Analysis of Functional Domains of the *v-fms*-Encoded Protein of Susan McDonough Strain Feline Sarcoma Virus by Linker Insertion Mutagenesis

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The Susan McDonough strain of feline sarcoma virus contains an oncogene, *v-fms*, which is capable of transforming fibroblasts in vitro. The mature protein product of the *v-fms* gene (gp140^{fms}) is found on the surface of transformed cells; this glycoprotein has external, transmembrane, and cytoplasmic domains. To assess the functional role of these domains in transformation, we constructed a series of nine linker insertion mutations throughout the *v-fms* gene by using a dodecameric *Bam*HI linker. The biological effects of these mutations on the function and intracellular localization of *v-fms*-encoded proteins were determined by transfecting the mutated DNA into Rat-2 cells. Most of the mutations within the external domain of the *v-fms*-encoded protein eliminated focus formation on Rat-2 cells; three of these mutations interfered with the glycosylation of the *v-fms* protein and interfered with formation of the mature gp140^{fms}. One mutation in the external domain led to cell surface expression of *v-fms* protein even in the absence of complete glycosylational processing. Cell surface expression of mutated *v-fms* protein is probably necessary, but is not sufficient, for cell transformation since mutant *v-fms* protein was found on the surface of several nontransformed cell lines. Mutations that were introduced within the external domain had little effect on in vitro kinase activity, whereas mutations within the cytoplasmic domain all had strong inhibitory effects on this activity.

The *v-fms* oncogene, which is contained within the Susan McDonough strain (15) of feline sarcoma virus (SM-FeSV), is capable of transforming fibroblasts in vitro (10, 22). The *v-fms* oncogene is one of a subclass of oncogenes that possess an associated tyrosine-specific protein kinase activity (1, 19, 27); this activity is believed to be required for cellular transformation. Evidence suggests that *c-fms*, the cellular homolog of *v-fms*, is the receptor for colony-stimulating factor 1 (23). Thus, expression of *v-fms* in cells where it is not normally expressed (e.g., fibroblasts) may allow colony-stimulating factor 1 in serum (or produced by the fibroblasts) to stimulate the growth of these cells. Alternatively, *v-fms*-encoded proteins may function as modified growth factor receptors that are constitutively active, thereby leading to the unregulated growth of cells in the absence of growth factor.

The viral *gag* gene of SM-FeSV is partially deleted at the 3' end (8) and is fused in-frame to the *fms* gene. The initial protein product of the fused *gag-fms* gene is a 160-kilodalton (kDa) polyprotein that is cotranslationally glycosylated, resulting in a 180-kDa protein (gp180^{gag-fms}). The *gag* portion of this protein and the N-terminal 54 amino acids of *fms* are then cleaved (28), leaving a 120-kDa protein (gp120^{fms}) that undergoes additional glycosylational processing, resulting in the mature, 140-kDa protein (gp140^{fms}). This protein is expressed on the cell surface in association with coated pits (13) and is processed through endocytosis. The amino acid sequence of *v-fms*, deduced from the nucleotide sequence (8), indicates three distinct regions within the encoded protein: an external domain of 542 amino acids that projects out of the cell; a hydrophobic, 26-amino-acid transmembrane sequence embedded in the plasma membrane; and a 406-amino-acid cytoplasmic domain that contains the tyrosine kinase region. Cell surface expression of gp140^{fms}

appears to be necessary for cellular transformation (13, 18, 21), and complete carbohydrate processing appears to be necessary for cell surface expression (17).

Previous mutagenesis studies (2, 18, 20, 21, 28) on the *v-fms* gene have helped to define several aspects of the function of this gene. Most of these studies, however, involved making large-scale deletion mutations within the *v-fms* gene. We have constructed a series of nine linker insertion mutations within the fused *gag-fms* gene; these mutations result in the addition of four amino acids to the protein encoded by this fused gene. This approach has been successfully used to study functional domains within the *v-fps* (25) and *v-erbB* (16) oncogenes as well as the human glucocorticoid receptor (6). The mutations in the *v-fms* gene were found to affect the capacity of the gene to transform cells in vitro, as well as the processing, intracellular distribution, and tyrosine kinase activity of its encoded proteins.

MATERIALS AND METHODS

Cells and plasmids. The plasmid pB5, which contains the integrated SM-FeSV provirus (cloned into the *Eco*RI site of pBR328) was obtained from John Groffen (Oncogene Science, Mineola, N.Y.). The plasmid pDG510, which contains the herpes simplex virus type 2 thymidine kinase gene, was used as the selectable marker in our transfection experiments and was provided by Denise Galloway (Fred Hutchinson Cancer Research Center). The Rat-2 cells (26), which are thymidine kinase deficient (TK⁻), were obtained from Alan Senear (Fred Hutchinson Cancer Research Center). All cells were grown in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum.

Antisera. Polyclonal antisera to *v-fms*-encoded proteins were prepared from tumor-bearing rats as previously described (13) and were used for immunoprecipitations, kinase assays, and immunofluorescence experiments.

