Alternative splicing determines the carboxy terminus of the Epstein–Barr virus nuclear antigen 5 species expressed in the Burkitt's lymphoma cell line Daudi

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The Daudi strain of Epstein–Barr virus (EBV) possesses a genomic deletion, relative to the B95-8 EBV prototype, that removes the entire Epstein–Barr virus nuclear antigen 2 (EBNA2) open reading frame (ORF) and the sequences encoding the carboxy terminus of EBNA5. Immunoblot analysis carried out in this study indicates that two species of EBNA5 (31K and 37K) are expressed in Daudi cells. Nucleotide sequence analysis of Daudi cDNA clones has confirmed that, as a consequence of the genomic deletion, exons usually appearing further downstream in EBNA messages (exons U or HF) are spliced directly onto the truncated EBNA5 ORF. Furthermore, the use of alternative splicing suggests that the two EBNA5 species expressed in Daudi cells possess different carboxy termini.

Infection of human B lymphocytes with Epstein–Barr virus (EBV) predominantly results in a latent infection, with little production of virions, and a concomitant immortalization of the infected cells. There are at least nine virus-encoded proteins expressed during latent infection, and the genes encoding six of these, Epstein–Barr virus nuclear antigen 1 to 6 (EBNAs 1 to 6) are transcribed from a complex transcriptional unit spanning the left-hand 100 kbp of the viral genome, as conventionally depicted. Transcription in most B cells is driven by either of two promoters, one in the BamHI C fragment (Cp) and the other in the BamHI W fragment (Wp). Transcripts initiating at Wp and Cp contain one and two unique exons at their 5' ends, respectively. These are spliced to a variable number of repeated exons (W1 and W2) encoded within the BamHI W repeat. The W2 exons splice either to another W1 exon, or to two or three exons encoded within the adjacent BamHI Y fragment (Y1, Y2 and Y3). The Y2 or Y3 exon is in turn spliced to, or contiguous with, unique 3' sequences which include those encoding EBNA1, 2, 3, 4 and 6 (reviewed in Speck & Strominger, 1989). The major part of the remaining member of the EBNA family, EBNA5, is encoded by the W1 and W2 exons, and its carboxy terminus is specified by exons Y1 and Y2 (Dillner et al., 1986; Sample et al., 1986; Speck et al., 1986; Wang et al., 1987). Thus, the EBNA5 open reading frame (ORF) is present in the 5' leader sequences of messages that specify the other EBNA5s. It is noted, however, that such messages can only encode EBNA5 when a translational initiation codon is created by a splicing event at the 5' end of the mRNA (Sample et al., 1986; Speck et al., 1986; Rogers et al., 1990). Both the Daudi and P3HR-1 strains of EBV possess non-identical but similar deletions in their genomes that remove the BamHI Y fragment and adjacent sequences, resulting in the fusion of sequences in BamHI W and H (Jeang & Hayward, 1983; Jones et al., 1984). The deletions include the YH exon encoding EBNA2, and exons Y1 and Y2 encoding the carboxy terminus of EBNA5 (Fig. 1). In the case of Daudi, the last W2 exon is also absent. Deletion of the W2 and Y exons is predicted to result in the splicing of exons that usually appear further downstream directly onto W1, thus endowing the Daudi EBNA5 ORF with a novel 3' end. The aim of this study was to identify these exons, allowing the prediction of the amino acid sequence of Daudi EBNA5.

First, to confirm that the Daudi cell line under investigation did indeed express EBNA5, protein extracts were prepared from these cells and subjected to immunoblotting using either the JF186 monoclonal antibody (MAb) (Finke et al., 1987) or an affinity-purified antibody preparation against EBNA5 (Gregory et al., 1990). The affinity-purified antibody reacted with three protein species in Daudi cells, of M, 29K, 31K and 37K. A species of 29K was, however, also detected in proteins extracted from the EBV-negative cell line, Ramos (Fig. 2). It was assumed, therefore, that the protein of similar size in Daudi cells was a cross-reacting...
Fig. 1. A deletion in the Daudi EBV genome that results in the fusion of sequences in BamHI W to those in BamHI H with a loss of the entire BamHI Y fragment. (a) Physical map of the genome from the prototype B95-8 strain of EBV. EcoRI and BamHI restriction endonuclease maps are shown, together with the approximate location of the deletions in the Daudi and P3HR-1 EBV strains. (b) Schematic representation of the BamHI WYH region of the B95-8 EBV genome illustrating the BamHI sites (B), ORFs and other salient features. The sequences in this region that encode exons present in latent cycle mRNAs (W0, W1, W2, Y1 to Y3, YH, H and HF) are indicated. Above and below the double line, bent and vertical arrows represent potential promoters and polyadenylation signals, respectively. The locations of the lytic cycle replication origin (ori Lyt), the duplicated sequence left (DSL) and those sequences deleted in the Daudi and P3HR-1 EBV genomes are also indicated. (c) Schematic representation of the BamHI WH region of the Daudi viral genome illustrating the features that are retained in this strain. In particular, note that the deletion removes BYRF1 and the Y exons which encode EBNA2 and the carboxy terminus of EBNA5, respectively. The scale and features are as shown in (b).
Short communication

