Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 1603–1607, March 1989
Genetics

Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction

(gene families/DNA amplification *in vitro*/degenerate oligonucleotides)

ANDREW F. WILKS

Ludwig Institute for Cancer Research, Royal Melbourne Hospital, Parkville, Victoria 3050, Australia

Communicated by G. J. V. Nossal, November 28, 1988

ABSTRACT **The pivotal role that protein-tyrosine kinases (PTKs) play in the growth regulation of eukaryotic cells is manifest in the frequent appearance of members of the PTK family as growth factor receptors or as the transforming agents of acutely transforming retroviruses. A feature common to all members of the PTK family is a highly conserved catalytic domain which is characteristic of the group as a whole and whose activity appears to be tightly regulated within the cell by other domains of the PTK. Degenerate oligonucleotide probes corresponding to two invariant amino acid sequence motifs within the catalytic domains of all PTK family members were synthesized and employed in the polymerase chain reaction (PCR) to amplify cDNA sequences between them. An M13 PCR library was produced in this way from cDNA prepared against mRNA from the murine hemopoietic cell line FDC-P1. The PCR library was then screened by DNA sequencing for PTK-related sequences. Two sequences were identified that, on the basis of sequence comparison with known PTKs, may encode representatives of a new class of PTK.**

Protein-tyrosine kinases (PTKs; EC 2.7.1.112) are believed to play an important role(s) in the metabolism of the cell, most probably as components of signal-transduction pathways. Indirect evidence in support of this presumption is found in the frequent identification of members of the PTK family as cellular receptors for certain growth factors (1–8) and as products of the oncogenes of many of the acutely transforming retroviruses (7–12). Hanks *et al.* (13) recently suggested that the protein kinases could be mapped phylogenetically, based on the conservation of the amino acid sequences of their catalytic domains. Similar phylogenetic mapping of the PTK family suggested that there may be clusters of related subfamilies, such as the Src family [including c-Yes, c-Lyn, and hemopoietic cell kinase (HCK), among others], the Fes family (currently containing only c-Fes), and the colony-stimulating factor 1 (CSF-1) receptor family [including c-Kit and the platelet-derived growth factor (PDGF) receptor]. Such speciation is also evident in the noncatalytic domains of the PTKs; it is highly probable that the PTK catalytic domain has been recombined with a wide variety of noncatalytic domains, so that the use of the tyrosine kinase activity can be deployed in a regulated manner in as wide a variety of situations as possible.

Several approaches have been tried in order to identify novel members of the protein kinase family, such as low-stringency screening of cDNA libraries with previously characterized cDNA probes (14) or the use of degenerate oligonucleotide probes based on the highly conserved regions of the protein kinase catalytic domain (15). Each of these approaches has been successfully applied. However, more recently, a technique has been developed that is capable of greatly facilitating the isolation of any gene for which some sequence data are known. The polymerase chain reaction (PCR) (16, 17) technique has already demonstrated considerable flexibility in its application to a wide variety of diagnostic and molecular biological situations. The technique is based on the enzymatic amplification of sequences residing between two oligonucleotide primers that define the 5′ and 3′ borders of the amplified segment. The technique has recently been applied to the cloning of cDNA sequences defined by mixed oligonucleotides derived from selected amino acid sequences (18). A natural extension of this application is in cloning members of gene families, such as those comprising the PTK family. To detect members of the PTK family expressed in hemopoietic cells, the technique has been applied to cDNA from a murine factor-dependent hemopoietic cell line, FDC-P1 (19), and PTK-related sequences have been isolated.* The isolation of new PTK-related sequences will aid in the understanding of the response of these cells to growth factors such as the colony-stimulating factors, a response that is known to be accompanied by changes in the patterns of intracellular protein phosphotyrosine metabolism (20).

