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RESEARCH

Cloning and Characterization of a Novel Human Gene Related to Vascular Endothelial Growth Factor

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This paper describes the cloning and characterization of a new member of the vascular endothelial growth factor (VEGF) gene family, which we have designated VRF for VEGF-related-factor. Sequencing of cDNAs from a human fetal brain library and RT–PCR products from normal and tumor tissue cDNA pools indicate two alternatively spliced messages with open reading frames of 621 and 564 bp, respectively. The predicted proteins differ at their carboxyl ends resulting from a shift in the open reading frame. Both isoforms show strong homology to VEGF at their amino termini, but only the shorter isoform maintains homology to VEGF at its carboxyl terminus and conserves all 16 cysteine residues of $VEGF_{165}$. Similarity comparisons of this isoform revealed overall protein identity of 48% and conservative substitution of 69% with $VEGF_{189}$. VRF is predicted to contain a signal peptide, suggesting that it may be a secreted factor. The *VRF* gene maps to the DIIS750 locus at chromosome band 11q13, and the protein coding region, spanning ~5 kb, is comprised of 8 exons that range in size from 36 to 431 bp. Exons 6 and 7 are contiguous and the two isoforms of VRF arise through alternate splicing of exon 6. *VRF* appears to be ubiquitously expressed as two transcripts of 2.0 and 5.5 kb; the level of expression is similar among normal and malignant tissues.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a secreted, covalently linked homodimeric glycoprotein that specifically activates endothelial tissues (Keck et al. 1989; Leung et al. 1989; Senger et al. 1993). This factor is involved in a variety of physiological processes, including normal angiogenesis, formation of the corpus luteum (Yan et al. 1993), placental development (Sharkey et al. 1993), regulation of vascular permeability (Senger et al. 1993), inflammatory angiogenesis (Sunderkotter et al. 1994), and autotransplantation (Dissen et al. 1994), as well as pathological conditions such as tumor-promoting angiogenesis (Plate et al. 1992; Christofori et al. 1994),

VEGF is a distant relative of the platelet-derived growth factor (PDGF) gene family with many of the cysteine residues involved in dimerization of these proteins conserved in position (Leung et al. 1989; Keck et al. 1989). A more closely related homolog of VEGF is placenta growth factor (PlGF) (Maglione et al. 1991), which shares 39% amino acid identity and 62% conservative substitution. Furthermore, VEGF and PlGF contain 8 cysteine residues in homologous positions, occur as dimeric proteins, and are therefore likely to have similar tertiary structures (Maglione et al. 1991). VEGF and PlGF have been found to occur together as heterodimers in vivo (DiSalvo et al. 1995). No other closely related homologs of the two proteins have yet been reported.

While attempting to identify candidate genes for multiple endocrine neoplasia type 1 (MEN1), which maps to chromosomal region 11q13 (Lars-

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son et al. 1988), we isolated a panel of cDNAs using cosmid cCLGW4 (D11S750) known to map to this region (Larsson et al. 1992). This cosmid was found to contain two previously described genes, *PLCB3* (Weber et al. 1994) and *FKBP2* (Grimmond et al. 1995), as well as novel genes (Lagercrantz et al. 1995a,b). Here we describe the cloning and characterization of a differentially spliced gene from the D11S750 locus, encoding a protein that we have designated VRF (VEGF-related factor), that has striking sequence homology with VEGF.

