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The Myristylation Signal of p60$^{v\text{-}src}$ Functionally Complements the N-Terminal *fps*-Specific Region of P130$^{gag\text{-}fps}$

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The P130$^{gag\text{-}fps}$ protein-tyrosine kinase of Fujinami sarcoma virus contains an N-terminal *fps*-specific domain (N*fps*) that is important for oncogenicity. The N-terminal 14 amino acids of p60$^{v\text{-}src}$, which direct myristylation and membrane association, can replace the *gag*-N*fps* sequences of P130$^{gag\text{-}fps}$ (residues 1 to 635), producing a highly transforming *src-fps* polypeptide. Conversely, *gag*-N*fps* can restore modest transforming activity to a nonmyristylated v-*src* polypeptide. These results emphasize the modular construction of protein-tyrosine kinases and indicate that N*fps*, possibly in conjunction with *gag*, functions in the subcellular localization of P130$^{gag\text{-}fps}$.

Cytoplasmic protein-tyrosine kinases contain two adjacent regions of sequence homology, corresponding to the kinase and SH2 domains (Fig. 1), that are bounded by N- and C-terminal sequences which are structurally distinct in the different protein-tyrosine kinases. p98$^{c\text{-}fps}$ and its oncogenic counterpart P130$^{gag\text{-}fps}$ have an *fps*-specific N-terminal domain (N*fps*) of approximately 250 amino acids (Fig. 1). Insertions or deletions in N*fps* destroy P130$^{gag\text{-}fps}$ transforming activity in Rat-2 cells and impair its ability to transform chicken embryo fibroblasts but have no apparent effect on kinase activity (1, 37, 38). In contrast, removal of *gag*-encoded sequences does not abolish v-*fps* transformation of chicken embryo fibroblast or Rat-2 cells (12; I. Sadowski and T. Pawson, unpublished results). Several observations suggest that N*fps* participates in the subcellular localization of P130$^{gag\text{-}fps}$. In particular, the *fps*-containing virus PRCII, which is poorly oncogenic (2), encodes a *gag-fps* polypeptide which has a deletion in N*fps* (17) and has a subcellular distribution different from that of Fujinami sarcoma virus P130$^{gag\text{-}fps}$ (42).

Wild-type p60$^{v\text{-}src}$ and P130$^{gag\text{-}fps}$ have distinct distributions within the cell. p60$^{v\text{-}src}$ is concentrated predominantly in adhesion plaques (34) and at intercellular junctions (29, 33, 41) and is tightly associated with the plasma membrane (6, 23, 24). P130$^{gag\text{-}fps}$ appears cytoplasmic by immunofluorescence (28, 42); subcellular fractionation reveals a salt-dependent association with the plasma membrane and cytoskeleton (11, 28).

To investigate whether N*fps* is involved in subcellular localization, we have constructed hybrid v-*src* to v-*fps* coding sequences in which the N-terminal 14 amino acids of p60$^{v\text{-}src}$ are exchanged with the *gag*-N*fps* sequences of P130$^{gag\text{-}fps}$. The first seven residues of p60$^{v\text{-}src}$ are sufficient to specify the addition of myristate through an amide linkage to the N-terminal glycine (21) and are required for attachment to the plasma membrane (3, 4) and transforming activity (7, 20). The first 14 amino acids take heterologous proteins, including an intact transforming v-*fps* polypeptide, to the plasma membrane (30). We asked whether this p60$^{v\text{-}src}$ membrane localization signal would confer transforming activity on the C-terminal SH2 and kinase domains of P130$^{gag\text{-}fps}$. Similarly, we investigated whether *gag*-N*fps* sequences would functionally substitute for the p60$^{v\text{-}src}$ myristylation signal. The results indicate that the N-terminal regions of v-*fps* and v-*src* peform analogous functions.

