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Monoclonal Antibodies to the Transformation-Specific Glycoprotein Encoded by the Feline Retroviral Oncogene v-*fms*

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Monoclonal antibodies prepared to epitopes encoded by the transforming gene (v-*fms*) of the McDonough strain of feline sarcoma virus were used to study v-*fms*-coded antigens in feline sarcoma virus-transformed rat and mink cells. These antibodies reacted with three different polypeptides (gP180*gag-fms*, gp140*fms*, and gp120*fms*), all of which were shown to be glycosylated. Protein blotting with [^{125}I]-labeled monoclonal immunoglobulin G's was used to determine the relative steady-state levels of these glycoproteins in transformed cells and showed that gp120 and gp140 were the predominant products. Immunofluorescence assays and subcellular fractionation experiments localized these molecules to the cytoplasm of transformed cells in quantitative association with sedimentable organelles. Thus, v-*fms*-coded glycoproteins differ both chemically and topologically from the partially characterized products of other known oncogenes and presumably transform cells by a different mechanism.

Oncogenes responsible for malignant transformation are now recognized to be transmitted in the germ lines of normal animals (3, 6, 10, 13–15, 21, 23, 25–28). It has been suggested that these genes act in cellular growth and differentiation and that their altered expression in susceptible target cells can lead to tumor formation. Of critical importance are questions concerning the number of potential oncogenes, the functions of their protein products, and the placement of these proteins within a defined cellular metabolism. At least 14 of the known oncogenes have been transduced by retroviruses, conferring upon the recombinant viruses the ability to induce acute diseases. Several of these genes (now designated *src*, *fps*, *yes*, *ros*, *abl*, and *fes*) encode phosphoproteins with associated, tyrosine-specific protein kinase activities, whereas another gene (*ras*) specifies a polypeptide with guanine nucleotide-binding properties; the functions of other retroviral transforming proteins remain unclear (for review of nomenclature, see reference 5). In an effort to characterize the viral oncogene (v-*fms*) transduced by the McDonough strain of feline sarcoma virus (SM-FeSV) (20), we prepared monoclonal antibodies reactive to the v-*fms*-coded protein and developed sensitive assays for its detection in transformed cells. We show that the v-*fms*-coded protein is rapidly glycosylated, post-translationally cleaved, and remains quantitatively associated with sedimentable cytoplasmic organelles.

The order of genes in the SM-FeSV provirus is 5′ Δ*gag*, *fms*, *env* 3′, in which the 3′ end of the helper virus-derived *gag* gene has been deleted (9; see Table 1, footnote *a*, for nomenclature of retroviral genes). When a v-*fms* probe was used, only full-genome-length SM-FeSV mRNA molecules were detected in cloned mink and rat cells transformed by the virus. The product of the fused *gag* and v-*fms* genes is a glycosylated polyprotein (see below) of about 180 kilodaltons (kd), here designated gP180*gag-fms* (2, 22, 30). Two lower-molecular-weight products representing the amino-terminal (p60*gag*) and carboxy-terminal (gp120*fms*) portions of the molecule appear to result from proteolytic cleavage at a preferred site near the *gag-fms* junction (2, 22). However, the possibility that translation of gp120*fms* is initiated at an internal AUG codon cannot be formally excluded. The 3.0- ± 0.3-kilobase-pair v-*fms* gene is sufficient to specify the gp120*fms* portion of the polyprotein (9) which cannot be precipitated with antisera to *gag* gene-coded products (2, 9, 22, 30).

Antisera to gP180*gag-fms* were previously prepared by inoculating SM-FeSV-transformed rat cells into syngeneic Osborne-Mendel rats

TABLE 1. Specificity of monoclonal antibodies for epitopes encoded by the *v-fms* gene[a]

