Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 6595–6599, October 1985
Genetics

Isolation and chromosomal localization of the human *fgr* protooncogene, a distinct member of the tyrosine kinase gene family

(acute transforming retrovirus/heteroduplex analysis/*in situ* hybridization)

STEVEN R. TRONICK*, NICHOLAS C. POPESCU†, MARC S. C. CHEAH*, DAVID C. SWAN*, SUZANNE C. AMSBAUGH†, CAROLE R. LENGEL*, JOSEPH A. DIPAOLO†, AND KEITH C. ROBBINS*

*Laboratory of Cellular and Molecular Biology and †Laboratory of Biology, National Cancer Institute, Bethesda, MD 20205

Communicated by Robert J. Huebner, May 24, 1985

ABSTRACT **The cell-derived domain of Gardner–Rasheed feline sarcoma virus (GR-FeSV) consists of a γ-actin- and a tyrosine-specific protein kinase-encoding sequence designated v-*fgr*. By utilizing a v-*fgr* probe, it was possible to detect related sequences present at low copy number in DNAs of a variety of mammalian species and to isolate a human *fgr* homologue. Comparative studies revealed that this human DNA clone represented all but 200 base pairs of v-*fgr*. Analysis of human genomic DNA demonstrated that the *fgr* protooncogene was distinct from the cellular homologues of other retrovirus *onc* genes. In addition, the *fgr* protooncogene was localized to the distal portion of the short arm of human chromosome 1 at p36.1–36.2 by *in situ* hybridization. Taken together, our findings establish that the *fgr* protooncogene is a unique member of the tyrosine kinase gene family.**

Acutely transforming retroviruses have arisen in nature as the result of apparently rare recombinational events involving replication-competent type C retroviruses and a small set of evolutionarily conserved cellular genes termed protooncogenes. Such transduced cellular (*onc*) sequences confer upon the recombinant virus the ability to rapidly induce malignancies. Recent studies have indicated that protooncogenes can be activated as oncogenes within human tumor cells in the absence of retrovirus involvement and that such oncogenes may very well play a role in the development of malignancy. Thus, understanding the functions of protooncogenes as well as the mechanisms by which they acquire transforming properties is likely to be critical for deciphering the neoplastic process.

Recent findings regarding the relationship of retrovirus *onc* genes to proteins of known function have strongly implicated growth factor-mediated regulatory pathways in the steps leading to malignant transformation. The simian sarcoma virus transforming gene, v-*sis*, encodes a protein closely related in amino acid sequence (1, 2), secondary structure (3), and antigenic properties (3, 4) to human platelet-derived growth factor (PDGF) (5, 6), a potent mitogen for connective tissue cells (7, 8). In addition, the amino acid sequences of receptors for epidermal growth factor (EGF) and insulin have been shown to possess striking similarity with the tyrosine-specific protein kinases encoded by the v-*erbB* and v-*ros* transforming genes, respectively (9, 10). Several other acutely transforming retroviruses encode protein kinases that are related to one another (11, 12) and possess amino acid sequence homology with EGF (9) and insulin (10, 59) receptors. Moreover, receptors for EGF (13), insulin (14–16), and PDGF (17–20) all respond to their respective growth factors by autophosphorylation on tyrosine residues. Thus, certain growth factor receptors share functional and structural relationships with retrovirus-encoded tyrosine kinases.

Recently we have described the molecular organization of Gardner–Rasheed feline sarcoma virus (GR-FeSV) (21), an acutely transforming retrovirus (22) that encodes a tyrosine-specific protein kinase (23). Nucleotide sequence analysis has revealed that the GR-FeSV kinase is related to other retrovirus-encoded protein kinases, in particular, the products of avian v-*src* and v-*yes onc* genes (24). In the present study, isolation and structural characterization of the human *fgr* protooncogene has made it possible to determine its chromosomal localization and relationship to other members of the tyrosine kinase gene family.