MATERIALS AND METHODS

Nucleic Acids. Poly(A)$^+$ RNA was prepared by the method of Gonda *et al.* (21). In brief, cells were washed in isotonic phosphate-buffered saline and resuspended at 10^7 per ml in 0.1 M NaCl/10 mM Tris, pH 7.5/1 mM EDTA. Before homogenization at high speed in a Polytron (Selby–Anax), the cell lysate was made 1.0% with respect to SDS, and proteinase K was added to a final concentration of 200 μg/ml. After digestion for 4 hr at 37°C, poly(A)$^+$ RNA was isolated by the addition of 500 mg of oligo(dT)-cellulose (Sigma), followed by low-salt elution. RNA was recovered by ethanol precipitation. Oligonucleotides were synthesized on an Applied Biosystems 380A oligonucleotide synthesizer using standard chemistry; the crude oligonucleotide preparation was not purified further.

DNA Amplification. Poly(A)$^+$ mRNA from the growth factor-dependent hemopoietic cell line FDC-P1 and an Amersham cDNA-synthesis kit were used to generate oligo(dT)-primed double-stranded cDNA (1 μg). Five microcuries of [α-^{32}P]dATP (3000 Ci/mmol; 1 Ci = 37 GBq) was included in the reaction mixture to label the cDNA product. PCR was performed with a Geneamp kit (Cetus), using the manufacturer's buffers and modified conditions, and the PTK I and PTK II oligonucleotides (1 μg each). The PCR cycle was 1.5 min at 95°C (denaturation), 2 min at 37°C (annealing), and 3 min at 63°C (elongation). An additional 5 μCi of [α-^{32}P]dATP was included to label the PCR product. For cloning, the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PCR, polymerase chain reaction; PTK, protein–tyrosine kinase.

*The sequences reported in this paper have been deposited in the EMBL/GenBank data base (accession no. J04523).

amplified DNA was ethanol-precipitated, air-dried, and resuspended in 20 μl of double-distilled water before being digested in *Eco*RI buffer with *Eco*RI and *Bam*HI (20 units each) for 4 hr at 37°C. The DNA was then purified on an Elutip-d column (Schleicher & Schuell) and ligated into *Eco*RI- and *Bam*HI-cleaved M13mp19. Sequencing was carried out by the dideoxy chain-termination method (22), using a Sequenase kit (United States Biochemical). In all cases [α-[^{35}S]thio]dATP (Amersham, catalogue no. SJ304) was the preferred radionucleotide.

RNA Analysis. Poly(A)$^+$ mRNA samples were prepared as described (21), Aliquots (1 μg) were electrophoresed in 1% agarose gels containing 2.2 M formaldehyde, 20 mM Mops (pH 6.8), 1 mM EDTA, and 5 mM sodium acetate, and the electrophoretically separated RNAs were transferred to Hybond N (Amersham, catalogue no. RPN303N) or nitrocellulose (Schleicher & Schuell, BA85, catalogue no. 401196) membranes. Filters were prehybridized for 4 hr in 50% formamide containing 3× SSC (1× is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), 5× Denhardt's solution (1× is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 10 mM Hepes (pH 7.0), poly(C) at 100 μg/ml, denatured herring sperm DNA at 100 μg/ml, *Escherichia coli* DNA at 10 μg/ml, and 0.1% SDS and were hybridized for 18 hr at 42°C in the same solution with nick-translated ^{32}P-labeled DNA insert. Filters were washed to a final stringency of 0.2× SSC/0.1% SDS at 68°C before exposure to Kodak XAR-5 x-ray film with two intensifying screens.

RESULTS AND DISCUSSION

Strategy. The basic strategy of the approach is outlined in Fig. 1. Three regions of the catalytic domain of the PTKs were used to derive oligonucleotide primers. To select suitable regions from which to generate PTK oligonucleotide primers, careful consideration was given to identifying the most highly conserved regions of the PTK catalytic domain. Fig. 2 shows a comparison of 13 PTK amino acid sequences, from which consensus sequences have been derived from particularly highly conserved regions. Of these, two particular sequences designated PTK I and PTK II were selected for the derivation of oligonucleotide primers. The sequence -IHRDL- (PTK I) at position 392 in c-Src defined the 5′ border of the region of the cDNA to be amplified, and the sequence -DVWSFG- (PTK II) located at position 453 in c-Src defined the 3′ border. Between these two sequences is a characteristic and highly conserved sequence, -P(V/I)-(K/R)W(M/T)APES-, which served as a reference point with which to identify potential PTK sequences. The amino acid sequences of known PTKs in this region, although broadly conserved across the family of PTKs, are sufficiently diverse to permit the designation of any candidate PTK clones as previously known or novel and tentatively provided a more precise assignment—for example, to the cytoplasmic (Src-like) or the growth factor receptor subfamily.

