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Isolation and Oncogenic Potential of a Novel Human *src*-Like Gene

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We have isolated cDNA molecules representing the complete coding sequence of a new human gene which is a member of the *src* family of oncogenes. Nucleotide sequence analysis revealed that this gene, termed *slk*, encoded a 537-residue protein which was 86% identical to the chicken proto-oncogene product, $p60^{c\text{-}src}$, over a stretch of 191 amino acids at its carboxy terminus. In contrast, only 6% amino acid homology was observed within the amino-terminal 82 amino acid residues of these two proteins. It was possible to activate *slk* as a transforming gene by substituting approximately two-thirds of the *slk* coding sequence for an analogous region of the v-*fgr onc* gene present in Gardner-Rasheed feline sarcoma virus. The resulting hybrid protein molecule expressed in transformed cells demonstrated protein kinase activity with specificity for tyrosine residues.

Normal cellular genes which have given rise to retrovirus *onc* sequences represent the most abundant class of genes capable of acquiring oncogenic properties. In addition to these, dominant transforming genes present in certain tumor cells have been found by their ability to induce foci of transformation when transfected into susceptible assay cells (for a review, see reference 1). More recently, other genes implicated in the malignant process by virtue of their amplification in tumor cells have been identified because of their genetic relatedness to known oncogenes (15, 17, 32). Of the transforming genes identified to date, roughly half encode products which possess structural or enzymatic relatedness to protein-tyrosine kinases. This family includes altered versions of genes encoding cell surface receptors for certain growth factors (8, 35, 40), as well as a much larger number that have not yet been linked to normal cellular functions.

The prototype protein-tyrosine kinase gene v-*src* (6) and its close relatives, v-*yes* (16) and v-*fgr* (23), were each identified initially within oncogenic retroviruses as components derived from distinct cellular genes (38). In an effort to search for new *src*-related genes within the human genome, we have taken a more general approach not reliant upon the identification of novel oncogenic viruses or transforming genes, but dependent only upon normal gene expression. In the present study, we report the isolation of cDNA molecules representing the complete coding sequence of a new human *src*-related gene and demonstrate that the gene can acquire transforming properties by substituting a portion of its coding sequence for a related *onc* gene in a retrovirus vector.

MATERIALS AND METHODS

Cells. Continuous mouse NIH/3T3 (13) and human umbilical vein endothelial cell lines (19) have been described previously.

cDNA libraries. A complementary DNA library prepared from simian virus 40-transformed human fibroblasts has been described previously (25). Similar cDNA libraries prepared from normal human fibroblasts or human umbilical vein endothelial cells were gifts from H. Okayama and G. Ricca, respectively.

Analysis of cellular RNA and DNA. DNAs were digested with restriction enzymes, fractionated by electrophoresis through agarose gels, and blotted onto nitrocellulose filters as described previously (38). Cellular RNAs were fractionated in the presence of formaldehyde by agarose gel electrophoresis and transferred to nitrocellulose filters (20). Filters were hybridized with nick-translated pv-*fgr*-1 DNA as described by Wahl et al. (41) and visualized by autoradiography.

Nucleotide sequence determination. Restriction enzyme fragments derived from cDNA clones were transferred into the polylinker regions of pUC13, pUC18, or pUC19 vectors. Recombinant plasmid DNAs were alkaline denatured and sequenced directly (31). Sequencing reaction products were analyzed in buffer gradients (5) or 6% polyacrylamide gels. In each case, the nucleotide sequences for both strands were determined.

Transfection assays and protein analysis. DNA transfection of NIH/3T3 cells was performed by the calcium phosphate precipitation technique (10) as modified by Wigler et al. (42). Transformed foci were scored at 2 to 3 weeks. Subconfluent cultures (around 10^7 cells per 10-cm petri dish) were labeled for 3 h at 37°C with 4 ml of phosphate-free Dulbecco modified Eagle medium containing 1 mCi of $^{32}P_i$ (carrier free; New England Nuclear Corp.) per ml or with 4 ml methionine-free Dulbecco modified Eagle medium containing 125 μCi of [^{35}S]methionine (New England Nuclear) per ml. Radiolabeled cells were lysed with 1 ml of lysing buffer (10 mM sodium phosphate [pH 7.5], 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1 mM phenylmethylsulfonyl fluoride) per petri dish, clarified by centrifugation at 100,000 × *g* for 30 min, and divided into five identical aliquots. Extracts were incubated with antiserum, and immunoprecipitates were recovered with the aid of *Staphylococcus aureus* protein A bound to Sepharose beads (Pharmacia Fine Chemicals). Precipitated proteins were analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gradient gels as previously described (2).

