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The Amino-Terminal Domain of the v-*fms* Oncogene Product Includes a Functional Signal Peptide That Directs Synthesis of a Transforming Glycoprotein in the Absence of Feline Leukemia Virus *gag* Sequences

ESTHER F. WHEELER,[1] MARTINE F. ROUSSEL,[1] ANNIE HAMPE,[2] MARY H. WALKER,[1] VICTOR A. FRIED,[3] A. THOMAS LOOK,[1,4] CARL W. RETTENMIER,[1] AND CHARLES J. SHERR[1]*

Departments of Tumor Cell Biology,[1] Hematology-Oncology,[4] and Biochemistry,[3] St. Jude Children's Research Hospital, Memphis, Tennessee 38105, and Laboratoire d'Hematologic Experimentale, Centre Georges Hayem, Hopital St. Louis, Paris, Cedex 75010, France[2]

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The nucleotide sequence of a 5′ segment of the human genomic c-*fms* proto-oncogene suggested that recombination between feline leukemia virus and feline c-*fms* sequences might have occurred in a region encoding the 5′ untranslated portion of c-*fms* mRNA. The polyprotein precursor gP180$^{gag\text{-}fms}$ encoded by the McDonough strain of feline sarcoma virus was therefore predicted to contain 34 v-*fms*-coded amino acids derived from sequences of the c-*fms* gene that are not ordinarily translated from the proto-oncogene mRNA. The (gP180$^{gag\text{-}fms}$) polyprotein was cotranslationally cleaved near the *gag-fms* junction to remove its *gag* gene-coded portion. Determination of the amino-terminal sequence of the resulting v-*fms*-coded glycoprotein, gp120$^{v\text{-}fms}$, showed that the site of proteolysis corresponded to a predicted signal peptidase cleavage site within the c-*fms* gene product. Together, these analyses suggested that the linked *gag* sequences may not be necessary for expression of a biologically active v-*fms* gene product. The *gag-fms* sequences of feline sarcoma virus strain McDonough and the v-*fms* sequences alone were inserted into a murine retroviral vector containing a neomycin resistance gene. Both constructs were biologically active when transfected into NIH 3T3 cells and produced morphologically transformed foci at equivalent efficiencies. When transfected into a cell line (Ψ2) expressing complementary viral gene functions, G418-resistant (Neo^r) cells containing either of these vector DNAs produced high titers of transforming viruses. Analysis of proteins produced in cells containing the vector lacking *gag* gene sequences showed that gP180$^{gag\text{-}fms}$ was not synthesized, whereas normal levels of both immature gp120$^{v\text{-}fms}$ and mature gp140$^{v\text{-}fms}$ were detected. The glycoprotein was efficiently transported to the cell surface, and it retained wild-type tyrosine kinase activity. We conclude that a cryptic hydrophobic signal peptide sequence in v-*fms* was unmasked by *gag* deletion, thereby allowing the correct orientation and transport of the v-*fms* gene product within membranous organelles. It seems likely that the proteolytic cleavage of gP180$^{gag\text{-}fms}$ is mediated by signal peptidase and that the amino termini of gp140$^{v\text{-}fms}$ and the c-*fms* gene product are identical.

The product of the c-*fms* proto-oncogene is related to the receptor for the mononuclear phagocyte colony-stimulating factor, CSF-1 (31), and is a member of a class of proto-oncogene products and growth factor receptors that encode tyrosine-specific protein kinases (5). The mature cell surface glycoproteins encoded by the c-*fms* proto-oncogene and the v-*fms* oncogene of the McDonough strain of feline sarcoma virus (SM-FeSV) are closely related in their biochemical and topological properties. Both proteins are active as tyrosine-specific protein kinases in immune complexes (3, 22), and both are able to specifically bind CSF-1 (26a, 31). The fact that CSF-1 is produced by fibroblasts (34) and that SM-FeSV produces fibrosarcomas in cats (17, 27) raises the possibility that viral transformation is due to an autocrine mechanism that depends upon transduction of a competent v-*fms*-coded receptor into fibroblasts. Alternatively, the v-*fms* gene product may be activated as an oncogene by alterations in its coding sequences which deregulate its activity as an enzyme or change its interaction with critical physiologic substrates (reviewed by C. J. Sherr and E. R. Stanley, *in* T. Graf and P. Kahn, ed., *Oncogenes and Growth Control*, in press). The two mechanisms are not mutually exclusive.

The SM-FeSV v-*fms* gene is expressed as a fusion polyprotein encoded, in part, by *gag* sequences derived from feline leukemia virus (FeLV) (4, 21, 26, 35). Insertion of c-*fms* sequences into the *gag* open reading frame results in the production of a glycosylated polyprotein precursor (gP180$^{gag\text{-}fms}$) that is proteolytically cleaved to yield an amino-terminal *gag*-coded fragment (p55^{gag}) and a carboxy-terminal v-*fms*-coded glycoprotein (gp120$^{v\text{-}fms}$) (4, 26). The latter glycoprotein is transported to the cell surface, undergoing concomitant remodeling of its N-linked oligosaccharides and thereby increasing in its apparent molecular weight (1, 2). Cell surface expression of the mature form of the glycoprotein (gp140$^{v\text{-}fms}$) is required for transformation (24). Since the 5′ end of the c-*fms* gene must have been truncated by recombination with FeLV in the formation of SM-FeSV, the v-*fms* gene products, gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$, could differ from their c-*fms*-coded counterparts at their amino termini. These genetic alterations might

* Corresponding author.

potentially activate c-*fms* as an oncogene and confer the ability of the v-*fms* gene products to transform cells.

We have investigated the differences in the 5′ ends of the v-*fms* and c-*fms* gene products by determining the nucleotide sequence of a 5′ portion of the human c-*fms* gene and comparing the predicted amino acid sequences with the experimentally determined N-terminal sequences of $gp120^{v\text{-}fms}$ and $gp140^{v\text{-}fms}$. Our results predicted that the c-*fms* and v-*fms* gene products have similar amino termini and suggested, in turn, that *gag* gene sequences might be dispensable in the synthesis of transforming v-*fms*-coded glycoproteins. We demonstrate that an SM-FeSV mutant lacking *gag* gene sequences encoded molecules indistinguishable from $gp120^{v\text{-}fms}$ and $gp140^{v\text{-}fms}$; these are biologically active as transforming proteins.

MATERIALS AND METHODS

Retroviral constructs. To engineer a retroviral vector containing the intact Δ*gag-fms* coding region joined to a selectable neomycin resistance gene (*neo*), we used two previously described plasmid subclones containing different portions of the SM-FeSV provirus (24). The first of these (designated pSM-FeSV.5′) contains the 5′ long terminal repeat (LTR), the Δ*gag* sequences, and the 5′ portion of v-*fms*, terminating at a unique *Cla*I site within the viral oncogene; the second plasmid (pSM-FeSV.3′) contains the remaining 3′ portion of SM-FeSV, including v-*fms* sequences, the SM-FeSV *env* gene, and the 3′ LTR. In addition to proviral DNA, both subclones contain mink cell sequences that flanked sites of proviral insertion in the cells from which the provirus was originally cloned (7). pSM-FeSV.5′ contains a single *Sac*I site downstream of the LTR and 5′ to the Δ*gag* sequences (7, 11). The plasmid was digested with *Sac*I, treated with the Klenow fragment of DNA polymerase I, and ligated to *Bam*HI linkers. After digestion with *Bam*HI, the plasmid was recircularized, and the modified plasmid was digested with *Cla*I and ligated to *Cla*I-treated pSM-FeSV.3′ DNA to regenerate an intact SM-FeSV provirus. A *Bam*HI fragment of ca. 5 kilobase pairs (kbp) containing the complete Δ*gag-fms* open reading frame was excised and inserted into the unique *Bam*HI cloning site of the *neo*-containing retroviral vector, pZIP*neo*SV(X)-1 (6) (see Fig. 3). Supercoiled plasmids containing the Δ*gag-fms* gene in the sense and nonsense orientations were isolated and purified as previously described (24).

