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Epstein-Barr virus mRNAs produced by alternative splicing

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ABSTRACT

The structure of Epstein-Barr virus mRNAs transcribed in B95-8 cells has been studied by cDNA cloning and sequencing. We present here the analysis of four cDNAs. The corresponding mRNAs are probably transcribed from a single promoter located in the US region. They are produced by alternative splicing of exons transcribed from the US, IR and UL regions. The exons are spread over 100 kbp. The exons from the IR region constitute a unit which is repeated several times. The cDNAs share the exons from the US and IR regions. Some of the cDNAs also share some of the exons from the UL region. Each cDNA contains a long open reading frame or the 5' end of a long open reading frame which ends several hundred nucleotides downstream on the viral genome. The 5' untranslated regions are unusually long. Three mRNA species differing in their 5' untranslated regions may encode for the nuclear antigen EBNA-1. The other mRNAs encode for polypeptides which may not have any common region.

INTRODUCTION

Epstein-Barr virus (EBV) is a herpesvirus ubiquitous in humans (for reviews, see 1 and 2). The virus is associated with infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma. *In vitro*, the virus immortalizes human and simian B-lymphocytes. Some fragments of the viral genome immortalize monkey epithelial cells (3), others transform established rodent cells (4). Cell lines established from EBV-associated Burkitt's lymphoma and from *in vitro*-infected lymphocytes harbour the viral genome in an episomal or in an integrated state. They produce viral antigens and some of them produce virus.

The viral genome is nearly 175 kbp long. The genome of the B95-8 virus, which is taken as a prototype, was sequenced (5). Two clusters of tandemly-repeated sequences, designated TR and IR, delimit the US and UL regions (Figure 1A). The structure of

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viral mRNAs was studied by S1 mapping and primer extension experiments (6 - 9) and by cDNA cloning and sequencing (10 - 12). Two cDNAs, designated T1 and JYK2, contain exons transcribed from the IR and UL regions (10, 11). They share the exons from the IR region and some of the exons from the UL region. The T1 cDNA contains the 3' end of an open reading frame which should encode for a repetitive polypeptide (10). The JYK2 cDNA contains the 5' end of the open reading frame coding for the nuclear antigen EBNA-1 (11). A third cDNA, designated T2, contains exons transcribed from the US, IR and UL regions (12). The T2 cDNA shares the exons from the IR region with the T1 and JYK2 cDNAs. We describe here four additional cDNAs, designated T3, T4, T5 and T6, containing exons transcribed from the US, IR and UL regions. They differ among themselves and from the T1, T2 and JYK2 cDNAs with respect to the exons from the UL region.

MATERIALS and METHODS

Construction of the cDNA library. B95-8 cells (13) were grown in RPMI 1640 medium with 10% fetal calf serum (Gibco Laboratories). Cytoplasmic RNAs were prepared (14, 15) and polyadenylated RNAs were selected by chromatography on oligo(dT)-cellulose (15, 16). Double-stranded cDNA molecules were synthesized from polyadenylated RNAs (17) and made blunt-ended by treatment with S1 nuclease and Klenow fragment of *E. coli* DNA polymerase I. The cDNA molecules were cloned in the λgt10 bacteriophage as described (18), with modifications (12). They were methylated by EcoRI methylase before ligation with EcoRI linkers and digestion by EcoRI restriction endonuclease. They were separated from the remaining linkers by chromatography on Sephadex G-75. The longest cDNA molecules were selected by centrifugation through a sucrose density gradient and ligated with the EcoRI arms of the λgt10 bacteriophage. The resultant molecules were packaged *in vitro* (19). The library was amplified on the *HflA150* strain BNN102.

Screening of the cDNA library. The cDNA library was screened by *in situ* hybridization (15). The probe was prepared by nick-translation (20).

