# Cell Surface Expression of Membrane-anchored *v-sis* Gene Products: Glycosylation Is Not Required for Cell Surface Transport

**Mark Hannink and Daniel J. Donoghue**

Department of Chemistry, University of California at San Diego, La Jolla, California 92093

*Abstract.* The v-*sis* gene is able to transform cells by production of a growth factor that is structurally related to platelet-derived growth factor. This growth factor has been detected in the conditioned media of v-*sis* transformed cells, and is able to stimulate the autophosphorylation of the platelet-derived growth factor receptor. We have used the v-*sis* gene product to analyze the role of protein-encoded signals in cell surface transport. We constructed several gene fusions that encode transmembrane forms of the v-*sis* gene product. These membrane-anchored forms of the v-*sis* gene product are properly folded into a native structure, as indicated by their dimerization, glycosylation, and $NH_2$-terminal proteolytic processing. Indirect immunofluorescence demonstrated that several of these membrane-anchored gene products are transported to the cell surface. Removal of the N-linked glycosylation site from the v-*sis* gene product did not prevent cell surface transport. Several of these mutant genes are able to induce focus formation in NIH3T3 cells, providing further evidence that the membrane-anchored proteins are properly folded. These results demonstrate that N-linked glycosylation is not required for the cell surface transport of a protein that is in a native, biologically active conformation. These results provide a correlation between cell surface expression of the membrane-anchored v-*sis* gene products and transformation.

THE problem of protein sorting and transport in the cell is of major importance to cell biology. The current model postulates distinct protein-encoded signals that govern their sorting and transport (Blobel, 1980). Evidence for this model has been obtained from the analysis of the biosynthesis and transport of secretory and integral membrane proteins. These proteins are typically synthesized with a hydrophobic $NH_2$-terminal leader sequence that serves as a signal for their translocation across the membrane of the rough endoplasmic reticulum (RER).[1] Cleavage of the leader sequence and the addition of a high mannose oligosaccharide to the consensus site for N-linked glycosylation occur cotranslationally. Translocation can be halted by the action of a stop transfer sequence, typically a hydrophobic stretch of 20 or so amino acids followed by several charged residues (Yost et al., 1983). Successive utilization of leader sequences and stop transfer sequences can account for the transmembrane orientation of proteins that have multiple membrane spanning domains (Friedlander and Blobel, 1985).

The signals that direct the further sorting and transport of these proteins during their passage from the RER to their final destination are not understood. Recent evidence has suggested that N-linked glycosylation of the polypeptide chain is one signal that allows for the cell surface transport of integral membrane proteins. In one example, the G protein of vesicular stomatitis virus (VSV) was found to require glycosylation at one of its two sites in order for cell surface transport (Machamer et al., 1985). Another series of experiments demonstrated that a rat growth hormone–G hybrid protein also required the addition of an N-linked oligosaccharide for transport to the cell surface (Guan and Rose, 1984; Guan et al., 1985). However, one limitation of these studies was the inability to assay the proteins for proper folding into a native configuration. In this report we have used the v-*sis* gene product as a model system for the study of protein transport and sorting mechanism. The facile dimerization of the v-*sis* gene product and its requirement of dimerization for biological activity allow in vivo analysis of the native conformation of the polypeptide chain.

The v-*sis* oncogene was isolated as the transforming gene of simian sarcoma virus, (SSV) (Theilen et al., 1971; Devare et al., 1983). It contains an *env-sis*–fused open reading frame of 813 nucleotides, encoding a protein of 271 amino acids. This protein can be divided into four different regions on the basis of the origin of the coding sequences and on the presence of a region of homology with the amino acid sequence of platelet-derived growth factor (PDGF) (Doolittle et al., 1983; Waterfield et al., 1983; Deuel et al., 1983). The first

1. *Abbreviations used in this paper*: EGF, epidermal growth factor; Endo H, endoglycosidase H; MuLV, murine leukemia virus; PDGF, platelet-derived growth factor; RER, rough endoplasmic reticulum; SSV, simian sarcoma virus; VSV, vesicular stomatitis virus.

region includes residues 1–51, which are encoded by the *env*-derived portion of the open reading frame, and contains the signal sequence of the *env* gene product from simian sarcoma-associated virus. The second region extends from residue 52 to a basic dipeptide Lys-Arg at residues 110–111. A high mannose oligosaccharide is added to Asn residue 93 during the translocation of the nascent polypeptide chain across the membrane of the RER (Robbins et al., 1985; Hannink and Donoghue, 1986) The third region includes residues 112–220, and is 96% identical to the amino acid sequence of the B chain of human PDGF (Johnsson et al., 1984). A total of eight cysteine residues are found within this region, and dimerization is required for the biological activity of both the v-*sis* gene product and PDGF (Owen et al., 1984; King et al., 1985; Hannink et al., 1986). The minimal transforming region of the v-*sis* gene product is located within this region, consisting of 88 amino acids from residue 127 to 214 (Sauer et al., 1986). The fourth region of the v-*sis* gene product consists of the COOH-terminal 51 residues, 221–271. These are conserved with respect to the predicted amino acid sequence of the human c-*sis* gene product, but are not found in the amino acid sequence of PDGF (Josephs et al., 1984).

In this study we have constructed mutants of the v-*sis* gene in which a coding region for a membrane anchor domain is fused to the v-*sis* gene. These gene fusions result in the conversion of the v-*sis* gene product from a secretory protein to an integral membrane protein. Our results demonstrate that the addition of a COOH-terminal transmembrane domain to a secretory protein does not interfere with proper folding of the polypeptide chain. Furthermore, a membrane-anchored v-*sis* gene product is transported to the cell surface in the absence of N-linked glycosylation. Finally, these results suggest that the membrane-anchored v-*sis* gene products must be transported to the cell surface in order to manifest their transforming potential.

## Materials and Methods

### Construction of the v-sis-G Fusions

The v-*sis* gene was cloned into a pBR322 derivative, pMH100, in which the v-*sis* coding sequences have a unique XhoI site at their 3′ end. This plasmid was digested with BstEII, and the 5′ overhang was filled in with the Klenow fragment of *Escherichia coli* DNA polymerase I and the four deoxynucleoside triphosphates. The plasmid was then recut with XhoI, and the large fragment containing the bacterial vector sequences and the 5′ end of the v-*sis* gene was isolated. An AluI to XhoI fragment, containing the coding sequences for the COOH-terminal 49 amino acids of the G protein, was isolated from pSVGL (Rose and Bergmann, 1982) and ligated into the v-*sis* fragment. The blunt-end ligation between the filled in BstEII site and the AluI site restores the BstEII site, and maintains the correct reading frame across the junction. The resulting plasmid, pMH107, encoded the v-$sis^{239}$-G gene. The v-$sis^{239}$-$G^{IR}$ gene was constructed in a similar fashion, except that the AluI to XhoI fragment was from $pSVGL^{IR}$ (Adams and Rose, 1985).

In order to construct the v-$sis^{223}$-G gene, pMH100 was digested with SmaI and XhoI, followed by ligation of the AluI to XhoI fragment from pSVGL. To construct the v-$sis^{239}$-$G^{338}$ gene, pSVGL was digested with KpnI, followed by treatment with Klenow fragment and the deoxynucleoside triphosphates to remove the 3′ overhang. The plasmid was recut with XhoI, and the KpnI to XhoI fragment containing the 3′ end of the G gene was isolated. This fragment was ligated into a filled-in BstEII to XhoI fragment containing the 5′ end of the v-*sis* gene, generating pMH54. The v-$sis^{127-239}$-G gene was constructed by the ligation of a SalI to BstEII fragment containing the 5′ portion of the v-$sis^{127}$ gene into a SalI to BstEII fragment from pMH107, containing the 3′ end of the G gene and bacterial vector sequences. Finally, the v-$sis^{239NS}$-G gene was constructed by the swapping of the SstI to BstEII fragment of the v-$sis^{NS}$ gene for the corresponding fragment of the v-$sis^{239}$-G gene. The respective v-*sis*-G genes were inserted into a murine leukemia virus (MuLV)-derived expression vector to allow for their expression in NIH3T3 cells as previously described (Hannink and Donoghue, 1984), and were inserted into pJC119 (Sprague et al., 1983) to provide for their expression in COS-1 cells.

