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"Replacement" of COOH-terminal truncation of v-*fms* with c-*fms* sequences markedly reduces transformation potential

(protooncogenes/gene transfer/cell transformation)

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ABSTRACT **Protooncogenes when transduced by retroviruses may undergo structural modifications that render their gene products oncogenic. The c-*fms* gene encodes a transmembrane protein with tyrosine kinase activity that is very similar or identical to the receptor for the monocyte-macrophage colony-stimulating factor. Its transforming homologue (v-*fms*) in the Susan McDonough strain feline sarcoma virus causes fibrosarcomas in cats. Molecular cloning and sequence analysis of the cDNA that encodes the cytoplasmic domain of the human c-*fms* gene shows that the product of the transduced viral homologue, v-*fms*, is truncated at the COOH-terminal end. The COOH-terminal 40 amino acids of the c-*fms* gene product are replaced in the v-*fms* gene product by 11 amino acids encoded by the retroviral genome. Hybrid v-*fms*/c-*fms* genes, in which either the entire cytoplasmic domain or the COOH-terminal coding sequences of the v-*fms* gene were replaced by the corresponding segments of the c-*fms* gene, had a reduced ability to transform fibroblasts despite a high level of encoded protein on the cell surface. These data indicate that the COOH-terminal modifications contribute to the transforming potential of the v-*fms* viral oncogene product.**

Protooncogenes have critical roles in cell growth and differentiation. However, when transduced by retroviruses, protooncogenes are modified to yield transforming oncogenes (1, 2). The introns of the cellular genes are removed by RNA splicing of the retroviral genome. More important are alterations in the coding regions—e.g., modifications of the 5′ and/or 3′ end of the coding sequence, deletions, and point mutations. Recombination with coding regions of a retroviral genome may generate a translation product that is a fusion protein. Thus, retroviral oncogenes exhibit several structural differences from their cellular homologues potentially relevant to their capacity for cell transformation. Our work has focused on the comparison of the c-*fms* protooncogene to its transforming retroviral homologue, v-*fms*.

The c-*fms* protooncogene encodes a 165-kDa transmembrane glycoprotein that is very similar or identical to the receptor for a hematopoietic growth factor, monocyte-macrophage colony-stimulating factor (M-CSF) (3). M-CSF acts on progenitor and mature cells of the monocyte-macrophage lineage to induce cell proliferation, maintain cell viability, and induce biochemical differentiation (4–7). The c-*fms* gene product is also expressed in placenta and choriocarcinoma cells and indeed may have a wider role in cell growth and differentiation beyond the monocyte-macrophage lineage (8, 9). The v-*fms* oncogene is part of the genome of the Susan McDonough strain of feline sarcoma virus (SM-FeSV), a virus that causes fibrosarcomas in cats (10–12). The v-*fms* gene product is synthesized as part of a 180-kDa fusion protein that includes NH_2-terminal sequences encoded by the *gag* region of the retroviral genome. The 180-kDa product is enzymatically cleaved to yield a partially glycosylated 120-kDa protein; that is further glycolsylated to yield a transmembrane glycoprotein of 140 kDa that binds M-CSF (13) and is found in clathrin-coated pits and endocytic vesicles (14). Membrane expression of the v-fms protein is necessary for cell transformation (14–18). Both the v-*fms* and c-*fms* gene products have tyrosine kinase activity (19, 20).

Comparison of other cellular and viral oncogene homologues within the tyrosine kinase family reveal differences at the 5′ or 3′ end or point mutations relevant to the transforming potential of the viral oncogene (21–32). We have molecularly cloned the cDNA sequences encoding the transmembrane and cytoplasmic domains of the human c-*fms* gene product. Structural and functional comparison suggest that modifications at the COOH-terminal end of the v-*fms* oncogene are relevant to its transforming potential.

