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Enhanced Expression of the Epstein–Barr Virus Latent Membrane Protein by a Recombinant Vaccinia Virus

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SUMMARY

The complete coding sequence of the Epstein–Barr virus strain B95-8 latent membrane protein (LMP) was cloned using a Raji cell cDNA library and genomic B95-8 DNA. The clone was characterized by sequencing and then used to make a recombinant vaccinia virus. This virus (VLMP) was shown to express a relatively high level of LMP in an authentic fashion. Antisera raised in rabbits against VLMP were shown to react with B95-8 LMP as well as cross-reacting with a 50K cellular protein.

Epstein–Barr virus (EBV) is one of six known human herpesviruses and has a near ubiquitous presence in all populations. Interest in EBV has been stimulated by its association with several human cancers including Burkitt's lymphoma, polyclonal B-cell lymphoproliferations in the immunosuppressed and nasopharyngeal carcinoma. Infection of B lymphocytes with EBV *in vitro* leads to immortalization producing lymphoblastoid cell lines which express a restricted set of EBV genes (for reviews, see Dambaugh *et al.*, 1986; Dillner & Kallin, 1988). Such latent gene products include the nucleus-associated antigens EBNA 1, EBNA 2, the EBNA 3 family and EBNA-leader protein. There are also two membrane proteins, latent membrane protein (LMP) and the terminal protein (Laux *et al.*, 1988).

Two forms of LMP are encoded by the BNLF 1 open reading frame (Fennewald *et al.*, 1984). Full-length LMP mRNA, a latent transcript, is assembled from three exons and a shorter, lytic, mRNA consists of the majority of the third exon (Hudson *et al.*, 1985). The predicted products of these transcripts have been identified as proteins having an apparent M_r by SDS–PAGE of approximately 60K and 50K (Hennessy *et al.*, 1984; Modrow & Wolf, 1986; Rowe *et al.*, 1987). Biochemical studies have shown that full-length LMP is an integral membrane phosphoprotein with a cytoplasmic carboxyl terminus (Mann *et al.*, 1985; Mann & Thorley-Lawson, 1987). In addition, computer-assisted analysis and protease digestions indicate that the amino terminus of LMP is located in the cytoplasm adjacent to six hydrophobic transmembrane domains and three short external loops (Liebowitz *et al.*, 1986). One of these external loops appears to be a target for EBV-specific cytotoxic T lymphocytes. Thus LMP may be important in the host immune response against EBV (Thorley-Lawson & Israelsohn, 1987; Murray *et al.*, 1988*a, b*). Several lines of evidence suggest that LMP may play a fundamental role in the association between EBV and cell transformation. Transfection of the LMP gene into rodent and murine fibroblasts induces transformation of these cells (Wang *et al.*, 1985, 1988). In addition, LMP is expressed in lymphoblastoid cell lines, some Burkitt's lymphoma-derived cell lines and some nasopharyngeal carcinoma biopsies (Modrow & Wolf, 1986; Rowe *et al.*, 1986; Rowe *et al.*, 1987; Tugwood *et al.*, 1987; Young *et al.*, 1988).