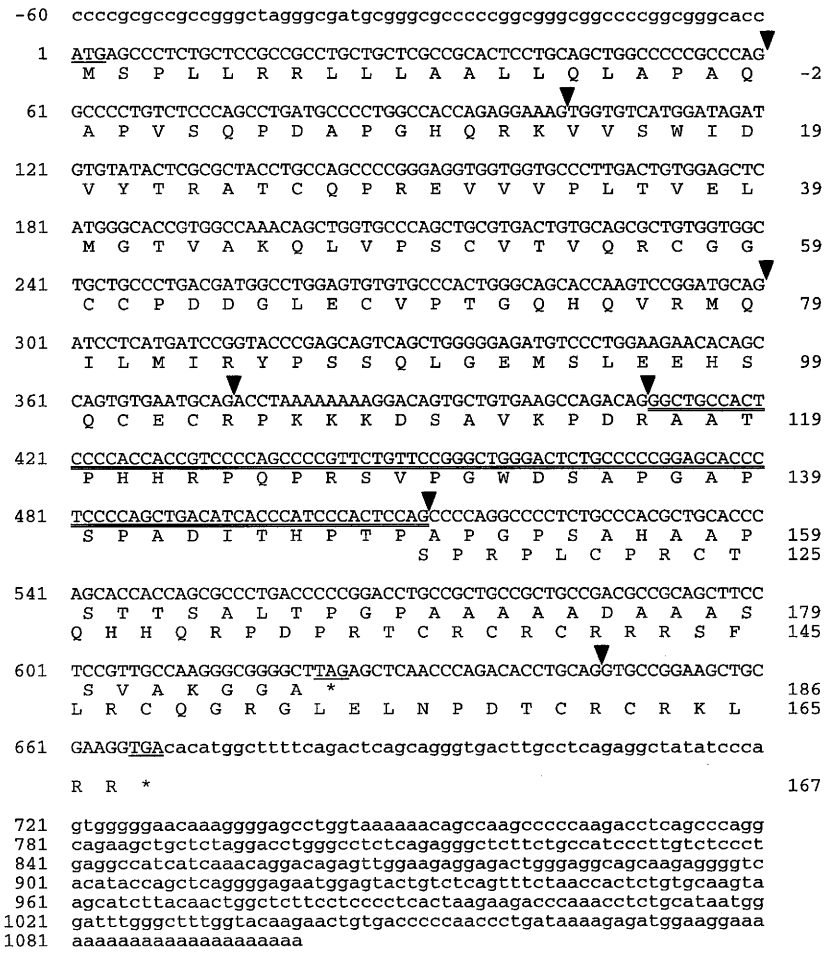

Figure 1 Nucleotide and predicted peptide sequences of VRF derived from the cDNA clone pSOM175 (nucleotides –2 to poly(A) tract) or genomic DNA (nucleotides –60 to –3 inclusive). The numbering of nucleotides is given at *left* starting from the A of the initiation codon. Amino acids are numbered at *right*, starting from the first residue of the predicted mature protein after the putative signal peptide has been removed. The alternately spliced region is double underlined, and the resulting peptide sequence from each mRNA is included. Start and stop codons are underlined. The positions of intron/exon boundaries are indicated by inverted arrowheads.

RESULTS

Cloning of *VRF* cDNAs

The original *VRF* cDNA, termed pSOM175, was isolated by screening a human fetal brain library (Stratagene) with the cosmid D11S750 (Larsson et al. 1992). cDNA library screening with pSOM175 recovered several partial but overlapping cDNAs for *VRF*. A composite sequence of the entire coding region was determined and found to consist of a 621-bp open reading frame (ORF), 412 bp of 3′ untranslated region (UTR), and 2 bp of 5′ UTR (Fig. 1, GenBank accession no. U43368). Attempts to isolate cDNAs with longer 5′ UTRs were unsuccessful; therefore, the corresponding region from genomic DNA was sought. An 850-bp *Pst*I restriction fragment from cosmid cCLGW4 (D11S750) (Larsson et al. 1992), which contained exon 1 and an undetermined amount of the 5′ UTR, was cloned and partially sequenced, from which ~60 bp of the 5′ UTR immediately upstream of the initiation codon was determined (GenBank accession no. U43370). To confirm that the sequences upstream of the ATG were 5′ UTR, an ExoIII deletion subclone of this region (corresponding approximately to nucleotide positions –250 to –750 with respect to the initiation codon) was used to screen Northern blots. Bands corresponding in size to messages obtained with *VRF* cDNA probes were observed (data not shown).