Cell line	Species	Viral proteins immunoprecipitated		
		Goat antiserum to FeLV	Tumored rat antiserum (TB-D6 rat)	Monoclonal antibodies
SM-FeSV transformed				
G-2/M	Mink	gP180*gag-fms* p60*gag* gPr85*env*[b]	gP180*gag-fms* gp120*fms* p60*gag*	gP180*gag-fms* gp120*fms*
G-2/NRK	Rat	gP180*gag-fms* p60*gag* gPr85*env*	gP180*gag-fms* gp120*fms* p60*gag*	gP180*gag-fms* gp120*fms*
TSM3	Mouse	gP180*gag-fms* p60*gag* gPr85*env*	gP180*gag-fms* gp120*fms* p60*gag*	gP180*gag-fms* gp120*fms*
GA-FeSV-transformed F3C17	Mink	P95*gag-fes*	P95*gag-fes*	None
ST-FeSV transformed				
B3T-1	Mink	P85*gag-fes*	P85*gag-fes*	None
ST/NRK	Rat	P85*gag-fes*	P85*gag-fes*	None
FeLV infected				
FB64	Mink	Pr180*gag-pol* Pr65*gag* gPr85*env*	Pr180*gag-pol* Pr65*gag*	None
FC64	Mink	Pr180*gag-pol* Pr65*gag* gPr85*env*	Pr180*gag-pol* Pr65*gag*	None
Uninfected controls				
CCL64	Mink	None	None	None
NRK	Rat	None	None	None
NIH/3T3	Mouse	None	None	None
FEF	Cat	None	None	None

[a] The viral gene products detected in these studies included the known FeLV precursor proteins (Pr180*gag-pol*, Pr65*gag*, and gPr85*env*), the Snyder-Theilen and Gardner-Arnstein strains of the FeSV polyproteins encoded by *gag* and *fes* sequences (P85*gag-fes* and P95*gag-fes*), and various SM-FeSV-encoded products (gP180*gag-fms*, gp120*fms*, gPr85*env*, and p60*gag*). By convention (5), the proteins are abbreviated "p" for protein, "gp" for glycoprotein, "Pr" for precursor, and "P" for polyprotein, followed by the apparent molecular weight of the polypeptide in kd. Underlined subscripts refer to the viral genes encoding each product and include the genes required for viral replication (*gag*, group specific antigens; *pol*, polymerase; *env*, envelope glycoprotein) and two different FeSV oncogenes, *fes* and *fms*. Confluent T75 flasks or 100-mm petri plates were labeled for 30 min with either 200 μCi of [^{3}H]leucine per ml (58 Ci/mmol) or 250 μCi of [^{35}S]methionine per ml (400 Ci/mmol) in 2 to 6 ml of leucine-free or methionine-free medium. After lysis and sedimentation of nuclei, 4×10^6 to 6×10^6 acid-precipitable cpm of each extract (1 ml) were reacted with the designated antiserum (5 to 15 μl) or with cell-free supernatants (100 μl) of cultures producing monoclonal antibodies 1.32.6, 3.19.4, or 5.15.4 plus rabbit anti-rat IgG; immune complexes were then coprecipitated with *Staphylococcus aureus* strain Cowan I as described previously (9). Washed precipitates were denatured and subjected to electrophoresis in 7% linear or 6 to 12% gradient polyacrylamide slab gels containing SDS (17), and the position of precipitated radiolabeled proteins was determined by autofluorography of the dried gel slab (4). Each monoclonal antibody preparation rendered similar results, as shown in the last column of the table. G-2/M, G-2/NRK, F3C17, B3T-1, and ST/NRK are transformed nonproducer cells derived by infection and cell cloning procedures (22). TSM3 is a transformed NIH/3T3 derivative obtained after transfection with molecularly cloned SM-FeSV proviral DNA (9). The mink FB64 and FC64 cell lines produce FeLV, subgroups B and C, respectively (22, 24).

[b] SM-FeSV contains the complete FeLV-derived *env* gene and encodes a protein indistinguishable in size and antigenicity from the *env* gene precursor gPr85*env*.

(22). Although these sera recognize v-*fms*-coded antigens, they also precipitate *gag* gene-coded antigens and contain antibodies to rat cellular proteins. To obtain monoclonal antibodies with defined specificities to v-*fms*-coded epitopes, spleen cells from a hyperimmune donor animal (rat D6) were fused to Y3-Ag1.2.3 rat myeloma cells (12). Supernatants from lymphocyte-myeloma hybrid cultures were tested by immunoprecipitation and gel electrophoresis for

antibodies reactive to metabolically labeled gP180*gag-fms* and gp120*fms* but not to p60*gag*. Four positive hybrids were cloned twice in soft agar and established as stable lines with previously published procedures (11). Three of these lines (clones SM1.32.6, SM3.19.4, and SM5.15.4) produced immunoglobulin G (IgG) (γ1) and one (clone SM4.23.5) produced IgM. The three IgGs were used in subsequent studies.

