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Recombinant vaccinia virus induces neutralising antibodies in rabbits against Epstein-Barr virus membrane antigen gp340

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The Epstein-Barr virus membrane antigen gene gp340 was isolated, inserted into several strains of vaccinia virus and expressed under the control of a vaccinia virus promoter. The EBV-derived protein which was produced by the recombinant vaccinia viruses was heavily glycosylated, readily labelled with threonine, could be detected at the surface of infected cells and had a mol. wt. of ~340 kd, all of which are properties of the authentic gp340. Polyclonal rabbit antisera against gp340 and an EBV-neutralising anti-gp340 monoclonal antibody both recognised cells infected with the recombinant vaccinia viruses. Moreover, rabbits vaccinated with one of the recombinants produced antibodies that recognised EBV-containing lymphoblastoid cells and neutralised EBV.

Key words: Epstein-Barr virus/vaccinia virus recombinants/glycoprotein gp340/vaccines

Introduction

Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis and is closely associated with two human malignancies, Burkitt's lymphoma (de Thé *et al.*, 1978) and nasopharyngeal carcinoma (Simons and Shanmugaratnam, 1982). Although Burkitt's lymphoma affects relatively few individuals, nasopharyngeal carcinoma is a significant world health problem, being the most common tumour of males in southern China. The possibility of controlling these EBV-associated neoplasms by developing a vaccine against EBV infection has been discussed for some time (Epstein, 1970, 1976) and following recent technical advances this objective has now become feasible.

To be effective, an antiviral vaccine should recognise an external component of the virus. EBV membrane antigen (MA) is detected on the envelopes of intact virions and on the plasma membrane of virus producer cell lines. It consists of at least two glycoproteins of mol. wts. 340 000–350 000 daltons and 220 000–270 000 daltons (gp340/220) which share antigenic determinants (Qualtiere and Pearson, 1979, 1980; Strnad, *et al.*, 1979; Thorley-Lawson and Edson, 1979; North *et al.*, 1980). Antibodies raised against the plasma membranes of EBV-producer cell lines or EBV envelopes have virus-neutralising activity (Thorley-Lawson, 1979; North *et al.*, 1982; Thorley-Lawson and Geilinger, 1980). In addition, monoclonal antibodies or monospecific antisera which recognize EBV membrane antigen will also neutralise the virus (Hoffman *et al.*, 1980; Thorley-Lawson and Geilinger, 1980; Franklin *et al.*, 1981). These data suggest that it should be possible to produce an anti-EBV vaccine based on gp340/220.

Although an EBV-neutralising response was obtained in animals vaccinated with gp340/220 incorporated into liposomes (North *et al.*, 1982) only relatively small amounts of gp340/220 are obtainable from EBV-producer cell lines. Thus this approach would be difficult to apply to mass vaccination programmes unless alternative, richer sources of the antigen were available. The recent determination of the complete nucleotide sequence and genomic location of the DNA that codes for gp340/220 (Biggin *et al.*, 1984; Hummel *et al.*, 1984) opens up the possibility of using genetic manipulation to produce gp340/220 or for alternative strategies of vaccination.

Potential new vaccines based on the expression of foreign genes in vaccinia virus have recently been developed (Paoletti *et al.*, 1984; Smith *et al.*, 1983a, 1983b). Since vaccinia virus was used so effectively in the global eradication of smallpox, recombinant viruses based on vaccinia offer many potential advantages for the control of other diseases. We have investigated the possibility of producing a vaccine against EBV by constructing vaccinia virus recombinants that express EBV gp340. We show that rabbits which have been vaccinated with such recombinants produce antibodies which have EBV-neutralising activity.

Results

Experimental design

To simplify and generalise the construction of vaccinia virus recombinants that efficiently express foreign genes, several insertion vector plasmids have been generated (Mackett *et al.*, 1984). These vectors incorporate the unique vaccinia transcriptional control sequences. One of these vectors, pGS20, has been used to insert and express a variety of exogenous genes in vaccinia virus (Smith *et al.*, 1983a, 1983b, 1984; Mackett *et al.*, 1985). This vector contains a single *Bam*HI site adjacent to the promoter and transcriptional initiation site of a vaccinia virus early gene. These sequences are flanked by vaccinia virus DNA derived from the thymidine kinase (TK) gene (Figure 2). When a continuous protein-coding sequence with its own translational initiation codon is inserted into this *Bam*HI site its expression is controlled by the vaccinia virus promoter. Insertion of the chimeric gene into vaccinia by homologous recombination *via* the TK sequences results in transcription originating at an authentic vaccinia initiation site and the resulting message is translated into the authentic foreign protein. Thus we engineered the EBV gp340 gene such that its translational initiation codon was adjacent to the end of a 2.75-kb *Bam*HI fragment which contained the entire coding sequence of gp340 (Figures 1 and 2). This enabled the gp340 gene coding sequence to be placed adjacent to the vaccinia 7.5 K gene promoter in pGS20 and the resultant plasmid was used to insert the gene into vaccinia virus.