**WUL1, WUE3.1 and WHF1** indicated that either exon U or exon HF can be spliced directly onto W1. The consequence of this for EBNA5 structure is that the 89 amino acids in the prototype (B95-8) protein that are encoded by exons W2, Y1 and Y2 (removed by the genomic deletion in Daudi) are substituted by either two amino acids encoded by the U exon or 23 amino acids encoded by HF (see Fig. 4). Mannick *et al.* (1991) have recently demonstrated that the deletion of the Y1 and Y2 exons markedly impairs the ability of EBV to immortalize B cells *in vitro*; however, the precise role played by EBNA5 in the immortalization process is unclear. It is of interest, in the context of the function of EBNA5, that Jiang *et al.* (1991) have shown that EBNA5 co-localizes with the retinoblastoma protein within the nucleus of EBV-immortalized B cells. EBNA5 has no homology to the retinoblastoma protein, nor does it possess the predicted binding sites for the retinoblastoma gene product (Dyson *et al.*, 1989). At present, the significance of the predicted substitution of carboxy-terminal sequences in Daudi cells is unknown. The demonstration of multiple EBNA5 species in a single EBV-infected B cell line containing a normal BamHI WYH region is probably due to their expression from mRNAs containing different numbers of W exons (Dillner *et al.*, 1986; Speck *et al.*, 1986). Since none of the cDNAs characterized in this study represent full-length copies of mRNAs, the exact number of W exons is unknown. However, the size difference between the two observed Daudi EBNA5 species (6K, corresponding to the size of a peptide encoded by one copy of the W1/W2 exon pair) would be consistent with this hypothesis. An alternative explanation that cannot be excluded, and is supported by data presented here, is that the two EBNA5 species are encoded by different messages containing the same number of W exons, but possessing alternative 3' coding exons, the larger protein being encoded by a message with a W1/HF exon junction (such as the one represented by the WHF1 clone), and the smaller species being encoded by messages exhibiting a W1/U exon junction (such as those represented by the WUL1 and WUE3.1 clones). The difference in the Mr of the HF and U-encoded peptides (calculated to be approximately 3K), however, is too small to explain the observed difference in size of the two Daudi EBNA5 species, assuming that the structures of these proteins (Fig. 4) do not result in anomalous migration on polyacrylamide gels. In this regard, it is interesting that the carboxy-terminal amino acid from the HF exon is a potentially reactive cysteine residue. The inclusion of portions of the L and E3 exons in clones WUL1 and WUE3.1 suggests that these represent parts of the Daudi EBNA3 and EBNA6 messages, respectively. Similarly, the presence of HF exon sequences in clone WHF1 may indicate that

**most of the B95-8 W1-encoded sequence**, it is possible that these mutations explain the lack of reactivity of this antibody with either the P3HR-1 or Daudi EBNA5 species (this study; Finke *et al.*, 1987).

**Since clones WFH1, WUL1 and WUE3.1 do not contain the splice junction creating the translational initiation codon required for EBNA5 expression**, it is unclear whether these clones represent true EBNA5 mRNAs. To date, analysis of cDNA clones has shown that all mRNA species capable of encoding EBNA5 also contain the EBNA2 exon (Sample *et al.*, 1986; Speck *et al.*, 1986). However, it is possible that EBNA5-encoding mRNAs may also contain one of the other EBNA ORFs instead of EBNA2. The detection of EBNA5 species in Daudi and P3HR-1 cells (this study; Rowe *et al.*, 1987; Wang *et al.*, 1987) clearly demonstrates that these proteins can be expressed from mRNAs lacking the EBNA2 ORF. Characterization of cDNA clones

