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Short Communication

Expression of the Epstein–Barr virus major membrane proteins in Chinese hamster ovary cells

(Recombinant DNA; eukaryotic expression vector; dihydrofolate reductase gene; nasopharyngeal carcinoma)

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SUMMARY

The gene of the major membrane antigen (gp250/350) of the Epstein–Barr virus (EBV) was isolated and inserted under the control of the SV40 early promoter into a eukaryotic expression vector, which allows selection for dhfr$^+$ phenotype. Following transfection with this vector, Chinese hamster ovary cells express on their surfaces proteins immunologically similar to the major EBV membrane antigen. The transcript encoding gp250/350 is processed by partial splicing similarly but more efficiently than in B95-8 cells from which the DNA originates.

INTRODUCTION

EBV can cause infectious mononucleosis after primary infection and can consequently be detected lifelong in B-lymphocytes. The virus is believed to be an etiological factor in the development of Burkitt's lymphoma and is to 100% associated with the undifferentiated form of NPC, which is one of the most common tumors amongst the Southern Chinese population (for review, see Epstein and Achong, 1979). The prevention or delay of EBV infection through vaccination would reduce the risk of developing NPC and EBV-related lymphomas and would be of substantial benefit (Epstein, 1976). Large-scale preparation of virus for a vaccine, based on inactivated viral particles or fractions derived from it, would not be feasible due to the low, inefficient replication rate of the virus in cell culture. A second reason to circumvent this type of vaccine production from whole virus is the potential transforming activity of the viral genome, which would require preparations essentially free of viral transforming genes.

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Abbreviations: bp, base pair(s); CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; *dhfr*, dihydrofolate reductase gene; gp, glycoprotein; Ig, immunoglobulin; kb, kilobase(s) or 1000 bp; MA, membrane antigen; mcs, multiple cloning site; NPC, nasopharyngeal carcinoma; nt, nucleotide(s); ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl, 0.015 M Na_3 · citrate, pH 7.6; SV40, simian virus 40.