Plasmid constructions

The EBV gp340/220 gene has been mapped to the *Bam*HI L fragment of strain B95-8 DNA (Hummel *et al.*, 1984) and the DNA sequence and transcriptional map of this fragment have been determined (Biggin *et al.*, 1984). Figures 1 and 2 indicate graphically the strategy employed to isolate the gp340/220 gene in a bacterial vector containing the appropriate vaccinia se-

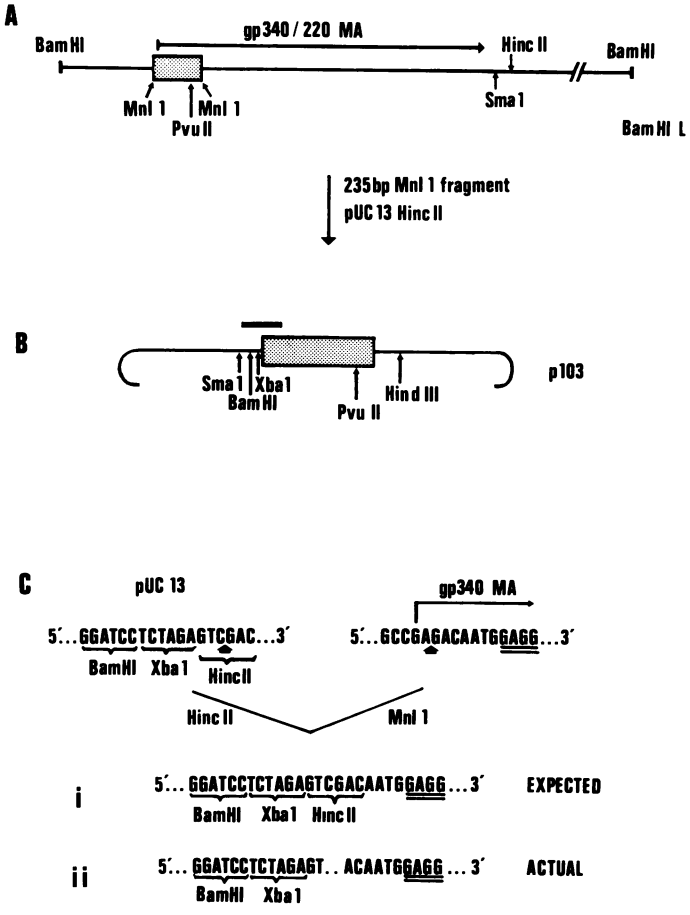

Fig. 1. Isolation of the 5′ end of EBV MA gp340/220 gene. (**A**) The 5.05-kb *Bam*HI L fragment of EBV DNA from B95-8 virus (Arrand *et al.*, 1981) (not to scale). The indicated restriction sites show only those cleavage points which were important for the plasmid construction and do not constitute a complete map. The hatched box represents an *Mnl*I DNA fragment that contains the 5′ end of the EBV MA gp340/220 gene. The direction of transcription of the gene is indicated. (**B**) The 235-bp *Mnl*I fragment containing the 5′ end of the gene was cloned into the *Hinc*II site of pUC13 and the resultant plasmid designated p103. The bold line indicates the DNA sequence shown in (**C**). (**C**) Part of the polylinker sequence of pUC13 and the sequence around the 5′ end of the EBV MA gene is indicated. The arrow indicates the direction of the transcription and the major RNA initiation site of gp340. The double underlined GAGG immediately to the right of the ATG translational start site is the *Mnl*I restriction enzyme recognition sequence and the short arrow seven base pairs upstream is the site at which *Mnl*I cleaves. The short arrow in the pUC13 sequence is the cleavage site of *Hinc*II. Blunt end ligation of the *Mnl*I fragment to the *Hinc*II site of pUC13 should give the expected sequence (i). Sequencing of the junction indicated that 2 bp were deleted in the cloning process and this is indicated by the actual sequence (ii).

