Hum Genet (1996) 98 : 666–671 

ORIGINAL INVESTIGATION

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# Isolation and characterization of the full-length 3′ untranslated region of the human von Hippel-Lindau tumor suppressor gene

Received: 10 April 1996

**Abstract** We have isolated the 3′ untranslated region (3′UTR) of the human von Hippel-Lindau (VHL) tumor suppressor gene from a P1 phage containing the entire VHL genomic sequence. Several putative noncanonical (ATTAAA) poly(A) signals were identified, and the functional significance of these signals was examined by preparing VHL mammalian expression constructs with this DNA fragment and the previously isolated partial cDNA. Northern blot analysis from transfected renal carcinoma cells showed that both the endogenous and transgene VHL transcripts were the same length. Use of VHL transgene deletion mutants indicated that an ATTAAA sequence located between nucleotide (nt) +4237 and nt +4379 most likely serves as an active poly(A) signal in renal carcinoma cells, yielding a 3.6-kb 3′UTR. This work indicates that, together with the 5′UTR and the coding region, these sequences comprise the full-length human VHL cDNA. Sequence analysis revealed a 300- to 600-bp region conserved in human, murine, and rat VHL UTRs. In addition, the human 3′UTR was extremely rich in *Alu* repetitive elements.

## Introduction

The von Hippel-Lindau (VHL) disease tumor suppressor gene was isolated several years ago using positional cloning techniques (Latif et al. 1993), and was subsequently implicated in playing a causal role in the development of a variety of inherited and spontaneous neoplastic disorders. Analysis of lymphocyte DNA from VHL families has shown mutations in approximately 60% (Chen et al. 1995; Crossey et al. 1994) of individuals tested. In addition, analysis of tumor DNA from sporadic clear cell kidney cancer patients has shown that collectively mutations and hypermethylation in the VHL gene account for over 75% of these cases (Foster et al. 1994; Gnarra et al. 1994; Herman et al. 1994; Shuin et al. 1994).

The full-length VHL transcript has a ubiquitous 4.4–5.0 kb length in all human tissues analyzed. In addition, the promoter region has been mapped and the major transcription initiation site identified (Kuzmin et al. 1995). However, only a partial cDNA has ever been obtained. This 1.62-kb cDNA (group 7) appears to include, in three exons, the entire VHL coding sequence (642 bp) and a truncated 3′ untranslated region (UTR). Extensive screening of over $6 \times 10^6$ clones from cDNA libraries prepared from both poly dT and random primed templates, using RNA from different sources, did not yield a full-length cDNA containing the entire 3′UTR. Although some clones were isolated through cDNA walking containing pieces of UTRs, none of them produced long uninterrupted sequences, and the entire UTR could not be pieced together.

The 3′UTRs of mRNA often contain signals for controlling eukaryotic gene expression by affecting polyadenylation, stability, localization, and translation of RNA transcripts (Decker and Parker 1995, for review). In addition, expansions of a trinucleotide repeat in the 3′UTR of the gene responsible for myotonic dystrophy has been shown to cause the disease (Brook et al. 1992). We decided to clone the relatively large and elusive VHL 3′UTR from a genomic fragment. Although this sequence was similarly not detected in a representative cosmid library, it was found on a P1 phage, previously isolated from a genomic library (Kuzmin et al. 1994; Latif et al. 1993). Isolation of the VHL 3′UTR sequence provides the materials necessary to search for mutations in this region.

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## Materials and methods

### Isolation of the VHL 3′UTR, and subclone construction

P1 phage 191, isolated from human genomic DNA library (Genome Systems, St. Louis, Mo.) and containing the entire VHL gene, served as a template for an extra long polymerase chain reaction (PCR) amplification of exon 3. The primers YH1A (5′-TTC CTT GTA CTG AGA CCC TAG T-3′) located in the second intron, starting 55 bp upstream of exon 3, and P1V (5′-GGC *TCT AGA* TAA TAC GAC TCA CTA TAG GG-3′) located adjacent to the cloning site in the P1 vector, and included a 5′ overhang containing a *Xbal* site for cloning (underlined), were used with rTth DNA polymerase, XL (Perkin Elmer), as directed by the manufacturer, with 5 m*M* $Mg^{+2}$, and yielded a strong 9.4-kb PCR product. We employed a PCR protocol as follows: 94° C for 1 min; 16 cycles of 94° C for 15 s; 62.5° C for 30 s, and 72° C for 7 min 30 s; 16 cycles of 94° C for 15 s, 62.5° C for 30 s, and 72° C for 7 min 30 s + 15 s/cycle; 72° C for 10 min; 4° C, hold; in a Perkin Elmer 9600 GeneAmp PCR system. The PCR product was purified, and the first *Xbal* site in the exon 3 coding region that overlaps a dam site (GATC) was methylated with DAM methylase (New England Biolabs) to silence the restriction site. The modified PCR product was then digested with *Bsp*El/*Xbal*, and the resultant 5.3-kb fragment was gel purified, electroeluted, and ligated into a *Psp*lA/*Xbal*-digested pBluescript II SK (Stratagene) and subsequently called pBX6. This insert was sequenced in both directions using AmpliTaq with an Applied Biosystems 373 automated DNA sequencer.

