line (IB-4; 15, 33). Northern (RNA) blot and nuclease S1 analyses with RNA from partially permissive (B95-8) cells provided evidence that the 2.3- and 2.0-kb EBV RNAs in these cells are spliced (14); the sequence of a cDNA clone for the 2.3-kb RNA from these cells was recently reported (18), while the studies described

in this manuscript were in progress. The recently reported cDNA clone sequence starts at nucleotide 166607, at the right end of the linear EBV genome, and consists of one exon from the right end and eight exons from the left end of the linear EBV genome. Nuclease S1 mapping data suggested that the first exon of the 2.3-kb RNA in these partially permissive cells begins at nucleotide 166498, 28 base pairs (bp) downstream of a TTATTTT sequence which could be a Goldberg-Hogness box (2, 18). We now report the characterization of nearly full-length cDNA clones for the 2.3- and 2.0-kb RNAs from a latently infected cell line, IB-4. Translation of the 2.3- and 2.0-kb RNAs in vitro yields integral membrane proteins which we have tentatively designated LMP-2A and LMP-2B, respectively.

These experiments began with the screening of IB-4 cell cDNA libraries (28, 32) with probes from the U1 domain of EBV DNA. Seven cDNA clones were isolated. The DNA sequences of two cDNAs, relative to the genomic sequence (1), indicated that they were derived from two related and highly spliced mRNAs that begin within the U5 region of the EBV episome and extend across the fused terminal repeats into the U1 region of the genome (Fig. 1). Based on the cDNA sequences, the two mRNAs consist of a unique 5' exon from the U5 region of the genome followed by eight common exons derived from the U1 region. Three shorter cDNAs, two beginning within exon 3 and one beginning within exon 6, were also characterized and could represent either mRNA. The complete nucleotide sequence of the shortest cDNA was determined, whereas only the terminal sequences were determined for the cDNAs beginning within exon 3. Restriction endonuclease analysis was performed to confirm the structures of these two cDNAs. The 5'-terminal sequences of the two remaining cDNAs indicated that these represented RNAs with at least exons 1 and 2 of the 2.3- or

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FIG. 1. Structures of the 2.3- and 2.0-kb mRNAs. Exons of the mRNAs are shown relative to the region of the EBV genome to which they map, as determined from the nucleotide sequences of their cDNAs (top). Open exons represent noncoding segments of the mRNA, while solid exons represent coding regions. The proteins encoded by the 2.3- and 2.0-kb mRNAs have been tentatively designated LMP-2A and LMP-2B, respectively. The position of the likely initiation codon (ATG) for each protein is indicated. The bottom of the figure illustrates the partial structure of the circularized EBV genome with respect to largely unique (U1 and U5) and major repeat (IR1, IR4, and TR) elements of the DNA. Stippled boxes represent repeated elements.

2.0-kb mRNA. The 3' terminus of either of these cDNAs, however, did not match EBV DNA or each other. These cDNAs are likely to be the result of a cloning artifact or, alternatively, they may represent RNAs derived by transcription across the junction of EBV and cellular DNA at the site of integration of the EBV genome in IB-4 cells (23). Each splice junction conforms to the N/GT . . . AG/N rule for eucaryotic mRNA splicing (25). The genomic coordinates of each exon are presented in Table 1. Sequence analysis confirmed that there is one less T residue in the cDNAs than in the B95-8 EBV genomic sequence (1, 18). The extra base in the genomic sequence occurs at position 270, 271, 359, or 360 (there are two consecutive T residues immediately before and after the splice junction of exons 2 and 3).

The longest cDNA representing the 2.3-kb RNA is 1,958

TABLE 1. Genomic coordinates for the exons of the 2.3- and 2.0-kb EBV mRNAs^a

Exon	mRNA	Genomic coordinate ^b	
		5'	3'
1	2.3	166548	166916
1	2.0	169752	169906
2	2.3 and 2.0	57	271
3	2.3 and 2.0	359	458
4	2.3 and 2.0	540	788
5	2.3 and 2.0	871	951
6	2.3 and 2.0	1026	1196
7	2.3 and 2.0	1280	1495
8	2.3 and 2.0	1574	1682
9	2.3 and 2.0	5408	5856 and 5863

^a Determined by comparison of cDNA and genomic nucleotide sequences.