For in vitro kinase assays, immunoprecipitates from unlabeled cells were suspended in 0.025 ml of 20 mM Tris hydrochloride (pH 7.5)–5 mM $MnCl_2$ containing 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol). After incubation at 30°C for 10 min, phosphorylated products were isolated by preparative gel electrophoresis and subjected to partial acid hydrolysis. Hydrolysates mixed with unlabeled phosphoserine, phosphothreonine, and phosphotyrosine were fractionated by two-dimensional electrophoresis as described previously

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(12). Plates were exposed at −70°C to Kodak XR-5 film for 18 h with an intensifier screen.

RESULTS

Isolation of a new human *src*-like gene. In an effort to identify new, transcriptionally active members of the protein-tyrosine kinase gene family, we utilized a v-*fgr* probe to screen cDNA libraries prepared from cells not expressing *fgr* proto-oncogene mRNA. Thus, complementary DNA libraries derived from normal human endothelial cells or fibroblasts as well as transformed fibroblasts were screened with a DNA segment representing the protein-tyrosine kinase coding sequence of v-*fgr* (38). Several cDNA clones, ranging in size from 1.1 to 2.5 kilobase pairs (kbp), were isolated and compared with each other. Identical constellations of restriction enzyme sites present within each cDNA clone demonstrated that all of the cDNAs were overlapping and represented the same transcript. The longest clone, designated T10, was chosen for further analysis.

Previous studies have shown that in spite of the high degree of nucleotide sequence relatedness among v-*fgr*, v-*yes*, and v-*src* genes, each is represented within the human genome as a unique proto-oncogene distinguishable on the basis of Southern analysis by using each viral *onc* gene as a probe (38, 43). To determine whether the T10 cDNA clone was derived from the transcript of one of these known proto-oncogenes, restriction enzyme digests of human genomic DNA were fractionated on agarose gels and analyzed by hybridization with T10 DNA. T10-related bands of 12.5, 8.8, 4.8, and 4.5 kbp or 25, 13.5, 3.9, and 3.2 kbp were detected in human DNA treated with *Eco*RI or *Eco*RV, respectively (data not shown). When the same DNAs were hybridized with v-*fgr*, v-*yes*, or v-*src* probes, bands readily distinguishable from those detected with T10 probe were observed. These findings strongly indicated that T10 did not represent human *fgr*, *yes*, or *src* proto-oncogenes.

Nucleotide sequence of T10 cDNA. The nucleotide sequence of the T10 cDNA clone was determined by the method of Sanger et al. (31) (Fig. 1). Analysis of this sequence revealed that the T10 cDNA clone was 2,435 base pairs (bp) in length and contained a long open reading frame of 1,611 nucleotides (Fig. 1). The ATG codon at positions 371 through 373 conformed to Kozak's rules for translation initiation (18) and was preceded by a TAG termination signal just two codons upstream. Thus, the methionine codon at position 371 most likely initiates translation of a T10 translational product. The amino acid sequence predicted from the long open reading frame was 537 residues in length, terminating with a TAA codon at nucleotide positions 1982 through 1984. A computer homology search revealed that the carboxy-terminal 191 amino acids of the putative T10 translational product were highly related to analogous regions of proteins specified by v-*yes*, v-*fgr*, and v-*src* at levels of 85, 75, and 74% identity, respectively (Fig. 2A). Very recently a murine *src*-like gene, lsk^T has been isolated (21). The amino acid sequence of the lsk^T protein is only 68% homologous to the carboxy terminal 191 amino acids of the T10 translational product. A lesser degree of homology was observed with other protein-tyrosine kinases including v-*erbB* and v-*fms*, which represent known growth factor receptors.