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Immunofluorescence microscopy. Immunofluorescence was performed both on viable cells, to detect *v-fms*-encoded proteins on the cell surface, and on permeabilized, methanol-fixed cells. The technique used was that of Manger et al. (13), modified as described below. Viable cell fluorescence was done at 4°C to prevent the internalization of cell surface proteins. Briefly, the cells were washed in ice-cold phosphate-buffered saline containing 0.5 mM MgCl₂ and 0.9 mM CaCl₂ (PBSCM) and then incubated for 30 min in PBSCM containing rat anti-*v-fms* antiserum. The cells were then rinsed in PBSCM to remove unbound antiserum, and a rhodamine-conjugated goat anti-rat antibody (Jackson ImmunoResearch Laboratories, Inc.) was placed on the cells for an additional 30 min. The cells were then rinsed again in PBSCM to remove unbound antibodies, and the cover slips on which the cells were grown were mounted directly in Elvanol. Fluorescence labeling of methanol-fixed cells was performed as previously described (13), and the cells were photographed on a Zeiss Photomicroscope.

Construction of linker insertion mutations in the *v-fms* gene. The basic strategy used to construct a series of in-phase linker insertion mutations in the *v-fms* gene was based on the method of Stone et al. (25). A 20-μg sample of pB5 DNA was partially digested (in the presence of 20 μg of ethidium bromide per ml) with either *Rsa*I or *Alu*I restriction enzyme for 30 min. Ethidium bromide was used to limit DNA digestion; the major digestion products of this reaction were a circularly permuted population of full-length, linearized DNA molecules. The digested DNA was electrophoresed through 1.2% agarose (containing 5 μg of ethidium bromide per ml), and the DNA band corresponding to full-length linear DNA (16.1 kilobases) was identified by comigration with pB5 (linear) DNA cut at a single *Pvu*I site. The DNA in this band was then electroeluted from the agarose into dialysis tubing as described (14). Nonphosphorylated *Bam*HI linkers [5'-d(CGCGGATCCGCG), from New England Biolabs] were ligated onto the linearized DNA by linker tailing (12). Excess linkers were removed by chromatography over a Bio-Gel P-60 column (Bio-Rad), and the DNA was recircularized. A sample of this DNA was used to transform competent *Escherichia coli* HB101 prepared by the method of Hanahan (9). A total of 248 ampicillin-resistant bacterial colonies were screened for the presence of plasmids containing a new *Bam*HI site. Digestion of pB5 DNA with *Bam*HI produces fragments of 7.1, 5.0, and 4.0 kilobases; insertion of a linker within the fused *gag-fms* gene results in the cleavage of the 7.1-kilobase fragment. The site of the inserted linker was mapped by digesting each plasmid DNA with *Bam*HI, *Bam*HI-*Xho*I, *Bam*HI-*Bgl*II, and, when necessary, *Bam*HI-*Hinc*II and *Bam*HI-*Sph*I. Comparison of the sizes of the generated fragments with those predicted from the published DNA sequence of *v-fms* (8) allowed us to identify unambiguously the restriction site containing the inserted *Bam*HI linker. Several of the plasmids were sequenced through the region of the inserted linker (data not shown) to confirm that the linkers were properly inserted. In addition, plasmids containing the inserted linkers were digested with *Pst*I to determine whether any small insertions or deletions had taken place during the mutagenesis procedure; all mutant plasmids (along with pB5 DNA) produced identical size fragments from the *gag-fms* region. We were able to identify full-length, *v-fms*-encoded proteins in all of the cell lines transfected with the different mutated plasmids (see below).

Cell culture and transfection. Transfection experiments were performed essentially as described by Stone et al. (25).

Dishes (10 cm) of TK⁻ Rat-2 cells were transfected 2 days after seeding, when the cells were about 95% confluent. DNA precipitates were formed by dissolving 10 μg of plasmid DNA (containing an inserted linker in the fused *gag-fms* gene) and 0.5 μg of pDG510 selectable marker DNA (TK⁺) in 0.5 ml of 0.25 M CaCl₂. To this was added 0.5 ml of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1.5 mM NaHPO₄, and 280 mM NaCl (pH 7.05), and a precipitate was allowed to form for 30 min at room temperature. This solution was then added to a dish of Rat-2 cells containing 10 ml of medium. After 8 h the medium was changed, and the cells were incubated for an additional 36 h. The cells were then trypsinized as follows: 40% of the cell suspension was divided between two plates containing Dulbecco modified Eagle medium plus 10% heat-inactivated fetal bovine serum, and 40% of the suspension was divided between two plates containing the above medium supplemented with HAT (100 × 10⁻⁶ M hypoxanthine, 0.4 × 10⁻⁶ M aminopterin, and 16 × 10⁻⁶ M thymidine). The media were changed every 4 days. Cells growing in the Eagle medium without HAT were scored for foci 17 days after transfection. Colonies remaining after HAT selection were counted 18 days after transfection; a number of these colonies were subcloned, screened for expression of *v-fms* proteins, and then used to establish cell lines.

