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Cell surface expression of fusogenic vesicular stomatitis virus G protein from cloned cDNA

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Vesicular stomatitis virus (VSV) enters the host cell by the receptor-mediated endocytotic pathway. This brings the virus particle into acidic vesicles inside the cell where infection occurs through a fusion event between the viral and the host vesicle membrane. In this work we have shown that the VSV glycoprotein (G) carries the fusion activity of this virus. The G protein was expressed on the surface of baby hamster kidney 21 cells from cloned cDNA which had been engineered into an expression vector and introduced into cell nuclei with the aid of a glass microneedle. A short (60 s) treatment with acid (pH ≤6.0) medium induced fusion of cells having G protein on their surface. For efficient G protein expression and cell-cell fusion we had to trim the 5′ end of the G cDNA and to use as promoter the long terminal repeat of the mouse Moloney sarcoma virus.

Key words: vesicular stomatitis virus/G protein/fusion activity/cDNA expression/microneedle injection

Introduction

Simple enveloped viruses like alpha-, myxo-, paramyxo- and rhabdoviruses package their RNA genome into a nucleocapsid which is surrounded by a lipid bilayer carrying glycoprotein spikes. The viral envelope is acquired from the plasma membrane (PM) of the infected host cell during virus budding (Simons and Garoff, 1980). For infection, the enveloped viruses must be disassembled and the genome introduced into the cell cytoplasm. Recent studies have shown that this occurs through fusion between the viral and the host lipid membranes (White *et al.*, 1983). This event introduces the viral nucleocapsid with the genome into the cell cytoplasm where infection can start. In the cases of alpha-, myxo- and paramyxoviruses there is good evidence for the glycoprotein spikes acting as catalysts for the membrane fusion (White *et al.*, 1983). In the case of rhabdoviruses, the single glycoprotein species, G, is likely to be a 'fusion protein' but so far this has not been shown experimentally. The present work shows cell surface expression of the vesicular stomatitis virus (VSV) G protein from cloned cDNA which has been engineered into an eucaryotic cDNA expression vector downstream from a mouse Moloney sarcoma virus (MSV) promoter, and which was introduced into cell nuclei of baby hamster kidney (BHK) cells by microneedle injection. The expressed G protein induces cell-cell fusion after a brief low pH (≤6.0) treatment. This result shows unambiguously that the G protein carries the fusion activity of VSV. The VSV G protein has been expressed from cloned cDNA before but membrane fusion activity has not been documented (Rose and Bergman, 1982).

Results

We have earlier used an SV40 early region based vector (pSV2) for transient expression of the Semliki Forest virus (SFV) structural proteins from cloned cDNA (Kondor-Koch *et al.*, 1983). Cells microinjected with the recombinant DNA were shown to fuse with each other after a brief low pH treatment. Here we have used the same strategy for the expression of fusogenic G protein from the cDNA.

Plasmids pSV2-G and pSV2-G del

The cDNA molecule encoding the G protein has been cloned into the *Pst*I site of the *Escherichia coli* plasmid pBR322 by J.Rose using the G/C homopolymer tailing technique (Rose and Gallione, 1981). The whole G cDNA can easily be transferred into the pSV2 expression plasmid using the regenerated *Pst*I sites in pBR322-G but, as we expected interference of the included homopolymer regions with transcription (and possibly translation) activity, we looked for an engineering strategy which excluded these regions. An examination of the restriction endonuclease cleavage map of the G cDNA showed a convenient *Hae*III site in the 3′-untranslated region. No useful cleavage site was available in the corresponding 5′ region. The homopolymer at the latter end was therefore removed by exonuclease digestion (*Bal*31) and a new cleavage site (*Hind*III) was introduced at the point of the deletion with a synthetic oligonucleotide. For practical purposes the engineering was done in the plasmid pUC8 after inserting the G cDNA with the 5′ homopolymer into the polylinker region of this plasmid (see Figure 1 for details). Deleted G cDNA molecules were then, together with an untreated control, transferred into the expression vector pSV2. After initial characterization of the 5′ region of the G cDNA, six clones were chosen as having suitable deletions and these were further analyzed by DNA sequencing. The five longest clones were shown to have 40, 35, 30, 15 and 5 nucleotides, respectively between the *Hind*III site, at the point of deletion, and the translation initiation codon for the G protein (Figure 2a). The shortest molecule had all of the 5′-untranslated region of the G cDNA removed, including the A of the initiation codon. The G/C homopolymer was only partially removed from the three longest molecules. These still contained 20, 15 and 5 residues of the homopolymer, respectively.