Sequencing of the cDNAs. DNA was prepared from the isolated bacteriophages (15) and the EcoRI inserts were cloned in the

pUC13 plasmid (21). Restriction fragments were prepared from the recombinant plasmids and cloned in the mp8, mp9, tg130 and tg131 derivatives of the M13 bacteriophage (22, 23). DNA sequencing was performed as described (24, 25), with modifications (26).

RESULTS

We have constructed a cDNA library from the cytoplasmic polyadenylated RNAs of B95-8 cells in the λgt10 bacteriophage. About 1 x 10^6 recombinants were screened by in situ hybridization. The pDK10 plasmid (27) was used as a probe. It contains the B95-8 BamHI-C fragment, which is located at the junction of the US and IR regions (Figure 1, A and B). Four of the isolated cDNAs, designated T3, T4, T5 and T6, are described here. They are 1.5, 1.7, 2.1 and 1.5 kbp long, respectively. Restriction maps and sequencing strategies are shown in Figure 2.

The nucleotide sequence of the four cDNAs was compared with the nucleotide sequence of the B95-8 viral genome (5). This comparison has shown that the corresponding mRNAs contain exons transcribed from the US, IR and UL regions (Figure 1, A and C).

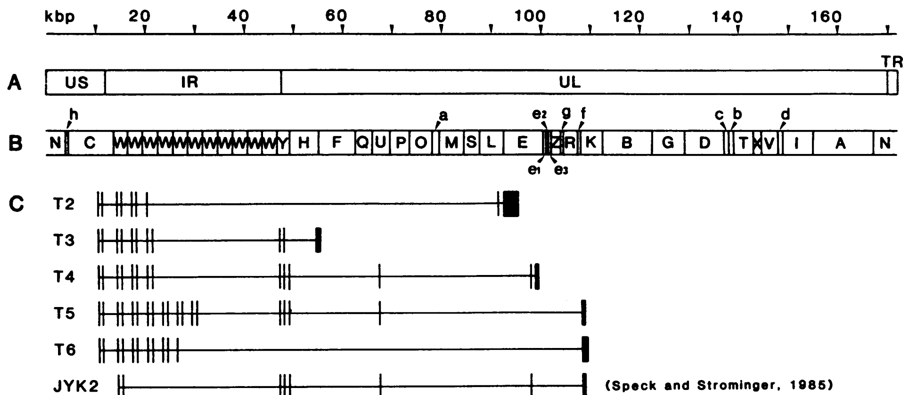

Figure 1 : A/ Linear representation of the EBV genome. Two clusters of tandemly-repeated sequences, designated TR and IR, delimit the US and UL regions. B/ BamHI restriction map of the B95-8 viral genome. C/ The structure of the T3, T4, T5 and T6 cDNAs and of the previously-described JYK2 and T2 cDNAs (11, 12). The exons are represented by vertical bars and positionned onto the viral genome. The exons from the IR region are positionned arbitrarily relative to the leftmost copies of the BamHI-W fragment.

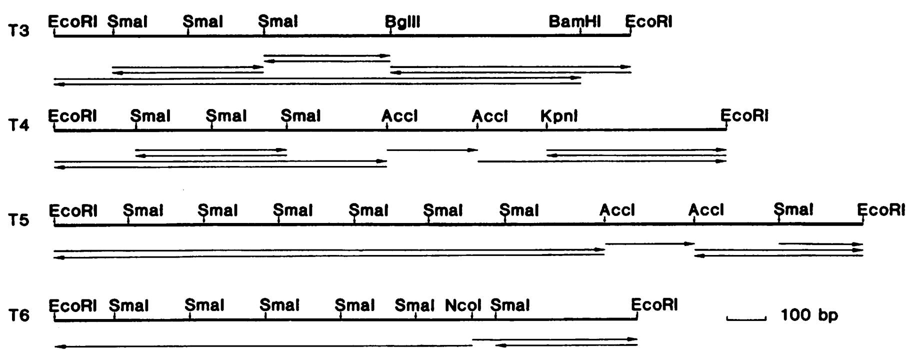

Figure 2 : Restriction maps and sequencing strategies of the T3, T4, T5 and T6 cDNAs. The horizontal arrows represent the restriction fragments which were cloned in the mp8, mp9, tg130 and tg131 bacteriophages and the direction of DNA synthesis in the sequencing reactions.