pSM-FeSV.5′ contains two *Nco*I sites, one within host cell sequences flanking the 5′ LTR and the other within the v-*fms* gene, close to the *gag-fms* junction. To insert the v-*fms* gene lacking *gag* sequences into the retroviral vector, pSM-FeSV.5′ was digested with *Nco*I, treated with the Klenow fragment of DNA polymerase I, and ligated to a synthetic *Bam*HI linker (5′-GCGGATCCGC-3′; see Results). After *Bam*HI digestion, the plasmid was recircularized to create a deletion mutant lacking 5′ LTR and Δ*gag* sequences. The modified plasmid was digested with *Cla*I and ligated to pSM-FeSV.3′ as described above, and the *Bam*HI fragment (ca. 3 kbp) containing the v-*fms* gene was excised and inserted in both orientations into pZIP*neo*SV(X)-1 (see Fig. 3).

DNA transfection and isolation of virus-producing cell lines. Plasmid DNA was assayed for transforming activity on NIH 3T3 cells by transfection with calcium phosphate DNA precipitates as previously described (7, 24). An NIH 3T3 cell line (Ψ2) containing a murine leukemia provirus defective in its ability to package viral RNA into virions (15) was transfected by similar methods. Ψ2 cell lines transfected with pZIP*neo-gag-fms* or pZIP*neo*-v-*fms* DNA were treated with G418 (800 μg/ml) 48 h later to select cells that had acquired the *neo*-containing constructs (32). Cells transfected with pSM-FeSV were cotransformed with a *neo*-containing plasmid (pSV*neo*) (32) and selected similarly. Cells cultured in the presence of G418 for 2 weeks were subsequently maintained in medium containing a lower concentration (400 μg/ml) of neomycin. After selection, cultures were subjected to single-cell cloning in microtiter wells. Cells were plated at a limiting dilution in 96-well culture dishes, and colonies derived from single cells were obtained. Ψ2 cells transfected with pSM-FeSV DNA in the absence of pSV*neo* were subjected to single-cell cloning without previous G418 selection.

Virus-producing cell lines were assayed for transforming viruses by titrating filtered culture supernatants onto NIH 3T3 cells as described previously (30). Confluent T75 flasks of Ψ2 producer lines were grown for 18 h in 10 ml of culture medium, and the culture fluids were collected, filtered, and serially diluted into medium containing 1 μg of polybrene per ml (33). NIH 3T3 cells seeded at 10^5 cells per dish (diameter, 60 mm) were infected and fed every 3 days thereafter. Cells were stained with Giemsa to enumerate foci 14 days after infection. All cell cultures were maintained in Dulbecco modified minimal essential medium containing 10% fetal calf serum.

Analysis of v-*fms*-coded glycoproteins. Subconfluent cultures of transformed cells in T75 flasks were incubated in 5 ml of methionine-free medium for 30 min and then labeled for 45 min in 2 ml of the same medium containing 250 μCi of L-[^{35}S]methionine per ml. An equal volume of complete medium was added, and the incubation was continued for an additional 45 min. Cells were rinsed in ice-cold phosphate-buffered saline and lysed in RIPA buffer (50 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 20 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.5% sodium dodecyl sulfate [SDS]) containing 2% aprotinin and 1% phenylmethylsulfonyl fluoride as protease inhibitors. Immune precipitation with antiserum to the v-*fms* gene products or to FeLV *gag*-coded determinants (p30) was performed as described previously (2), and the washed precipitates were denatured and analyzed by electrophoresis on polyacrylamide gels containing SDS (SDS-PAGE) (2). Under the above labeling conditions, all three forms of the v-*fms*-coded glycoprotein were observed, but $p55^{gag}$, which has a short half-life (2), was not readily detected. Shorter labeling intervals were necessary to demonstrate the presence of the *gag*-coded fragment (1, 2, 4, 26). Thus, $p55^{gag}$ was observed in some experiments (see Fig. 5 and 6) but not in others (see Fig. 4).

For labeling in the presence of tunicamycin, cells were incubated in 2 μg of the antibiotic per ml 6 h before and during the 1-h labeling period. Kinetic experiments, digestion of immune precipitates with glycosidases (2), the immune complex kinase assay, flow cytometric detection of cell surface epitopes (24), and cell surface iodination with lactoperoxidase (2) have been described in detail previously. For subcellular fractionation, cells metabolically labeled with [^{35}S]methionine were homogenized in hypotonic buffer; the nuclei were removed, and membranes were recovered by centrifugation (23). The membrane fraction and proteins in the soluble cytosol were treated with RIPA buffer before immunoprecipitation and analysis by SDS-PAGE.