Pulse-chase analysis of *v-fms*-encoded proteins. Dishes (35 mm) of confluent cells were pulse-labeled in methionine-free medium with 200 μCi of [³⁵S]methionine per ml for 20 min and then chased for either 0, 0.5, 1, 2, 4, or 8 h with medium containing 10 times the normal concentration of methionine. The medium was then removed, and cells were rinsed twice in ice-cold PBSCM and then frozen at -70°C. The cells were later thawed and lysed in phosphate RIPA (0.15 M NaCl, 0.05 phosphate [pH 7.2], 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 300 Kallikrein units of trypsinol per ml); these lysates were then centrifuged at 27,000 × *g* for 30 min to remove insoluble materials. The supernatants were used for radioimmunoprecipitation essentially as described by Gentry et al. (5), with protein A-Sepharose CL4B (Pharmacia) used to precipitate the immunocomplexes. Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 7.5% gels by the method of Laemmli (11). The gels were fixed, treated with En³Hance (New England Nuclear Corp.), dried, and fluorographed.

Growth in soft agar. Cells were tested for their capacity to form colonies in soft agar as previously described (4). Colonies were counted 10 days after the cells were seeded.

Morphological analysis. Cells were examined 3 and 5 days after being passaged and were photographed on plastic tissue culture dishes, using a Zeiss photomicroscope.

In vitro kinase activity. Confluent 60-mm dishes of cells were labeled in methionine-free medium with 100 μCi of [³⁵S]methionine per ml for 4 h and then washed, frozen, thawed, and lysed as described above for pulse-chase analysis. The lysates were equalized for the amount of protein they contained (24), and these lysates were then immunoprecipitated as described above. After the immunoprecipitates were washed (5), they were used in an in vitro kinase activity assay as previously described (30). After the kinase assay, the phosphorylated proteins were separated by polyacrylamide gel electrophoresis as described above. The dried gel was autoradiographed using an intensifying screen. Areas of the gel corresponding to the gp120^{fms} band were identified by laying the autoradiogram over the dried gel; these sections were then excised and solubilized in Protosol

FIG. 1. Sites within the fused *gag-fms* gene of SM-FeSV that were mutagenized by insertion of a dodecameric *Bam*HI linker. The restriction sites were determined by analysis of the published sequence (8); the numbers indicate the position of the first nucleotide of the restriction site. The cross-hatched area within the cytoplasmic domain indicates the region of homology with the kinase domain of other tyrosine kinase oncogenes; the stippled area within this region is a 71-amino-acid insert that is unique to the *v-fms* oncogene.

(New England Nuclear Corp.), scintillation fluid was added, and [35 S]methionine and [32 P]phosphate in the gel were quantitated by liquid scintillation spectrometry.

RESULTS

Construction of linker insertion mutations. Our general approach was to identify regions of the *v-fms* protein that are functionally required for transforming activity by constructing a series of linker insertion mutations along the length of the *v-fms* gene. Mutagenesis was conducted (as described in Materials and Methods) on a plasmid, pB5, which contains the entire SM-FeSV provirus. Structural features of this provirus, the fused *gag-fms* gene, and the sites of linker insertion within this fused gene are shown in Fig. 1. Two enzymes, *Rsa*I and *Alu*I, were used independently to cut the pB5 plasmid to ensure a good distribution of linkers inserted throughout the fused *gag-fms* gene. Stone et al. (25) found restriction site hot spots with this technique, whereas Ng and Privalsky (16) reported no bias in cleavage of *Alu*I sites. Under the conditions that we used to generate the linear plasmid molecules, cutting was nonrandom; some *Alu*I and *Rsa*I sites were more readily cut than others (data not shown). Of the potentially available sites within the fused *gag-fms* gene, only 5 of 9 *Rsa*I sites (128 clones screened) and 9 of 16 *Alu*I sites (120 clones screened) were found to contain a single inserted linker. Nine mutant plasmids (of 14 identified) containing a single inserted linker at an *Rsa*I or *Alu*I restriction site (Fig. 1) were chosen for further analysis.

In contrast to the approach used by Stone et al. (25), we used the linker tailing procedure of Lathe et al. (12) to attach the *Bam*HI linkers. This method limits linker insertion to a single copy of the linker per plasmid molecule. We confirmed that our mutated plasmids did not contain multiple linker inserts by digesting the plasmids with the restriction enzyme *Bss*HII, which would cut the sequence d(GCGCGC) formed by two adjacent *Bam*HI linkers. None of the mutant plasmids contained a new *Bss*HII site, indicating that only a single linker had been inserted at each of the restriction sites (data not shown). The disadvantage of using linker tailing was that it did not allow us to compare the effects of small versus large inserts at a single restriction site. Insertion of a single *Bam*HI linker into the DNA does not create a stop codon, irrespective of the reading frame or the sequence of nucleotides surrounding the linker. The nucleotide sequence of the *gag-fms* gene determined by Hampe et al. (8) was used

to predict the amino acid sequence of the mutated protein that resulted from linker insertion (Table 1). At five restriction sites, insertion occurred between two amino acid codons, whereas at four sites insertion was within a codon.

