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K-*sam* gene encodes secreted as well as transmembrane receptor tyrosine kinase

(heparin-binding growth factor receptor/fibroblast growth factor receptor/secreted receptor)

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ABSTRACT **K-*sam* was first identified as a gene amplified in the stomach cancer cell line KATO-III. The size of the major transcript of the K-*sam* gene was 3.5 kilobases in KATO-III cells, and we have previously shown that K-*sam* encodes a receptor tyrosine kinase that belongs to the heparin-binding growth factor receptor, or fibroblast growth factor receptor, gene family. The K-*sam* gene expresses multiple sizes of mRNAs in brain tissue, the immature teratoma cell line NCC-IT, and KATO-III. RNA blot analyses with a variety of K-*sam* probes indicate that there are at least four classes of K-*sam* mRNAs. Three types of K-*sam* cDNAs in addition to the previously reported type of K-*sam* cDNA were isolated, and their nucleotide sequences encode a full-length transmembrane receptor, a secreted receptor with a tyrosine kinase domain, and a secreted receptor without a tyrosine kinase domain.**

The K-*sam* gene (1) was first identified as amplified DNA fragments in the stomach cancer cell line KATO-III (2) by the in-gel DNA renaturation method (3–5). K-*sam* cDNA, corresponding to a 3.5-kilobase (kb) mRNA, encodes a receptor tyrosine kinase. The K-*sam* cDNA is homologous to mouse *bek* (6), mouse keratinocyte growth factor (KGF) receptor (7), chicken Cek3 (8), human *bek* (9, 10), and human TK14 (11) cDNAs. N-*sam* is a K-*sam*-related gene. N-*sam* cDNAs (1) are highly homologous to cDNAs for chicken basic fibroblast growth factor (FGF) receptor (12)/Cek1 (13) and mouse basic FGF receptor (14, 15), and N-*sam* is identical with *flg* (10, 16, 17). Heparin-binding growth factors, or the FGF family, include acidic and basic FGFs, INT2 protein (18), HST1 protein (19–21), FGF5 (22), HST2 protein/FGF6 (23–25), and KGF (26). The K-*sam*/*bek* and N-*sam*/*flg* genes establish a gene family of heparin-binding growth factor receptors or FGF receptors.

The K-*sam* gene is amplified preferentially in poorly differentiated types of stomach cancer (5). K-*sam* mRNA of 3.5 kb is overexpressed in stomach cancer cells with K-*sam* amplification (1). During studies of K-*sam* gene expression, multiple sizes of K-*sam* mRNA were found. We describe here molecular cloning and characterization of three additional K-*sam* cDNAs. K-*sam* cDNA type I (K-*sam*-I) was obtained from a cDNA library of human brain, K-*sam* cDNA type III (K-*sam*-III) was obtained from a cDNA library of the immature teratoma cell line NCC-IT (27), and K-*sam* cDNA type IV (K-*sam*-IV) was obtained from a cDNA library of KATO-III cells. The previously published K-*sam* cDNA derived from KATO-III cells was designated K-*sam* cDNA type II (K-*sam*-II). K-*sam*-I, corresponding to a 4.5-kb K-*sam* mRNA, encoded a transmembrane receptor with a tyrosine kinase domain, identical with the previously reported human Bek protein. Sequence analyses predicted that K-*sam*-III encoded a secreted receptor with a tyrosine kinase domain and that K-*sam*-IV encoded a secreted receptor without a tyrosine kinase domain.‡