Hybrid *src-fps* expression vectors. Hybrid *src-fps* coding sequences were constructed by manipulation of Rous sarcoma virus Schmidt-Ruppin A v-*src* and the RX18m insertion mutant of Fujinami sarcoma virus (Fig. 1). An *Xho*I linker in RX18m defines an insertion-tolerant site previously suggested as demarcating the C-terminal border of the N*fps* domain (38). P64$^{src\text{-}fps}$ has the myristylation signal of p60$^{v\text{-}src}$ (v-*src* codons 1 to 14) joined to the C-terminal SH2 and kinase domains of P130$^{gag\text{-}fps}$ (*gag-fps* codons 639 to 1184). The reciprocal fusion protein, P126$^{gag\text{-}fps\text{-}src}$, contains at its N-terminus residues 1 to 636 of P130$^{gag\text{-}fps}$ joined to residues 16 to 526 of p60$^{v\text{-}src}$ (Fig. 1).

The v-*src*, RX18m *gag-fps*, and hybrid coding sequences were subcloned into the simian virus 40-based pECE expression vector (9) and tested by transfection into COS-1 cells. All four plasmids encoded proteins of the expected size (data not shown).

Transforming activities of hybrid *src-fps* oncogenes. To test their transforming activities, these expression vectors were transfected into Rat-2 cells (39) by the calcium phosphate coprecipitation technique (14). *src*-transfected cells formed foci in 1 week of transfection, and *gag-fps*- and *src-fps*-transfected cells formed foci within 2 weeks (Fig. 1A). Cells obtained from several independent foci induced by v-*src*, RX18m *gag-fps*, or *src-fps* all formed large colonies when cultured in soft agar. The morphologies of clonal cell lines established from these colonies are shown in Fig. 2. S7a cells transformed by v-*src* were very rounded and refractile. F19a cells transformed by RX18m v-*fps* were more fusiform, whereas SF6s cells transformed by the *src-fps* hybrid were intermediate in morphology. These cells were subsequently shown to express the appropriate proteins (see below). The ability of P64$^{src\text{-}fps}$ to induce rapid focus formation and anchorage-independent growth indicated that it was highly transforming.

In contrast, the *gag-fps-src* hybrid gene failed to produce foci within 1 month. In subsequent transfections of Rat-2

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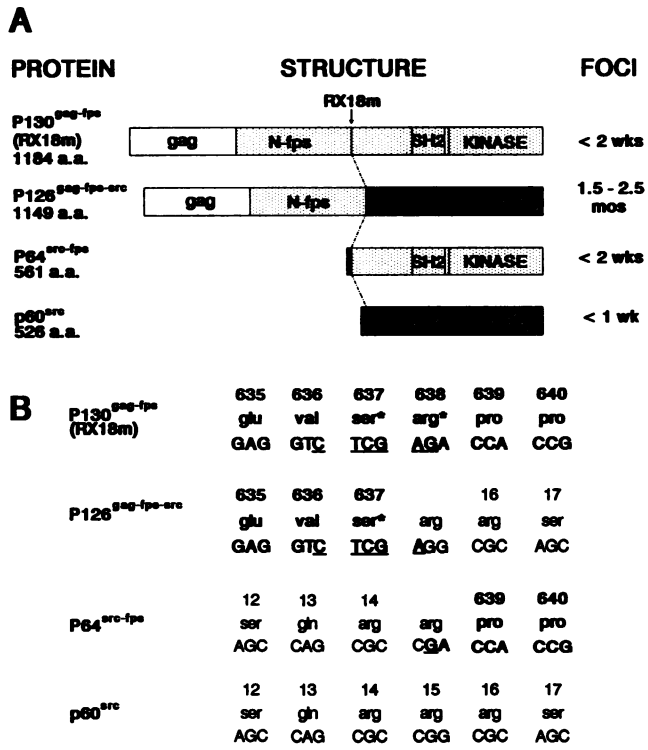

