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Mapping of the Gene Coding for Epstein-Barr Virus-Determined Nuclear Antigen EBNA3 and Its Transient Overexpression in a Human Cell Line by Using an Adenovirus Expression Vector

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The open reading frame which lies within the Epstein-Barr virus (EBV) T2 cDNA isolated by Bodescot et al. (M. Bodescot, O. Brison, and M. Perricaudet, Nucleic Acids Res. 14:2611–2620, 1986) was inserted into a eucaryotic expression vector containing a strong adenovirus promoter. The T2 cDNA contains viral genomic sequences from the short BLRF3 open reading frame fused to the adjacent BERF1 long open reading frame. After transfection of human cells, the recombinant plasmid directed the expression of a 140-kilodalton protein. The expressed protein had the same molecular weight, subcellular localization, and immunological characteristics as the EBV-determined nuclear antigen EBNA3, which is made in lymphocytes latently infected with EBV. Immunoprecipitation of extracts of transfected cells labeled with [^{32}P]phosphoric acid showed that the EBNA3 protein is phosphorylated.

Epstein-Barr virus (EBV) is a human herpes virus associated with two malignant tumors, Burkitt's lymphoma and nasopharyngeal carcinoma (for a review, see reference 6). EBV can infect primate B lymphocytes, conferring upon the cells the ability to grow permanently in culture (13). In these immortalized cells, a family of several EBV-determined nuclear antigens (EBNAs) is characteristic of EBV infection. EBNA1 (a 65- to 75-kilodalton [kDa] protein), encoded by BKRF-1 (*Bam*HI fragment K rightward reading frame 1) of the viral genome (15, 26), is a sequence-specific DNA-binding protein which binds to the EBV origin of replication (ori-P) and supports replication of plasmids containing ori-P sequences (21, 22, 29, 30). EBNA2 (80 to 90 kDa) is encoded by BYRF-1 (*Bam*HI fragment Y rightward reading frame 1) (5). This protein is suspected to have a role in the immortalization of B cells (8, 23). EBNA3 (140 kDa) has been described recently by several investigators (14, 16, 24, 25). It has been shown that the open reading frame BERF1 (*Bam*HI fragment E rightward open reading frame 1), which lies within the *Bam*HI E restriction fragment of the viral genome, codes at least in part for this protein (14, 16). EBNA4 is a 148- to 180-kDa protein whose gene and function have not been identified (18). EBNA5 (41 to 70 kDa) is encoded by exons from the *Bam*HI W, Y, and H restriction fragments of the EBV genome (7). The function of this protein has not been determined.

From a cDNA library made from cytoplasmic RNAs of the EBV-producing marmoset B-cell line B95-8, Bodescot et al. (3) have isolated a full-length cDNA, designated T2, whose structure is shown in Fig. 1. The cDNA contains two exons transcribed from the *Bam*HI C fragment, five exons transcribed from the *Bam*HI W fragments, and two exons transcribed from the *Bam*HI L and E fragments of the viral genome. The last two exons contain, respectively, most of the open reading frames BLRF3 (*Bam*HI fragment L rightward open reading frame 3) and BERF1 (*Bam*HI fragment E rightward open reading frame 1) (1), which are joined through splicing.

To express the open reading frame which lies within the T2 cDNA, we subcloned it (using the restriction sites shown in Fig. 1) into the eucaryotic expression vector pMLP10, which was constructed by A. Ballay, M. Levrero, and P. Perricaudet. This vector contains a chimeric association of the very left end of the adenovirus type 5 genome and the major late promoter of the adenovirus type 2 genome joined to its tripartite leader sequence (2; A. Ballay, M. Levrero, and P. Perricaudet, for publication).

The equivalent of the T2 cDNA sequence was reconstructed (for technical ease) by using the 5′ region from the cDNA and the intron-free 3′ region from the viral genome (Fig. 2). The resulting chimeric plasmid pMLP10 T2 harbors the whole coding sequence of the cDNA downstream from the adenovirus promoter (Fig. 2).

The ability of the pMLP10 T2 plasmid to direct the synthesis of an EBV protein was tested in a transient expression assay after transfection of 293 cells. 293 cells (11) were transfected by the calcium phosphate precipitation method (10) with 10 μg of recombinant plasmid DNA and 10 μg of salmon sperm carrier DNA. Forty-eight hours later, the cell monolayer was rinsed with 40 mM Tris–150 mM NaCl–1 mM EDTA (pH 7.4), scraped into the same buffer, and pelleted by centrifugation. The cell pellet was suspended in Laemmli sample buffer and sonicated. Proteins were separated by 9% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate (20). Immunoblotting was performed as described previously (4, 17, 27). Human anti-EBV sera were used as the source of antibodies. The characteristics of the sera used in this study are given in Table 1.