MATERIALS AND METHODS

Isolation and Structural Characterization of the Human c-fms cDNA. A library of human liver cDNA, cloned into λGT11 (Meloy Laboratories, Springfield, VA) was screened with nick-translated DNA fragments (33, 34) of the v-*fms* oncogene (11). Hybridization and washing conditions were as described (29) except that the filters were washed at 65°C. DNA fragments were isolated from phage clones positive on tertiary screening, subcloned into pUC12, mapped using several restriction enzymes, and sequenced, with the dideoxy-chain termination (35, 36) and Maxam–Gilbert (37) methods of analysis. To create hybrid v-*fms*/c-*fms* genes, specific restriction endonuclease fragments derived from the human c-fms cDNA were substituted for the corresponding fragments of the v-*fms* gene in the SM-FeSV proviral genome (11).

Analysis for Cell Transformation. NIH 3T3 cells were passaged in medium containing 10% fetal calf serum, while the transforming focus assay (38, 39) was performed in 2.5% fetal calf serum. Plasmid DNA was introduced by the calcium phosphate precipitation technique (40). Four hours after transfection, the precipitate was removed and cells were given a glycerol shock (41). Twenty-four hours later the cells were trypsinized and split 1:8. After 10–14 days of incubation, transformed foci were scored by staining the plates with Giemsa solution. In certain experiments, 10^6 cells were harvested 48 hr after transfection by trypsinization and injected into nude mice (42, 43). The animals were scored for tumor formation 6 weeks later. Clones expressing either the v-fms protein or one of the hybrid v-fms/c-fms proteins were obtained after cotransfection of NIH 3T3 cells with a plasmid

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Abbreviations: M-CSF, monocyte-macrophage colony-stimulating factor; SM-FeSV, Susan McDonough strain feline sarcoma virus; kb, kilobase(s).

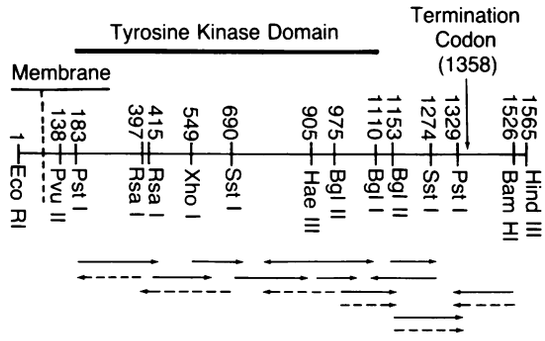

FIG. 1. Restriction endonuclease map and sequencing strategy for the c-fms cDNA clone. Shown above the map are the landmarks of protein structure; the transmembrane and tyrosine kinase domains and the termination codon. The positions of these landmarks were deduced from the DNA sequence and its comparison to the v-*fms* gene (12). The numbering system begins arbitrarily at the 5′ end of the cDNA clone. The segment at the right end of the map between the *Bam*HI and *Hin*dIII sites is part of the polylinker of the pUC vector into which the *Eco*RI/*Bam*HI c-*fms* fragment had been subcloned. The solid and dashed lines shown below indicate opposite strands read from the DNA sequencing gels.

containing the appropriate proviral genome and pSV2neo with subsequent selection in geneticin (G-418) (44).

Characterization of the Hybrid v-fms/c-fms Protein. A rat monoclonal antibody (2.6.3) directed against the external domain of the v-fms protein was obtained from C. Sherr (45). For immunofluorescence analysis, goat anti-rat IgG labeled with fluorescein isothiocyanate was used, whereas unlabeled goat anti-rat IgG was used for immunoprecipitations (Calbiochem). For flow cytometric analysis with the Epics 753 system, single cell suspensions were prepared by exposure of monolayers to phosphate-buffered saline (PBS) without calcium and magnesium, and containing 1 mM EDTA. These cells were incubated at 4°C with the monoclonal antibody, 2.6.3, and then with the anti-rat IgG fluorescein isothiocyanate (46, 47). For analysis of protein synthesis, confluent monolayers of cells were incubated with [^{35}S]methionine (500 μCi/ml; >1000 Ci/mmol; 1 Ci = 37 GBq) in methionine-free medium for 3 hr. Immunoprecipitates were formed from cell extracts by standard methods (15–17, 45) and analyzed on a 7.5% polyacrylamide gel.