FIG. 1. (A) Structure of *src*, *fps*, and *src-fps* hybrid proteins. Symbols: ▨, *src* sequences; ▩, *fps* sequences; □, *gag* sequences. The latency of focus formation on Rat-2 monolayers is indicated. Hybrid genes were constructed from wild-type Rous sarcoma virus Schmidt-Ruppin A and RX18m *gag-fps* and then incorporated into the simian virus 40-based expression vector pECE by standard molecular cloning procedures (27). (B) Nucleotide and amino acid sequences at the junctions of the *src-fps* and *gag-fps-src* hybrids. Shown is *fps*-derived (bold face) and *src*-derived information. Underlined nucleotides are those that resulted from the RX18m insertion; asterisks indicate amino acids that are present in RX18m but not in wild-type $P130^{gag\text{-}fps}$. a.a., Amino acids.

cells with 10 μg of plasmid DNA, *gag-fps-src* induced foci after nearly 2 months but with very low efficiency (approximately 2 foci per μg of DNA, compared with approximately 250 foci per μg of DNA for other constructs). Five morphologically transformed cultures isolated from *gag-fps-src* foci and expressing $P126^{gag\text{-}fps\text{-}src}$ failed to grow in soft agar, which showed that this protein is only weakly transforming in vitro. One of these nonclonal cultures (GFS19; Fig. 2) was selected for further study. GFS19 contained cells of an elongated spindlelike shape in a background of Rat-2 cells. The elongated cells became more prominent after multiple passages, but this cell line remained unable to grow in soft agar.

Expression of hybrid *src-fps* proteins in transformed Rat-2 cells. Transformed cell lines or parental Rat-2 cells were metabolically labeled with $^{32}P_i$ (Fig. 3) or [^{35}S]methionine (data not shown) and immunoprecipitated with antibodies to *src*, *fps*, or *gag* epitopes. Anti-*src* monoclonal antibody 327 (MAb 327), which binds an epitope in the amino-terminal half of both viral and cellular $p60^{src}$ (25), identified $p60^{v\text{-}src}$ in S7a v-*src*-transformed cells and $P126^{gag\text{-}fps\text{-}src}$ in GFS19 cells. As expected, MAb 327 recognized Rat-2 $p60^{c\text{-}src}$ but failed to immunoprecipitate $P130^{gag\text{-}fps}$ or $P64^{src\text{-}fps}$. Anti-*fps* rat tumor serum immunoprecipitated $P64^{src\text{-}fps}$, RX18m $P130^{gag\text{-}fps}$, and $P126^{gag\text{-}fps\text{-}src}$ from the appropriate cells. The relatively inefficient immunoprecipitation of $P126^{gag\text{-}fps\text{-}src}$ by anti-*fps* rat tumor serum (Fig. 3) was presumably due to the absence of C-terminal v-*fps* epitopes in this hybrid protein. Finally, anti-$p19^{gag}$ MAb R254E (19) recognized RX18m $P130^{gag\text{-}fps}$ and $P126^{gag\text{-}fps\text{-}src}$. Thus, proteins of the expected size and antigenicity were synthesized in cells transformed by v-*src*, *gag-fps*, *src-fps*, and *gag-fps-src*.

Novel 110-kilodalton protein containing *src* and *fps* sequences. One of the foci induced by transfection of the *src-fps* plasmid into Rat-2 cells, and subsequently cloned by passage in soft agar, expressed a 110-kilodalton protein (P110) rather than the expected $P64^{src\text{-}fps}$. The morphology of this cell line, SF5a, is shown in Fig. 2. These cells formed colonies in soft agar and tumors in Fischer rats slightly more rapidly than did cells expressing authentic $P64^{src\text{-}fps}$ (data not shown). P110 was immunoprecipitable with anti-*fps* rat tumor serum and with a rabbit antiserum raised against a bacterial *trpE*–v-*fps* fusion protein containing $P130^{gag\text{-}fps}$ residues 822 to 1184 (data not shown). P110 had therefore retained C-terminal v-*fps* sequences. This variant sequence must have been generated during transfection, presumably by recombination with plasmid or carrier Rat-2 DNA. P110 was shown to be myristylated (see below) and therefore may have retained the *src*-derived region of $P64^{src\text{-}fps}$ or acquired part of a cellular myristyl protein.