The putative start codon (Fig. 1) matches the vertebrate consensus sequence [(GCC)GCC{A/G}CCATGG] described by Kozak (1987). Furthermore, based on *VRF*'s homology to *VEGF* (see below), this ATG is likely to represent the genuine translation start site. However, in-phase stop codons were not identified upstream of this site. An out-of-frame ATG is located at position –37 but is not part of a Kozak consensus sequence.

The 3′ end of the cDNA contained a long poly(A) tail that was not preceded by a canonical polyadenylation signal (AATAAA) (Birnstiel et al. 1985); a related sequence, GATAAA, is ~18 nucleotides upstream of the poly(A) tail (Fig. 1).

A second isoform of VRF [designated VRF_{167} in keeping with the nomenclature for VEGF (Tischer et al. 1991; Houck et al. 1991) whereby the isoforms are identified by the amino acid lengths of the mature proteins once the signal peptides have been cleaved from the NH_2-termini] was identified after sequencing PCR products generated from human fetal brain cDNA lysates and RT–PCR products from a renal cell carcinoma. VRF_{167} (GenBank accession no. U43369) differs from VRF_{186} as a result of a 101-bp deletion between positions + 411– + 511 (inclusive) in the cDNA encoding the latter (Fig. 1). This not only deletes ~33 amino acids from within VRF_{186} but also results in a different carboxy-terminal peptide sequence through the introduction of a frameshift within the ORF that terminates at a new site downstream of the stop codon utilized in generating VRF_{186}.

Comparison of VRF Isoforms to the VEGF Family

The nucleotide sequences and predicted translation products of *VRF* cDNAs (Fig. 1) were compared against peptide and nucleotide data bases with BLAST. Four expressed sequenced tags (ESTs) (GenBank accession nos. H28025, H39505, R56770, T08411) were identified as having regions of identity with *VRF*. Significant homology was observed with *VEGF* and other gene family members. Nucleotide alignment of the respective cDNAs revealed regions of sequence identity on the order of 59% (124/212 bp). The amino acid homology between VRF_{186} and $VEGF_{189}$ was 32% identity and 49% similarity over the entire peptide. However, it was notable that no similarity was observed over the carboxy-terminal quarter of the proteins. Sequence alignments of the VRF_{167} isoform showed greater overall similarity to members of the VEGF gene family than VRF_{186}. Peptide homology comparisons revealed 48% identity and 69% similarity between VEGF and VRF_{167}, respectively. This increase in VEGF homology relative to VRF_{186} was attributable to additional conservation of several distinct regions located toward the carboxyl-terminus of the protein (Fig. 2).

The predicted peptide lengths for the two VRF isoforms (Fig. 1) and the four isoforms of VEGF (Houck et al. 1991) are similar, and a region homologous to the signal peptide at the amino terminus of VEGF (von Heijne 1986; see Fig. 2) is also present in both VRF isoforms. The nomenclature of the VRF isoforms has been derived assuming that the signal peptide is cleaved from the preprotein in the same place as VEGF (Keck et al. 1989; Leung et al. 1989), that is, after alanine 21 in VRF or alanine 26 of VEGF (Fig. 2). Cysteine residues were found to be highly conserved between VRF_{167} and other members of the VEGF gene family. Both VRF isoforms contained the 8 cysteines maintained among VEGF, PlGF, and the PDGFs, but an additional 8 cysteine residues were conserved among VRF_{167}, $VEGF_{189}$, and PlGF, all of which were located within the divergent carboxy-terminal end of VRF_{167}. The striking conservation of number and position of these residues suggests that these three proteins are likely to have very similar tertiary structures.