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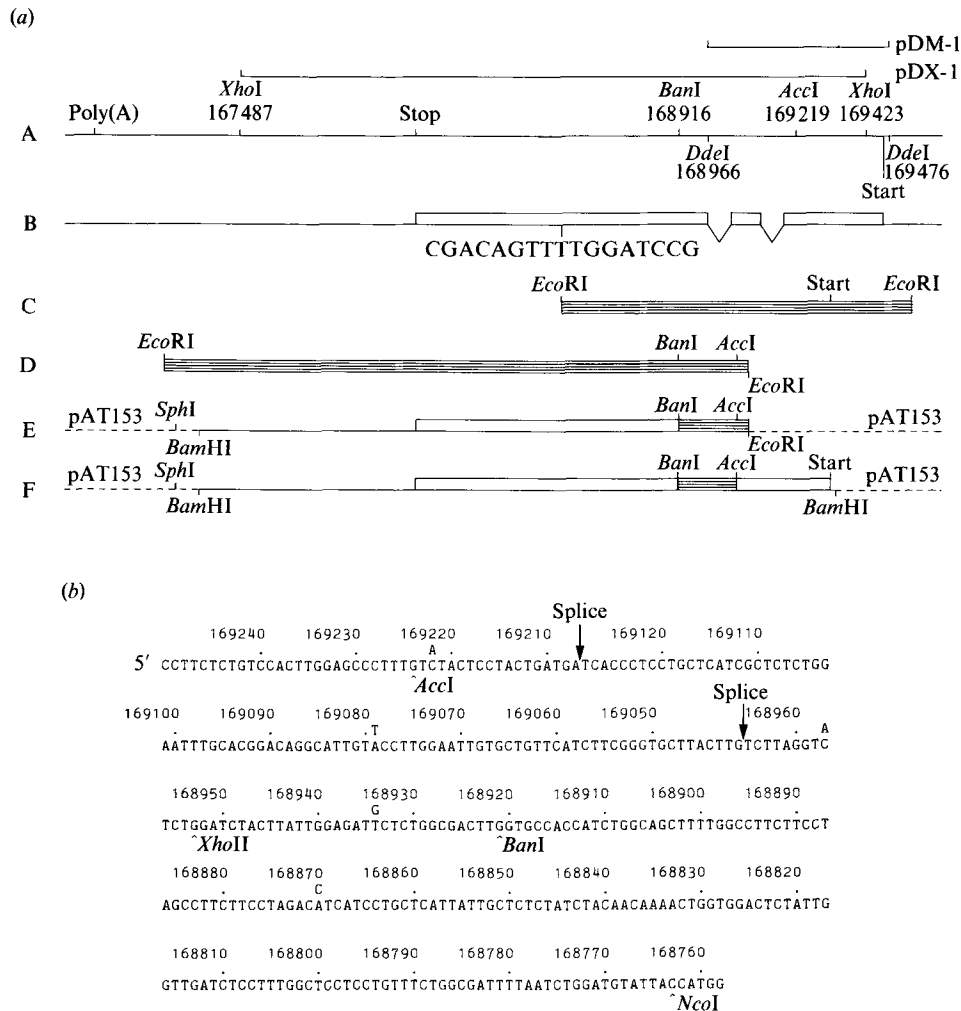


Fig. 1. (a) Construction of the LMP coding sequence. Line A represents the *EcoRI* Dhet region of EBV with the coding sequence of LMP shown below by the open boxes in B [numbered according to the B95-8 sequence of Baer *et al.* (1984)]. Both pDM-1 and pDX-1 were derived from cloned B95-8 DNA (Arrand *et al.*, 1981). pDM-1 is the *DdeI* fragment (168966 to 169476) inserted into the *HinII* site of pUC9. pDX-1 (kindly provided by Dr A. Goodeve) is the *XhoI* fragment (167487 to 169423; with *BamHI* linkers attached) inserted into the *BamHI* site of pUC13. Poly(A)⁺ Raji cell RNA was used to construct a cDNA library (Gubler & Hoffman, 1983) in bacteriophage lambda gt10 (Stratagene) utilizing an oligonucleotide (shown in B) and oligo(dT)₁₅ as primers. The library was probed for the 5' end of LMP cDNA using the short *AccI*-*XhoI* subfragment of pDX-1. C and D show the lengths and positions of the cDNAs isolated, as determined by restriction analysis. Clone C contained the complete 5' end of the gene, but since the *BanI* site had been lost, this clone was not used. Because of restriction site anomalies at the 3' end of clone D, only the *BanI*-*AccI* fragment spanning the two introns was used in the final construction. In order to complete the gene, B95-8 DNA was fused to the 3' and 5' ends of the cDNA fragment. Firstly, a *BanI*-*XhoI* fragment from pDX-1 was ligated to the *BanI*-*EcoRI* fragment from clone D and inserted between the *BamHI* and *EcoRI* sites of pAT153 (E). Next an *AccI*-*BamHI* fragment (*BamHI* site from pUC9) from pDM-1 was simultaneously ligated along with the *SphI*-*EcoRI* fragment from clone E into *SphI*- plus *BamHI*-cut pAT153 to create the final LMP construct (F). (b) Sequence around the splice junctions in Raji LMP cDNA. A fragment from the *EcoRI* linker at the 5' end of the cDNA clone D to the *XhoI* site at position 168948 was inserted into M13 mp18 and 19 and sequenced by the chain termination method of Sanger *et al.* (1977). Sequence downstream from the *XhoI* site was obtained by similar sequencing of the *AccI* (169219)-*NcoI* (168758) fragment. The splice junctions of the exons are indicated by the arrows. Sites of sequence variation between Raji and B95-8 are shown by the corresponding B95-8 nucleotide above the Raji sequence.