Effect of linker insertion mutations on focus formation. Plasmid DNAs containing mutations within the fused *gag-fms* gene were cotransfected into Rat-2 cells (which are TK⁻) by calcium phosphate precipitation (7), along with a plasmid (pDG510) containing the TK selectable marker gene. The transfected cells were passaged and subcultured into nonselective medium (for quantification of transformed foci) as well as into medium containing HAT (for quantification of HAT-resistant colonies). Growth of cells in both selective and nonselective media was used to control for experimental variability in the transfection procedure itself. For example, transfection with a plasmid that produced no foci but did produce colonies indicates that the lack of focus-forming capacity of the plasmid was not a result of poor transfection efficiency. All mutations were analyzed by establishing cell lines from HAT-resistant colonies expressing *v-fms*-encoded proteins.

The capacity of the plasmids containing linker insertion mutations to transform Rat-2 cells in a focus assay was variable (Table 1). Most of the mutations in the external domain of the *fms* protein (*Alu*I-2109, -2655, and -2948 and *Rsa*I-2740) completely eliminated focus formation. An exception was the mutation at *Alu*I-1405, which was not surprising, given that the site of this mutation is upstream of the *gag-fms* cleavage site (28) and therefore the inserted amino acids should not appear in the mature gp140^{fms} protein. In agreement with this, we found that the *gag* portion of the cleaved *gag-fms* fusion protein from CL1405-F cells had a higher molecular weight on polyacrylamide gels than the *gag* protein from the other cell lines (data not shown). The increase in size of the *gag* protein in this cell line was consistent with the insertion of four amino acids in this part of the protein. A mutation at *Alu*I-3003, which is at the junction of the external and transmembrane domains, had no effect on the focus-forming activity of the plasmid. Most likely this mutation merely displaces the entire external domain but does not affect its conformation. In contrast, a mutation at the junction of the transmembrane and cytoplasmic domains (*Rsa*I-3079) completely eliminated focus formation.

Mutations in the cytoplasmic domain had variable effects

TABLE 1. Transfection of Rat-2 cells with mutagenized pB5 DNA^a

Plasmids	Amino acid change		Foci/plate	HAT-resistant colonies/plate	Cell line	Growth in soft agar (colonies/field ± SD)
	From:	To:				
With inserted linker						
<i>AluI</i> -1405 ^b	A	ARIRA	5; 9	10; 18	CL1405-F	2 ± 2
<i>AluI</i> -2109	–	RGSA	0; 0	30; 38	CL2109-H	0
<i>AluI</i> -2655	–	RGSA	0; 0	4; 6	CL2655-B	0
<i>RsaI</i> -2740	Y	SRIRD	0; 0	7; 14	CL2740-K	0
<i>AluI</i> -3003	–	RGSA	19; 20	22; 27	CL3003-B	7 ± 2
<i>RsaI</i> -3079	Y	SRIRD	0; 0	30; 33	CL3079-D	0
<i>AluI</i> -3146	S	SADPR	11; 14	16; 17	CL3146-U	0
<i>RsaI</i> -3246	–	RGSA	0; 0	46; 50	CL3426-K	0
<i>AluI</i> -3723	–	RGSA	9; 11	25; 30	CL3723-A	0
Control plasmids						
No DNA			0; 0	0; 0		
pB5 (SM-FeSV) (10 μg)			51; 53	0; 0		
pDG510 (TK ⁺) (10 μg)			0; 0	>200; >200		
pDG510 (TK ⁺) (0.5 μg)			0; 0	82; 89		
pB5 (10 μg) + pDG510 (0.5 μg)			24; 30	14; 20	pB5 I Rat-2	93 ± 12 0

^a A 10- μ g sample of plasmid DNA (pB5) containing an inserted *Bam*HI linker at the indicated restriction site was cotransfected along with 0.5 μ g of pDG510 DNA (TK plasmid) into Rat-2 cells. The transfected cells were passaged and grown in medium (foci quantitated after 17 days) or medium supplemented with HAT (colonies quantitated after 18 days). All cell lines were derived from the HAT-resistant colonies as described in the text. Four amino acids are added to the protein as a result of linker insertion; a dash indicates insertion between two amino acid codons, whereas a letter indicates the amino acid codon (single-letter code) into which the linker was inserted. The resulting amino acids in the protein are listed in single-letter code. The last column lists the growth capacity in soft agar of the cell lines expressing mutagenized *v-fms* proteins. Numbers shown are the mean number of colonies (larger than six to eight cells) in a total of eight microscope fields, $\pm$ standard deviation.