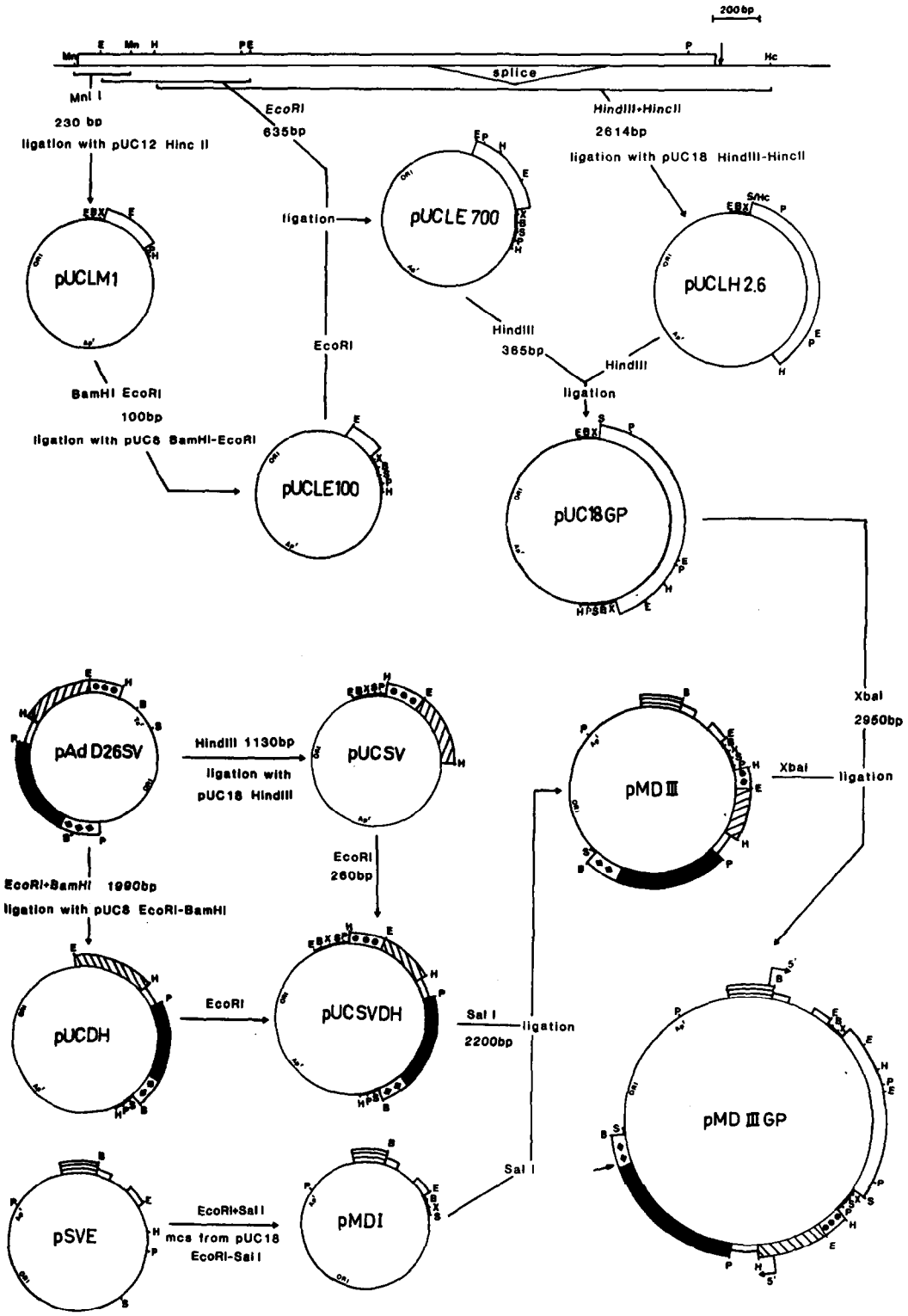

Fig. 1. Construction scheme of the eukaryotic expression vector pMDIIIGP carrying the *dhfr* gene and gp250/350-encoding gene with transcriptional regulation sequences. At the top a part of the leftward strand of the *Bam*HI-L of the EBV B95-8 strain is shown. The open bar represents the coding region for the gp250/350 (ORF BLLF1, Baer et al., 1984) with restriction enzyme sites (abbreviations see below) used for engineering in pUC plasmid vectors (Yanisch-Perron et al., 1985). The fragment spliced out from the mRNA to yield gp250 is indicated by "splice" and lines. The lines below represent sequences which were subcloned as indicated. The 230-bp fragment

Investigation of the composition of the viral membrane proteins have identified at least two glycoproteins of M_rs 250000 and 350000 (gp250/350) which have common antigenic determinants (Qualtiere and Pearson, 1979; 1980; Thorley-Lawson and Edson, 1979). The reading frame encoding both proteins is found within the *Bam*HI-L fragment of EBV. The differences in M_r are caused by an incomplete splice event which removes an internal part from the mRNA encoding gp350 to yield a smaller message encoding gp250 in the same frame as gp350 (Beisel et al., 1985). Epstein et al. (1985) showed that an immunization of cotton top tamarins with the purified gp350 from EBV-producing cells protects animals from a lethal challenge of EBV. They advocate the use of this protein as a subunit vaccine. Recently, Mackett and Arrand (1985) showed the expression of gp350 in eukaryotic cells infected by recombinant vaccinia virus. We report here the expression in an eukaryotic system which produces both proteins with their glycosylations. As host cells we used CHO cells deficient of the *dhfr* gene and an expression vector carrying this gene and the gp250/350-encoding sequences under the control of the SV40 early gene promoter.

EXPERIMENTAL AND DISCUSSION

(a) Cloning of the glycoprotein encoding sequence and construction of the expression vector pMDIIIGP.