The specificity of monoclonal antibodies for v-*fms*-coded antigens was confirmed by immunoprecipitation of metabolically labeled products from different transformed or virus infected cell lines (Table 1). The three monoclonal IgGs precipitated only gP180*gag-fms* and gp120*fms* from lysates of cell lines transformed by SM-FeSV, including a mouse cell transformant derived by transfection with molecularly cloned proviral DNA (9). None of these antibodies reacted with polyproteins encoded by two other FeSV isolates (the Snyder-Theilen and Gardner-Arnstein strains), each of which carries an oncogene (v-*fes*) unrelated to v-*fms* (2, 9, 22, 30). Control experiments showed that the monoclonal IgGs did not precipitate viral gene products from cells infected with different feline leukemia virus (FeLV) subgroups, even though these proteins were readily detected with appropriate antisera (Table 1, columns 1 and 2).

The v-*fms* proteins are functionally distinct from those oncogene products which have associated tyrosine-kinase activities and are themselves phosphorylated in tyrosine. Immune precipitates formed with the SM-FeSV gene products have shown either limited (1) or no (30) in vitro tyrosine kinase activity under conditions in which immunoprecipitates containing v-*fes*-encoded polyproteins exhibit very high kinase activities. Both gP180*gag-fms* and gp120*fms* were poorly labeled in vivo (in serine and threonine [1]) with [^{32}P]phosphoric acid (24), whereas P85*gag-fes* and P95*gag-fes* showed very high levels of tyrosine phosphorylation (1, 24, 30). Infection of SM-FeSV mink cell transformants with the Snyder-Theilen strain of FeSV (FeLV), followed by in vivo metabolic labeling with [^{32}P]phosphoric acid, has also enabled the ready detection of P85*gag-fes* in the same cells in which gP180*gag-fms* and gp120*fms* were not detectably phosphorylated (S. J. Anderson and C. J. Sherr, unpublished data). In addition, no increase in the total level of phosphotyrosine has been found in SM-FeSV transformants as compared with untransformed cells (1).

A second major difference between v-*fms*-coded products and other transforming proteins is glycosylation. We previously found that both gP180*gag-fms* and gp120*fms* could be metabolically labeled with [^{3}H]mannose (24). To firmly establish the glycoprotein nature of these molecules, their synthesis was studied in the presence of tunicamycin, an antibiotic which interferes with glycosylation (16). Figure 1A shows the typical pattern of precipitation obtained with lysates from SM-FeSV-transformed cells radiolabeled for 30 min with [^{35}S]methionine. gP180*gag-fms* and p60*gag* were detected with an anti-gag (p30) serum (lane 1) or TB-D6 rat serum (lane 3); the latter serum also precipitated gp120*fms*. As expected, gP180*gag-fms* and gp120*fms* were detected with monoclonal antibody SM5.15.4, whereas p60*gag* was not (lane 4). When tunicamycin-treated cells were labeled with [^{35}S]methionine (Fig. 1B), the apparent molecular weights of the proteins precipitated by both tumored rat serum (lane 3) and monoclonal IgG (lane 4) were 155 and 95 kd, respectively, whereas the mobility of p60*gag* was unchanged. The quantities of radiolabeled, immunoprecipitated products were reduced in tunicamycin-treated cells, presumably owing to the mild toxicity of the drug. The other two preparations of monoclonal IgG also reacted with p155 and p95 (data not shown), indicating their specificities for v-*fms* protein and not carbohydrate antigenic determinants.

