Human FLT3/FLK2 Gene: cDNA Cloning and Expression in Hematopoietic Cells

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The human FLT3 cDNA was cloned from a pre-B-cell line and characterized. The deduced amino acid sequence shows that FLT3 codes for a receptor-type tyrosine kinase of 993 residues, presenting a strong similarity with the corresponding mouse FLT3/FLK2 protein as well as with the receptors for colony-stimulating factor 1 (CSF1R/FMS) and steel locus factor (SLFR/KIT). An analysis of the expression of the gene using amplification of reverse transcribed FLT3 mRNA by polymerase chain reaction shows that FLT3 is expressed in various lymphohematopoietic cells and tissues, including a series of immature cell lines and leukemias of lymphocytic origin. © 1993 by The American Society of Hematology.

Understanding the maturation and differentiation processes of the hematopoietic cells will have important consequences at both the clinical and fundamental levels. Intercellular communications via secreted or membrane-bound signalling molecules are of primary importance in these processes.1-4 A group of receptors for signalling molecules belongs to the tyrosine kinase superfamily.5 Three of these receptor-type tyrosine kinases (RTKs) and their ligands play central roles in hematopoiesis. They are the receptors for colony-stimulating factor 1 and steel factor, respectively, encoded by the FMS and KIT proto-oncogenes,6-8 and the product of the FLT3/FLK2 gene.9-11 The ligand of which is an as yet unidentified factor probably synthesized by stromal cells within the hematopoietic microenvironment. Together with the two receptors for platelet-derived growth factor, they are sometimes referred to as class III RTKs.5

Class III RTKs share structural characteristics such as the presence of five Ig-like domains in their extracellular regions, and an interrupted kinase domain in their intracellular regions. Two other RTK classes, comprising the fibroblast growth factor receptors and the products of the FMS, KIT, and FLT4 genes, have a similar structure but present a different number of Ig-like domains, respectively three and seven.9-15 FMS, KIT, and FLT3 are expressed in hematopoietic cells and in other tissues such as placenta, brain, cerebellum, and gonads. They are also variably expressed in hematopoietic malignancies.16-21

The Flt3/Flk2 gene was first identified and characterized in the mouse, after a search for either new class III RTK genes2-11 or stem cell–associated tyrosine kinase genes.9 The mouse gene encodes a bona fide tyrosine kinase of 1,000 amino acids residues, with an apparent molecular weight of 135 to 155 Kd.22 FLT3 is closely linked to the FLT1 gene encoding the receptor for vascular endothelial growth factor,23 and both genes are located in the mouse on chromosome 5, and in humans on chromosomal band 13q12.24

Within the hematopoietic system, and in contrast to CSF1R/FMS whose expression is lineage specific and SLFR/KIT that seems to have distinct early and late functions, the interaction of FLT3/FLK2 with its ligand is thought to regulate pluripotent stem cells, early progenitor cells, and immature lymphocytes.9 This suggests a unique and potentially crucial role in modulating stem cell function. However, very few clues as to the normal function or possible pathologic alterations of FLT3 have yet been gathered. The mouse Kit gene is allelic with the W locus.25-26 Mice mutated at this locus exhibit defects in the development of germ cells, pigment cells, and hematopoietic cells.7 Unfortunately, no such spontaneously occurring mutant is known at the Flt3 locus, perhaps because such mutation has lethal effects. Furthermore, in contrast to Fms and Kit, no oncogenic retroviral form of Flt3 has yet been isolated.

As a new step toward the definition of the structural and functional features of FLT3, we now describe the isolation and nucleotide sequence of the human FLT3 cDNA, and the expression of the gene in various normal and malignant lympho-hematopoietic cells and tissues. This characterization will allow the development of useful reagents to explore the function of FLT3 in the human model.

