Expression of Epstein–Barr Virus Membrane Antigen gp340/220 in Mouse Fibroblasts Using a Bovine Papillomavirus Vector

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SUMMARY

Epstein–Barr virus (EBV) membrane antigen glycoproteins gp340 and gp220 are encoded by a single gene. We have inserted this gene into a bovine papillomavirus (BPV) vector and expressed gp340/220 in mammalian cells under the control of the mouse metallothionein promoter. The proteins produced were of similar Mr, showed similar antigenic specificity and were transported to the same subcellular location as the authentic gp340/220. The inclusion of heavy metal ions in the medium had no effect on the levels of gp340/220, which were approximately the same as those found in standard EBV-transformed lymphoblastoid cell lines, e.g. B95-8. Cells that expressed gp340/220 were selected by several rounds of fluorescence-activated cell sorting, but on passage they rapidly lost the ability to express this glycoprotein. In contrast to this we found that BPV-transformed cells expressing a truncated version of gp340/220 still produced it at significant levels after extended passage.

The incidence of two human malignancies, endemic Burkitt's lymphoma (de Thé et al., 1978) and nasopharyngeal carcinoma (Simons & Shanmugaratnam, 1982) is closely associated with infection by Epstein–Barr virus (EBV). It has therefore been proposed that prevention of EBV infection may be an effective means of controlling these virus-associated neoplasms (Epstein, 1976). An estimated 85,000 new cases of these two tumours arise annually (Parkin et al., 1984), indicating the importance of attempts to develop an EBV vaccine.

The best characterized potential vaccine antigens for EBV are the two major envelope glycoproteins gp340 and gp220. These glycoproteins are derived from a single gene by splicing, without a change in reading frame. Much of the EBV-neutralizing activity in human sera is directed against gp340/220 (Pearson et al., 1970; Thorley-Lawson & Poodry, 1982) and monoclonal antibodies (MAbs) as well as polyclonal antisera directed against gp340 neutralize virus (Thorley-Lawson & Geilinger, 1980; Hoffman et al., 1980). Moreover, immunization with purified gp340 has protected cottontop tamarins against EBV-induced lymphomas (Epstein et al., 1985; Morgan et al., 1988).

Large quantities of gp340/220 will be required for mass vaccination campaigns and it is unlikely that lymphoblastoid cell lines transformed by EBV would be an adequate source. Since the genomic location and DNA sequence of gp340/220 are known (Hummel et al., 1984; Biggin et al., 1984) it should be possible to engineer an alternative source of gp340/220 using recombinant DNA technology. Prokaryotic systems are unable to carry out some post-translational modifications such as glycosylation, and as 50% of the mass of gp340 consists of carbohydrate we have opted to express gp340/220 in a eukaryotic system. Previously we reported the generation of a recombinant vaccinia virus which expresses gp340 (Mackett & Arrand, 1985). In an effort to develop a non-lytic system we have produced gp340/220 in continuous fibroblast cell lines using a bovine papillomavirus (BPV) vector system.
Several features of the BPV system make it a useful expression vector. BPV DNA is capable of morphologically transforming mouse fibroblasts, thus facilitating the identification of cells harbouring this DNA or constructs containing it, and the resulting transformed cells usually maintain the BPV DNA as an episomal plasmid at high copy number. Thus BPV vectors have been used successfully to express several viral glycoproteins that have proved difficult to produce in *Escherichia coli*, e.g. influenza virus haemagglutinin (Sambrook *et al.*, 1985).