The G protein expression from all six G cDNA deletion molecules was studied using indirect immunofluorescence analysis of cells microinjected with the various constructions. These will be called pSV2-G del −40, −35, −30, −15, −5 and +2 according to the deletion. The untrimmed version is referred to as pSV2-G. About 100 BHK cell nulcei were injected with each construct and then incubated for 8 h before analysis for intracellular antigens (Figure 2b). The highest level of protein expression was obtained with pSV-2 del −15 and −5. Using this DNA, ~10% of the 100 injected cells

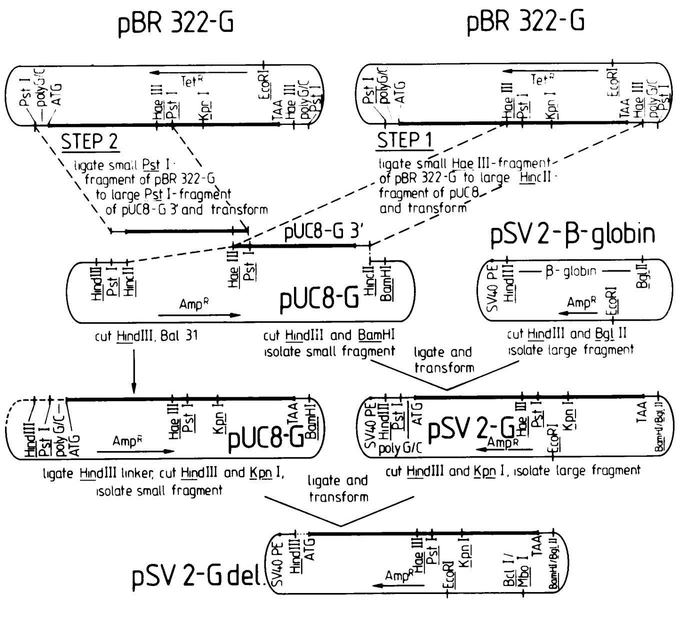

Fig. 1. Scheme for the construction of the pSV2-G expression vectors. The sequence coding for the VSV G protein is represented by a heavy line. The G cDNA was inserted into the polylinker region of the pUC8 in two steps. In this plasmid the 5′ homopolymer was removed using *Bal*31 and then transferred to the expression plasmid pSV 2 downstream from the SV40 early promoter (PE).