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MATERIALS AND METHODS

RNA Blot Analysis. RNA blot analyses were performed under high-stringency conditions (19, 20). Poly(A)$^+$ RNA of postmortem human brain (28) was kindly provided by H. Kobayashi and S. Tsuji of Niigata University. Poly(A)$^+$ RNAs of NCC-IT cells and KATO-III cells were prepared as described (1). RA0.7 is a specific probe for the K-*sam* gene (1) and corresponds to the 5′ noncoding region and a small portion of the coding region of K-*sam*-II (nucleotides 1–719 of K-*sam*-II). The EC probe was made by polymerase chain reaction (PCR) and corresponds to the region of K-*sam*-I encoding the extracellular domain (nucleotides −46 to 1071). The ATM probe was made by PCR and corresponds to the region of K-*sam*-II encoding the transmembrane domain (nucleotides 1259–1595). The SR0.5 probe corresponds to the region encoding the second part of the K-*sam*-II tyrosine kinase domain (nucleotides 2071–2604) and cross-hybridizes with N-*sam* cDNAs (1). The DD0.4 probe was made by *Dra* I restriction endonuclease digestion and corresponds to the 3′ noncoding region of K-*sam*-I (nucleotides 2927–3275). The SKT probe was made by PCR and corresponds to the 3′-terminal region of K-*sam*-IV (nucleotides 751–1110).

Isolation of K-*sam* cDNA Clones. A human brain cDNA library was constructed from poly(A)$^+$ RNA of postmortem human brain (28) and cloned into λgt10. Size fractionation of cDNA inserts (>0.5 kb) was added to the procedure described previously (20). An NCC-IT cDNA library was constructed and cloned into λgt10. A KATO-III cDNA library was constructed and cloned into λZAPII (Stratagene). These cDNA libraries were screened with appropriate probes under high-stringency conditions (20).

DNA Sequencing. cDNA sequences were determined by the dideoxy chain-termination method as described (1).

cDNA PCR. cDNA PCR was carried out as described (1). The DNA sequences of primers were as follows: primer U1 (sense), 5′-CTGACAAGGGAAATTATACC-3′ (corresponding to nucleotides 671–690 of K-*sam*-I); primer U2 (sense), 5′-GACTGCCGGCAAATGCCTCCA-3′ (corresponding to nucleotides 782–802 of K-*sam*-I) with a *Sal* I site added to the 5′ terminus; primer D1 (antisense), 5′-TTTGCACAGAGGAAATAGATGCC-3′ (corresponding to nucleotides 1110–1088 of K-*sam*-IV); primer D2 (antisense),

Abbreviations: FGF, fibroblast growth factor; KGF, keratinocyte growth factor.

‡The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M87770 (K-*sam*-I), M87771 (K-*sam*-III), and M87772 (K-*sam*-IV)].

5'-ACCAGCGGGGTGTTGGAG-3' (corresponding to nucleotides 1334–1317 of K-*sam*-I) with a *Sal* I site added to the 5' end; primer D3 (antisense), 5'-ATCATCTTCATCATCTCCAT-3' (corresponding to nucleotides 1622–1603 of K-*sam*-I).

RESULTS

K-*sam* Gene Expression. RNA blot hybridization with a specific probe for the K-*sam* gene, RA0.7, showed bands of various sizes (Fig. 1, lanes 1–3). RNA from postmortem human brain showed bands of 4.5, 4.0, and 1.6 kb. RNA from NCC-IT cells gave bands of 4.5, 4.0, 3.2, and 1.6 kb. The intensity of the 4.0-kb band was weak in human brain RNA, whereas it was as strong as that of the 4.5-kb band in NCC-IT RNA. In RNA from KATO-III cells, the major bands were at ≈3.5 kb, ranging from 4.2 to 3.2 kb, and less intense bands of 4.5 kb and ≈1.6 kb (1.8 to 1.4 kb) were also detected.