Of all the protein-tyrosine kinases described to date, the putative T10 translational product was most closely related to the chicken c-*src* encoded protein, $p60^{c\text{-}src}$. A single site for tyrosine phosphorylation first identified within $p60^{v\text{-}src}$ (36) and present in $p60^{c\text{-}src}$ at position 416 is homologous to the tyrosine residue at amino acid position 420 of the T10-coded protein (Fig. 2B). Regions of $p60^{c\text{-}src}$ thought to be responsible for ATP binding at positions 274 through 279 and 295 (3, 30) are also present in the T10 translational product at positions 278 through 283 and 299. In addition, the tyrosine residue at position 531 is homologous to $p60^{c\text{-}src}$ tyrosine 527, a possible regulatory site for protein kinase activity. Moreover, these two proteins are almost identical in size and share significant homology over a region of 455 amino acids (Fig. 2B).

Despite its high degree of relatedness to $p60^{c\text{-}src}$, the amino-terminal region of the predicted T10 gene product, 82 amino acids in length, showed no significant homology with $p60^{c\text{-}src}$ or any other previously described protein (Fig. 2B). Thus, this region, which corresponds to that encoded by the first two exons of c-*src* (37), may be involved in determining unique interactions of these related proteins with specific cellular targets. The single feature in this amino-terminal domain shared with $p60^{c\text{-}src}$ or $p60^{v\text{-}src}$ was a glycine residue at amino acid position 2, followed by lysine residues at positions 7 and 9. This region has been shown to be important for the addition of a myristic acid residue to the amino terminus or $p60^{v\text{-}src}$. This post-translational modification appears to mediate attachment of $p60^{v\text{-}src}$ to the inner face of cell membranes (28) and to play a critical role in v-*src*-induced transformation (29).

The 5′ untranslated region of T10 cDNA was 370 bp in length and highly G+C rich. The first 250 bp was especially G+C rich (81%), and computer analysis for secondary structure of mRNA (14) showed several possible stem or stem-and-loop structures of high stability (>100 kcal [ca. 418.4 kJ]) in this region. Downstream of the termination codon was an untranslated stretch 452 bp in length. Within this region, we detected a consensus polyadenylation signal 33 bp upstream of the site of polyadenylation. Based upon the presence of a polyadenylation signal as well as a poly(A) stretch in the cDNA clone, we conclude that T10 contains the 3′ extent of this gene.

Size of the T10 transcript. Recent studies have shown that human c-*yes* transcripts can be detected in a variety of human cells as a 4.8-kilobase (kb) mRNA (33), whereas expression of c-*src* (26) and c-*fgr* (7) mRNAs, species of 5.0 and 3.0 kb, respectively, appear to be more limited. To determine the length of the T10 transcript, we hybridized size-fractionated mRNA isolated from normal human endothelial cells and fibroblasts with a probe representing the T10 sequence. A transcript of 3.3 kb was readily detected in poly(A)-positive endothelial cell RNA as well as a similar preparation from normal human fibroblasts (Fig. 3). When these same RNAs were analyzed by using a v-*yes* sequence as a probe, a 4.7-kb mRNA species was detected (Fig. 3). In contrast, human c-*fgr* mRNA was not expressed at detectable levels in the cells examined (data not shown). These findings demonstrated that the T10 sequence was expressed as a 3.3-kb mRNA and provided additional evidence that T10 was distinct from human *src*, *fgr*, and *yes* proto-oncogenes.