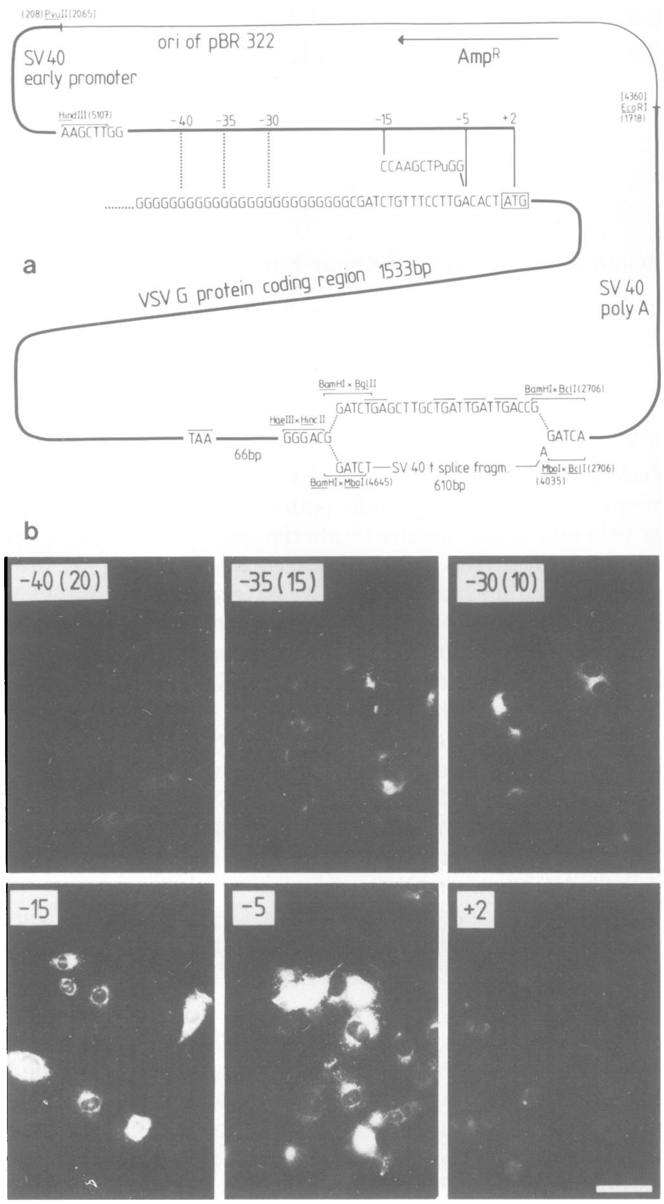

Fig. 2. (**a**) Map of pSVG expression vectors. The fragment taken from pBR322 is represented by a thin line, all other sequences by heavy lines. The numbering at the ligation points is done according to the map of pBR322 of Sutcliffe (1979) or to the map of SV40 of Reddy *et al.* (1978), respectively. The different deletion mutants are labelled according to the distance from the ATG codon (A = +1) to the endpoint of the *Bal*31 deletion. For mutant −15, 10 nucleotides were shown to differ from the cDNA sequence, possibly due to ligation of an altered *Hind*III linker molecule. Downstream of the cDNA, the vector contained either a DNA fragment containing the SV40 t splice signals (pSV2-G), possibly a *Mbo*I restriction fragment (see Mulligan and Berg, 1980) or instead a 27-bp fragment comprising translation stop codons (pSV-G) in all reading frames (Kondor-Koch *et al.*, 1983). (**b**) Comparative expression analysis of different pSV2-G deletion mutants in BHK cells by indirect immunofluorescence. Cells were internally stained 8 h after microinjection of DNA by treatment with a specific anti-VSV serum. The numbers refer to the deletion mutants as described in **a**. The length of GC homopolymers when present are given in brackets. The bar corresponds to a length of 60 μm.

showed positive fluorescence. pSV2-G del −30 and −35 showed fewer positive cells and no expression at all was detected with pSV2-G, pSV2-G del −40 and +2. The reason for the variation in G expression was not further analysed but it is possible that the presence of the G/C homopolymer indeed interferes with transcription and/or translation activity. In the case of pSV2-G del +2 the lack of expression must be due to the missing initiation codon for translation (the second AUG triplet is 92 bases downstream and in wrong reading frame; see Rose and Gallione, 1981).

Cell-surface staining of cells microinjected with pSV2-G del −15 and −5 showed that the G protein was present in the plasma membrane (not shown). However, a brief treatment of microinjected cells with pH 5.7 medium did not induce cell-cell fusion. Such a treatment readily results in cell-cell fusion when VSV particles have been bound to the surface of confluent BHK 21 cells (White *et al.*, 1981). Several possibilities could account for the lack of fusion, i.e. (i) the expressed G protein is incorrectly synthesized and/or processed (we tried to characterize the G protein expressed from pSV2-G del −5 by polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting but without success), (ii) the amount of G protein produced is insufficient for cell-cell fusion or (iii) the VSV induced fusion event is not a function of the G protein alone.

Plasmid pSVG

One way by which incorrect G protein could be produced, we reasoned, was by mis-splicing of the G mRNA, which in our expression system is produced in the cell nucleus and not in the cytoplasm as during infection. Aberrant splicing could for instance take place if a (cryptic) donor splice site is present in the G coding region and this combines with the acceptor site in the small t splice region, which is located downstream from the G cDNA in the pSV2 vector (Figure 2a). Such a mechanism has been previously postulated to explain the production of anomalous E1 membrane protein from this same vector harbouring the SFV cDNA molecule (Kondor-Koch *et al.*, 1983). We therefore engineered the G cDNA del −5 into a pSV2 vector lacking the small t splice region (see Figure 2a).