MATERIALS AND METHODS

Molecular Clones. Two DNA fragments representing nucleotides 915–1756 or 711–915 of the gene encoding the primary GR-FeSV translational product (24) were cloned into pBR322. The former clone, designated pv-fgr-1 contained only tyrosine kinase-encoding sequences, whereas the latter clone, designated pv-fgr-2, contained 3′ γ-actin- and 5′ tyrosine kinase-encoding sequences. A pBR322 clone of integrated GR-FeSV has been described (21). A DNA clone containing v-*src* sequences (pEcoRIB) (25) was obtained from the American Type Culture Collection (no. 41005). Unintegrated Y73 avian sarcoma virus DNA provided by K. Toyoshima was used to prepare a 1.15-kilobase-pair (kbp) v-*yes*-specific probe that encompassed nucleotides 1652–2801 (26).

Isolation of v-*fgr*-Related Human Sequences. A bacteriophage library of human fetal liver DNA was provided by T. Maniatis (27). The bacteriophage were plated at a density of 2×10^4 plaque-forming units per dish with *Escherichia coli* strain BNN45, which was a gift from K. Shimizu and M. Wigler. Nitrocellulose filter replicas of the infected bacterial lawns, prepared by the method of Benton and Davis (28), were hybridized at 62°C for 24 hr in a solution containing ^{32}P-labeled pv-fgr-1 probe (specific activity, 2×10^8 cpm/μg), 6× NaCl/Cit (1× NaCl/Cit = 0.15 M NaCl/0.015 M sodium citrate), 5× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% bovine serum albumin/0.02% Ficoll), and 100 μg of sheared salmon testes DNA per ml. Filters were washed at 45°C in 0.1 × NaCl/Cit containing 0.1% $NaDodSO_4$ and autoradiographed at −70°C for 24 hr with Kodak XAR film and Dupont Cronex Lightning Plus screens.

Analysis of Cloned and Genomic DNAs. DNAs were digested with restriction enzymes, fractionated by electrophoresis through agarose gels, and blotted onto nitrocellulose filters as

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Abbreviations: FeSV, feline sarcoma virus; GR-FeSV, Gardner–Rasheed-FeSV; kbp, kilobase pair(s).

described (29). DNA fragments were labeled with [^{32}P]dCTP by nick translation to a specific activity of 2×10^8 cpm/μg and incubated with nitrocellulose filters in a buffer containing 20 mM sodium phosphate (pH 6.4), 50% formamide, 10% dextran sulfate, 5 mM EDTA, 2× Denhardt's solution, 5× NaCl/Cit, and 50 μg of sonicated single-stranded salmon testes DNA per ml. After hybridization at 42°C for 24–48 hr, filters were washed sequentially at room temperature in 2× NaCl/Cit and then at 42°C in 0.1× NaCl/Cit containing 0.1% $NaDodSO_4$. Filters were autoradiographed as described above.

Heteroduplex Analysis of Cloned DNAs. Linearized plasmid DNAs were hybridized in the presence of 50% formamide at 22°C for 4–6 hr. Samples were spread onto a distilled-water hypophase and prepared for electron microscopy as described (30). Contour lengths were determined by comparison to ϕX174 single- and double-stranded DNAs with the aid of a Zeiss Videoplan 2 image analysis system.

***In Situ* Chromosome Hybridization.** Cultured peripheral blood lymphocytes from a normal male donor were stimulated with phytohemagglutinin and synchronized with methotrexate (0.1 μM) prior to harvest (31). Chromosomes were prepared by a standard air-dried method after hypotonic treatment in 0.075 M KCl and several fixations in methanol/glacial acetic acid, 3:1 (vol/vol). The method described by Harper and Saunders (32) was used for *in situ* hybridization. Chromosome preparations were hybridized with 1 μg of [^{3}H]DNA probe (specific activity, 2.2×10^7 cpm/μg) per ml, washed, covered with nuclear track emulsion NTB2 (Kodak), and stored dessicated at 4°C for 14 days. Autoradiographs were developed, rinsed briefly in water, and fixed; air-dried slides were stained for 5 min with 0.25% Wright stain in 60 mM phosphate, 1:3 (vol/vol) at pH 6.8 (32). Slides were then destained through an alcohol series and restained with Wright stain to improve the quality of G-bands. Grains were counted directly on banded chromosomes.