The oligonucleotide probes derived (Fig. 3) were based upon the DNA sequences encoding the two consensus amino acid sequences. The DNA sequences encoding these motifs exhibited considerably less degeneracy than a simple decoding of the amino acid sequences would predict. This is presumably due to a high degree of evolutionary codon selection. The PTK I and PTK II probes were thus mixtures of 64 and 8 different oligonucleotide sequences, respectively. The predicted t_m range of the oligonucleotides was 39–45°C for PTK I and 45–49.2°C for PTK II. Although this was well below the 63°C elongation temperature selected for the PCR reaction, it was anticipated that the 37°C annealing step incorporated into the protocol would additionally serve as an extension phase, during which the oligonucleotides would be elongated sufficiently to remain hybridized as the temperature increased to 63°C. Each oligonucleotide was additionally designed to include a restriction enzyme-specific linker flanking the consensus PTK sequence, facilitating the subsequent directional cloning of amplified fragments into M13 phage.

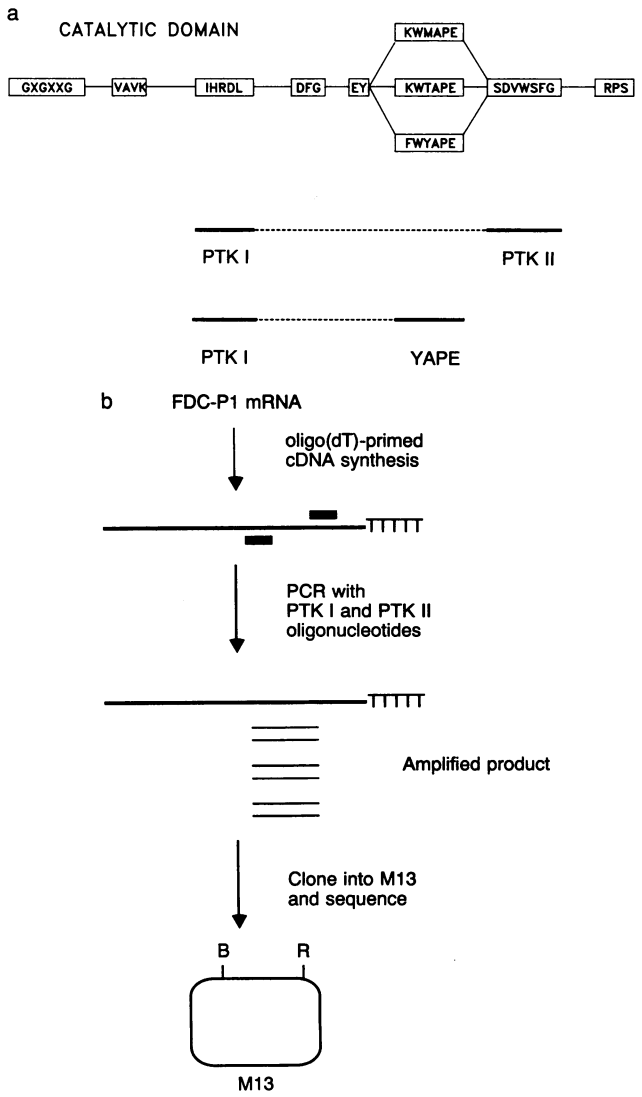

FIG. 1. Strategy for cloning PTK-related sequences. (*a*) Conserved regions of PTK catalytic domains. Blocks of identity held in common between the catalytic domains of PTKs are shown as boxes, with the conserved motif written in one-letter code within. Oligonucleotide probes were initially derived from the -IHRDL- motif (PTK I) and the -DVWSFG- motif (PTK II). A further round of PCR was performed using PTK I and the FWYAPE-based (YAPE) oligonucleotide. (*b*) Summary of the PCR cloning strategy. After synthesis of FDC-P1 cDNA, the cDNA was amplified by using PTK I and PTK II oligonucleotides as primers. After codigestion with *Bam*HI and *Eco*RI the amplified fragment was ligated into M13mp18, and sequence analysis was performed on 200 clones. B, *Bam*HI site; R, *Eco*RI site.

