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U2 region of Epstein–Barr virus DNA may encode Epstein–Barr nuclear antigen 2

(herpesvirus/nuclear antigens/sequence diversity/growth transformation/latency)

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ABSTRACT **Sequence analysis of the U2 regions of the B95-8 and AG876 Epstein–Barr virus (EBV) isolates reveals divergence within a long open reading frame previously identified as encoding 1.5 kilobases of the 3′ end of a viral RNA expressed in latently infected, growth-transformed, B-lymphocyte cell lines. Differences among EBV isolates within the U2 open reading frame are shown to correlate with differences in an EBV nuclear antigen, EBNA2. B95-8, W91, Raji, Cherry, and Lamont EBV isolates have similar U2 domains and encode similar-size EBNA2 proteins, while AG876, Jijoye, and P3HR-1 have variant or absent U2 domains and variant or absent EBNA2 proteins. The AG876 U2 open reading frame and EBNA2 protein are both shorter than those of B95-8. These data indicate that the U2 open reading frame encodes EBNA2.**

Epstein–Barr virus (EBV) causes infectious mononucleosis or mild respiratory syndromes. Thereafter, the virus usually persists in a latent state in B lymphocytes. These latently infected lymphocytes can proliferate indefinitely in culture and can form polyclonal lymphomas in immunosuppressed individuals. EBV is believed to be an etiologic factor in the development of African Burkitt lymphoma and anaplastic nasopharyngeal carcinoma, since the cells of these tumors characteristically harbor EBV. Additionally, infection of primate B lymphocytes with this virus is known to induce cell proliferation (for reviews, see refs. 1–4).

The EBV genome is a 170-kilobase (kb) linear DNA molecule that consists of largely unique DNA domains (U1, U2, U3, U4, and U5), internal tandem direct repeats (IR1, IR2, IR3, and IR4), and terminal repeats (TR) (for review, see ref. 4). In latently infected cells, the complete viral genome persists as episomes (5) or integrated DNA (6). Expression of the viral genome is limited (7–10). However, at least three sites on the viral genome are characteristically transcribed. Three transcripts—from IR1 into U2, from U3 through IR3 into U4, and from U5—are each spliced into a cytoplasmic polyadenylylated RNA that is presumed to encode protein (11–15). The U3–IR3–U4 RNA is known to encode an Epstein–Barr nuclear antigen (EBNA) (EBNA1), and the U5 RNA encodes a membrane protein (12, 14, 16–18, 47).

No protein has been assigned to the IR1–U2 RNA. This RNA is initiated by a promoter in any of the copies of IR1, transcribed through subsequent copies of IR1, terminated in U2, polyadenylylated, and spliced into a 3-kb cytoplasmic RNA (11, 13). Hybridizations with the separated DNA strands of IR1 and U2 indicate that 5′ exons of the RNA must be encoded by IR1 (13). These putative 5′ exons have not been identified, however. Furthermore, the principal open reading frame in IR1, identified by DNA sequence analysis (19), does not hybridize to the 3-kb cytoplasmic RNA, suggesting a complex splicing program for the 5′ exons. The principal 3′ exons have been mapped to contiguous DNA fragments near the center of the U2 domain (13). The importance of this gene in growth transformation is suggested by the finding that a nontransforming EBV isolate, P3HR-1 (20), has undergone a deletion of the U2 region (21–24). Other reportedly transformation-competent isolates are, however, known to vary in their central U2 sequence (22).

An EBNA-positive human serum that reacts poorly with the EBNA1 protein detected a second nuclear protein, designated EBNA2, in nuclei of each of five latently infected cell lines that were tested (16). EBNA2 was absent from noninfected continuous B-cell lines (16). The EBNA2 protein is 85 kDa, while EBNA1 varies in size from 68 to 85 kDa, depending on the length of the EBV IR3 repeat in each EBV isolate (14, 16). Antisera raised in rabbits against EBNA1–*lacZ* or EBV membrane–*lacZ* fusion proteins react with either EBNA1 or the membrane protein (16, 47) and not with EBNA2, indicating that EBNA2 is not encoded by the U3–IR3–U4 or U5 RNA.