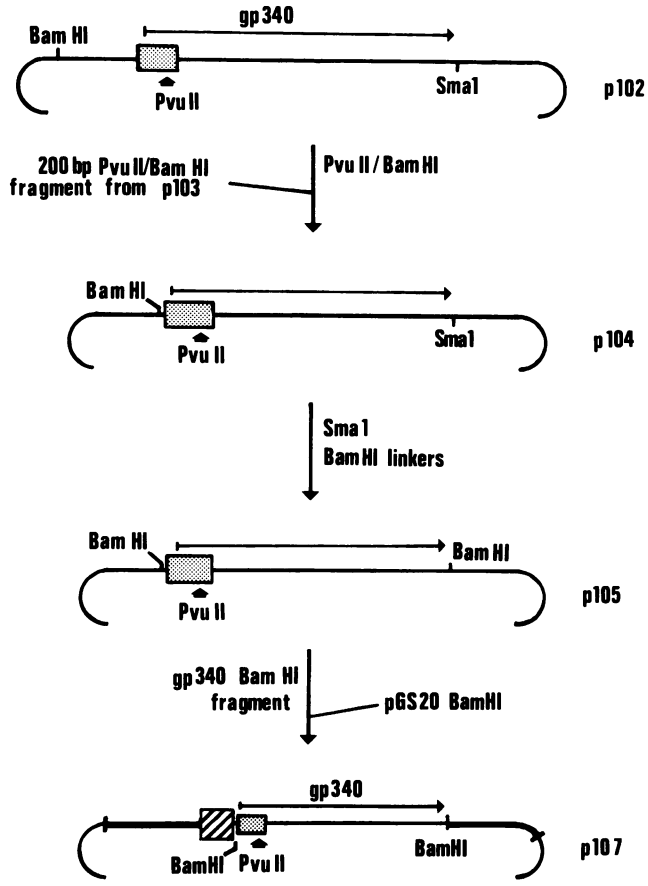

Fig. 2. Construction of a vector for insertion of the EBV MA gp340/220 coding sequence into vaccinia virus. The 3.5-kb *Bam*HI-*Hinc*II fragment from EBV *Bam*HI L (Figure 1a) was inserted into *Bam*HI + *Nru*I cleaved pAT153; this has the effect of destroying both the *Hinc*II and *Nru*I sites in the resultant plasmid, p102. The hatched box represents an *Mnl*I fragment which contains the 5′ end of the gp340 gene (Figure 1a) and the arrow indicates the direction of transcription. An ~200-bp *Bam*HI-*Pvu*II fragment from p103 (Figure 1b) containing the 5′ end of the gp340/220 gene adjacent to the *Bam*HI site was inserted between the unique *Bam*HI and *Pvu*II sites of p102. The resulting plasmid designated p104 has a unqiue *Sma*I site at the 3′ end of the gp340/200 gene. This was converted to a *Bam*HI site with the use of synthetic *Bam*HI linkers. The plasmid p105 contains the entire gp340/220 coding sequence bounded by *Bam*HI sites. This *Bam*HI fragment was inserted into pGS20 at its unique *Bam*HI site, to give p107 which contains the EBV MA gene coding sequence adjacent to a vaccinia promoter and transcriptional start site (of a gene that codes for a protein of 7.5 kd) flanked by sequences derived from the vaccinia virus thymidine kinase gene (bold lines).

quences. Figure 1a shows a map of the *Bam*HI L fragment of EBV DNA. Examination of the DNA sequence at the 5′ end of the gp340/220 gene showed that the translational initiation codon was only 6 bp away from the message start site (Figure 1c). Fortuitously, an *Mnl*I cleavage site between the transcriptional and translational initiation points allowed separation of the coding sequence and promoter. A 235-bp *Mnl*I fragment containing the 5′ end of the gene, devoid of its promoter, was inserted into pUC13 at its *Hinc*II site (Figure 1b). The *Mnl*I fragment should leave a GAC at its 5′ end which when ligated to the *Hinc*II cleaved site (GTC) would regenerate the *Hinc*II site (Figure 1c,i). We screened several plasmids that were positive by DNA hybridisation for the *Mnl*I fragment but found that the *Hinc*II site was absent. DNA sequence analysis revealed that during the cloning procedure a base pair had been deleted from both the pUC13 sequence and the *Mnl*I fragment (see Figure 1c,ii).

Although this deletion destroys the *Hinc*II site it does not affect the juxtapositioning of the *Bam*HI site and the translational initiation point of the gp340/220 gene.

The remaining steps of the construction are shown in Figure 2. The final plasmid, p107, contained the entire gp340/220 gene under the control of the vaccinia 7.5 K promoter and was used to express the MA gene in vaccinia virus strains WR and Wyeth.

Construction and characterisation of vaccinia recombinants that express gp340

CV1 cells infected with vaccinia virus strain WR or Wyeth were transfected with p107. The cells were harvested and TK^- virus was isolated by plaque assay on TK^- cells in the presence of 25 μg/ml BUdR. Recombinant viruses, TK^- by virtue of inser-