Evidence that T10 possesses transforming potential. Structural relatedness between T10 and members of the v-*src* transforming gene family suggested that T10-coded protein might possess oncogenic potential. To test this possibility, we constructd a chimeric molecule utilizing a biologically active Gardner-Rasheed feline sarcoma virus (GR-FeSV) plasmid DNA as a vector. Our strategy was based upon the presence of a common *Apa*I restriction enzyme site at position 1028 in T10 and at position 1066 in the GR-FeSV

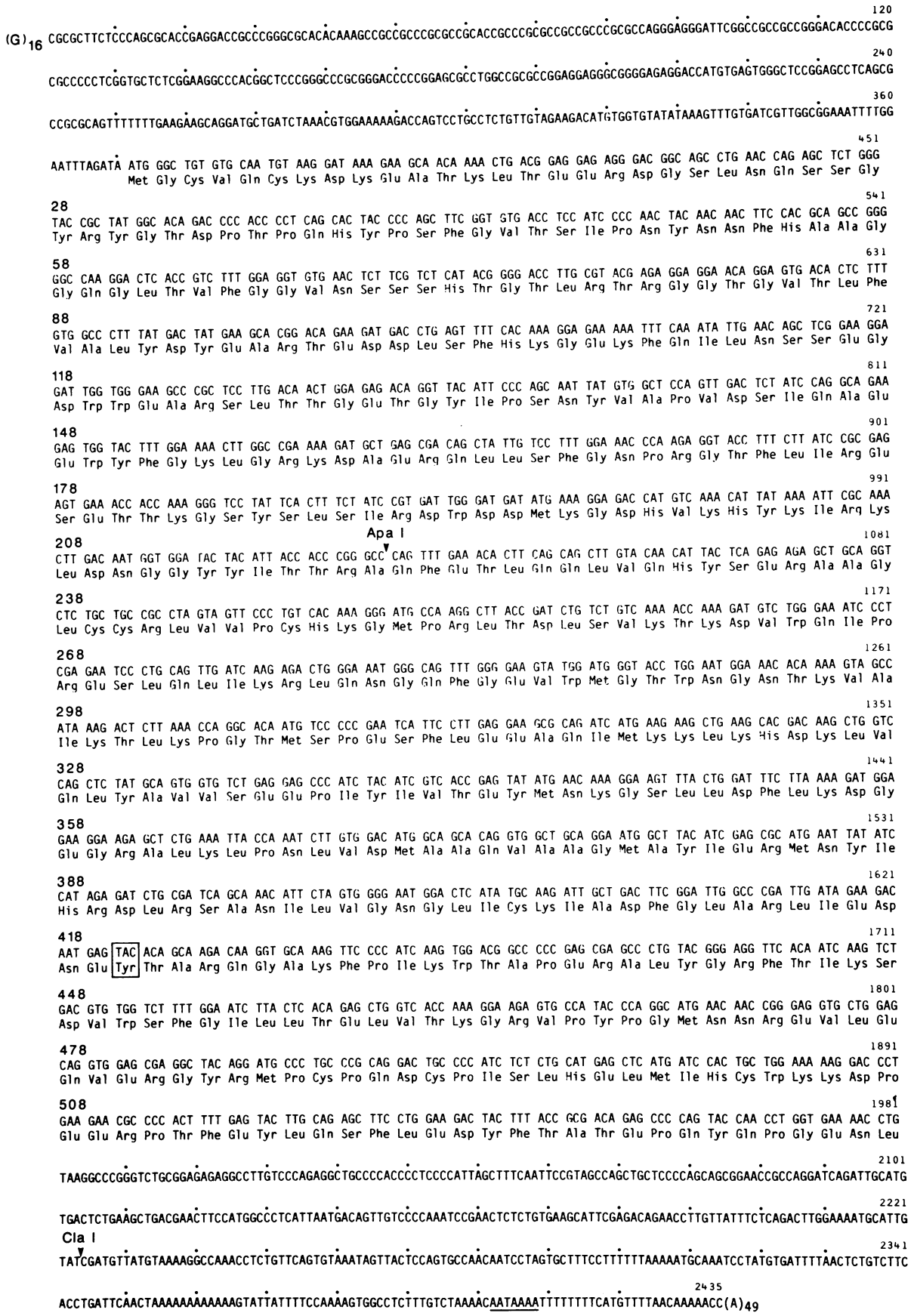

FIG. 1. Nucleotide sequence of T10 cDNA. The nucleotide sequence is shown in capital letters. Amino acids predicted from the long open reading frame are designated by their three-letter codes. Nucleotide positions are indicated on the right, whereas amino acid positions are indicated on the left. Restriction enzyme sites used in constructions are also indicated.