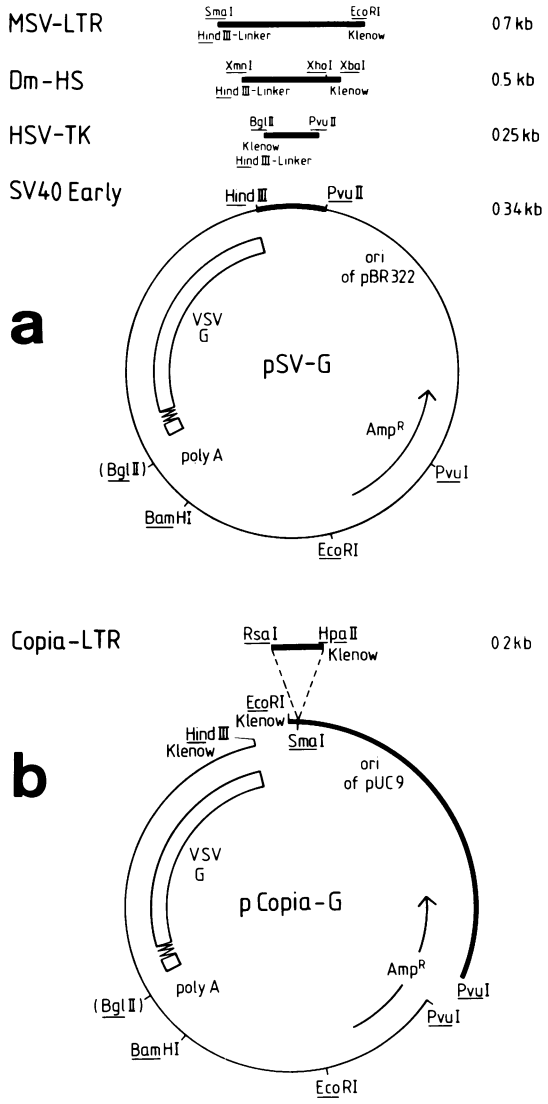

Fig. 3. Introduction of different eucaryotic promoter regions into the pSV-G vector. **(a)** The different promoter fragments shown by heavy lines were substituted for the 0.34 kb *Hind*III-*Pvu*II fragment containing the early SV40 promoter. The VSV G protein coding region is shown by a divided box. The division indicates the membrane spanning region of the protein close to the C-terminal end of the protein. **(b)** The *copia*-LTR was introduced into the vector using a *Eco*RI-*Pvu*I fragment (heavy line) cut out of pUC9, which contained the first 140 bp of the LTR cloned into the *Sma*I site (Steller and Pirrotta, 1984).

The new recombinant plasmid was called pSVG. The level of G protein expression from pSVG was not significantly higher than the corresponding construct bearing the small t splice region, and no cell-cell fusion between microinjected cells could be observed (data not shown). We also tested the pSVG (and the pSV2-G del −5) for G expression in mouse 3T3 cells, monkey CVI cells and MDCK cells. In all cases the level of G expression, as judged by immunofluorescence of microinjected cells, was lower than in BHK cells.

Plasmid pMSVG and other promoter constructions

As a way to increase the level of G protein expression we tested several different promoters. These were the mouse Moloney sarcoma virus (MSV) long terminal repeat (LTR), the thymidine kinase (TK) promoter from herpes simplex virus (HSV) and the heat-shock (HS) promoter and the *copia*-LTR element both from *Drosophila melanogaster*. The MSV promoter was available as a 0.7-kb *Sma*I-*Eco*RI fragment (3′-5′), the HSV-TK promoter as a 0.25-kb *Bgl*II-*Pvu*II fragment, the *Drosophila melanogaster* HS promoter as a 0.5 kb *Xmn*I-*Xba*I fragment and the *copia*-LTR as a 0.2-kb *Rsa*I-*Hpa*II fragment (Figure 3a). In brief, the engineering was done as follows: the 3′ end of the promoter fragment was converted into a *Hind*III site using a *Hind*III linker molecule and the 5′ end was made blunt. The promoter fragment was then exchanged for the *Hind*III-*Pvu*II fragment of pSVG containing the SV40 early promoter (Figure 3). The *copia*-LTR element was introduced in a more complicated way (see Figure 3b). The various constructions were called pMSV-G, pTK-G, pHS-G and p*copia*-G. In all constructions the predicted 5′ end of the transcripts contained the initiation codon for the G protein as the first AUG triplet.

When tested for G protein expression using immunofluorescence analyses of microneedle injected cells, the pMSV-G construction gave by far the best result; more than half of the injected cells showed a strong intracellular fluorescence (not shown). The other constructions resulted in G protein expression levels similar to those earlier obtained with pSVG (pTK-G and p*copia*-G) or less (pHS-G). The G protein expression from pHS-G was induced by a 30 min 42°C heat shock given 3 h after microinjection. Analyses of expressed G protein by the PAGE-immunoblot technique from cells transfected ($CaPO_4$ coprecipitation) with the various promoter constructions showed, in the pMSV-G sample, a clear band co-migrating with the G protein of purified VSV and VSV-infected cell lysate (Figure 4). None of the other samples showed any detectable G protein band. No G protein was found in the media of the transfected cells. The co-migration of the G protein expressed from pMSV-G with that of VSV suggests that it has been correctly synthesized and processed. The reason for the superior G protein expression from pMSV-G DNA in comparison to the other constructions is not clear but it is likely to be due to a more efficient transcription in BHK cells from the MSV promoter compared with the other ones. Another possibility would be that the 5′ end of the mRNA transcribed from pMSV-G forms a better translation initiation complex with the ribosome than the mRNAs expressed from the other constructs.

Immunofluorescence analyses of intact cells microinjected with pMSV-G DNA showed a strong surface staining for the G protein (Figure 5), thus indicating a high concentration of this protein in the plasma membrane. When neighbouring cells in a confluent monolayer of BHK cells were injected with pMSV-G DNA, incubated for 8 h and then treated for 1 min with medium of different pH (these were pH 6.6, 6.3, 6.0, 5.7 and 5.4) fusion of injected cells was observed at pH 6.0 or lower (Figure 6). The pH dependence of the fusion reaction when expressed from G cDNA is thus similar to the cell-cell fusion observed with complete virions added to monolayer cell cultures (White *et al.*, 1981). The polykaryon formation was specific for the injected cells; fused cells were not found elsewhere on the cell monolayer. The number of fused cells represented 30–50% of the injected cell number. This fraction is somewhat lower than the amount of positive cells observed in the immunofluorescence analyses of pMSV-G injected cells.