Analysis of mRNA. Total cellular RNA was purified from tissues by a modification of the guanidine hydrochloride method (33) as described by Adams *et al.* (34). Poly(A)-containing RNA was isolated by chromatography on oligo(dT)-cellulose columns. RNAs were fractionated in the presence of formaldehyde by agarose gel electrophoresis, transferred to nitrocellulose filters (35), and hybridized with nick-translated pv-fgr-1 DNA as described by Wahl *et al.* (36).

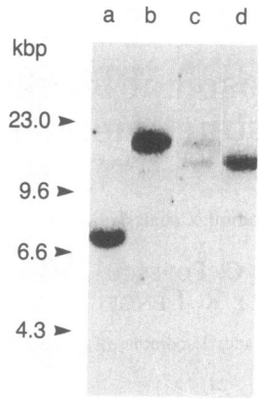

FIG. 1. Detection of v-*fgr*-related sequences in mammalian DNAs. DNAs isolated from normal cat (lane a), raccoon (22) (lane b), rhesus monkey (lane c), and human (lane d) cells were digested with *Hin*dIII, fractionated by agarose gel electrophoresis, and subjected to Southern analysis with the pv-fgr-1 probe. *Hin*dIII digests of bacteriophage λ DNA were used as molecular size standards.

RESULTS

v-*fgr* Is Derived from an Evolutionarily Conserved Cellular Gene. Previous studies have shown that the primary GR-FeSV translational product possesses a tyrosine-specific protein kinase activity encoded by a sequence, v-*fgr*, which is not related to the genome of its parental helper virus (21). To examine the relationship of v-*fgr* to the mammalian genome, a DNA fragment representing v-*fgr* was subcloned into a plasmid vector for use as a probe. This probe, designated pv-fgr-1, detected related sequences in cellular DNAs from a variety of species (Fig. 1). Single bands were observed in *Hin*dIII digests of cat, racoon, and human DNA, and two bands were detected in rhesus monkey DNA. These findings demonstrated that v-*fgr* was cell-derived and conserved within the mammalian genome at low- or single-copy number.

Isolation of v-*fgr*-Related Sequences from Human DNA. In an effort to isolate cellular sequences related to v-*fgr*, a human fetal liver DNA λ phage library (27) was screened with pv-fgr-1 probe. A single hybridizing phage clone was isolated and shown to contain 17 kbp of human sequence that was not actin-related (data not shown). Recombinant phage DNA, treated with *Eco*RI, was examined by agarose gel electro-