Several peptide regions within VEGF that are believed to be associated with protein dimerization are maintained between VEGF and both VRF isoforms. The strongest areas of homology include regions located in the mature protein after amino acids 49–71 (PSCVxxxRCGGCCxDxGLECVPT) and 101–107 (CECRPKK) of VRF. In addition, VRF_{167} also displays homology to VEGF at the extreme carboxy-terminal end (TCRCxKxRR; amino acids 159–167).

```
                                                  ↓
VRF167    -21 MSPLLRRLL..LAALLQLAPA...QAPVSQPDAPGHQRKVVSWIDVYTRA 24
VEGF189   -26 MNFLLSWVHWSLALLLYLHHAKWSQAAPMAEGGGQNHHEVVKFMDVYQRS 24

VRF167     25 TCQPREVVVPLTVELMGTVAKQLVPSCVTVQRCGGCCPDDGLECVPTGQH 74
VEGF189    25 YCHPIETLVDIFQEYPDEIEYIFKPSCVPLMRCGGCCNDEGLECVPTEES 74

VRF167     75 QVRMQILMIR.YPSSQLGEMSLEEHSQCECRPKKKDSAVKPDSPRP.... 119
VEGF189    75 NITMQIMRIKPHQGQHIGEMSFLQHNKCECRPKKDRARQEKKSVRGKGKG 124

VRF167    120 ..............LCPRCTQHHQR...PDPRTCRCRCRRRSFLRCQGR 151
VEGF189   125 QKRKRKKSRYKSWSVPCGPCSERRKHLFVQDPQTCKCSCKNTDS.RCKAR 173

VRF167    152 GLELNPDTCRCRKLRR 167
VEGF189   174 QLELNERTCRCDKPRR 189
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Figure 2 Homology comparison between VRF_{167} and $VEGF_{189}$ peptide sequences. The arrow marks the signal peptide cleavage site of VEGF. Identical amino acids are indicated by vertical bars and conservative substitutions by colons. The numbering of amino acids is as described in the legend to Fig. 1.

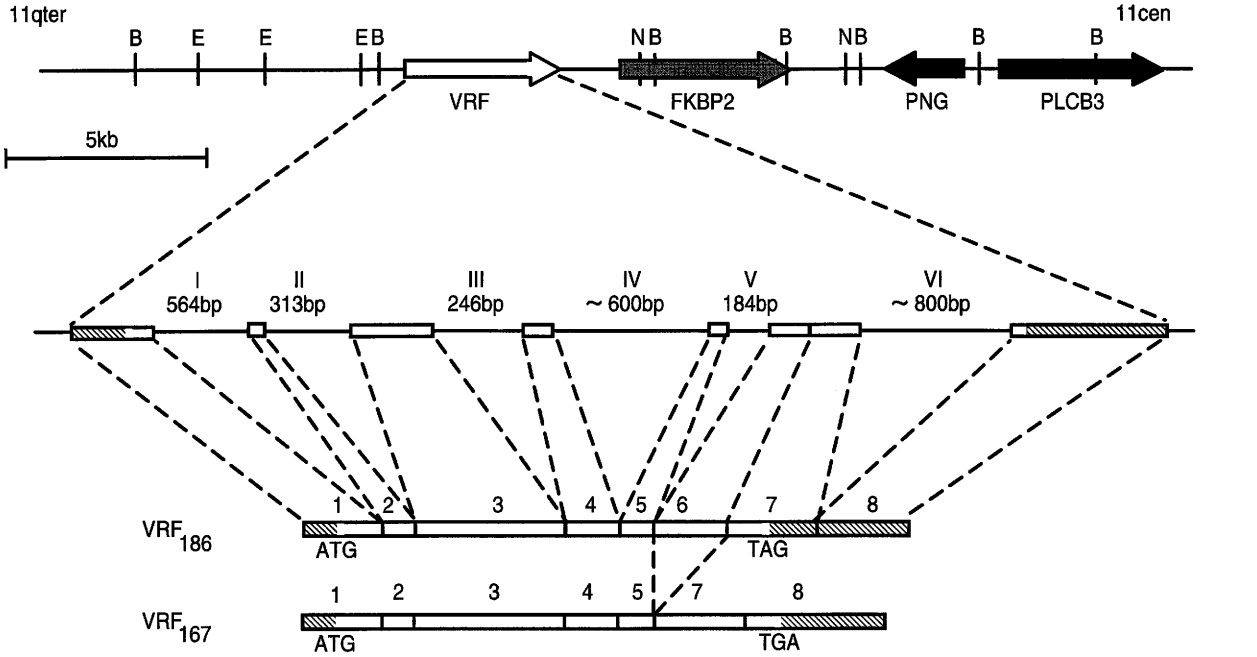

Figure 3 Genomic restriction map (B, E, and N represent restriction sites *Bam*HI, *Eco*RI, and *Not*I, respectively) and intron/exon structure of the *VRF* gene together with its orientation relative to other genes within cosmid cCLGW4 (D11S750). Sizes of introns and alternatively spliced RNAs are indicated.

The putative heparin binding clusters located at positions 121–135 of mature VEGF (Leung et al. 1989) are not conserved within the VRF isoforms. However, a noncontiguous clustering of basic residues located at the far carboxyl terminus of the $VEGF_{121}$ peptide, which is believed to account for its heparin binding ability (Cohen et al. 1995), is present in VRF_{167}.

Characterization of the *VRF* Gene

We have shown above that the *VRF* gene is alternately spliced to yield two major mRNA and protein isoforms. From establishing the intron/exon structure of the protein coding region of this gene (Fig. 3; Table 1) we have found that the VRF_{167} isoform is generated by the removal of exon 6 from pre-mRNA prior to translation (Fig. 3). The hypothesis that VRF_{167} (pSOM175-6) was derived by alternate splicing of *VRF* and not another closely related gene was further confirmed by hybridizing a *VRF* cDNA to Southern blots of human genomic DNA. As the genomic region of the *VRF* gene had been restriction mapped previously (Fig. 3), genomic DNA was digested with restriction enzymes (*Eco*RI, *Bam*HI) that were known not to cut within *VRF*, hybridized with pSOM175-6, and revealed a single band of the expected size. The *VRF* gene was also mapped against a human–hamster hybrid panel, confirming single-gene copy number and localization to 11q13 (data not shown).