Protein-tyrosine kinase activity of *src-fps* hybrid proteins. $P130^{gag\text{-}fps}$, $p60^{v\text{-}src}$, $P64^{src\text{-}fps}$, P110, and $P126^{gag\text{-}fps\text{-}src}$ were immunoprecipitated with appropriate antibodies and introduced into immune complex kinase reactions. All of these proteins were able to autophosphorylate and to phosphorylate enolase in vitro (data not shown). Phosphoamino acid analysis of the wild-type and hybrid proteins immunoprecipitated from ^{32}P-labeled cells (Fig. 3) revealed that these polypeptides all contained phosphotyrosine as well as phosphoserine in vivo (data not shown).

Myristylation of $P64^{src\text{-}fps}$ and P110. Transformed cells expressing wild-type or hybrid proteins, or the aberrant P110 tyrosine kinase, were metabolically labeled with [^{3}H]myristic acid, and the oncoproteins were immunoprecipitated with anti-*src* or anti-*fps* antibody (Fig. 4). $p60^{v\text{-}src}$ from S7a cells was radiolabeled after incubation with [^{3}H]myristic acid, as was $p60^{c\text{-}src}$ from Rat-2 cells. No label was detected in $P130^{gag\text{-}fps}$ or $P126^{gag\text{-}fps\text{-}src}$. However, both $P64^{src\text{-}fps}$ and P110 were labeled during incubation with [^{3}H]myristic acid. Treatment of the gel with hydroxylamine under conditions that remove fatty acids attached to proteins through ester linkage did not remove the label from $P64^{src\text{-}fps}$ or P110 (data not shown). These results suggest that $P64^{src\text{-}fps}$ and P110, like $p60^{v\text{-}src}$, contain N-terminal amide-linked myristate.

Subcellular localization of hybrid proteins. To investigate the subcellular localization of the hybrid proteins, we first used immunofluorescence with anti-*src* MAb 327 and anti-*gag* MAb R254E (Fig. 5). $p60^{v\text{-}src}$ in S7a cells was concentrated in spots at the level of the substratum and, more distinctively, in regions of intercellular contact (Fig. 5, panel 1). This latter distribution has been previously noted in chick (33) and rat (36) cells. Adhesion plaques may be few in number in these very rounded cells. $P130^{gag\text{-}fps}$, visualized with anti-$p19^{gag}$ antibody in F19a cells, showed a general cytoplasmic distribution (Fig. 5, panel 3) similar to that shown by Woolford and Beemon for Fujinami sarcoma virus-transformed chicken embryo fibroblasts (42). $P126^{gag\text{-}fps\text{-}src}$ showed a cytoplasmic spatial distribution similar to that of $P130^{gag\text{-}fps}$ (Fig. 5, panel 5) and quite distinct from that of $p60^{v\text{-}src}$.