Materials and Methods

Cells and tissue samples. The following cell lines were obtained from the American Type Culture Collection and cultured as recommended: JAR, BeWo, KATO III, HBL-100, MDA-MB-134, MCF7, 5637, MIA PaCa-2, A549, IMR-90, CCRF-SB, Daudi, Na-malwa, MOLT-4, KG-1, KG-1a, and THP-1. BLIN-1 and its sub-line 1E8P7-28 were gifts from Dr LeBien (Medical School, University of Minnesota, MN) and were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS). The same culture conditions were used for Jurkat cells. YU S1 T17 was a gift from Dr Halaban (Yale Medical School, New Haven, CT) and was cultured in HAM-12 medium supplemented with 10% newborn calf serum. The Nalm 6,69 and 207 cell lines31 as well as E29-24 and 2SP5, respectively, derived from Epstein-Barr virus (EBV)-transformed fetal bone marrow cells and peripheral blood lymphocytes, were grown in RPMI-1640 supplemented with 20% FCS,32 BL2, a Burkitt lymphoma cell line33 and JY, an EBV-transformed B-cell line, were cultured in RPMI 10% FCS. Dal and Per cell lines are described in.34 Abou.SV.D1 is a SV40-transformed stromal cell line...
obtained by the methodology described in34 (F. Birg, unpublished results, January 1993). These three latter cell lines were grown in Iscove's modified Dulbecco's medium supplemented with 10% FCS. All leukemic samples were obtained and classified according to cell morphology and immunophenotyping, as previously described.22 Numbering of the leukemic cases was conserved with respect to the one used in this reference. Human tissues were obtained from Drs Boubli (Hôpital de la Conception, Marseille, France) and Metras (Hôpital de la Timone, Marseille, France).

Fractionation of peripheral blood cells. Peripheral blood cells were isolated from blood samples obtained from healthy donors (Centre Regional de Transfusion Sanguine, Marseille, France) and fractionated into the four major hematopoietic nucleated populations (B and T lymphocytes, granulocytes, and monocytes) as previously described.23

DNA probes. The TH3L probe was a 0.7-kb EcoRI fragment of human FLT3 cDNA corresponding to the second portion of the tyrosine kinase domain.10 The EC probe consisted of two Pst I fragments of mouse Flt3 cDNA coding for the EC region of the receptor.24 The 1EU2 probe was a 1.8-kb BamHI-EcoRI fragment corresponding to the 5' portion of human pre-B cDNA clone 1EB. The Gapdh probe was a 1.3-kb EcoRI-HindIII fragment derived from a mouse pseudogene.35 Plasmid preparations were performed as described previously.36

cDNA cloning. A human pre-B cell cDNA library was constructed from 1E8 poly(A)+ RNA in lambda gt10 using the Amersham (Arlington Heights, IL) cDNA synthesis and cloning kits. The 3 × 10⁷ plaques were screened with the TH3L and EC probes (Fig 1). One clone, named 1EB, hybridizing to both probes was isolated and characterized. Further repeated screenings of the 1E8 library failed to identify longer clones. A human testis cDNA library (Clontech, Palo Alto, CA) was screened with the 1EU2 probe, derived from the 1EB clone (Fig 1). The cDNA clone named TH9, containing FLT3 sequences extending 5' to clone 1EB, was characterized. Hybridizations were performed at 68°C in 6× SSC and 1× Denhardt's, and washings were performed at 68°C in 2× SSC and 0.1% sodium dodecyl sulfate (SDS). To isolate 1EB cDNA sequences corresponding to clone TH9, reverse transcription (RT) and polymerase chain reaction (PCR) amplification was applied to 1E8 mRNA. Oligonucleotides 1 (5'-CTGTGGAAHTGGATGTATCTGC-3'), derived from TH9 as sense primer (positions 239-261 on the sequence), and 2 (5'-TGATCGCAAAGCCACTTCC-3'), derived from 1EB sequence as antisense primer (positions 582-604), were used (Fig 1). Lastly, additional 5' sequences were obtained by PCR amplification of 1E8 cDNA, using oligonucleotide 3 (5'-CGCGCTTCCGGAGGCCATG-3'), a mouse Flt3 cDNA-derived sense primer (positions 67-85 on mouse Flt3 cDNA),11 and oligonucleotide 4 (5'-CTCCAGCTTGGGTTTCTGTCAT-3'), an antisense primer (positions 398-420).