Discussion

The spike glycoproteins of simple enveloped viruses appear to be the key factors involved in both virus assembly and dis-

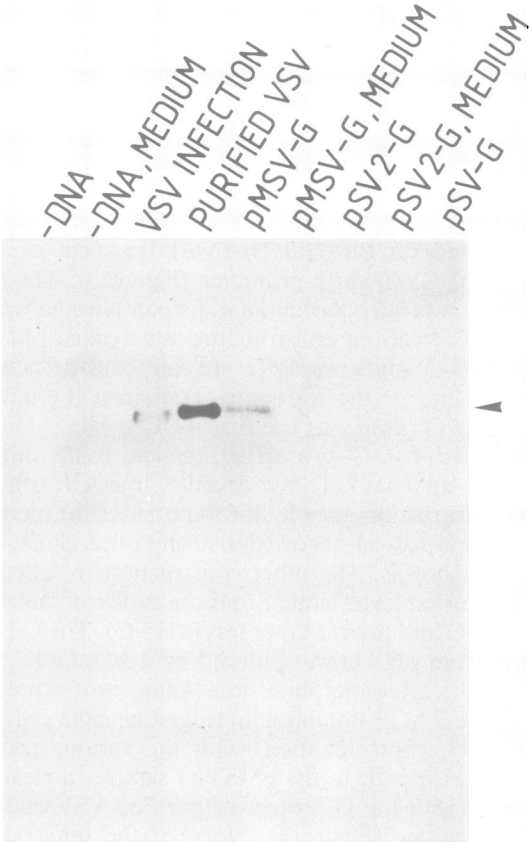

Fig. 4. Comparative expression analysis of the VSV G cDNA under control of different eucaryotic promoters by Western blotting. 30 h after transfection of BHK cells with the constructs (pMSVG, pSV2-G and pSVG), membrane proteins were extracted with Triton X-114, separated on SDS-polyacrylamide gels, transferred to nitrocellulose filters and detected by staining with a specific anti-VSV serum followed by an immunoperoxidase reaction. Proteins in the culture media were analysed after precipitation with trifluoroacetic acid. As controls, proteins of untransfected cells (−DNA), VSV infected cells or purified VSV were analysed. The arrow shows the position of the VSV G protein. Plasmids pTK-G, p*copia*-G and pHS-G were also analysed in a similar way but no G protein band could be detected.

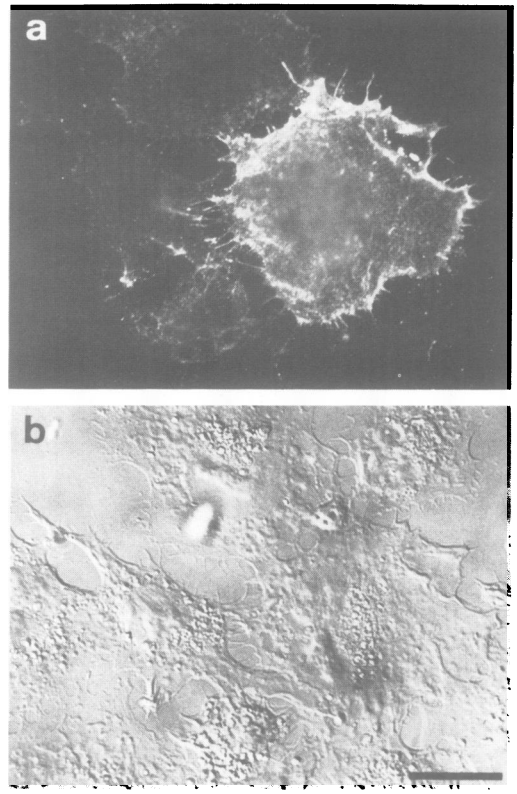

Fig. 5. (a) Cell surface immunofluorescence of VSV G protein in a cell that has been microinjected with pMSV-G DNA and incubated at 37°C for 8 h. **(b)** Same area of cell monolayer as in **(a)** when studied with Nomarski optics.

assembly. During virus maturation the cytoplasmic domain of the (membrane-) spanning viral glycoprotein is thought to interact with the nucleocapsid and thereby drive budding (Simons and Garoff, 1980). When the virus enters the cell the external portion of the spike glycoprotein induces membrane fusion between the virus and the host so that the viral nucleocapsid can be released into the cell cytoplasm and initiate infection (White *et al.*, 1983). The fact that the fusion in most cases is induced only at low pH provides a convenient way for the virus to control the assembly and disassembly process; it is when the virus has been taken up by the endocytic pathway into the target cell that low pH is first encountered and thus fusion can take place (Marsh *et al.*, 1982).