The eukaryotic expression vector was constructed in *Escherichia coli* from three parental plasmids. As backbone, the plasmid pSVE (Breathnach and Harris, 1983) was used. It contains the bacterial sequences necessary for maintenance in *E. coli* (origin of replication and β-lactamase gene), an SV40 early promoter and the rabbit globin splice donor and acceptor sites. The *dhfr* together with SV40 enhancer sequences, the adeno major late promoter, a splice acceptor site and the SV40 early polyadenylation site originate from pAdD26SV(A) (Kaufman and Sharp, 1982). The third parental plasmid contains the gp250/350 encoding *Bam*HI-L fragment of the EBV genome from the producing lymphoid cell line B95-8 (Skare and Strominger, 1980).

At the top of Fig. 1 the coding region for gp250/350 with restriction enzymes used for subcloning is shown. We engineered the gp250/350 gene such that its translational initiation codon is located at the end of a fragment which could be excised from the pUC plasmid vector by digestion with *Bam*HI, *Xba*I or *Sal*I. The strategy to obtain this plasmid, pUC18GP, is graphically indicated in Fig. 1. According to the sequence, the blunt end ligation of the

derived from *Mnl*I digestion was inserted into pUC12 linearized with *Hin*cII. The resulting plasmid, pUCLM1, carries the translational start of the gp gene plus three additional bp as it was found by DNA sequence analysis (not shown). A 100-bp *Eco*RI-*Bam*HI fragment was isolated from it and inserted into pUC8 (pUCLE100). From *Bam*HI-L the 635-bp *Eco*RI fragment was inserted into this clone to obtain pUCLE700. A 2614-bp *Hin*dIII-*Hin*cII fragment including the translational stop and the polyadenylation site (arrow) was isolated from *Bam*HI-L and inserted into pUC18 (pUCLH2.6). The 5′ part of the gene was excised as 365-bp *Hin*dIII fragment from pUCLE700 and combined with pUCLH2.6 to obtain the construct pUC18GP. From this clone the gp250/350 coding sequence can be excised with *Bam*HI, *Xba*I or *Sal*I. The *dhfr* gene was recloned from pAdD26SV(A) (Kaufman and Sharp, 1984) by insertion of a 1130-bp *Hin*dIII fragment into pUC18 (pUCSV) and of a 1990-bp *Eco*RI-*Bam*HI fragment into pUC8 (pUCDH). A 260-bp *Eco*RI fragment of pUCSV was isolated and inserted into pUCDH to obtain the construct pUCSVDH, from which the *dhfr* with its transcriptional regulation sequences can be isolated as a *Sal*I fragment. This sequence was combined with the vector pSVE previously engineered by insertion of the mcs of pUC18 (pMDI). As final step the gp250/350-encoding *Xba*I fragment of pUC18GP was integrated to obtain pMDIIIGP. Open segment, gp250/350-encoding sequence; filled-in segment, *dhfr*; segment with dark dots, SV40 enhancer sequence; hatched segment, adeno major late promoter and splice donor site; thin open segment, splice signal sequences (upstream from the gp250/350 gene, β-globin splice donor and acceptor sites; Van den Berg et al., 1978, intron sequence as thin line; upstream from the *dhfr*, splice acceptor site from an antibody heavy chain variable region, Bothwell et al., 1981; segment with concentric lines, SV40 early promoter; open segment with diamonds, SV40 polyadenylation site; heavy line, sequences derived from the pUC-encoded mcs. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; Mn, *Mnl*I; P, *Pst*I; S, *Sal*I; X, *Xba*I. Only in pMDIIIGP the transcriptional starts of mRNAs encoding gp250/350 and the DHFR are indicated as angular arrows, polyadenylation sites as arrows. Except for the coding region in the upper part of the figure the schemes are not drawn to scale.