The strong conservation of exon/intron organization between members of the VEGF family (Houck et al. 1991; Tis-

Table 1. Intron/Exon Boundaries of the Human *VRF* Gene

5'UTR.....	Exon 1 (Xbp*)	GCCCAG	gtacgtgcgg	Intron I (564bp)
tctcccacag	GCCCCT Exon 2 (43bp)	GGAAAG	gtaatactta	Intron II (313bp)
ctgctcccag	TGGTGT Exon 3 (197bp)	ATGCAG	gtcctgggca	Intron III (246bp)
ctgagcacag	ATCCTC Exon 4 (74bp)	ATGCAG	gtgccagcca	Intron IV (~600bp)
tactttccag	ACCTAA Exon 5 (36bp)	AGACAG	gtgagtcttt	Intron V (184bp)
tcctccctag	GGCTGC Exon 6 (101bp)			(No intron)
CCCACTCCAG	CCCCAG Exon 7 (135bp)	CTGCAG	gtgaggcgtc	Intron VI (~800bp)
ccctcctcag	GTGCCG Exon 8 (431bp)	GGAAGG		

Upper- and lowercase letters denote exonic and intronic sequences, respectively.
*The 5′ end of exon 1 has not yet been determined.

cher et al. 1991) was similarly extended to the genomic structure of *VRF*. In nearly every case, the exon/intron boundaries (Table 1) were found to be in the same location as the *VEGF* gene. The exception was exon 6 of *VRF*, which was contiguous with exon 7 (i.e., no intervening sequence but conservation of the exon/intron boundary position). This suggests that exon 6 in the *VRF* gene is derived from a partially retained intron.

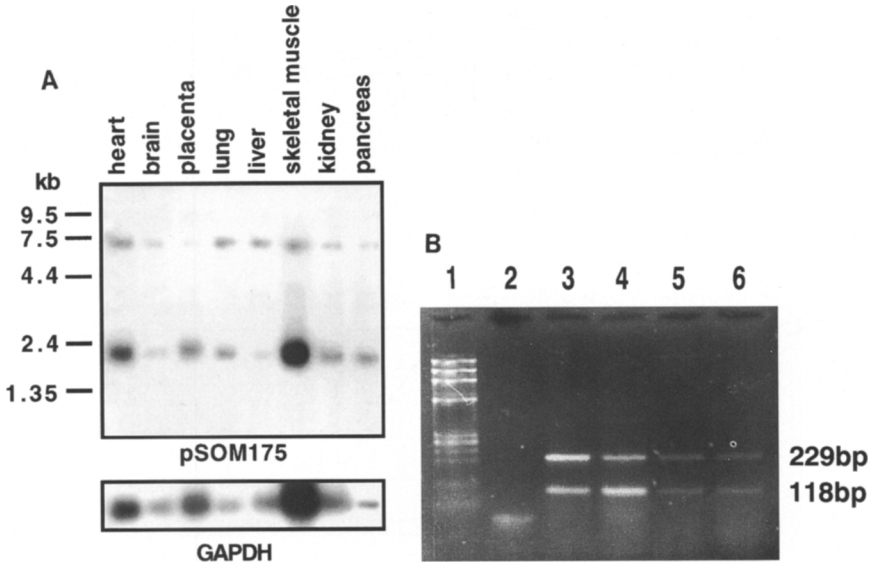

Figure 4 (*A*) Autoradiograph of multiple tissue Northern blot hybridized with the *VRF* cDNA clone pSOM175. Size markers are indicated in kilobases at *left*. Two transcripts of 5.5 and 2.0 kb were detected in all samples. Results of control hybridization of the same blots using GAPDH cDNA are included in the lower panel. (*B*) RT–PCR of alternative splice forms of *VRF* in normal human tissue mRNAs. (Lane *1*) Size markers (ΦX174 DNA cut with *Hae*III); (lane *2*) negative control; (lane *3*) normal kidney; (lane *4*) normal lung; (lane *5*) normal pancreas; (lane *6*) normal colon.

Orientation of the *VRF* Gene

The location and orientation of the human *VRF* gene (Fig. 3) within cosmid cCLGW4 (the D11S750 locus), which maps to chromosome 11q13 (Larsson et al. 1992), was determined by PCR between primers from either end of the *VRF* cDNA and a primer located within the 5′ end of *FKBP2*. Only an exon 7-specific *VRF* primer and a primer within the 5′ UTR of *FKBP2* gave a specific amplification product using both genomic DNA and cCLGW4 as template. Direct sequencing of the termini confirmed the specificity of this product (data not shown).

Expression Studies of *VRF*

Northern blot analysis of a total of 20 normal human tissues as well as cultured fibroblasts and lymphoblastoid cell lines revealed that *VRF* was expressed in all samples studied, with no obvious predominance in any tissue after normalization with GAPDH (Fig. 4A). Two bands of 5.5 and 2.0 kb were visible in all samples assayed. We assessed *VRF* expression in normal endocrine tissues, an insulinoma, and a medullary thyroid carcinoma. *VRF* was expressed in all samples, although the level in both tumors was reduced by 