^b Nucleotide number of the B95-8 EBV genome (1).

bp long (Fig. 2). Since this RNA appears to be 2.2 kb, as determined by denaturing agarose gel electrophoresis and Northern (RNA) blot analysis (Fig. 3A), and since the poly(A) tail is likely to be approximately 200 nucleotides, the cDNA is nearly full length; the previously described exon begins 50 bp 5' to the start of this cDNA clone (18) and is likely to be the cap site of this RNA. This RNA has one principal open reading frame (ORF) which begins at the putative cap site. Translation is expected to initiate at the first methionine codon in this ORF, since this codon is in a favorable context (17) for translational initiation (Fig. 2). This ORF spans the first eight of the nine exons of the 2.3-kb mRNA.

The longest cDNA representing the 2.0-kb RNA is 1,744 bp long and is also likely to be nearly full length, since this RNA [including the poly(A) tract] is 1.9 kb, slightly smaller than the 2.0-kb 18S rRNA (Fig. 3A). The cDNA begins at nucleotide 169752, 43 bp downstream of the third of three closely spaced TAGAAA sequences which could be Goldberg-Hogness boxes for the initiation of transcription of this mRNA. There is no potential splice acceptor sequence between the TAGAAA and the start of the cDNA, making it likely that the cap site for this RNA is near the start of the cDNA. This would position the cap site approximately 200 bp 5' to the latent-infection membrane protein (LMP) mRNA cap site (6), raising the possibility that there are common or overlapping regulatory domains for transcription of the 2.0-kb and the LMP mRNAs in opposite directions. The unique 5' exon of the 2.0-kb mRNA is at least 155 nucleotides long and is spliced from base 169906 of the genome to exon 2 at base 57, the acceptor site of exon 2 of the 2.3-kb mRNA. The first favorable-context methionine codon of the 2.0-kb mRNA cDNA occurs two nucleotides after the splice acceptor site of exon 2 (Fig. 2). Two preceding methionine codons are either unfavorable translational initiation sites or are soon followed by a stop codon. Since the methionine codon in common exon 2 is in-frame with the ORF of the larger RNA as well, translation of the 2.0-kb mRNA is predicted to result in an amino terminally shorter (119 amino acids) version of the same protein as that encoded by the 2.3-kb mRNA. A hydrophilicity analysis of the predicted amino acid sequence of each ORF revealed that these mRNAs would encode two hydrophobic proteins which would differ in the presence of a hydrophilic amino terminus encoded by the larger RNA. Both proteins would have 12 highly hydrophobic domains of at least 17 amino acids (Fig. 2) which could be membrane-spanning domains (16). Assuming a model with an even number of membrane-spanning domains and a cytoplasmic orientation for the amino and carboxy termini, all positively charged amino acids except two would be on the cytoplasmic side of the membrane. The translation products would be 53,011- and 40,383-dalton integral membrane proteins. The shorter protein would lack almost all of the amino-terminal cytoplasmic domain.

A cDNA probe consisting of exons 6 to 9 detected 2.2- and 1.9-kb RNAs in Northern blots of IB-4 or B95-8 RNA (Fig. 3A, lanes 1 and 2). A 4.4-kb RNA was also detected. Unlike the 2.3- and 2.0-kb RNAs, the 4.4-kb RNA increased in abundance in 12-*O*-tetradecanoylphorbol-13-acetate-treated B95-8 cells (compare lanes 2 and 3 in Fig. 3A). This transcript, therefore, is most likely the previously characterized 4.75-kb late-replicative-cycle mRNA which is partially homologous to the 2.3- and 2.0-kb mRNAs and contains the BNRF1 ORF (1, 14, 15). Since the 2.3- and 2.0-kb mRNAs did not increase in abundance, they are more likely to be transcripts of latent-infection genes. Because of their prob-

LMP-2A →

CGCTGCTGCAGCTATGGGGTCCCTAGAAATGGTGCATGGGCGGGGTCCCCCTAGCCCCGGCGGGGATCCGGATGGGT
 MetGlySerLeuGluMetValProMetGlyAlaGlyProProSerProGlyGlyAspProAspGlyT 80