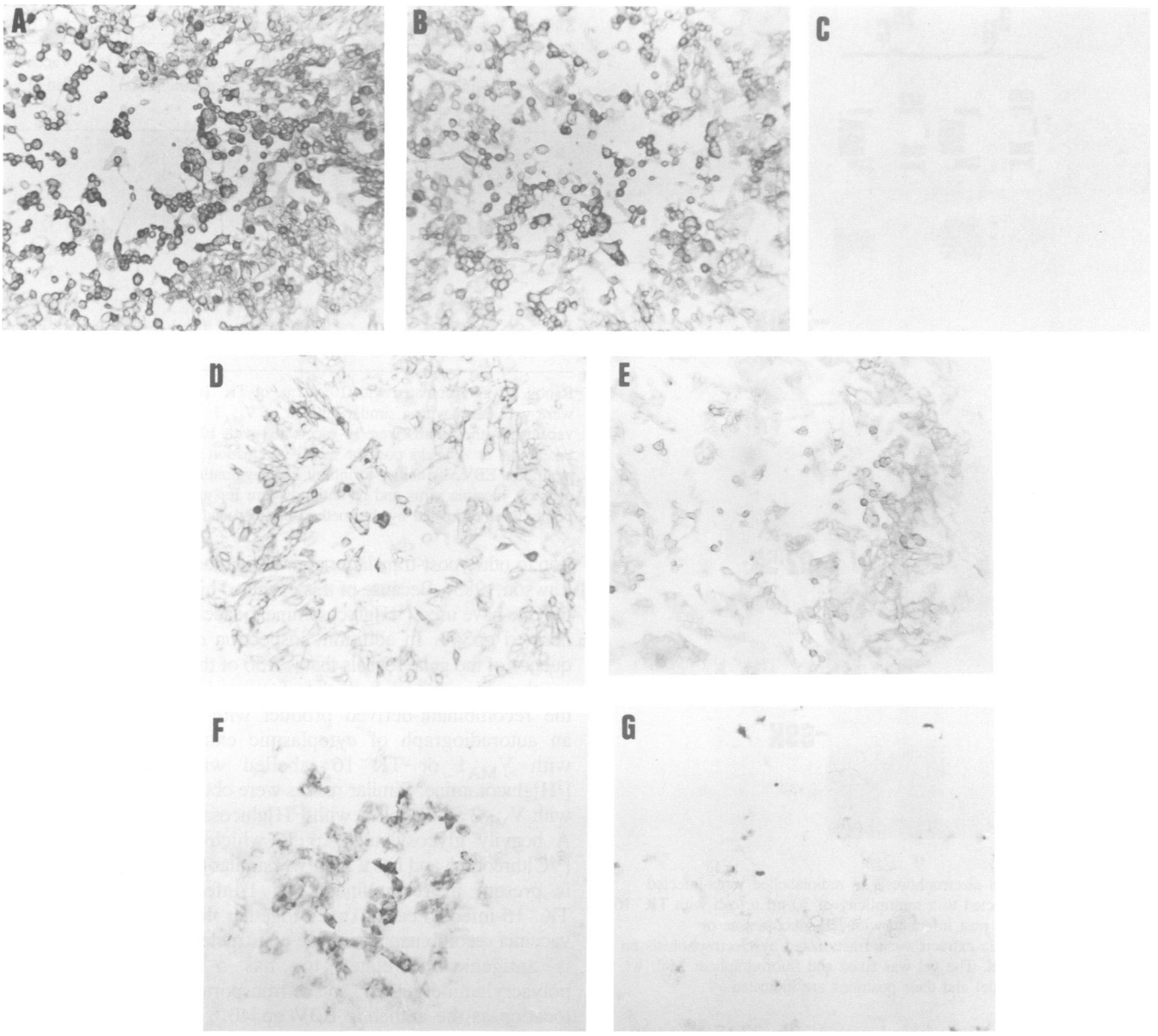

Fig. 3. Immunoperoxidase staining of recombinant virus-infected cells. CV1 cells infected with vaccinia viruses $V_{MA}1$ (**A,B**), $V_{MA}2$ (**D,E**), TK^-16 (**C**) or V Blu27 (**F,G**) were fixed either with formaldehyde (**B,E,G**) or methanol (**A,C,D,F**) and stained by an indirect immunoperoxidase method. **A**–**E** were initially incubated with a monoclonal antibody 72A1 (Hoffman *et al.*, 1980, which recognises the EBV MA gp340/220 gene) and subsequently with a peroxidase conjugated rabbit anti-mouse IgG antibody. **F** and **G** were initially incubated with a pool of three different monoclonal antibodies to β-galactosidase (Wellcome Biotechnology) and subsequently with a peroxidase conjugated rabbit anti-mouse IgG antibody. The cells binding the initial monoclonal antibodies were visualised by the development of a black precipitate by peroxidase using hydrogen peroxide and 4-chloro-1-naphthol. All photographic exposures were for equivalent times.

tion of the gp340/220 gene within the vaccinia TK gene, were identified by screening for the presence of EBV gp340/220 DNA in the case of WR recombinants (Mackett *et al.*, 1982) and for expression of the gp340/220 gene in the case of Wyeth recombinants (Mackett *et al.*, 1985). WR recombinant virus, positive for gp340/220 DNA, designated $V_{MA}1$ and Wyeth recombinant virus, positive for gp340/220 expression, designated $V_{MA}2$ were plaque purified once more in TK^- cells in the presence of BUdR and then large stocks were prepared under non-selective conditions in HeLa S3 spinner cells.