DNA Amplification. Fig. 4 shows the PCR-amplified product of cDNA generated by using FDC-P1 mRNA as a template. After 22 cycles of amplification an obvious ethidium bromide-stained DNA band of the expected size (≈210 base pairs) was detected. This DNA fragment was radiolabeled during the PCR process, confirming its origin as part of the amplification process. Additionally, the background radioactivity present in the track was increased, probably due to nonspecific priming of the original template cDNA. Approximately 1 μg of amplified DNA was generated. After

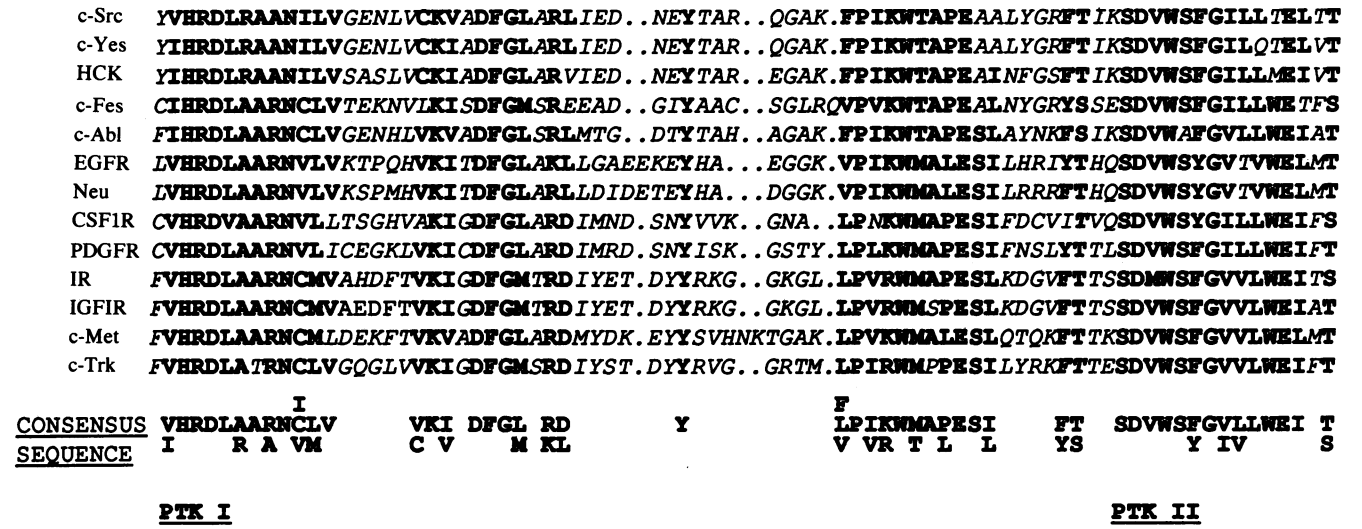

FIG. 2. Derivation of consensus sequences for PCR primers. Alignment of amino acid sequences from conserved regions of various PTK catalytic domains. The appropriate regions of murine neuronal c-Src [amino acids (aa) 391–466; ref. 9], human c-Yes (aa 392–367; ref. 10), murine HCK (aa 354–429; ref. 14), murine c-Fes (aa 677–753; ref. 11), human c-Abl (aa 359–434; ref. 12), human epidermal growth factor receptor (EGFR, aa 809–885; ref. 7), human Neu (aa 841–917; ref. 1), murine colony-stimulating factor 1 receptor (CSF1R, aa 772–848; ref. 8), human platelet-derived growth factor receptor (PDGFR, aa 789–886; ref. 2), human insulin receptor (IR, aa 1116–1192; ref. 3), human insulin-like growth factor I receptor (IGFIR, aa 1101–1177; ref. 4), human c-Met (aa 1118–1192; ref. 5), and human c-Trk-2[h] (aa 289–365; ref. 6) are shown. Dots correspond to gaps introduced to optimize sequence alignment, and each dot represents one amino acid. Conserved amino acids are shown in bold type and the derived consensus sequences are shown below. Amino acid sequences chosen for the derivation of PCR primers are designated PTK I and PTK II.