N-terminal protein sequence analysis. Five tissue culture

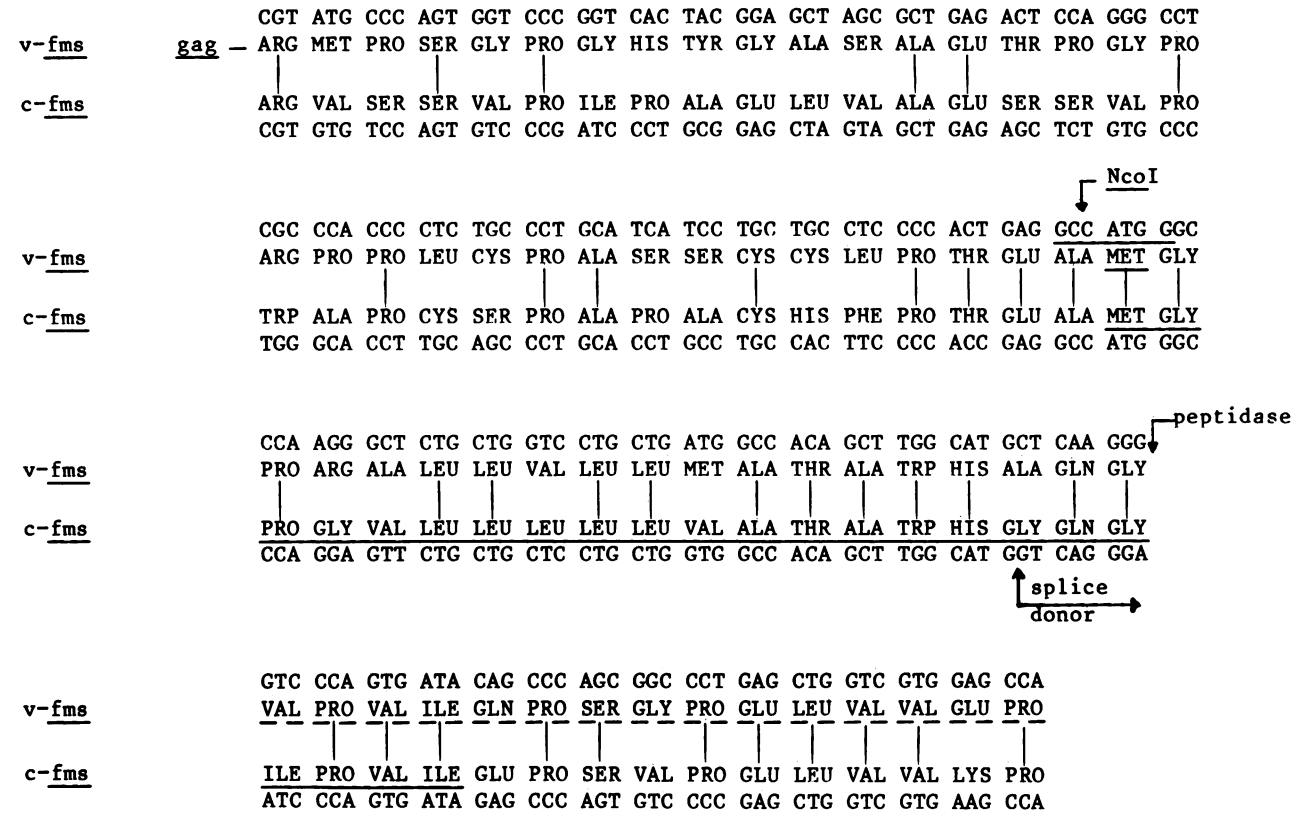

FIG. 1. Homology between 5′ v-*fms* and human c-*fms*. The nucleotide sequence of a portion of the human c-*fms* gene corresponding to the region of recombination between FeLV and feline c-*fms* is shown. The position of the *gag*-v-*fms* junction is marked. The splice donor site marks the end of the sequenced exon in the genomic clone. The nucleotide sequence downstream of the splice donor site was taken from a human cDNA sequence of others and includes a sequence of the next exon (6a). Although the c-*fms* genomic sequences 5′ to the splice site are translated to show their potential homology to v-*fms* amino acids, the first ATG codon in this open reading frame is 102 nucleotides from the site of *gag-fms* recombination (see the text). This ATG codon and the consensus flanking sequences are underlined and include a site of restriction for *Nco*I that was used to construct v-*fms* mutants lacking *gag* sequences. Amino acid sequencing analysis defined the amino termini of gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$ which correspond to a site of proteolytic cleavage (marked peptidase) within a predicted hydrophobic leader peptide (underlined) of the c-*fms* gene product. The sequences in the v-*fms* amino terminus that were experimentally determined are underscored with dashed lines.

dishes (diameter, 150 mm) of confluent, transformed cells were lysed in RIPA buffer and immunoprecipitated with antiserum to v-*fms*-coded determinants. The denatured immunoprecipitates were separated by preparative SDS-PAGE, and gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$, which were visualized by Coomassie blue staining (see Fig. 2, inset), were eluted from the gels (13). The quantity of eluted protein was estimated by comparison with known protein standards run on parallel gels stained with Coomassie blue. The electroeluted samples were precipitated (37) and solubilized in 2% SDS before sequencing. The N-terminal sequences of the eluted proteins were determined by automated Edman degradation on a gas-phase sequencer (model 470A; Applied Biosystems, Inc.) with a 03RPTH run program and the cartridge temperature adjusted to 47°C. The phenyl thiohydantoin residues were identified and quantitated by using an on-line microbore high-pressure liquid chromatography system (model 120A; Applied Biosystems, Inc.). Approximately 40% of the sample was analyzed at each cycle, and the total net yields were calculated after correction for background signals. Repetitive yields were calculated by linear regression analysis for all quantitated residues except the serine residue at position 7.

Molecular cloning and DNA sequence analysis. Recombinant bacteriophages containing human c-*fms* sequences were obtained as previously described (25), and a 5′ 2.6-kbp *Eco*RI fragment containing the site corresponding to the *gag-fms* junction was subcloned into pBR322. The nucleotide sequences of this fragment were determined by the procedure of Maxam and Gilbert (16). Both strands were independently analyzed several times and from different restriction sites. All restriction sites used as starting points were also analyzed as internal sites within overlapping fragments. A portion of the sequence corresponding to a 5′ region of the v-*fms* gene is shown in Fig. 1.

RESULTS

Predicted relationship between the amino-terminal ends of the v-*fms* and c-*fms* gene products. Recombination between FeLV and c-*fms* sequences in normal cat DNA led to the insertion of the v-*fms* gene into the open reading frame of the FeLV *gag* gene (7, 11). Because SM-FeSV encodes a polyprotein containing fused *gag*-coded and v-*fms*-coded amino acids, the amino-terminal end of the c-*fms* gene product appeared to have been replaced by *gag*-coded amino acids. The human c-*fms* sequences have been molecularly cloned (12, 25), and limited nucleotide sequencing analysis

(19, 36) suggested that the human proto-oncogene is closely homologous to SM-FeSV v-*fms* sequences. We therefore reasoned that an analysis of the 5′ coding sequences of the cloned human c-*fms* locus might render insight into the nature of the recombination event that led to the formation of SM-FeSV.

A radiolabeled probe prepared to the most 5′ v-*fms* sequences was used to identify a 2.6-kbp *Eco*RI fragment at the 5′ end of the human c-*fms* locus (12, 25), which was predicted to contain a site analogous to the site of recombination between FeLV and the feline c-*fms* gene. The complete nucleotide sequence of this fragment of human DNA was determined, and relevant portions of the sequence are shown in Fig. 1. A single open reading frame of 229 base pairs containing sequences partially homologous to 5′ v-*fms* sequences was identified and compared with the v-*fms* sequence to define the putative site of FeLV recombination. This comparison showed that the recombination site was 151 base pairs upstream of a splice donor sequence in the c-*fms* gene (Fig. 1). The open reading frame 5′ to the *gag* recombination site included an additional 78 base pairs (not shown in Fig. 1) which were not preceded or interrupted by a canonical splice acceptor site. The simplest interpretation, then, is that the open reading frame represents the first coding exon of the human c-*fms* gene.

The c-*fms* nucleotide sequence shows that the first and only ATG codon in the entire open reading frame was flanked by consensus sequences typical of initiator codons (i.e., GCCATGG, underlined in Fig. 1) (14). The sequence directly downstream of the first ATG triplet encoded a continuous run of hydrophobic amino acids that could serve as the signal peptide for translation of the c-*fms* gene product on membrane-bound polyribosomes. Taken together, these data predict that recombination between FeLV and c-*fms* occurred in a region of the cellular gene encoding 5′ untranslated sequences of c-*fms* mRNA. The SM-FeSV polyprotein should therefore contain 34 amino acids at the *gag-fms* junction which are not ordinarily translated from c-*fms* mRNA. Similar conclusions have been reached by Coussens and co-workers (6a), who recently determined the complete nucleotide sequence of a human c-*fms* cDNA.

An unusual feature of the processing of the glycosylated *gag-fms* polyprotein (gP180$^{gag\text{-}fms}$) is the cleavage of the *gag* portion of the molecule (p55^{gag}) to generate the carboxy-terminal v-*fms*-coded glycoprotein, gp120$^{v\text{-}fms}$ (4, 26). This glycoprotein undergoes modification of its N-linked oligosaccharide chains during intracellular transport to the cell surface, giving rise to a mature glycoprotein of apparently greater molecular weight (gp140$^{v\text{-}fms}$) (1, 2). The *gag* portion of the polyprotein contains a potential signal sequence for translation of gP180$^{gag\text{-}fms}$ on membrane-bound ribosomes (10, 11), which was presumed to direct translocation of the amino-terminal portion of the polypeptide into the cisternae of the endoplasmic reticulum (23). As predicted (28), proteolysis liberates p55^{gag} within the lumen of the endoplasmic reticulum (23). Kinetic experiments indicate that gP180$^{gag\text{-}fms}$, gp120$^{v\text{-}fms}$, and p55^{gag} are metabolically labeled simultaneously and show no discernable precursor-product relationship, suggesting that proteolysis is a cotranslational event (2, 4, 23). To determine the exact site(s) of proteolytic cleavage within gP180$^{gag\text{-}fms}$, gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$ were purified from polyacrylamide gels, and their N-terminal amino acid sequences were determined (Fig. 2). A sequence of 15 amino acids was unambiguously determined, and corresponded exactly to a region of the v-*fms*-coded polypeptide shown in Fig. 1 (dashed underline). The same amino