### Focus Assays and Viral Titers

The plasmid DNAs were introduced into NIH3T3 cells using the calcium phosphate coprecipitation method. The transfections were carried out in the presence of p836, a replication-competent DNA clone of MuLV (Hoffman et al., 1982). Typically, 1 μg of the plasmid DNA, 0.5 μg of p836, and 20 μg of sheared calf thymus DNA was precipitated with EtOH. The precipitate was resuspended in 400 μl of 2 × Hepes (50 mM Hepes, 280 mM NaCl, 1 mM sodium phosphate, pH 7.0), and bubbled into 400 μl of 0.25 M $CaCl_2$. The calcium phosphate precipitate was allowed to form for 30–45 min at room temperature before being added to NIH3T3 cells grown on 60-mm dishes. The precipitate was left on the cells overnight and the next day the cells were refed with DME plus 10% calf serum. The cells were then split onto 4 × 10-cm dishes, and allowed to become confluent. The cells were refed every 2–3 d with DME plus 10% calf serum, and scored for the presence of foci after 7–10 d. The conditioned media from the focus assays was collected and assayed for the presence of focus-forming virus. Serial dilutions of the conditioned media were added to NIH3T3 cells in 60-mm dishes in the presence of 2 μg/ml of polybrene. The virus was allowed to adsorb for 1 h, then the cells were washed and refed. The cells were allowed to become confluent and scored for the presence of foci after 5–7 d.

### COS-1 Transfections, Labelings, and Immunoprecipitations

COS-1 cells (Gluzman, 1981) were transfected using the DEAE-Dextran method basically as described (Hannink et al., 1986). 60-mm dishes containing COS-1 cells at 70–80% confluency were washed twice with Tris-saline (10 mM Tris 7.4, 150 mM NaCl). A solution of the DNA in Tris-saline containing DEAE-dextran (2 × $10^6$ molecular mass) at 500 μg/ml was added to the cells. The cells were kept at 37° for 15–20 min before aspirating off the DEAE-dextran solution. A 10% solution of DMSO in Tris-saline was added to the plates for 1 min, followed by two washes with Tris-saline. The cells were refed with DME plus 10% calf serum containing 100 μM chloroquine. After 2 h the chloroquine was removed and the cells were allowed to grow for 48 h.

The cells were labeled with 100 μCi each of [$^{35}$S]cysteine and [$^{35}$S]methionine in DME lacking cysteine and methionine. The usual labeling period was 2 h, except for the pulse-chase experiment in which a 30-min labeling period was used before the chase with DME plus 10% calf serum. The cells were washed three times in Tris-saline and lysed in 0.5 ml of Ripa buffer (10 mM sodium phosphate, pH 7.0, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1% Trasylol). Cellular debris was pelleted and the lysate was aliquoted for immunoprecipitation. The immunoprecipitate was allowed to form for 1 h on ice, and collected using fixed *Staphylococcus aureus* bacteria. The pellet was washed three times with Ripa before resuspending in 1 × sample buffer (50 mM Tris, pH 6.8, 2% SDS, 20% B-mercaptoethanol, 10% glycerol). For samples to be analyzed under nonreducing conditions, the B-mercaptoethanol was omitted from the sample buffer. The immunoprecipitates were boiled for 2 min before electrophoresis on 15% SDS-polyacrylamide gels. The gels were processed for fluorography to visualize the radioactive proteins. For endoglycosidase H (Endo H) digestions, the immunoprecipitates were resuspended in 50 mM sodium phosphate, pH 6.1, 25 mM EDTA, 0.5% Nonidet P-40, 0.5% B-mercaptoethanol, and boiled for 1 min. 10 mU of Endo H (Boehringer Mannheim Biochemicals, Indianapolis, IN) was added and the reaction was allowed to proceed for 16–18 h before quenching in 2 × sample buffer and analysis by SDS PAGE. Tunicamycin treatment was done for a period of 2 h before the labeling period at a concentration of 2 μg/ml. Tunicamycin was included in the labeling media during the 2-h labeling period.

### Immunofluorescence

COS-1 cells were grown on coverslips and transfected as described above. 48 h after transfection, the cells were fixed in 3% paraformaldehyde in phosphate-buffered saline. For external staining, the cells were reacted with a 1:100 dilution of rabbit antisera against the bacterially synthesized v-*sis* gene product, followed by a commercial rhodamine-conjugated goat anti-rabbit IgG. The cells were then permeabilized with Hepes-buffered saline containing 1% Triton X-100 and incubated with a 1:200 dilution of a mouse

monoclonal raised against a peptide corresponding to residues 496–511 of the G protein (a gift of Tom Kreis, European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany). Biotinylated goat anti-mouse IgG was added, followed by fluorescein-labeled streptavidin. The coverslips were then mounted in 90% glycerol–10% 1 M Tris 8.0. NIH3T3 cells were infected at a multiplicity of infection greater than one, and plated onto coverslips the next day. The cells were processed for immunofluorescence as described above 2 d after infection.

## Results

### Construction of Membrane-Anchor Mutants of the v-sis Gene

To alter the cytological location of the v-*sis* gene product from a secretory protein to a membrane bound protein, we have constructed several gene fusions between the v-*sis* gene and the G gene of VSV (Fig. 1). Our first concern was to characterize the dimer formation and biological activity of the v-*sis* gene product when anchored in the membrane. The minimal size of the biologically active v-*sis* gene product is 88 amino acids, located within the PDGF-related portion of the v-*sis* gene product from residues 127 to 214 (Sauer et al., 1986). This truncated form of the v-*sis* gene product, when expressed with an $NH_2$-terminal signal sequence to direct its translocation across the membrane of the RER, is fully active in transformation. This protein contains 8 of the 10 cysteine residues found in the v-$sis^{wt}$ gene product, and exists as a dimer when analyzed under nonreducing conditions. Further truncations of the v-*sis* gene product, at either its COOH terminus or $NH_2$ terminus, result in loss of biological activity and failure to dimerize. This minimal transforming region of the v-*sis* gene was included in all of the fusions we constructed in order to ensure that the resultant gene product encoded sufficient v-*sis* sequences for dimerization and transformation.

Second, we wanted to be able to alter the transport of the membrane-anchored v-*sis* protein such that transport to the cell surface would be blocked. It has been proposed that glycosylation of transmembrane proteins is an important signal for their transport to the cell surface (Guan et al., 1985). The v-$sis^{wt}$ gene encodes a single site for N-linked glycosylation at residues 93–95. We therefore used a mutant of the v-*sis* gene in which residues 58–126 have been deleted. This $NH_2$-terminal mutant of the v-*sis* gene retains full biological activity although it lacks the site for N-linked glycosylation (Sauer et al., 1986). This mutant v-*sis* gene was also fused to the transmembrane domain of G, with the expectation that its gene product would remain localized to an intracellular organelle, and fail to be transported to the cell surface. Since alterations in the transmembrane and cytoplasmic tail of the G protein have been shown to affect its transport, we also constructed a gene fusion between the v-*sis* gene and a mutant G gene, in which an arginine for isoleucine substitution was introduced into the hydrophobic transmembrane domain (Adams and Rose, 1985). This mutant form of the G protein was shown to be a transmembrane protein, but its transport to the cell surface is blocked at some point in the Golgi apparatus.