By using [^{3}H]mannose and longer labeling periods (1 to 2 h), both gP180*gag-fms* and gp120*fms*, but not p60*gag*, were detected with tumored rat serum; in addition, another polypeptide of approximately 140 kd was seen (Fig. 1C). The latter glycoprotein was precipitated with monoclonal antibodies to v-*fms*-coded determinants but not with anti-*gag* sera (data not shown), suggesting that, like gp120, gp140 is derived from the carboxy-terminal portion of the SM-FeSV polyprotein. The simplest model suggests that the primary translation product, P155*gag-fms*, is immediately glycosylated to yield gP180*gag-fms* and coordinately cleaved into NH_2-p60*gag* and gp120*fms*-COOH. The latter product might then be modified by either further addition of sugar residues or other post-translational mechanisms to yield gp140*fms*. The failure to metabolically label p60*gag* with [^{3}H]mannose or to alter its apparent molecular weight after tunicamycin treatment shows that carbohydrate addition occurs only within the v-*fms* moiety.

The availability of monoclonal antibodies has enabled us to use a sensitive protein blotting assay to detect the various v-*fms*-encoded species in SM-FeSV-transformed cells and to study the physical association of these molecules with different subcellular compartments. Lysates of SM-FeSV transformants were subjected to electrophoresis on polyacrylamide slab gels containing sodium dodecyl sulfate (SDS), and the separated proteins were transferred to nitrocellulose filters (29). Filters containing bound proteins