^b Designation: Letters, restriction site; numbers, nucleotide position of inserted linker.

on focus formation. Insertion of a linker at *AluI*-3146 did not affect the capacity of the plasmid to form foci. Two mutations are within the kinase domain: insertion at *RsaI*-3426 eliminated focus formation, whereas insertion at *AluI*-3723 did not. It should be noted that after transfection, the cells were passaged at a 1/5 dilution; therefore, the number of foci per plate reported (Table 1) actually represents only 20% of the total foci induced by the DNA. It should also be noted that the absolute numbers of both foci and colonies in these transfection experiments were somewhat variable when these experiments were repeated; however, plasmids that gave no foci after transfection did so repeatedly, whereas plasmids that yielded foci did so each time the experiment was repeated.

Establishment of cell lines expressing *v-fms*-encoded proteins. Cells from HAT-resistant colonies cloned in the transfection assay were labeled with [³⁵S]methionine and screened by immunoprecipitation for the presence of *v-fms*-encoded proteins. Although the cotransfections were done at a ratio of mutated *v-fms* plasmid (pB5) DNA to selectable marker plasmid (pDG510) DNA of 20/1, only a small percentage (5 to 20%) of the HAT-resistant colonies were actively expressing *v-fms*-encoded proteins. We were able to establish a cell line that expressed *v-fms*-encoded proteins for each of the mutations described above (Table 1); however, the amounts of these proteins produced by the cell lines varied considerably (see below).

Growth in soft agar. Since growth in soft agar is considered to be a critical measure of transformation, all of the cell lines expressing mutant *v-fms*-encoded proteins were tested (along with Rat-2 cells) for this capacity (Table 1). Rat-2 cells failed to form colonies in soft agar, whereas pB5 I cells readily formed large colonies. Only two of the mutant cell lines, CL1405-F and CL3003-B, were able to grow in soft agar. These were the same two cell lines that were morphologically transformed (Fig. 2). The number of colonies formed by these cells, however, was less than 1/10 of the number of colonies formed by the pB5 I cells.

Effect of mutations on cell morphology. The morphology of cells expressing mutant *v-fms*-encoded proteins was examined at different densities as well as different temperatures. At 37°C and at subconfluent densities, several of the cell lines exhibited morphologies different from that of wild-type Rat-2 cells. When the cells became confluent, however, these divergent morphologies converged and resembled the normal phenotype of Rat-2 cells, a flat, swirling monolayer (Fig. 2A). In contrast, two cell lines, CL1405-F and CL3003-B (Fig. 2C and D), were morphologically transformed at all densities and resembled the pB5 I cell line (transfected with wild-type *v-fms* DNA; Fig. 2B); all of these cells were spindle shaped, not contact inhibited, and overgrew each other (Fig. 2). The morphology of pB5 I, CL1405-F, and CL3003-B cells was examined (along with that of Rat-2 cells) at 40°C (at several densities) to determine whether the phenotype of any of these lines was temperature sensitive. All of these cell lines grew more slowly at the higher temperature; the Rat-2 cells remained morphologically normal, whereas the wild-type pB5 I cells and the mutant cell lines CL1405-F and CL3003-B were still morphologically transformed (data not shown).

In vitro kinase activity. Wild-type *v-fms* proteins encode a tyrosine-specific in vitro protein kinase activity (1, 19, 27). Cell lines expressing mutant *v-fms*-encoded proteins were lysed in phosphate RIPA (see Materials and Methods), and the lysates were tested for kinase activity (Table 2). The specific kinase activity of the gp120^{fms} protein was compared in the various cell lines since this is the more abundant of the two *v-fms* proteins (gp120^{fms} and gp180^{gag-fms}) that are found in all of the cell lines. The mutations, however, affected the kinase activity of all three forms of the *fms* protein (data not shown); changes in the levels of gp120^{fms} kinase activity were paralleled by alterations in the activity of gp180^{gag-fms} and gp140^{fms} (in cells in which it was made).

Kinase activity was not detectable in the parental Rat-2 cell line (data not shown); however, kinase activity in gp120^{fms} was detectable in most of the mutant cell lines

FIG. 2. Morphology of several cell lines derived from HAT-resistant colonies after transfection with mutagenized pB5 DNA. (A) Normal Rat-2 cells; (B) transfected Rat-2 cells (pB5 I cells) expressing wild-type *v-fms* protein. The cell lines CL1405-F (C) and CL3003-B (D) produce *v-fms* proteins mutagenized at the sites indicated in Fig. 1.

(Table 2). When the specific kinase activity of gp120^{fms} was determined, it was found that mutations in the external domain of the protein had only a slight inhibitory or stimulatory effect on kinase activity. Of the mutations within the cytoplasmic region of the protein, two mutations (*Rsa*I-3079 and *Rsa*I-3146) were upstream of the generally accepted protein kinase domain, while two mutations (*Rsa*I-3426 and *Alu*I-3723) were within the kinase domain. In contrast to the mutations in the external domain, mutations within the cytoplasmic domain of *v-fms*-encoded proteins had a large inhibitory effect on kinase activity. In particular, the mutation at *Rsa*I-3426, which is within the region of amino acid homology with other kinase oncogenes, resulted in very low levels of kinase activity in the transfected cell lysates.