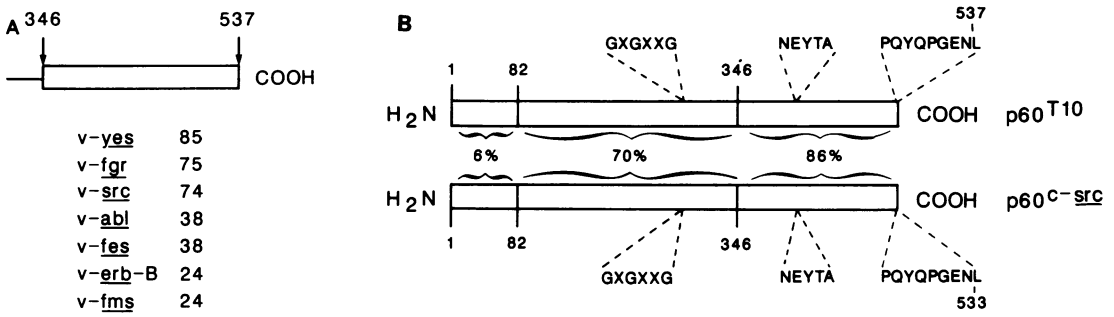

FIG. 2. Relationship of the T10 translational product to protein-tyrosine kinases. (A) The amino acid sequence of the T10-coded protein deduced from nucleotide sequence analysis was compared from position 346 to its carboxy terminus with analogous regions of the proteins specified by the retrovirus *onc* genes shown. Values represent the percentage of positions which are identical. (B) Comparison of T10 translational product with chicken $p60^{c\text{-}src}$. Some amino acid positions are given as references. Percentages refer to amino acid identities in each domain indicated. Sites of tyrosine phosphorylation (NEYTA), regions of each protein possibly involved in ATP binding (GXGXXG), and respective carboxy termini (PQYQPGENL) are shown in single-letter amino acid designations.

onc gene v-*fgr* (Fig. 4). The resulting construct, designated pv-*fgr*/T10, contained the GR-FeSV 5′ long terminal repeat, p15, and γ-actin-coding sequences as well as 219 nucleotides of v-*fgr* (22). The contribution of T10 consisted of 945 bp of coding sequence as well as a portion of its 3′-untranslated stretch. The pv-*fgr*/T10 plasmid induced foci of transformation upon transfection of NIH/3T3 cells with a specific focus-forming activity which was within fourfold of that displayed by pv-*fgr* (Fig. 4). In contrast, the vector plasmid alone, pv-*fgr* Δ3′, was unable to induce morphologic transformation in these same assay cells. These findings demonstrate that pv-*fgr*/T10 has oncogenic properties and suggest that T10 can be activated as a transforming gene.

In an effort to detect the protein specified by the chimeric transforming DNA, cells transformed with pv-*fgr*/T10 were metabolically labeled with [^{35}S]methionine and examined by immunoprecipitation with anti-feline leukemia virus p15 serum, which recognizes the amino-terminal region of the major GR-FeSV translational product, $P70^{gag\text{-}actin\text{-}fgr}$ (22). Cells transformed by the chimeric molecule expressed a 71,000-dalton protein, designated $P71^{gag\text{-}actin\text{-}T10}$, which could be clearly distinguished from $P70^{gag\text{-}actin\text{-}fgr}$ (Fig. 5). This size difference was consistent with the fact that $P71^{gag\text{-}actin\text{-}T10}$ is predicted to possess seven additional residues at its carboxy terminus as compared with the GR-FeSV translational product. Additional lower-molecular-weight proteins were detected in pv-*fgr* or pv-*fgr*/T10 transfectants. However, with this same antiserum, no smaller proteins have been observed in GR-FeSV-transformed nonproducer cells (22). Thus, it is likely that these smaller proteins are products of rearranged plasmid molecules generated during the process of transfection. In other experiments, the $P71^{gag\text{-}actin\text{-}T10}$ hybrid protein was detected when these same cells were metabolically labeled with ^{32}P demonstrating that this molecule was a phosphoprotein (data not shown).

