Isolation of Stable Mouse Cell Lines That Express Cell Surface and Secreted Forms of the Vesicular Stomatitis Virus Glycoprotein

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ABSTRACT We have characterized two stable transformed mouse cell lines (CG1 and CTG1) that express either the normal vesicular stomatitis virus glycoprotein (G) or a truncated form of the G protein (TG) that lacks the COOH-terminal anchor sequences and is secreted from the cells. These cell lines were obtained using a hybrid vector consisting of the transforming DNA fragment of bovine papilloma virus linked to a segment of the SV40 expression vector pSV2 containing cloned cDNA encoding either the normal or truncated form of the vesicular stomatitis virus G protein. Using indirect immunofluorescence we have found that >95% of the cells in each line express the G protein(s), although the level of expression within the population is variable. The normal G protein expressed in these cells obtains its complex oligosaccharides in <30 min and is transported to the cell surface. In contrast, the TG protein obtains its complex oligosaccharides with a half-time of about 2.5 h. Immunofluorescence data show an apparent concentration of the TG protein in the rough endoplasmic reticulum. These data together suggest that transfer of this anchorless protein from the rough endoplasmic reticulum to the Golgi apparatus is the rate-limiting step in its secretion. We observed, in addition to normal G protein, two smaller G-related proteins produced in the CG1 cell line. We suggest that these proteins could result from aberrant splicing from sites within the G mRNA sequence to the downstream acceptor in the pSV2 vector.

The vesicular stomatitis virus (VSV)[1] glycoprotein (G) has served as an important model system for studying the synthesis, processing, and transport of an integral membrane glycoprotein (reviewed in reference 6). G protein can be divided into four primary domains: the signal sequence, the main body of the protein, the transmembrane domain, and the cytoplasmic tail (17). The amino terminal signal sequence presumably directs the initial interactions between the ribosome-mRNA-nascent polypeptide complex and the cytoplasmic side of the rough endoplasmic reticulum (rough ER) membrane. After this initial interaction, the body of the nascent protein is inserted into the rough ER membrane and the signal sequence is cleaved (2, 7, 11, 15). As transfer proceeds, glycosylation of two asparagine residues (amino acid residues 178 and 335) occurs through a dolichol-mediated transfer of high mannose oligosaccharide complexes (14, 17, 19). Transfer through the rough ER membrane stops after the hydrophobic transmembrane domain is inserted into the lipid bilayer. This leaves the highly charged COOH-terminal domain of G protein on the cytoplasmic side (2, 8, 18, 25). G protein is then transported to the Golgi apparatus (1), where the N-linked high mannose oligosaccharides are trimmed and converted into complex oligosaccharides (6). G protein is then transported to the plasma membrane where it is normally incorporated into virions.

Using DNA vectors containing SV40 promoters, splice

[1] *Abbreviations used in this paper:* BPV-1, bovine papilloma virus; DME, Dulbecco-Vogt's modified Eagle's medium; endo H, endonuclease H; ER, endoplasmic reticulum; FBS, fetal bovine serum; G glycoprotein, normal vesicular stomatitis virus glycoprotein; TG, truncated form of G protein; VSV, vesicular stomatitis virus.

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sites, and polyadenylation signals, we have obtained transient expression of a cDNA clone encoding the VSV G protein in both mouse L cells and monkey COS-1 cells (16). These experiments have shown that synthesis, processing, and transport of G protein occur normally in the absence of any other VSV proteins. A derivative of the G cDNA clone that does not encode the last 79 amino acids of G protein was also expressed in COS-1 cells (16). This deletion removes DNA encoding both the hydrophobic transmembrane domain and the highly charged cytoplasmic tail of G. This truncated form of G protein (TG protein) appeared to accumulate in the rough ER, although it was found to be secreted slowly.

The major expression system that we used previously for the G protein employed the SV40 vector JC119 (23). Although this system is rapid and convenient, it has several drawbacks. It limits one to a single monkey cell line COS-1 (which provides SV40 large T antigen, reference 3) and the expression is short-lived because replication of the vector results in cell death. Also, only a small fraction of cells (2–5% in most experiments) actually express the protein. To overcome these problems we constructed a vector that would allow us to obtain mouse cell lines expressing normal and mutant G proteins. This vector contains the subgenomic transforming fragment of the bovine papilloma virus (BPV-1) genome (12) linked to a segment from the pSV2G or pSV2TG DNA (13) that we had used to obtain transient expression of G or TG proteins after microinjection into mouse L cells (16). Transcription from the early promoter in the pSV2 vector is independent of SV40 T antigen and therefore should occur in any cell line. The BPV genome is known to replicate stably as an episome in the nuclei of transformed cells with ~20–100 copies per cell (10). It has also been linked to other genes and shown to direct their replication and expression (20). We report here that these hybrid vectors containing either the TG or G cDNA insert are capable of transforming mouse C127 cells, which then produce either the TG or normal G proteins.