A total of six different gene fusions were constructed, as shown in Fig. 1. Four of these constructs differ in the v-*sis*–derived sequences present in the gene fusion, and three encode altered transmembrane domains. The v-$sis^{239}$-G gene is the prototype of these gene fusions, containing a coding

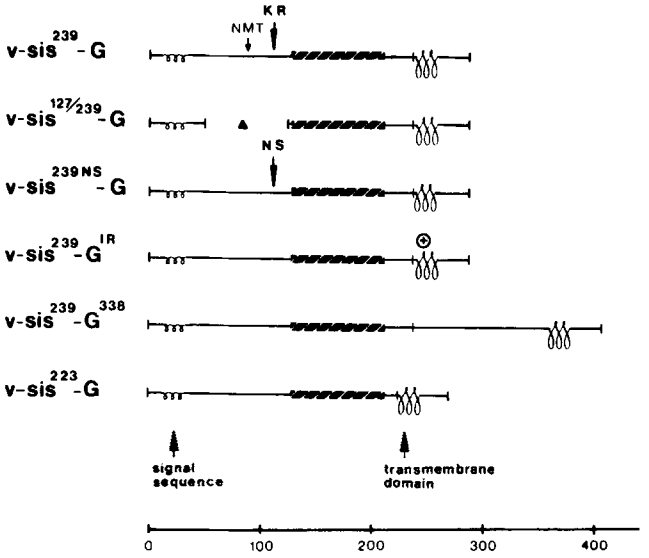



*Figure 1.* Construction of the v-*sis*-G gene fusions. The six different gene fusions that were constructed are shown. The signal sequence of the v-*sis* gene and the transmembrane domain of the G protein are indicated by small and large curliques, respectively. The minimal transforming region of the v-*sis* gene is indicated by the cross-hatched box. The sites for N-linked glycosylation (N-M-T) and for proteolytic processing (K-R) are indicated above the v-$sis^{239}$-G fusion. These sites are present in the other fusions also, except as indicated by deletion in the v-$sis^{127-239}$-G gene and by substitution in the v-$sis^{239NS}$-G gene. The superscripted number indicates the amino acid codon in the respective gene at which the fusion was made. The scale is indicated on the bottom of the figure.

region for 239 amino acids of the v-*sis* gene product fused in frame to the coding region for the 49 COOH-terminal amino acids of the VSV G gene. The v-$sis^{127-239}$-G gene fusion encodes a protein that lacks residues 58–126 of the v-*sis* gene product. The v-$sis^{239NS}$-G gene fusion encodes an Asn-Ser substitution for the Lys-Arg processing site at residues 110–111. This results in the loss of the processing site and the addition of a new site for N-linked glycosylation. The parental v-$sis^{NS}$ gene is also fully biologically active in transformation, in spite of the addition of a new N-linked oligosaccharide to its gene product at residue 110. (Hannink and Donoghue, 1986). The v-$sis^{223}$-G gene encodes the identical transmembrane domain fused into the v-*sis* gene after the codon for amino acid 223. Two other v-*sis*-G fusions were constructed with altered anchor domains. The v-$sis^{239}$-$G^{IR}$ gene encodes the COOH-terminal 49 amino acids from the mutant $G^{IR}$ protein containing an isoleucine to arginine substitution in the hydrophobic region. The v-$sis^{239}$-$G^{338}$ gene contains the first 239 codons of the v-*sis* gene fused to the COOH-terminal coding region of the G gene at codon 338, resulting in the addition of the COOH-terminal 117 amino acids of the G protein to the v-*sis* gene product.

### Dimerization of the Transmembrane v-sis Gene Product

We first wished to determine if the v-*sis*-G gene fusions encoded proteins that were capable of dimerization. For these experiments we used a transient expression assay in COS-1 cells, similar to that used by other investigators to study signals involved in protein processing and transport (Rose and Bergmann, 1982; Warren and Shields, 1985). This system uses an expression vector in which the desired gene is ex-