We have in this report described the expression from cloned cDNA of the VSV G protein in a fusogenic form. Several facts show that the fusion reaction is specific for the G protein; (i) cell-cell fusion occurred only in microinjected cells of the monolayer, (ii) injections of plasmid constructs which result in lower level of G protein expression than pMSV-G (e.g., pSVG del −5) did not lead to fusion, and (iii) the pH dependence of the fusion reaction was very similar to that observed when using VSV particles (White *et al.*, 1981). We have therefore unambiguously shown that the G protein alone can express the fusion activity of VSV. No other viral components are needed for the expression of this function. A similar approach has been taken before by White *et al.* (1982) and our group (Kondor-Koch *et al.*, 1983) to show that the influenza hemagglutinin (HA) molecule and the SFV spike glycoproteins are responsible for the fusion activity of these viruses.

In the cases of influenza and Sendai viruses, there is evidence for the involvement of the N-terminal peptides of the HA_2 and F_1 glycoprotein subunits, respectively, in the membrane fusion event. Both peptides show strong amino acid sequence conservation and have an overall hydrophobic composition (Gething *et al.*, 1978; Scheid *et al.*, 1978; Richardson *et al.*, 1980; Porter *et al.*, 1979; Ward and Dopheide, 1980; Dopheide and Ward, 1980; Gething *et al.*, 1980; Winter *et al.*, 1981; Hiti *et al.*, 1981; Fang *et al.*, 1981). The HA_2 and F_1 N termini are produced by proteolytic cleavage of the non-fusogenic precursor molecules HA and F_0, respectively (Homma and Ohuchi, 1973; Scheid and Choppin, 1974; Lazarowitz and Choppin, 1975; Klenk *et al.*, 1975; Huang *et al.*, 1981; Maeda *et al.*, 1981; White *et al.*, 1981; Ohuchi *et al.*, 1982). One hypothesis for the myxo- and paramyxovirus induced fusion is that the N-terminal peptides of HA_2 and F_1, respectively, interact with the lipid bilayer of the target membranes and thereby promote fusion. In the case of influenza virus, the HA molecule has to undergo a conformational change induced by low pH, probably in the host cell endosome, to expose its 'fusion peptide'. Studies on the HA conformation after acid treatment has confirmed the exposure of a (new) hydrophobic region (Skehel *et al.*, 1982).