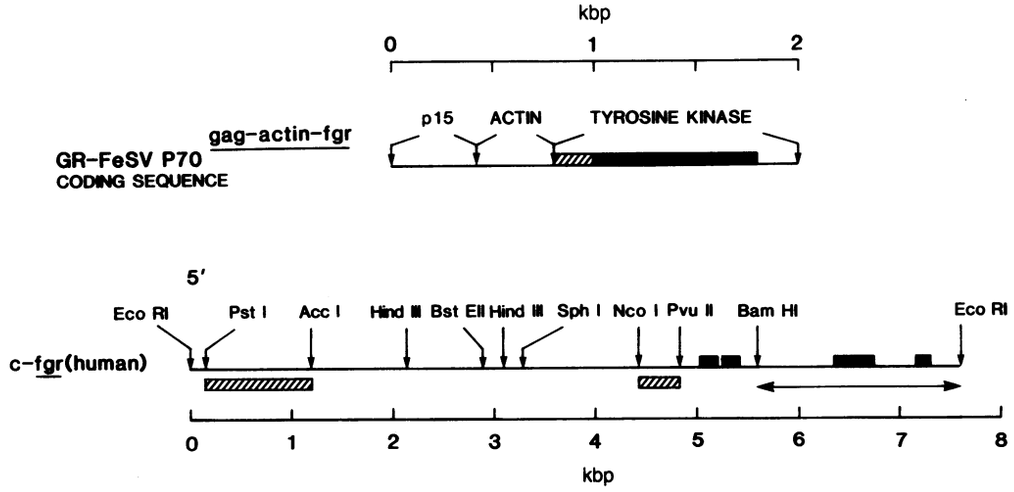

FIG. 2. Structure of the human *fgr* protooncogene. The location of pv-fgr-1 and pv-fgr-2 probes within GR-FeSV are indicated by the black and cross-hatched boxes, respectively. Regions of c-*fgr* (human) that were hybridized by pv-fgr-1 or -2 probes are similarly indicated; "5'" represents the orientation of human *fgr*-related sequences with respect to the GR-FeSV genome. A subclone containing the 2-kbp region defined by *Bam*HI and *Eco*RI sites at 5.6 and 7.6 kbp, respectively, and indicated by the double-headed arrow was designated pc-fgr BE2. Not all *Pvu* II sites are shown. The *Eco*RI site at 7.6 kbp on the c-*fgr* (human) map is a linker inserted during the construction of the library (27).

phoresis and Southern blotting with pv-fgr-1 as a probe. Of the four human DNA fragments observed, only one was hybridized. This fragment, 7.6 kbp in length, was isolated by cloning and designated pc-fgr E7.6. This clone was further analyzed by restriction enzyme mapping and hybridization with pv-fgr-1 and a probe designated pv-fgr-2, which contained sequences representing both actin- and tyrosine kinase-encoding portions of the GR-FeSV genome. As summarized in Fig. 2, the pv-fgr-2 probe detected DNA fragments that localized to both terminal regions of the 7.6-kbp human DNA fragment, whereas pv-fgr-1 probe hybridized fragments derived from only one end. This analysis oriented the human v-*fgr*-related sequence with respect to the GR-FeSV genome (Fig. 2). Furthermore, these data suggested that v-*fgr*-related sequences were arranged within the human genome in a manner consistent with a cellular gene possessing noncontiguous coding regions.

Comparison of the Human *fgr* Sequence with GR-FeSV DNA by Heteroduplex Analysis. In order to obtain more detailed information concerning the size and distribution of the v-*fgr*-related sequences present in our human DNA clone, pc-fgr E7.6 was compared with GR-FeSV DNA by heteroduplex analysis. Four stretches of pc-fgr E7.6 were related to GR-FeSV DNA (Fig. 3). This homology was localized to the same region of pc-fgr 7.6 that has been identified by hybridization with the pv-fgr-1 probe. Those stretches detected by the pv-fgr-2 probe (Fig. 2) were not observed by heteroduplex analysis, suggesting that such regions were either too short or not well-enough conserved to form a stable heteroduplex under the conditions utilized. These data revealed that all but ≈200 bp at the 3′ terminus of v-*fgr* was present within pc-fgr E7.6 DNA.