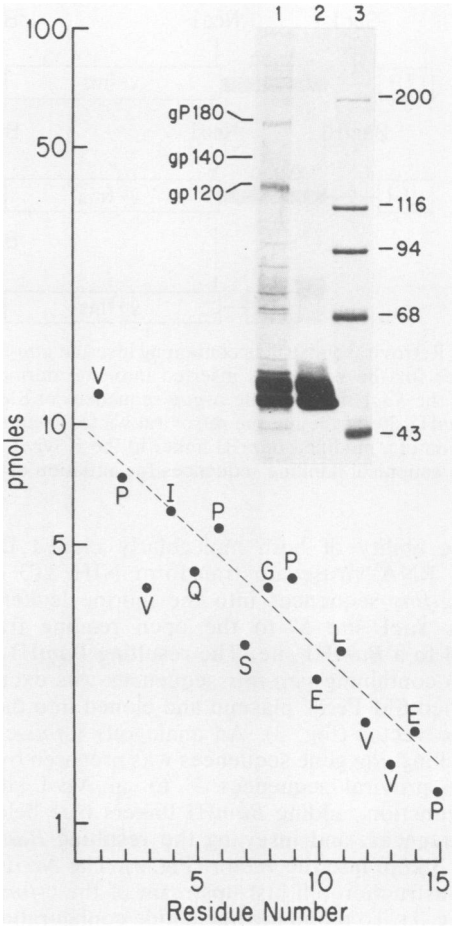

FIG. 2. Determination of the amino-terminal sequences of gp120$^{v\text{-}fms}$. The v-*fms*-coded glycoproteins were immunoprecipitated from lysates of SM-FeSV-transformed mink lung epithelial cells, separated by electrophoresis in SDS-polyacrylamide gels, visualized by staining with Coomassie brilliant blue (insert), and electroeluted from the appropriate gel slice. Approximately 17 pmol of gp120$^{v\text{-}fms}$ was subjected to automated Edman degradation to yield the indicated amino acid sequence based on recoveries calculated by linear regression analysis. Amino acids: E, glutamic acid; Q, glutamine; G, glycine; I, isoleucine; L, leucine; P, proline; S, serine; V, valine. (Insert) Lane 1, precipitates from lysates incubated with polyvalent rat antisera to v-*fms*-coded glycoproteins; lane 2, precipitates obtained with nonimmune rat sera. The mobilities of the v-*fms*-coded glycoproteins are noted in the left margin. The migration of marker proteins of known molecular mass (shown in kilodaltons) are indicated at the right margin.

acid sequence was obtained for gp140$^{v\text{-}fms}$. These results indicate that proteolytic cleavage occurred upstream of a valine residue (marked as a peptidase in Fig. 1) 18 amino acids carboxy terminal to the predicted initiator methionine. An analogous sequence of c-*fms*, predicted from nucleotide sequence analysis of human genomic and cDNA clones, would suggest that this site in gP180$^{gag\text{-}fms}$ corresponds to a putative site of cleavage for a signal peptidase. Thus, the amino-terminal portions of the v-*fms* and c-*fms* products appear to be identical.

Construction of v-*fms* retroviral vectors lacking *gag* sequences. The above results suggested that *gag* gene sequences might not be required for transformation by SM-FeSV. We therefore inserted the v-*fms* gene, with or without *gag* sequences, into a murine leukemia virus vector and

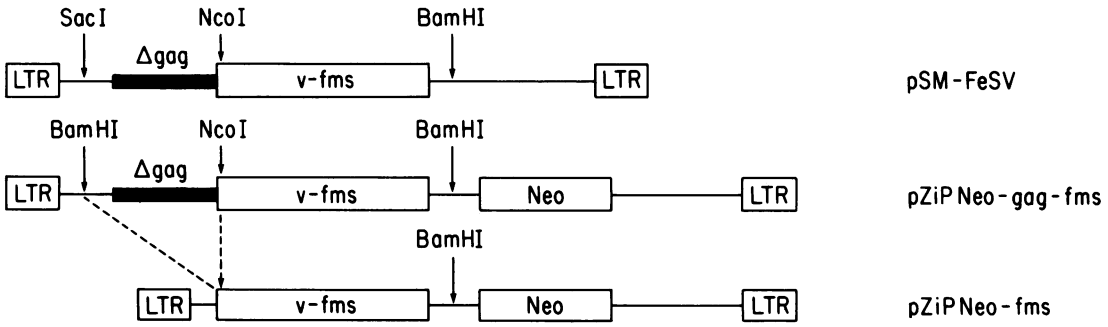

FIG. 3. Retroviral constructs containing inserted *gag-fms* sequences or v-*fms* sequences alone. The SM-FeSV genome is shown as a frame of reference for the sequences inserted into the murine retroviral vector, pZIP*neo*SV(X)-1. The *gag-fms* construct was engineered by converting the *Sac*I site 5′ to the Δ-*gag* sequences of SM-FeSV to a *Bam*HI site, enabling insertion of the Δ-*gag*-v-*fms* sequences into the unique *Bam*HI site of the murine retroviral vector. Sequences encoding only the v-*fms* gene were inserted into the murine retroviral vector after addition of a modified *Bam*HI linker to the 5′ *Nco*I site of the gene. In addition to the *Bam*HI site, the linker contains nucleotides that restore the canonical flanking sequences for initiation of protein synthesis when ligated to the *Nco*I-cleaved 5′ terminus.

tested the ability of both molecularly cloned DNA and rescuable RNA viruses to transform NIH 3T3 cells. To insert *gag-fms* sequences into the murine leukemia virus vector, a *Sac*I site 5′ to the open reading frame was converted to a *Bam*HI site. The resulting *Bam*HI fragment (ca. 5 kb) containing *gag-fms* sequences was excised from the modified SM-FeSV plasmid and cloned into the *Bam*HI site of the vector (Fig. 3). An analogous v-*fms*-containing vector lacking *gag* gene sequences was prepared by deleting SM-FeSV proviral sequences 5′ to an *Nco*I site at the *gag-fms* junction, adding *Bam*HI linkers (see below) 5′ to v-*fms* sequences, and inserting the resulting *Bam*HI fragment (ca. 3 kbp) into the vector (Fig. 3). The *Nco*I site used in this construction fell just upstream of the c-*fms* initiator codon (Fig. 1). To retain the nucleotide configuration around this ATG codon, we appended a palindromic linker sequence (5′-GCGGATCCGC-3′), containing a site of restriction for *Bam*HI, to the blunt-ended *Nco*I site. These manipulations created a *Bam*HI site just 5′ to the *Nco*I site and restored the original initiator consensus sequences. As controls, the 5-kbp *gag-fms* and 3-kbp v-*fms* *Bam*HI fragments were also inserted into the cloning site of the vector in the opposite orientations. The vector used for these studies contained a neomycin resistance gene (*neo*) whose product is encoded by a spliced mRNA (6).

Biological activity of molecularly cloned proviral DNA. DNA-mediated gene transfer was used to introduce the plasmids containing vector DNA into NIH 3T3 cells. Both pZIP*neo-gag-fms* and pZIP*neo-fms* (Fig. 3) produced foci of transformed cells that were morphologically indistinguishable from those obtained in parallel experiments with pSM-FeSV DNA. Foci could be first observed by 8 days and were enumerated 14 days after transfection. The efficiencies of transformation were equivalent for pZIP*neo-gag-fms* and pZIP*neo-fms* and were equal to that obtained with pSM-FeSV (ca. 20,000 focus-forming units per μg of DNA). At low DNA concentrations (0.01 to 0.05 μg per plate), the efficiency of focus formation was independent of the size of the DNA dose, suggesting that, for each of the constructs tested, all of the information required for transformation resided on a single class of DNA molecules. Neither of the vectors containing *gag-fms* or v-*fms* sequences in the opposite orientation gave rise to detectable foci.