ACGATGGCGGAAACAACTCCCAATATCCATCTGCTTCTGGCTCTTCTGGGAACACCCCCACCCACCGAAGCATGAGGAA
 yrAspGlyGlyAsnAsnSerGlnTyrProSerAlaSerGlySerSerGlyAsnThrProThrProProAsnAspGluGlu 160

CGTGAATCTAATGAAGAGCCCCACCGCCTTATGAGGACCATATTTGGGCAATGGCGACCGTCACTCGGACTATCAACC
 ArgGluSerAsnGluGluProProProProTyrGluAspProTyrTrpGlyAsnGlyAspArgHisSerAspTyrGlnPr 240

ACTAGGAACCCCAAGATCAAAGTCTGTACTTGGGATTGCAACACGACGGGAATGACGGGCTCCCTCCCCCTCCCTACTCTC
 oLeuGlyThrGlnAspGlnSerLeuTyrLeuGlyLeuGlnHisAspGlyAsnAspGlyLeuProProProProTyrSerP 320

LMP-2B →

CACGGGATGACTCATCTCAACACATATACGAAGAAGCGGCGAGGAAGTATGAATCCAGTATGCCTGCCTGTAATGTGTT
 roArgAspAspSerSerGlnHisIleTyrGluGluAlaGlyArgGlySerMetAsnProValCysLeuProValIleVal 400

GCGCCCTACCTCTTTTGGCTGGCGGCTATTGCCGCTCGTGTTCACGGCCTCAGTTAGTACCGTTGTGACCGCCACCGG
 AlaProTyrLeuPheTrpLeuAlaAlaIleAlaAlaSerCysPheThrAlaSerValSerThrValValThrAlaThrGI 480

CTTGGCCTCTCACTTCTACTCTTGGCAGCAGTGGCCAGCTCATATGCCGCTGCACAAAGGAACTGCTGACACCGGTGA
 yLeuAlaLeuSerLeuLeuLeuAlaAlaValAlaSerSerTyrAlaAlaAlaGlnArgLysLeuLeuThrProValT 560

CAGTGCTTACTCGGCTGTCACTTCTTTGCAATTTGCCTAACATGGAGGATTGAGGACCCACCTTTAATTCCTCTCTG
 hrValLeuThrAlaValValThrPhePheAlaIleCysLeuThrTrpArgIleGluAspProProPheAsnSerLeuLeu 640

TTTGATCTGTGGCGGCTGGCGGACTACAAGGCATTACGTTCTGGTGATGCTTGTGCTCCTGATACTAGCGTACAG
 PheAlaLeuLeuAlaAlaAlaGlyGlyLeuGlnGlyIleTyrValLeuValMetLeuValLeuLeuIleValAlaValAr 720

AAGGAGATGGCGCGCTTGTACTGTTTGTGGCGGCATCATGTTTGGCATGTGTACTTGTCTCATCGTCGACGCTGTTT
 gArgArgTrpArgArgLeuThrValCysGlyGlyIleMetPheLeuAlaCysValLeuValLeuIleValAlaValVal 800

TGCAGCTGAGTCCCTCCTTGGAGCTGTAACCTGTGGTTTCCATGACGCTGCTGCTACTGGCTTTCGCTCCTGGCTCTCT
 euGlnLeuSerProLeuLeuGlyAlaValThrValValSerMetThrLeuLeuLeuAlaPheValLeuTrpLeuSer 880

TCGCCAGGGGGCTAGGTACTCTTGTGTCAGCCCTTTTAACTTGGCAGCAGCTCTGGCACTGCTAGCGTCACTGATTTT
 SerProGlyGlyLeuGlyThrLeuGlyAlaAlaLeuLeuThrLeuAlaAlaAlaLeuAlaLeuAlaSerLeuIleLe 960

GGGCACACTTAACCTGACTACAATGTTCTCTCATGCTCCTATGGACACTTGTGGTTCTCCTGATTGTCTCTCGTGCT
 uGlyThrLeuAsnLeuThrThrMetPheLeuLeuMetLeuLeuTrpThrLeuValValLeuLeuIleCysSerSerCys 1040