*Bam*HI/*Eco*RI digestion of the PCR product the DNA was cloned into *Bam*HI/*Eco*RI-cleaved, phosphatase-treated M13mp19 replicative-form DNA.

Initially, 24 M13 clones were fully sequenced by the dideoxy method (22), and the encoded protein sequences were compared with known PTK sequences. Subsequently, a total of 200 clones were examined by comparison of single base sequencing reactions and novel clones were fully sequenced; the protein sequences of representative clones are shown in Fig. 5. Of these 200 clones, 133 contained a PTK-related sequence, 55 contained no insert, and the remainder contained head-to-tail oligonucleotide dimers or sequences unrelated to PTKs. Clone FD15 was identical to murine c-*fes* (11). Clone FD16 showed 95% amino acid sequence identity to the human insulin-like growth factor I (IGF-I) receptor (4) and 92% amino acid sequence identity to the human insulin receptor (3) and probably represented a murine equivalent of the IGF-I receptor. Clone FD19 was 98% identical to human c-*met* (5) and is thus a strong candidate for the murine homologue of this gene. Finally, clone FD175 showed 100% amino acid identity to a murine c-*lyn* clone described by others (S. McEwen, D. Holtzman, and A. R. Dunn, personal communication).

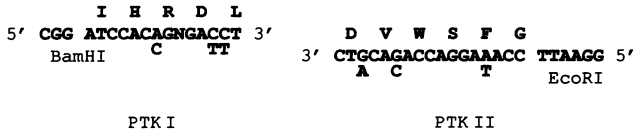

FIG. 3. Oligonucleotide sequences employed in the PCR amplification of PTK sequences. Nucleotide sequences encoding the PTK I and PTK II consensus sequences were derived from the amino acid sequences of the PTKs listed in Fig. 2. Amino acid sequences are shown in one-letter code above the oligonucleotide sequences; alternative nucleotides are shown below the sequence. The N at position 12 in PTK I denotes the use of all four bases. The PTK II coding sequence was reversed and complemented before synthesis of the corresponding oligonucleotide. Restriction sites for *Bam*HI and *Eco*RI have been built into the PTK I and PTK II oligonucleotides, respectively.

Unassigned PTK-Related Sequences. Clones FD17 and FD22 currently remain without a known cellular homologue. The presence of an "acidic" tyrosine residue 10–12 amino acids C-terminal to the conserved -DFG- motif is invariant in the PTK family and is encoded in both FD17 and FD22. The FD17 and FD22 sequences are thus highly similar to other members of the PTK family. Comparison of the amino acid sequences of FD17 and FD22 showed them to be more closely related to each other (73% identical) than to any other sequences in the data bases searched (EMBL/GenBank, National Biomedical Research Foundation, Swiss Oncogene, Melbourne; the highest score was with v-*abl* at 50% vs. FD17 and 51.5% vs. FD22). Of particular interest is the sequence around the highly conserved PTK-specific motif -(K/R)-W(M/T)APES- (Fig. 2). Two of the most highly conserved features of this motif (the basic residue, lysine or arginine, preceding the tryptophan, and the methionine or threonine residue following the tryptophan) are replaced in the FD17/FD22 motif with phenylalanine and tyrosine, respec-

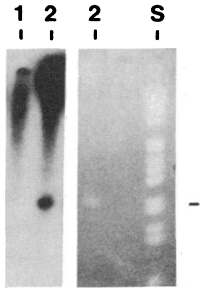

FIG. 4. Gel electrophoresis of amplified PTK sequences. After 22 cycles, the PCR product was electrophoresed in a 2.2% agarose gel and the gel was dried and autoradiographed. (*Left*) Autoradiogram shows the radiolabeled cDNA before PCR (lane 1) and the labeled PCR product of the same material (lane 2). (*Right*) Ethidium bromide staining of the same gel; PCR product (lane 2) and size standards [BRL 1-kilobase (kb) ladder] (lane S) are shown. Marker at right points to an ≈210-base-pair DNA fragment amplified in the PCR reaction.