MATERIALS AND METHODS

Construction of Plasmids: Restriction endonucleases were purchased from New England Biolabs (Beverly, MA) or Bethesda Research Laboratories (Gaithersburg, MD). DNA fragments for ligation were purified by electrophoresis on 1% agarose gels or 6% polyacrylamide gels. The recombinant DNA methodology was essentially as described previously (16). Synthetic DNA linkers containing the Bam HI site were purchased from Collaborative Research Inc. (Lexington, MA). To convert the unique Pvu II sites in pSV2G and pSV2TG (13, 16) to Bam HI sites the DNAs were linearized with Pvu II and then joined with T4 DNA ligase in the presence of a 50-fold molar excess of Bam HI linkers. Plasmids obtained from cells transfected by this DNA were screened for the presence of the Bam HI site. The Bam HI fragments excised from these plasmids were then ligated into the unique Bam HI site in pBPV52-1 (generously provided by Peter Howley, National Institutes of Health).

Transfection and Cloning of Mouse Cells: Mouse C127 cells and all transformed lines derived from them were maintained in Dulbecco-Vogt's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (FBS). Monolayers of C127 cells were transfected with the appropriate DNA via calcium phosphate precipitation as described previously (16, 26) with the following modifications. Briefly, 20 μg of plasmid DNA was added to 0.25 ml of 0.25 M $CaCl_2$. An equal volume of 2 × HEPES buffer containing 280 mM NaCl, 50 mM HEPES, and 1.5 mM $NaPO_4$, pH 7.4 was added. The precipitate was allowed to form for 30 min at room temperature and was then added to 5-cm plates of cells (containing 3 ml of DME plus 10% FBS per plate) that were ~50% confluent. After 4 h at 37°C the medium was removed and 1 ml of 15% glycerol in 1× HEPES buffer was added. The glycerol solution was removed after 3 min, the monolayers were washed with DME and incubated further with DME plus 10% FBS. 24 h later the cells from a single dish were transferred to three 10-cm dishes and grown to confluence. Transformed foci of cells were removed from the dish with trypsin and replated. These cells were then subcloned in soft agar. Bottom agar (0.5% agar in DME plus 10% FBS) was poured into 5-cm plates and allowed to cool. Cells were added to 2.5 ml of top agar (0.33% agar in DME plus 10% FBS) and layered over the bottom agar so that ~100 or 500 cells were added per plate. Plates were incubated at 37°C until colonies were clearly visible. Colonies were picked and replated and the resulting cloned cell lines were maintained in DME plus 10% FBS.

Labeling and Immunoprecipitation: Confluent cell monolayers were labeled with 100 μCi of [^{35}S]methionine per milliliter of culture media for various lengths of time as described in the figure legends. Extracts were prepared and immunoprecipitated as described previously (16). Medium was removed from the plate and then 1 ml of detergent solution (1% NP-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4) was added for ~1 min. The lysate was transferred from the plate to a 1.5-ml Eppendorf tube, and the nuclei were pelleted. The supernatant was carefully removed to avoid the pellet and adjusted to 0.3% SDS. Then 2 μl of rabbit VSV antiserum was added and the mixture incubated at 37°C for 15 min. After incubation with antiserum, 15 μl of washed, fixed *Staphylococcus aureus* bacteria was added, and the mixture was incubated for another 15 min at 37°C. Precipitates were washed three times with RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl pH 7.4) and subjected to electrophoresis as previously described.

Endoglycosidase H Digestion: Immunoprecipitates were resuspended by boiling in 10 μl of 1% SDS, 50 mM Tris, pH 6.8 for 2 min. The fixed *S. aureus* bacteria were pelleted, and the supernatant was saved. Endoglycosidase H (endo H, 34.5 U/mg, 87 μg/ml, Health Research Incorporated, Albany, NY) was diluted 1:10 in 0.15 M NaCitrate (pH 5.3) and then 10 μl was added to an equal volume of the immunoprecipitated sample which had been boiled for 2 min in 10 mM Tris-HCl, pH 6.8, 1% SDS. Digestion was carried out at 37°C for 16 h. The samples were then electrophoresed on SDS polyacrylamide gels (16).

Antibodies and Immunofluorescence Staining: The procedures for indirect immunofluorescence staining were performed as described previously (16), with the following exceptions. In some experiments we used unfractionated rabbit anti-VSV serum (diluted 1:300) for surface staining followed by incubation with fluorescein-conjugated goat anti-rabbit IgG diluted 1:200 (M. L. Cappel Laboratories, Cochranville, PA). Internal staining after permeabilization with 1% NP-40 was done with unfractionated guinea pig anti-VSV serum (diluted 1:300) followed by incubation with rhodamine-conjugated goat anti-VSV IgG diluted 1:200 (N. L. Cappel Laboratories). The unfractionated antisera gave results that were comparable to those obtained previously with affinity-purified antibodies (16).