We also examined the capacity of immunoprecipitated *v-fms* proteins from the various cell lines to phosphorylate two exogenous substrates, angiotensin II (29) and enolase

(3), which have previously been shown to be substrates for other protein tyrosine kinases. Neither of these substrates, however, was phosphorylated to any appreciable degree over that seen with control Rat-2 immunoprecipitates (data not shown). This result was not surprising since *v-fms* has been shown to be incapable of phosphorylating casein in vitro (19) and is considered to be a poor kinase on exogenous substrates.

Effect of mutations on processing of *v-fms*-encoded proteins. Processing of *v-fms*-encoded proteins in the mutant cell lines was studied by pulse-chase analysis. The initial product of the fused *gag-fms* gene is a glycosylated 180-kDa protein (gp180^{*gag-fms*}). Cleavage of the *gag* portion of this protein, which is thought to occur cotranslationally, occurs 54 amino acids downstream of the *gag-fms* junction (28). This results in a glycosylated 126-kDa protein which is then processed by removal of terminal sugar residues (17) down to 120 kDa.

TABLE 2. Specific kinase activity of gp120^{fms}^a

Cell line	[³⁵ S]methionine cpm	[³² P]PO ₄ cpm	Ratio, [³² P]PO ₄ cpm/ [³⁵ S]methionine cpm	% of wild-type activity
pB5 I	136	34	0.25	100
CL1405-F	241	54	0.22	88
CL2740-K	1,160	373	0.32	128
CL3003-B	65	21	0.32	128
CL3079-D	204	9	0.04	16
CL3146-U	41	NS ^b	<0.07 ^c	<28 ^c
CL3426-K	521	NS	<0.01 ^c	<4 ^c
CL3723-A	356	29	0.08	32

^a Specific kinase activity was determined as described in the text. Incorporation of [³⁵S]methionine into the gp120^{fms} band from CL2109-H and CL2655-B cells was too low to enable us to quantitate the specific kinase activity of this protein.

^b NS, Not significantly above background (which was subtracted from all values shown). Background values were: [³⁵S]methionine, 63 cpm; [³²P]phosphate, 5 cpm.

^c Numbers were calculated by using a value for [³²P]PO₄ counts per minute of 50% above background, which was about the limit of detection.

Terminal sugar residues are then added, resulting in the gp140^{fms} molecule; this mature *v-fms* protein is the species found on the cell surface (13).

The proteins produced by the mutated *v-fms* genes in five of the cell lines (CL1405-F, CL3003-B, CL3079-D, CL3146-U, and CL3723-A) were fully processed to the mature gp140^{fms} species (Fig. 3); the diffuse appearance of this band on polyacrylamide gels is thought to result from carbohydrate heterogeneity. Production of an apparently mature but mutated gp140^{fms} protein is not sufficient for transformation, since the cell lines CL3079-D, CL3146-U, and CL3723-A, which produced this protein, were not transformed. None of the mutations introduced within the *v-fms* gene disrupted any of the potential sites for N-linked glycosylation.

Three of the mutations, at *RsaI* sites 2655 and 3426 and at *AluI* site 2740, apparently interfere with the capacity of the protein to be fully processed, since CL2655-B, CL2740-K, and CL3426-K cells did not produce normal amounts of the mature, 140-kDa *fms* protein. We thought that a block in glycosylation of the protein would interfere with the transport of the molecule to the cell surface; however, these cell lines differ in expression of *v-fms* protein on the cell surface (see below). Incorporation of radioactive methionine into the remaining cell line (CL2109-H) was too low during the 20-min pulse to allow us to study processing of *v-fms* proteins. However, the *v-fms*-encoded proteins in these cells

could be detected by labeling the cells for a longer period of time with [³⁵S]methionine (data not shown) and by fluorescence labeling of methanol-fixed cells (see Fig. 5).

Expression of mutant *v-fms* proteins on the cell surface. We looked for *v-fms*-specific fluorescence on the surface of cells expressing mutant *v-fms*-encoded proteins because cell surface expression is thought to be required for transformation (13, 18, 21). Six of the nine cell lines were found to express *v-fms* protein on the cell surface (Fig. 4); these included all five cell lines that completely processed *fms* proteins to the mature 140-kDa species (Fig. 3). In addition, the CL2740-K cells, which did not produce detectable amounts of mature gp140^{fms}, did show cell surface fluorescence. This is in contrast to the CL2655-B and CL3426-K cell lines, which did not produce the 140-kDa mature *v-fms* protein (Fig. 3) and did not exhibit cell surface fluorescence.

Permeabilized cell fluorescence. Cells were fixed with methanol and then examined for *v-fms*-specific immunofluorescence within the cells. The intensity of immunofluorescence varied widely between the various cell lines (Fig. 5). There was, however, an excellent correlation between the intensity of immunofluorescence in the cell lines and the amount of immunoprecipitable *v-fms* proteins (data not shown). In all of the cell lines, whether morphologically normal or transformed, the *v-fms*-encoded proteins were distributed throughout the cytoplasm but excluded from the nucleus. This distribution of protein is probably indicative of

FIG. 3. Glycosylational processing of mutagenized *v-fms*-encoded proteins from cell lines listed in Table 1; the figure shows the location of the mutation in *v-fms* for each of the cell lines. Cells were pulse-labeled with [³⁵S]methionine for 20 min and then chased for the indicated number of hours in medium containing 10 times the normal concentration of methionine. Several of the cell lines (CL2655-B, CL2740-K, and CL3426-K) did not produce mature, fully glycosylated gp140^{fms} protein (autoradiographs above the drawing in the figure).