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**Fig. 2. Expression of EBNA5 in Daudi cells.** Protein extracts from 10⁶ IB4, Daudi and Ramos cells were subjected to electrophoresis on a 10% SDS-polyacrylamide gel, and immunoblotted with a 1/10 dilution of an affinity-purified antibody preparation (Gregory *et al.*, 1990), reactive with EBNA5. The positions of Mr standards are indicated. The presence of a 29K species in the EBV-negative cell line Ramos, as well as in IB4 and Daudi, indicates that this is a cross-reacting cellular protein. The two EBNA5 species expressed in Daudi cells are indicated by arrows.
Fig. 3. The *in vitro* amplification of Daudi cDNAs. The top line represents a schematic representation of portions of the Daudi viral genome, showing the relative positions of exons (W0, W1, W2, HF, U, L, E1, E3 and E4) and the primers used to amplify cDNAs by PCR (designated a to h). To conserve space, exons U to E4 are drawn to one-quarter of the scale of exons W0 to HF. The exons encoding EBNA3, EBNA6 and the BHRF1-encoded proteins are indicated. Cytoplasmic RNA (1 µg), prepared from Daudi cells, was ‘reverse-transcribed’ into cDNA using random hexamer oligonucleotides. The products of this reaction were then subjected to PCR amplification with primers a to h. The structures of five cDNA clones isolated, designated WOW1, WOW1'ATG, WHF1, WUL1 and WUE3.1, are shown below the genome. The WOW1 and WOW1'ATG clones were amplified using primers a (5' GTTCTCGAGCGCAAGGAGTCCA 3') and b (5' GTGCCTTCTTAGGAGCTGTC 3'), and differ solely by possession of either a W1 exon or a truncated form of this exon, known as W1'. The selection of the W1' acceptor creates the EBNA5 translational initiation codon. The WHF1 clone was amplified using primers c (5' ATTGAATTCTAAGAAGGCACGGTCG 3') and d (5' CCCGAATTCTGGTTACAACAAATGTCG 3'). Clones WUL1 and WUE3.1 were generated by ‘nested’ PCR, WUL1 being generated by subjecting the products of amplification with primers a and f (5' CGTGAATTCTCCGACGATCTATGGC 3') to a second round of amplification with primers c and e (5' TCCCAAGCTTCCGCCGATACCT 3'). In a similar fashion, clone WUE3.1 was generated by subjecting the products of amplification with primers a and h (5' TATCAAGCTTGATAGCGCTTG 3') to a second round of amplification with primers c and g (5' CTCTGAATTCATCACCCCTGCT 3'). Exons are indicated by solid bars and splicing is represented by the thin lines between the exons. An asterisk denotes that an incomplete copy of that particular exon exists in the clone. Note that, for conservation of space, only one copy of each of the W exons is shown in the representation of WHF1. This clone actually possessed four copies.

Fig. 4. Alternative splicing of RNA is predicted to result in the expression of EBNA5 species possessing different carboxy termini. (a) A hypothetical schematic representation showing the splicing of either the U or HF exons onto the EBNA5 ORF in Daudi mRNAs. Exons are indicated by boxes and splicing is represented by the thin lines between the exons. An asterisk denotes that an incomplete copy of that particular exon exists in the cDNA clone. Only one copy of each of the W2/W1 exon repeat is shown. Shading denotes the EBNA5 ORF. (b) The amino acid sequences of the two forms of EBNA5 predicted from nucleotide sequences of the Daudi cDNA clones. The arrows indicate the exon boundaries in the mRNAs. The amino acids indicated by asterisks denote substitutions relative to the prototype B95-8 strain.
this clone represents a message for the BHRF1-encoded protein. Since the clones obtained in this study were not derived from a cDNA library, but by PCR amplification, the clones may not represent the predominant EBNA transcripts expressed in Daudi cells.

It is noteworthy that multiple EBNA5 species have also been detected in P3HR-1 cells (Rowe et al., 1987; Wang et al., 1987). Since the P3HR-1 virus possesses a similar genomic deletion to that in the Daudi strain of EBV, it is conceivable that the different P3HR-1 EBNA5 proteins are also encoded by messages containing different 3' exons. EBNA5 species have been reported that are larger in size than that predicted from the number of BamHI W fragments in an individual viral genome (Finke et al., 1987; Rooney et al., 1988; Allan & Rowe, 1989). This also may be due to their carboxy termini being encoded by exons other than those derived from BamHI Y. Since EBNA5 clearly contributes to the efficiency of the B cell immortalization process (Hammerschmidt & Sugden, 1989; Mannick et al., 1991; Allan et al., 1992), the biological significance of the mutated EBNA5 species in Daudi (or P3HR-1) cells warrants further investigation.

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**References**


JONES, M. D., FOSTER, L., SHEEDY, T. & GRIFFIN, B. E. (1984). The EB virus genome in Daudi Burkitt's lymphoma cells has a deletion similar to that observed in a non-transforming strain (P3HR-1) of the virus. *EMBO Journal* 3, 813–821.


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