RESULTS

Construction of the Expression Vector and Establishment of Transformed Cell Lines

To obtain stable expression of normal and mutant VSV G proteins we first constructed the plasmid diagrammed in Fig. 1. We had previously constructed a plasmid (pSV2G) containing the early promoter of SV40, the cloned VSV G cDNA, the small t splice site, and the SV40 early polyadenylation signal (13, 16). These sequences were excised from the pSV2G plasmid and ligated into the plasmid pBPV52-1, which contains pBR322 sequences and the BPV transforming fragment. The identical construction was carried out with the plasmid pSV2TG carrying a deleted form of the G gene that lacks sequences encoding the COOH-terminal 79 amino acids of G protein. These two plasmids are called pSVBPVG and pSVBPVTG, respectively. Because specific pBR322 sequences apparently prevent replication of BPV DNA (12), the plasmids were digested with Xho I before transfection onto mouse cells. The digestion leaves the SV40, G gene sequences, and the BPV sequences intact but removes the pBR322 sequences. Transformed foci of mouse C127 cells were picked and then subcloned in soft agar. The two transformed cell lines obtained that we have characterized extensively are designated CG1 and CTG1. The CG1 line carries

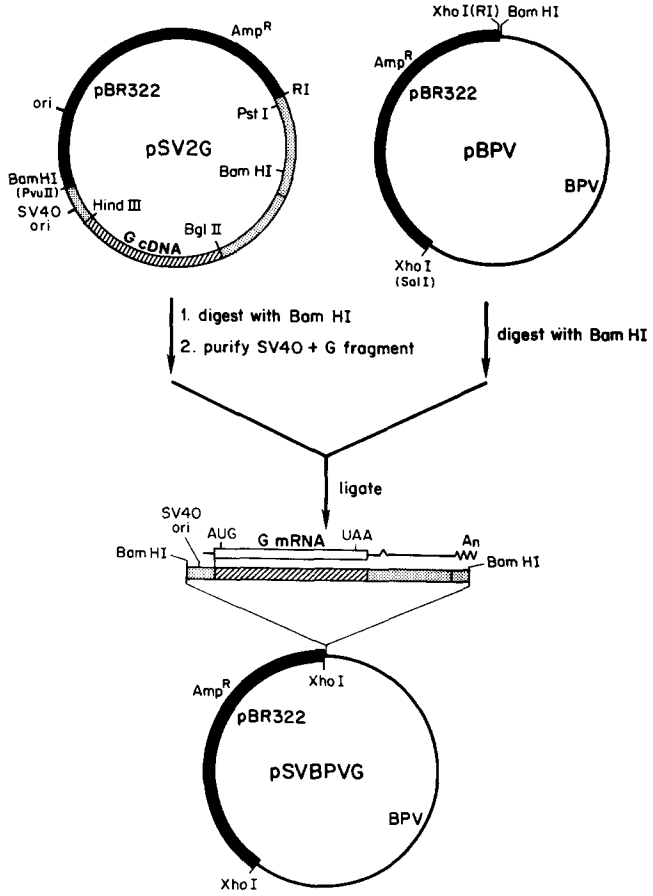


FIGURE 1 Construction of pSVBPVG. Solid bar in pSV2G-2 is a pBR322 segment containing the origin of replication and the ampicillinase gene. Stipled bars are SV40 segments as described by Mulligan and Berg (13). Hatched bars represent the cloned G cDNA segment. This plasmid is identical to pSV2G except that the Pvu II site was converted to a Bam HI site with Bam HI linkers. Solid bar in pBPV52-1 is the Eco RI-Sall fragment of pBR322 containing the ampicillinase gene. The 69% transforming fragment of BPV DNA (thin line) was joined to the pBR322 segment with Xho I linkers. The Bam HI fragment of pSV2G-2 containing the G cDNA was ligated at the Xho I site in pBPV52-1 as indicated. The mRNA expected to encode G protein is diagrammed above the Bam fragment. The small t intron of SV40 is shown (single peak to the right of *UAA*) as is the SV40 polyadenylation signal (A_n) for the early mRNA. An essentially identical construction was performed starting with pSV2TG to generate pSVBPVTG.

sequences for expression of normal VSV G protein, while the cell line CTG1 carries sequences for expression of the TG protein.

Immunofluorescence of CTG1 and CG1 Cells

We used indirect immunofluorescence to determine the percentage of CTG1 and CG1 cells expressing the TG or G proteins and to examine the intracellular location of the respective proteins. Examples of the results obtained are shown in Fig. 2. Greater than 95% of the cells in both cell lines were found to be positive for the expression of TG or G proteins. However, there was considerable variability in the level of fluorescence within both cell populations.