We next tested P71 in an immune complex assay for protein kinase activity. Phosphorylation of P71 and heavy-chain immunoglobulin G (IgG) molecules was observed when P71 was immunoprecipitated with feline leukemia virus p15 antiserum from lysates of pv-*fgr*/T10 DNA transformants (data not shown). To determine whether the observed in vitro kinase activity exhibited specificity for tyrosine residues, phosphorylated P71 and IgG heavy chains were subjected to phosphoaminoacid analysis. Both molecules were detectably phosphorylated only on tyrosine residues (Fig. 6), demonstrating that $P71^{gag\text{-}actin\text{-}T10}$ was a protein-tyrosine kinase. All of these findings, combined with the observed structural relatedness of the T10 translational product to known protein-tyrosine kinases, strongly suggested that the normal T10 gene encoded a protein-tyrosine kinase.

DISCUSSION

In the present study, we have described the isolation and characterization of a previously unknown human cellular gene, designated *slk*, for *src*-like kinase. Although highly related to members of the *src* family of transforming genes, it was possible to demonstrate that *slk* was distinct from human *src*, *fgr*, and *yes* proto-oncogenes. The high degree of similarity between the predicted amino acid sequence of the *slk* translational product and that of known protein-tyrosine kinases suggested that the *slk*-encoded protein also possessed this enzymatic activity. Experiments showing that a hybrid protein molecule containing the carboxy-terminal

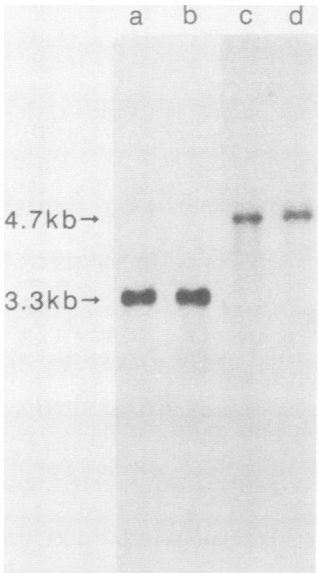

FIG. 3. Detection of T10 mRNA. Five micrograms of poly(A)-containing RNA isolated from normal human endothelial cells (lanes a and c) or fibroblasts (lanes b and d) were analyzed in the presence of formaldehyde by agarose gel electrophoresis and blotting. Nitrocellulose filters were hybridized with nick-translated T10 (lanes a and b) or v-*yes* (lanes c and d) DNA and autoradiographed. The sizes of transcripts detected are indicated.