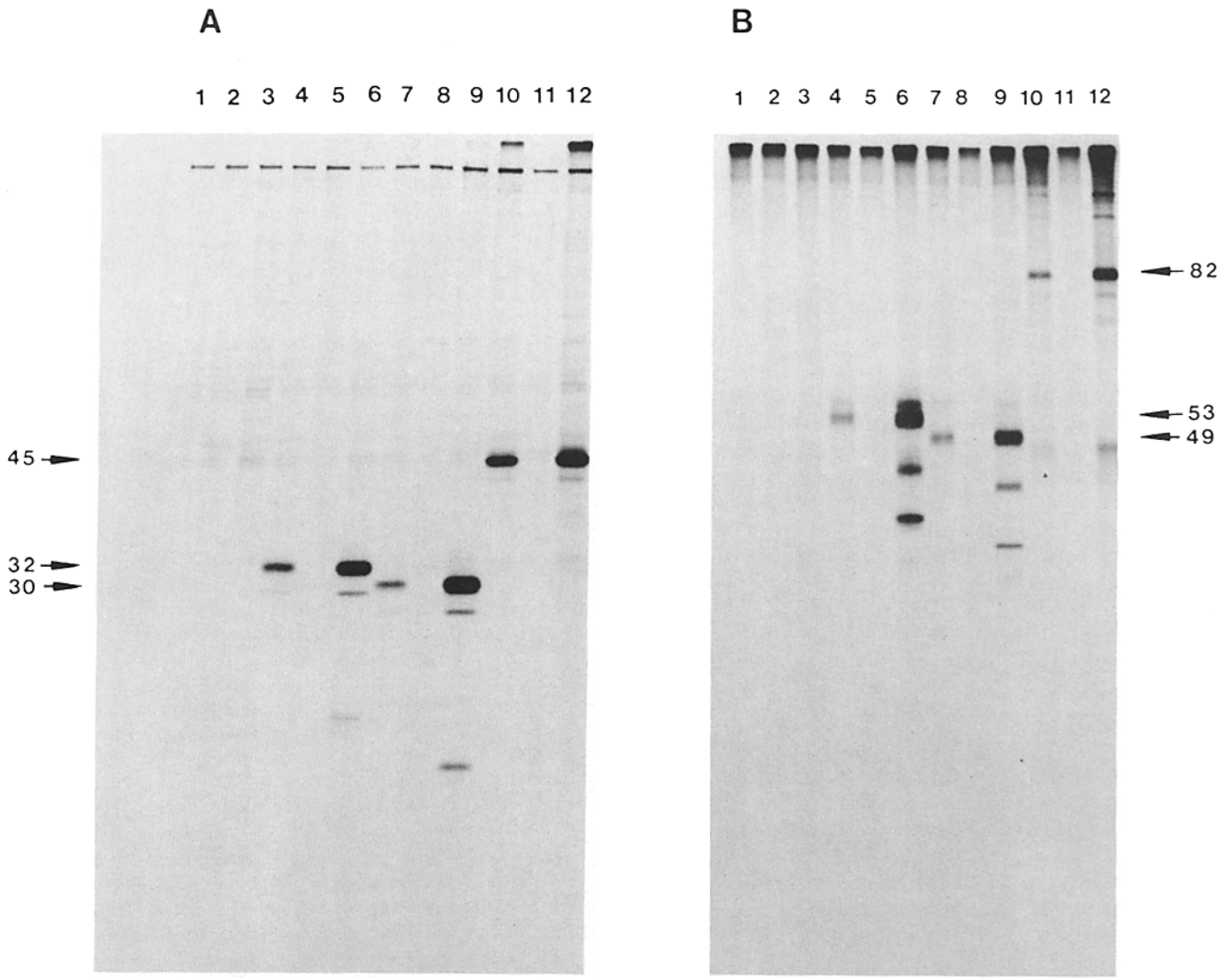



*Figure 2.* Dimerization of the v-*sis*-G gene products. COS-1 cells were transfected with the following v-*sis*-G genes inserted in pJC119, an SV40 expression vector. Lanes *1–3* are from mock transfected cells; lanes *4–6* are from cells transfected with the v-$sis^{239}$-G fusion; lanes *7–9* are from cells transfected with the v-$sis^{223}$-G fusion; and lanes *10–12* are from cells transfected with the v-$sis^{239}$-$G^{338}$ fusion. Cell lysates were immunoprecipitated with antisera against the $NH_2$terminus of the v-*sis* gene product, shown in lanes *1*, *4*, *7*, and *10*; or with $NH_2$-terminal sera that was blocked with preincubation of peptide, lanes *2*, *5*, *8*, and *11*; or with antisera against the COOH terminus of the G protein, lanes *3*, *6*, *9*, and *12*. The immunoprecipitates were analyzed by SDS PAGE under reducing (*A*) and nonreducing (*B*) conditions, and the gel was fluorographed to visualize the labeled proteins. The arrows indicate the unprocessed immunoprecipitated proteins of 32, 30, and 45 kD in *A*. In *B*, the arrows indicate the v-*sis*-G dimers of 53, 49, and 82 kD. Molecular weights were determined by comparison with commercially available protein standards.

pressed from the SV40 late promoter (Sprague et al., 1983). Three of the fusion genes were introduced into COS-1 cells and radiolabeled cell lysates were immunoprecipitated with antipeptide antiserum directed against the $NH_2$ terminus of the v-*sis* gene product, or with antipeptide antiserum directed against the COOH terminus of the G protein. The v-$sis^{239}$-G gene was found to encode a protein of 32 kD that was specifically recognized by both antisera, and whose immunoprecipitation was blocked when the $NH_2$-terminal serum was incubated with an excess of peptide before immunoprecipitation (Fig. 2 *A*, lanes *4–6*). The v-$sis^{223}$-G and the v-$sis^{239}$-$G^{338}$ genes were also found to encode the expected proteins of 30 and 45 kD (Fig. 2 *A*, lanes *7–12*). Smaller proteins were also immunoprecipitated by the anti-G serum from the lysates (Fig. 2 A, lanes *6*, *9*, and *12*). These proteins arise from proteolytic processing at residues 110–111 of the v-*sis* gene product. When the identical immunoprecipitates were analyzed under nonreducing conditions, proteins with a molecular mass consistent with dimerization were found (Fig. 2 *B*). The other v-*sis*-G gene products were also found to exist as dimers when analyzed under nonreducing conditions (data not shown). This result indicates that the v-*sis*-G proteins do dimerize, in spite of the addition of a transmembrane domain, and therefore are potential transforming gene products.

### *Glycosylation of the Transmembrane v-sis Gene Product*

The v-*sis* gene encodes a single site for the addition of an N-linked oligosaccharide, an Asn-Met-Thr sequence located at residues 93–95. Previous work has demonstrated

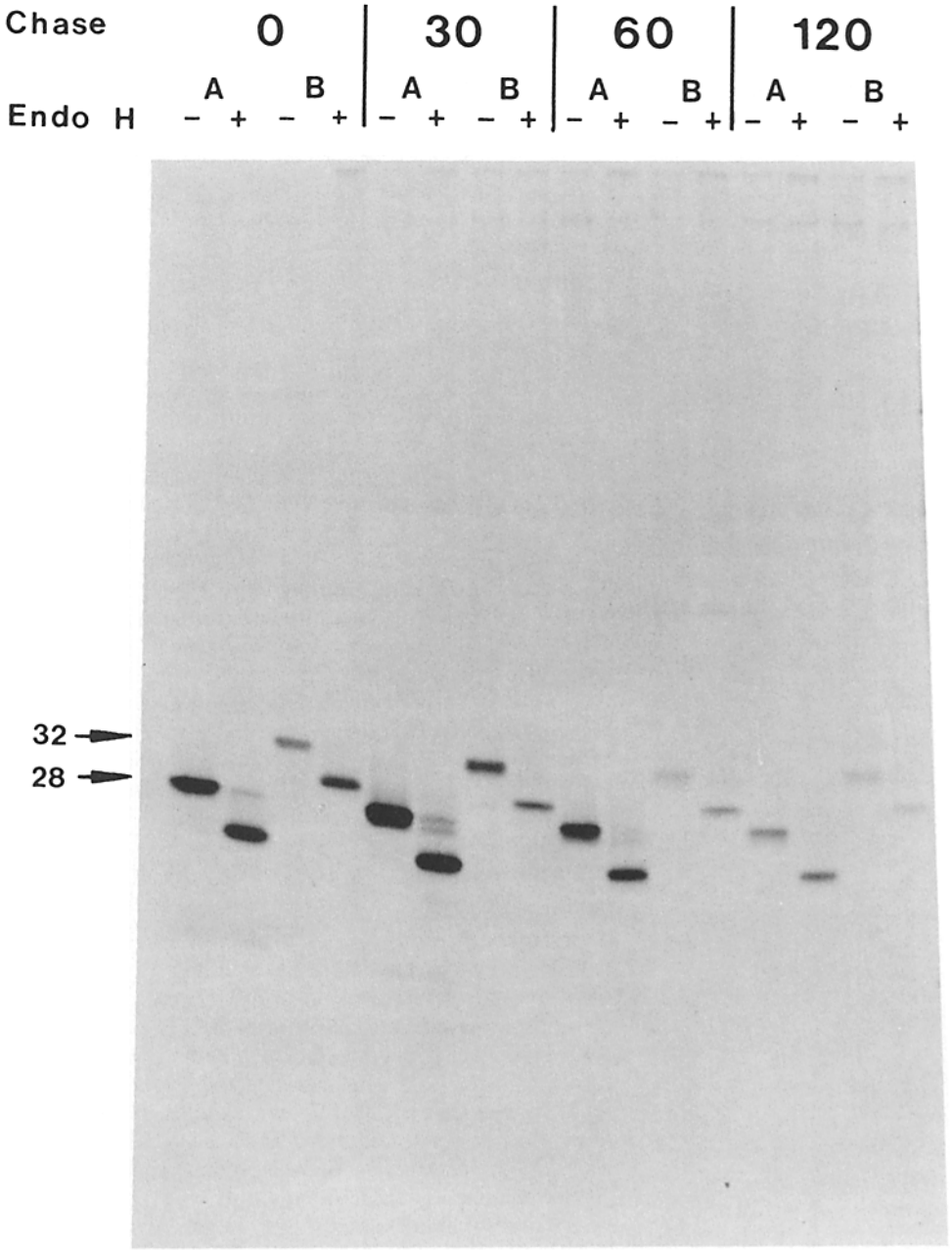



*Figure 3.* Glycosylation of the v-*sis*-G gene product. COS-1 cells were transfected with the v-$sis^{239}$ gene (*A*) or the v-$sis^{239}$-G gene (*B*) in the SV40 expression vector. The cells were labeled for 30 min, then chased with unlabeled media for the indicated times. Immunoprecipitates were prepared and treated (+) or not treated (−) with Endo H. The immunoprecipitates were then analyzed under reducing SDS PAGE conditions, and fluorography was performed to visualize the labeled proteins. The arrows indicate the v-$sis^{239}$ protein of 28 kD and the v-$sis^{239}$-G protein of 32 kD (before Endo H treatment).

that the v-*sis* gene product is modified by the addition of a high mannose oligosaccharide during its translocation across the membrane of the RER. To investigate the modification of the transmembrane forms of the v-*sis* gene product by glycosylation, a pulse-chase experiment was performed in which the sensitivity of the oligosaccharide to Endo H was examined. The transfected COS-1 cells were labeled for 30 min, then the radiolabel was chased for periods of 30, 60, and 120 min. The v-$sis^{239}$-G gene product remained sensitive to Endo H during the labeling period and the chase (Fig. 3, lane *B*), as did the v-$sis^{239}$ gene (Fig. 3, lane *A*). The pattern of glycosylation of the other v-*sis*-G gene products was similar. The v-$sis^{239NS}$-G gene, containing two sites for N-linked glycosylation, was also found to remain sensitive to Endo H during a 2-h chase (data not shown).