We now report the sequence of the U2 domain of the B95-8 and AG876 isolates of EBV. The B95-8 U2 DNA sequence reveals a 1545-base-pair (bp) open reading frame. The U2 domains of some isolates such as W91, Raji, Lamont, and Cherry are quite closely related to the B95-8 U2, while others such as AG876 and Jijoye are divergent from B95-8 U2 (22). These nucleotide sequence differences among EBV isolates correlate with differences in their EBNA2 proteins, indicating that the U2 open reading frame encodes EBNA2.

MATERIALS AND METHODS

Cell Cultures and Viral DNAs. Viral DNA was purified from the supernatant virions of induced B95-8 (25), AG876 (26), or HVPapio (27) cultures or from cells of Jijoye (20) or HVPan (28) cultures by isopycnic centrifugation (21).

Electrophoretic Blotting. Nuclei were prepared by resuspending washed cells in 2% sucrose/10 mM Tris·HCl, pH 7.5/10 mM NaCl/3 mM $Mg(OAc)_2$/1 mM phenylmethylsulfonyl fluoride/0.7% Nonidet P-40. Nuclei or cells from the EBV-positive IB4 (7), Namalwa, W91 (29), Jijoye (20), P3HR-1 (20), and AG876 (26) cell lines and the EBV-negative Loukes (11) cell line were boiled in sample buffer (16), electrophoresed on a preparative polyacrylamide gel (30), and electrophoretically transferred to nitrocellulose (31). Blots were routinely immunostained by reaction with a 1:100 dilution of human serum for 18 hr at 4°C followed by 2 hr at 22°C, washed, allowed to react with ^{125}I-labeled staphylococcal protein A (Amersham), and washed again.

Recombinant DNAs, DNA Sequence Analysis, and Southern Blot Hybridizations. The procedures used for restriction endonuclease cleavage, Southern blotting, isolation of discrete DNA fragments from agarose gels, and nick-translation of DNA have been detailed elsewhere (32). The cloned *Bam*HI

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

X (pDF322) and *Bam*HI H (pDK286) DNA fragments from the B95-8 EBV U2–IR2 region and the cloned *Bam*HI D1 fragment (pDA113) from the AG876 EBV U2–IR2 region have been previously described (21, 22, 32, 33). The sequence of the first 1851 bp of B95-8 DNA in Fig. 1 was determined by the method of Maxam and Gilbert (34) and confirmed in part by phage M13 dideoxynucleotide chain termination sequence analysis (35) of *Hin*cII fragments of *Bam*HI X subcloned in M13mp9 and M13mp10 (36). The DNA sequences of the U2–IR2 and IR2–U3 junction regions in B95-8