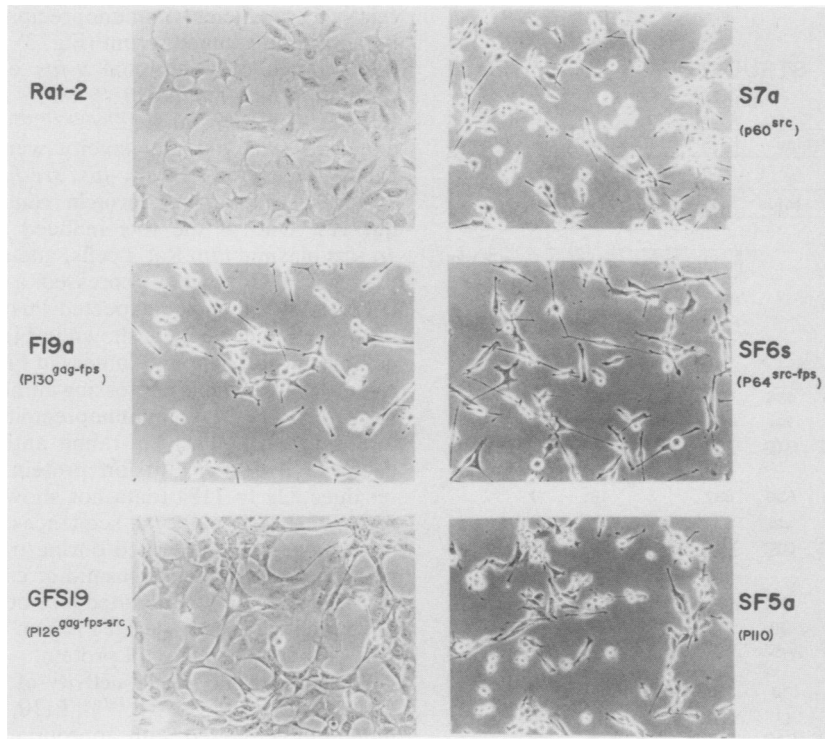

FIG. 2. Morphology of Rat-2 cells transformed by v-*src*, *gag-fps*, or hybrid genes. Normal Rat-2 cells are compared with S7a ($p60^{v\text{-}src}$), SF6s ($P64^{src\text{-}fps}$), SF5a (P110 *src-fps* variant), GFS19 ($P126^{gag\text{-}fps\text{-}src}$), and F19a (RX18m $P130^{gag\text{-}fps}$) cells. Transformed cells (except GFS19, which is not a clonal line) were cloned as colonies from soft agar.