The exons are designated according to the BamHI fragment of the viral genome from which they are transcribed and to their size. For example, C32 is a 32 nucleotide-exon transcribed from the BamHI-C fragment of the viral genome. When the cDNA contains an incomplete copy of the exon, the size of the exon cannot be deduced from the analysis of the cDNA. In this case, the number is replaced by 5' or 3', depending on the part of the exon which is present in the cDNA. For example, C3' designates the 3' end of an exon transcribed from the BamHI-C fragment of the viral genome. The coordinates of the exons relative to the B95-8 viral genome are indicated in Table 1.

The four cDNAs share the C3' and C32 exons, which are transcribed from the US region (Figure 3). They also share the W66 and W132 exons, which are transcribed from the IR region. These two exons constitute a unit which is repeated several times. Although the units of the T5 and T6 cDNAs were not all sequenced, the restriction enzyme analysis suggests that they are identical. The T3 to T6 cDNAs contain respectively 3, 3, 6 and 4 copies of the unit. The T6 cDNA contains an additional copy of the W66 exon, which joins the repeats to the exons from the UL region. Some of the four cDNAs also share some of the exons transcribed from the UL region. The T3 to T5 cDNAs contain the Y33 and Y122 exons. The T4 and T5 cDNAs contain the Y59 and U172 exons. The T4 cDNA contains the E367 exon. The T3 and T4 cDNAs

Table 1

Exons	5' end	3' end
C32	11,626	11,657
C3'		11,479
E367	98,364	98,730
E5'	98,805	
HF5'	54,335	
K5'	107,942	
U172	67,478	67,649
W66	14,554	14,619
W132	14,701	14,832
Y33	47,761	47,793
Y122	47,878	47,999
Y59	48,386	48,444

The coordinates of the exons of the T3, T4, T5 and T6 cDNAs relative to the viral genome. The exons are designated as described in Results. The numbers refer to the position of the nucleotide located at the 5' or the 3' end of the exons relative to the B95-8 viral genome (5). The coordinates of the W66 and W132 exons are given relative to the leftmost copy of the BamHI-W fragment.

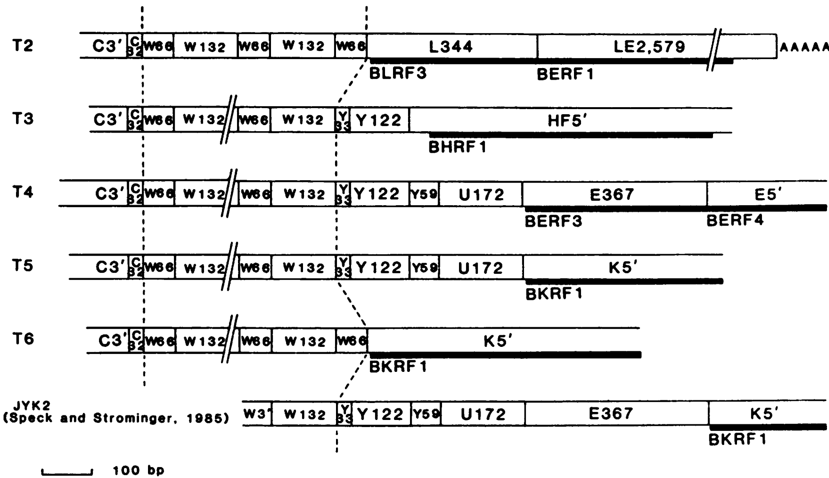

Figure 3 : The structure of the T3, T4, T5 and T6 cDNAs and of the previously-described JYK2 and T2 cDNAs (11, 12). The exons are represented by boxes and designated as described in Results. The dotted lines delimit the exons from the US, IR and UL regions. The W66 and W132 exons constitute a unit which is repeated several times. Although the T3, T4, T5 and T6 cDNAs contain respectively 3, 3, 6 and 4 copies of the unit, only two copies are shown. The T2 and T6 cDNAs contain an additional copy of the W66 exon. Only a part of the LE2,579 exon of the T2 cDNA is shown. The long open reading frames present in the cDNAs are represented by thick lines and designated as described (5).