The construction of a plasmid, designated p107, which contains the gp340/220 coding sequence as a 2.75 kb *BamHI* fragment, was described previously (Mackett & Arrand, 1985). The *BamHI* fragment containing the gp340/220 coding sequence was inserted into the unique *BglII* site of plasmid p341 (Pavlakis & Hamer, 1983). This placed the mouse metallothionein gene promoter adjacent to the translational initiation codon of the gp340/220 gene and eliminated the *BglII* and *BamHI* sites. Transcripts from this promoter should terminate and be polyadenylated due to the presence of simian virus 40 (SV40) sequences at the 3' end of the gp340/220 gene. The resulting plasmid, designated p108, was cleaved with *BamHI* and the entire 7.95 kb genome of BPV type 1 (BPV-1) was inserted to generate p109 and p110. p109 contains the BPV-1 genome such that the direction of transcription of the BPV-1 is the same as the gp340/220 coding sequence; p110 contains the BPV-1 sequences in the opposite orientation to p109. A *BamHI* linker was introduced at the *SacI* site of the gp340/220 gene in p107 and the resulting 2.5 kb *BamHI* fragment coded for a polypeptide 47 amino acids shorter than the full-length gp340/220. The truncated gene was inserted as a *BamHI* fragment into the *BglII* site of p341. The resulting plasmid, designated p108Δ, was cleaved with *BamHI*, and the gp340/220 coding sequence was inserted into the same orientation as p110 to generate p110Δ. p109 and p110 were used to transform mouse C127 cells and approx. 10 transformed cell foci produced by each plasmid were picked and expanded for preliminary characterization. Low Mr DNA was extracted from these clones, cleaved with restriction enzymes, transferred to nitrocellulose and probed with 32P-labelled BPV-1 and gp340/220 DNA sequences. A high percentage of these transformed cell clones harboured episomally replicating plasmids containing sequence rearrangements (data not shown). However, two cell clones, C114 and C19 derived by p109 and p110 transformation respectively, had maintained their original structure. These foci were single cell-cloned by limiting dilution, expanded and examined for expression of gp340/220. Fig. 1 shows that cells fixed with formaldehyde bound the MAb 72A1, which recognizes gp340/220. This indicated that recombinant plasmid-produced gp340/220 was located on the cell surface, the normal subcellular location of gp340/220 in lymphoblastoid cell lines. We noted that there was a wide spectrum in the extent of expression of gp340/220 when individual cells were compared and that, although cloned, there was variability in the morphology of different cells within the population.

In order to examine the recombinant gp340 produced, we radioiodinated cell surfaces of B95-8, C127, C114 and C19 cells using lactoperoxidase, and the labelled proteins were immunoprecipitated with MAb 72A1 or polyclonal rabbit anti-gp340 antisera. Fig. 2 shows an autoradiograph of the SDS–polyacrylamide gel used to separate the proteins. As can be seen, a single approx. 340K protein was precipitated from B95-8 cells whereas no detectable proteins were immunoprecipitated from surface-labelled mouse C127 cells. However, C114 and C19 produced two high Mr, glycoproteins that reacted with anti-gp340 antisera. Lower percentage polyacrylamide gels run for longer times show a slight difference in the migration of gp340 from B95-8 cells and C114 and C19 cells. This is probably due to different extents of glycosylation in different host cells, C127 cells being of mouse origin and B95-8 cells of marmoset origin. It is of particular interest that both gp340 and gp220 were produced in similar amounts, since B95-8 cells produce exclusively gp340 and B95-8 virus DNA was the source of the recombinant gene. gp340 expression was not enhanced by adding 1 mM-CdCl2, a stimulator of expression from the metallothionein promoter (data not shown). We also found that it was possible to surface-label gp340/220 in C19 and C114 without detaching them from the monolayer (data not shown). Immunoperoxidase staining followed by a counter stain with rhodamine B allowed closer examination of the cells expressing gp340/220. We observed large multinucleated cells, arising from two, three or four cells, which expressed low levels of gp340/220 and in general high levels
Fig. 1. Immunofluorescence staining of cells expressing EBV membrane antigen gp340/220. Cells transfected with plasmid p110 (a) or parental C127 cells (b) were surface-fixed with formaldehyde and incubated with MAb 72A1, which recognizes gp340/220. They were subsequently incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse antibody and visualized by excitation at 450 to 490 nm using a u.v. photomicroscope.