*Mnl*I fragment with pUC12 should regenerate the *Hin*cII or *Sal*I site since the *Mnl*I digestion should leave a GAC 5′ of the transcriptional start. However, this site was absent and by nt sequence analysis we showed that during the cloning procedure one base (G) had been deleted from the EBV *Mnl*I fragment. The sequence from the pUC12 polylinker (from *Xba*I) to the start codon of gp250/350 reads: TCT AGA GTC ACA ATG. Since the deletion does not affect the translational initiation we used this clone for further engineering (Fig. 1).

The plasmid pAdD26SV(A) was reconstructed such that the *dhfr* gene with its transcriptional signals could be excised from the resulting plasmid, pUCSVDH as a cassette with *Sal*I (Fig. 1).

Between the *Eco*RI and *Sal*I sites of pSVE we inserted the mcs of pUC18. This clone, pMDI, was used as the backbone of the expression vector. The *dhfr* sequences were integrated into the *Sal*I site and the gp250/350 encoding fragment was inserted 3′ of the SV40 promoter and splice signals to yield the final vector, pMDIIIGP.

(b) Transfection of CHO cells and detection of gp250/350

Cultured *dhfr*$^-$ CHO cells were transfected (Graham and Van der Eb, 1973) with the plasmids pMDIIIGP and as control with pMDIII, which lacks the gp250/350 gene. After selection in medium