CTTCATGTCCACTGAGCAAGATCCTTCTGGCAGCAGTGTTCCTATATGCTCTCGCACTCTTGTGTAGCCTCCGCGCTA
 erSerCysProLeuSerLysIleLeuLeuAlaArgLeuPheLeuTyrAlaLeuAlaLeuLeuLeuAlaSerAlaLeu 1120

ATCGCTGGTGGCAGTATTTTGGCAAACTTCAAGAGTTTAAAGCAGCACTGAATTTATACCCAATTTGTTCTGCATGTT
 IleAlaGlyGlySerIleLeuGlnThrAsnPheLysSerLeuSerSerThrGluPheIleProAsnLeuPheCysMetLe 1200

ATTACTGATTGTGCTGGCATACTCTTCTTCTTCTGCTATCTGACGAATGGGGCAGTGGAAATAGAACATACGGTCCAG
 uLeuLeuIleValAlaGlyIleLeuPheIleLeuAlaIleLeuThrGluTrpGlySerGlyAsnArgThrTyrGlyProV 1280

TTTTTATGTGCTCGGTGGCTGCTCACCATTGGTAGCCGGCTGTGTGGCTGACGGTGATGTCTAACACGCTT.TGTCT
 alPheMetCysLeuGlyGlyLeuLeuThrMetValAlaGlyAlaValTrpLeuThrValMetSerAsnThrLeuLeuSer 1360

GCCTGGATTCTTACAGCAGGATTCCTGATTTCTCTTCTTCTTGGCTTGGGGTATTAGATGCTGCCGCTACTG
 AlaTrpIleLeuThrAlaGlyPheLeuIlePheLeuIleGlyPheAlaLeuPheGlyValIleArgCysCysArgTyrCy 1440

CTGCTACTACTGCCTTACACTGGAAAGTGAGGAGCGCCACCGACCCCATATCGCAACTGTATAAAGAAATGCCACCA
 sCysTyrTyrCysLeuThrLeuGluSerGluGluArgProProThrProTyrArgAsnThrVal 1520

GATCGCTGCCACTTCCACAGCAATGGCAGGATGCTGGCGCTTTGCTATGAATTATCCAAGAAACCCACGGAGCAGG 1600

GCAACATTGCAGGGCTCTGTTACGCGATGGTCGTCATCTGGCTCTCCTGTGTGACCCCTCACTTTGTACAGACTTTTGG 1680

CAATGGGAGCACATTTCCCCCGCCTTTGGGCACCCACGGGGTGCTCCCCCTGGACACTTATGTTTCAAGCAGCTCACCT 1760

ATGGTCACTCAGGCACGGTCGCCCCCTCCGAGTGACCACTCAGCTTCCAGACTATGCATACACTGAATTTAGCCTGATATT 1840

GTCCCCCTAGCCCCGGGCCAGCCCTCCTCAGAAACTCTGCATGGAGAAGCTGGACGTGAACCTCCCCCCAGACCTGT 1920

GTGCTGTATTACAAACACTACAATAAACCCCAATGTGC (A)_n

FIG. 2. Nucleotide sequence and translation of the longest cDNA representing the 2.3-kb mRNA. Exon junctions are denoted by arrowheads. The initiation methionine codon of putative LMP-2B lies within the longer LMP-2A ORF and is shown in boldface type. The 12 potential minimal-length transmembrane segments (16) are underscored. The canonical hexanucleotide polyadenylation signal sequence (30) and the site of polyadenylation (nucleotide 5856 of the genome) are also shown in boldface type. An alternative site of polyadenylation (nucleotide 5863) was also used.

able association with latent infections, we have tentatively designated the proteins which would be encoded by the 2.3- and 2.0-kb mRNAs LMP-2A and LMP-2B, respectively. A 5' exon probe from the larger cDNA detected the 2.3- but not the 2.0-kb mRNA (Fig. 3A, lane 6), confirming that the 2.3-kb RNA is the template for the larger cDNAs. An additional RNA of 5 kb was detected with this probe but was also detected on blots of RNA from EBV-negative lymphocytes (data not shown) and is most likely the result of cross-hybridization of the probe to 28S rRNA. Only the 2.0-kb transcript was detectable in Raji poly(A)⁺ RNA (Fig.