of expression were observed only in isolated fibroblast-type cells. We also noted that on continued passage the majority of the cell population ceased to express gp340/220. To investigate this phenomenon we subjected C114 and C19 to two rounds of fluorescence-activated cell sorting and obtained populations of both clones in which all the cells expressed gp340/220. After 3 months in culture (resulting in 50 cell doublings) very few cells expressing gp340/220 were discernible. Inclusion of 5-azacytidine in the medium did not increase gp340/220 expression in late passage cells, suggesting that methylation was not responsible for switching off expression of the gene (data not shown). High and low Mr DNA was prepared from early and late passage cells. Southern analysis indicated little change in the state of the foreign DNA on passage in these cells (data not shown). Other workers have reported difficulty in expressing gp340/220 to high levels in Chinese hamster ovary cells and in yeast, suggesting that the protein is toxic for eukaryotic cells (Motz et al., 1986, 1987; Schultz et al., 1987). To circumvent these problems we have constructed a BPV vector which contains a deleted form of the gp340/220 coding sequence lacking the membrane anchor region of the protein. This truncated gp340/220 should be secreted into the medium of transformed cells in culture. p110Δ was used to transform mouse C127 cells and approx. 10 cell foci were picked and expanded for preliminary characterization. Low Mr DNA was extracted from these clones and analysed by Southern blotting. Four out of six clones initially examined had maintained the plasmid p110Δ in its original configuration (data not shown). Three of these clones (1A, 2A and 6A) were passaged at a 1:30 split over a 3-month period giving approximately 50 cell doublings. Fig. 3 shows a Western blot analysis of supernatants derived from these clones at passages 1 and 9. Expression of a secreted gp340/220 was not expected from clone 2A as analysis of low Mr DNA from this clone revealed rearrangements in the BPV vector. However, in contrast to our previous constructs, significant levels of the truncated gp340/220 were detected in late passages of clones 1A and 6A as well as in early passages. Although quantification is difficult, we estimate that between 10 and 50 μg/10⁶ cells was produced over a 3-day period. The cells can be maintained as a monolayer in roller bottles for up to 4 weeks, and a further advantage of this system is that the
Fig. 2. Analysis of surface proteins from recombinant and parental cell lines. Cell membranes from CI27 (lanes 1 to 3), clone 9 (lanes 4 to 6), clone 14 (lanes 7 to 9) and B95-8 (lanes 10 to 12) cells were labelled using $^{125}$I and lactoperoxidase, and proteins were immunoprecipitated using normal rabbit serum (lanes 1, 4, 7 and 10), MAb 72A1 (lanes 2, 5, 8 and 11) or a polyclonal rabbit anti-EBV serum (lanes 3, 6, 9 and 12). $M_r$ ($\times 10^{-3}$) markers were run in parallel and their positions are indicated.

Fig. 3. Western blot analysis of culture supernatant from CI27 cells transformed with p110. Culture supernatants (15 $\mu$l) from clones 1A, 2A and 6A at passage 1 (lanes 1, 2 and 3 respectively), clones 1A, 2A and 6A at passage 9 (lanes 4, 5 and 6 respectively) were run on a 5% polyacrylamide gel and transferred to nitrocellulose. The blots were incubated with a 1:50 dilution of a polyclonal rabbit anti-gp340/220 antisem and subsequently with 1 $\mu$Cl of $^{125}$I-labelled Protein A. An autoradiograph of the blot is shown.
cells can be grown in serum-free medium for several weeks thus greatly simplifying purification of the gp340/220 from cell supernatants.

Motz et al. (1986, 1987) and Whang et al. (1987) have achieved expression of gp340/220 in rodent and primate cells, either as the complete molecule or as a secreted truncated molecule, under the control of the SV40 early promoter. In all cases expression of the gene was lost on passage of the cells, even using a methotrexate amplification system necessitating the use of selectable markers and periodic cloning. In agreement with this we have also observed a loss of expression of the membrane-bound gp340/220 on passage. However, in the BPV system, where we have used a mouse metallothionein promoter to express the secreted gp340/220, stable expression over extended passage has been achieved. The high Mr of gp340/220, its resistance to proteolysis and denaturation, and the fact that the molecule has large amounts of carbohydrate and sialic acid make it relatively simple to purify (David & Morgan, 1988). Once purified, the protein itself can be studied or used in ELISAs for determining antibody to gp340 in receptor-binding studies (Tanner et al., 1987) or in competitive radioimmunoassays to measure quantities of gp340/220 produced in other cell lines. It will also be useful in analysing cytotoxic T cell responses to gp340/220 during natural infection with EBV. Although this material could be used for generating polyclonal or monoclonal antisera, it would require extensive characterization before it could be used in human trials as it is derived from a cell line containing a transforming virus. Having established that cell lines can stably express the truncated version of gp340/220, it should be possible to generate other suitable stable cell lines which will produce sufficient quantities of the protein for human vaccine trials.

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REFERENCES


Short communication


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