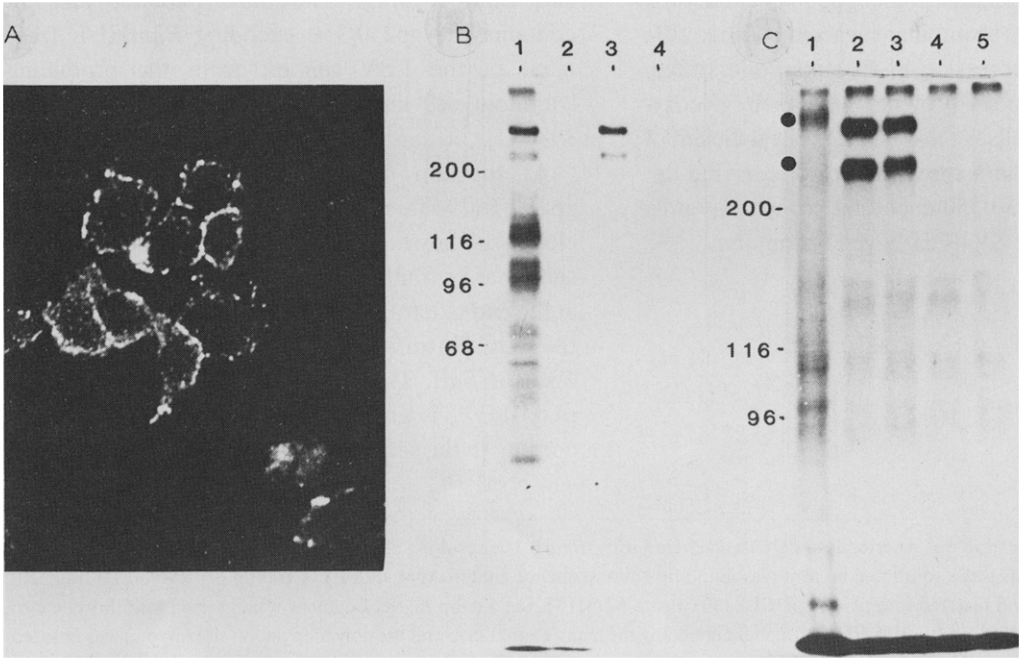

Fig. 2. Detection of the gp250/350 in CHO cells transfected with pMDIIIGP and selected for dhfr$^+$ phenotype. (A) Immunofluorescence. Cells were initially incubated with a monoclonal antibody specific for gp250/350 (11D7; W.J., unpublished) and subsequently with a fluorescein-labeled rabbit anti-mouse IgG antibody. Examination of the cells under UV light shows a membrane fluorescence which indicates the expression of the gp250/350 and its localization in the cytoplasmic membrane. (B) Identification and localization. Surfaces of immunofluorescence-positive cells were labeled with ^{125}I (Jilg and Hannig, 1981) and solubilized with NP40. The EBV-related MA was immunoprecipitated with the mentioned monoclonal antibody and analysed through SDS–10% PAGE and autoradiography (Jilg and Wolf, 1985). Lanes: (1) aliquot of the NP40 extract; (2) aliquot of the NP40 unsoluble pellet; (3) immunoprecipitation of proteins from the NP40 extract with the monoclonal antibody specific for gp250/350; (4) same as lane (3), but with a monoclonal antibody directed against a viral capsid component of EBV. Sizes at the left margin in kDa. (C) Reactivity with antibodies of NPC patients, M_r and ratio of gp250-gp350 compared with the authentic gp of B95-8. NP40 extracts of ^{125}I-labeled surface proteins were precipitated with a serum pool from NPC patients and analysed through SDS–7.5% PAGE and autoradiography. Lanes: (1) B95-8 cells; (2) and(3) immunofluorescence-positive CHO clones; (4) CHO clone transfected with control plasmid pMDIII; (5) EBV-negative BJA cells. The recombinant gps have sligthly smaller M_rs than those from B95-8 cells, which mainly produce the 350-kDa component (both proteins are marked by a black dot).

lacking all nt the appearance of DHFR-positive cells was clearly visible after two to three weeks. The cells transfected with the control plasmid had a normal growth behavior and could be picked from the plates very easily, whereas the colonies derived from transfection with pMDIIIGP showed very slow growth and most of them died after reaching a colony size of about 100–200 cells. Only a few could be picked from the plates and grown to a larger scale. Finally, after several passages the cells approached normal division rates. Four clones derived from the transfection with pMDIIIGP were expanded and two of them were found to be positive in an immunofluorescence test for EBV membrane-specific fluorescence (Fig. 2A). The identification and localization of the recombinant produced gp250/350 is shown in Fig. 2B. A CHO cell clone positive in the immunofluorescence was surface-labeled with ^{125}I. Through NP40 extraction only membrane proteins become solubilized and the appearance of two iodinated proteins in the expected M_rs of 250000 and 350000 in the extract but not in the insoluble fraction indicates a localization in the cell membrane. Both proteins are recognized by the monoclonal antibody used for immunofluorescence but not by an antibody specific for a viral capsid antigen (anti-p150; W.J., unpublished). The reactivity of human sera derived from NPC patients and the size comparison to the authentic proteins from the EBV producer cell line B95-8 are shown in Fig. 2C. The proteins produced by the CHO clones were recognized by the NPC sera as the authentic EBV MA, but have M_rs significantly lower than the gp250/350 of B95-8 cells. This may be caused by a decrease of posttranslational glycosylation as compared to the lymphoid B95-8 cell. The ratio of the two proteins is nearly equal and indicates that the CHO post-transcriptional system recognizes the splice signals more efficiently than B95-8 cells, where only a minor fraction of the gp-encoding mRNA is processed to yield gp250.

We have no exact explanation for the very low yield of stable MA-producing clones. It seems possible, that especially in the selection phase, when the cells are in a depleted medium, the integration of the foreign glycoprotein into the cell membrane impedes the viability of these recombinants.