Nucleotide sequencing. Restriction fragments derived from cDNA clones were cloned into appropriate Bluescript vectors (Stratagene, LaJolla, CA). PCR products were blunt-ended with T4 DNA polymerase before cloning into SmaI-digested vectors. Nucleotide sequences were determined by the dideoxynucleotide method, using single- or double-stranded templates, and the modified T7 polymerase (Sequenase) protocol (US Biochemicals, Cleveland, OH). Sequence analysis was performed using PC-gene software (Intelligenetics, Mountain View, CA).

Fig 1. Organization of the cloned human FLT3 cDNA. (A) Schematic representation of the domains of the putative FLT3 protein: SP, signal peptide; TM, transmembrane domain; JM, juxtamembrane domain; TK1 and TK2, tyrosine kinase sub-domains; KI, kinase insert; CT, C-terminus. (B) Structure of the FLT3 cDNA coding sequence, represented by an open box. (C) cDNA clones obtained: 1EB from the 1EB library, TH9 from the tests library (the broken line indicates the portion of the clone the sequence of which was not determined). The four oligonucleotide primers used in PCR experiments (40 cycles, annealing at 58°C) to obtain the 5' portion of the cDNA as discussed in the text are indicated by arrows numbered 1 to 4. CR2906 and CR2511 are two resulting PCR products. (D) Probes used in the screening of cDNA libraries.
RNA preparation and Northern blot analysis. Total RNA was prepared from frozen hematopoietic cells or tissues by lysis in guanidium isothiocyanate and centrifugation over a cesium chloride cushion. For Northern blot analysis, 10 µg aliquots were electrophoresed in 1% agarose/10% formaldehyde gels and transferred onto Nytran membranes (Schleicher and Schuell, Dassel, Germany). TH3L was used as a probe, after radioactive labeling by random priming. Blots were hybridized for 20 hours and washed as described previously. Membranes were hybridized with a mouse Gapdh cDNA probe to control the nondegradation and loading of the RNA samples. A blot filter containing 2 µg per lane of poly(A)+ RNA extracted from various human tissues was purchased from Clontech (Palo Alto, CA), and hybridized with the TH3L probe, using the conditions recommended by the manufacturer.

Reverse transcriptase-PCR analysis. cDNA was prepared from 2 or 5 µg of total RNA, using random hexamers as primers and the MMLV reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD). PCR was performed in a Perkin Elmer-Cetus apparatus (Norwalk, CT), according to the instructions of the manufacturer, and using an equivalent of 500 ng of reversed transcribed RNA (or 0.25 ng, for the actin control) and 50 pmol of each oligonucleotide as primers. The first cycle was run as follows: denaturation at 94°C for 2 minutes, annealing at 55 to 65°C (depending on the melting temperature of the primers) for 2 minutes, and synthesis at 72°C for 3 minutes. The next 28 or 38 cycles were run using the same conditions except that the denaturation step was only 1 minute. For the last cycle, synthesis time was 10 minutes. Oligonucleotides for FLT3 expression analysis were: sense primer 5’-TCAAGTAGCTGTGCGATACATTCC-3’ (positions 1534-1558 on the sequence), antisense primer 5’-CACCCTGTACCTGTAGGTGCT-3’ (positions 1719-1744). Sense primer 5’-TACCACCTGGCATCGTGATGGACT-3’ and antisense primer 5’-TCTCTTTGCACTCCGTGCGCAAT-3’ were used for the actin control. One tenth of the amplified products were run in 2% agarose gels in 1X Tris borate buffer (TBE). For FLT3 expression analysis, electrophoresed PCR products were transferred onto Nytran membranes in 20X SSC. Hybridization was performed at 42°C in 6X SSC and 1X Denhardt’s, and washings were performed at 42°C in 2X SSC and 0.1% SDS. The probe was an internal oligonucleotide [gamma 32P] ATP-labeled with T4 polynucleotide kinase. Sense primer 5’-CATCTCATTTGATGGACT-3’ (positions 1623-1647).