Further analyses were performed with other K-*sam* probes to predict the structure of the multiple mRNAs derived from the K-*sam* gene. The EC probe, corresponding to the region of K-*sam*-I encoding the extracellular domain, showed the same pattern in RNA blot hybridization as RA0.7 with RNA from KATO-III (data not shown). The ATM probe, corresponding to the region of K-*sam*-II encoding the membrane-spanning domain, hybridized to 4.5- and 4.0-kb bands in NCC-IT RNA and to 4.5- and 3.5-kb bands in KATO-III RNA (Fig. 1, lanes 4 and 5). SR0.5, corresponding to the region of K-*sam*-II encoding the second part of the tyrosine kinase domain, hybridized to 4.5-, 4.0-, and 3.2-kb bands in NCC-IT RNA and to 4.5- and 3.5-kb bands in KATO-III RNA (Fig. 1, lanes 6 and 7).

Isolation and Characterization of K-*sam*-I. To isolate K-*sam* cDNAs corresponding to the 4.5-kb mRNA in normal tissue, a human brain cDNA library was screened with RA0.7. A clone that contained a 4.2-kb insert was isolated out of 2 × 10^5 clones and designated K-*sam*-I.

K-*sam*-I contains a large open reading frame that encodes a protein of 821 amino acids with calculated M_r of 92,024 (Fig. 2*A*). The N-terminal 21 amino acid residues of the K-*sam*-I product correspond to the signal peptide, while the following 354, 23, and 423 residues constitute the extracellular, transmembrane, and cytoplasmic domains, respectively. The extracellular domain of the K-*sam*-I product contains three immunoglobulin-like (Ig-like) domains (29). An uninterrupted stretch of acidic residues (12) exists between the first and second Ig-like domains of the K-*sam*-I product. The cytoplasmic domain contains a tyrosine kinase domain with a 14-amino acid kinase insert region (30). The 3' noncoding region of K-*sam*-I has an internal polyadenylylation signal (31) in addition to the three terminal polyadenylylation signals.

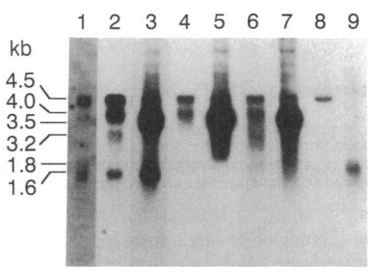

FIG. 1. K-*sam* gene expression. Poly(A)$^+$ RNA (2 μg per lane) was fractionated in agarose gels, transferred to Nitroplus membranes (MSI), and probed with ^{32}P-labeled RA0.7 (lanes 1–3), ATM (lanes 4 and 5), SR0.5 (lanes 6 and 7), DD0.4 (lane 8), or SKT (lane 9). Lane 1, human brain; lanes 2, 4, and 6, NCC-IT; lanes 3, 5, and 7–9, KATO-III. Films of lanes 1, 2, 4, 6, and 9 were exposed for 24 hr at −70°C, and those of lanes 3, 5, 7, and 8 for 9 hr at −70°C.

The nucleotide sequence of K-*sam*-I is almost identical with that of human *bek*. The reported nucleotide sequence of human *bek* terminates at nucleotide 3236 of K-*sam*-I. Although the nucleotide sequence of K-*sam*-I and that of human *bek* differ at nucleotides −179, −129, −126, −103, 159, and 2724 of K-*sam*-I, the deduced amino acid sequence of K-*sam*-I is identical with that of human *bek*.

K-*sam*-I differs from K-*sam*-II in four regions. The first Ig-like domain and 6 base pairs (bp) in the juxtamembrane domain of K-*sam*-I (nucleotides 110–376 and 1282–1287, respectively) are absent from K-*sam*-II (Fig. 3). The region encoding the second half of the third Ig-like domain of K-*sam*-I (nucleotides 940–1083) is replaced by a nucleotide sequence without significant homology in K-*sam*-II. The C-terminal coding region and the 3' noncoding region of K-*sam*-I (nucleotides 2277–3995) is also replaced by a different sequence in K-*sam*-II. The reason for the change in the C-terminal tail and the 3' noncoding region remains unknown.