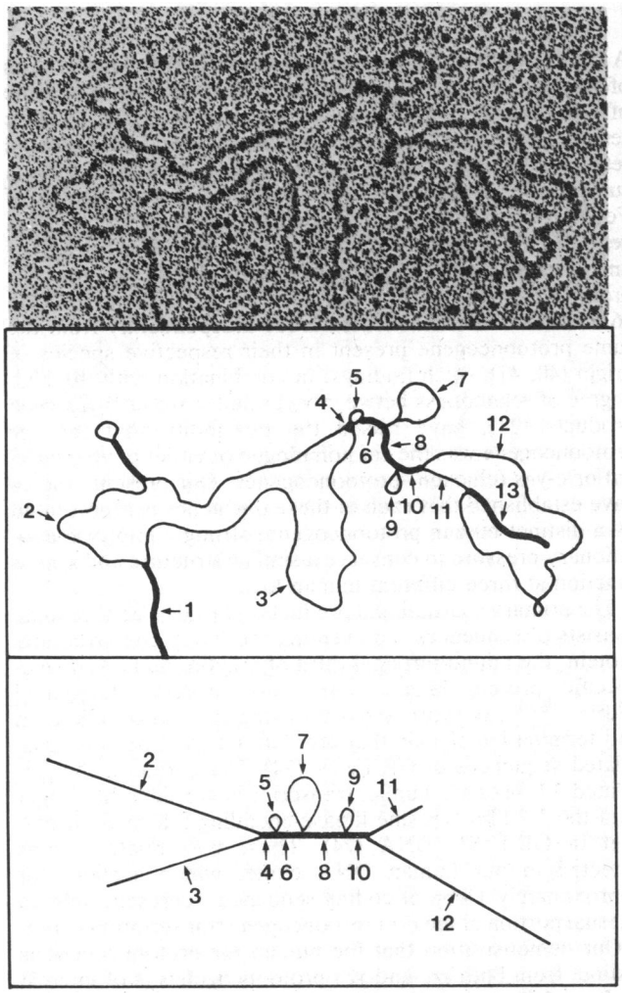

FIG. 3. Heteroduplex analysis of the human *fgr* protooncogene. (*Top*) A representative heteroduplex formed between c-*fgr* E7.6 and GR-FeSV plasmid DNAs. (*Middle*) Tracing of this structure. (*Bottom*) Interpretive sketch (not to scale). Double-stranded regions are indicated by thick lines, and single-stranded regions, by thin lines. Features 1 and 13 are plasmid vector arms (feature 1 is not shown in its entirety). Features 2 and 4–11 represent c-*fgr*; 3, 4, 6, 8, 10, and 12 represent v-*fgr*. Contour lengths (in kbp) of nonplasmid features are as follows: 2, 5.0; 3, 3.8; 4, 0.13; 5, 0.05; 6, 0.13; 7, 0.97; 8, 0.38; 9, 0.41; 10, 0.12; 11, 0.3; and 12, 3.2.

The Human *fgr* Protooncogene Is Distinct from *src* and *yes* Protooncogenes. In an effort to establish that the v-*fgr*-related sequences present in pc-fgr E7.6 represented the *fgr* protooncogene, human genomic DNA was examined by the Southern technique with pv-fgr-1 and a repetitive sequence-free region of our human DNA clone, designated pc-fgr BE2, as probes. Each probe detected identical bands of 14.5 kbp in *Hin*dIII digests or 2.6 kbp in *Bam*HI digests (Fig. 4), conclusively demonstrating that pc-fgr E7.6 represented the human *fgr* protooncogene.

The highly conserved nature of the predicted products of feline-derived v-*fgr* and avian-derived v-*src* and v-*yes* (24) has raised the possibility that the *fgr* protooncogene is the cat homologue of either c-*src* (chicken) or c-*yes* (chicken) protooncogenes. To address this question, human DNA was subjected to Southern analysis with v-*src* and v-*yes* probes. Each probe detected related sequences in either *Hin*dIII or *Bam*HI digests (Fig. 4). The v-*src* probe hybridized bands of 10.5 and 2.8 kbp in the *Hin*dIII digest and bands of 8.7, 2.8, and 0.8 kbp in *Bam*HI digests. In contrast, the v-*yes*-related bands of 4.8, 3.7, 3.2, 2.65, 2.0, and 1.48 kbp as well as 10.5 kbp were detected in *Hin*dIII- or *Bam*HI-treated DNAs, respectively. Since none of the human DNA fragments hybridized by v-*src* or v-*yes* probes corresponded to those detected with *fgr* probes (Fig. 4, lanes A), the human genome must contain distinct protooncogenes related to each of these tyrosine kinase-encoding *onc* genes.

Chromosomal Localization of c-*fgr* (Human). The availability of a characterized human *fgr* protooncogene probe made it possible to attempt localization of this sequence within the

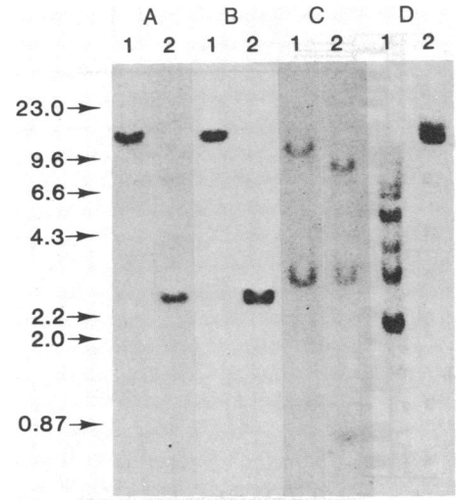

FIG. 4. The human *fgr* protooncogene is distinct from human v-*src*- and v-*yes*-specific sequences. Twenty-five micrograms of normal human DNA were digested with either *Hin*dIII (lanes 1) or *Bam*HI (lanes 2) and subjected to agarose gel electrophoresis and Southern analysis with pv-fgr-1 (lanes A), pc-fgr BE2 (lanes B), v-*src* (lanes C) or v-*yes* (lanes D) as probes. A *Hin*dIII digest of bacteriophage λ DNA was used to provide molecular size standards in kbp for lanes A–C. Sizes of bands in lanes D are given in the text.