RESULTS

Isolation and Characterization of a c-fms cDNA Clone. A 1.53-kilobase (kb) c-fms cDNA clone was isolated from the human liver cDNA library (Fig. 1). Using the strategies displayed in Fig. 1, the DNA sequence of 1346 nucleotides was determined extending from the *Pst* I site (position 183) to the *Bam*HI site. After this work was completed, the sequence of the human c-fms cDNA was published (48). The region we sequenced is homologous to the entire cytoplasmic domain and transmembrane portion of the v-*fms* encoded protein.

Shown in Fig. 2 is a comparison of the v-*fms* and c-*fms* COOH-terminal coding sequences beginning at a position corresponding to nucleotide 1059 on Fig. 1. There was 92% homology at the nucleotide sequence level and 95% homology at the amino acid sequence level (data not shown) until the sequences diverge near the 3′ end of the coding region. The homology ends 33 nucleotides before the v-fms termination codon. Hence the last 11 amino acids of the v-fms protein are different from the amino acids found in the corresponding region of the c-fms protein. The coding region of the c-*fms* gene continues 87 nucleotides beyond the point that corresponds to the end of the coding region of the v-*fms* gene. Therefore, the c-fms protein is 29 amino acids longer at the COOH-terminal end than the v-*fms* gene product.

Transforming Potential of the v-fms Protein Requires the COOH-Terminal Modifications. Hybrid genes were created that combine coding sequences of both genes (Fig. 3). Two constructions were made; in the first, the coding sequences for the entire cytoplasmic domain of the v-fms protein were replaced with the corresponding coding sequence of the c-*fms*