To further test the transforming potential of the v-*fms* constructs, pSM-FeSV and the various vector DNAs were transfected into Ψ2 cells (15). The Ψ2 cell line contains an integrated copy of a modified murine leukemia provirus that provides *gag*, *pol*, and *env* gene functions in *trans*. The Ψ2 provirus lacks sequences necessary to package its own RNA into virions so that transfected Ψ2 cells generate helper-free pseudotype viruses carrying retroviral RNA genomes encoded only by vector DNAs. Because Ψ2 cells are morphologically transformed, eliminating the possibility of directly scoring foci after transfection, cells transfected with vector DNAs were treated with G418 to generate colonies expressing the neomycin resistance gene. Since pSM-FeSV lacks a linked Neo^r marker, pSM-FeSV DNA was cotransfected with a separate plasmid (pSV*neo*) (32) containing *neo*. Because both pSM-FeSV and pSV*neo* DNAs were coprecipitated with calcium phosphate, the majority of cells acquiring one plasmid also acquired the other (20).

Filtered supernatants from the transfected, G418-resistant Ψ2 cell cultures were tested for the presence of focus-forming viruses by infection of NIH 3T3 cells. Cells transfected with pSM-FeSV, pZIP*neo-gag-fms*, and pZIP*neo-fms* all produced focus-forming viruses at titers of $\geq 5 \times 10^4$/ml. Cells transfected with vectors containing v-*fms* sequences in the nonsense orientation did not produce detectable transforming virus. G418-resistant Ψ2 cells were subcloned from single cells in microtiter wells, and culture supernatants from the individual cell lines were also screened for virus production. All vector-containing subclones produced transforming viruses, and virus titers in excess of 10^6 focus-forming units per ml were obtained from several independent subclones. Even higher titers (3×10^7 focus-forming units per ml) were obtained with several Ψ2 subclones containing pSM-FeSV. These results, taken together with the data from transfection analyses, indicated that v-*fms* sequences with or without the *gag* gene were equally efficient in the transformation of an established fibroblast cell line.

Analysis of v-*fms* gene products in transformed NIH 3T3 cells. The protein products encoded by the transforming v-*fms* constructs were analyzed by metabolically labeling transfected NIH 3T3 cultures with [^{35}S]methionine for 1.5 h. Cell lysates were prepared and immunoprecipitated with antibodies directed to v-*fms*-encoded or *gag* gene-coded epitopes. The immunoprecipitates were electrophoretically separated in polyacrylamide gels, and labeled proteins were visualized by fluorography. The patterns of v-*fms*-coded polypeptides in cells transformed by pZIP*neo-gag-fms* and pZIP*neo-fms* are shown in Fig. 4A. Like cells transformed

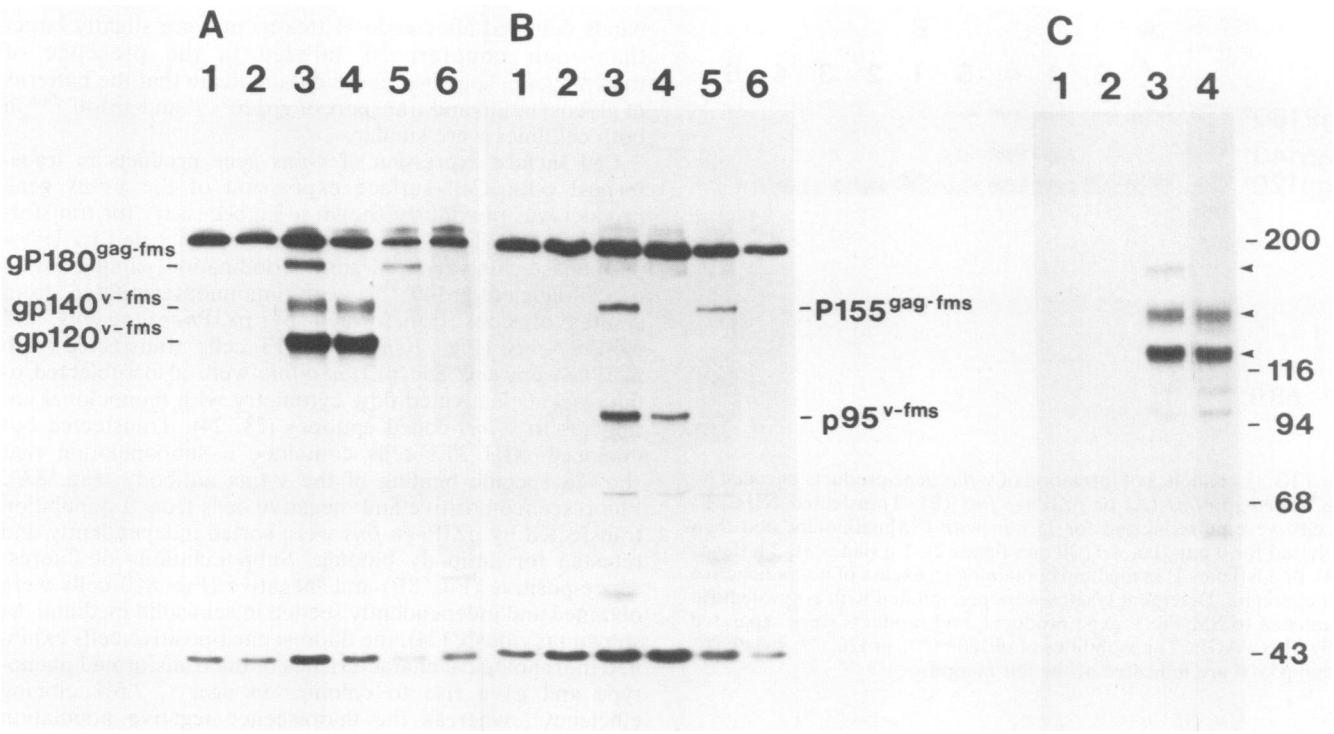

FIG. 4. Transforming gene products of pZIP*neo-gag-fms* and pZIP*neo-fms* constructs. Immunoprecipitated proteins were subjected to SDS-PAGE and detected by fluorography or autoradiography of the dried polyacrylamide slab. (A and B) NIH 3T3 cells transfected with either pZIP*neo-gag-fms* (lanes 1, 3, and 5) or pZIP*neo-fms* (lanes 2, 4, and 6) were metabolically labeled with [^{35}S]methionine for 90 min in either the absence (A) or presence (B) of 2 μg of tunicamycin per ml. Detergent lysates were precipitated with nonimmune rat serum (lanes 1 and 2), polyvalent rat antiserum to SM-FeSV gene products (lanes 3 and 4), or antiserum to FeLV *gag*-coded p30 structural protein (lanes 5 and 6). The mobilities of the v-*fms*-coded glycoproteins are noted at the left margin, and those of the unglycosylated polypeptides labeled in the presence of tunicamycin are indicated at the right margin. (C) Immune complex kinase assays of lysates from NIH 3T3 cells transfected with either pZIP*neo-gag-fms* (lanes 1 and 3) or pZIP*neo-fms* (lanes 2 and 4). Detergent lysates were precipitated with either nonimmune rat serum (lanes 1 and 2) or polyvalant rat antiserum to SM-FeSV gene products (lanes 3 and 4) before the kinase reaction was begun. The mobilities of marker proteins of known molecular mass are indicated in kilodaltons at the right margin, and the v-*fms*-coded glycoproteins (arrows) are indicated.