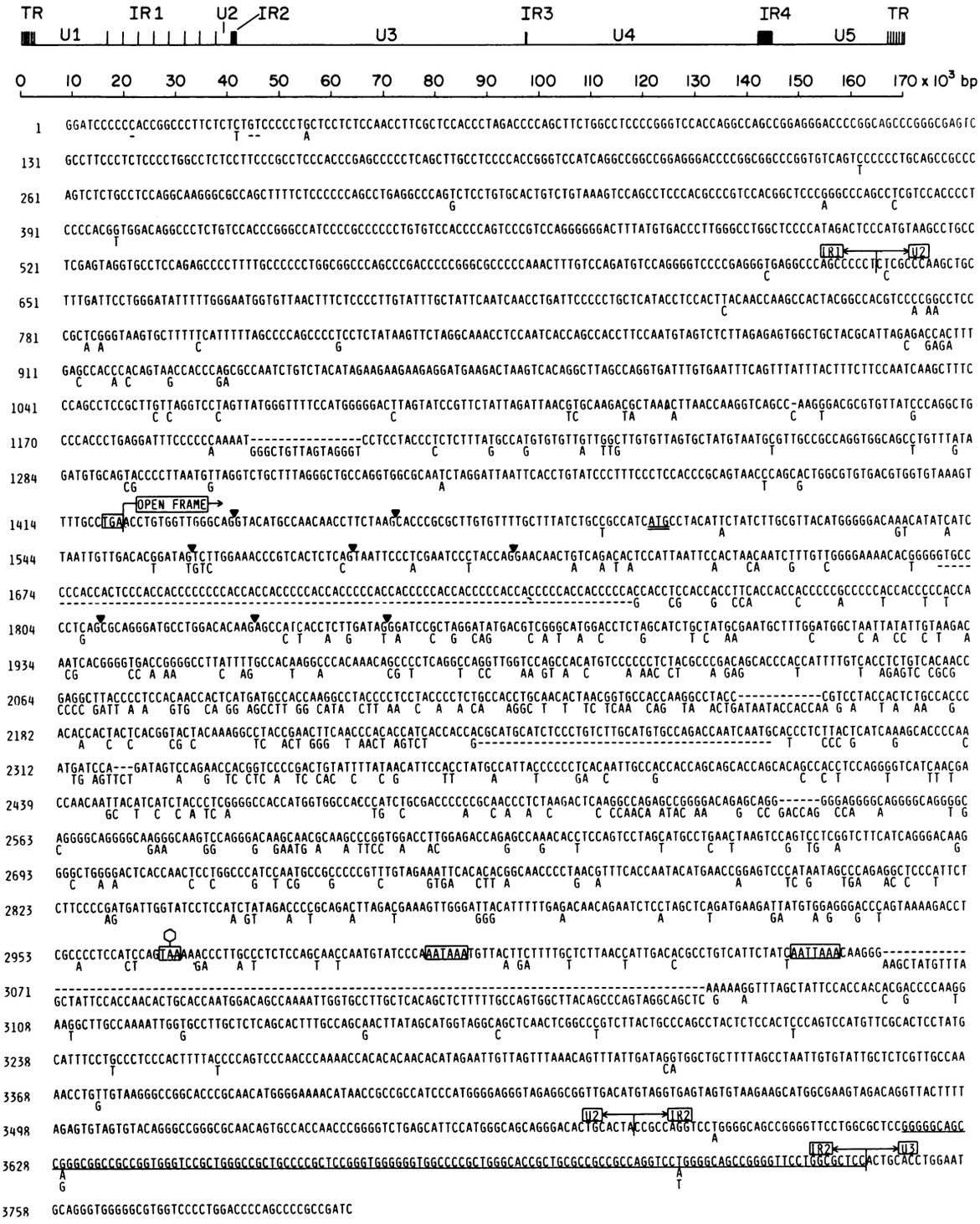


FIG. 1. Comparison of U2 DNA sequences of B95-8 and AG876 EBV. At the top is a schematic diagram of the EBV DNA, indicating unique sequence domains U1–U5, internal repeat sequences IR1–IR4, and the terminal repeats TR (for review see ref. 4). Below the diagram, the sequence of the B95-8 U2 domain and adjacent regions is shown on the upper lines. Differences with AG876 are indicated immediately below the B95-8 nucleotide. A hyphen indicates a deleted base. The 5′ end of the longest open reading frame for both isolates is indicated by the labeled box. The 3′ end of the open reading frame is indicated by the hexagon. Two possible polyadenylylation signals 3′ to the reading frame are enclosed in boxes. The solid arrowheads indicate possible splice acceptor sites near the 5′ end of the U2 open reading frame. A putative initiation methionine codon within the open frame is indicated by the double underline. The sequence of the 125-bp IR2 element is underlined. The coordinates given are relative to the DNA sequence for B95-8.

*Bam*HI H and in AG876 *Bam*HI D1 were determined by M13 dideoxynucleotide chain termination analysis of both strands of specific and overlapping random (37) fragments subcloned into M13mp9 and M13mp10. Sequence data were compiled and analyzed by using the DATBAS program (38), modified to run on a DEC-20 computer. The translation products of the B95-8 and AG876 U2 open frames were characterized by using previously described criteria (39).

