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Putative tyrosine kinases expressed in K-562 human leukemia cells

(chronic myeloid leukemia/differentiation/erythroleukemia/oncogene/phylogeny)

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ABSTRACT **Tyrosine phosphorylation is important in the transmission of growth and differentiation signals; known tyrosine kinases include several oncoproteins and growth factor receptors. Interestingly, some differentiated cell types, such as erythrocytes and platelets contain high amounts of phosphotyrosine. We analyzed tyrosine kinases expressed in the K-562 chronic myelogenous leukemia cell line, which has a bipotential erythroid and megakaryoblastoid differentiation capacity. Analysis of 359 polymerase chain reaction-amplified cDNA clones led to the identification of 14 different tyrosine kinase-related sequences (JTK1–14). Two of the clones (JTK2 and JTK4) represent unusual members of the fibroblast growth factor receptor gene family, and the clones JTK5, JTK11, and JTK14 may also belong to the family of receptor tyrosine kinases but lack a close relationship to any known tyrosine kinase. Each of these different genes has its own characteristic expression pattern in K-562 cells and several other human tumor cell lines. In addition, the JTK11 and JTK14 mRNAs are induced during the megakaryoblastoid differentiation of K-562 cells. These tyrosine kinases may have a role in the differentiation of megakaryoblasts or in the physiology of platelets.**

The frequent appearance of protein tyrosine kinases as oncogenes and growth factor receptors suggests that tyrosine phosphorylation plays an important role in the transmission of cell growth signals. Since the discovery of tyrosine phosphorylation by v-*src*, the transforming gene of the Rous sarcoma virus (1), the number of known tyrosine kinases has increased with an accelerating pace (2, 3). The remarkable conservation of the primary structure of the tyrosine kinase catalytic domain has substantially aided the cloning of tyrosine kinase genes. At present, tyrosine kinases form a large gene family, which can be divided into functional and evolutionary subfamilies (3). The conservation of certain tyrosine kinase sequences between organisms as divergent as invertebrates and mammals also emphasizes the importance of these signal transducers.

Most known tyrosine kinases transmit signals regulating cell growth or differentiation. Less is known about tyrosine phosphorylation in other cellular processes. However, some terminally differentiated cells, such as neurons, platelets, and erythrocytes, contain intriguingly high levels of phosphotyrosine and tyrosine kinase activity (4–6). It has also been reported that thrombin activation of platelets increases their tyrosine-specific protein phosphorylation (5) and that the c-*src* kinase is concentrated in certain secretory vesicles inside platelets (7). Tyrosine kinases might, thus, play a role in the secretion of platelet contents as well as in platelet aggregation induced by various agonists.

K-562 is a chronic myelogenous leukemia cell line that has a bipotential differentiation capacity: the cells have features of erythroleukemia (8, 9), but treatment with tumor promoters induces them to differentiate into the megakaryoblastoid direction (10–12). We were interested in tyrosine kinases expressed in these cells because of their potential role in platelet/erythrocyte-specific tyrosine phosphorylation. Polymerase chain reaction (PCR) application (13, 14) was used to detect members of the tyrosine kinase family expressed in the K-562 cells. Here we report the identification of 14 different tyrosine kinase sequences,‡ several of which represent unusual putative tyrosine kinases. We have also studied their expression in the K-562 cells and in other human tumor cell lines.

MATERIALS AND METHODS

Cell Culture. Cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum. K-562 erythroleukemia (15) and Dami megakaryoblastic leukemia cells (16) were induced to differentiate with 1–2 nM phorbol 12-myristate 13-acetate (PMA, ref. 12).

PCR Cloning. The first strand of cDNA was synthesized from 1 μg of poly(A)$^+$ RNA using avian myeloblastosis virus reverse transcriptase (Pharmacia) and oligo(dT) priming. One-tenth of the first-strand cDNA preparation was used as a template for amplification of the tyrosine kinase-related sequences, as described by Wilks (13). The major PCR products were cloned into GEM3Zf(+) plasmid (Promega).