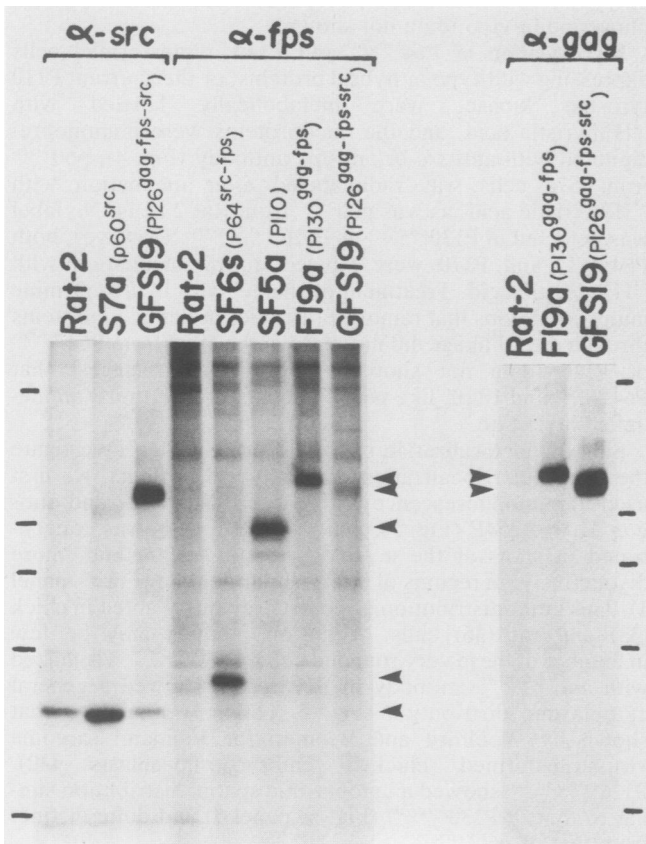

FIG. 3. In vivo phosphorylation of *src-fps* hybrid proteins. Normal Rat-2 cells or cells expressing $P130^{gag\text{-}fps}$ (F19a), $P126^{gag\text{-}fps\text{-}src}$ (GFS19), P110 (SF5a), $P64^{src\text{-}fps}$ (SF6S), or $p60^{v\text{-}src}$ (S7a) were metabolically labeled with $^{32}P_i$. Cell lysates were immunoprecipitated with anti-*src* MAb 327 (α-*src*), anti-*fps* rat antiserum (α-*fps*), or anti-*gag* MAb R254E (α-*gag*). Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiography was for 12 h. An intensifying screen was used for the α-*gag* panel. Positions of immunoprecipitated oncoproteins are indicated by arrows. Marker proteins (outside of panels) were 205, 116, 92, 66, and 45 kilodaltons in size.

Despite repeated attempts with affinity-purified antibodies and rat tumor sera, we were unable to generate anti-*fps* antibodies that would reliably detect $P64^{src\text{-}fps}$ or P110 by immunofluorescence. To circumvent this problem, we used subcellular fractionation to assay for membrane association of the *src-fps* hybrid. We found that 86% of [^{35}S]methionine labeled $P64^{src\text{-}fps}$ showed a salt-insensitive association with the P100 (membrane) fraction of the cells (data not shown). This association is very similar to that of $p60^{v\text{-}src}$ with the crude membrane fraction (4), which indicates that $p60^{v\text{-}src}$ and $P64^{src\text{-}fps}$ are associated with membranes to the same extent.

Function of the N*fps*-specific domain. Previous work has shown that the v-*fps* oncoprotein has an N-terminal region (N*fps*) that is important for oncogenicity. A number of insertions or deletions in this domain abolish transforming activity in Rat-2 cells (1, 37). In contrast, transforming activity is preserved after removal of the N-terminal avian *gag* sequences (12). The data presented here show that the N-terminal 14 amino acids of $p60^{v\text{-}src}$ can replace the N-

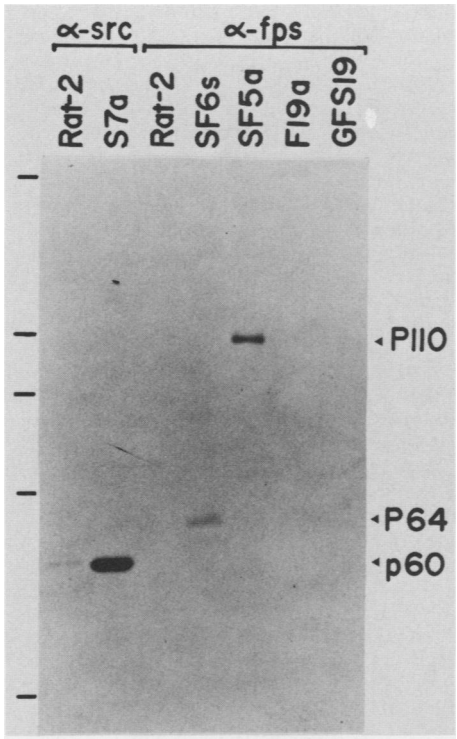

FIG. 4. Radiolabeling of *src-fps* hybrid proteins with [^{3}H]myristic acid. Normal Rat-2 cells and cells expressing $p60^{v\text{-}src}$ (S7a), $P64^{src\text{-}fps}$ (SF6s), P110 (SF5a), $P130^{gag\text{-}fps}$ (F19a), or $P126^{gag\text{-}fps\text{-}src}$ (GFS19) were metabolically labeled with 1 mCi of [^{3}H]myristic acid per ml for 12 h as described by Sefton et al. (35). Cell lysates were immunoprecipitated with anti-*src* MAb 327 (α-*src*) or with anti-*fps* rat tumor serum (α-*fps*). Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by fluorography for 1 week. Mobilities of $p60^{src}$, $P64^{src\text{-}fps}$, and P110 are indicated. Marker proteins (left) were 205, 116, 92, 66, and 45 kilodaltons in size.

terminal 639 amino acids of $P130^{gag\text{-}fps}$ in conferring transforming potential on the v-*fps* SH2 and kinase domains. This functional complementation of *gag*-N*fps* by a known membrane localization signal strongly supports the hypothesis that these sequences are crucial for localization of $P130^{gag\text{-}fps}$.