RESULTS

Human FLT3 is expressed in pre-B-cell lines. FLT3 expression has been found in mouse lymphocyte progenitors (Rosnet et al, unpublished data, January 1993). We therefore looked for expression of FLT3 in cell lines representative of human immature lymphocytes. Total RNAs, prepared from four pre-B-cell lines, were analyzed for FLT3 expression by Northern blot hybridization. A transcript of 3.7 kb was evidenced in the 1E8 cell line (Fig 2). Weak signals were also detected in two other pre-B-cell lines, Nalm6 and 697, but not in 207. In non-pre-B-cell lines, we detected FLT3 transcripts in THP-1 (Fig 2), Daudi, and JY (not shown). In tissues, an FLT3 transcript was found in total RNA extracted from thymus and lymph nodes (Fig 2). Finally, poly(A)+ RNA from various human tissues (Clontech, San Diego, CA) were hybridized with an FLT3 probe. A weak 3.7-kb signal was observed in kidney and pancreas, whereas heart, liver, placenta, and skeletal muscle were negative (not shown).

Clone of human FLT3 cDNA from 1E8 mRNA. A cDNA library constructed from 1E8 RNA was screened with probes previously isolated. Clone 1EB (Fig 1) was isolated and characterized. Subsequent rounds of screening did not yield any clones extending 5’ from 1EB. An alternative strategy was therefore devised to obtain a full-length cDNA. It included a round of screening of a testis cDNA library, and cloning of PCR products (see Materials and Methods). As testicular transcripts are often aberrant, we did not contemplate isolating a full-length FLT3 cDNA from the testis library. The sequence of testis clone TH9 was used to derive oligonucleotide primers. The 5’ end of the cDNA was then obtained by PCR amplification of 1E8 reverse transcribed RNA, using an oligonucleotide designed from the mouse sequence corresponding to the ATG initiation codon preceded by a sequence of fifteen 5’ untranslated nucleotides (primer 3 in Fig 1), and an oligonucleotide designed from the TH9 sequence (primer 4 in Fig 1). PCR product CR2511 was obtained. In addition, TH9-derived primer 1 and 1EB-derived primer 2 were used in PCR on 1E8 cDNA to obtain the FLT3 cDNA fully representative of the 1E8 single transcript. PCR product CR2906 was obtained. Complete FLT3 nucleotide sequence was then determined using cDNA clones and several PCR subclones.

Nucleotide sequence analysis of FLT3 cDNA. The nucleotide sequence of FLT3 cDNA and deduced amino acid...
Fig 3. Nucleotide sequence of the FLT3 cDNA and deduced amino acid sequence of the FLT3 gene product. Nucleotides, and corresponding amino acids (single-letter code) shown below, are numbered at the beginning of each lane. Special features indicated are: the putative signal peptide and transmembrane domains (double underlining), and the putative asparagine linked glycosylation sites (single underlining).
sequence of the FLT3 protein are shown in Fig 3. The sequence extends over 3422 nucleotides. It possesses a polyadenylation signal 17 bp from its end but no poly(A) stretch. The largest open reading frame starting from the ATG codon terminates at a TAG codon at position 2980-2983. It is therefore able to code for a primary translational product of 993 amino acids.

**Deduced amino acid sequence of the FLT3 protein.** The FLT3 protein shows all the characteristics of a transmembrane receptor-type tyrosine kinase, with the presence of two stretches of respectively 23 and 21 hydrophobic amino acids (Fig 3, double underlining), representative of putative signal peptide and transmembrane domain, respectively. The extracellular region of FLT3 is predicted, according to its primary amino acid sequence, to fold into five Ig-like domains, a characteristic of class III RTKs. It is 541 amino acids long and contains nine potential asparagine-linked glycosylation sites.

**Comparison of FLT3 sequences with related genes and proteins.** As shown in Fig 4, the deduced amino acid sequence of the human FLT3 protein is closely related to that of the mouse Flt3 gene product. Human FLT3 is 7 residues shorter than the mouse receptor. There is an 86% overall amino acid sequence identity between the two proteins. This identity is observed all along the molecule, including the extracellular region, kinase insert, and C-terminus, regions that are usually less conserved among different class III RTKs. A shift in the position of the termination codon is caused by the insertion of a C at position 2970, not present in the mouse sequence. The accuracy of the sequence at this position was confirmed by analysis of the corresponding human genomic DNA (not shown). The similarity of the human and mouse nucleotide sequences extends into the 3' untranslated regions, including an AT-rich region possessing an ATTAA motif possibly responsible for a rapid turnover of the messenger.