FIG. 4. Cell surface fluorescence of *v-fms*-encoded proteins in cell lines derived by transfection of Rat-2 cells with mutagenized *v-fms* DNA (Table 1). Cells were treated at 4°C with polyclonal rat anti-*v-fms* antiserum, followed by rhodamine-conjugated goat anti-rat antiserum as described in the text. (A) Negative control Rat-2 cells; (B) positive control pB5 1 cells.

endoplasmic reticulum localization, as demonstrated for the wild-type *v-fms* protein by electron microscopy (13).

Phenotype of cells expressing mutant *v-fms* proteins. The data presented in the preceding figures and tables are summarized in Table 3. Of the nine cell lines that expressed mutated *v-fms* proteins, only two of the lines (CL1405-F and CL3003-B) appeared, by the three criteria examined, to be transformed.

DISCUSSION

The technique of insertional mutagenesis has been used here to analyze functional domains of *v-fms*-encoded proteins. It should be noted that, depending on the reading frame and the surrounding nucleotides, different amino acids were inserted at different sites (Table 1). Thus, effects of mutations may include both positional and compositional changes. We examined the morphology and growth properties of transfected Rat-2 cells and compared the expression, glycosylational processing, kinase activity, and intracellular localization of wild-type and mutated *v-fms*-encoded proteins. Seven of the nine mutations introduced in the *v-fms* gene eliminated the transforming capacity of this gene (see above); this apparently resulted from either a failure to complete glycosylational processing, a lack of expression at the proper intracellular location, or a reduction in kinase activity of the mutated *v-fms* proteins.

Although several of the mutated *v-fms* plasmids (with the *Bam*HI linker inserted at *Alu*I site 3146 or 3723) gave rise to

multiple foci (Table 1), cell lines derived from the corresponding colonies did not appear morphologically transformed, grow in soft agar, or possess an associated *in vitro* kinase activity. This result suggests that the HAT-resistant clones we picked had relatively lower levels of *v-fms* protein (and therefore kinase activity) than the cells in the foci and that this subthreshold amount of kinase activity in our clones was insufficient for cell transformation. However, it must be emphasized that we have not examined any of the foci that arose from transfection of the mutated plasmid DNA for level of *v-fms* expression (by immunoprecipitation or immunofluorescence), kinase activity, or capacity to grow in soft agar.

The major drawback of linker insertion mutagenesis as performed here is the variable levels of expression of *v-fms*-encoded proteins in the various cell lines. This problem of variable protein expression was avoided by Stone et al. (25) by establishing cell lines from foci in the transfection experiment instead of from HAT-resistant colonies. Clones picked from foci would, by definition, be expected to produce relatively large amounts of transforming proteins. However, this approach did not allow for analysis of mutations that abolish focus formation, as most of ours did. These mutations are likely to reveal more about the mechanism of cellular transformation than those mutations that do not interfere with focus formation. All of our cell lines were established from HAT-resistant colonies that were selected at random and screened for the production of *v-fms*-encoded proteins. This avoids the problem of using cell lines estab-

FIG. 5. Internal fluorescence of *v-fms*-encoded proteins in cell lines derived by transfection of Rat-2 cells with mutagenized *v-fms* DNA (Table 1). Cells were fixed and permeabilized in ice-cold methanol and then exposed to polyclonal rat anti-*v-fms* antiserum followed by rhodamine-conjugated goat anti-rat antiserum (see the text). (A) Negative control Rat-2 cells; (B) positive control pB5 I cells.

lished on the basis of a biological parameter (e.g., focus formation) that may have arisen as a result of gene rearrangement, activation of another oncogene by promoter insertion, or other mechanism.

Glycosylational processing of *v-fms* proteins occurs concomitantly with its transport to the cell surface (17), and carbohydrate addition can influence the conformation of a protein and its transport to the cell surface. In the CL2740-K cell line, even though the *v-fms* protein was not completely processed (Fig. 3) to mature gp140^{fms}, *v-fms*-encoded proteins were found on the cell surface (Fig. 4). In contrast, the

CL2655-B cells, which also did not produce normal amounts of gp140^{fms}, showed no detectable *v-fms* proteins on the cell surface. We presume that a change in the protein conformation as a result of the inserted four amino acids prevented the protein from being recognized by glycosyltransferases or proteins that direct its transport through the glycosylational machinery. It is unlikely that the incomplete processing results from inactivation of a processing enzyme gene (by integration of the provirus during transfection) because mutation at a nearby site (in the CL2655-B cell line) also prevented terminal glycosylation. Both of these mutations