To confirm the absence of any N-linked oligosaccharides on the v-$sis^{127-239}$-G protein, COS-1 cells expressing the fusion genes were treated with tunicamycin before and during the labeling period. The inhibition of N-linked oligosaccharide formation resulted in a shift of the v-$sis^{223}$-G and the v-$sis^{239}$-$G^{338}$ proteins to a lower molecular mass form (Fig. 4, lanes *1–4*), while no difference in the v-$sis^{127-239}$-G protein was detected (Fig. 4, lanes *5* and *6*).

### *Cellular Localization of the Transmembrane v-sis Gene Product*

To determine the cellular localization of the v-*sis*-G gene products, double-label immunofluorescence experiments were performed. In these experiments, transfected COS-1 cells were fixed, then incubated with anti-*sis* serum followed by rhodamine-labeled conjugate before permeabilization.

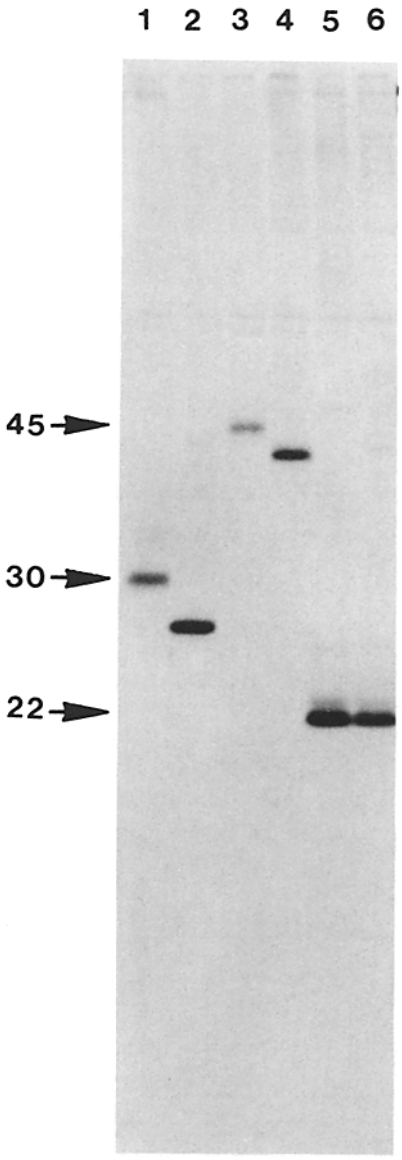



*Figure 4.* Tunicamycin treatment of the v-*sis*-G gene products. COS-1 cells were transfected with the v-*sis*-G gene in the SV40 expression vector. The cells were treated (lanes *2*, *4*, and *6*), or not treated (lanes *1*, *3*, and *5*) with tunicamycin at a concentration of 2 μg/ml for 2 h before and during the labeling period. Cell lysates were immunoprecipitated with the anti-*sis* serum and the immunoprecipitates were analyzed under reducing SDS PAGE conditions, followed by fluorography to visualize the labeled proteins. The v-*sis*-G genes used are as follows: the v-$sis^{223}$-G gene (lanes *1* and *2*); the v-$sis^{239}$-$G^{338}$ gene (lanes *3* and *4*); and the v-$sis^{127\text{-}239}$-G gene (lanes *5* and *6*). The arrows indicate the glycosylated proteins of 30 and 45 kD that are encoded by the v-$sis^{223}$-G and the v-$sis^{239}$-$G^{338}$ genes, and the nonglycosylated protein of 22 kD encoded by the v-$sis^{127\text{-}239}$-G gene.

After permeabilization, the cells were incubated with the anti-G peptide serum, followed by fluorescein-labeled conjugate. The v-$sis^{239}$-G gene product was found to be transported to the cell surface (Fig. 5, *C* and *D*), as was the v-$sis^{223}$-G gene product (data not shown). The v-$sis^{127\text{-}239}$-G gene product was also found to be transported to the cell surface in spite of its lack of N-linked glycosylation (Fig. 5, *E* and *F*). The intensity of cell surface staining with the anti-*sis* serum was variable in these cases, indicating that not all of the cells transported the v-*sis*-G gene products equally. However, in the case of the v-$sis^{239NS}$-G gene product, (Fig. 5, *G* and *H*), little cell to cell variation was observed, and the intensity of cell surface staining was consistently greater. Cell surface staining of cells expressing the v-$sis^{239}$-$G^{IR}$ gene (Fig. 5, *I* and *J*), and the v-$sis^{239}$-$G^{338}$ gene (data not shown) was not found.

The cell surface transport of these proteins in NIH3T3 cells was also examined by double-label indirect immunofluorescence. Acutely infected NIH3T3 cells were found to express the v-$sis^{239NS}$-G protein on the cell surface (Fig. 6) when stained with anti-*sis* serum. NIH3T3 cells infected with the other constructs used in this study were not found to express detectable levels of the *sis* protein at the cell surface, although internal staining with either the anti-*sis* or the anti-G sera displayed a similar Golgi staining (data not shown). These results are in agreement with the immunofluorescence experiments in COS-1 cells, where the v-$sis^{239NS}$-G protein was found to have the highest level of cell surface expression. Lower levels of protein expression in the infected NIH3T3 cells as compared with the transient expression assays in COS-1 cells is the most likely explanation for the inability to detect cell surface expression of the other *sis*-G proteins in NIH3T3 cells. Alternatively, the v-$sis^{239NS}$-G protein may have a slower rate of turnover or internalization than the other fusion proteins, thereby increasing the level of detectable cell surface protein.

### *Transforming Activity of the Transmembrane v-sis Gene Product*

To assay the fusion genes for their biological activity, the respective fusion genes were inserted into a retroviral vector to provide for expression in NIH3T3 cells. The resultant plasmids were introduced into NIH3T3 cells using the calcium phosphate coprecipitation method. Since the expression vector is replication-defective, a DNA clone of replication-competent MuLV was included in the transfections to allow for viral spread and rescue. The v-$sis^{239}$-G, v-$sis^{239NS}$-G, and the v-$sis^{127\text{-}239}$-G genes were found to have comparable biological activities only slightly lower than v-$sis^{wt}$ gene. (Table I). The v-$sis^{223}$-G gene and the v-$sis^{239}$-$G^{IR}$ gene were found to have further reduced levels of focus formation, while the v-$sis^{239}$-$G^{338}$ gene was found to be biologically inactive. In all cases the conditioned media of the focus assays was found to contain rescuable focus-forming virus at levels consistent with the biological activities of the respective genes.