CLONE	SEQUENCE	IDENTITY	NO. CLONES
15	IHRDLAARNCLV*TEKNVLKI*SDFGM*SREEA*.D*GIYAACS*...*GLRQVP*VKNTAPEALNYGRYS*SE*SDVW	c-fes	49
16	IHRDLAARNCM*VAEDFTVKIGDFGMTRDIY.ETDYYRKG..GKGL.LPVRWMSLESLKDGVFTTHSDVW	IGFI-R	15
19	IHRDLAARNCM*LDEKFTVKVADFGLARDMY.DKEYYSVHNKTGAK.LPVKWMALESLQRQKFTTKSDVW	c-met	4
175	IHRDLRAANVLV*SESLMCKIADFGLARVIE.DNEYTAR...EGAK.FPIKWTAPEAINFGCFTIKSDVW	c-lyn	3
17	IHRDLATRNILVENENRVKIGDFGLTKVLPQDKEYYKVK..EPGE.SPIFWYAPESLTESKFSVASDVW	?	45
22	IHRDLAARNVLVESEHQVKIGDFGLTKAIETDKEYYTVK..DDRD.SPVFWYAPECLIQCKFYIASDVW	?	17

FIG. 5. Alignment of amino acid sequences encoded by clones isolated from the PCR library. Examples of each species of clone isolated from the PCR library were aligned to maximize homology. The conventional one-letter amino acid code is used; conserved residues are displayed in bold type and nonconserved residues are in italic. The identities of those clones corresponding to known PTK sequences are shown in the third column, and the number of individual isolates of each type in the 200 clones sequenced is shown in the right-hand column. Clones FD17 and FD22 have not previously been described and are assumed to represent novel PTK-related sequences.

tively. Moreover, these changes are conserved in all of the FD17-type and FD22-type clones, including a human homologue of FD17 (C. M. Hovens and A.F.W., unpublished data). This modified motif has very different charge and hydrophilicity properties than the usual motif, and the consequences with respect to the substrate specificity and biological properties of these molecules remain to be elucidated. In this motif, the presence of a methionine following the tryptophan is invariant in the growth factor receptor group, whereas in the Src-like PTKs, threonine is always found. Thus FD17 and FD22 may represent members of a novel PTK subfamily.

To isolate additional members of this putative subfamily of PTKs, a similar PCR-based strategy was employed that used oligonucleotides tailored to the isolation of sequences with the distinctive FD17/22-specific -FWYAPE- motif; this approach is outlined in Fig. 1. The use of the PTK I and YAPE oligonucleotides to amplify sequences from murine genomic DNA was unsuccessful, and it is probable that the location of intron sequences in this region renders the amplification process inviable. However, by using murine brain cDNA as the most complex source of cDNA sequences available, in combination with the same oligonucleotides, efficient amplification of an ≈160-base-pair fragment was observed (data not shown). This DNA was cloned into M13 as before, and analysis of 50 randomly selected clones revealed the presence of only FD17 and FD22 sequences (data not shown). Because of the complexity of brain RNA and the sensitivity of the amplification technique employed, it can be tentatively concluded that the existence of other related sequences is unlikely, although the existence of a very rare or highly tissue-specific related sequence cannot be ruled out.