Starting at amino acid position 957, a difference between the published murine FLT3 and FLK2 sequences has been noticed. The comparison of the mouse and human FLT3 sequences highlights the fact that the murine FLT3 sequence is probably the correct one.

The FLT3 gene product is related to CSF1R/FMS and SLFR/KIT, as schematized in Fig 5. FLT3 shows respectively 18% and 19% identity with KIT and FMS in the extracellular region, and 63% and 64% in the tyrosine kinase domain. Noticeably, FMS and KIT are more closely related to each other, because they are 27% and 71% identical in their extracellular regions and kinase domains, respectively. Together, FLT3, KIT, and FMS share only 9% identity in their extracellular regions, but this identity includes eight cysteines possibly involved in intramolecular bonds responsible for the Ig-like folding of the second, third, and fifth Ig-like domains. They are 57% identical in their tyrosine kinase domains.

The CSF1R and SLFR receptors, although not exclusively expressed in the hematopoietic system, form a subgroup whose functions are important in hematopoiesis. Subsequent analysis of FLT3 expression suggested a similar role for FLT3.

**Expression of FLT3 in human cells and tissues.** To gain
knowledge of the function of FLT3/FLK2 in hematopoiesis, we analyzed the distribution of FLT3 mRNA in a variety of human lympho-hematopoietic cells, cell lines, and tissues, using RT-PCR amplification of mRNAs (see Materials and Methods).

Reverse transcription was performed and the resulting cDNA was amplified by PCR using primers specific for a 210-bp sequence of FLT3, as determined from the sequence herein. As control, a 506-bp sequence from the beta-actin cDNA was also amplified. The amplified materials were then electrophoresed through 2% agarose gels (Fig 6). The PCR products were also blotted and hybridized with an internal FLT3 primer and showed specificity for FLT3 (not shown).

We first screened various normal lympho-hematopoietic tissues including fetal and adult tissues by RT-PCR. As shown in Fig 6A, a PCR product was detected in all tissues tested, including liver, spleen, thymus, and bone marrow. A faint band was also observed in human placenta. Mature purified blood cells (B and T lymphocytes, monocytes, and granulocytes) were also tested for FLT3 expression. No PCR product was evidenced in B and T lymphocytes, whereas monocytes and granulocytes were weakly positive (Fig 6A). We previously showed that Northern blot analysis of these mature blood cell populations did not show any FLT3 transcripts. It is therefore probable that FLT3 transcripts are not present in mature B and T lymphocytes, rare in monocytes and granulocytes, or only present in subpopulations of these cells or in contaminating populations.

We next looked for FLT3 expression in hematopoietic cell lines of various origins, including pre-B-, B-, T-, erythroid- and myelo-monocytic cell lines. An FLT3-specific PCR product was obtained from most pre-B-cell lines, including the three initially found positive by Northern blot analysis (Fig 2). Consistently lower or undetectable signals were associated with the mature B-cell lines (Fig 6B). Variable signals were found in T- and myeloid cell lines. In addition, the HL60 promyeolocytic cell line was found to be strongly positive, whereas three erythroid cell lines, TF1, HEL, and K562 were negative for FLT3 expression (not shown).

We analyzed the expression of FLT3 in a series of leukemia samples including 10 pre-B-, 2 pre-T-cell leukemias, and one T-cell leukemia. All the samples showed the presence of an FLT3-specific PCR product at a high level (Fig 6C).

Finally, we looked for the expression of FLT3 in other tissues of by using the RT-PCR screen on cell lines of various origins (Fig 6D). A low level of the FLT3 PCR product was present in the MCF7 breast tumor and 5637 bladder carcinoma cell lines. When compared with the actin control, FLT3 expression in both cell lines was equivalent. All the other tumor cell lines were negative. The three stromal cell lines were also found to be negative.