by SM-FeSV, lysates from cells transformed by pZIP*neo-gag-fms* expressed three forms of the v-*fms*-coded glycoprotein, including gP180$^{gag\text{-}fms}$, gp120$^{v\text{-}fms}$, and gp140$^{v\text{-}fms}$ (Fig. 4A, lane 3). As expected, only the polyprotein precursor was precipitated with antiserum to *gag*-coded determinants (Fig. 4A, lane 5). The p55^{gag} fragment was not readily detected under these labeling conditions (see Methods). In contrast, the lysate from cells transfected with pZIP*neo-fms* contained gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$ at levels comparable to those seen in pZIP*neo-gag-fms* transformants but lacked the gP180$^{gag\text{-}fms}$ polyprotein precursor (Fig. 4A, lane 4). The antiserum to *gag* determinants also failed to precipitate the polyprotein (Fig. 4A, lane 6). In the absence of *gag* sequences, then, the immature glycoprotein, gp120$^{v\text{-}fms}$, and the mature cell surface form of the glycoprotein, gp140$^{v\text{-}fms}$, were each expressed at levels equivalent to those seen in cells transformed by pZIP*neo-gag-fms* (Fig. 4A) or by pSM-FeSV (data not shown). Immunoprecipitated gp120$^{v\text{-}fms}$ molecules encoded by pZIP*neo-gag-fms* and pZIP*neo-fms* were recovered from preparative SDS slab gels and subjected to automated Edman degradation. The amino-terminal sequences of both molecules were identical to that shown for gp120$^{v\text{-}fms}$ of SM-FeSV in Fig. 2.

In the presence of the antibiotic tunicamycin, addition of N-linked oligosaccharides to the v-*fms* gene products is inhibited, and the apparent molecular weights of gP180$^{gag\text{-}fms}$ and gp120$^{v\text{-}fms}$ are reduced by ca. 25 kilodaltons (1, 2). Metabolic labeling in the presence of tunicamycin yielded the expected gene products in cells transformed by pZIP*neo-gag-fms* (Fig. 4B, lanes 3 and 5), whereas only the unglycosylated analog of gp120$^{v\text{-}fms}$ (p95^{fms}) was detected in pZIP*neo-fms* transformants (lanes 4 and 6). The unglycosylated forms of gp120$^{v\text{-}fms}$ in cells transfected with either vector construct were identical in size.

The v-*fms*-coded molecules exhibit an associated tyrosine-specific protein kinase activity that phosphorylates the v-*fms* gene products in immune complexes (3). Immune complexes from cells transfected with pZIP*neo-gag-fms* catalyzed the transfer of the γ phosphate of [γ^{32}P]ATP to the three forms of the glycoprotein (Fig. 4C, lane 3). In contrast, immune complexes prepared from cells containing the *gag*-deleted mutant contained phosphorylated forms of only gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$ (Fig. 4C, lane 4). Phosphoamino acid analyses (not shown) indicate that all forms of the glycoprotein were phosphorylated exclusively on tyrosine residues.

Kinetic studies of the formation of the different v-*fms*-coded polypeptides were performed by labeling cells for 15 min with [^{35}S]methionine, removing the labeling medium, and incubating the cells in complete medium containing an excess of nonradioactive methionine for various periods of time. Cell lysates prepared at various time intervals were precipitated with antiserum to SM-FeSV gene products and analyzed on denaturing polyacrylamide gels. Results obtained with cells transformed by pZIP*neo-gag-fms* are

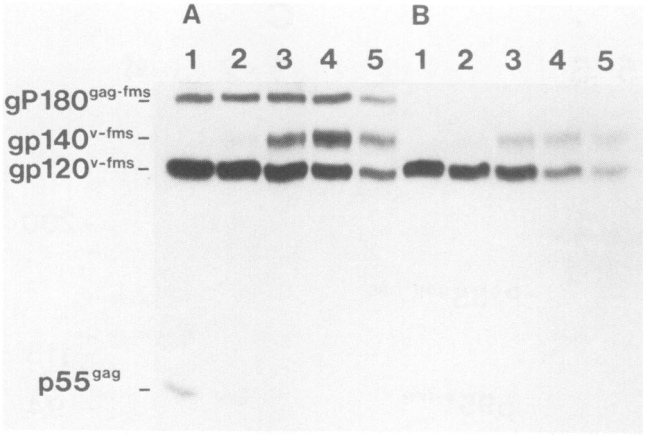

FIG. 5. Kinetics of formation of v-*fms* gene products encoded by pZIP*neo-gag-fms* (A) or pZIP*neo-fms* (B). Transfected NIH 3T3 cells were pulse-labeled for 15 min with [^{35}S]methionine and then chased for 0 min (lanes 1) 30 min (lanes 2), 1 h (lanes 3), 2 h (lanes 4), or 3 h (lanes 5) in medium containing an excess of nonradioactive methionine. Detergent lysates were precipitated with polyvalent rat antisera to SM-FeSV gene products, and products were separated by SDS-PAGE. The mobilities of gP180$^{gag\text{-}fms}$, gp120$^{v\text{-}fms}$, gp140$^{v\text{-}fms}$ and p55^{gag} are indicated at the left margin.

shown in Fig. 5A, lanes 1 through 5. Whereas gP180$^{gag\text{-}fms}$ and gp120$^{v\text{-}fms}$ were visualized after the 15-min labeling period, gp140$^{v\text{-}fms}$ first appeared after 30 min of chase and was subsequently degraded. Note that in contrast to the data of Fig. 4A, the p55^{gag} fragment was detected after a short labeling period and rapidly turned over. Very similar results were previously obtained for other cell lines transformed by SM-FeSV (2, 24, 26). In lysates from cells transfected with pZIP*neo-fms*, gp120$^{v\text{-}fms}$ was rapidly labeled and turned over with similar kinetics (Fig. 5B, lanes 1 through 5). Lysates from the latter cells contained no gP180$^{gag\text{-}fms}$ polyprotein, nor was the p55^{gag} cleavage product evident. Thus, the absence of the *gag* polypeptide did not alter the normal synthesis, processing, or turnover of gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$.

Since the v-*fms* gene products are transmembrane glycoproteins, their synthesis, glycosylation, and transport are membrane-associated processes. Cell lysates prepared from cultures labeled for 30 min with [^{35}S]methionine were separated into membrane and cytosolic fractions and studied for immunoprecipitable products. All of the v-*fms* gene products remained membrane associated regardless of whether they were translated as polyproteins (Fig. 6). Moreover, the p55^{gag} fragment was also recovered exclusively in the membrane fraction in agreement with previous studies (23).

To demonstrate that the v-*fms* gene products from the two transformed cell lines were glycosylated in an equivalent manner, the immunoprecipitated glycoproteins from cultures metabolically labeled with [^{35}S]methionine were subjected to digestion with endoglycosidase H (endo H) or neuraminidase. The N-linked oligosaccharides of the immature glycoprotein, gp120$^{v\text{-}fms}$, are sensitive to digestion with endo H and resistant to neuraminidase, whereas those of the mature glycoprotein, gp140$^{v\text{-}fms}$, show the opposite pattern of sensitivity (2). gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$ from both cell lines showed the expected patterns of sensitivity to these two glycosidases (Fig. 7A and B). In addition, in cells transformed by pZIP*neo-gag-fms*, gP180$^{gag\text{-}fms}$ was sensitive to endo H and resistant to neuraminidase. In all cases, the bands detected after endo H treatment were slightly larger than their counterparts labeled in the presence of tunicamycin. Together, these data indicate that the patterns of glycosylation and transport of gp120$^{v\text{-}fms}$ and gp140$^{v\text{-}fms}$ in both cell lines were similar.