Screening of the PCR Library and Nucleotide Sequence Analysis. Tyrosine kinase-related sequences obtained by initial sequencing of 59 randomly picked clones were screened out of 300 clones by colony hybridization, and the nucleotide sequences of the nonhybridizing ones were determined. Inserts of altogether 86 recombinant plasmids were sequenced using the dideoxynucleotide chain-termination method (17). Data bases were searched using FASTA and WORDSEARCH programs (18).

Determination of Phylogenetic Relationships. The phylogeny program PAUP (19) was used to calculate the most parsimonous trees from the alignment of a 77-character (amino acids and introduced gaps)-long sequence corresponding to the amplified domain. The alignment of Hanks *et al.* (3) was extended to include recently cloned sequences. The multistate step matrix for amino acid differences was inverted from an SG (Structure-Genetics) similarity matrix (20), and the internal inconsistencies were resolved to the direction of the odd-logarithmic matrix of Dayhoff (21). Heuristic approaches were used to find the shortest trees.

RNA and DNA Blot Analyses. Poly(A)$^+$ RNA and restriction endonuclease-digested human DNAs were isolated and analyzed as described (22).

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Abbreviations: PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; FGFR, fibroblast growth factor receptor.
‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M37781 and M37782).

RESULTS AND DISCUSSION

Identification of Additional Tyrosine Kinase-Related cDNA Sequences. The strategy of Wilks (13) was used to amplify tyrosine kinase sequences after reverse transcription of polyadenylylated RNA of K-562 cells. As a source of cDNA for sequence amplification, we also used tumor promoter-treated K-562 cells, which have been shown to undergo megakaryoblastoid differentiation (11, 12). Two degenerate oligonucleotides corresponding to the IHRDL and DVWSFG (single-letter code) conserved amino acid sequences and with flanking restriction enzyme-cleavage sites were used for the amplification. The PCR product was analyzed by electrophoresis in 2% agarose, where bands corresponding to the expected reaction products of ≈210 base pairs (bp) were seen. In addition, there was a smaller amount of amplified DNA migrating at ≈100 bp, which was not analyzed further.

The major reaction products were cloned into a plasmid, and altogether 359 clones were screened. Sequence analysis resulted in the identification of exclusively tyrosine kinase-related sequences, representing altogether 14 distinct cDNAs, called JTK1–14. Six of the JTK clones were identified as known tyrosine kinase cDNAs, namely those encoding the *lyn* (23), *hck* (24, 25), c-*abl* (26), the insulin-like growth factor I receptor (IGFI-R; ref. 27), the platelet-derived growth factor receptor type β (PDGF-β-R; refs. 28 and 29), and TYK2 (30). PDGF-β-R has not been previously detected in hematopoietic cells; yet, its mRNA was induced upon PMA treatment of the K-562 cells in our experiments (data not shown). The sequences for the remaining eight putative tyrosine kinases could not be found in the data bases searched (GenBank, release 63.0; EMBL, release 22.0; SwissProt, release 11.0; and National Biomedical Research Foundation, release 21.0). The deduced amino acid sequences of the JTK1–14 cDNAs are shown in Fig. 1*A*.

Several tyrosine kinase-specific sequence motifs are present in these sequences: the sequence DLRAAN or DLAARN (in one-letter code) beginning with the amino acid sequence derived from the left-hand oligonucleotide is present in all JTK clones. This is a characteristic feature used to distinguish tyrosine kinases from serine and threonine kinases that have the DLKPEN consensus sequence in this region (3). The tyrosine residue at position 35 or 36 (arrow in Fig. 1*A*) corresponds to the autophosphorylation site Y-416 in the chicken c-*src* protein (38) and is present in all JTK sequences. However, instead of the conserved (M/T/Y)A-(P/L)E amino acid sequence at positions 49–52, JTK6, JTK11, and JTK14 use the sequences CPPE, IAIE, and MAIE, respectively. Also, it is of interest that JTK5 has the amino acid triplet DNA (sic) at positions 22–24, instead of the highly conserved DFG motif found in all other known tyrosine kinases.