Serum 1 detected EBNA1, EBNA2, and EBNA3 proteins in a whole-cell extract of IB-4 cells (19) (latently infected with B95-8 virus) (Fig. 3, lane 2) but not in a BL2 (EBV-negative Burkitt's lymphoma cell line) cell extract (lane 1). A protein with an apparent molecular mass of 140 kDa was

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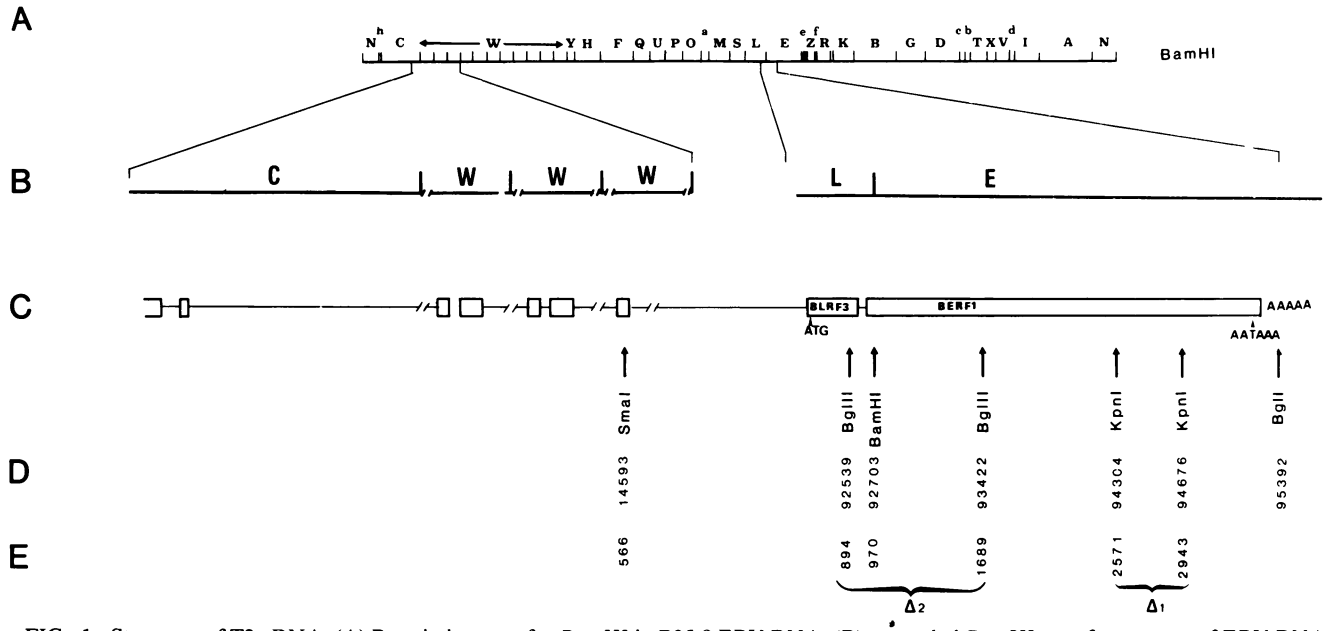

FIG. 1. Structure of T2 cDNA. (A) Restriction map for *Bam*HI in B95-8 EBV DNA; (B) expanded *Bam*HI map from a part of EBV DNA; (C) map of exons (open boxes) in T2 cDNA; (D) coordinates of restriction sites on EBV genome (1); (E) coordinates of restriction sites on T2 cDNA (3). The two open reading frames BLRF3 and BERF1 lie within the last two exons of the cDNA. The positions of the putative initiation codon and the polyadenylation signal are shown along the cDNA. Only the restriction sites of interest for the construction of the recombinant plasmids are indicated.