Evidence for the expression of the MA gene and an indication of the purity of the recombinant viruses were obtained by the binding of antibodies (raised against the MA gene product) to virus-infected cells in plaques. Figure 3 shows virus-infected cells fixed either with methanol (A,C,D,F) or formaldehyde (B,E,G), incubated with the monoclonal antibody 72A1 (which binds to EBV MA gp340/220, Hoffman *et al.*, 1980) and visualised using a peroxidase-conjugated antibody colour test. All the infected cells in the plaques produced by $V_{MA}1$ (A,B) and $V_{MA}2$ (D,E) bound 72A1. However, cells infected with a WR-derived TK^- virus (TK^-16), (C), or Wyeth virus (not shown), the parental virus of $V_{MA}2$, did not bind 72A1. $V_{MA}1$ plaques also bound polyclonal rabbit sera raised against the EBV MA gp340/220 (data not shown). Cells infected with $V_{MA}1$ or $V_{MA}2$ that had been fixed with formaldehyde, which fixes the cell surface, also bound 72A1 (B,E) indicating that the recombinant virus-produced gp340 is localised on the cell membrane, the normal subcellular location of gp340/220 in lymphoblastoid cell lines containing

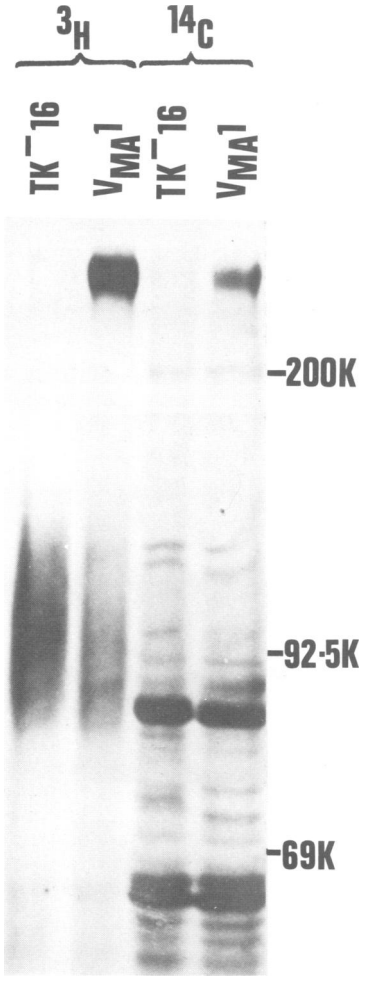

Fig. 4. Polyacrylamide gel electrophoresis of radiolabelled virus-infected cells. CV1 cells were infected to a multiplicity of 30 p.f.u./cell with TK^-16 or $V_{MA}1$ and labelled 2 h post infection with [^{3}H]glucosamine or [^{14}C]threonine. Cytoplasmic extracts were fractionated by electrophoresis on a 7.5% polyacrylamide gel. The gel was fixed and fluorographed. Mol. wt. markers were run in parallel and their positions are indicated.

EBV. As a control, cells infected with V Blu 27 (S.Newton *et al.*, unpublished), a recombinant vaccinia virus that expresses *Escherichia coli*, β-galactosidase was either fixed with methanol which permeabilises the cell (F) or surface fixed with formaldehyde (G) and incubated with a pool of three monoclonal antibodies against β-galactosidase followed by the colour test. β-Galactosidase should be located in the cell cytoplasm as it does not have the required signal sequence for transport to the cell surface. Figure 3 (G and H) shows that methanol-fixed infected cells bind the monoclonal antibodies to β-galactosidase, whereas formaldehyde fixed cells do not.

Characterisation of the recombinant gene product

The DNA sequence of the gp340 gene predicts an unmodified mol. wt. polypeptide of ~95 kd. However the authentic gp340 gene product had an apparent mol. wt. on polyacrylamide gels of ~340 kd. The difference between these figures is presumably due to extensive glycosylation. This is anticipated from an examination of the DNA sequence because the gp340/220 gene contains 37 Asp-X-Thr/Ser tripeptides (the classical N-linked glycosylation site) and there are >100 serine residues which could act as sites for O-linked glycosylation. Indeed extensive N- and O-linked glycosylation has been found in gp340 in addition to other post-translational modifications (Edson and Thorley-Lawson,1983). Because of this expected high degree of glycosylation we have used [^{3}H]glucosamine to label the recombinant virus-derived gp340. In addition, inspection of the nucleic acid sequence of the gene reveals that >150 of the amino acids in gp340 are threonine and we have therefore used this amino acid to label the recombinant-derived product with ^{14}C. Figure 4 shows an autoradiograph of cytoplasmic extracts of cells infected with $V_{MA}1$ or TK^-16 labelled with [^{14}C]threonine or [^{3}H]glucosamine. Similar results were obtained with cells infected with $V_{MA}2$ and labelled with [^{3}H]glucosamine (data not shown). A heavily glycosylated protein which also labels well with [^{14}C]threonine and has a mol. wt. similar to authentic EBV gp340 is present in recombinant $V_{MA}1$-infected cells but not in TK^-16-infected cells. Taken together the results show that the vaccinia recombinants we have constructed express a protein that is antigenically related to, has a similar mobility on polyacrylamide gels to, and is transported to the same cellular location as the authentic EBV gp340.