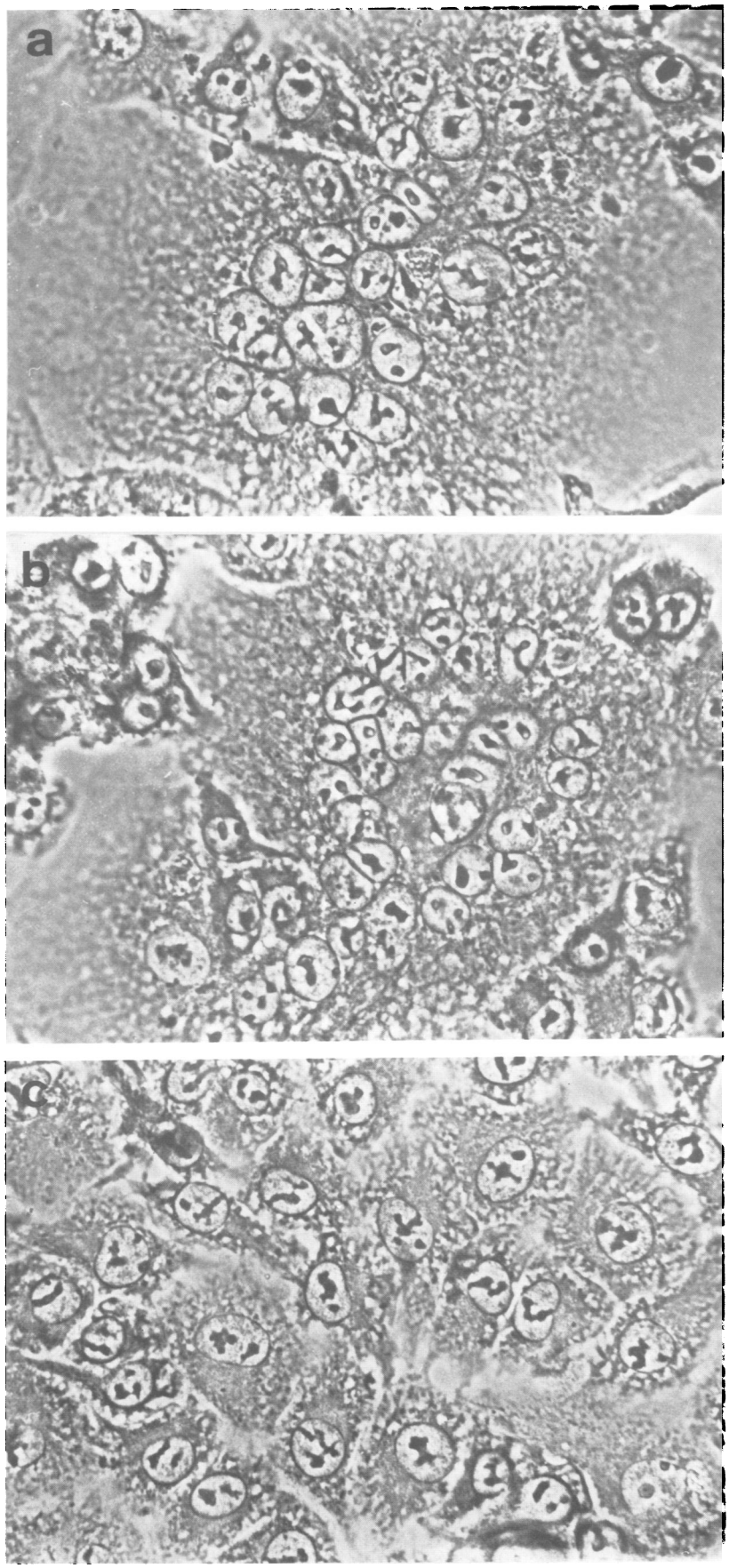

Fig. 6. Cell-cell fusion induced by VSV G protein expressed from pMSV-G DNA. **(a)** Polykaryon induced by pH 6.0 medium and **(b)** by pH 5.7 medium. **(c)** Control cells which have received pSVG DNA and incubated with pH 6.0 medium. For each experiment 50 adjacent cells in a confluent monolayer of BHK-21 cells were injected, incubated for 8 h at 37°C and then treated with low pH medium for 60 s. The cells were stained, with Giemsa after a 1 h incubation in normal medium.

The fusion mechanism postulated for the myxo- and paramyxoviruses cannot however directly apply to VSV. The VSV G protein, unlike the HA and F_1 molecules, does not have a late proteolytic processing event which would reveal a hydrophobic N-terminal 'fusion peptide' (Lingappa *et al.*, 1978). The N terminus of the mature G protein itself is not especially apolar and, moreover, is not conserved when compared with the G protein of the related rabies virus (Rose and Gallione, 1981; Analionis *et al.*, 1981; Rose *et al.*, 1982). An examination of the VSV G primary structure does not reveal any outstanding hydrophobic sequences which could serve as internal 'fusion peptides' as has been postulated for the E1 protein of alpha viruses (Garoff *et al.*, 1980). It would be very surprising, however, if the mechanism for G protein-induced fusion differed from that of other simple enveloped viruses. Clearly much more remains to be learned about the mechanism by which viral spike glycoproteins induce membrane fusion. One important step in this direction is to characterize in detail the 'fusion structures' or 'peptides' of the rhabdo- and alpha-virus spike glycoproteins, as well as those of myxo- and paramyxoviruses. This can for instance be done by the generation and characterization of fusion mutants. The fusion assay, as presented here for the VSV G protein, should be most useful for the investigation of G protein mutants made by *in vitro* mutagenesis.

Materials and methods

Materials

Various plasmids were obtained: pSV2-β-globin from Mulligan and Berg (1980), the VSV G cDNA in pBR322 from Rose and Gallione (1981) and the plasmid pUC8 Vieira and Messing (1982) via Hans Lehrach (EMBL).

Plasmid DNA containing eucaryotic promoter regions were provided: *copia*-LTR and *Drosophila* 70-K heat-shock gene by Hermann Steller (EMBL); MSV-LTR originally by Dhar *et al.* (1980) and MSV TK originally by McKnight (1980), both subcloned by Alan Colman (University of Warwick, England).

The cell line BHK 21 was obained from Kai Simons (EMBL); NIH 3T3 from Michael Wigler (Cold Spring Harbor, NY) via Günter Schütz (DKFZ, Heidelberg); CV_1 (African green monkey kidney) from the US Cell Culture Collection; and MDCK from Daniel Louvard (Institut Pasteur, Paris). VSV stock virus and the IgG fraction of a specific rabbit VSV antiserum were gifts from Karl Matlin (EMBL). An affinity purified goat anti-rabbit antibody conjugated to rhodamine were kindly provided by Hilkka Virta (EMBL). A Fab-IgG fraction of a sheep anti-rabbit serum labelled with a peroxidase activity was purchased from the Institut Pasteur (Paris).