In previous studies on LMP, reagents have been derived from portions (e.g. C-terminal peptides) of the protein. We wished to express LMP in an entirely native configuration in order to produce antisera recognizing extracellular as well as intracellular epitopes. To achieve this objective and provide reagents for use in studying cell-mediated immunity we constructed a recombinant vaccinia virus expressing LMP. Vaccinia virus recombinants are well established as an effective system for expression of eukaryotic proteins (Mackett *et al.*, 1985), induction of antisera (Mackett & Arrand, 1985) and studies on cell-mediated immunity (Borysiewicz *et al.*, 1988). However, as vaccinia virus is unable to splice transcripts, it was necessary to isolate an LMP cDNA. This was achieved by screening a cDNA library prepared from Raji cell RNA. A partial LMP cDNA was isolated and a full-length gene was assembled using EBV genomic B95-8 DNA as shown in Fig. 1(a).

The 5' end of the cDNA to the *Nco*I site at position 168 758 (Fig. 1 b) was sequenced to ensure that the two splice junctions were present. This confirmed that the splicing pattern of the LMP mRNA was precisely as predicted by earlier genomic sequencing and S1 mapping (Bankier *et al.*, 1983; Hudson *et al.*, 1985). Within this relatively short stretch of DNA there are only five nucleotide changes between the Raji and B95-8 sequences (see Fig. 1 b). Four of these lie within the region incorporated into the final construct and cause conservative amino acid substitutions in the encoded protein: two Ile to Leu changes, one Phe to Tyr and one Met to Ile. This last change (position 168934) removes the translational initiation site for truncated LMP. These nucleotide changes are in complete agreement with those observed in Raji genomic DNA (Hatfull *et al.*, 1988).

It has been postulated (Modrow & Wolf, 1986) that Raji cells express only the truncated form of LMP. However, our data suggest that the full-length protein is synthesized in these cells since we were able to clone from Raji mRNA a cDNA corresponding to the 5' end of the message for the full-length protein (Fig. 1 a; line C) and this Raji LMP messenger lacks the initiation codon for the truncated form.

The *Bam*HI fragment containing the LMP gene was incorporated into vaccinia virus via the plasmid insertion vector pGS20 using standard techniques (Mackett *et al.*, 1985). Plaques formed by virus lacking thymidine kinase (TK⁻) were picked and recombinants were detected by DNA dot blot hybridization using the LMP cDNA as probe. An LMP DNA-positive recombinant (VLMP) was analysed further for expression of protein by immunostaining of infected cells and Western blotting. Both experiments employed the pooled anti-LMP monoclonal antibodies (MAbs) CS1 to 4 (Rowe *et al.*, 1987) as a probe for LMP. Two cell lines were used: DG-75 which is derived from an EBV-negative Burkitt's lymphoma (Ben-Bassat *et al.*, 1977) and human TK⁻ 143 cells (Rhim *et al.*, 1975). The immunostaining experiments to determine the localization of LMP in cells infected with the vaccinia-LMP recombinants are shown in Fig. 2. No reaction was seen in uninfected cells or in cells infected with a recombinant (VBILF2) known to express the glycoprotein product of the EBV open reading frame BILF2 (M. Mackett, unpublished). In EBV-infected lymphocytes, anti-LMP staining appears as intense membrane-associated patching accompanied by a weaker, more diffuse cytoplasmic staining (Mann *et al.*, 1985; Liebowitz *et al.*, 1986). The pattern of staining observed in VLMP-infected HTK⁻ cells (Fig. 2c) is thus exactly like that reported for native LMP.

Western blotting of infected TK⁻ 143 cells was carried out as described by Rowe *et al.* (1987) except that the immunoperoxidase method of detection was used (Hsu & Soban, 1982). The results in Fig. 3(a) clearly show that recombinant LMP is expressed as a polypeptide of around 60K in VLMP-infected cells but not in mock-infected cells or in cells infected with VBILF2. No reaction was seen on an identical blot probed with an isotype-matched negative control MAb (results not shown).

In Fig. 3(b) the recombinant LMP is compared by Western blotting with the authentic LMP from B95-8 cells. B cell lines were used at 10⁶ cells/lane and VLMP-infected fibroblasts at 10⁴ cells/lane. The method was as described by Rowe *et al.* (1987) and used the CS1 to 4 MAbs as probe. It can be seen that the vaccinia virus-expressed LMP (lane 3) migrates slightly faster than authentic, full-length (58K) LMP (lane 2). This could be due to differences in post-translational modification or, more likely, to the four amino acid differences between the recombinant LMP