Table I. Response of rabbits to vaccination with $V_{MA}1$ or TK^-16

Animal	Weeks post initial vaccination	Anti-gp340 titre	EBV neutralisation	Vaccinia anti-HA titre
6	0	–	–	–
15	0	–	–	–
37	0	–	–	–
6	4	–	–	1:32
15	4	1:50	+	1:8
37	4	1:1000	++	1:32
6	8	–	–	1:128
15	8	1:200	+++	1:32
37	8	1:1000	+++	1:128

Rabbit 6 was vaccinated with 10^8 p.f.u. of TK^-16 and rabbits 15 and 37 were vaccinated with a similar amount of $V_{MA}1$. At 4 weeks post vaccination the rabbits were re-vaccinated with 10^8 p.f.u. of $V_{MA}1$. Serum taken at 4 or 8 weeks post the initial vaccination was tested for its ability to neutralise EBV, its ability to inhibit haemagglutination of fowl red blood cells by vaccinia virus and its antibody titre for gp340. Serum titre end-points were estimated by the method of Reed and Muench (1938).

Vaccination of rabbits

To determine whether the $V_{MA}1$ recombinant vaccinia virus would elicit an appropriate antibody response, rabbits were inoculated intradermally with 10^8 p.f.u. of $V_{MA}1$ or TK^-16 virus. Following vaccination all the rabbits developed typical local skin lesions. At 28 days post vaccination they were re-vaccinated intradermally with 10^8 p.f.u. of $V_{MA}1$. Following this boost lesions were not observed. Serum taken at 4 weeks and 3 months past the initial vaccination from the two rabbits (15 and 37) vaccinated with $V_{MA}1$ had antibody that recognised B95-8 (or W91) cells but not Raji cells as shown by indirect immunofluorescence (data not shown). Serum from the rabbit vaccinated with TK^-16 virus did not bind to B95-8, W91 or Raji cells. (B95-8, W91 and Raji cells are lymphoblastoid cell lines that contain EBV genomes. Only B95-8 and W91 are positive for EBV MA gp340/220.) Table I shows the response of the rabbits to vaccination. From both the vaccinnia anti-haemagglutinin titre at 4 weeks post vaccination and the fact that all three animals had typical skin lesions, virus replication can be seen to have taken place. Rabbit 15 seems to have responded less well than rabbits 6 and 37. The second vaccination at 4 weeks had boosted the anti-

haemagglutinin titre in all animals ~4-fold by week 8. Serum taken before vaccination showed no vaccinia antibodies anti-gp340 antibodies or any EBV neutralisation. Rabbit 6 vaccinated with TK^-16 showed no anti-gp340 antibodies and no EBV neutralisation. However, sera from both rabbits vaccinated with $V_{MA}1$ neutralised EBV and had significant anti-gp340 antibody levels. Higher gp340 antibody levels were found in rabbit 37 than in rabbit 15, paralleling the anti-vaccinia antibody levels. Thus in rabbits the recombinant $V_{MA}1$ elicits a high level of antibodies to gp340 and furthermore these antibodies are EBV neutralising. This augurs well for the potential use of gp340 recombinant viruses for vaccination against EBV infection.

Discussion and Conclusions

We have described a vaccinia virus recombinant which expressed the EBV MA gp340 gene under the control of a vaccinia virus promoter. The recombinant EBV gp340 was antigenically similar and corresponded in size to the authentic EBV MA. Glycoprotein gp340 produced in $V_{MA}1$-infected cells was detectable on the cell surface, the normal subcellular localisation of gp340 in lymphoblastoid cell lines containing EBV. Many EBV genome-containing lymphoid cell lines which produce the virus have two high mol. wt. glycoproteins on their cell surface, gp340 and gp220. These are specified by the same gene-coding sequence and gp220 is translated from a spliced gp340 message (Beisel *et al.*, 1985; Biggin *et al.*, 1984). The vaccinia recombinants that we describe produce only the gp340 and no gp220. This is probably due to the apparent lack of a splicing mechanism in the vaccinia virus transcriptional system (Wittek *et al.*, 1980; Cooper *et al.*, 1981). It should be noted that the DNA used to engineer this gene was derived from the B95-8 strain of EBV. Cells containing B95-8 virus produce only the high mol. wt. form of the glycoprotein gp340. However, mouse C127 cells transformed with a bovine papilloma virus vector which contained the same gene as that inserted into the vaccinia virus recombinants produced two high mol. wt. proteins of 340 and 220 kd (our unpublished observations) suggesting that the mouse transcriptional system can produce spliced mRNA from this gene. Furthermore, rabbits vaccinated with the recombinant viruses $V_{MA}1$ and 2 produced antibodies that recognised EBV gp340 and neutralised EBV. These results show that this approach fulfils the primary requirements of a vaccine against EBV, i.e., an antibody response that will neutralise *in vitro* the pathogen to be protected against *in vivo*. The next step in showing the efficacy of this prototype anti-EBV vaccine is to demonstrate protection against EBV-associated disease in an animal model system. The cottontop tamarin is the only reliable animal model system which develops a lymphoma when inoculated with EBV. It remains to be seen whether a vaccinia virus recombinant which expresses gp340 will protect tamarins against EBV challenge. However, vaccinia virus recombinants expressing foreign genes have protected against other virus infections (Smith *et al.*, 1983b; Paoletti *et al.*, 1984; Moss *et al.*, 1984; Kieny *et al.*, 1984) and it may be anticipated that this recombinant or a similar one expressing higher levels of gp340 will protect the tamarins against EBV.