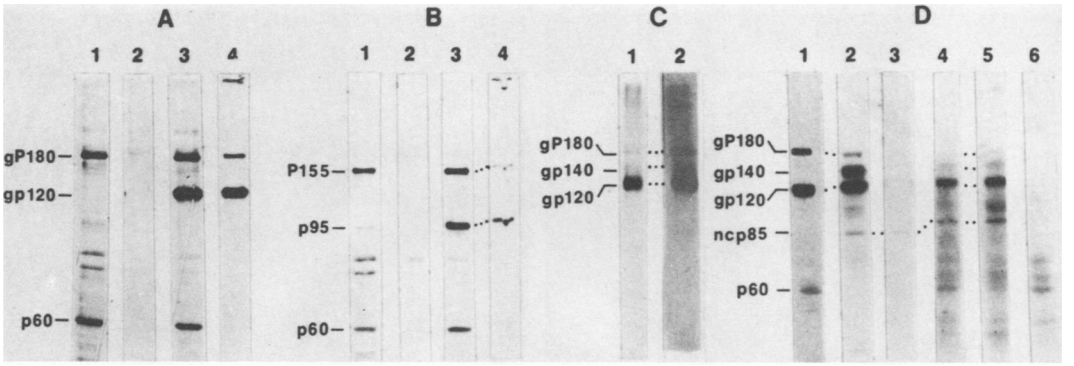

FIG. 1. (A) Confluent 100-mm plates of G-2/M cells labeled for 30 min with 250 μCi of [^{35}S]methionine per ml in 2 ml of methionine-free medium, lysed with detergent, and precipitated with antisera. Coprecipitated immune complexes were denatured and subjected to electrophoresis on 7% polyacrylamide gels containing SDS, and the positions of the proteins were determined by autoradiography of the dried gel slab (4, 17). The antisera used were: goat antiserum to FeLV p30 (lane 1), normal rat serum (lane 2), tumored rat antiserum to SM-FeSV-transformed cells (lane 3), and monoclonal IgG SM5.15.4 (lane 4). (B) Confluent 100-mm plates were treated for 60 min with 25 μg of tunicamycin (Calbiochem) per ml and then labeled in the presence of the drug for 30 min. The antisera and labeling conditions were identical to those described in (A). The composite data shown in (A) and (B) derive from different autoradiograms of the same gel run with appropriate molecular-weight standards. (C) Confluent T75 flasks of G-2/M cells were labeled for 60 and 120 min with 200 μCi of [^{3}H]mannose per ml (lanes 1 and 2, respectively) in 6 ml of glucose-free medium. Labeled products were precipitated with TB-D6 rat serum, and the coprecipitated complexes were analyzed on 6 to 12% gradient slab gels as described above. (D) Culture flasks containing cells were lysed directly in SDS-polyacrylamide gel sample buffer (10 mM Tris-hydrochloride [pH 8] containing 1% SDS, 1% 2-mercaptoethanol, 1 mM EDTA, 5% glycerol, and 0.02% bromophenol blue [9]). The extracts were boiled for 4 min and centrifuged at 150,000 × *g* for 30 min to pellet DNA, and samples containing 30 μg of protein were applied to 6 to 12% polyacrylamide linear gradient slab gels (9). Alternatively, cells were fractionated after homogenization with a tight-fitting Dounce homogenizer in 0.02 M Tris-hydrochloride (pH 7.5) containing 0.02 M NaCl and 2 mM $MgCl_2$. The nuclei were sedimented for 10 min at 5,000 × *g*, suspended in buffer containing 0.5% Nonidet P-40, and resedimented, and the supernatant (perinuclear fraction) was saved. Microsomes were pelleted at 150,000 × *g* for 60 min, disrupted in buffer containing 0.5% Nonidet P-40 and 0.5% sodium desoxycholate, and saved. Proteins in the above fractions and in the postmicrosomal supernatant were quantitated (19) and denatured as described above, and representative samples were subjected to electrophoresis. Separated proteins in the gel slab were electrophoretically transferred to 0.2-μm nitrocellulose filters (29) and preadsorbed for 3 h at 37°C in TNE buffer (20 mM Tris-hydrochloride (pH 7.5), 0.15 M NaCl, and 1 mM EDTA) containing 0.1% Nonidet P-40 (Shell Chemicals), 0.3 g% bovine serum albumin (Cohn fraction IV), and 0.1 g% bovine gamma globulin (Cohn Fraction II; both from Sigma Chemical Co.). Cells producing monoclonal IgG were shifted to serum-free medium containing transferrin and bovine serum albumin as the only proteinaceous molecules (11). Monoclonal IgGs were purified to apparent homogeneity from serum-free culture supernatants by salt fractionation and chromatography on DEAE cellulose, and individual preparations from different antibody-producing cultures were externally radiolabeled with ^{125}I (8). Preadsorbed nitrocellulose papers were reacted overnight at 4°C with preadsorption buffer containing 3 × 10^6 dpm of ^{125}I-labeled monoclonal IgG per ml and washed five times (20 min per wash at 22°C) with TNE buffer. Dried papers were subjected to autoradiography at −70°C with Kodak Lightning-Plus intensifying screens (4). Lane 1, Control [^{3}H]leucine-labeled gP180*gag-fms*, gp120*fms*, and p60*gag* immunoprecipitated from a lysate of G-2/M cells (30-min pulse) were separated electrophoretically and transferred to nitrocellulose. Dried papers were impregnated with fluor and autofluorographed (4). Extracts of SM-FeSV-transformed G-2/M cells (lane 2) and of untransformed CCL64 mink cells (lane 3) were analyzed directly by blotting with ^{125}I-labeled monoclonal IgG SM3.19.4. Samples (20 μg of protein per lane) (19) from the perinuclear extract (lane 4), the microsomes (lane 5), and the postmicrosomal supernatant (lane 6) were similarly analyzed and were each derived from fractionation of transformed G-2/M cells; approximately 65% of the total cytoplasmic protein was found in the postmicrosomal supernatant, 25% in the microsomes, and 10% in the perinuclear extract for the experiment shown.

were reacted with purified, ^{125}I-labeled monoclonal antibodies, washed to remove unbound radioactivity, and subjected to autoradiography. The major advantage of protein blotting is that it does not rely on kinetic radiolabeling and accurately measures the total steady-state pools of immunoreactive molecules.

In a control experiment, it was shown that ^{3}H-labeled gP180*gag-fms* and gp120*fms*, recovered by immunoprecipitation after a 30-min pulse and