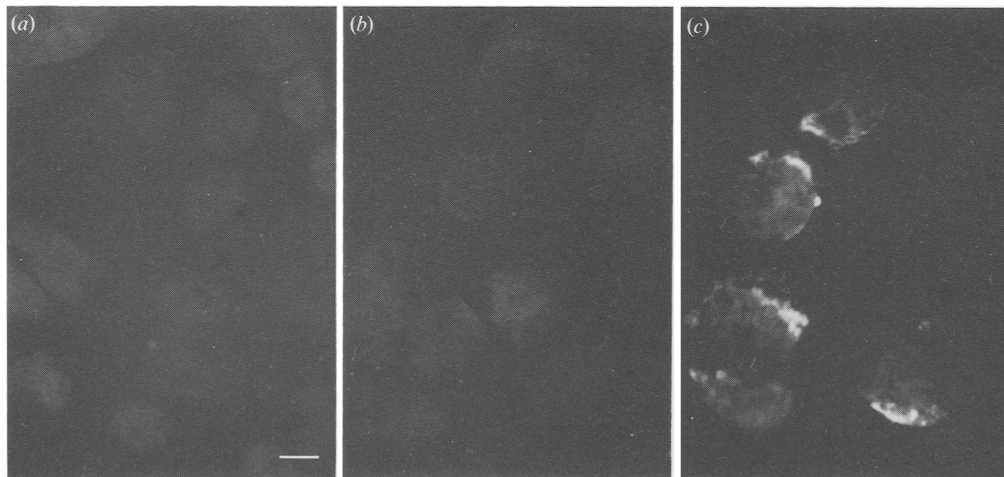


Fig. 2. Cytostaining of mock-infected and recombinant-infected cells using MAbs CS1 to 4. Recombinant vaccinia virus-infected cells were fixed using methanol and stained using the alkaline phosphatase monoclonal anti-alkaline phosphatase technique (Cordell *et al.*, 1984). (a) Mock-infected cells; (b) cells infected with VBILF2; (c) cells infected with VLMP. Bar marker represents 10 nm.

and B95-8 LMP. In this context it has been shown that even single amino acid changes can significantly influence the electrophoretic mobility of polypeptides in SDS-polyacrylamide gels (Matlashewski *et al.*, 1987; Leon *et al.*, 1988). A 41K cross-reactive protein is observed in the lymphoid cell extracts including (lane 4) the EBV-negative B cell line BALL-1 (Hiraki *et al.*, 1977). This has been reported before (Rowe *et al.*, 1987) and may correspond to one of the LMP-related cellular proteins described by Hatzubai *et al.* (1988).

Fig. 3(b) also demonstrates that VLMP-infected human TK⁻ cells express substantially more LMP than do 100-fold more B95-8 cells and could therefore provide a useful source of this protein for biochemical studies. In previous studies degradation products of LMP have often been seen, suggesting that this protein is susceptible to cleavage by endogenous proteases (Liebowitz *et al.*, 1987; Wang *et al.*, 1988). However, no such fragments were observed in our experiments.

To determine whether the VLMP recombinant vaccinia virus would elicit an appropriate antibody response, two half-lop rabbits were inoculated intradermally with 10^8 p.f.u. of VLMP. At 65 days post-vaccination they were re-vaccinated intradermally with a further 10^8 p.f.u. of VLMP. Serum samples were taken before vaccination and at 33 days and 110 days past the initial vaccination. When tested in an indirect immunofluorescence assay using the cell lines B95-8, Raji and DG-75 (EBV-negative control) the pre-vaccination sera were uniformly negative but the post-vaccination sera gave rise to fluorescence on both the EBV-positive and EBV-negative cells (data not shown). This result most probably reflects reactivity with LMP-related host cell proteins as has been previously observed (Hennessy *et al.*, 1984; Hatzubai *et al.*, 1988).

In order to circumvent the problems of host protein cross-reactivity and the low abundance of LMP in EBV-positive lymphoid cell lines, an extract of 2.5×10^7 B95-8, M-ABA (Crawford *et al.*, 1979) or BALL-1 cells was immunoprecipitated with sera from the vaccinated rabbits, fractionated on a polyacrylamide gel and analysed by Western blotting using pooled anti-LMP MAbs as described by Rowe *et al.* (1988). The results in Fig. 3(c) show that in extracts of B95-8 and M-ABA cells a polypeptide of 58K is recognized by sera from the vaccinated rabbits but that this protein is absent from corresponding extracts of BALL-1 cells. A cross-reacting polypeptide of about 50K is observed in both cell types. Again, no reaction was seen when an identical blot was probed with an isotype-matched negative control antibody (results not shown).