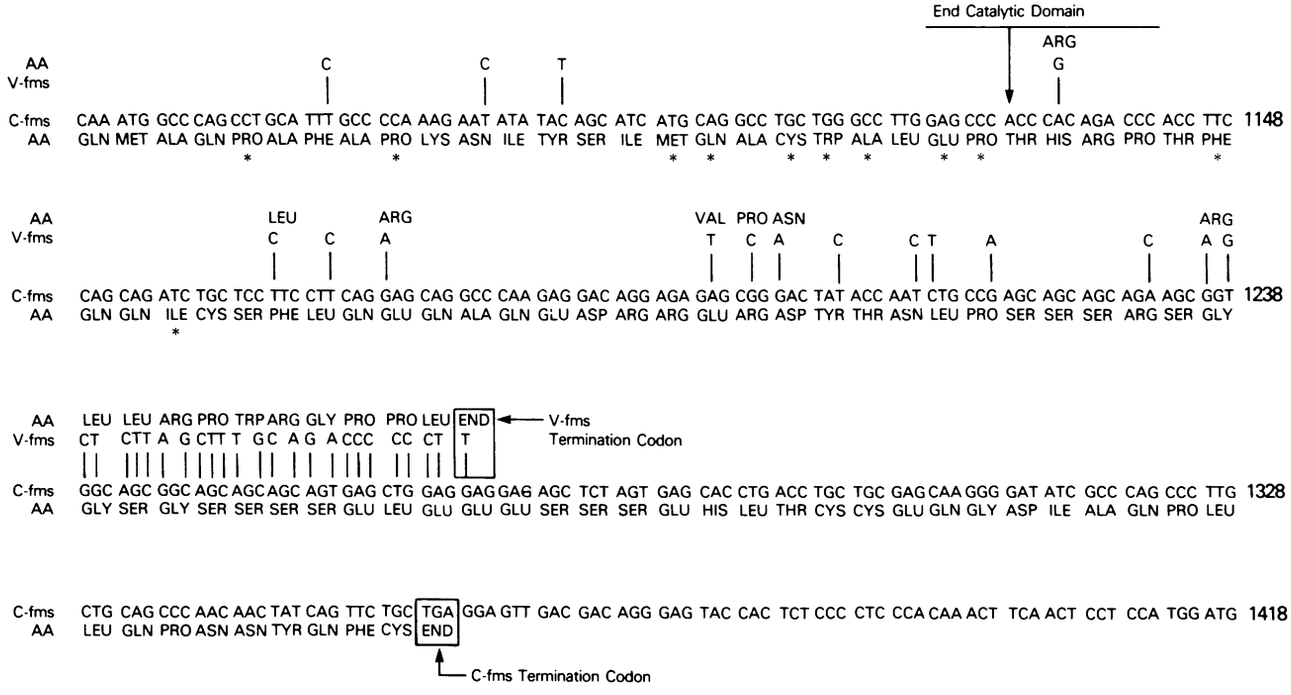

FIG. 2. A comparison of the c-*fms* and v-*fms* coding sequences corresponding to the COOH-terminal regions of the protein. The nucleotide sequence begins at position 1059 on the map shown in Fig. 1. This position corresponds to amino acid 1364 in the v-*fms* sequence as published by Hampe *et al.* (12). Asterisks below amino acids indicate conserved residues in proteins with tyrosine kinase activity (30, 55). Nucleotide 1110 is the *Bgl* I site used to construct PVC-196.

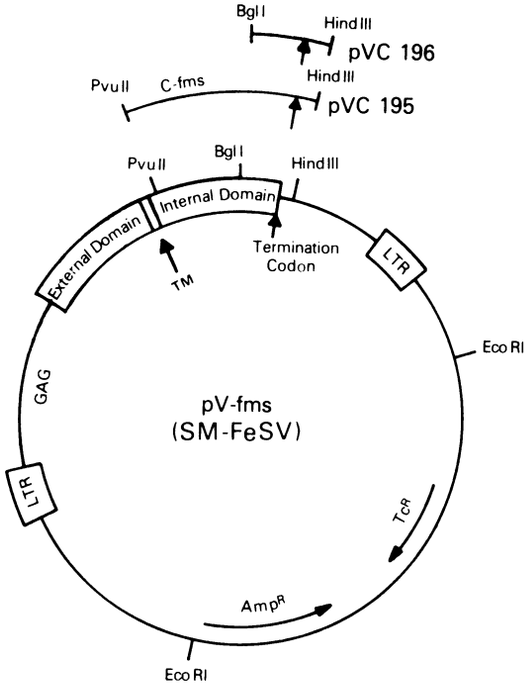

FIG. 3. Construction of the v-*fms*/c-*fms* hybrid genes. The constructions were made in the parent plasmid, pSM-FeSV (10, 11), that contains the entire proviral genome. pVC-195 as constructed in two steps. First, the 1365-base-pair *Pvu* II/*Hin*dIII fragment containing the c-*fms* coding sequences (Fig. 1) was subcloned into a 9.3-kb vector fragment that extends from the *Pvu* II site in the coding sequences to the *Hin*dIII site in the plasmid vector of pSM-FeSV. In the second step, the 4.6-kb *Hin*dIII fragment that includes the 3' long terminal repeat (LTR) was added. pVC-196 was also constructed in two steps. In the first, an 8.8-kb *Cla* I/*Hin*dIII vector fragment, a 2.1-kb *Cla* I/*Bgl* I fragment containing most of the coding sequences of the v-*fms* gene, and a 0.45-kb *Bgl* I/*Hin*dIII fragment from the c-*fms* gene (Fig. 1) were ligated together and a subclone was obtained. To this subclone was added the 4.6-kb *Hin*dIII fragment that includes the 3' LTR. The structure of pVC-195 and pVC-196 was verified by a series of restriction endonuclease digestion. Particularly useful were *Sst* I and *Xho* I; these enzymes cut at different sites in the c-*fms* compared to the v-*fms* coding sequences.

gene (Fig. 3, pVC-195); in the second, the v-fms tyrosine kinase domain was left intact and only the coding sequences for the COOH-terminal end of the protein were replaced with the corresponding portion of the c-*fms* gene (Fig. 3, pVC-196).