Phylogenetic Relationships of JTK Sequences. To determine the interrelationships of our unusual putative tyrosine kinases, we used the parsimony principle presented in *Materials and Methods*. The resulting phylogeny of JTK sequences and some previously known tyrosine kinases is shown in Fig. 2. The phylogenetic tree was constructed using only the amino acid sequence corresponding to the PCR-amplified domain. A step matrix based on both similarities of codons and structural relations between corresponding amino acids gave a phylogenetic tree consistent with the

A

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                                        10         20         30         40         50         60
PDGFB-R   JTK 12  IHRDLAARNV LICEGKLVKI CDFGLARDIM RD-SNYISKG STFLPLKWMA PESIFNSLYT TLSDVWSFG
IGF1-R    JTK 13  IHRDLAARNC MVAEDFTVKI GDFGMTRDIY ET-DYYRKGG KGLLPVRWMS PESLKDGVFT TYSDVWSFG
HCK       JTK  9  IHRDLRAANI LVSASLVCKI ADFGLARVIE DN--EYTARE GAKFPIKWTA PEAINFGSFT IKSDVWSFG
LYN       JTK  8  IHRDLRAANV LVSESLMCKI ADFGLARVIE DN--EYTARE GAKFPIKWTA PEAINFGCFT IKSDVWSFG
ABL       JTK  7  IHRDLAARNC LVGENHLVKV ADFGLSRLMT GD--TYTAHA GAKFPIKWTA PESLAYNKFS IKSDVWSFG
TYK2      JTK  1  IHRDLAARNV LLDNDRLVKI GDFGLAKAVP EGHEYYRVRE DGDSPVFWYA PECLKEYKFY YASDVWSFG
          JTK  3  IHRDLAARNV LVESEHQVKI GDFGLTKAIE TDKEYYTVKD DRDSPVFWYA PECLMQSKFY IASDVWSFG
          JTK 10  IHRDLATRNI LVENENRVKI GDFGLTKVLP QDKEYYKVKE PGESPIFWYA PESLTESKFS VASDVWSFG
          JTK  6  IHRDLAARNC LVREAGVVKV SDFGMARYVL DD--QYTSSS GAKFPVKWCP PEVFNYSRFS SKSDVWSFG
          JTK  5  IHRDLAARNC VIDDTLQVKI TDNALSRDLF PM-DYHCLGD NENRPVRWMA LESLVNNEFS SASDVWSFG
          JTK 11  IHRDLAARNC MLNENMSVCV ADFGLSKKIY NG-DYYRQGR IAKMPVKWIA IESLADRVYT SKSDVWSFG
          JTK 14  IHRDLAARNV LVGENLASKI ADFGLSRGEE V----YVKKT MGRLPVRWMA IESLNYSVYT TKSDVWSFG
          JTK  2  IHRDLAARNV LVTEDNVMKI ADFGLARGVH HI-DYYKKTS NGRLPVKWMA PEALFDRVYT HQSDVWSFG
          JTK  4  IHRDLAARNV LVTEDNVMKI ADFGLARDVH NL-DYYKKTT NGRLPVKWMA PEALFDRVYT HQSDVWSFG
CONSENSUS         IHRDLaarNv lvted.vvki aDfglargie .d-dyytkke .gklPvkWma pEsl.dsvft ikSDVWSFG
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B

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                                                  50                                                  100
JTK2  ACCGGGACCT GGCTGCCCGC AATGTGCTGG TGACTGAGGA CAATGTGATG AAGATTGCTG ACTTTGGGCT GGCCCGCGGC GTCCACCACA TTGACTACTA
JTK4  ..A..... ..........  .........  ....C.....  ...C.....  .....C..A.  ....C.....  ......G.A.  ..G...A..C  .C........
FLG   ....A.....  ...A...A.G  .....C....  ....A.....  .........  .....A..A.  .......C..  C..A..G.A.  A.T.......  .C........
CEK   .........  ...G...A.G  ..C.......  ....C.....  ...C.....  .....C....  ....C.....  ........A.  A.........  .C..T.....
BEK   .T..A..TT.  .......A.A  ..C...T...  .A..A..AA.  .........  .....A..A.  .......C..  ....A.G.AT  A.CA..A...  .A........