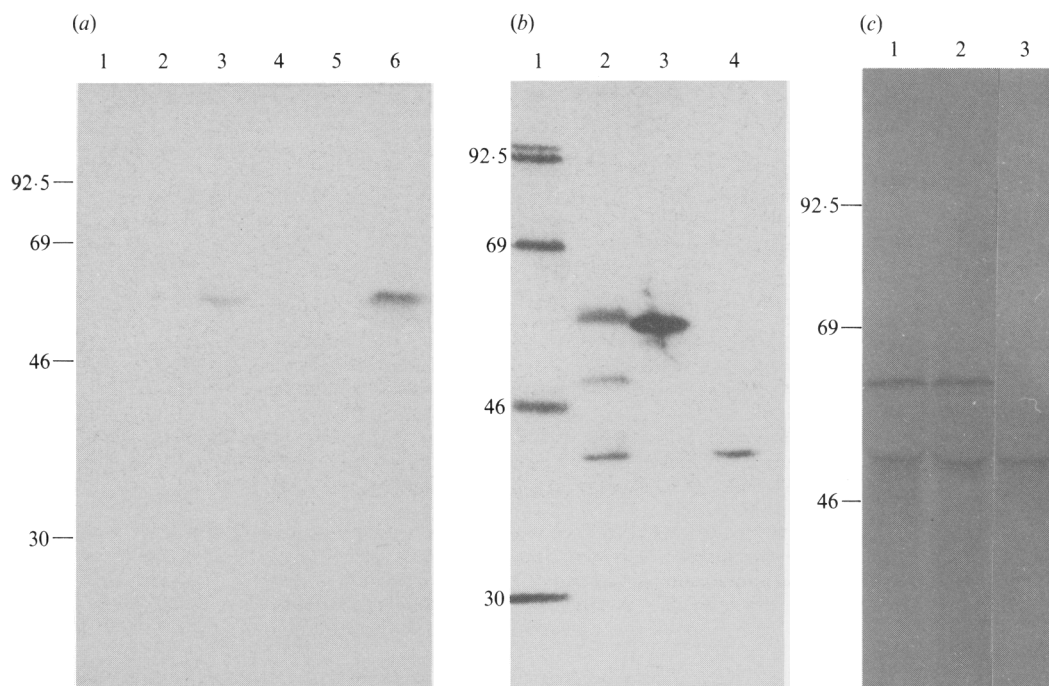


Fig. 3. Analysis of recombinant and authentic LMPs using MAbs CS1 to 4 and rabbit anti-VLMP sera. (a) Western blot analysis of mock-infected and vaccinia recombinant virus-infected cell lines using MAbs CS1 to 4. Cell lines used (10^4 cells/lane) were (lanes 1 to 3) DG-75 and (lanes 4 to 6) human TK-143. Lanes 1 and 4, mock-infected cells; lanes 2 and 5, cells infected with VBILF2; lanes 3 and 6, cells infected with VLMP. M_r markers are indicated ($\times 10^{-3}$). (b) Western blot analysis of B cell lines and the VLMP recombinant using MAbs CS1 to 4. Lane 1, ^{14}C -labelled protein M_r markers ($\times 10^{-3}$; Amersham); lane 2, B95-8 cells; lane 3, VLMP-infected human TK-143 cells; lane 4, BALL-1 cells. (c) Immunoprecipitation and immunoblotting analysis of cell extracts using rabbit anti-VLMP sera and MAbs CS1 to 4. The method was based on that of Rowe *et al.* (1988). Cells were resuspended in RIPA buffer at a concentration of 5×10^7 per ml. Sera from rabbits vaccinated with the VLMP recombinant were mixed with the extracts at a 1:25 dilution and incubated overnight at 0°C . Immune complexes were precipitated using fixed *Staphylococcus aureus* bacteria and solubilized in 100 μl of SDS-PAGE sample buffer. Samples (50 μl /lane) were electrophoresed and electroblotted. Blots were then probed using MAbs CS1 to 4 and processed as described in (a). Lane 1, B95-8; lane 2, M-ABA; lane 3, BALL-1.

Only the full-length form of LMP is observed in this experiment whereas two forms are seen by Western blotting using the CS1 to 4 MAbs. This is probably due to truncated LMP having the same apparent M_r as the cross-reactive band and therefore reactivity to truncated LMP may be obscured.

In this work the LMP gene was cloned in a form suitable for insertion and expression in vaccinia virus. Recombinant viruses containing this sequence expressed relatively large amounts of a protein of similar M_r to the authentic LMP produced in B95-8 cells, and the cellular localization of the product within cells infected with the recombinants was the same as that of the native protein. In addition, following vaccination of rabbits, the VLMP recombinant virus could elicit anti-LMP antibodies against the authentic protein from B95-8 and M-ABA cells. However, cross-reactive components of these rabbit antisera preclude their routine use as specific reagents. Vaccinia virus recombinants have been used with some success in MAb production (Yilma *et al.*, 1987) and thus the VLMP recombinant may well be of value for making novel MAbs against LMP.

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