Neither pVC-195 or pVC-196 exhibited significant transforming potential (Fig. 4 and Table 1). Various proportions of pSM-FeSV DNA were mixed with either pVC-195 or pVC-196 DNA and a total of 20 μg was used to transfect 10^6 3T3 cells. The number of foci observed was directly proportional to the content of pSM-FeSV DNA (Fig. 4). The hybrid v-*fms*/c-*fms* genes lacked transforming potential when assayed *in vivo* in nude mice. All mice injected with 3T3 cells transfected with pSM-FeSV developed tumors, whereas no tumors were formed in mice injected with cells transfected with either pVC-195 or pVC-196 (Table 1).

The Hybrid v-fms/c-fms Proteins Are Processed and Expressed at the Cell Surface. The proviral genome contained in plasmid pSM-FeSV, pVC-195, or pVC-196 was introduced into 3T3 cells by cotransfection with pSV2neo and selection in G-418. After 12 days, cell-surface expression of the fms proteins was determined on cells derived from ≈100 G-418-resistant colonies using the fluorescence-activated cell sorter (Fig. 5). There was a significant shift in the mean fluorescence of cells transfected with plasmids containing the v-*fms* oncogene or one of the hybrid v-*fms*/c-*fms* genes compared

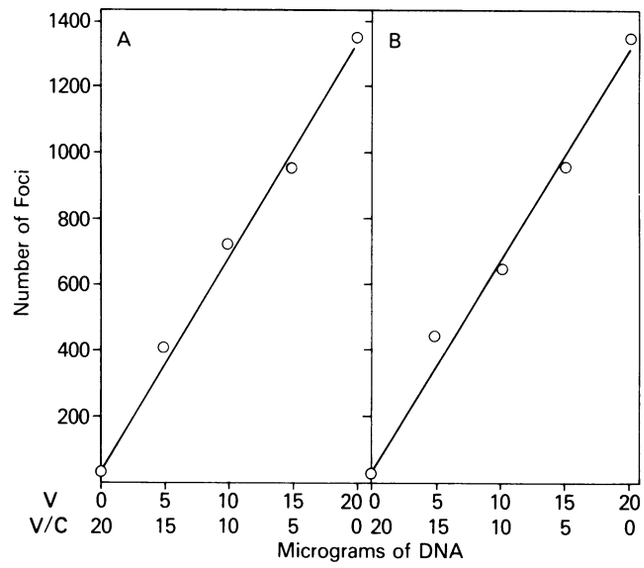

FIG. 4. Assay of the transforming potential of the v-*fms* and hybrid v-*fms*/c-*fms* genes by focus formation in 3T3 cells. Cells were transfected with the indicated proportions of pSM-FeSV and pVC-195 (*A*) or pVC-196 (*B*). Foci were scored 10 days later.

to fluorescence of control cells that had not been incubated with the *fms*-specific monoclonal antibody. In contrast, cells transfected with pSV2neo alone exhibited no shift in mean fluorescence when the *fms*-specific antibody was added (Fig. 5*A*).

Individual clones of G-418 cells expressing either the v-fms or hybrid v-fms/c-fms proteins were isolated. Immunoprecipitates of [^{35}S]methionine-labeled cell extracts were analyzed by NaDodSO$_4$ gel electrophoresis. A band analogous to the glycoprotein 120-kDa v-*fms*-encoded protein was seen in cells expressing the hybrid v-*fms*/c-*fms* genes, although its molecular mass was increased slightly, consistent with its longer COOH terminus. The glycoprotein 140-kDa band was obscured by a comigrating radiolabeled band seen in extracts of control 3T3 cells (Fig. 6, lane 1), although a distinct glycoprotein 180-kDa band was observed in extracts of cells expressing the v-fms or a hybrid v-fms/c-fms protein (lanes 2 and 3). These data indicate that the hybrid v-fms/c-fms proteins are processed and expressed on the cell surface in a manner quite analogous to that of the v-*fms* oncogene product. Expression of the hybrid v-fms/c-fms proteins resulted in a reduced capacity to induce cellular transformation.

DISCUSSION

The v-fms protein is modified at the COOH-terminal end compared with the protein encoded by the c-*fms* gene. The

Table 1. Comparison of transformation potential of the v-fms and hybrid v-fms/c-fms proteins

Plasmid	3T3 cell foci*	Tumor formation in nude mice†
pSM-FeSV	1200 ± 150	5/5
pVC-195	30 ± 4	0/5
pVC-196	32 ± 4	0/5

*Expressed as foci per 20 μg of DNA per 10^6 cells. This assay was performed four times, and the number of foci shown represent the mean ± SD.