end with the HF5' and E5' exons, respectively. The T5 and T6 cDNAs end with the K5' exon. The C3' exons of the T3 to T6 cDNAs are respectively 0.08, 0.14, 0.12 and 0.08 kbp. The HF5' and E5' exons of the T3 and T4 cDNAs are respectively 0.65 and 0.24 kbp. The K5' exons of the T5 and T6 cDNAs are respectively 0.4 and 0.55 kbp. The nucleotide sequences of the viral genome corresponding to the junctions between the exons and the introns (Table 2) follow the previously-established rule (28, 29).

Each of the four cDNAs contains a long open reading frame or the 5' end of a long open reading frame which ends several hundred nucleotides downstream from the 3' end of the cDNA on the viral genome (Figure 3). The reading frames are designated according to the BamHI fragment of the viral genome in which they start (5). For example, BHRF1 is a long open reading frame which starts in the BamHI-H fragment of the viral genome. The T3 cDNA contains the BHRF1 reading frame, which is located in the HF5' exon. The corresponding polypeptide is 191 amino acids long and has a predicted molecular weight of 22 kD. It contains 40 % nonpolar, 35 % polar, 12 % acidic and 13 % basic side chains. Two

Table 2

		Intron	Exon	Intron	
C32	11,615	TTCCCTCTAG	GA...CAT	GTATCT	11,664
C3'			...ACC	GTAAGT	11,486
E367	98,353	ATATTTTCAG	AC...AAG	GTGAGT	98,737
E5'	98,794	TTAATTTTAG	CA...		
HF5'	54,324	GGTTTTCTAG	TT...		
K5'	107,931	TCTCTTTTAG	TG...		
U172	67,467	ATTTCTGCAG	GT...AAG	GTGCTG	67,656
W66	14,543	GCCATCCAAG	CC...GAG	GTAAGT	14,626
W132	14,690	CCCGTCTCAG	GG...GGG	GTAAGT	14,839
Y33	47,750	TACAACCAAG	CC...CGG	GTAAGT	47,800
Y122	47,867	TCCAATGTAG	TC...CAG	GTGATT	48,006
Y59	48,375	CCACCCGCAG	TA...CAG	GTACAT	48,451
		CCCCCC C	G A	A	
		X AG	G ... AG	GT AGT	
		TTTTTT T	T C	G	

The nucleotide sequences of the viral genome located around the acceptor and donor sites corresponding to the T3, T4, T5 and T6 cDNAs. The exons are designated as described in Results. The numbers refer to the position of the adjacent nucleotide in the B95-8 viral genome (5). The position of the junctions corresponding to the W66 and W132 exons are given relative to the leftmost copy of the BamHI-W fragment. The consensus sequences (28, 29) are underlined.