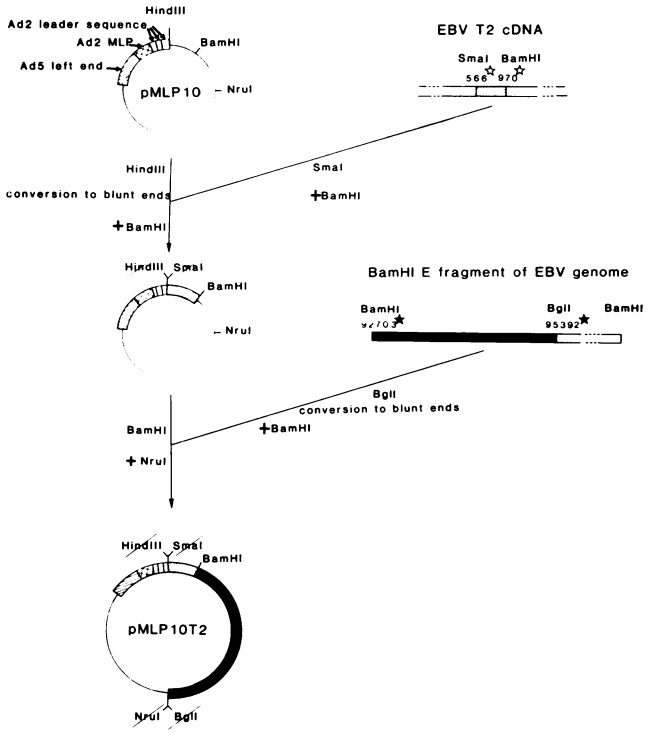

FIG. 2. Construction of pMLP10 T2 plasmid. The *Sma*I-*Bam*HI fragment of the EBV T2 cDNA (positions 566 to 970) and the *Bam*HI-*Bgl*I fragment of the EBV genome (positions 92703 to 95392) were inserted between the *Hin*dIII and *Nru*I restriction sites of pMLP10 after blunt ending of the *Hin*dIII and *Bgl*I ends. ☆, Coordinates on T2 cDNA (3); ★, Coordinates on EBV genome (1). Ad2, Adenovirus type 2; Ad5, adenovirus type 5.

detected in the 293 cells transfected with the pMLP10 T2 plasmid (Fig. 3, lane 3). This protein comigrated with the EBNA3 protein present in the IB-4 cell extract and was absent in mock-transfected cells. These results strongly suggested that the mRNA transcribed from the T2 cDNA encodes the EBNA3 protein. To confirm this hypothesis, deletions were made in the recombinant plasmid in two different sequences of the open reading frame. The recombinant plasmid pMLP10 T2Δ1 was obtained by deleting the *Kpn*I restriction fragment which maps between positions 2506 and 2947 in the T2 cDNA (Fig. 1). The deleted plasmid was expected to direct the expression of an EBNA3 protein with an internal deletion of 147 amino acids near the C-terminal end. A second recombinant plasmid, pMLP10 T2Δ2, was made by deleting the *Bgl*II restriction fragment which maps between positions 894 and 1689 in the cDNA (Fig. 1). This deleted plasmid was expected to direct the expression of an EBNA3 protein with an internal deletion of 245 amino acids near the N-terminal end. The plasmids pMLP10 T2Δ1 and pMLP10 T2Δ2 were transfected into 293

TABLE 1. Reactivities of human EBNA3-positive sera with different EBV antigens

Serum	Reactivity with antigen:						
	VCA[a]	EA[a]	EBNA[a]	EBNA1[b]	EBNA2[b]	EBNA3[b]	EBNA3[a]
1	40	5	320	+	+	+	ND[c]
2	1,280	ND	1,280	ND	ND	+	ND
3	1,280	ND	1,280	ND	ND	+	ND
4	1,280	20	1,280	ND	ND	+	ND
5	1,280	ND	1,280	ND	ND	ND	+
6	1,280	ND	1,280	ND	ND	ND	+

[a] Antigens measured by immunofluorescence; the numbers are reciprocals of the serum dilutions.
[b] Antigens visualized on immunoblots.
[c] ND, Not determined.