## *Discussion*

We have constructed a series of gene fusions between the v-*sis* gene and the G gene of VSV. The gene products encoded by these v-*sis*-G fusions result in the conversion of the v-*sis* gene product from a secretory protein to an integral membrane protein. The membrane-anchored v-*sis*-G gene products exist as dimers when analyzed under nonreducing conditions. They are proteolytically processed and glycosylated in the same manner as the v-$sis^{wt}$ gene. By these criteria, the membrane-anchored v-*sis* gene products are properly folded into a native conformation. Certain of the

membrane-bound forms of the v-*sis* gene product, including one that lacks the site for N-linked glycosylation, were transported to the cell surface. Differences among the gene fusions were also found in their ability to induce focus formation in NIH3T3 cells.

### *Implications for Protein Transport*

An important difference between these results and the results of previous experiments that examined protein-encoded signals for cell surface transport is the lack of a requirement of glycosylation for cell surface transport. This is demonstrated by the fact that the nonglycosylated v-$sis^{127\text{-}239}$-G gene product was transported to the cell surface equally as well as the glycosylated v-$sis^{239}$-G gene product. One explanation for this difference may be the importance of the native structure of the protein for proper transport. Initial evidence for the role of native protein structure in protein transport came from the analysis of *ts* mutants of the G protein, in which the defect responsible for protein transport has been shown to be a single amino acid change of phenylalanine to serine at residue 204 (Gallione and Rose, 1985). In this case, the possible denaturation of the protein at the restrictive temperature was invoked as an explanation for its defect in transport. In this study, the membrane-bound v-*sis*-G proteins were found to possess a native structure, as judged by both their capacity to dimerize and by their ability to induce focus formation in NIH3T3 cells. A limitation of other studies, which have implicated glycosylation as a determinant for cell surface transport, was the inability to assay the hybrid proteins for proper folding into a native conformation. Our results suggest that it is not glycosylation per se, but rather the effect of glycosylation on protein structure that is important for cell surface transport. The importance of glycosylation for the formation and maintenance of the native structure of proteins needs to be investigated further.

The importance of the nature of the oligosaccharide for proper transport is not known. It has been demonstrated with specific glucosidase I inhibitors that cell surface transport of the v-*fms* protein can be blocked within the RER/Golgi compartment. (Nichols et al., 1985). Therefore, failure to process the high mannose oligosaccharide that is added during translocation to a specific complex type can block further transport of the protein. It is not known if the high mannose oligosaccharide of the v-*sis* gene product is subject to the action of glucosidase I upon transit from the RER to the Golgi. However, the oligosaccharide that is added to the v-$sis^{wt}$ or the v-*sis*-G proteins is not modified to the complex type during a 2-h chase period. Yet these proteins are transported to the cell surface, demonstrating that modification of the high mannose oligosaccharide to the complex type is not a general requirement for cell surface transport. The differences regarding the importance of oligosaccharide modification for transport suggest that there are multiple pathways of transport through the Golgi. The nature of the discrimination among proteins for these various pathways is not known at this time, but is clearly of importance in the understanding of protein-sorting mechanisms.

### *Implications for Transformation by v-sis*

The v-*sis* gene product is able to transform cells by virtue of its ability to activate the PDGF receptor in an autocrine fashion (Huang et al., 1984). Cells transformed by the v-*sis* gene secrete a mitogenic factor into the media; this protein has been identified as the 20-kD protein resulting from $NH_2$-terminal processing (Garret et al., 1984). This secreted form has been shown to cause autophosphorylation of the PDGF receptor (Owen et al., 1984; Garret et al., 1984). Since exogenous PDGF is not sufficient for transformation (Assoian et al., 1984), it is not clear how the endogenous synthesis of a PDGF-related growth factor leads to transformation. The simplest explanation is that chronic stimulation of the PDGF receptor by the v-*sis* gene product results in transformation. Antibodies to PDGF have been shown to inhibit transformation by SSV, suggesting that a secreted or a cell surface-associated v-*sis* gene product is responsible for transformation (Johnsson et al., 1985). An alternative possibility is that the site of interaction of the v-*sis* gene product with the PDGF receptor may be important (Huang et al., 1984; Heldin and Westemark, 1984). When PDGF binds to the PDGF receptor and stimulates its tyrosine kinase-associated activity, it does so on the outside of the cell. In contrast, the v-*sis* gene product can potentially interact with the PDGF receptor inside the cell, during transport through the Golgi and secretory vesicles. In support of this model, an SSV-transformed cell line that does not secrete a mitogenic factor into the media, and whose growth is not affected by the addition of anti-PDGF IgG, is still able to form tumors in nude mice. The tumor size of different SSV-transformed cell lines did correlate with the level of secreted mitogen (Huang et al., 1984). Therefore, at least a portion of the biological activity of the v-*sis* gene product may be mediated by an intracellular form.

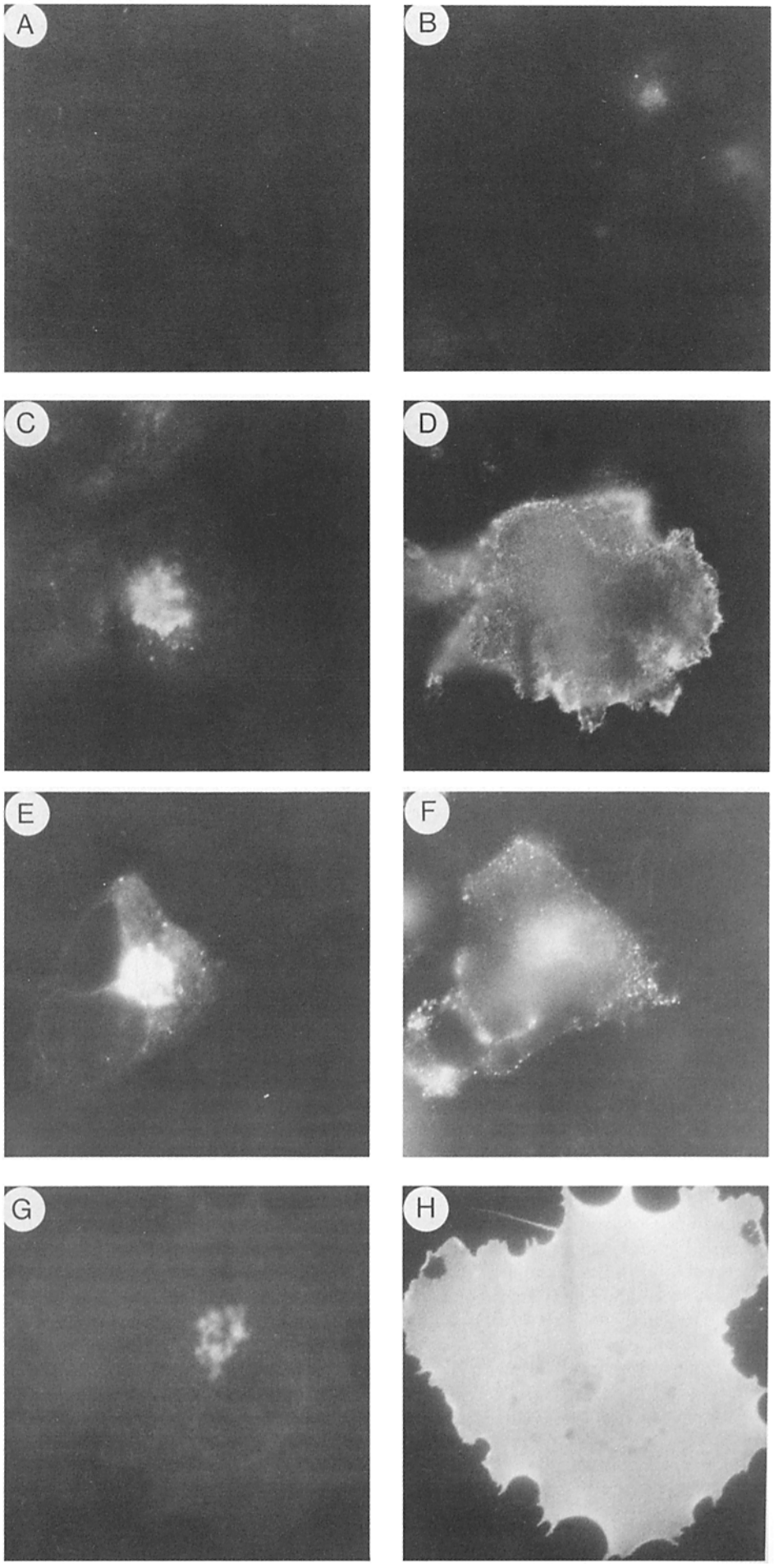
In the results presented here, we have found differences in both the biological activities of the v-*sis*-G genes and in the level of cell surface transport of their respective proteins. In particular, the v-$sis^{239}$-G gene and others with identical COOH-terminal coding regions were found to have similar levels of biological activity. Their proteins were all found to be transported to the cell surface. This is in contrast to the absence of detectable cell surface transport of the v-$sis^{239}$-$G^{IR}$ and the v-$sis^{239}$-$G^{338}$ proteins. The genes encoding these latter proteins were also found to have reduced or nonexistent levels of focus-forming activity. This correlation suggests that cell surface expression of the membrane-anchored v-*sis* gene products is required for transformation. The v-$sis^{223}$-G