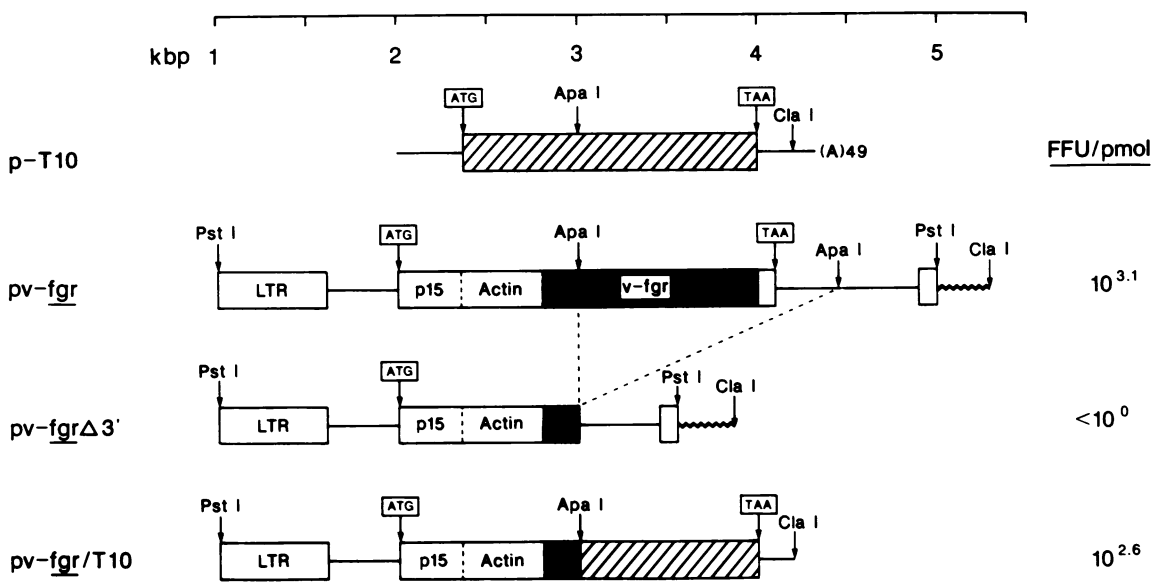

FIG. 4. Focus-forming activity of pv-*fgr*/T10 chimeric DNA. The structures of DNA molecules were confirmed by restriction enzyme analysis and are shown schematically. Focus formation was scored 14 to 21 days after transfection. Abbreviations: LTR, GR-FeSV 5' long terminal repeat; v-*fgr*, GR-FeSV *onc* gene; FFU/pmol, focus-forming units per picomole of insert DNA.

two-thirds of the *slk* translational product specifically phosphorylated tyrosine residues provided further evidence for this possibility. Moreover, the high-titered transforming activity displayed by our retrovirus construct containing the *slk* gene established that normal *slk* could acquire transforming properties under these experimental conditions, suggesting its oncogenic potential.

Within the larger protein-tyrosine kinase family are the genes encoding receptors for epidermal growth factor (8, 40),

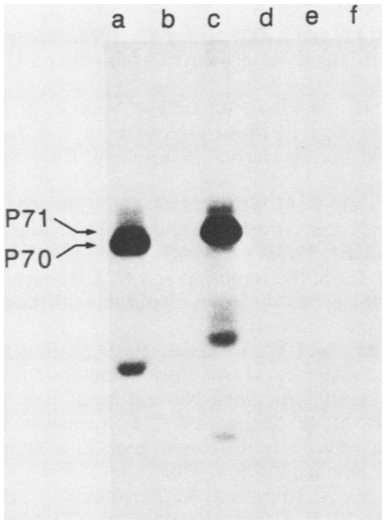

FIG. 5. Detection of the pv-*fgr*/T10 translational product in transfected NIH/3T3 cells. Cells transformed by pv-*fgr* (lanes a and b) or pv-*fgr*/T10 (lanes c and d) or control NIH/3T3 cells (lanes e and f) were metabolically labeled with [^{35}S]methionine. Extracts were incubated with anti-FeLV p15 (lanes a, c, and e) or preimmune (lanes b, d, and f) sera. Immune complexes were precipitated with the aid of protein A-bound Sepharose beads and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The locations of $P70^{gag\text{-}actin\text{-}fgr}$ and $P71^{gag\text{-}actin\text{-}T10}$ are indicated.

the mononuclear phagocyte growth factor CSF-1 (35), insulin (9, 39), and possibly other growth-promoting polypeptides (24). Recently, two protein-tyrosine kinase encoding retrovirus *onc* genes have been defined as altered versions of epidermal growth factor and CSF-1 receptor genes (8, 35, 40). These findings have suggested that tyrosine kinases specified by other retrovirus-transforming genes might also represent growth factor receptors. Our comparisons have revealed that $p60^{c\text{-}src}$ and the putative *slk* translational product, $p60^{slk}$, are almost identical in size and are highly related in amino acid sequence over a stretch which accounts for 85% of their extent. In contrast, even within their conserved tyrosine kinase domains, only distant relationships exist between either protein and known growth factor receptors. Moreover, $p60^{slk}$ does not possess a hydrophobic domain capable of spanning cellular membranes. Such domains are hallmarks of the known polypeptide growth factor receptors described to date. Thus, like $p60^{c\text{-}src}$ and recently described c-*abl* (4) and lsk^{T} (21) gene products, $p60^{slk}$ would appear to represent a protein-tyrosine kinase whose structure is not consistent with that of known polypeptide growth factor receptors.