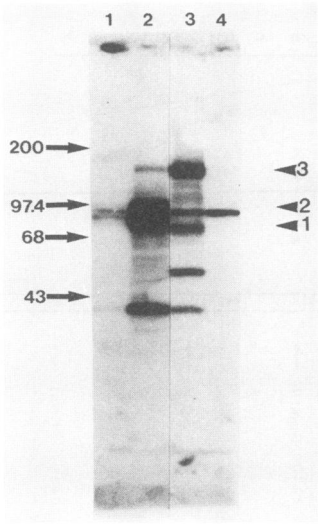

FIG. 3. Immunological detection of EBNA3 in 293 cells transfected with pMLP10 T2 recombinant DNA by using an anti-EBNA human serum. Twenty microliters of proteins corresponding to 2×10^6 cells was run on 9% sodium dodecyl sulfate-polyacrylamide gels. After blotting onto nitrocellulose filters (4, 29), the nonspecific antibody binding sites were blocked by incubation with low-fat milk (17). Specific proteins were detected with a 1/200 dilution of anti-EBNA human serum. The antigen-antibody complexes were visualized after incubation with 1 μCi of ^{125}I-labeled protein A. Lanes: 1, BL2 cells; 2, IB-4 cells; 3 and 4, 293 cells transfected with pMLP10 T2 or salmon sperm carrier DNA, respectively. Molecular mass estimates in kilodaltons are shown in the left margin and were determined with prestained standard proteins (Bethesda Research Laboratories, Inc.). The positions of EBNA1 (1), EBNA2 (2), and EBNA3 (3) are indicated in the right margin.

cells, and the proteins were assayed in a Western blot (immunoblot) experiment; proteins of approximately 115 and 100 kDa were detected by the anti-EBNA3 serum 2 in pMLP10 T2Δ1- and pMLP10 T2Δ2-transfected cells, respectively (Fig. 4). This result clearly shows that the 140-kDa protein is encoded by the open reading frame present in the cDNA, since deletions within the coding sequence led to the expression of shorter polypeptides that were still recognized by the anti-EBNA3 serum. The EBV T2 cDNA clone contains an open reading frame which is made up of BLRF3 and BERF1 sequences. Assuming that the ATG located at position 598 in the T2 cDNA is used as an initiation codon, the mRNA should encode a 944-amino-acid protein with a theoretical molecular mass of 103 kDa. Analysis by polyacrylamide gel electrophoresis showed that the EBNA3 protein in fact migrated as a protein of higher molecular mass. The high percentage of proline residues (13%) in the protein might account for this abnormal electrophoretic mobility. A similar discrepancy has been observed by Hennessy et al. (16), who reported that the expression of BERF1 (which could encode an 812-amino-acid polypeptide) leads to the synthesis of a 120- to 130-kDa protein, as determined by polyacrylamide gel electrophoresis, whereas the theoretical molecular mass is 92.5 kDa.

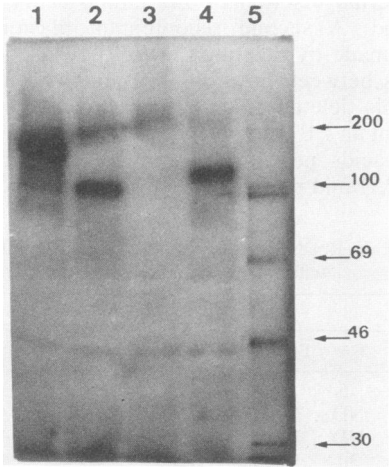

FIG. 4. Western blot analysis of proteins extracted from 293 cells transfected with pMLP10 T2Δ1 and pMLP10 T2Δ2 recombinant plasmids. The experiment was performed as described in the legend to Fig. 3. 293 cells were transfected with pMLP10 T2 (lane 1), pMLP10 T2Δ2 (lane 2), carrier DNA (lane 3), or pMLP10 T2Δ1 (lane 4). Molecular mass estimates in kilodaltons are shown in the margin.

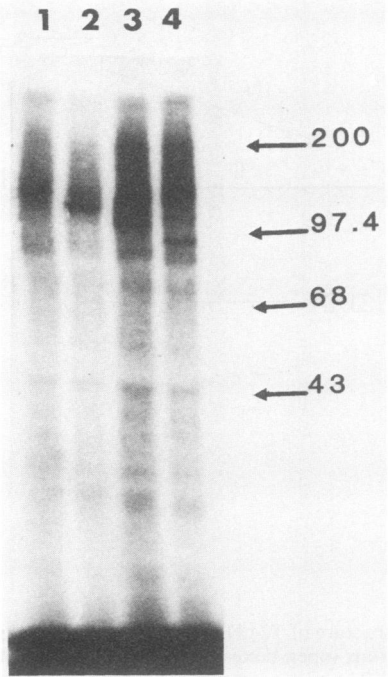

FIG. 5. Immunopreciptation of transfected 293 cells metabolically labeled with [^{32}P]phosphoric acid. Cell protein (corresponding to 3×10^6 cells) was incubated with 4 μl of serum 1 at 4°C, and then immune complexes were bound to protein A-Sepharose (Pharmacia). The bound proteins were eluted by boiling in Laemmli sample buffer, analyzed by polyacrylamide gel electrophoresis, and autoradiographed. Shown are extracts of 293 cells transfected with carrier DNA (lane 1), pMLP10 T2 (lane 2), pMLP10 T2Δ1 (lane 3), or pMLP10 T2Δ2 (lane 4). Molecular mass estimates in kilodaltons are shown in the right margin and were determined as described in the legend to Fig. 3.