The large capacity of vaccinia virus for foreign DNA (Smith and Moss, 1983) suggests the possibility of constructing recombinants that express up to as many as 20 foreign genes. The advantage of this is that it becomes feasible to protect against more than one pathogen using a single recombinant virus that expresses more than one protective foreign protein. For example, areas of southern China that have a high incidence of nasopharyngeal carcinoma also have a high incidence of hepatitis B. It would be highly desirable to construct recombinant viruses that simultaneously express hepatitis antigens and EBV genes. Using this technology it would also be possible to express other EBV membrane proteins, e.g., the 85-kd component of the membrane-antigen complex. Recombinants expressing both gp340 and the 85-kd protein may be more effective in controlling EBV infection.

Very little is known about the extent to which the biological or pathological properties of vaccinia virus strains are altered by insertion and expression of a foreign gene. Having the gp340 gene inserted into two different virus strains should allow assessment of the effect of the foreign gene by comparison of the biological properties of both parental strains with their recombinants. Such data will be of great value prior to use of any vaccinia recombinant-based vaccine in humans.

Materials and methods

Cells and virus

The human lymphoid cell lines Raji (Pulvertaft, 1965), B95-8 (Miller and Lipman, 1973) and W91 (Miller *et al.*, 1976) were grown in RPMI 1640 medium containing 10% fetal calf-serum (FCS). The mouse monoclonal cell line 72A1 (Hoffman *et al.*, 1980) which secretes an IgG1 class antibody that recognises EBV gp340/220 was grown in RPMI 1640 medium supplemented with 10% FCS, 13.611 mg/l hypoxanthine, 0.176 mg/l aminopterin and 3.876 mg/l thymidine. Human TK^-143 cells maintained in minimal essential medium (MEM) containing 25 μg/ml 5-bromodeoxyuridine (BUdR) and 5% FCS were used for selection of TK^- recombinants. Before use the cells were passaged twice in MEM + 5% FCS without BUdR. CV1 monkey kidney cells were passaged in Dulbecco's modified MEM containing 5% FCS. For large stocks of virus, HeLa S3 spinner cells maintained in Joklik's suspension medium supplemented with 10% horse serum were used. Vaccinia virus strains WR, Wyeth (New York City Board of Health) or TK^-16 (Weir and Moss, 1983) and V Blu27, were grown and purified essentially as described by Joklik (1962). V Blu27 is a Wyeth recombinant which expresses the gene for *E. coli* β-galactosidase.

Plasmid constructions

Routine procedures were similar to those described in detail by Maniatis *et al.* (1982). Recombinant plasmids were prepared using the vectors pAT153 (Twigg and Sherratt, 1980), pUC13 (Vieira and Messing, 1982) or pGS20 (Mackett *et al.*, 1984) and purified as described by Birnboim and Doly (1979). The EBV-membrane antigen coding sequence was excised from the *Bam*HI L clone of B95-8 virus DNA (Arrand *et al.*, 1981). Enzymes were purchased from various molecular biology companies and used according to their instructions. DNA sequencing was by the method of Maxam and Gilbert (1980).

Generation of recombinants

Recombinants were obtained essentially as outlined previously (Smith *et al.*,1983a; Mackett *et al.*, 1984, 1985). A plasmid vector was constructed which contains, inserted into the body of the vaccinia virus TK gene, a vaccinia virus transcription start site upstream of the EBV-membrane antigen coding sequence (p107, see Results and Figures 1 and 2). This plasmid was then used to recombine the membrane antigen gene into the vaccinia virus genome in a manner similar to that performed for marker rescue (Weir *et al.*, 1982). Briefly, cells were infected with wild-type (TK^+) vaccinia virus, either the WR laboratory strain or the Wyeth vaccine strain, at a multiplicity of 0.1 – 0.05 p.f.u. per cell. At 2 h post infection, calcium-phosphate precipitated chimeric plasmid, p107 DNA was added and cells were harvested at 48 h post infection. Spontaneously generated TK^- virus and recombinant viruses were selected from infected cell lysates by plaque assay on TK^- 143 cells with 25 μg/ml of BUdR in the agar overlay. Total TK^- virus was then screened for EBV gp340 DNA by a dot blot hybridisation protocol (Mackett *et al.*, 1982), or for expression of gp340 as detected by antibody binding to plaques (see below). Virus which was positive for gp340 DNA or expression of gp340 was plaque purified twice and characterised more fully.