RNA Expression. To examine the range of expression of the clones isolated, a panel of mRNA samples were examined by blot hybridization analysis (Fig. 6). As reported elsewhere (11), the c-*fes* oncogene was expressed as a 2.8-kb mRNA species (data not shown), whereas the candidate c-*met* probe hybridized to an ≈8.5-kb mRNA species expressed in FDC-P1, WEHI-3B D$^-$, and WEHI-265 myeloid cells and in 3T3 fibroblasts (data not shown), a result consistent with that reported for the human c-*met* RNA (5). The expression of this gene in hemopoietic cells has not been noted previously. The expression of c-*lyn* has been examined in detail and will be described elsewhere (S. McEwen, D. Holtzman, and A. R. Dunn, personal communication). The two novel PTK sequences showed overlapping patterns of expression. Clone FD17 hybridized to two mRNA species of 4.8 kb and 4.4 kb present in myeloid cells and T cells. Although both mRNA species were expressed in FDC-P1 cells, the highest levels of FD17 mRNA were seen in WEHI-3B D$^+$, a myelomonocytic leukemic cell line representing an intermediate blast-cell stage of hemopoietic differentiation, which can be induced to differentiate by certain physiological stimuli, such as granulocyte-colony-stimulating factor (23). Lower levels of FD17 expression were detected in WEHI-3B D$^-$ (a nondifferentiating variant of WEHI-3B D$^+$; ref. 24), WEHI-265 (a monocytic cell line; ref. 25), and EL4 (a T-cell line); 3T3 fibroblasts had no detectable FD17 mRNA. The basis for the vast difference between the expression of this mRNA in WEHI-3B D$^+$ compared to WEHI-3B D$^-$, two presumably highly related cell lines, is unknown, although the phenomenon remains intriguing. Clone FD22 hybridized to a 4.9-kb mRNA in all of the cell lines examined, although slightly higher levels were detected in the source mRNA of the PCR library, FDC-P1 (19).

Prospects. Hunter (26) suggested that kinases may function in a role analogous to that of the microchips of computers, receiving input from their millieu and relaying the signal to their substrates. It was suggested that the structure of a given kinase ordains it for a particular metabolic niche within the cell. Thus, a philosophical basis for the structural polymorphism of PTKs has been laid; PTK-related sequences are found in combination with a wide variety of noncatalytic domains. The intriguing differences between the known PTKs and the sequences presented here suggest further flexibility in the use of the basic PTK motifs, and it will be of considerable interest to define the biology of these molecules.

It seems unlikely that the precise protocol employed in this study provides an exhaustive catalogue of all of the PTKs present within a cell, and many variations suggest themselves as potential improvements to the approach. Longer, more degenerate oligonucleotides may yield additional candidate PTKs, as may the use of different conserved sequence motifs for the derivation of primer sequences.

A widely applicable approach has been developed to uncover new PTKs in hemopoietic cell lines. It can be anticipated that similar PCR-based methodologies will fur-

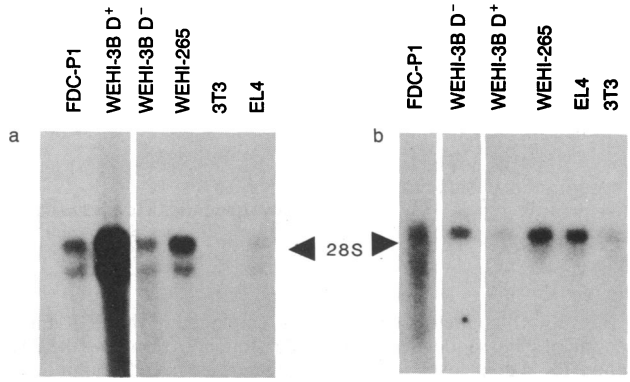

FIG. 6. Blot analysis of mRNA species encoded by FD17 and FD22. Filters were probed with ^{32}P-labeled FD17 (*a*) or FD22 (*b*) DNA. The filters were washed to a temperature of 68°C in 0.2× SSC/0.1% SDS and exposed to x-ray film overnight at −70°C with two intensifying screens. The position of the 28S ribosomal RNA species is indicated. The source of the RNA sample in each lane appears above that lane. See text for further explanation.

ther extend the list of PTK-related sequences. The approach may also have a broader application in the discovery of members of other gene families such as those encoding sequences related to protein kinase C (27), Myc (28), Fos (29), Ras (30), and steroid hormone receptors (31).

I wish to thank Raja Kurban for excellent technical assistance, Gavin Reid and Richard Simpson for preparation of the oligonucleotides, and Steve Ralph and Chris Hovens for critical reading of the manuscript. I am indebted to Tony Burgess, Ashley Dunn, and Patricia Wilks for advice, support, and encouragement.