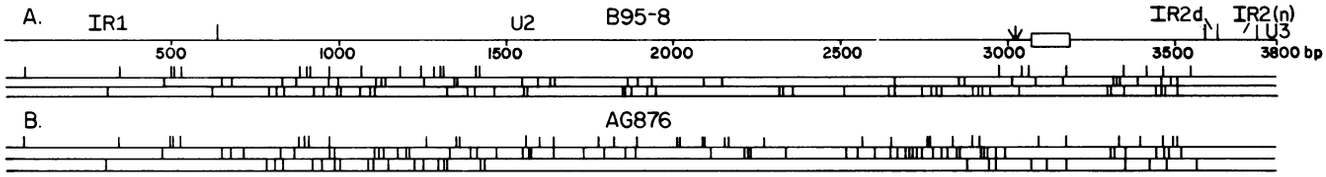
RESULTS

Nucleotide Sequence of the B95-8 U2 Domain. The nucleotide sequence of the B95-8 U2 and adjacent regions (Fig. 1) was determined as described in *Materials and Methods*. The important features of the sequence are (*i*) a 1545-base open translational frame (Fig. 2*A*) which coincides with the part of U2 previously shown to be protected by latently infected cell cytoplasmic polyadenylylated RNA in S1 nuclease experiments (13); (*ii*) an ATG codon 78 bases into the open reading frame (Fig. 1, position 1498) which is flanked by nucleotides associated with translational initiation sites (40); (*iii*) two close matches with consensus splice acceptor sites 5′ to the potential translational initiation site (41); and (*iv*) two polyadenylylation signals 36 and 89 bp 3′ to the termination codon of the open reading frame. Attempts to precisely map the splice site by the "prime cut" method (42) have been unsuccessful owing to the few copies of the RNA per cell (13). Other features of the DNA sequence are (*i*) a CCC-CCA-CCA triplet repeat element within the open reading frame sequence at positions 1672–1758 in Fig. 1, (*ii*) an AGG-GGC repeat element within the open reading frame at position 2545–2574, and (*iii*) an incomplete copy of the 125-bp IR2 repeat (24, 32) at the U2–IR2 junction.

Unusual features of the protein that would be translated from the open reading include a polyproline domain (Fig. 2; amino acids 84–128) encoded by the CCC-CCA-CCA triplet repeat element, a domain consisting of arginine or lysine alternating with glycine or serine (Fig. 2; amino acids 363–378) encoded in part by the AGG-GGC repeat, and an acidic carboxyl terminus (Fig. 2; amino acids 445–494). The amino acid sequence translated from the open reading frame was compared to sequences stored in the National Biomedical Research Foundation data bank (March 1984) (39). No statistically significant matches were noted.

EBNA2 Protein. IB4, Cherry, Lamont, and Raji cells, each latently infected with a different EBV isolate, had previously been shown to have an 85-kDa EBNA2 protein (16) and are known to be similar to B95-8 in their U2 domains (22). Among cell lines previously untested for EBNA2, W91 has a B95-8-like U2 region, while Jijoye and AG876 have variant U2 domains related to each other but distantly related to B95-8 by DNA hybridization (refs. 21 and 22 and Fig. 5), and P3HR-1 has no U2 domain (21–24). If U2 encodes part of EBNA2, W91 cells would be expected to have an 85-kDa EBNA2, Jijoye and AG876 cells a variant EBNA2, and P3HR-1 no EBNA2. All would be expected to have an EBNA1 protein. Accordingly, immunoblots were done with proteins of P3HR-1, Jijoye, and AG876, using protein extracts of IB4 and Namalwa cells as positive controls and with proteins of Loukes cells, an EBV-negative B-lymphoblast cell line, as a negative control. The results (Fig. 3) are that an 85-kDa EBNA2 protein was easily detected in W91 (not shown) and in Namalwa and IB4 cells. Their respective EBNA1 proteins were 77, 79, and 75 kDa. EBNA1 was identified with EBNA1-reactive human sera or with serum from a rabbit immunized with the glycine-alanine copolymer domain of EBNA1 (16). Although a 72-kDa EBNA1 could be detected in Jijoye and P3HR-1, no EBNA2 protein could be detected. An AG876 EBNA2 was demonstrated by weak reactivity at 75 kDa, similar in size but distinct from the 77-kDa AG876 EBNA1 (Fig. 4).

Nucleotide Sequence of the AG876 U2 Domain. If U2 encodes EBNA2, the smaller AG876 EBNA2 should be encoded by a shorter open reading frame in the AG876 U2 DNA. The nucleotide sequence of the AG876 U2 domain is

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B95 TCGWAGTCQQPSKHPRLCFALSAAIMPTFYLALHGGQTYHLIVDTDSLGNPSLSVIPSNPYQEQLSDTPLIPLTIFVGENTGVPPPLPPPPPPPPPPPPPPPPPPPPPPPPPPPSPPPPPPP
AG8 TCGWAGTCQQPSKHPRLCFALSAAIMPTYYLALHGGQSYNLIVDTDMSGNPSLSVIPTNPYQEQLSNNPLIQLQIVVGENTG----------APAPPQPPPPPPPP

126 PPPQRRDAWTQEPSPLDRDPLGYDVGHGPLASAMRMLWMANYIVRQSRGDRGLILPQGPQTAPQARLVQPHVPPLRPTAPTILSPLSQPRLTPPQPLMMPPRPTPPTPLPPATLTVPPRPT----
 97 PPPERRDAWTQEPLPLDMNPLGSDASQGPLASSIRMLCMAQYLLRNARGQQGLLRPLGPQTRSQVTLERQPVHNPRQEAPIILLQSPAPPRFTPVPMVALGHTLQPTPPPRPTLPQPRIPLIIPP