The DD0.4 probe hybridized to a 4.5-kb band, but not to a 3.5-kb band, in KATO-III RNA (Fig. 1, lane 8). DD0.4 hybridized to a 4.5-kb band, but not to a 4.0-kb band, in NCC-IT RNA (data not shown). K-*sam*-I most likely corresponds to a 4.5-kb mRNA.

Isolation and Characterization of K-*sam*-III. An NCC-IT cDNA library was screened with SR0.5 and RA0.7, and a clone that hybridized to both probes was isolated (1). The nucleotide sequence of the cDNA was almost identical with that of K-*sam*-I, except that the membrane-spanning region of K-*sam*-I (nucleotides 940–1287, Fig. 2*A*) was deleted. The cDNA was designated K-*sam*-III and was analyzed.

The nucleotide sequence of K-*sam*-III terminates 19 bp downstream of the internal polyadenylylation signal of K-*sam*-I (nucleotide 3105), and adenine at position −235 of K-*sam*-I is replaced by cytosine in K-*sam*-III. K-*sam*-III encodes a secreted receptor with a tyrosine kinase domain and consisting of 705 amino acids with calculated M_r of 79,211.

cDNA PCR with primers U2 and D3 revealed 846-bp bands from poly(A)$^+$ RNA of human brain, NCC-IT, and KATO-III (Fig. 4). The 846-bp bands are likely to be derived from K-*sam* mRNA encoding a receptor with a membrane-spanning region. A 498-bp band was also amplified from poly(A)$^+$ RNA of NCC-IT, but not from RNA of human brain and KATO-III (Fig. 4). Both RA0.7 and SR0.5 hybridized to a 3.2-kb K-*sam* mRNA, but ATM did not (Fig. 1). These results show the existence of a K-*sam* mRNA that encodes a secreted receptor with a tyrosine kinase domain in NCC-IT.

Isolation and Characterization of K-*sam*-IV. A 1.6-kb band hybridized to RA0.7 and EC, but not to ATM or SR0.5 (Fig. 1), suggesting the existence of a class of K-*sam* mRNA that encodes a secreted receptor without a tyrosine kinase domain. A KATO-III cDNA library in λZAPII was screened to isolate clones that hybridized to EC but not to ATM. Fourteen clones were isolated out of 2 × 10^5 clones. Restriction enzyme mapping revealed that two clones, with 1.5-kb and 2.2-kb inserts, had different 3'-terminal sequences. Neither of the 3'-terminal sequences of these two inserts was contained in K-*sam*-I, K-*sam*-II, or K-*sam*-III. The 1.5-kb cDNA was designated K-*sam*-IV and was analyzed.

The nucleotide sequence of K-*sam*-IV is identical with that of K-*sam*-I amino acid codons 1–249, but further 3' the two sequences differed (Fig. 2*B*). From residue 250, the open reading frame of K-*sam*-IV continues downstream for another 5 amino acids and is then followed by a stop codon, TGA. The 3' noncoding region of K-*sam*-IV, 504 bp, includes a polyadenylylation signal 30 bp upstream from the 3' end. The deduced amino acid sequence of K-*sam*-IV contains the signal peptide, the first Ig-like domain, the acidic region, the second Ig-like domain, and the additional 5 amino acids but