                                                  150
JTK2  TAAGAAAACC AGCAACGGCC GCCTGCCTGT GAAGTGGATG GCGCCCGAGG CCTTGTTTGA CCGGGTGTAC ACACACCAGA GTGACGTGTG GTCCTTT
JTK4  C.....G..A .C........ .G.....C.. .......... .....T.... .......... ...A..C... ..T....... .......C.. .......
FLG   ...A..G..A .C........ .A........ .......... ..A....... .A..A..... ....A.C... ..C....... ....T..... ...T..C
CEK   C.....G..G .CA....... ..T....G.. .......... ..C..G.... .TC....C.. ...AA.A... ..C..T.... ....T..T.. ...G..C
BEK   ...A..G..C .CA..T..G. .A..T..A.. C......... ..T..T..A. ..C.T..... TA.A..T... ..T..T.... .C..T.TC.. ......C
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FIG. 1. (*A*) Deduced amino acid sequences of the JTK clones. (Upper) JTK sequences corresponding to known tyrosine kinases appear, followed by the noncorresponding sequences. The most-conserved residues are shown with uppercase letters, and commonly used residues are shown with lowercase letters in the consensus sequence at bottom. Note that the IHRDL and DVWSFG sequences at the amino and carboxyl terminal ends (underlined) are derived from the PCR primers and, therefore, may not represent true mRNAs. Arrow points to the location of the conserved tyrosyl residue homologous to the autophosphorylation site Y-416 in the *src* kinase (38). In JTK5 this tyrosine is positioned one residue to the left in comparison with the other JTK clones. (*B*) Comparison of fibroblast growth factor receptor (FGFR)-related tyrosine kinase nucleotide sequences. Shown are the amplified domains of the JTK2 and JTK4 clones and the corresponding sequences from the human FLG (32–34), chicken FGFR (*cek-1*; refs. 35 and 36), and mouse *bek* tyrosine kinase (37) cDNAs.

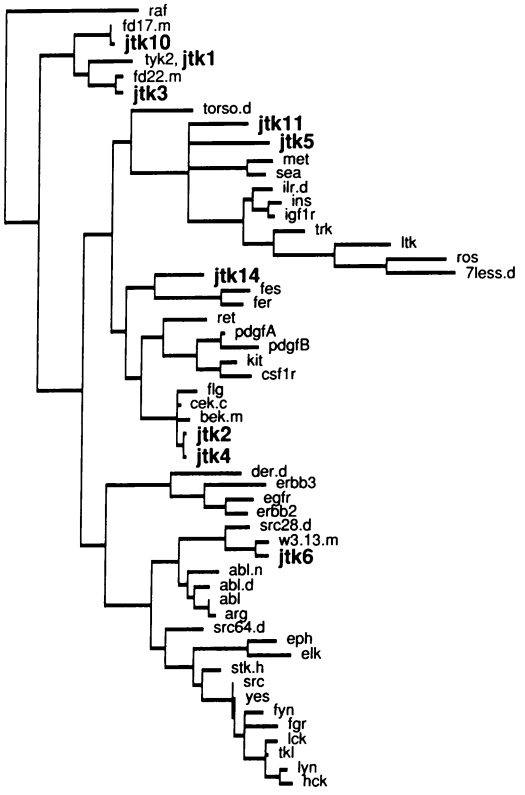

FIG. 2. Phylogenetic tree of known tyrosine kinases. The tree is rooted in the serine/threonine kinase *raf*, which was included as an outgroup. Tyrosine kinase sequences from species other than human are indicated after the appropriate name as follows: .m, mouse; .c, chicken; .n, *Caenorhabditis elegans* (nematode); .d, *Drosophila melanogaster*; .h, *Hydra attenuata*.

established phylogeny of tyrosine kinases (3). The latter phylogeny is based on comparison of amino acids from the entire tyrosine kinase domain by using an alternative approach of distance matrix.