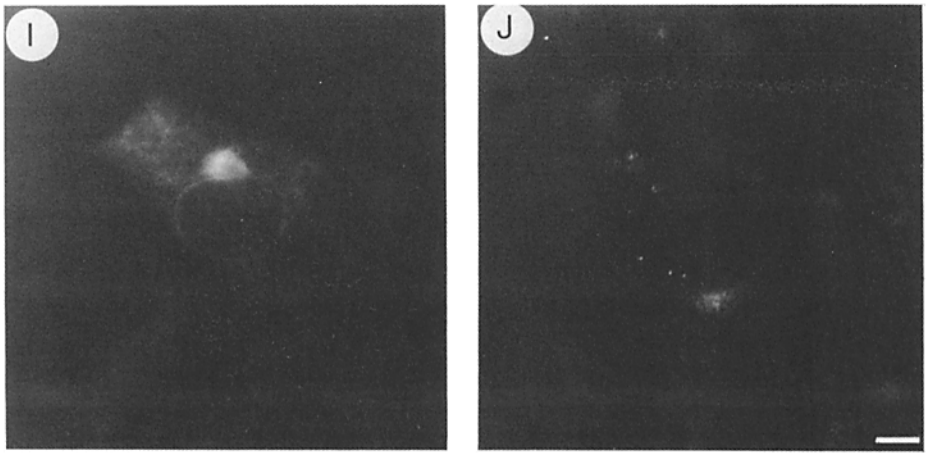
*Table I. Biological Activities of the v-sis-G Fusion Genes*

| Gene fusion | Focus assay | Titer |
|---|---|---|
| | *FFU/pmol* | *FFU/ml* |
| v-$sis^{234}$ | $1.0 \times 10^4$ | $3.5 \times 10^6$ |
| v-$sis^{239}$-G | $4.7 \times 10^3$ | $1.0 \times 10^5$ |
| v-$sis^{127\text{-}239}$-G | $3.7 \times 10^3$ | $1.1 \times 10^5$ |
| v-$sis^{239NS}$-G | $4.8 \times 10^3$ | $1.5 \times 10^5$ |
| v-$sis^{223}$-G | $5.0 \times 10^2$ | $3.0 \times 10^3$ |
| v-$sis^{239}$-$G^{IR}$ | $4.1 \times 10^2$ | $2.0 \times 10^3$ |
| v-$sis^{239}$-$G^{338}$ | $<1.0 \times 10^1$ | $<5.0 \times 10^1$ |

The v-*sis*-G genes were inserted into a MuLV-derived retroviral vector and introduced into NIH3T3 cells using the calcium phosphate coprecipitation technique. The biological activities of the respective v-*sis*-G fusion genes are given in focus-forming units per picomole (FFU/pmole) of transfected plasmid DNA. The levels of rescuable focus-forming virus that was detected in the conditioned media of the focus assays are given in focus-forming units per milliliter (FFU/ml) of conditioned media. The v-$sis^{234}$ gene was included in these assays as a representative deletion mutant (Hannink et al., 1986).

*Figure 5.* Indirect immunofluorescence of the v-*sis*-G gene products. COS-1 cells were transfected with the following v-*sis*-G genes in the SV40 expression vector and processed for immunofluorescence as described in Materials and Methods. *A* and *B* show the inside and outside staining, respectively, of mock-transfected cells; *C* and *D* are the inside and outside staining of cells transfected with the v-$sis^{239}$-G gene; *E* and *F* are the inside and outside staining of cells transfected with the v-$sis^{127\text{-}239}$-G gene; *G* and *H* are the inside and outside staining from cells transfected with the v-$sis^{239NS}$-G gene; *I* and *J* (*right page*) are the inside and outside staining of cells transfected with the v-$sis^{239}$-$G^{IR}$ gene. An automatic exposure setting of 3,200 was used for the inside staining, and a setting of 1,600 was used for the outside staining. Bar in J, 10 μm.






protein is transported efficiently to the cell surface, yet also has a reduced level of focus formation. The reason for this is not clear. This protein has a shorter distance between the minimal transforming region and the membrane anchor domain than the v-*sis*$^{239}$-G proteins. This may affect either the ability of the v-*sis*$^{223}$-G protein to interact directly with the PDGF receptor or the release of a PDGF-related protein from the membrane-anchored protein.

Since transformation by the v-*sis* gene product is mediated through autocrine stimulation of the PDGF-receptor, one interpretation of our results is that a transforming interaction can occur between two integral membrane proteins. The principal objection to this interpretation is the possibility that the transmembrane form of the v-*sis*-G gene product is not biologically active, but that a small amount of a PDGF-related protein is released into the extracellular matrix instead, allowing its interaction with the receptor and consequent transformation.

A form of the G protein, $G_s$, which is secreted from VSV-infected cells, has been identified. This protein has been shown to be formed during or shortly after translation both in vivo and in vitro, and its production in vitro was not affected by the presence of protease inhibitors. These results suggest that the $G_s$ protein arises from an altered viral mRNA specifically encoding $G_s$, although such an altered mRNA has not been detected in VSV-infected cells. Alternatively, this secreted form may arise from a protein that lacks the transmembrane domain as a result of a reading frameshift of the G mRNA (Graeve et al., 1986). In view of the differences in the levels of focus formation that was found, we consider it unlikely that a similar v-*sis*-$G_s$ protein is responsible for the observed biological activities of the v-*sis*-G genes.