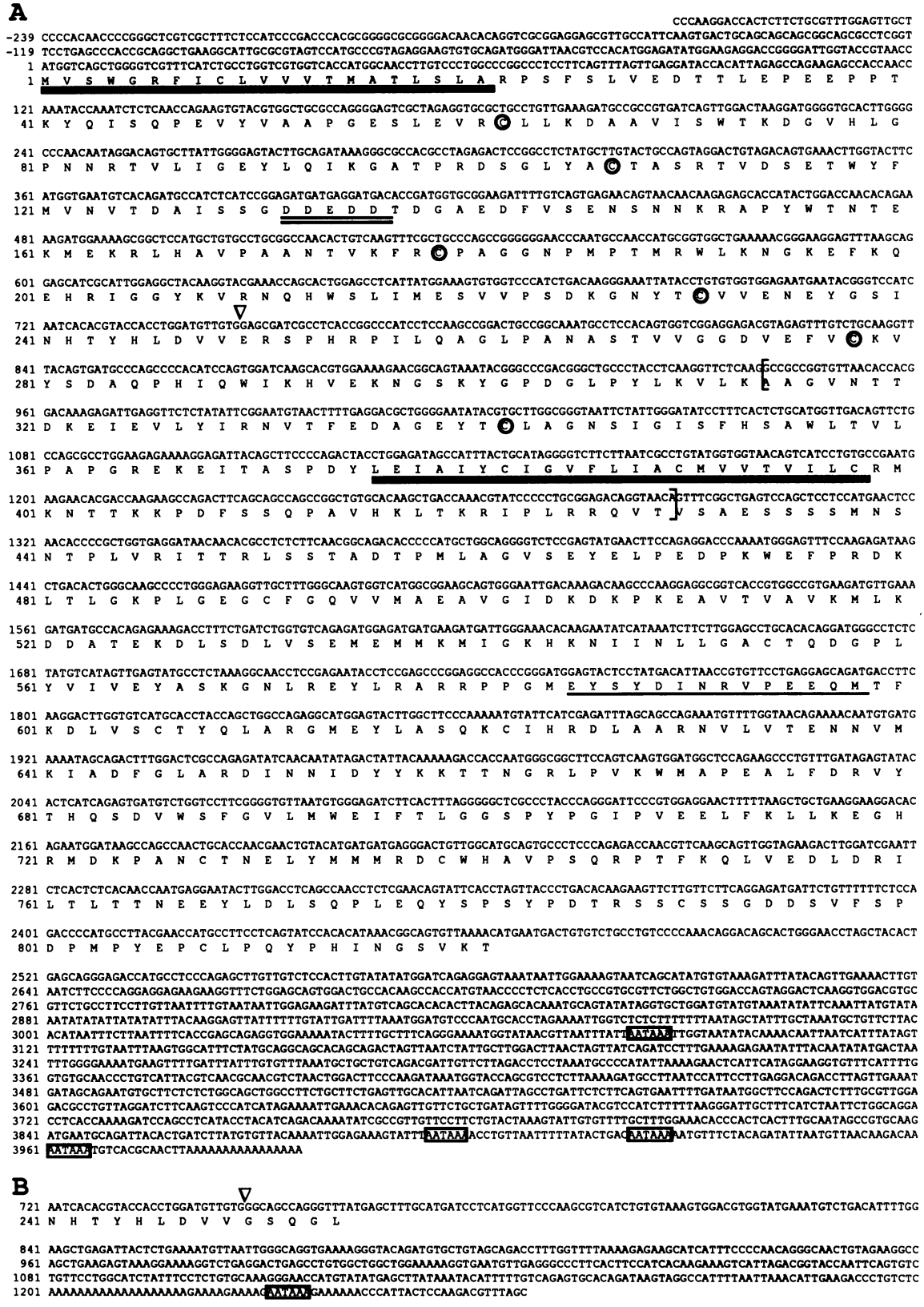

FIG. 2. Nucleotide sequence and deduced amino acid sequence of K-*sam* cDNAs. (*A*) K-*sam*-I. Nucleotides are numbered at left, as are the deduced amino acids, which are shown below the nucleotide sequence. Heavy underlines indicate the putative hydrophobic signal peptide and the transmembrane domain. Underline indicates the kinase insert region in the tyrosine kinase domain. Double underline indicates an uninterrupted stretch of acidic residues (DDEDD). Cysteine residues that define Ig-like domains are circled. The region deleted in K-*sam*-III is bracketed. Open arrowhead indicates the point at which sequence identity between K-*sam*-I and K-*sam*-IV diminishes. Polyadenylylation signals are boxed. (*B*) K-*sam*-IV. Nucleotide sequence of K-*sam*-IV is identical with that of K-*sam*-I at positions −272 to 747. Nucleotide sequence downstream from nucleotide position 720 is shown.

lacks the transmembrane domain and tyrosine kinase domain. Thus, K-*sam*-IV seems to encode a secreted receptor that lacks a tyrosine kinase domain and consists of 254 amino acids with calculated M_r of 28,298.