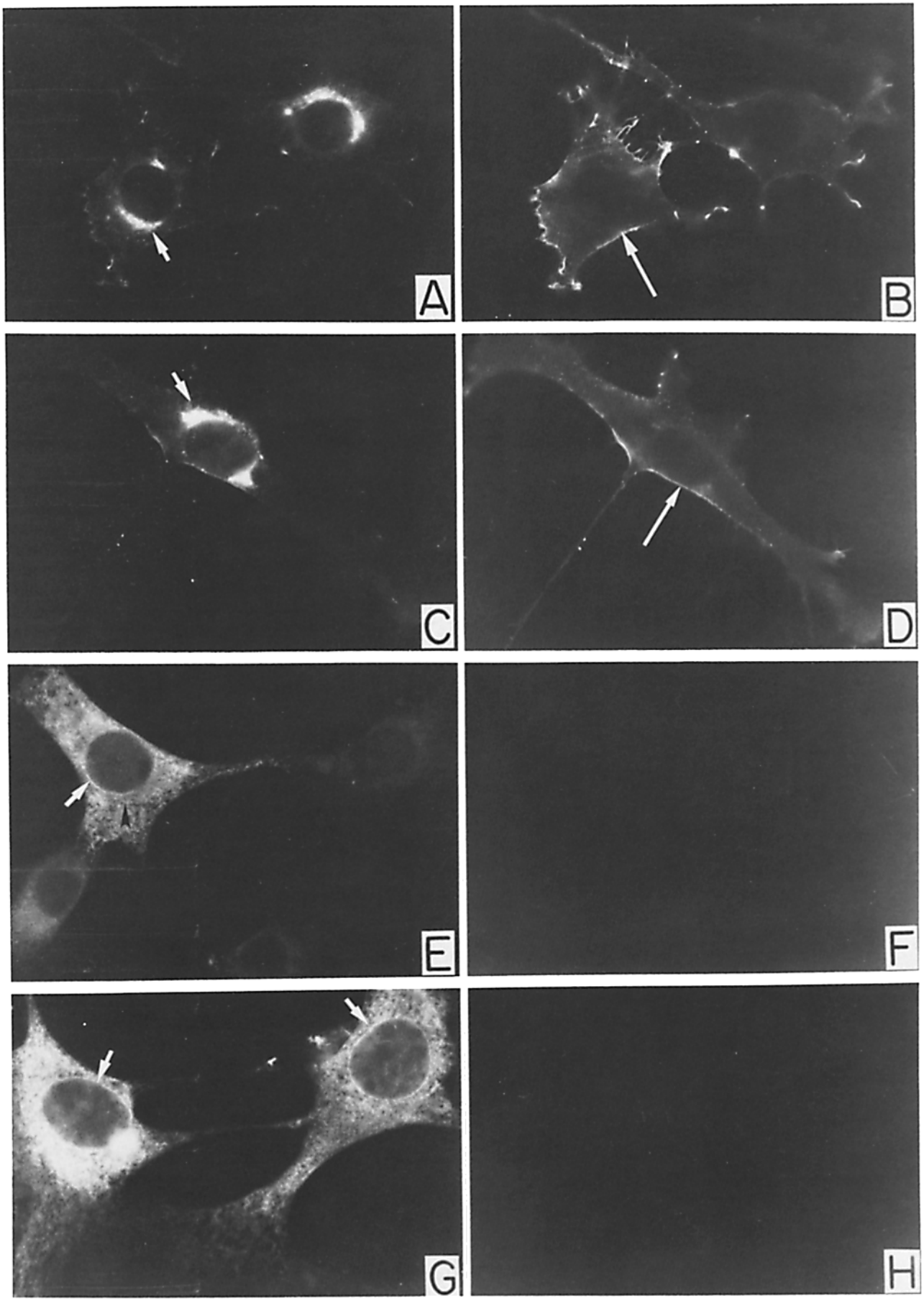
In the CTG1 cells the diffuse lattice-like cytoplasmic fluorescence and the nuclear ring stain suggested a concentration of TG protein within the rough ER, as we observed previously for this protein in COS-1 cells and mouse L cells (16). No cell surface fluorescence was detectable, presumably because the TG protein lacks the transmembrane domain that anchors normal G protein into the plasma membrane. The immunofluorescence data are also consistent with the slow acquisition of endo H resistance by TG protein (see below), suggesting slow transport from the rough ER to the Golgi apparatus.

As was seen in the CTG1 cells, there was a distinct reticular fluorescence in the CG1 cells, suggesting that G protein was associated with the rough ER. There was, as well, a distinct concentration of fluorescence near the nucleus that is typical of the Golgi apparatus. The cell surface labeling showed that G protein had been transported to the plasma membrane. The majority of the cells in the population showed only faint internal and surface fluorescence for G protein. However, there was a wide range of intensities, including some very bright cells. The distribution of fluorescence within the CG1 cells suggested that the G protein synthesized was being processed and transported through the rough ER, the Golgi apparatus, and then inserted into the plasma membrane as in a VSV-infected cell.

Detection of TG Protein and Normal G Protein by Immunoprecipitation

To determine whether the cell lines that had been cloned were expressing the VSV G or TG proteins of the appropriate sizes, we analyzed expression in the CG1 and CTG1 cell lines by immunoprecipitation. Cells were labeled with 100 μCi of [^{35}S]methionine for 1 h, lysed, and the cytoplasmic proteins were immunoprecipitated. We also carried out immunoprecipitations on media from these cells to determine whether any proteins were secreted from the cells. Proteins were then analyzed by electrophoresis on SDS polyacrylamide gels (Fig. 3). A single species of the TG protein was found in the media, and a single species was found in the cytoplasmic fraction from the cells. These results are similar to what we observed when expressing the TG protein in COS-1 cells using the vector JC119. By comparison to those of marker VSV proteins, the apparent molecular weights of the cell-associated and secreted forms of TG protein are calculated to be 61,250 and 62,500, respectively. We presume that the increase in the apparent molecular weight (1,250) of the secreted form of TG is due to addition of terminal sugar residues (probably sialic acid) before secretion (see below).