Mutation of the $p60^{v\text{-}src}$ myristylation signal by substitution of the N-terminal glycine with alanine converts $p60^{v\text{-}src}$ to a soluble, nontransforming protein (4, 20). The *gag*-N*fps* sequences of $P130^{gag\text{-}fps}$, when placed N terminal to residue 16 of $p60^{v\text{-}src}$, were able to restore weak transforming activity to the $p60^{v\text{-}src}$ SH2 and kinase domains. The cytoplasmic pattern of immunofluorescence displayed by $P126^{gag\text{-}fps\text{-}src}$ is equivalent to that of wild type $P130^{gag\text{-}fps}$, consistent with the interpretation that *gag*-N*fps* directs localization. The reasons for the poor transforming ability of $P126^{gag\text{-}fps\text{-}src}$ are not yet established. The $p60^{v\text{-}src}$ kinase domain may function effectively only when tightly associated with the plasma membrane. Alternatively, the N*fps* domain may extend more C terminally than does the RK18m insertion site, perhaps encompassing the 150-amino-acid region that lies between the RX18m site and the border of SH2.

The success of these domain-switching experiments emphasizes the modular design of protein-tyrosine kinases and our growing understanding of their structure and evolution.

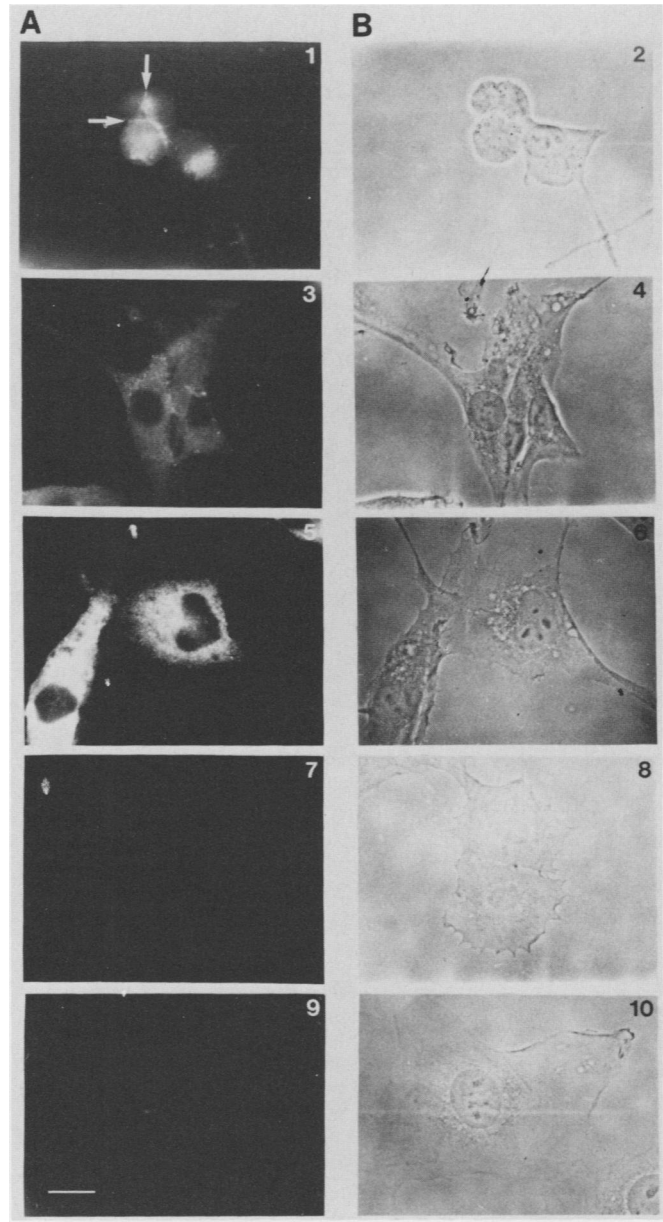

FIG. 5. Immunofluorescent staining of cells expressing v-*src*, *gag-fps*, or *gag-fps-src* proteins. (A) Immunofluorescence micrographs; (B) corresponding phase-contrast photos. Panels: 1 and 2, S7a cells expressing $p60^{v\text{-}src}$ stained with α-*src* MAb 327 (20 μg/ml); 3 and 4, F19a cells expressing $P130^{gag\text{-}fps}$ stained with α-*gag* MAb R254E (1:400 dilution); 5 and 6, GFS19 cells expressing $P126^{gag\text{-}fps\text{-}src}$ stained with α-*gag* MAb R254E; 7 through 10, Rat-2 cells stained with α-*src* MAb 327 (7 and 8) or α-*gag* MAb R254E (9 and 10). Arrows indicate cell-cell junctions; bar represents 20 μm. The immunostaining procedure was based on the method of Heggeness et al. (16). Cells were rinsed, fixed for 20 min in 3% paraformaldehyde in phosphate-buffered saline, and permeabilized during a 3-min incubation with 0.1% Triton X-100 in phosphate-buffered saline. After treatment with 0.1 M glycine in phosphate-buffered saline for 10 min, cover slips were incubated with primary antibody for 20 min. After four rinses of 5 min each, cells were treated with a fluorescein-conjugated anti-mouse immunoglobulin (Jackson Laboratories, Pa.). After additional rinses, cover slips were mounted in 90% glycerol–0.1 M Tris hydrochloride (pH 8) and photographed with a Zeiss photomicroscope III equipped for epifluorescence.