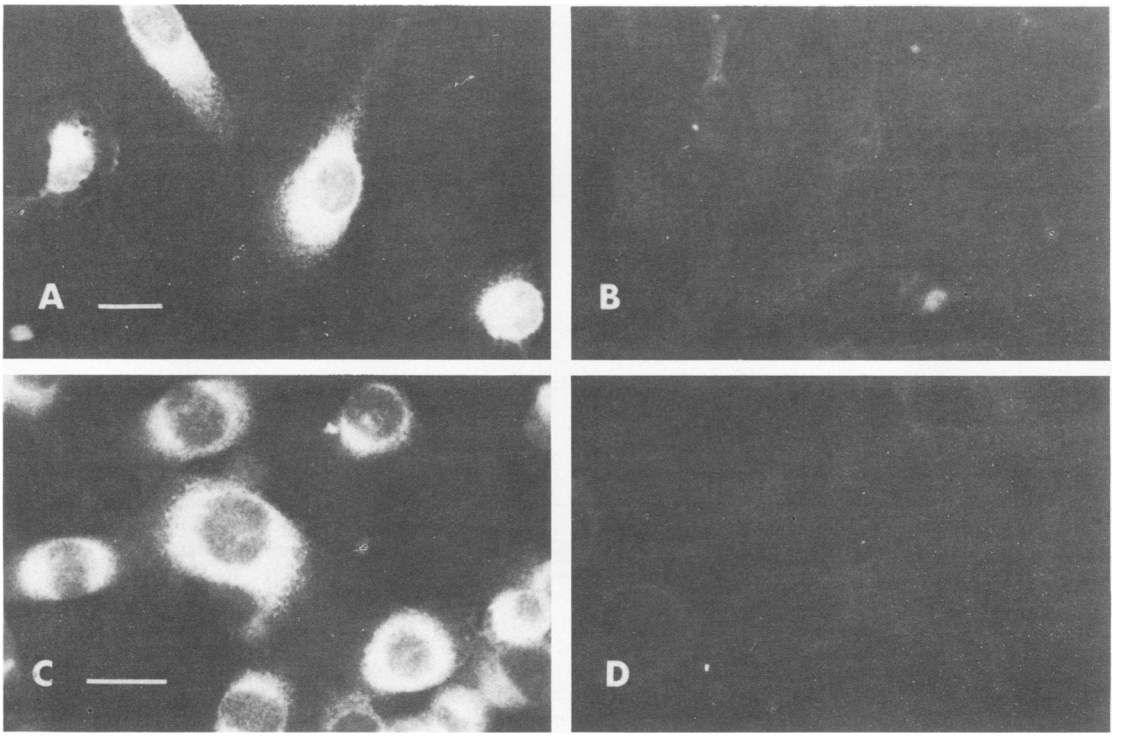

FIG. 2. Localization of v-*fms* antigens in transformed cells by immunofluorescence. Cells nonproductively transformed by SM-FeSV and uninfected control cells were plated on wells of gelatin-coated multi-test slides (Flow laboratories, Inc.) and grown overnight at 37°C. The cells were fixed in absolute methanol for 4 min at −20°C, rinsed with 80% acetone, and air dried. Prior fixation with 3.7% formaldehyde in phosphate-buffered saline for 10 min at room temperature did not affect the results. The fixed cells were incubated for 45 min at 37°C with purified monoclonal antibodies diluted in phosphate-buffered saline containing 0.4 g% bovine serum albumin, washed with phosphate-buffered saline, and incubated for 45 min at 37°C with affinity-purified, rhodamine-conjugated rabbit antibodies against rat IgG. (The rabbit antibodies were a generous gift of Jurgen Wheland of the National Cancer Institute, Bethesda, Md.) After extensive washing with phosphate-buffered saline, the cells were mounted with Elvanol under no. 1 cover slips and observed with a Zeiss microscope equipped with epifluorescence optics. (A) SM-FeSV-transformed CCL64 mink cells (G-2/M) stained with 100 μg of SM5.15.4 IgG per ml and photographed with a ×63 objective. (B) Uninfected CCL64 mink cells stained and photographed as described in (A) with matched exposure and developing time. (C) SM-FeSV-transformed rat NRK cells (G-2/NRK) stained with 20 μg of SM3.19.4 IgG per ml and photographed with a ×100 objective. (D) Uninfected NRK cells stained and photographed as described in (C) with matched exposure and developing time. Bars, 20 μm.

separated electrophoretically, could be transferred to nitrocellulose at equal efficiencies (e.g., compare results with ^{3}H-labeled molecules before [Fig 1A, lane 3] and after [Fig 1D, lane 1] transfer). After electrophoresis and transfer of unlabeled proteins from a total extract of the same cells, gP180 and gp120 were also detected after reaction of the filters with ^{125}I-labeled antibodies. However, the relative quantity of gp120 appeared significantly greater in the blot, and gp140 was also seen (Fig. 1D, lane 2). Blotting of the immunoprecipitated proteins (labeled in the same experiment as that shown in Fig. 1D, lane 1) readily detected both gp120 and gp140 at levels similar to those shown in Fig. 1D, lane 2 (data not shown). The apparently elevated levels of gp120 and gp140 in the blot as compared with the concentrations shown by kinetic radiolabeling must therefore reflect their steady-state pools in transformed cells. Thus, the major v-*fms* products which accumulate are the processed, glycosylated forms.