Cell surface expression of v-*fms* gene products in transformed cells. Cell surface expression of the v-*fms* gene product was previously shown to be necessary for transformation (24). When viable cells were radiolabeled by lactoperoxidase-catalyzed cell surface iodination, similar levels of ^{125}I-labeled gp140$^{v\text{-}fms}$ were immunoprecipitated from lysates of cells transformed by pZIP*neo-gag-fms* and pZIP*neo-fms* (Fig. 7C). NIH 3T3 cells transfected with pZIP*neo-gag-fms* and pZIP*neo-fms* were also subjected to fluorescence-activated flow cytometry with monoclonal antibodies to v-*fms*-coded epitopes (23, 24). Transfected but uncloned NIH 3T3 cells contained a subpopulation that showed specific binding of the v-*fms* antibody (Fig. 8A). Fluorescence-positive and -negative cells from a population transfected by pZIP*neo-fms* were sorted independently and retested for antibody binding. Subpopulations of fluorescence-positive (Fig. 8B) and -negative (Fig. 8C) cells were obtained and independently seeded in semisolid medium. As shown previously (24), the fluorescence-positive cells exhibited morphological characteristics of the transformed phenotype and gave rise to colonies in agar (>7.5% cloning efficiency), whereas the fluorescence-negative population failed to form colonies (cloning efficiency, <0.5%). Thus,

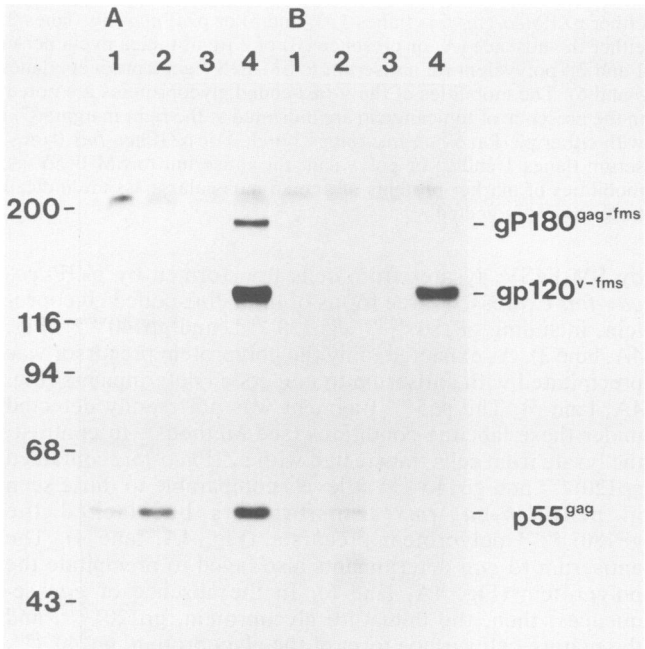

FIG. 6. Subcellular fractionation of newly synthesized v-*fms* gene products encoded by pZIP*neo-gag-fms* (A) and pZIP*neo-fms* (B). Transfected NIH 3T3 cells were labeled with [^{35}S]methionine for 30 min and then homogenized in hypotonic buffer in the absence of detergent. Cytosolic (lanes 1 and 2) and membrane (lanes 3 and 4) fractions were prepared by sedimentation, lysed in detergent, and precipitated with either nonimmune rat serum (lanes 1 and 3) or polyvalent antiserum to SM-FeSV gene products (lanes 2 and 4). Products were separated by SDS-PAGE and detected by fluorography. The mobilities of marker proteins of known molecular mass are indicated in kilodaltons at the left margin, and those of the membrane-associated gP180$^{gag\text{-}fms}$, gp120$^{v\text{-}fms}$, and p55^{gag} are at the right margin.

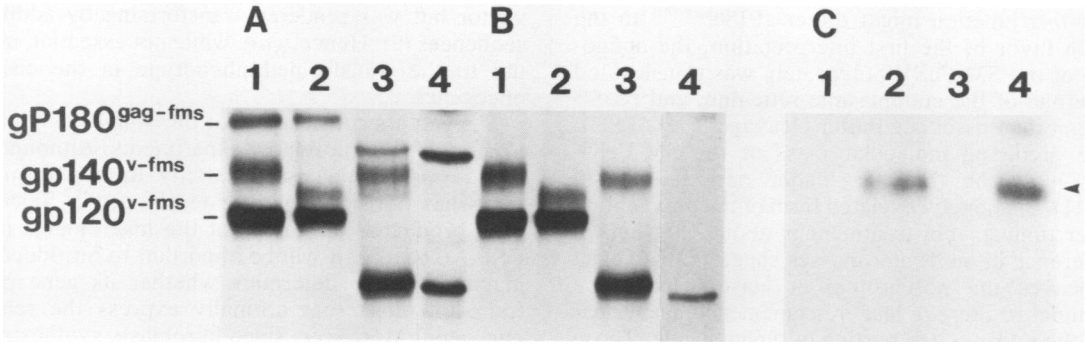

FIG. 7. Processing and cell surface expression of v-*fms* gene products in NIH 3T3 cells transfected with pZIP*neo* constructs. (A and B) Cells transfected by pZIP*neo-gag-fms* (A) or pZIP*neo-fms* (B) were metabolically radiolabeled with [^{35}S]methionine and immunoprecipitated with antiserum to v-*fms*-coded glycoproteins. Immune complexes were incubated with no enzyme (lanes 1), neuraminidase (lanes 2), or endo H (lanes 3) for 20 h at 37°C. In each panel, unglycosylated v-*fms*-coded gene products labeled in the presence of tunicamycin are shown in lane 4. (C) Lactoperoxidase-catalyzed surface radioiodination of cells transfected by pZIP*neo-gag-fms* (lanes 1 and 2) or pZIP*neo-fms* (lanes 3 and 4). Detergent lysates were precipitated with nonimmune rat serum (lanes 1 and 3) or antiserum to SM-FeSV gene products (lanes 2 and 4). The mobility of gp140$^{v\text{-}fms}$ is indicated by the arrow at the right margin.

cells transformed by pZIP*neo-fms* encoded a mature glycoprotein whose expression at the cell surface correlated with the transformed phenotype. We conclude that, for each of the parameters tested, cells transformed by the *gag*-negative mutant were biologically similar to those transformed by SM-FeSV.

DISCUSSION

The N-terminal amino acid sequences of the c-*fms* gene product appear to have been retained in the recombination event that generated SM-FeSV. The SM-FeSV-coded polyprotein, gP180$^{gag\text{-}fms}$, contained *gag*-specified amino acids as well as 34 amino acids encoded by a 5′ region of the v-*fms* gene that is not normally translated. Proteolytic cleavage at a site near the *gag-fms* junction removed the latter amino acids and generated a v-*fms*-coded glycoprotein, gp120$^{v\text{-}fms}$, whose amino terminus appeared to be identical to its c-*fms*-coded counterpart. The site of cleavage within gP180$^{gag\text{-}fms}$ was 53 amino acids carboxy terminal to the *gag-fms* junction and occurred just downstream of a 19-amino acid hydrophobic leader that is predicted to represent the amino-terminal signal peptide of the c-*fms* gene product. Thus, cleavage of the SM-FeSV polyprotein occurred as a cotranslational event and appeared to be mediated by signal peptidase.