50% which corresponded to the loss of one chromosome 11 allele (Weber et al. 1994). Because VEGF has been shown previously to be overexpressed in highly malignant tumors (Plate et al. 1992) we assayed levels of *VRF* mRNA in a panel of 11 glioblastomas, 13 metastasizing and 12 nonmetastasizing breast carcinomas, and 34 renal cell carcinomas. Compared with their normal counterparts or nonmalignant cell lines, elevated transcription was not found in any of the tumors.

As it was not possible to differentiate between the alternately spliced VRF_{186} and VRF_{167} mRNAs by Northern analysis owing to the small size difference (101 nucleotides), RT–PCR was performed to confirm further the presence of both messages in normal and tumor tissues. A region corresponding to the carboxy-terminal end of the ORF (nucleotide positions + 362– + 590; see Fig. 1) was amplified from a panel of matched human normal tissue/tumor mRNAs with two major products being identified (Fig. 4B). Direct sequencing of these products confirmed that they represented the two different VRF isoforms.

DISCUSSION

We have cloned and characterized a new member of the VEGF gene family, which we have designated VRF. The strong homology between VEGF, PlGF, and VRF reflects conservation of structural

motifs important for peptide function (i.e., homo/heterodimerization and heparin binding).

As yet, the various roles of VRF in vivo remain to be elucidated. We have shown here that VRF possesses strong homolgy to several angiogenic factors, and investigations into its effect on endothelial cell function are ongoing. Furthermore, its ubiquitous expression pattern suggests that its role may extend beyond the endothelium. In light of the recent report that VEGF and PlGF form heterodimers in vivo (DiSalvo et al. 1995), it is possible that VRF may also interact with one or both of these factors in a similar fashion. As VRF proteins have divergent carboxy-terminal ends, with the longer isoform lacking some of the motifs involved in VEGF stability and function, it is tempting to speculate that this isoform could act as an antagonist/regulator of the shorter isoform.

Recent studies of VEGF function have reported the importance of heparin binding that is involved in dimerization and transport and assists in binding of the protein to some receptors such as *flt1* (Gengrinovitch et al. 1995). One of the major heparin binding domains (basic cluster of residues at position 121–135; see Fig. 2) of VEGF (Leung et al. 1989; Ferrara et al. 1992) is absent from both VRF isoforms. However, VRF_{167} may still be capable of heparin binding through a region of basic amino acids at its carboxyl terminus, provided the tertiary structure of the protein allows the clustering of these noncontiguous residues.