247 RPTTLPPTPLLTVLQRPTELQPTPSPPRMHLPVLHVPDQSMHPLTHQSTPNDP-DSPEPRSPTVFYNIPPMPLPPSQLPPPAAPAQPPPGVINDQQLHHLPSGPPWWPPICDPPQPSKTQGQSRG
212 RHTNQPATTPPTAPQRLTLGHQLSLPP----------HPPPHQSTPHCSSDSTGLPPPPTSYSIPSMTLSPEPLPPPAAPAHPLPGVIYDQQALPPTPGPPWWPPVRDPTPTTQTPPTNTK

371 QSR--GRGRGRGRGKGKSRDKQRKPGGPWRPEPNTSSPSMPELSPVLGLHQGQGAGDSPTPGPSNAAPVCRNSHTATPNVSPIHEPESHNSPEAPILFPDDWYPPSIDPADLDESWDYIFETT
323 QGPDQGQGRGRWRGRGRSKGRGRMHKLPEPRRPGPDTSSPSMPQLSPVVSLHQGQGPENSPTPGPSTAGPVCRVTPSATPDISPIHEPESSDSEEPPFLFPSDWYPPTLEPAELDESWEGIFETT

494 ESPSSDEDYVEGPSKRPRPSIQ
448 ESHSSDEENVGGPSKRPRTSTQ
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FIG. 2. Comparison of B95-8 and AG876 U2 reading frames and translation products. (*A* and *B*) Schematic diagrams of the B95-8 and AG876 U2 regions, indicating by vertical lines the positions of termination codons in the three possible reading frames for the transcribed sequence. The locations of two possible polyadenylylation signals are indicated by the vertical arrow. The open box shows the position of an imperfect 105-bp repeat represented once in B95-8 U2 and twice in AG876 U2. IR2d represents the 38-bp partial copy of the IR2 sequence adjacent to U2. IR2(n) signifies a variable number of direct tandem copies of the IR2 sequence; in B95-8, $n = 11$; for AG876, $n = 13$. Below are the translations (standard one-letter code) of the longest open reading frames from B95-8 and AG876 U2 indicated in *A* and *B* (positions 1423–2967 in Fig. 1). Hyphens indicate deleted amino acids after maximal alignment of the DNA (Fig. 1) and amino acid sequences. Vertical broken lines indicate identical residues. Coordinates are indicated for both the B95-8 and AG876 amino acid sequences. Amino acid 26 is a potential translation initiation site (40).