†Cells (10^6) transfected with 20 μg of DNA were injected subcutaneously into each animal and scored for the tumor formation after 6 weeks.

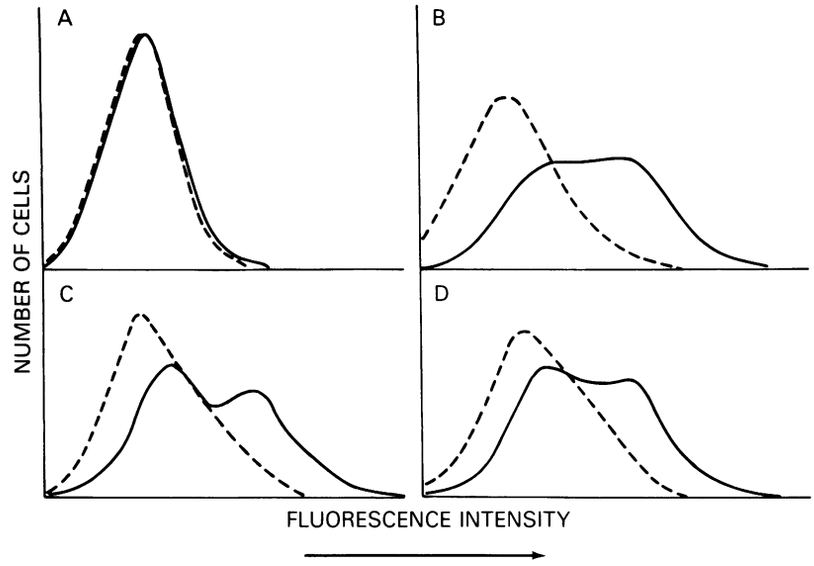

FIG. 5. Surface expression of the v-fms and hybrid v-fms/c-fms proteins as determined by fluorescence analysis. In each experiment, 2×10^6 3T3 cells (two 10-cm plates) were transfected with 20 μg of pSV2neo (*A*), or 4 μg of pSV2neo and 16 μg of pSM-FeSV (*B*), pVC-195 (*C*), or pVC-196 (*D*). After 24 hr, each plate was split 1:4 and G-418 was added to a concentration of 1 mg/ml. The medium was changed every 3 days; after 9 days, there were 40–50 G-418-resistant colonies per plate. The colonies were removed by trypsinization, passaged, and grown again to confluency prior to harvesting for fluorescence analysis. The cells were recovered in PBS containing EDTA and prepared for flow cytometric analysis. Shown are the results obtained with cells that underwent reaction with the fluorescein isothiocyanate goat anti-rat IgG only (---) or first with the fms-specific monoclonal antibody 2.6.3 followed by the fluorescein isothiocyanate-labeled antibody (——).

terminal 40 amino acids of the c-fms-encoded protein are replaced by 11 different amino acids in the v-fms protein. Other than the striking changes at the COOH-terminal end, the cytoplasmic domains of the v-*fms*- and c-*fms*-encoded products are highly conserved, including those residues that are part of the tyrosine kinase catalytic unit. Reconstruction of the fms gene to "correct" the COOH-terminal modifications by assembly of hybrid v-*fms*/c-*fms* genes reduced the transforming potential. Tumor formation in nude mice was abolished. This reduction of transforming capacity occurred even though the hybrid v-fms/c-fms proteins were expressed on the cell surface as transmembrane proteins. In addition, the v-fms/c-fms hybrid proteins have been shown to exhibit *in vitro* protein kinase activity (D. Kaplan and P.J.B., unpublished observations).