JTK1, JTK3, and JTK10 form a distinct subfamily with no well-characterized members. During our studies we learned that JTK1 is identical with the recently cloned TYK2 cDNA (30). JTK3 and JTK10 sequences are similar to the FD22 and FD17 mouse cDNAs, respectively, reported by Wilks (13) and may represent the human homologues of these genes.

JTK5, JTK11, and JTK14 seem to belong to the receptor branches of the tree. These sequences lack an intimate relationship with other known tyrosine kinases; they have relatively weak homologies to many tyrosine kinase growth factor receptors. In the phylogenetic tree the cytoplasmic tyrosine kinases *fes* and *FER* are located closest to JTK14. However, JTK14 contains the RWMAIE motif, where M (methionine) seems characteristic of many receptor tyrosine kinases. Furthermore, data base searches gave highest similarity scores for JTK14 with several receptor tyrosine kinases. The location of *fes* and *FER* among receptor tyrosine kinases might result from the use of limited sequence information for the construction of the phylogenetic tree.

JTK2 and JTK4 belong to the fibroblast growth factor receptor (FGFR) gene family. Fig. 1*B* shows the homology of JTK2 and JTK4 sequences to other members of the FGFR family. These include human *FLG* (hFGFR-1), originally cloned from a human endothelial cell cDNA library (31–33), its mouse homologue (34), and the corresponding chicken gene *cek-1* (39, 40) and another member of the FGFR family, the mouse *bek* gene (41). The presence of several FGFR-related mRNA sequences in the K-562 cells is particularly exciting, as there is also a gene family encoding several different fibroblast growth factors (35–37, 42–46).

In our phylogenetic tree only JTK6 is located in the subfamily of cytoplasmic tyrosine kinases. It is highly homologous with mouse W3.13M cDNA (47) and *Drosophila src28* gene (48), and it may be the human homologue of these genes. Thus, JTK6 represents another example of the evolutionary conservation of tyrosine kinase sequences.

JTK cDNAs Represent mRNA Transcribed from Distinct Genes in K-562 Cells. Potential errors accumulated during amplification were controlled by sequencing multiple clones of the same JTK DNA from the original PCR libraries. Only a few PCR point mutations were found; otherwise, the distinct clones representing each of the JTK sequences gave identical results. This finding suggests that the nucleic acid sequences we obtained faithfully represent the corresponding mRNA sequences. Furthermore, screening of an oligo(dT)-primed K-562 cell cDNA library (a gift from Gerard Grosveld, Erasmus University, Rotterdam, The Netherlands) has allowed the isolation and analysis of cDNA clones that independently authenticate the different JTK sequences obtained by PCR cloning (unpublished results).

Southern blotting and hybridization of *Eco*RI- and *Bam*HI-digested human peripheral blood DNA gave rise to bands of distinct molecular sizes for each of the JTKs (Table 1). The JTK2 and JTK4 probes showed multiple bands in all analyses (Fig. 3). The nucleotide sequence and Southern hybridization data together suggest the existence of a closely related tyrosine kinase subfamily characterized by the JTK2 and JTK4 sequences. The other JTK sequences seem to be present as single-copy genes in the human genome.

JTK mRNAs Are Differentially Regulated. Northern (RNA) hybridization analysis of these different JTK mRNAs is shown in Fig. 4. For induction of megakaryoblastic differentiation the K-562 and Dami leukemia cells were treated with PMA for 2 days; JTK11 and JTK14 mRNAs increased during this treatment. The 4.9-kb JTK11 mRNA was heavily induced in the PMA-treated K-562 cells, whereas very little expression was seen in the Dami cells. In contrast, the 4.4-kilobase (kb) JTK14 mRNA was only weakly expressed, even by the induced K-562 cells but was expressed much more strongly in the Dami cells. The lack of significant expression of the JTK11 and JTK14 sequences in the non-treated K-562 cells is consistent with their isolation exclusively and repeatedly from the PCR library from PMA-

Table 1. Southern blot analysis of the genomic JTK clones

	Restriction fragment length, kbp	
	*Eco*RI	*Bam*HI
JTK1	18	5.6
JTK2 and JTK4	25	26
	20	8.9
	10	5.1
	4.7	2.5
	2.3*	0.5*
JTK3	20	7.2
	8.3	
JTK5	13	25
	6.7	10
JTK6	16	19
		3.0
JTK10	6.5	17
	2.3	
JTK11	12	19
JTK14	8.9	

kbp, Kilobase pairs.
*Specific for JTK4 probe.