In the cell line CG1 three immunoprecipitable bands were observed in the cells and one was found in the medium (Fig. 3). The largest species found in the cell co-migrated with authentic G protein (apparent molecular weight 66,300) from VSV-infected C127 cells. The two additional, faster-migrating species have apparent molecular weights of 64,000 and 60,250, respectively. According to their increasing gel mobility we have designated the three species of cell-associated G protein as G, G_1, and G_2. The calculated molecular weight of the single G-related protein species in the medium is 65,000. This band is designated G_r (G released). Bands larger than VSV L protein present in immunoprecipitates from media and cytoplasmic fractions are nonspecific background proteins that are also found in immunoprecipitates from untransformed parental C127 cells. The profiles of immunoprecipitable G-related species found in the CG1 or CTG1 cells have remained unchanged during 6 mo of cell passage.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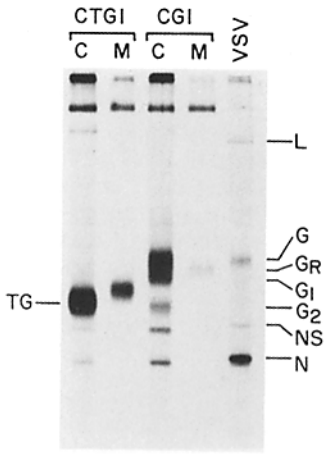

FIGURE 3 Analysis of immunoprecipitates from CTG1 and CG1 cells. Cells were labeled with 100 μCi of [^{35}S]methionine for 1 h, and G protein was immunoprecipitated and subjected to electrophoresis on a 15% SDS polyacrylamide gel. The gel was dried and then fluorographed for 48 h. Immunoprecipitates of cell (*C*) and media (*M*) fractions from CTG1 cells expressing TG protein or from CG1 cells expressing G and G-related proteins are shown. [^{35}S]Methionine-labeled VSV virion proteins from virus infected C127 cells are shown as markers. VSV proteins *L*, *G*, *NS*, and *N* are indicated. G-related proteins G_1, G_2, and G_R are indicated for CG1 cells and TG protein is indicated for the CTG1 cells. The sharp bands migrating just ahead of the *NS* and *N* markers in the two "*C*" lanes are variable background bands from the immunoprecipitation, as are larger molecular weight bands (above *L*) which are also present in nontransformed cells.

Kinetics of Secretion and Acquisition of Endoglycosidase H (endo H) Resistance of TG Protein

To determine the rate of secretion of the TG protein expressed in the CTG1 cell line, we labeled CTG1 cells with [^{35}S]methionine for 15 min and then chased with unlabeled methionine for various lengths of time. Cells and media were harvested separately, immunoprecipitated, and analyzed by electrophoresis on SDS polyacrylamide gels (Fig. 4*a*). Quantitation of the data in Fig. 4*a* is shown as an insert plotted as a percentage of the total radioactivity in each band. After the 15-min labeling period TG protein was found exclusively in the cytoplasmic fraction of the cells. After ~2.5 h, ~50% of the immunoprecipitable TG protein was found in the medium. At the latest times examined, nearly all of the TG protein was found in the medium. A minor band of TG protein migrating more slowly than the major species found within the cell co-migrated with the single species of TG found in the medium. Only traces of the slower-migrating species of TG protein was found within the cell, suggesting that export occurs rapidly after conversion to the larger form. The half-time of secretion from the CTG1 cell line was nearly identical to that previously determined in transient expression experiments for a similar protein in monkey COS-1 cells (16).

To determine whether the size difference between the major cell-associated and -secreted forms of the TG protein could be due to the extent of glycosylation, we analyzed the sensitivity of the oligosaccharide chains to endo H digestion in a pulse-chase experiment. CTG1 cells were pulse labeled with