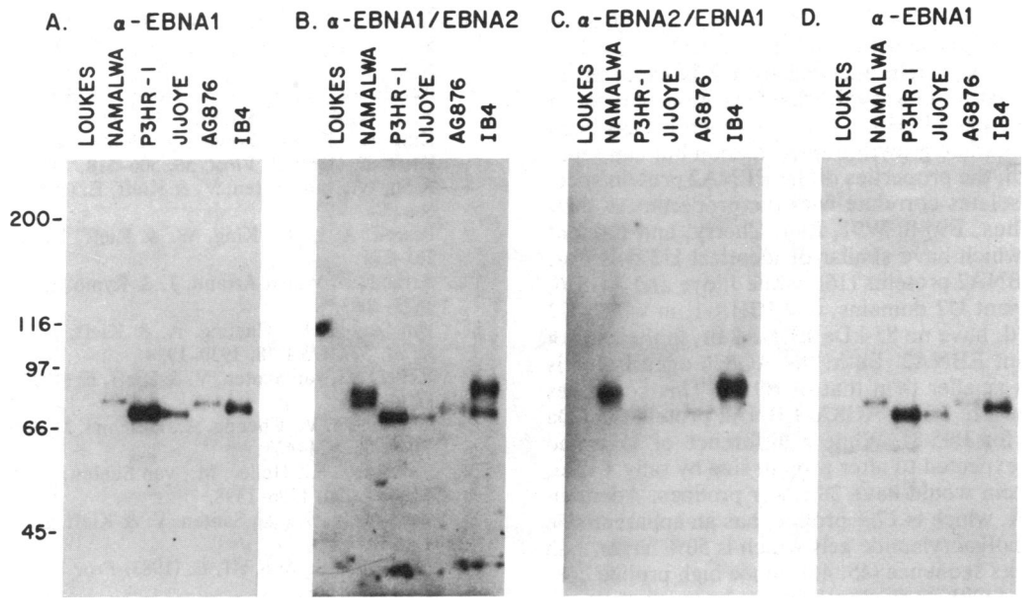

FIG. 3. EBNA2 in cells infected with different EBV isolates. Electroblots of total cellular proteins from the indicated cell lines were immunostained with human sera (*A*, *B*, and *C*) or rabbit anti-EBNA1 serum (*D*). The relative reactivities of each serum toward EBNA1 or EBNA2 are indicated above each blot (α = anti). Size standards are indicated in kDa.

shown in Fig. 1 in comparison to that of B95-8 U2. Most importantly, AG876 has a similar but slightly shorter open reading frame than B95-8. The B95-8 and AG876 IR1 and U2 sequences 5′ to the beginning of the open reading frame are highly conserved except for occasional single base changes and very small deletions. The sequence is also highly conserved at the beginning and end of the open frame but poorly conserved at the center. Within the open frame, AG876 has deletions of 87 nucleotides and 42 nucleotides, while B95-8, relative to AG876, has deletions of 12, 3, and 6 nucleotides. Thus, the AG876 open reading frame is 36 codons shorter than B95-8. After the polyadenylylation signals at positions 3006 and 3058, AG876 and B95-8 are almost identical except for the presence in AG876 of two tandem imperfect copies of a 105-bp nucleotide sequence represented once in B95-8 U2 DNA. The U2–IR2 junction, IR2 repeat, and IR2-U3 junction of the two DNAs are also almost identical. Adjacent copies of the IR2 repeat from AG876 exhibit occasional single base changes (24, 32).

Origin of Diversity Between B95-8 and AG876 U2 Domains. Previous data indicated that Jijoye, HVPan, and HVPapio are divergent from B95-8 U2 at the site of AG876 divergence (22). Three experiments were done to investigate the origin of the diversity between AG876 and B95-8. First, the divergent B95-8 and AG876 U2 *Hinc*II fragments described in the legend to Fig. 5 were labeled and hybridized to blots of uninfected lymphocyte DNA. No signal was obtained despite detection of single-copy B95-8 or AG876 DNA added to cell DNA (data not shown). Second, in Southern blot hybridizations, the Jijoye EBV U2 DNA was shown to be more related to AG876 than to B95-8 (Fig. 5). Third, HVPapio DNA hybridized more to the AG876 U2 fragment, while HVPan hybridized about equally to both B95-8 and AG876 (Fig. 5). Thus, B95-8, AG876, HVPan, and HVPapio U2 domains appear to have derived from a common origin and not by recent recombination with cell DNA.

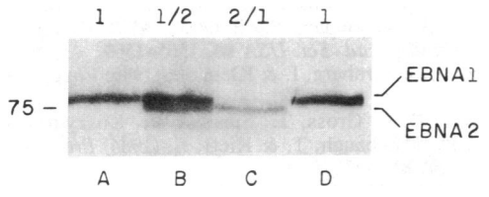

FIG. 4. EBNA2 sera recognize a variant EBNA in the AG876 isolate. Adjacent strips of blotted AG876 nuclear proteins were immunostained with EBNA-positive sera. The same sera were used in lanes A, B, C, and D as in the corresponding panels of Fig. 3. The relative reactivities of each serum toward EBNA1 (1) and EBNA2 (2) are indicated above each strip. Size is indicated in kDa.

DISCUSSION

The IR1–U2 gene was initially identified by hybridizations with nuclear and cytoplasmic RNAs from latently infected growth-transformed cells as one of the three viral mRNAs (7–15). Since the nucleotide sequence of IR1 has been previously reported (19), these analyses complete the nucleotide sequence of the gene. The importance of the IR1–U2 gene in growth transformation is further suggested by the inability of an EBV U2 deletion derivative, P3HR-1, to transform cells (21–24, 43) and by the activity of U2 in stimulating cell DNA synthesis after transfection (44).