Restriction endonucleases were generous gifts from Vincenzo Pirrotta (EMBL) or purchased from New England Biolabs or Boehringer (Mannheim). Polynucleotide kinase was a gift from Hans Lehrach, T_4 DNA ligase from Ray Brown and Fritz Winkler (EMBL). The exonuclease *Bal*31 was obtained from the Bethesda Research Laboratories (Maryland) and DNA polymerase I and bacterial alkaline phosphatase from Boehringer (Mannheim). The decanucleotide CCAAGCTTGG (*Hind*III-linker) was purchased from Collaborative Research (Waltham, MA).

The *E. coli* K12 strain HB 101 was a gift from Hans Lehrach (EMBL).

DNA construction

All enzymes were used as recommended by the manufacturers. Genetic engineering was carried out by standard methods as described in the Laboratory Manual of Maniatis *et al.* (1982). Restriction fragments were purified using the method of Tautz and Renz (1983). DNA sequencing was performed as described by Maxam and Gilbert (1980). Plasmids were purified from *E. coli* according to Holmes and Quigley (1981) or Birnboim and Doly (1979).

Plasmids pSV2-G and pSV2-G del were constructed as shown in Figure 1. The plasmid pSVG, lacking the small t intron was obtained through a three fragment ligation involving: (i) the small *Kpn*I-*Bam*HI fragment of pUC8G (contains the 3′ part of the G cDNA) (see Figure 1), (ii) the small *Hind*III-*Kpn*I fragment of pSV2-G del −5 (contains the 5′ part of the G cDNA) and (iii) the large *Hind*III-*Bgl*I fragment of the plasmid pSVS-SFV. The latter fragment contains a version of the pSV2 vector where an oligonucleotide with stop translation signals is inserted in place of the small t intron fragment (Kondor-Koch *et al.*, 1983). The *Hind*III site next to the stop translation oligonucleotide on the 5′ side in pSVS SFV had been changed into a *Bgl*II site (Kondor-Koch, unpublished). The engineering of various promoters in front of the G cDNA is outlined in Figure 3.

Cell culture

MDCK and mouse 3T3 cells were grown at 37°C in 5% CO_2 and passaged twice a week in plastic bottles in Eagle's minimal essential medium with Earl's salts (Earl's MEM) supplemented with 10 mM Hepes, pH 7.3, 5% (v/v) fetal calf serum (FCS), penicillin (110 U/ml), streptomycin (100 μg/ml) and fungizone (0.025 μg/ml). For CU_1-cells the medium was modified according to Dulbecco (DMEM) containing 10% (v/v) FCS. For BHK cells the medium was modified (GMEM, Glasgow) by adding 2 mM glutamine and 10% (w/v) tryptose phosphate. Cells were released from the plastic using trypsin-EDTA (0.5 g trypsin and 0.2 g EDTA/l in Puck's saline). All reagents were purchased from Gibco Bio Cult. (Paisley, Scotland).

Introduction of vector DNA into cells

Circular forms of expression vector DNA were introduced into the nuclei of different cell lines either using microneedle injection or the calcium phosphate transfection technique. The microinjection method has been described in detail elsewhere (Timm *et al.*, 1983). Individual cells in a subconfluent monolayer on a coverslip (50–100/experiment) were injected with a glass needle in a paraffin oil chamber using a de Fonbrune micromanipulation set up at DNA concentration of 1 μg/μl. After incubation for 8 h, cells were processed for indirect immunofluorescence or cell-cell fusion.

The calcium phosphate precipitation technique was used according to the protocol of Graham and van der Eb (1973) and Kondor-Koch *et al.* (1983). Cells, which had received DNA precipitate, were incubated at 37°C for 30 h and then processed for analysis by SDS-polyacrylamide gel electrophoresis. If proteins in the culture medium were to be analysed the medium was changed to one lacking FCS 6 h in advance.

Western immunoblotting

SDS-PAGE and immunoblotting were done as described before (Kondor-Koch *et al.*, 1983). The cells and the media of transfected cells were processed separately. Cell lysates were extracted with Triton X-114 to enrich for membrane proteins (Bordier, 1981). The samples were run on 10% polyacrylamide gels as described by Maizel (1969) and proteins were blotted onto nitrocellulose filters by the procedure of Burnette (1981). VSV G protein was detected using a specific rabbit antiserum and peroxidase-conjugated sheep anti-rabbit IgG as described by Burke *et al.* (1982).

Immunofluorescence

Indirect immunofluorescence analysis was carried out using the method of Ash *et al.* (1977). The cells were stained either directly (to reveal surface antigens) or after permeabilization of cellular membranes with Triton X-100 (to show internal antigens).

Cell-cell fusion

Fifty adjacent cells in a confluent monolayer of BHK 21 cells were injected with DNA, incubated for 8 h to allow surface expression of the G protein, and then treated with low pH medium to induce cell-cell fusion (White *et al.*, 1981). This was observed using Giemsa staining of cells after a 1 h incubation at 37°C with normal medium.

Acknowledgements

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