In vivo ^{32}P labeling of EBNA3 protein. At 48 h posttransfection, 293 cells were labeled for 3 h with 200 μCi of [^{32}P]phosphoric acid (200 mCi/mmol; 1 mCi/ml; Amersham) as described previously (28); at the end of the labeling period, the cells (7×10^6) were washed twice with phosphate-buffered saline, suspended in 0.4 ml of immunoprecipitation buffer (0.1 M Tris [pH 8.3], 0.1% sodium dodecyl sulfate, 0.25% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM EDTA, 2mM phenylmethylsulfonyl fluoride), d. Full-

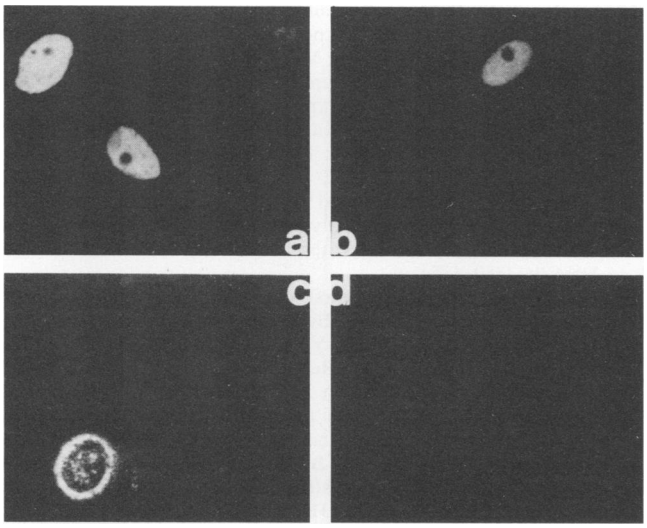

FIG. 6. Indirect immunofluorescence staining of transfected Vero cells. The cells were removed from plates 24 h posttransfection and treated as described previously (12); EBV-positive serum 5 (dilution, 1/10) was used to detect EBNA3 protein. The cells were transfected with pMLP10 T2 (a), pMLP10 T2Δ1 (b), pMLP10 T2Δ2 (c), or carrier DNA (d).

tated by using serum 1 as described previously (28). The EBNA3 proteins, as well as both deleted proteins, could be phosphorylated in vivo (Fig. 5). Phosphorylation sites could be either dispersed over the protein or restricted to the sequence common to both deleted proteins.

To determine the intracellular location of EBNA3 protein (as well as EBNA3 truncated polypeptides) synthesized during transient expresssion, immunofluorescence experiments were performed. In pMLP10 T2-transfected cells, fluorescence was localized in the nucleus; the patchy staining appears to be perinucleolar (Fig. 6a). The same result was obtained when pMLP10 T2Δ1 was used for transfection (Fig. 6b). On the other hand, when pMLP10 T2Δ2 was transfected into the cells, perinuclear staining was observed (Fig. 6c).

These results show that EBNA3 protein synthesized during transient expression is localized in the nucleus of transfected cells, as in EBV-transformed lymphocytes. Hennessy et al. (16) transfected rodent cells with BERF1 inserted in a eucaryotic expression vector; they also observed nuclear staining by using anti-EBNA3 antibodies. We showed that the deleted sequence Δ2 could be important in the process leading to nuclear localization of this antigen. Nuclear localization signal sequences have been identified in several nuclear proteins; however, no universal sequence or position within the primary protein sequence for the nuclear localization signal has been apparent (for a review, see reference 9). The shortest amino acid sequence conferring nuclear localization of EBNA3 protein could be determined by further deletion analysis of the T2 cDNA.

The use of our expression vector, pMLP10, allowed us to identify the EBV gene which codes for the EBNA3 protein and to overexpress it in eucaryotic cells. The availability of a plasmid able to direct the synthesis of high levels of the EBNA3 protein is a valuable tool to study the functions of the protein during latent infection and possibly in the immortalization process.

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