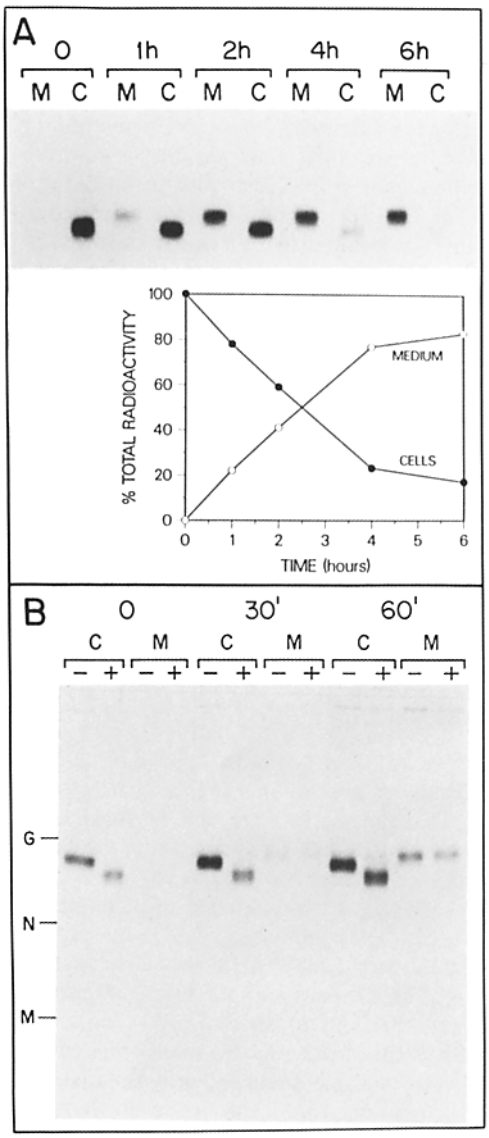

FIGURE 4 Kinetics of secretion and acquisition of endo H resistance of TG protein from CTG1 cells. (*A*) Five 5-cm plates of equal cell density were pulse labeled for 15 min with 100 μCi of [^{35}S]-methionine, then chased with DME plus 10 mM unlabeled methionine for the desired length of time. Immunoprecipitates from cell and media fractions, designated *C* and *M*, were subjected to electrophoresis on 15% SDS polyacrylamide gels. Quantitation of the radioactivity in each band is shown as an inset within *A*. (*B*) Immunoprecipitates of cytoplasmic and media fractions (*C* and *M*) from CTG1 cells were pulse labeled and chased as described above. Half of each sample was digested with endo H (+) after immunoprecipitation as described in Materials and Methods; the other half was not digested (−). Samples were electrophoresed on 15% SDS polyacrylamide gels and fluorographed for 48 h. The position of VSV virion proteins *G*, *N*, and *M* are shown as markers.

FIGURE 2 Detection of G protein and TG protein by immunofluorescence. Fixation and staining were performed as described in Materials and Methods. *A*, *B*, *C*, and *D* show CG1 cells labeled either internally with rhodamine-conjugated antibody (*A* and *C*), or the same cells labeled on the surface with fluorescine-conjugated antibody (*B* and *D*). *E*, *F*, *G*, and *H* show the same CTG1 cells labeled either internally (*E* and *G*) or on the cell surface (*F* and *H*). Short arrows in *A* and *C* point to presumed Golgi apparatus. Long arrows in *B* and *D* indicate cell surface staining. Short arrows in *E* and *G* indicate nuclear envelope staining and the arrowhead (*E*) indicates the reticular cytoplasmic staining.

[^{35}S]methionine for 15 min, then chased with cold methionine for various lengths of time. Cytoplasmic and media fractions were digested with endo H after immunoprecipitation, and then analyzed by electrophoresis on SDS polyacrylamide gels. Endo H cleaves the precursor (high mannose) oligosaccharides added during insertion into the rough ER membrane, but will not cleave complex oligosaccharides that result from processing of the precursor in the Golgi apparatus (6). Fig. 4*b* shows the results of this experiment. The single TG species found in the medium was completely resistant to digestion at all times examined, suggesting that its pathway of export included transport through the Golgi apparatus. Of the two bands of TG protein associated with the cell, the major (faster-migrating) species was sensitive to digestion with endo H and the minor (slower-migrating) species was resistant to endo H digestion. The apparent increase in molecular weight before secretion presumably correlates with the addition of the complex oligosaccharide chains (24). No size difference was observed between the secreted and cell-associated forms of TG protein made in COS-1 cells (16), although the exported form has complex (endo H-resistant) oligosaccharides (unpublished results). Presumably, the complex chains added in COS-1 cells differ somewhat from those added in C127 cells.

Multiple Species of G-related Proteins Synthesized in the CG1 Cell Line

Because we had observed two G-related proteins in addition to the normal G protein in CG1 cells, we considered the possibility that these cells were derived from some unusual transfection event. To test this possibility we examined four independent cell lines that had been transformed by the pSVBPVG plasmid DNA. Labeled proteins from each line were analyzed by electrophoresis on SDS polyacrylamide gels. We found the same pattern of two G-related proteins in addition to G in the cells and one G-related protein released into the medium (data not shown).

Since the identical pattern of G and G-related proteins was observed from multiple, independently isolated, transformed cell lines we assumed that it did not result from a rare DNA recombination event(s). To determine directly whether there were any rearrangements of the G or TG coding sequences within the episomal DNA in the transformed C127 cells, we analyzed DNA from a Hirt extract (4) by gel electrophoresis and Southern blotting (22). The data showed that the G or TG cDNA coding sequences could be excised from episomal DNA from CG1 or CTG1 cells to yield fragments identical to those from the parental plasmids. These results indicated that no DNA rearrangements of the TG or G coding sequences had occurred during replication in the C127 cells (data not shown).

To examine glycosylation of the G and G-related proteins we immunoprecipitated cytoplasmic and media fractions from CG1 cells and then digested portions with endo H. Cells were labeled for 1 h with [^{35}S]methionine, and the immunoprecipitated proteins were digested with endo H (Fig. 5). The G_1 and G_2 species were apparently sensitive to endo H digestion. The increase in gel mobility for both is consistent with a decrease in molecular weight of ~3,000. These decreases suggest the removal of two oligosaccharide chains from each protein (9). The species designated G was completely resistant to endo H digestion as is observed for normal G protein from VSV-infected cells after a 1-h labeling period. In these experiments G_r was also resistant to endo H digestion.