human genome. For this purpose, pc-fgr BE2 DNA (Fig. 2) was nick-translated and used for *in situ* hybridization of normal human lymphocyte chromosome preparations. The specific location of silver grains was assigned by chromosome banding after hybridization and autoradiography. A total of 324 grains were scored, 152 (47%) of which were observed on chromosome 1. Of these, 96 (63%) were localized beyond the distinctive G-positive band, p35, with the greatest accumulation of grains between p35 and p36.2 (Fig. 5). In contrast, grains not on chromosome 1 were randomly distributed among the other chromosomes (Fig. 5*B*). We conclude from these results that the *fgr* protooncogene is located on human chromosome 1 at p36.1–36.2.

Expression of the *fgr* Protooncogene in Human Cells. To determine whether the human *fgr* protooncogene was normally expressed, RNA isolated from human tissues was analyzed by blot-hybridization with pv-*fgr*-1 as a probe. A v-*fgr*-related transcript of 3.0 kb was detected in normal human lung but not in human liver RNA preparations (Fig. 6). As a control, each RNA preparation was hybridized to a similar extent by an actin probe (data not shown). These findings demonstrate that the *fgr* protooncogene is transcriptionally active in certain normal human cells.

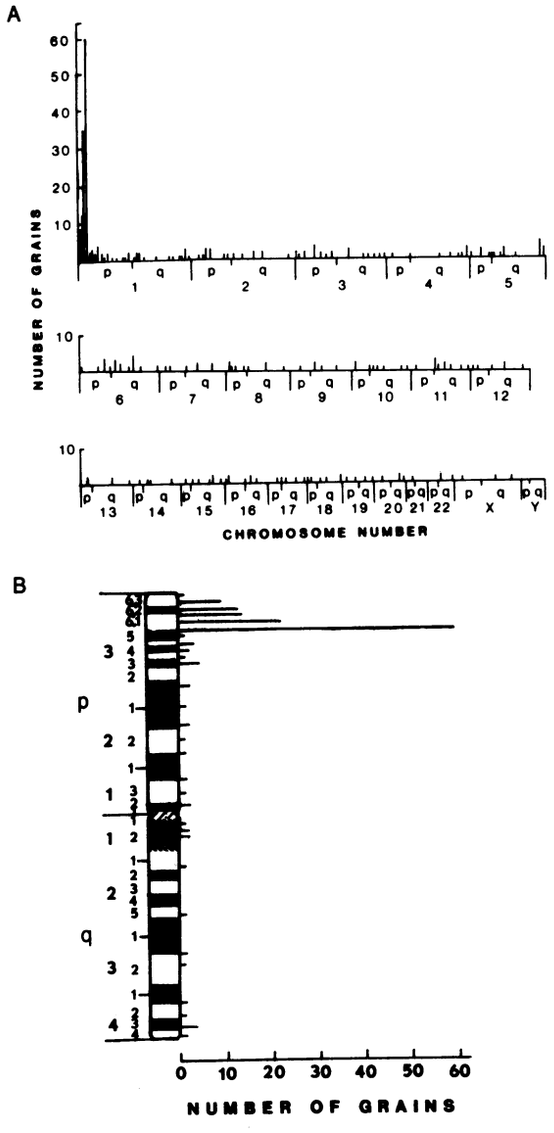

FIG. 5. Localization of the human *fgr* protooncogene by *in situ* hybridization. Human lymphocyte chromosomes were hybridized with pc-fgr BE2, G-banded, and scored. (*A*) Histogram of silver grain distribution in 102 metaphases based on localization on a 400-band ideogram (37). (*B*) Distribution of silver grains on chromosome 1.