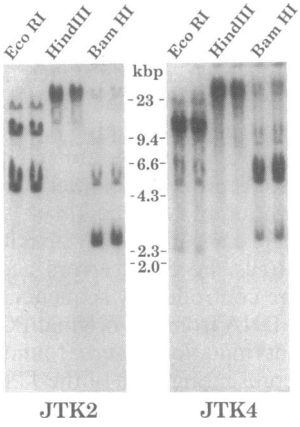

FIG. 3. Southern blotting and hybridization of normal human DNA from two individuals with the JTK2 and JTK4 probes. These cDNA inserts detect several major bands in *Eco*RI-, *Hin*dIII-, and *Bam*HI-digested DNA, suggesting the presence of a closely related gene family. kbp, Kilobase pairs.

induced K-562 cells. Single major species of JTK3, JTK5, and JTK6 mRNAs (5.0 kb, 3.4 kb, and 2.8 kb, respectively) were expressed at rather uniform levels in both cell lines regardless of PMA treatment. Overlapping, but somewhat different, sets of mRNA species were seen with the closely related JTK2 and JTK4 probes in both cell lines. The JTK2 probe showed a major band at 3.0 kb and minor bands at 4.8 and 7.5 kb. The JTK4 probe hybridized strongly with the 4.8-kb mRNA and less intensely with 3.0-, 5.2-, and 7.5-kb mRNAs. In addition to these mRNA species, a weak signal was seen with the JTK2 and JTK4 probes in Dami RNA at 2.5 kb. Three major JTK10 mRNA species (4.8, 5.5, and 7.3 kb) were present in the K-562 cells, and four (4.8, 5.5, 7.3, and 8.5 kb) were present in Dami cells. Multiple JTK10 mRNA species might result from differential splicing or polyadenylylation or both because the Southern data suggest that JTK10 represents a single-copy gene.

Besides the K-562 and Dami cells, various other hematopoietic and nonhematopoietic cell lines expressed the JTK

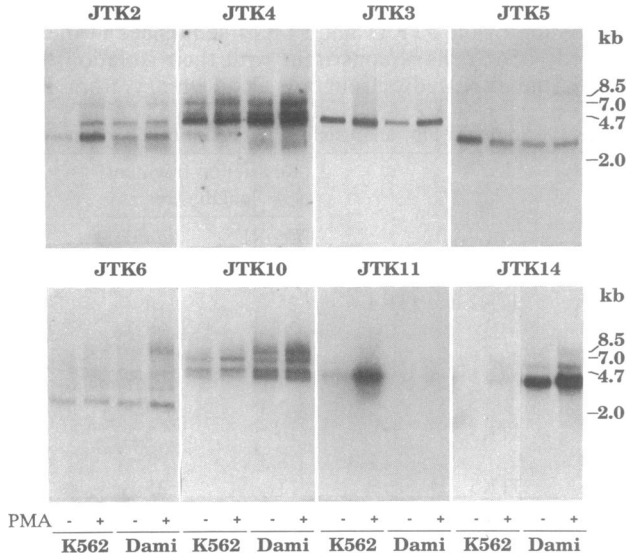

FIG. 4. Analysis of K-562 and Dami cells for JTK mRNA expression. The cells were grown with PMA for 2 days to induce megakaryoblastic differentiation, and their polyadenylylated RNA was extracted and analyzed with the JTK probes indicated. Note the faint JTK14 band in the induced K-562 cells. The mobilities of the mRNA from the retinoblastoma gene (4.7 kb), β-actin (2.0 kb), and *ABL* (6.0 kb, 7.0 kb, and 8.5 kb) are indicated at right.