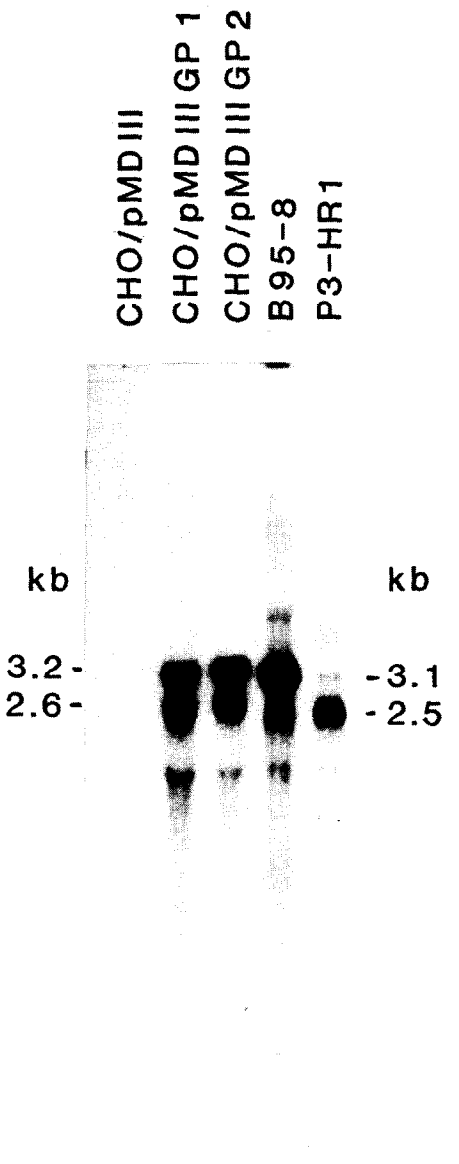

Fig. 3. Analysis of EBV-specific transcripts by Northern blotting of cytoplasmic RNA from two gp250/350 positive cell clones (carrying pMDIIIGP), from one negative clone (pMDIII) and from two EBV-producer cell lines (B95-8 and P3HR1). 10 μg RNA obtained according to Chirgwin et al. (1979) and pelleted through $CsCl_2$ (1.8 g/ml) was electrophoresed on a 1% agarose gel in the presence of 6% formaldehyde. After transfer to nitrocellulose, EBV-specific transcripts were hybridized with [^{32}P]pUC18GP in 50% formamide, 5 × SSC and 2 × Denhardt's solution at 50°C. The filter was washed 4 × 5 min with 2 × SSC and 0.1% SDS at room temperature and 2 × 15 min with 0.1 × SSC and 0.1% SDS at 68°C and finally exposed to an X-ray film for 24 h. To show the sizes of the transcripts (2.6 and 3.2 kb) in comparison to EBV-producing cells, RNA from B95-8 and P3-HR1 cells (2.5 kb and 3.1 kb) were also applied.

(c) Detection of EBV-specific DNA sequences and transcripts

The organization of the transfected sequences was studied by Southern blot analysis, using pMDIIIGP as probe. Complex patterns were observed, suggesting that DNA rearrangement had occurred (not shown). A Northern blot with cytoplasmic RNA hybridized with labeled pUC18GP shows the EBV-specific transcription products (Fig. 3). The mRNAs of positive CHO cells have slightly larger sizes than the transcripts from B95-8 cells. Beisel et al. (1985) determined the start of the gp250/350 encoding mRNA to be 19 nt 5′ of the translational start codon in B95-8 cells. The recombinant CHO cells, however, have different 5′-untranslated sequences. The SV40 promoter fragment (when the early promoter is used for initiation) contributes 60 nt (O'Hare et al., 1981), the β-globin fragment about 70 nt in its spliced form (Van den Berg et al., 1978) and the polylinker sequences of pUC18 about 30 nt. For that reason the resulting mRNAs from the CHO clones are about 130 nt larger in size. The distribution of the two transcripts reflects the situation found on the protein level: the CHO clones produce almost the same ratio, B95-8 cells mainly the gp350 variant and p3HR1 cells only the gp250 (Qualtiere and Pearson, 1980).

The amplification of the inserted sequences in the CHO clones with methotrexate and the purification of the gp250/350 are in progress. Since the glycoproteins are integrated in the cell membranes an amplification may lead to overcrowding of the cell surface. Amplification may also be difficult because of a possible toxicity of the glycoproteins which seemed to generate problems after transfection. To circumvent these problems constructs are now in work where the anchor sequence of the gp250/350 encoding sequence is removed and where the expression product should consequently be secreted into the medium.

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