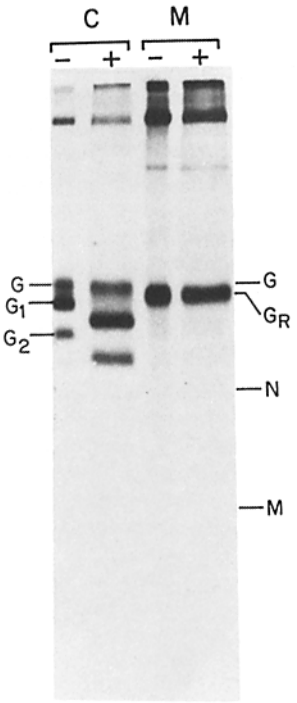

FIGURE 5 endo H sensitivity of G and G-related proteins from CG1 cells. CG1 cells were labeled for 1 h with 100 μCi of [^{35}S]methionine. Immunoprecipitates were prepared from both cytoplasmic (*C*) and media (*M*) fractions. Half of each sample was digested with endo H (+), half was not (−), and then both were analyzed by electrophoresis on 15% SDS polyacrylamide gels. The letters *G*, *N*, and *M* indicate positions of VSV virion marker proteins. The G and G-related proteins are labeled *G*, G_1, G_2 and G_r.

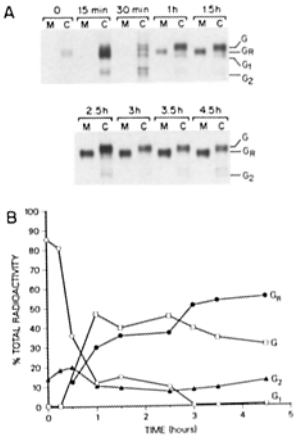

FIGURE 6 Pulse-chase analysis of G and G-related proteins made in the CG1 cell line. (*A*) Nine 5-cm plates of CG1 cells were pulse labeled with 50 μCi of [^{35}S]methionine followed by a chase with unlabeled methionine. G protein was immunoprecipitated and electrophoresed as described in Materials and Methods. The labels *G*, G_1, and G_2 indicate cell-associated proteins and G_r indicates the position of the protein found in the medium. The lighter bands at zero time presumably result from either random sample loss or variation in cell density on that particular plate and is not seen in all experiments. (*B*) To determine the relative proportions of the different proteins at each time point, the gel was scanned with a densitometer and the peak areas were determined. This quantitation is shown.

To examine possible kinetic relationships among the G-related proteins made in CG1 cells we pulse-labeled CG1 cells and chased for various lengths of time. Immunoprecipitated proteins were then analyzed by electrophoresis on SDS polyacrylamide gels (Fig. 6*a*). Quantitation of the data is plotted as percentage of the total radioactivity for each G and G-related protein species (Fig. 6*b*). After a 15-min pulse (zero time), only protein bands of G_1 and G_2 were present. After 15-min and 30-min chase periods, G_1 decreased significantly and G_r was evident in the medium. A band co-migrating with fully glycosylated G protein also appears at 30 min, as well as a band between G and G_1, which might be a precursor to G. We have therefore not quantitated G at 30 min because it is not clear whether both of these bands should be counted. At later times, G_1 disappears and G_r plateaus in the medium. The G protein remains relatively constant, decreasing slightly between 3 and 4.5 h. The G_2 band remains nearly constant

throughout the experiment and seems to resolve into two bands at most time points. These kinetic analyses suggest that the G_1 band is the precursor to the secreted species G_r and that at the earliest times it also contains a precursor (presumably partially glycosylated) of mature G protein.

These kinetic analyses suggested that G_r was not derived from G protein on the cell surface (by proteolysis for example). However, to test this directly we iodinated the G protein on the cell surface (5) and looked for any ^{125}I-labeled G or G-related protein(s) released into the medium. Cells were labeled with ^{125}I and lactoperoxidase, washed, and the appearance of immunoprecipitable labeled G-related proteins in the medium was examined for 4 h. We found that the cell-associated ^{125}I-labeled G protein remained essentially constant and that no ^{125}I-labeled G or G-related protein(s) were released into the medium. CTG1 cells labeled similarly did not yield any immunoprecipitable ^{125}I-labeled TG protein from either the cell or the media fractions analyzed. The results indicate that G_r does not result from proteolytic cleaveage and release of cell surface G protein into the medium (not shown).

DISCUSSION

We have described here a vector system that should prove useful for obtaining continuous stable expression of cloned cDNA in eucaryotic cells. This system employs a hybrid vector consisting of the transforming fragment of BPV (12) linked to a segment derived from the pSV2 vector (13). The pSV2 vector was designed for expression of cDNA clones under the control of the SV40 early promoter, and the BPV fragment is known to direct the replication of foreign DNA as an episome in mouse cells (20). By incorporating cDNA encoding either the VSV G protein or the TG protein into this vector and then selecting for cells transformed by it, we have been able to obtain cell lines (CG1 and CTG1) that express either normal G protein or the TG protein. The TG protein that lacks the C-terminal membrane anchor sequence is secreted from the CTG1 cells.