Detection of antibody binding to virus-infected cells or virus plaques

Monolayers of monkey kidney CV1 or Human 143 TK^- cells were used for plaquing virus. Monolayers of infected cells or monolayers showing well-separated plaques were fixed with cold methanol for 10 min. For cell-surface staining the monolayers were fixed with a fresh 1:10 dilution of stock 37% formaldehyde. The formaldehyde was washed away with cell-culture medium and the monolayers were incubated with 5 ml phosphate-buffered saline (PBS) containing 4% bovine

serum albumin (BSA), 0.02% sodium azide, at room temperature for 30 min, on a rocking platform. The solution was then supplemented with the monoclonal antibody 72A1 which recognises the EBV MA gp340/220 (Hoffman *et al.*, 1980) or with polyclonal anti-gp340 sera raised in rabbits and incubated on the rocking platform for 1 h. Monolayers were washed five times with PBS and incubated for a further 1 h with 1/100 dilution horseradish peroxidase-conjugated anti-mouse IgG (Dako) raised in rabbits or, in the case of the polyclonal rabbit sera, a 1/100 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Dako) raised in goats. The antibody binding was visualised by incubation with PBS containing 0.01% 4-chloro-1-naphthol, 0.003% hydrogen peroxide. A black colour develops in 5–30 min (Holland *et al.*, 1983; Smith *et al.*, 1981). 100 ml of substrate solution was made by dissolving 10 mg of 4-chloro-1-naphthol in 1 ml of ethanol and slowly adding this to the PBS. 100 μl of 3% hydrogen peroxide was then added. A second colour visualisation was also achieved by using a phosphatase-conjugated second antibody as a substitute for the peroxidase conjugate and Fast Red or Fast Blue BB as the substrate (Cordell *et al.*, 1984). Briefly, the alkaline phosphatase substrate was prepared by dissolving 2 mg of naphthol AS-MX (Sigma) in 0.2 ml of dimethyl formamide in a glass tube and then adding 9.8 ml of 0.1 M Tris-HCl, pH 8.2. Immediately before use Fast Red TR or Fast Blue BB (Sigma) was added to a final concentration of 1 mg/ml and the solution filtered directly onto the monolayer. It was found necessary to block endogenous alkaline phosphatase activity by adding 1 mM levamisole (Sigma) to the substrate.

Immunofluorescence staining

Slides containing air-dried, acetone-fixed B95-8, W91 or Raji cells were incubated at 37°C for 1 h in a humidified atmosphere with serial dilutions of the rabbit sera under test. Slides were washed in PBS for 3 × 10 min and subsequently incubated for 1 h at 37°C in a humidified atmosphere with the appropriate dilution of FITC-conjugated goat anti-rabbit antibody (Sigma). After washing three times with PBS as before, fluorescein-tagged cells were visualised by excitation at 450–490 nm using an u.v. photomicroscope.

EBV-neutralisation and membrane antigen ELISA

EBV neutralisation was carried out essentially as described by Moss and Pope (1972) and the gp340/220 membrane antigen ELISA was carried out as described by Randle and Epstein (1984).

Anti-vaccinia antibody

To monitor the anti-vaccinia antibody response of the rabbits to vaccination, their anti-vaccinia haemagglutinin antibody was assessed essentially as described by Naler *et al.* (1944). Vaccinia agglutinable fowl red blood cells were obtained from Salisbury Labs. Ltd., Downton, Salisbury,SP5 3JQ, UK.

Labelling of infected cells

Monolayers of CV1 cells were infected with 30 p.f.u. per cell of TK^-16 or $V_{MA}1$. After 2 h, the virus inoculum was replaced with MEM containing 50 μCi of [^{3}H]glucosamine or 50 μCi of [^{14}C]threonine. The cells were then incubated for a further 6 h at 37°C followed by washing three times with ice-cold PBS. Cytoplasmic extracts were produced by incubation of the infected cells with 0.1 M Tris-HCl pH 8.0, 0.1 M NaCl, 0.5% Nonidet P-40, 0.01% phenylmethylsulphonyl fluoride at 0°C for 10 min. 1–5% of the total extract was heated to 100°C with an equal vol. of 0.06 M Tris-HCl pH 6.8, 3% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue for 1 min and applied to 7.5% polyacrylamide gel. After electrophoresis, the gel was fixed, treated with Enhance (NEN) and fluorographed.

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