DISCUSSION

Cytokines and cell surface receptors interact, mediating intercellular communication, are central to the proliferation and differentiation of hematopoietic cells. Three iden-
The knowledge of the nucleotide sequence of FLT3 allowed us to design oligonucleotide primers. Thus, an RT-PCR-based analysis of FLT3 mRNA was conducted on lympho-hematopoietic cells and tissues, to establish a crude pattern of the expression of this gene in the human hematopoietic system. Expression was detected in all lympho-hematopoietic tissues tested. This confirmed the results of Northern blot hybridizations showing FLT3 expression in bone marrow, thymus and lymph nodes (Fig 2). Expression was also detectable in placenta by RT-PCR but not by Northern blot analysis. Thus, as in the mouse, FLT3 is expressed in all lympho-hematopoietic organs. In this species we detected the expression of the Flt3 mRNA and of the FLT3 protein in spleen, thymus, lymph nodes, fetal liver, placenta, kidney, brain, and cerebellum (deLapeyrière,
Rottapel and Rosnet, unpublished data, January 1993). In contrast to human, Flt3 is strongly expressed in mouse placenta.\textsuperscript{11}

\textit{FLT3} expression was observed in many different hematopoietic cell lines. It is consistently found in pre-B-cell lines, and to a lesser extent in B-, T-, and myelomonocytic cell lines but not in erythroid cell lines. Accordingly, it is found in all lymphoid leukemias, as previously reported.\textsuperscript{21} In contrast, \textit{FLT3} does not seem to be expressed in mature purified B and T cells. This is also in contrast with the expression of Flt3 observed in murine lymphocytes.\textsuperscript{11} Such discrepancies between the expressions in the mouse and in humans are likely to reflect species-specific involvement of the gene in some biological processes. Study of \textit{FLT3} expression in purified cell populations as well as in other human tissues will help in clarifying those differences.

The lack of expression of \textit{FLT3} in most nonhematopoietic cell lines is preliminary evidence for a possible restriction of the function of the \textit{FLT3} gene product to hematopoiesis, but will await further studies on nonhematopoietic fresh cells and tissues. Of course, this limited analysis does not preclude the possible importance of \textit{FLT3} expression in the nervous system, whereas its presence in kidney, already noticed in the mouse, and pancreas has to be further investigated. The negative results obtained with bone marrow stromal cell lines support the proposed model of an interaction between \textit{FLT3} and its ligand similar to that reported for KIT and SLF.\textsuperscript{8}

In our study, only the highest levels of \textit{FLT3} RT-PCR products are correlated with an expression detectable by hybridization of Northern blots prepared from 10 µg of total RNA. Thus, PCR analysis readily detected an \textit{FLT3} cDNA in cell lines such as 1E8, THP-1, JY, or Daudi, in which \textit{FLT3} transcripts were detectable by Northern blot hybridization but gave only a faint signal in cell lines such as KG-1 or Jurkat, in which no \textit{FLT3} mRNA was evidenced by Northern analysis. Despite the fact that some surface receptors can be active at low density, it seems likely that only
high levels of expression lead to the presence at the cell surface of functional FLT3 receptors. This conclusion can only be drawn for highly homogeneous cell lines and leukemic samples and not for tissues in which many cell types coexist.

Although it is difficult to draw firm conclusions from this preliminary survey of tissues, cell lines, and coarsely purified cells, our data suggest that FLT3 is predominantly expressed, and consequently probably important, in immature lymphoid and myeloid cells.

Further characterization of the human FLT3 clone and its encoded protein will contribute to a better understanding of the role of the FLT3 receptor. Its availability will permit derivation of useful reagents, such as MAbS or oligodeoxyribonucleotides, to study the early steps of normal human hematopoiesis. It will also be useful in the search for the human ligand, which in turn should prove to be an important factor for the stem cells. The respective roles of KIT and FLT3 will be interesting to compare. Furthermore, it will be possible to investigate a role for potential alterations of the FLT3 gene in leukemogenesis. Finally, we may wonder whether other genes of the FMS/KIT/FLT3 subgroup may exist, possibly in the vicinity of the FLT3 gene itself, and participate in the regulation of hematopoiesis.

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