The protein encoded by c-*fms*, possibly the M-CSF receptor (3, 8, 9, 20), has many structural similarities to the epidermal growth factor (EGF) receptor (29). Both are transmembrane proteins with a large external ligand binding domain and a cytoplasmic domain that contains a tyrosine kinase catalytic unit. Beyond the catalytic domain there are COOH-terminal segments of 254 amino acids in the EGF receptor and 74 amino acids in the M-CSF receptor. The EGF receptor gene has been transduced at least twice to create a retroviral oncogene, v-*erbB* (49, 50). The v-*fms* gene product resembles v-*erbB* in that both have undergone COOH-terminal modifications compared to the protein encoded by their cellular homologues. The terminal 32 amino acids of the EGF receptor are replaced by 4 amino acids encoded by the retroviral genome in the transforming v-*erbB* gene product (29). A similar modification has occurred on transduction of the c-*src* gene to create the v-*src* oncogene (23). Indeed, a tyrosine residue near the COOH-terminal end of c-*src* undergoes phosphorylation; this residue is absent in v-*src* (51). COOH-terminal modifications may contribute to oncogenic potential by eliminating the dependence on ligand binding for catalytic activity, by altering access of substrate to the catalytic domain, or by changing substrate specificity.

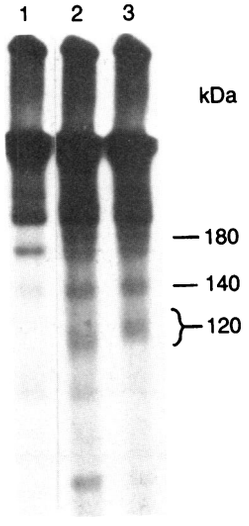

FIG. 6. Synthesis and processing of the v-fms and hybrid v-fms/c-fms proteins. Radiolabeled immunoprecipitates from control 3T3 cells (lane 1), from cells expressing the v-*fms* gene (lane 2), or a hybrid v-*fms*/c-*fms* gene (pVC-196) (lane 3) were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The molecular masses shown on the right were estimated by comparison to the migration position of simultaneously run high molecular weight protein standards (Bethesda Research Laboratories).

In most retroviral oncogenes, transduction is accompanied by several alterations in the coding sequences of the cellular homologue, any or all of which may contribute to transforming potential (21–32). In contrast to v-*erbB*, in which the external domain is missing, most or all of the ligand binding domain of c-*fms* is structurally preserved in v-*fms* (11, 48) and has been shown to bind M-CSF (13). Unlike the transmembrane and tyrosine kinase domains, the ligand-binding domain of human c-*fms* when compared to v-*fms* has 25% scattered amino acid differences. The c-*fms* sequence data are from the human genome, whereas the v-*fms* gene was transduced from the cat genome. Many of the amino acid differences are likely to represent neutral evolutionary substitutions. Our data appear to establish the overall importance of the COOH-terminal modification of the v-*fms* gene product with respect to the transforming potential, although more subtle assays may be required to evaluate the significance of specific amino acid substitutions. The frequent amino acid differences in the ligand-binding domains may also be functionally relevant to transformation by v-*fms* by uncoupling ligand activation of the kinase domain. Finally,

the tyrosine residue found that four amino acids from the end of c-*fms* could be a site for regulatory phosphorylation (30, 51); site-directed mutagenesis might be used to study the significance of this and other amino acid differences between c-*fms* and v-*fms*.

At present there is no direct evidence implicating mutations in the c-*fms* gene in human neoplasia. The v-*fms* gene causes fibrosarcomas in cats, but this species and tissue selectivity is likely to reflect properties of the viral envelope and transcriptional promoter. Expression of the c-*fms* gene in hematopoietic cells makes them potential targets for any oncogenic potential of mutated genes. The c-*fms* gene is deleted from the $5q^-$ chromosome found in patients with refractory anemia, who have a high risk for leukemic evolution (52, 53). A $5q^-$ chromosome is also often found in patients with secondary leukemia that develops after intensive chemotherapy (54). Perhaps deletion of one c-*fms* gene renders mutations in the residual c-*fms* allele more likely to contribute to neoplastic transformation. Study of c-*fms* gene structure in such patients might uncover mutations analogous to those found in v-*fms* that are relevant to the pathogenesis of leukemia.

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