Several lines of evidence indicate that the U2 open reading frame encodes most or all of EBNA2. First, U2 encodes contiguous exons of an RNA expressed in latently infected growth-transformed cells (13). EBNA2 is expressed in latently infected growth-transformed cells (16). The proteins encoded by the other principal viral RNAs in latently infected cells have already been identified (14–16). Second, the U2 exon coincides with an open translational reading frame. Third, the reading frame is likely to encode an important protein, since deletions in the B95-8 open frame relative to

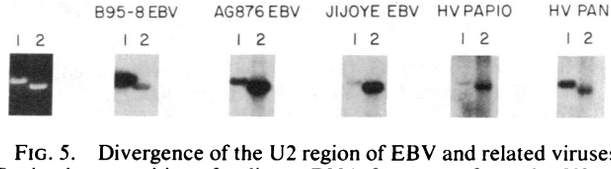

FIG. 5. Divergence of the U2 region of EBV and related viruses. Equimolar quantities of colinear DNA fragments from the U2 regions of B95-8 and AG876 were electrophoresed on adjacent sets of lanes in agarose gels (first strip), blotted, hybridized to ^{32}P-labeled viral DNA at 72°C, and washed. U2 fragments from B95-8 and AG876 (lanes 1 and 2, respectively) correspond to B95-8 nucleotides 1852–3445 and AG876 nucleotides 2006–3445 in Fig. 1.

AG876 and vice versa (Fig. 1) are all in multiples of three nucleotides, suggesting selection for maintenance of reading frame. Further, the protein encoded by U2 is likely to be important in initiation of growth transformation but not in virus replication, since P3HR-1 virus, in which U2 has been deleted, cannot initiate growth transformation but can replicate (43). Fourth, the properties of the EBNA2 protein specified by EBV isolates correlate with the properties of their U2 domains. Thus, B95-8, W91, Raji, Cherry, and Lamont EBV isolates, which have similar or identical U2 domains, have 85-kDa EBNA2 proteins (16), while Jijoye and AG876, which have variant U2 domains, and P3HR-1, in which U2 has been deleted, have no 85-kDa EBNA2 or, in the case of AG876, a variant EBNA2. Fifth, the AG876 open frame is 108 nucleotides smaller than that of B95-8. This correlates with the smaller size of the AG876 EBNA2 protein (75 kDa versus 85 kDa for B95-8). While a difference of 36 amino acids might be expected to alter protein size by only 4 kDa, the AG876 protein would have 28 fewer prolines. Adenovirus protein E1A, which is 17% proline, has an apparent size in $NaDodSO_4$/polyacrylamide gels which is 50% larger than predicted from its sequence (45, 46). Since high proline content is associated with anomalously slow electrophoretic migration in $NaDodSO_4$/polyacrylamide gels, deletion of 28 prolines might be expected to have more than a 4-kDa effect on the apparent size of the AG876 protein on $NaDodSO_4$/polyacrylamide gels.

RNA blot hybridizations indicated that IR1 encodes 5′ exon(s) of the 3-kb IR1–U2 RNA. These exons have not been defined. Thus, although the AUG identified near the beginning of the U2 open reading frame could be the codon for initiation of translation of EBNA2, we cannot exclude the possibility that translation is initiated in a 5′ exon. The 490 codons following the putative initiation codon near the beginning of the open reading frame encode a protein that is 28% proline. Anomalous migration of high proline content proteins could account for the discrepancy between 490 amino acids and the apparent size of B95-8 EBNA2 of 85 kDa.

Considering the evidence that the EBV, HVPan, and HVPapio U2 have a common origin, the extent of divergence between the EBV B95-8 and AG876 isolates is remarkable. First, protein-encoding regions of genes are usually conserved. Second, there might be expected to be selective conservation of a gene encoding a protein necessary for growth transformation or latency because of its likely significance for perpetuation of the virus in human populations. The divergence between AG876 and B95-8 could affect the ability of AG876 to induce growth transformation. Preliminary data suggest that AG876 may be defective in its ability to produce stable lymphocyte growth transformation.

David Lipman compared the DNA and putative protein sequences with the Genbank and Georgetown University data banks. Bart Barrel sent his B95-8 U2 sequence for comparison with ours prior to publication. Prescott Deininger provided instruction on random cloning and dideoxynucleotide sequencing. Alex Cobitz assisted in sequence derivation. This research was supported by Grants CA 19264 and CA 17281 from the National Cancer Institute and by Grant MV 32I from the American Cancer Society. K.H. is supported by National Research Service Award GM 07183. E.K. is a faculty research scholar of the American Cancer Society.

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