The strong sequence homology between VEGF, PlGF, and VRF reflect conservation of genomic structure between their genes with a similar number of exons, near identical intron/exon borders, and the existence of alternately spliced mRNA, particularly involving exon 6. One significant difference between *VRF* and the other *VEGF* gene family members is that the alternately spliced messages of *VRF* reported here give rise to proteins with different carboxyl termini. We show that this phenomenon arises through the retention or deletion of exon 6. Retention of intervening sequences in mRNA has been documented as a post-translational regulatory mechanism in several genes including P-transposase in *Drosophila* (for review, see Maniatis 1991) and bovine growth hormone pre-mRNA (Dirksen et al. 1995). The retention of an intron that results in a frameshift and different carboxyl termini is an uncommon phenomenon but has been reported recently for the β1-adrenergic receptor in the turkey (Wang and Ross 1995). In the case of β-adrenergic receptor, intronic retention gives rise to two receptor types and is involved in providing tissue specificity. The mechanisms that control intron retention in pre-mRNAs have been studied for some genes and involve specific splicing repressor factors (for review, see Maniatis 1991). Thus, studies to determine the possible role of such factors in regulation of the *VRF* gene appear warranted.

While the elucidation of all the possible roles of VRF continues, it is tempting to speculate that the two VRF protein isoforms act in an antagonistic or self-regulatory manner, similar to that reported for the turkey β-adrenergic receptor isoforms (Wang and Ross 1995).

The genomic localization of *VRF* at D11S750 places it within a 900-kb region known to contain the *MEN1* gene (Weber et al. 1994). In a large panel of tumors of endocrine and nonendocrine origin, a reduction in expression of *VRF* was only observed in those endocrine tumors known to be hemizygous for chromosome 11q, suggesting this was a gene dosage effect. Although *VRF* has not yet been excluded as a *MEN1* candidate by mutation analysis, its putative role as a growth factor makes it an unlikely candidate for the *MEN1* tumor suppressor gene.

METHODS

cDNA Cloning Sequencing, and Analysis

Screening of a human fetal brain library (Stratagene) with the cosmid D11S750 (Larsson et al. 1992) was performed as described (Viskochil et al. 1992). The 1.1-kb insert of SOM175 was used as a probe to isolate other cDNAs from a human fetal spleen library (Stratagene). The isolated cDNAs were sequenced on both strands using standard manual sequencing and automated sequencing protocols (PRISM, Applied Biosystems, Inc., model 373A). Oligonucleotides, nested deletions (Erase-a-base, Promega), and specific cDNA subclones were generated to complete total cDNA sequences. PCR products generated from the cDNAs were first purified from agarose gels (Qiagex gel purification columns, Qiagen) and then sequenced. Sequences were compared with the current GenBank data base at the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Altschul et al. 1990). Peptide homology alignments were performed using the program BESTFIT (GCG Wisconsin).

Northern Blot Analysis

Multiple tissue Northern blots (Clontech) containing poly(A)$^+$ RNA from heart, brain, placenta, lung, liver, skel-