mRNAs. Fig. 5 shows Northern (RNA) blotting and hybridization of RNAs isolated from a collection of cultured human tumor cell lines and from mouse embryos. The most uniform patterns of expression were observed for the JTK1, JTK3, and JTK5 mRNAs. The other JTK sequences showed a more variable or restricted pattern of expression. A clear JTK6 signal was obtained only from the K-562 cells, and JTK11 was highly expressed only in the A549 lung carcinoma cell line in addition to the PMA-treated K-562 cells. Interestingly, JTK14 gave a signal from mouse embryonic tissues and K-562 cells but not from the other human cell lines.

CONCLUSION

This work shows that a wide variety of different tyrosine kinases are expressed in a single cell type. Our PCR cloning revealed also several additional members of the tyrosine kinase gene family. It should be noted that this cloning method is selective: in addition to the genes represented in our PCR clones, the tyrosine kinases known to be expressed at similar levels in K-562 cells include c-*src*, c-*yes*, *fyn*, *FER*, and *trk* (49–52). Also, some additional kinases have emerged from the K-562 library during our search for the full-length JTK cDNA sequences. Thus, over 20 different tyrosine kinases are expressed in the K-562 cells. We do not know whether this unexpected variety of tyrosine kinase mRNAs reflects a tumor cell-specific deregulation of tyrosine kinase gene expression or whether a similar number of tyrosine kinase mRNAs is present in normal cells as well, as suggested by Hunter (2).

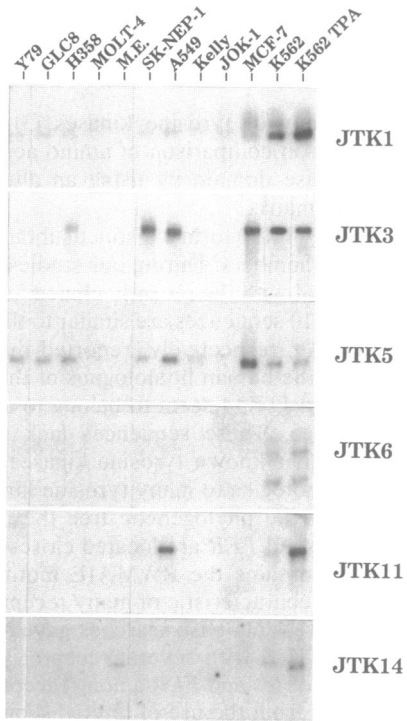

FIG. 5. Expression of JTK mRNAs in human tumor cell lines and in the mouse embryo (M.E.). Four micrograms of each RNA was loaded per lane, and the blots were rehybridized several times. The JTK14 signal was obtained only after a long autoradiographic exposure (compare the K-562 signals shown in Fig. 4). Origins of the cell lines are as follows: Y79, retinoblastoma; GLC8 and H358, small-cell lung cancer; MOLT-4, T-cell leukemia; SK-NEP-1, Wilms tumor; A549, lung carcinoma; Kelly, neuroblastoma; JOK-1, hairy cell leukemia; MCF-7, breast adenocarcinoma. Upper bands in the JTK6 panel represent residual signal from the previous hybridization with the JTK1 probe. TPA, PMA.

Many known tyrosine kinases are expressed widely in different tissues and cell types. This condition seems also the case with several of the JTK sequences, rendering functional analysis of these genes more difficult. JTK1–10 mRNAs were not significantly induced during the megakaryoblastic differentiation of K-562 cells. Although JTK11 is strongly induced during PMA treatment, its physiological function in megakaryocytes or platelets is questionable because it is not expressed in other megakaryoblastic leukemia cell lines. In contrast, expression of JTK14 in some fetal hematopoietic organs and specifically in the megakaryoblastoid leukemia cell lines is intriguing (unpublished results). JTK14 might be one of the kinases responsible for tyrosine phosphorylation in mature platelets.

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