Of the three labeled monoclonal IgGs tested for their ability to bind immobilized proteins, two (SM1.32.6 and SM3.19.4) were reactive and rendered similar data with lysates of transformed cells. Either of these labeled IgG preparations could be used to detect gP180, gp140, and gp120 in only 1 to 2 μg of total cellular extract from SM-FeSV-transformed mink cells

after 7 days of autoradiography. Radioimmunoassays of *gag* gene products indicate that gP180 and p60 together represent no more than 0.1% of the total cellular protein of these cells, suggesting that the blotting technique may be sensitive to subnanogram levels.

The blotting procedure also detected a protein of lower molecular weight (~85 kd) which was found not only in transformed cells but in uninfected cells as well (Fig. 1D, lane 3). This protein (designated ncp85 [for normal cell protein, 85 kd]) was reproducibly detected at very low levels with SM3.19.4 IgG but not Sm1.32.6 IgG. A protein of similar molecular weight was found in blots of canine kidney and African green monkey kidney cells and, to a lesser extent, in cat embryo fibroblasts, but not in several rodent cell lines (data not shown). This polypeptide could represent a physiologically unrelated molecule displaying an epitope recognized by the monoclonal antibody, or alternatively, it could reflect the presence of a gene product encoded by c-*fms* sequences.

Subcellular fractions from SM-FeSV-transformed mink cells were prepared which contained sedimentable cytoplasmic organelles and membranes. These fractions, as well as the soluble non-membrane-bound proteins in the cytosol, were separated electrophoretically and assayed for v-*fms* glycoproteins by blotting. The v-*fms* gene products (and ncp85) were detected exclusively in fractions containing sedimentable membranes and cytoplasmic organelles (Fig 1D, lanes 4 and 5). By contrast, although 65% of the total cytoplasmic protein fractionated with the cytosol, no soluble v-*fms*-coded products were detected (lane 6).

Immunofluorescent staining of fixed, permeabilized cells was used to locate v-*fms*-coded antigens in SM-FeSV transformants (Fig. 2). Intense granular, cytoplasmic fluorescence of SM-FeSV-transformed mink (Fig. 2A) and rat (Fig. 2C) cells was seen with each monoclonal IgG under conditions in which untransformed controls (Fig. 2B and D) were minimally visualized. Staining was most dramatic in the regions closest to the nuclei and radiated toward the cell periphery. Some weak staining of the cell borders was also seen. This pattern of cytoplasmic fluorescence differs from that found with cells transformed by the v-*ras* gene of Harvey sarcoma virus in which staining of plasma membranes is a major feature (11, 33).

We concluded that the v-*fms* transforming gene encodes molecules whose properties and localization within cells differ distinctly from the partially characterized products of other retroviral oncogenes. The rapid glycosylation of v-*fms* proteins and their quantitative association with sedimentable structures strongly suggest that their synthesis occurs on membrane-bound polyribosomes and that the molecules are rapidly sequestered within the endoplasmic reticulum of transformed cells. The presence of multiple v-*fms* products and the qualitative and quantitative differences between newly synthesized molecules versus those accumulated at steady-state levels are most compatible with multistep, post-translational processing of the glycoproteins within the endoplasmic reticulum-Golgi complex. The v-*fms*-encoded glycoproteins appear to concentrate and remain associated with cytoplasmic organelles or cytoskeletal networks. We cannot exclude that a minority of the molecules shuttle to and from the plasma membrane within vesicles and transiently gain access to the extracellular environment (32). These properties are unlike those of v-*src* pp60, which is synthesized on "free" polyribosomes (18) and then becomes associated with the inner surface of the plasma membrane (7, 31); of v-*ras* p21, which transiently appears in the cytosol and also becomes associated with the inner plasma membrane (33); and of the v-*abl* polyprotein, which spans the plasma membrane (34). Although functional interactions between v-*fms*-coded glycoproteins and the cellular counterparts of other oncogene products are not precluded, it is likely that the *fms* gene induces cellular transformation by a different mechanism.

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