Studies of *onc* genes closely related to *slk*, such as v-*fgr* (16) and v-*yes* (23), have shown that each of these transforming genes is altered at both the 5' and 3' ends as compared with their normal cellular counterparts. When sequences present in the v-*fgr* transforming gene were replaced by normal 3' *slk*-coding sequences, very little reduction in focus-forming activity was observed. The finding that alteration of the *slk* 3' coding sequence was not required for transforming activity of the v-*fgr*/*slk* construct suggests that abnormal retrovirus-derived carboxy termini of v-*fgr* or v-*yes* gene products might not be essential for their transforming functions.

In contrast to v-*fgr*- and v-*yes*-specified transforming proteins, the v-*src* gene product does not contain helper retrovirus sequences. The major structural difference between the v-*src* gene product and its nontransforming cellular homolog, $p60^{c\text{-}src}$, is the substitution of the last 19

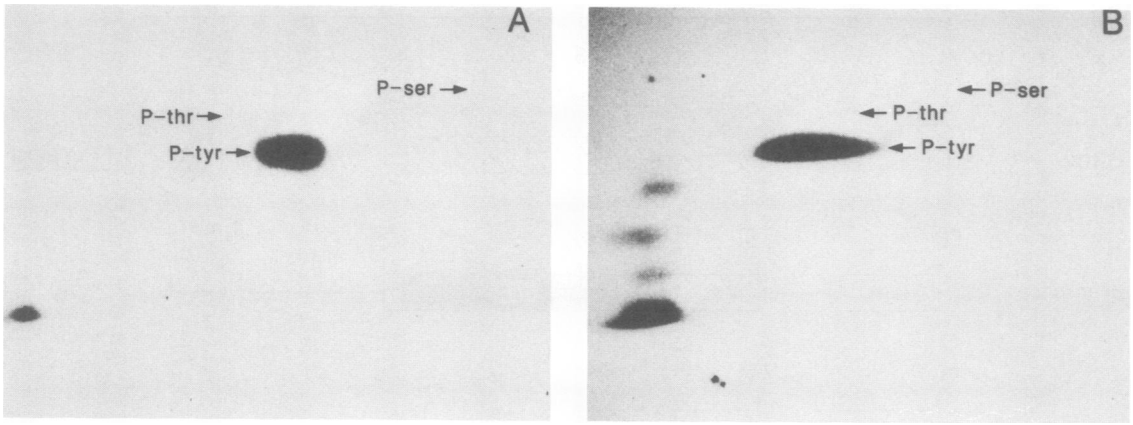

FIG. 6. Phosphoamino acid analysis of proteins labeled in immune complexes containing P71$^{gag\text{-}actin\text{-}T10}$. P71 (A) or IgG heavy chains (B) phosphorylated in vitro were isolated from preparative polyacrylamide gels and subjected to acid hydrolysis. Hydrolysates were mixed with unlabeled phosphoserine (P-ser), phosphothreonine (P-thr), or phosphotyrosine (P-tyr) and analyzed in two-dimensional gels. Migration of individual phosphoamino acids is shown.

carboxy-terminal amino acids of p60$^{c\text{-}src}$ for a new set of 12 amino acid residues (37). This alteration apparently greatly influences but does not alone determine *src* transforming activity (11, 27, 34). The carboxy-terminal 36 amino acids of p60^{slk} are identical to those of the chicken c-*src* gene product, with the exception of three amino acid differences, two of which are conservative changes. Thus, differences between normal and transforming *src* genes provide an excellent model for determining whether more subtle genetic alterations might also activate the *slk* gene. In any case, knowledge that this new gene can be activated as an oncogene under experimental conditions serves as a basis for efforts to search for its possible involvement in naturally occurring malignancies.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Semba et al. (K. Semba, M. Nishizawa, N. Miyajima, M. C. Yoshida, J. Sukegawa, Y. Yamanashi, M. Sasaki, T. Yamamoto, and K. Toyoshima, Proc. Natl. Acad. Sci. USA **83**:5459–5463, 1986) recently described a human gene (*syn*), the coding sequence of which we find to be identical to that of *slk*.

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