3A, lane 4). This was not unexpected, since the immediate upstream regulatory region and a portion of the 5' exon of the 2.3-kb mRNA are within a region of the EBV genome that is deleted from the Raji EBV genome (8, 29). Neither mRNA was detected on Namalwa RNA blots (Fig. 3A, lane 5). This was anticipated, since Namalwa cells contain only linear EBV genomes integrated via the terminal repeats (23), and these RNAs can only arise by transcription of EBV episomes.

For ascertaining whether these RNAs would translate to proteins with the predicted characteristics, the RNAs were

FIG. 3. Northern blot analysis and in vitro translation and membrane insertion. (A) Each lane of a 1.2% agarose-2.2 M formaldehyde gel (22) contained 5 μ g of poly(A)⁺ RNA from the indicated cells. The positions to which the 28S (5.0-kb) and 18S (2.0-kb) rRNAs migrated are indicated to the left of each lane by dots (not all blots were from the same gel). Each blot was probed with the same ³²P-labeled cDNA, except for the blot in lane 6, which was probed with a cDNA fragment representing the 5' exon of the 2.3-kb LMP-2A mRNA. The EBV replication cycle in B95-8 cells was induced (I) with TPA prior to RNA isolation. Numbers to the right represent kilobases. (B) RNAs for LMP-2A and LMP-2B were synthesized in vitro and translated in the presence of canine pancreatic microsomes. Equal percentages of alkaline-extracted (+, lanes 2 and 4) and nonextracted membranes (–, lanes 1 and 3) were loaded onto a sodium dodecyl sulfate-polyacrylamide (6 to 18%) gel. Numbers to the left and right represent kilodaltons.

synthesized in vitro from the two cDNAs off of the T3 polymerase promoter of the Bluescript KS+ plasmid (Stratagene Cloning Systems) and translated in a rabbit reticulocyte lysate in the presence of [³⁵S]methionine and canine pancreatic microsomes. The membranes from one-half of the translation reaction were alkaline extracted to remove proteins which were not inserted into the membranes. The alkaline-extracted and nonextracted membranes were then pelleted and washed through alkaline and neutral sucrose cushions, respectively (7). An equal fraction of each membrane pellet was solubilized in sodium dodecyl sulfate-urea sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20). The data clearly indicated that all of the LMP-2A found in the purified membranes (Fig. 3B, lane 1) was retained in the alkaline-extracted membranes (Fig. 3B, lane 2), indicating that LMP-2A is an integral membrane protein (7, 24). Identical results were obtained for LMP-2B (Fig. 3B, lanes 3 and 4). In addition, a comparison of the supernatant fraction with each membrane pellet indicated that >95% of the LMP-2A and LMP-2B synthesized in vitro was inserted into the membranes (data not shown). The apparent molecular weight of LMP-2A is 54,000, while that of LMP-2B is 40,000, values which agree with the predicted molecular weights of 53,011 and 40,383, respectively. The size of LMP-2A or LMP-2B was the same whether or not microsomal membranes were present in the translation reaction, indicating that no cleavage or glycosylation occurs upon insertion into membranes (data not shown).

Several EBV-immune human sera with unusually high reactivities to Epstein-Barr nuclear antigen or EBV replication-cycle-associated proteins were screened for their ability to immunoprecipitate LMP-2A and LMP-2B in vitro translation products. No specific immunoprecipitation was found. Rats were immunized with peptides corresponding to amino acid residues 42 to 55, 234 to 244, and 411 to 421 of LMP-2A, since these were predicted to be immunogenic epitopes (10). Peptides containing carboxy-cysteine residues were synthesized as described previously (11–13) and coupled to keyhole limpet hemocyanin by use of sulfo-MBS (Pierce Chemical Co.). Animals received six biweekly injections of 200 to 400 μ g of peptide-keyhole limpet hemocyanin conjugate. No antibody response could be detected by specific immunoprecipitation of in vitro-translated LMP-2A or by reactivity with immunoblots of the lysates of latently infected lymphocytes. Thus, we have no direct evidence as yet that these proteins are expressed in latently infected cells.

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