TABLE 3. Summary of data on the nine mutations within the *v-fms* gene^a

Restriction site of inserted linker	Focus formation	Cell line	Morphological transformation	Cell surface fluorescence	Permeabilized fluorescence	In vitro kinase activity	<i>v-fms</i> protein processing	Growth in soft agar	<i>v-fms</i> protein expression
No plasmid		Rat-2	—	—	—	—		—	
Plasmid alone	+	pB5 I	+	+	+	+	+	+	+++
<i>AluI</i> -1405	+	CL1405-F	+	+	+	+	+	+/-	+++
<i>AluI</i> -2109	—	CL2109-H	—	—	+	ND	ND	—	+
<i>AluI</i> -2655	—	CL2655-B	—	—	+	ND	—	—	+
<i>RsaI</i> -2740	—	CL2740-K	—	+	+	+	—	—	++++
<i>AluI</i> -3003	+	CL3003-B	+	+	+	+	+	+/-	++
<i>RsaI</i> -3079	—	CL3079-D	—	+	+	+/-	+	—	+++
<i>AluI</i> -3146	+	CL3146-U	—	+	+	+/-	+	—	+
<i>RsaI</i> -3426	—	CL3426-K	—	—	+	—	—	—	+++
<i>AluI</i> -3723	+	CL3723-A	—	+	+	+/-	+	—	+++

^a +, Positive; +/-, marginal; —, negative; ND, not determined.

occurred a short distance to the 5' side of a region of the gene that when deleted (21) also resulted in *v-fms* proteins that were not completely glycosylated and nontransforming. Therefore, this region of the protein is apparently important in controlling carbohydrate addition. We plan to determine what form of the *v-fms* protein (possibly gp120^{fms}) is on the surface of the CL2740-K cells and whether this cell surface protein undergoes internalization, as has been reported for wild-type gp140^{fms} (13).

It has been suggested that cell surface expression of *v-fms*-encoded proteins is necessary for cell transformation (13, 18, 21). We established several cell lines (CL3079-D, CL2740-K, CL3723-A) that express reasonably high levels of *v-fms*-encoded proteins on the cell surface (Fig. 5) and have an associated kinase activity, albeit at lower levels than wild type (Table 2). However, these cells were not morphologically transformed and did not grow in soft agar, and the plasmid DNA that contained the mutation did not (for CL3079-D and CL2740-K cells) produce foci. The mutations introduced apparently interfere with the production or transmission of a growth signal, perhaps by failure to phosphorylate a critical substrate, by preventing endocytosis, or by interfering with the association with a critical cell component.

Analysis of the kinase activity of the mutated *v-fms*-encoded proteins (Table 2) indicated that all of the mutations introduced in the cytoplasmic domain, not just those within the region of kinase homology, had an inhibitory effect on kinase activity. This decreased activity probably results from alterations in the conformation of that portion of the protein that is inside the cell. Mutations within the external domain had little effect on kinase activity of the protein, and the gp120^{fms} protein from the Rat-2-derived cell lines that appeared to be transformed (CL1405-F and CL3003-B) had relatively high levels of kinase activity. CL2740-K cells also had high levels of kinase activity, but the transformation defect in these cells is probably related to a defect in glycosylational processing (Fig. 3).

The CL3723-A cells produced relatively high amounts of *v-fms*-encoded protein which appeared to undergo normal glycosylation, was found on the cell surface, and possessed kinase activity, albeit at lower levels than wild type (Table 2). However, these cells were not transformed; this may result from a subthreshold level of kinase activity. Alternatively, it may be that this mutation in the kinase domain has altered the substrate specificity of the kinase activity such that it no longer phosphorylates a putative critical substrate *in vivo*. We plan to explore this possibility by labeling both CL3723-A and pB5 I cells with [³²P]phosphate and comparing the patterns of phosphorylated proteins in these two cell lines by two-dimensional gel electrophoresis.

Two of the linker insertion mutations flank the transmembrane region. The mutation at *AluI*-3003 occurs at the boundary of the external domain-transmembrane sequence junction; this mutation has little apparent effect on the transforming capacity of the plasmid. Presumably, the four amino acids inserted at this site displace the entire external domain outward, but do not cause any conformational changes within this region. The mutation at the junction of the transmembrane sequence and cytoplasmic domains (*RsaI*-3079), however, did destroy transforming activity of the pB5 plasmid. This was probably a result of the large decrease in kinase activity of the *v-fms* protein (Fig. 3) due to a change in the conformation of the cytoplasmic domain of the protein.

There is an excellent correlation of focus formation,

transformed morphology, and *in vitro* kinase activity among the pB5 I cells (wild-type *v-fms*) and the mutant clones CL1405-F and CL3003-B. Given this correlation, it is not clear why the CL1405-F and CL3003-B cells form many fewer colonies in soft agar than pB5 I cells. Levels of *v-fms* proteins are similar in all three cell lines (Fig. 5). We are currently testing the growth of these cell lines in nude mice to determine whether growth *in vivo* parallels growth in soft agar.

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