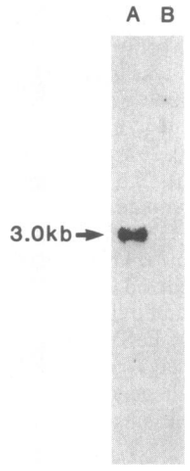

FIG. 6. Detection of a human *fgr*-related transcript. Ten micrograms of poly(A)-containing RNA isolated from normal human lung (lane A) or liver (lane B) were analyzed by blot-hybridization with pv-fgr-1 as probe. The transcript size is indicated in kb.

DISCUSSION

A family of retroviral *onc* genes has been defined on the basis of significant sequence similarities and by the ability of some of their translational products to phosphorylate tyrosine residues in protein substrates (11, 12). Independently isolated retroviruses belonging to this tyrosine kinase gene family, such as Snyder–Theilen and Gardner–Arnstein strains of FeSV, have captured the c-*fes* cellular sequence from the cat genome (38), whereas the Fujinami sarcoma virus has apparently transduced the chicken homologue of the *fes* protooncogene (38, 39). Similarly, the MH2 avian and the 3611 murine sarcoma viruses arose independently from the same protooncogene present in their respective species of origin (40, 41). Such findings, in combination with the high degree of relatedness between v-*fgr* and v-*src* or v-*yes* gene products (24), have raised the possibility that the *fgr* protooncogene was the cat homologue of either c-*src* (chicken) or c-*yes* (chicken) protooncogenes. Our present studies have established that each of these *onc* genes is represented by a distinct human protooncogene, strongly implying evolutionary pressure to conserve a similar structure and kinase function at three different human loci.

The primary translational product of the GR-FeSV genome consists of sequences representing the FeLV p15 structural protein, the amino-terminal third of γ-actin, and a tyrosine-specific protein kinase (24). This protein, designated $p70^{gag\text{-}actin\text{-}fgr}$, is synthesized by using translation initiation and termination signals that are derived from helper virus-related sequences of GR-FeSV (24). Furthermore, the *fgr*-related 3.0-kilobase human transcript is considerably larger than the 1.2-kbp tyrosine kinase-encoding information present in GR-FeSV DNA (24). Thus, v-*fgr*-related exons detected in our human DNA clone, which account for approximately 1 kbp of coding sequence, represent only an internal portion of the *fgr* protooncogene transcriptional unit.

Our demonstration that the human *fgr* protooncogene is distinct from both *src* and *yes* protooncogenes is of interest in view of the recent detection of two *src*-related human loci, one on chromosome 20 (42, 43) and the other residing on chromosome 1 at 1p34–36 (42), a location similar to that of the human *fgr* gene. Although it cannot be excluded that the *src*-related protooncogene detected on chromosome 1 is actually the c-*fgr* protooncogene, it is also possible that *src* and *fgr* protooncogenes map to distinct loci, which are indistinguishable at the level of *in situ* hybridization.

Many forms of cancer, including solid and hematopoietic tumors (44–46), display alterations affecting chromosome 1

(47–51). This chromosome is also known to contain a number of other genes that affect cell growth regulation, including the oncogenes B-*lym* (p32) (52), N-*ras* (p11–p13) (53–56) and *ski* (q12–qter) (57), as well as the gene encoding nerve growth factor (p21–p22) (58). Specific deletions involving 1p31–36 have been described in certain neuroblastomas and result in monosomies of the terminal region of 1p (46–51). Our present findings now make it possible to assess whether *fgr* protooncogene structure and/or expression is perturbed in human tumors possessing chromosome 1 alterations.

Note Added in Proof. A recently published physical map of c-*src-2*, one of two human v-*src*-related genes (60), shows striking similarity to the restriction map of the human c-*fgr* gene. In addition, the c-*src-2* nucleotide sequence is more homologous to v-*fgr* than to v-*src* or v-*yes*. Thus it appears that c-*src-2* and c-*fgr* (human) are the same gene.

We are grateful to S. A. Aaronson for his advice and support.

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