The FeLV *gag* gene has two 5′ methionine codons separated by an open reading frame for 77 amino acids that includes a hydrophobic signal sequence for membrane insertion (10). The second methionine codon marked the 5′ end of the sequence encoding the most amino-terminal *gag* polypeptide, p15. FeLV encodes a major *gag* gene precursor that is processed into virion structural proteins as well as a minor *gag*-coded glycoprotein that becomes membrane associated (29). Presumably, the ratio of the two *gag* gene products is determined by differential translational starts at the two initiation codons. Both methionine codons were represented in the 5′ *gag* sequences of SM-FeSV but, unlike FeLV, the first might serve as the preferred site of initiation of protein synthesis. Alternatively, the internal hydrophobic sequence

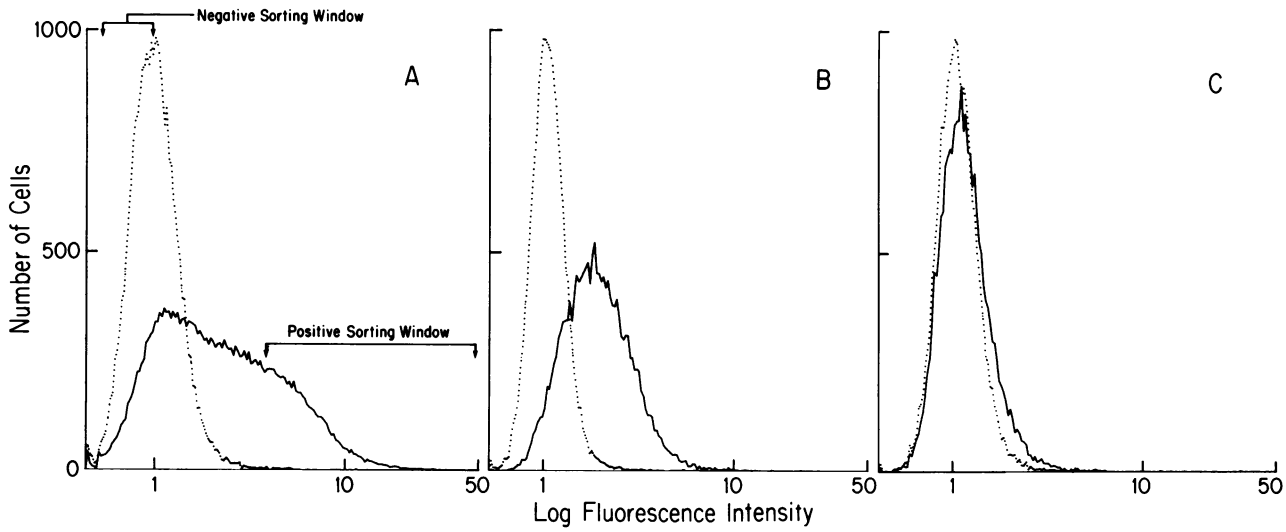

FIG. 8. Cell surface expression of v-*fms*-coded antigens on NIH 3T3 cells transfected with pZIP*neo*-v-*fms*. Cell cultures were reacted with a monoclonal antibody (SM 2.6.3) to a v-*fms*-coded epitope (1, 24) and subjected to flow cytometric analysis. (A) Fluorescence profile of uncloned NIH 3T3 cells transfected with pZIP*neo*-v-*fms*. The indicated positive (B) and negative (C) subpopulations were collected and resorted.

near the *gag-fms* junction might direct gP180$^{gag\text{-}fms}$ to the membrane. In favor of the first interpretation, the amino-terminal end of the SM-FeSV polyprotein was translocated into the cisternae of the endoplasmic reticulum and p55^{gag} remained membrane associated after cleavage (Fig. 6) (23). Whereas the predicted molecular mass of the SM-FeSV polyprotein, including the *gag* leader peptide, is 170 kilodaltons (11), the nonglycosylated form of the polypeptide detected after tunicamycin treatment is about 155 (Fig. 7) (1). This difference in molecular masses suggests that signal peptidase cleaves the polyprotein at least twice, once carboxy terminal to the *gag* leader segment and again near the 5′ end of the v-*fms*-coded portion of the molecule. Those gP180$^{gag\text{-}fms}$ molecules that do not undergo cleavage at the *gag-fms* junction persist within the cell for several hours and are slowly degraded (2, 4). Thus, proteolytic processing must occur cotranslationally or not at all.

We prepared murine retroviruses containing either the *gag-fms* or the v-*fms* sequence alone, either of which was active in transformation. The vector lacking the *gag* gene was engineered so as to remove all sequences of the SM-FeSV polyprotein 5′ to the cryptic initiation codon at the *gag-fms* junction. The construct lacking *gag* sequences was as efficient as its *gag-fms* counterpart in transforming NIH 3T3 cells after either DNA transfection or viral infection. The *gag*-negative mutant encoded a glycoprotein indistinguishable from gp120$^{v\text{-}fms}$ in each of its properties. The polypeptide was cotranslationally glycosylated, transported to the cell surface, similar in its turnover to the wild-type glycoprotein, and equally active as a tyrosine-specific protein kinase in immune complexes. The normal pattern of processing, transport, and cell surface expression of the mutant gene product implies that a functional hydrophobic signal sequence in the v-*fms* glycoprotein was unmasked by the *gag* deletion. This strongly supports the prediction that the recombination event between FeLV and the c-*fms* gene left the key amino-terminal features of the c-*fms* gene product intact. If genetic alterations in c-*fms* are required to activate its potential as an oncogene, these changes are most likely located in other regions of the gene. One such difference involves the carboxy-terminal ends of the v-*fms* and c-*fms* gene products, the former glycoprotein being truncated with respect to the latter (6a, 11).

The present experiments demonstrate that pZIP*neo-fms* contained all the v-*fms* coding sequences necessary to generate the intact transforming glycoprotein. Conceivably, initiation at the same methionine codon in wild-type SM-FeSV could directly result in the production of gp120$^{v\text{-}fms}$ molecules. Because the v-*fms* coding sequences are in the same reading frame as those of *gag*, de novo synthesis of gp120$^{v\text{-}fms}$ from SM-FeSV mRNA would require internal in-frame initiations, which are very rare in eucaryotic systems (14). Furthermore, spliced subgenomic mRNAs containing v-*fms* but lacking *gag* gene sequences are absent in SM-FeSV transformed cells (7). The presence of the p55^{gag} fragment, which is membrane-associated and rapidly degraded, suggests that gp120$^{v\text{-}fms}$ molecules are generated in SM-FeSV-transformed cells by cotranslational proteolysis of the gP180$^{gag\text{-}fms}$ polyprotein.

Several other oncogenes were transduced into the *gag* genes of retroviruses, and, at least in some systems, *gag* sequences are dispensable for transformation (8, 18). In the case of the avian Fujinami sarcoma virus, removal of *gag* gene sequences from the v-*fps* oncogene did not abrogate its transforming function (8). Interestingly, the c-*fps* gene product was nontransforming when tested in a similar retroviral vector but was rendered transforming by addition of *gag* sequences (9). Hence, *gag*, while not essential, may contribute to the transformed phenotype in the case of other oncogenes.

The vectors constructed for our studies may prove particularly useful for unrelated experiments. Although SM-FeSV transforms fibroblasts, its ability to transform other cell types has not been investigated in detail. Since the v-*fms* gene product is an analog of the macrophage receptor for CSF-1 (26a, 31), it will be important to introduce v-*fms* into macrophages to determine whether its gene product can transform cells that normally express the related proto-oncogene. Moreover, since fibroblasts synthesize CSF-1, it is conceivable that cells that do not produce the growth factor will be refractory to transformation. The ability to express v-*fms* in a murine retroviral vector containing a selectable marker should greatly facilitate such studies.

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