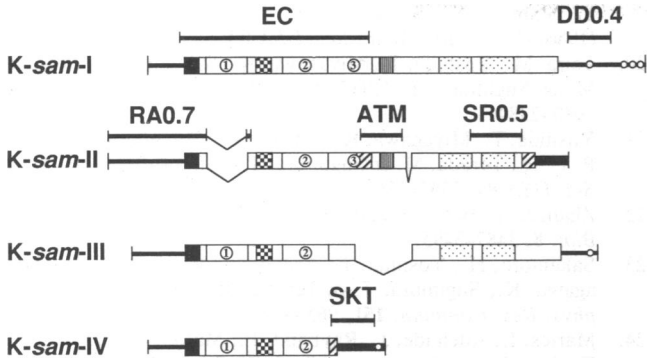

FIG. 3. K-*sam* cDNAs and probes. Noncoding region is indicated by bar. The 3′ noncoding regions specific for K-*sam*-II and K-*sam*-IV are indicated by thicker bars. Shown for each cDNA are the hydrophobic signal peptide (filled box), Ig-like domains (numbered open boxes), acidic region (checked box), transmembrane domain (striped box), tyrosine kinase domain (stippled box), divergent coding region of K-*sam*-II and K-*sam*-IV (hatched box), and polyadenylylation signal(s) [small circle(s)].

cDNA PCR with primers U1 and D1 amplified 440-bp products from poly(A)$^+$ RNA of human brain and KATO-III (data not shown). These PCR products showed the expected sizes and contained the expected internal *Pvu* II sites. PCR with primers U1 and D1 amplified 650-bp bands from genomic DNA of human placenta and KATO-III (data not shown), which also contained the internal *Pvu* II sites.

RNA blot hybridization with the SKT probe showed a 1.8-kb band in KATO-III (Fig. 1, lane 9) but not in human brain and NCC-IT (data not shown).

DISCUSSION

K-*sam* mRNAs of different sizes were present in various types of cells. K-*sam* mRNAs of 4.5, 4.0, and 1.6 kb were detected in normal tissue, human brain. K-*sam* mRNAs of 3.5 and 1.8 kb were found in KATO-III stomach cancer cells. K-*sam* mRNA of 3.2 kb was detected in NCC-IT teratoma cells. RNA blot analyses with a variety of K-*sam* probes, coupled with analyses of different types of K-*sam* cDNAs, indicate that there are at least four classes of mRNAs. The K-*sam* mRNA of 4.5 kb encodes the full-length receptor, which is identical with the human *bek* product. The K-*sam* mRNAs of 4.0 and 3.5 kb, which are truncated at least in the 3′ noncoding region, encode membrane-spanning receptors with a tyrosine kinase domain. The K-*sam* mRNA of 3.2 kb probably encodes a secreted receptor with a tyrosine kinase domain, and the K-*sam* mRNAs of 1.6 and 1.8 kb are likely

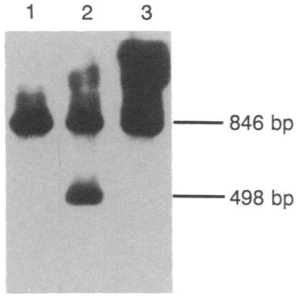

FIG. 4. cDNA PCR with primers U2 and D3. cDNA PCR products with primers U2 and D3 were fractionated in a 3% agarose gel, and transferred to Nitroplus membrane. The filter was hybridized with a ^{32}P-labeled probe made by PCR with primers U2 and D2 using K-*sam*-III as the template. Lane 1, human brain; lane 2, NCC-IT; lane 3, KATO-III.

to encode secreted receptors without a tyrosine kinase domain.