1. Coussens, L., Yang-Feng, T. L., Chen, E., Gray, A., McGrath, J., Liberman, T. A., Schlessinger, J., Franke, U., Levinson, A. & Ullrich, A. (1985) *Science* **230**, 1132–1139.
2. Yarden, Y., Escobedo, J. A., Kuang, W.-J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Franke, U., Fried, V. A., Ullrich, A. & Williams, L. T. (1986) *Nature (London)* **323**, 226–232.
3. Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.-H., Masiarz, F., Kan, Y. W., Godfine, I. D., Roth, R. A. & Rutter, W. J. (1985) *Cell* **40**, 747–758.
4. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T. L., Tsubkawa, M., Collins, C., Henzel, W., LeBon, T., Katthuria, S., Chen, E., Jacobs, S., Franke, U., Ramachandran, J. & Rujita-Yamaguchi, Y. (1986) *EMBO J.* **5**, 2503–2512.
5. Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A. & Vande Woude, G. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6379–6383.
6. Kozma, S. C., Redford, S. M. S., Xioao-Chang, F., Saurer, S. M., Groner, B. & Hynes, N. E. (1988) *EMBO J.* **7**, 147–154.
7. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Liberman, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D. & Seeburg, P. H. (1984) *Nature (London)* **309**, 418–425.
8. Rothwell, V. M. & Rohrschneider, L. R. (1987) *Oncogene Res.* **1**, 311–324.
9. Martinez, R., Mathey-Prevot, B., Bernards, A. & Baltimore, D. (1987) *Science* **237**, 411–414.
10. Sukegawa, J., Semba, K., Yamanishi, Y., Nishizawa, M., Miyajima, N., Yamamoto, T. & Toyoshima, K. (1987) *Mol. Cell. Biol.* **7**, 41–47.
11. Wilks, A. F. & Kurban, R. R. (1988) *Oncogene* **3**, 289–294.
12. Shivelman, E., Lifshitz, B., Gale, R. P., Roe, B. A. & Canaani, E. (1986) *Cell* **47**, 277–284.
13. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241**, 42–52.
14. Holtzman, D., Cook, W. D. & Dunn, A. R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8325–8329.
15. Hanks, S. K. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 388–393.
16. Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) *Science* **230**, 1350–1354.
17. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S., Higuch, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–491.
18. Lee, C. C., Wu, X., Gibbs, R. A., Cook, R. G., Muzny, D. M. & Caskey, C. T. (1988) *Science* **239**, 1288–1291.
19. Dexter, T. M., Garland, J., Scott, D., Scolnick, E. & Metcalf, D. (1980) *J. Exp. Med.* **152**, 1036–1047.
20. Morla, A. O., Schreurs, J. & Wang, J. Y. J. (1988) *Mol. Cell. Biol.* **8**, 2214–2218.
21. Gonda, T. J., Sheiness, D. K. & Bishop, J. M. (1982) *Mol. Cell. Biol.* **2**, 617–624.
22. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
23. Metcalf, D. & Nicola, N. A. (1982) *Int. J. Cancer* **30**, 773–780.
24. Warner, N. L., Moore, M. A. S. & Metcalf, D. (1969) *J. Natl. Cancer Inst.* **43**, 963–982.
25. Warner, N. L., Burchiel, S. W. & Walker, E. B. (1979) in *Immunobiology and Immunotherapy of Cancer*, eds. Terry, W. D. & Yamura, Y. (Elsevier/North-Holland, Amsterdam), pp. 223–243.
26. Hunter, T. (1987) *Cell* **50**, 823–829.
27. Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D. & Ullrich, A. (1986) *Science* **233**, 859–866.
28. Legouy, E., DePinho, R., Zimmerman, K., Collum, R., Yancopoulos, G., Mitsock, L., Kriz, R. & Alt, F. W. (1987) *EMBO J.* **6**, 3359–3366.
29. Cohen, D. R. & Curran, T. (1988) *Mol. Cell. Biol.* **8**, 2063–2069.
30. Touchot, N., Chardin, P. & Tavitan, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8210–8214.
31. Evans, R. M. (1988) *Science* **240**, 889–895.