potential N-linked glycosylation sites (30) are found at amino acids 22 and 118. The T4 cDNA contains the 5' end of a long open reading frame which is made up from the BERF3 and BERF4 reading frames. A part of BERF3 is joined to BERF4. It starts at the eighth nucleotide of the E367 exon. It ends 2.4 kbp downstream from the 3' end of the T4 cDNA on the viral genome. The corresponding polypeptide is 992 amino acids long and has a predicted molecular weight of 109 kD. It contains 41 % nonpolar, 36 % polar, 10 % acidic and 13 % basic side chains. Its proline residue content is 15 %, which is unusually high. This polypeptide contains tandem repeats of the PAAGP sequence. Six copies of this sequence are located at amino acid 567. A GPPAVGP and a AGPPAAGP sequence are located respectively immediately upstream and 8 amino acids downstream from the repeats. Both of these sequences correspond to a part of the tandem repeats. The V amino acid of the GPPAVGP sequence constitutes the only mismatch. This polypeptide contains also three copies of the PQAPYQGYQEPP sequence, which are located at amino acid 743 and separated by one amino acid. The T5 and T6 cDNAs contain the 5' end of the BKRF1 reading frame which is located in the K5' exon. It starts at the ninth nucleotide of the K5' exon. It ends 1.5 and 1.4 kbp downstream from the 3' end of the T5 and T6 cDNAs on the viral genome, respectively. The corresponding polypeptide is 641 amino acids long and has a predicted molecular weight of 56 kD. It contains 31 % nonpolar, 51 % polar, 7 % acidic and 11 % basic side chains. An unusual feature of this polypeptide is the presence of a stretch of 239 amino acids consisting exclusively of glycine and alanine (5, 32 - 34). It is located between amino acids 90 and 329. The glycine and alanine residue content of this polypeptide is 39 % and 16 %, respectively, which is unusually high.

DISCUSSION

We have studied the structure of EBV mRNAs transcribed in the B95-8 cell line. We report here the characterization of the T3, T4, T5 and T6 cDNAs. The corresponding mRNAs were produced by alternative splicing of exons transcribed from the US, IR and UL regions. These exons are spread over 100 kbp. The four cDNAs

share the exons from the US and IR regions. Some of the cDNAs also share some of the exons from the UL region. Each cDNA contains a long open reading frame or the 5' end of a long open reading frame which ends several hundred nucleotides downstream on the viral genome. The T4 and T5 cDNAs contain the same 5' untranslated exons. The T6 cDNA contains the same 5' untranslated exons as the previously-described T2 cDNA (12). The 5' untranslated exons and the first exon of the translated region of the T4 cDNA correspond to the 5' untranslated exons of the previously-described JYK2 cDNA (11). The 5' untranslated exons of the T3 cDNA correspond to the 5' end of the 5' untranslated region of the T4, T5 and JYK2 cDNAs. The polypeptides encoded by the mRNAs corresponding to the T2 to T4 cDNAs cannot share any common region. Indeed, a complete copy of a long open reading frame is present in the T2 and T3 cDNAs and the E5' exon is located downstream from the E2,579 and HF5' exons on the viral genome. On the contrary, the polypeptide encoded by the mRNA corresponding to the T4 cDNA may share a common region with those encoded by the mRNAs corresponding to the T5, T6 and JYK2 cDNAs. Indeed, the E5' exon is located upstream from the K5' exon. The T5, T6 and JYK2 cDNAs contain the 5' end of the BKRF1 reading frame, which encodes for the nuclear antigen EBNA-1 (31 - 36). Thus, the three corresponding mRNAs may encode for EBNA-1. Alternatively, some of these mRNAs may not contain the whole BKRF1 reading frame. Indeed, a part of the BKRF1 reading frame may be removed by splicing. In this case, the corresponding polypeptides would contain at least the N-terminal end of EBNA-1, including the stretch of glycine and alanine residues.

The T2 to T6 cDNAs contain similar C3' exons at their 5' end. A CCAAT and a TACAAAA sequence, 37 nucleotides apart, are present at nucleotides 11,267 and 11,305 of the B95-8 viral genome, respectively (5). They are located between 30 and 90 nucleotides upstream from the C3' exons on the viral genome. These sequences are close to the consensus sequences for RNA polymerase II promoters (28) and may be part of a promoter. This promoter probably enables transcription of the RNAs corresponding to the T2 to T6 cDNAs. The T3 to T6 cDNAs do not end with a poly(dA) tail and should represent incomplete copies of the