fps and *src* proteins appear to be regulated in quite different ways. $p60^{c\text{-}src}$, which, like $p60^{v\text{-}src}$, is myristylated and associated with the plasma membrane (5, 6), has at least two inhibitory domains that repress kinase activity and restrain transforming potential (22, 31, 32). Accumulation of point mutations in c-*src* during passage through the retrovirus life cycle results in rapid oncogenic activation (18). $p60^{c\text{-}src}$ is therefore under tight negative regulation and is activated when this repression is lifted.

$p98^{c\text{-}fps}$ lacks these inhibitory domains. $p98^{c\text{-}fps}$ is soluble in cultured myeloid cells (43), whereas $P130^{gag\text{-}fps}$ is associated with the particulate fraction of transformed fibroblasts (11, 42). High-level expression of c-*fps*/*fes* genes in avian or mammalian fibroblasts does not induce focus formation (13, 15). Furthermore, passage of c-*fps* in a retrovirus vector does not unmask its oncogenic potential. However, c-*fps* can be activated by N-terminal addition of *gag* sequences (13). Taking these observations into consideration, we speculate that *fps* proteins are controlled by positive rather than negative regulation.

A model consistent with the data presented here is that localization of *fps* proteins is modulated by the N*fps* domain. Association of the *fps* protein-tyrosine kinase with the membrane or cytoskeleton may allow access to critical substrates or may bring *fps* into contact with activating factors which interact with the SH2 domain (8). Alternatively, it could promote clustering of *fps* molecules, thereby promoting intermolecular autophosphorylation (40) and increased enzymatic activity. In its normal myeloid environment (10, 26), N*fps* may undergo a specific, transient interaction with a membrane or cytoskeletal component in response to external stimuli. In viral proteins, the addition of *gag* sequences and amino acid substitutions in N*fps* may directly enhance interaction with the cytoskeleton or membrane and thereby induce constitutive activation. Experiments to test this are in progress.

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