The other mechanism whereby a v-*sis* gene product could be released into the extracellular media is by COOH-terminal proteolysis of the v-*sis*-G fusion protein. The v-*sis* gene product has been reported to undergo COOH-terminal processing as well as $NH_2$-terminal processing, resulting in the formation of a PDGF-related protein that is detected as a 24-kD dimer under nonreducing conditions (Robbins et al., 1983). In our previous studies of v-*sis*–encoded gene products, particularly those with COOH-terminal deletions, we were unable to detect any evidence for the proposed COOH-terminal processing event (Hannink and Donoghue, 1986; Hannink, Sauer, and Donoghue, 1986). Thus the site and the biological importance of this COOH-terminal processing event is not known. COS-1 cells expressing the v-*sis*$^{239}$-G gene do not secrete a mitogenic factor into the media (data not shown). These results argue that a PDGF-related protein is not released from the v-*sis*-G proteins into the media as a result of COOH-terminal proteolysis. However, the sensitivity of the mitogen assay is not sufficient to detect low levels of secreted protein that nonetheless may be responsible for transformation when chronically produced in an autocrine fashion. Such a protein may not be freely secreted into the media in a manner that allows its detection in assays performed on serum-free conditioned media. In this respect, it is notable that considerably higher levels (50-fold) of anti-PDGF IgG was required to inhibit the growth of SSV-transformed cells than was necessary for the clearance of mitogenic activity from the conditioned media collected from these cells (Johnsson et al., 1985). In addition, it has been demonstrated that only a low level of the total mitogenic activity of SSV-transformed cells is released freely into the media, the remainder consisting of intracellular or plasma membrane–associated material (Robbins et al., 1985). Therefore, we can not rule out the possibility that the observed levels of focus formation of the v-*sis*-G genes are due to the presence of low levels of proteolytic release of a v-*sis* protein from the membrane-anchored v-*sis*-G molecule. It will be of interest to determine the effect of anti-PDGF IgG on the transformed phenotype and growth characteristics of cells transformed by the v-*sis*-G genes.

### *Other Transmembrane Growth Factor–related Proteins*

In these experiments we have constructed biologically active transmembrane mutants of the v-*sis* gene product. Recent evidence has come to light that similar transmembrane forms of growth factor–related proteins may have an important physiological role. In particular, the precursor to the epidermal growth factor (EGF) molecule has been hypothesized to have a transmembrane structure oriented in the same fashion as the v-*sis*-G proteins reported here. The importance of the transmembrane nature of the EGF precursor is not known, although several hypotheses have been advanced on the basis of homology between the EGF precursor and other proteins. These proteins include the low density lipoprotein receptor (Russell et al., 1984), and the proteins encoded by the *Notch*

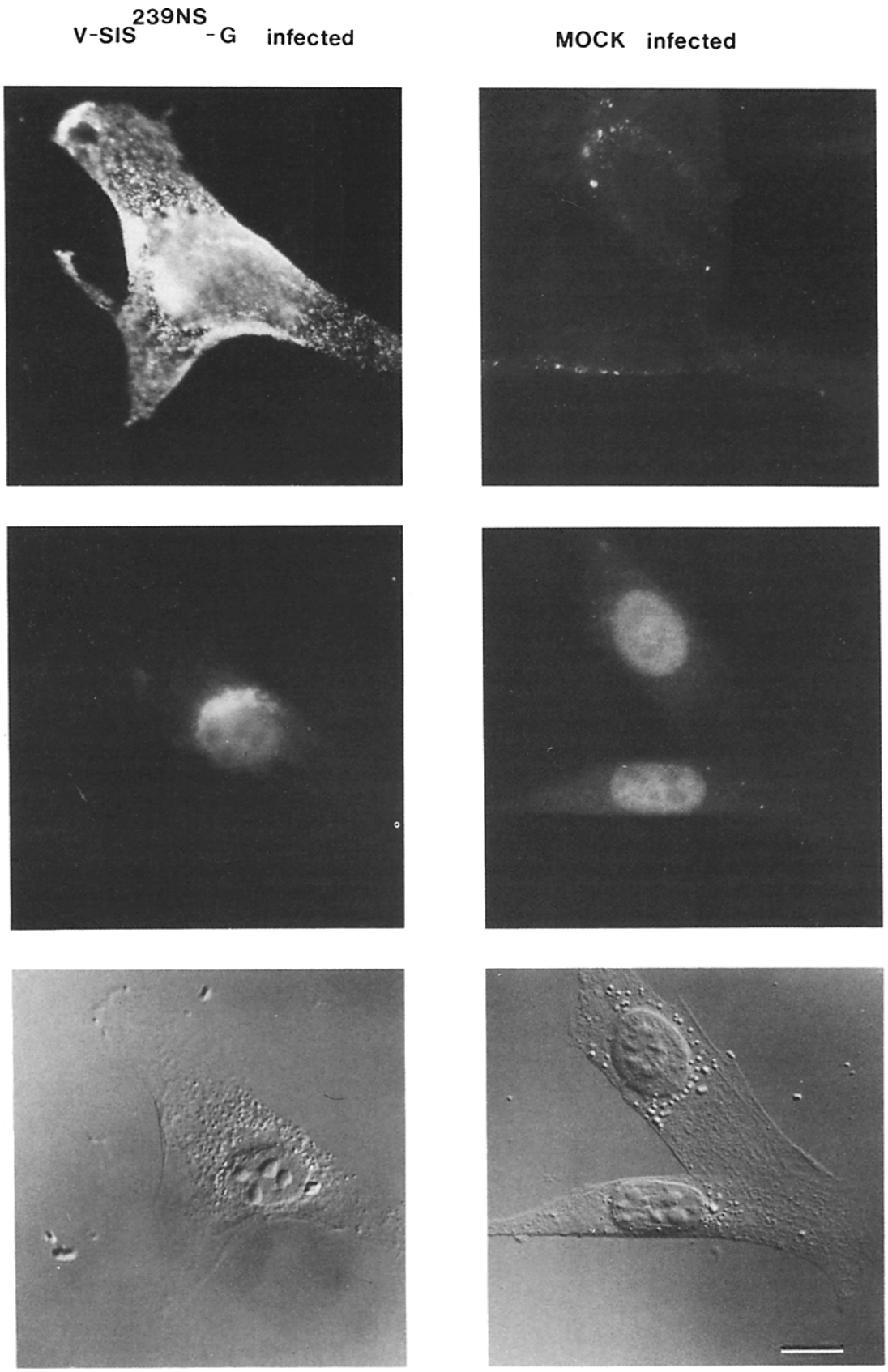



*Figure 6.* Indirect immunofluorescence in NIH3T3 cells. NIH3T3 cells were infected with the v-*sis*$^{239NS}$-G/MuLV virus stock or with the MuLV virus stock (Mock). The top photos show the outside staining with the anti-*sis* serum, the middle show the internal staining with the anti-G serum, and the bottom photos are the phase-contrast images of the same cells. An automatic exposure setting of 800 was used for these pictures. Bar, 10 μm.

locus of *Drosophila melanogaster* (Wharton et al., 1985) and by the *lin* locus of *Caenorhabditis elegans* (Greenwald, 1985). The basis for this homology is the spacing of six cysteine residues, which in the case of EGF, have been shown to be involved in intramolecular disulfide bonding. These proteins have up to 36 repeated units of an EGF-like domain, followed by a transmembrane domain and a variable intracellular domain. The *Notch* and *lin* proteins have been proposed to have a role in determining the fate of cells in a developing organism. Whether they function in an autocrine fashion by stimulation of the receptor on the same cell or in a paracrine fashion by either proteolytic release of an EGF-like factor or by interaction with a receptor on adjacent cells is not known. Our demonstration that membrane-anchored forms of the v-*sis* gene product are biologically active suggests that the transmembrane forms of these growth factor–related molecules may have physiological roles. Additional support of this comes from the cell autonomous behavior of the *Notch* locus in mosaic individuals, indicating that the effect of the *Notch* protein is mediated within the local environment of the cell, not as a widespread diffusable factor (Bender, 1985).

The plasma membrane has long been regarded as the site at which a cell receives signals from the extracellular matrix. A tremendous variety of signals interact with cell surface receptors, including mitogenic signals, growth inhibitory signals, and signals for the release of stored intracellular molecules. Perhaps the plasma membrane should also be regarded as a site for the reception of signals originating from the cell's interior, encoded by the cell's own genes.

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