The structures of K-*sam*-I and K-*sam*-II are different in four regions. The first Ig-like domain of K-*sam*-I is absent from K-*sam*-II, probably due to alternative splicing or cassette splicing (31). Such a variant receptor with two Ig-like domains was also reported for human *bek* (10). The second half of the third Ig-like domain of K-*sam*-I is replaced by another sequence in K-*sam*-II. The structures of *bek* and TK14 in this region have the K-*sam*-I pattern, whereas that of the KGF receptor has the K-*sam*-II pattern. According to the recently published partial genomic sequences around the third Ig-like domain of K-*sam*/human *bek* (32, 33), the difference in this region may be due to alternative splicing of a mutually exclusive exon (31). The human *bek* product exhibits high affinity for both acidic and basic FGFs (9), whereas the KGF receptor exhibits high affinity for both acidic FGF and KGF (7). Thus, the second half of the third Ig-like domain is important for ligand recognition. Six base pairs of K-*sam*-I that encode valine and threonine in the juxtamembrane domain are absent from K-*sam*-II, and this region is also absent from TK14. The 3′-terminal region of K-*sam*-I is replaced by another sequence in K-*sam*-II. The reason for the replacement remains to be determined. These changes in the extracellular domain and in the cytoplasmic domain may lead to altered ligand binding affinity and signal transduction.

The boundary of the region deleted in K-*sam*-III is also the boundary of structural difference between K-*sam*-I and K-*sam*-II (Fig. 3). The boundary is located at nucleotides 939/940 and 1287/1288 of K-*sam*-I. The nucleotide sequence of K-*sam*-I around the upstream boundary is AAG/GCC, which satisfied the consensus sequence of the 5′ and 3′ splice sites of an exon (34). The nucleotide sequence around the downstream boundary is CAG/GTAACA/GTT. As 6 bp, GTAACA, are absent from K-*sam*-II, the G at position 1288 may be the 3′ splice site. K-*sam* mRNA corresponding to K-*sam*-III lacks the membrane-spanning region, probably due to alternative splicing or cassette splicing.

K-*sam*-IV was isolated during studies on secreted receptors without a tyrosine kinase domain. K-*sam*-IV corresponded not to the 1.6-kb K-*sam* mRNA, but to the 1.8-kb K-*sam* mRNA in KATO-III. K-*sam* mRNA corresponding to K-*sam*-IV probably results from alternative splicing using alternative 3′-terminal exons (31).

Among many receptor tyrosine kinases, epidermal growth factor receptor (35) and basic FGF receptor (36) were reported to have secreted forms. The K-*sam* gene also is likely to generate secreted receptors and may generate a secreted receptor with a tyrosine kinase domain.

The biological significance of secreted receptors encoded by K-*sam* remains to be determined. It also remains unknown whether the secreted receptor with a tyrosine kinase domain and that without a tyrosine kinase domain have different biological functions. Secreted receptors may act as carrier proteins, as described for a secreted form of the growth hormone receptor that is also generated by alternative splicing (37, 38). Another possibility is that secreted receptors could modulate the response of target cells to ligands, heparin-binding growth factors, by acting as a trap for those growth factors or by competing with transmembrane receptors for their ligands. Similar mechanisms were postulated for cytokine receptors (39). Alternatively, a secreted receptor may transduce signals, as reported for the secreted interleukin 6 receptor, which may be able to transduce signals in association with the interleukin 6 signal transducer, gp130 (40, 41). Although the physiological role of secreted receptors encoded by K-*sam* remains to be elucidated, serological examination to detect such products could be a diagnostic

clue for certain types of stomach cancer with K-*sam* amplification.

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