etal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes were used to determine expression of *VRF* in normal human tissues. Northern filters from renal cell carcinomas and breast carcinomas were kindly provided from Drs. Ulf Bergerheim, Moraima Zelada, and Esther Schmidt. The extraction of poly(A)$^+$ RNA from normal adrenal, pancreas, thyroid, parathyroid, kidney, fibroblasts, lymphoblastoid cell lines, and endocrine tumors, the preparation of blots, and the hybridization conditions with cDNA probes were performed as described (Weber et al. 1994).

RT–PCR

Total RNA was isolated from a panel of human tumors and matching normal tissues (colon, lung, liver, kidney, pancreas), and cDNA synthesis reactions were carried out using 5 μg of RNA, random hexamers, and AMV reverse transcriptase (Promega) following methods recommended by the manufacturer. Five hundred nanograms of reverse-transcribed cDNA mixture (1 μl) was used in a PCR reaction to detect possible alternately spliced messages. Alternatively, 1 μl of high titer (>10^9 PFU/ml) cDNA library lysate was used as a template. The primers were 362F (5′-AGTGTGAATGCAGACCT-3′) and 590R (5′-GCGTCGGCAGCGGCAGCGG-3′). PCR products were visualized after electrophoresis through high percentage (3%) agarose gels stained with ethidium bromide. Alternately spliced products were confirmed by direct sequencing as described above.

Genomic Sequencing and Intron/Exon Mapping of the *VRF* Gene

Cosmid cCLGW4 (Larsson et al. 1992) was used as template and sequenced on both strands using both manual dideoxy sequencing methods and automated fluorescently labeled "dye terminator" (PRISM, Applied Biosystems, Inc.) cycle sequencing as described above, except that 2 μg of cosmid template and 20 pmoles of primer were used in each reaction. PCR products from genomic DNA were also sequenced using dye terminator cycle sequencing after purification of products from agarose gels using Qiagex gel purification columns (Qiagen). An oligonucleotide (19F, 5′-CGCCTGCTGCTCGCCGCACT-3′) was made to a region corresponding to nucleotides 19–38 with respect to the initiation codon, end-labeled with [γ-^{32}P] dATP, and hybridized to a Southern blot of a series of shotgun-cloned *Pst*I restriction fragments from cosmid cCLGW4 subcloned into pBluescript KS– (Stratagene). A single hybridizing clone with an 850 bp insert was sequenced on both strands as described above.

Intervening sequences were located by sequencing of cosmid cCLGW4 (Larsson et al. 1992) using oligonucleotide primers from the *VRF* cDNA sequence determined above. Comparison of cDNA and cosmid sequences revealed the exact location of each exon/intron boundary. The size of each intron was then determined by PCR amplification using flanking exonic primers and cCLGW4 or genomic DNA as template. Amplified products were gel purified and directly sequenced to confirm intron/exon boundaries. The intron sizes were determined either by complete sequencing of the intervening sequence or estimated by electrophoresis through high percentage agarose gels.

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