Using indirect immunofluorescence we have shown that G protein is produced and is transported to the cell surface in >95% of the CG1 cells. Likewise, >95% of the CTG1 cells produce the TG protein, which appears concentrated in the rough ER and is not anchored at the cell surface. We have determined by [^{35}S]methionine labeling (1-h pulse label) that TG protein represents 0.0024% of the total labeled CTG1 cell protein, while the G plus G-related proteins represent 0.0016% of the total cell protein in the CG1 cell line. In the CG1 cells G protein is processed rapidly (in <30 min) to an endo H–resistant form, a rate equivalent to that observed for normal VSV G protein in a VSV infection. The half-time for conversion of TG protein made in the CTG1 cell line to an endo H resistant form is 2–2.5 h, suggesting slow transfer to the Golgi apparatus compared to normal G protein. The half-time for secretion of this protein is also ~2.5 h, indicating rapid secretion after acquisition of endo H resistance. These characteristics of G protein and TG protein processing and transport are essentially identical to what we have observed during transient expression of these proteins in COS-1 cells under the control of the late SV40 promoter. The relatively slow processing of the TG protein suggests that it has lost an important domain required for rapid transport. This slow transport may result from random inclusion of the TG protein in vesicles budding from the rough ER and Golgi apparatus. If this is the case it seems surprising that the slow step appears to be confined only to transport from the rough ER to the Golgi apparatus.

Stable cell lines producing normal and mutant VSV glycoproteins will be especially useful for studying the structure and transport of this model transmembrane protein. The stable lines offer several experimental advantages over transient expression systems reported previously for G protein (16): (*a*) the expression does not require infection or transfection of cells before each experiment; (*b*) the cells remain "normal" during the experiment and are not killed by SV40 replication; (*a*) the level of expression is reproducible; (*d*) it should be possible to examine localization of mutant proteins by electron microscopy because virtually all cells express the protein; and (*e*) one is not limited to monkey cells, because BPV transforms mouse cell lines (12).

Although we observed stable expression of only a single species of TG protein in the CTG1 cells, the pattern of G protein expression in the CG1 cells was more complex. In addition to authentic G protein we observed two smaller G-related proteins (designated G_1 and G_2) and one G-related protein species released into the medium (designated G_r). G_1 and G_2 were found to have simple (endo H–sensitive) oligosaccharides, while on G protein the oligosaccharides were processed rapidly to the normal complex oligosaccharide (endo H–resistant) form. This suggests that this protein (G) has been processed through the Golgi apparatus. G_r protein (found in the medium) also had complex oligosaccharides, indicating that it too had been processed through the Golgi apparatus. Kinetic analyses indicated that G_1 was the intracellular precursor to the secreted G_r protein. Shortly after a pulse label the G_1 band also appears to contain the precursor (partially glycosylated) form of G protein that is rapidly processed to normal G protein.

Because we have observed only normal G protein in VSV-infected BPV-transformed cells, it seemed most likely that these "extra" G-related protein bands resulted from rearrangements in the DNA or RNA encoding them. We have analyzed the episomal DNA in both the CG1 and CTG1 cell lines and have found no rearrangements of the cDNA inserts or the 5′ and 3′ flanking sequences. These analyses have also indicated an average episome copy number of 20–30 per CTG1 cell and 30–40 per CG1 cell. The absence of DNA rearrangement suggests that aberrant splicing within the mRNA encoding the G protein could be responsible for generating the multiple G protein species. In fact, a perfect consensus donor splice sequence AGGTT (21), occurs at nucleotide 1393 in the G mRNA. If this donor sequence were spliced to the downstream splice acceptor of the small t intron, an mRNA would be produced that would encode a truncated, G-like protein lacking 55 amino acids from the COOH-terminus of the G protein (including the membrane anchor sequence) and terminating with an additional 43 amino acids specified by SV40 sequences. This protein would be just smaller than normal G protein (as are G_1 and G_r) and we would expect it to be secreted. An alternative model that G_r is derived from G protein by proteolytic cleavage (removing the membrane anchor) is difficult to reconcile with both the kinetic analysis and with the small apparent size differences between G_r and G (~10 amino acids). At least 29 amino acids would have to be removed from the COOH-terminus to result in release of a G protein (G_r) from the cell surface (unpublished results). We have accurate size markers for G proteins deleted to various extents at the COOH-terminus (16a) and therefore we believe the estimated molecular weight of the protein G_r.

Also, we were not able to detect release of iodinated G protein from the cell surface, suggesting that G_r was not derived by a cell-surface cleavage event. These data strongly suggest that G_r arises as a result of aberrant splicing of the G mRNA transcript in the CG1 cells. If this model is correct it may be possible to overcome the problem by deleting the small t intron from the vector.

Using indirect immunofluorescence and the fluorescence-activated cell sorter we have observed at least 30-fold variation in the level of G or TG proteins from cell to cell in our stable lines. Because these lines were derived from single cells cloned in soft agar the heterogeneity must develop spontaneously. This heterogeneity may result from variation in the copy number of the plasmid vector or from some variation in the cells such as the cell cycle. Although the heterogeneity develops spontaneously, it may still be possible to select high-level producer lines from the CG1 line using the fluorescence-activated cell sorter. Similar lines producing other viral glycoproteins in large quantities might be useful for the production of subunit vaccines.

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