corresponding mRNAs. However, downstream from the 3' end of the cDNAs, the viral genome contain sequences close to the AATAAA sequence characteristic of polyadenylation signals (37). A CATAAA sequence is located 50 nucleotides downstream from the BERF4 reading frame and should enable polyadenylation of the RNA corresponding to the T4 cDNA (5). Similarly, an AATAAA sequence is located 65 nucleotides downstream from the BKRF1 reading frame (5) and should enable polyadenylation of the RNA corresponding to the T5 and T6 cDNAs. An AATAAA sequence is located 570 nucleotides downstream from the BHRF1 reading frame (5). An AACCAAA and an ACTAAA sequence are located 113 and 387 nucleotides downstream from this reading frame, respectively (5). AATAAC sequences are located 161, 368 and 395 nucleotides downstream from this reading frame (5). One of these sequences should correspond to the signal which enables polyadenylation of the RNA corresponding to the T3 cDNA.

The T2 to T6 cDNAs contain tandem repeats of the unit constituted of the W66 and W132 exons. They contain respectively 2, 3, 3, 6 and 4 copies of the unit. Thus, the number of copies should vary depending on the mRNA species. Furthermore, we found a cDNA which is similar to the T5 cDNA, except that it contains three fewer copies of the unit (Bodescot and Perricaudet, unpublished results). Thus, similar mRNA species with different numbers of copies of the unit should exist. Whether the translation efficiency of the mRNAs varies depending on the number of repeats remains to be established. In the T3 to T5 cDNAs, the repeats are joined to untranslated exons from the UL region. On the contrary, in the T2 and T6 cDNAs, where an additional copy of the W66 exon is present, the exons from the IR region are joined to translated exons. The IR region of the viral genome contains eleven copies of a unit corresponding to the BamHI-W fragment (5). However, the cDNAs contain only 2 to 6 copies of the unit constituted of the W66 and W132 exons. Therefore, it is impossible to know how the precursor RNAs were spliced. Furthermore, DNA rearrangements might have occurred in bacteria so that the T2 to T6 cDNAs might be derivatives of cDNA molecules containing more repeats (38). The repeats of the unit constituted of the W66 and W132 exons are reminiscent of repeats

present in the late Polyoma virus mRNAs. Indeed, the 5' untranslated region of these mRNAs contains a tandemly-repeated exon, 57 nucleotides long (39).

The 5' untranslated regions of the T2 to T6 cDNAs are respectively 0.6, 0.9, 1.1, 1.7 and 1.0 kbp, which is unusually long. The mRNA coding for the mouse ornithine decarboxylase (40) and those coding for the mouse and the human myc proteins (41, 42) contain long 5' untranslated regions as well, which seem to be involved in regulation of the translation process (43, 44).

In the B95-8 cell line, some of the cells are engaged in production of virus, the others are not. The mRNAs corresponding to the cDNAs may be produced by all the cells. Alternatively, their production may be specific of the physiological state of the cell. The comparison of the structure of the JYK2, T5 and T6 cDNAs suggests that at least three mRNA species encode for the nuclear antigen EBNA-1. The translation of these mRNA species may be regulated differently. For example, this may enable the quantity of EBNA-1 to vary during the cell cycle or depending on whether the cell produces virus or not.

Both EBNA-1 and the ori-P region, which is located in the US region, are implicated in replication of the viral genome. Indeed, a plasmid which allows the production of EBNA-1 and contains the ori-P region is able to replicate autonomously in several cell lines (45). EBNA-1 binds to two loci within the ori-P region (46). The ori-P region is located 2.2 kbp upstream from the promoter which probably enables transcription of mRNAs coding for EBNA-1 and 99 kbp upstream from the exon coding for EBNA-1. EBNA-1 is thus reminiscent of the SV40 large T antigen. In this respect, we can speculate that the interactions of EBNA-1 with the region located upstream from the promoter may be of importance for regulation of the transcription process. For example, EBNA-1 may regulate its own production.

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