VHL mammalian expression vectors were constructed using a PCR3 based vector (Invitrogen) that contained the full VHL coding sequence beginning from the first in frame ATG, under the control of the CMV promoter. The bovine growth hormone poly(A) signal of the vector was removed by digestion with *BGl*III/*AFl*III, and was ligated to the *Bgl*III/*Afl*III 3′UTR containing fragment from pBX6, and termed pVHL3UTR. Unless otherwise mentioned, all standard cloning, electrophoresis, digestion, blotting and hybridization protocols were used as described (Sambrook et al. 1989).

### Cell lines and transfections

Human renal carcinoma cell line UMRC6 (Grossman et al. 1985) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco BRL, Bethesda, Md.). Transfections were performed using the cationic lipid, Lipofectamine (Gibco). A mixture of 12 μl lipofectamine/1.5 μg DNA was added to UMRC6 cells growing at 60–70% confluence in 3.5-cm plates, as suggested by the manufacturer. Geneticin (G418, Gibco BRL) selection (300 mg/ml) began 48 h after transfection. 30–50 G418 resistant colonies were pooled after 14 days of G418 selection and expanded in the presence of G418.

### RNA isolation and Northern analysis

Total RNA was isolated from cultured UMRC6 cells using RNAzol (Tel-Test), followed by purification with RNA TACK resin (Biotecx) as described by the manufacturer. A 10-μg RNA sample was run in each lane on a MOPS/formaldehyde gel, which was blotted to a nylon membrane and probed with a $^{32}$P-dCTP-labeled fragment derived from the exon 1 or exon 3 VHL coding region (as indicated in figure legends), and transcript size determination was made using a 0.24- to 9.5-kb RNA ladder (BRL).

### Mutation detection by direct DNA sequencing

The 350-bp region immediately downstream of the VHL coding region was directly sequenced with Sequence 2.0 (SUB) as directed by the manufacturer. Sequencing templates were generated using one 5′primer in the PCR, for magnetic strand separation with (Dynal, Oslo), as directed by the manufacturer. The dideoxy chain termination reaction used a nested primer, $^{35}$S-dATP, and the reactions were run on a 6% denaturing polyacrylamide gel, at 65V, dried and autoradiographed.

## Results

### Isolation and functional analysis of the VHL 3′UTR

Genomic P1 phage 191, which includes the entire VHL gene (Kuzmin et al. 1994), was used as a template for extra long PCR amplification of the entire third exon and downstream sequences. Using one primer from the 3′ region of the second intron and the other located on the P1 phage vector adjacent to the cloning site, we isolated a 9.4-kb PCR fragment, which presumably contained all of the 3′ portions of the VHL gene. This fragment was roughly mapped with several restriction enzymes, and a 5.3-kb fragment thought to be sufficiently large to contain the entire UTR was cloned into pBluescript and fully sequenced. Although the consensus polyadenylation signal AATAAA was not present in this sequence, five closely spaced noncanonical ATTAAA sequences were identified at nucleotide (nt) +3846, +4237, +4292, +4379, and +4873.

Nucleotide numbering in this communication is made in reference to the major transcription start site as nt +1, this corresponds to nt 148 in the nucleotide sequence appearing in the genebank (accession no. L15 409). The sequence of the 3′UTR beginning from the stop codon has been deposited in the genebank (accession no. *U49 746*).

To determine if any of these sequences encoded functional signals, we subcloned the putative 3′UTR into a modified PCR3 (Invitrogen) mammalian expression vector, which contained the full VHL coding region, but lacked the bovine growth hormone poly(A)signal present in the original vector. This construct, designated pVHL3UTR, now contained the full VHL coding region, putative 3′UTR and poly(A) signals, under the control of the cytomegalovirus (CMV) promoter, in addition to an SV40 controlled neomycin resistance gene (Fig. 1).

pVHL3UTR was transfected into the renal carcinoma cell line UMRC6, and after G418 selection and expansion of pooled colonies, RNA was harvested for Northern blot analysis. The UMRC6 cell line contains only one VHL allele, and it has a 10-bp deletion (Latif et al. 1993). This allele yields a mRNA with a weak 4.4-kb band. The transfected VHL gene, on the other hand, under the control of the strong CMV promoter, produced an intense band of identical size to the endogenous copy (Fig. 2 A, lanes 1 and 2).

We prepared two additional deletion mutants to demonstrate further that this transcript was derived from the exogenously introduced VHL gene construct, and to determine more precisely which of the several putative poly(A) signals was active. pVHL3UTR was digested with *Bgl*III/EcoRV (p3UBE) and *Bgl*II/Pstl (p3UBP9 to re-

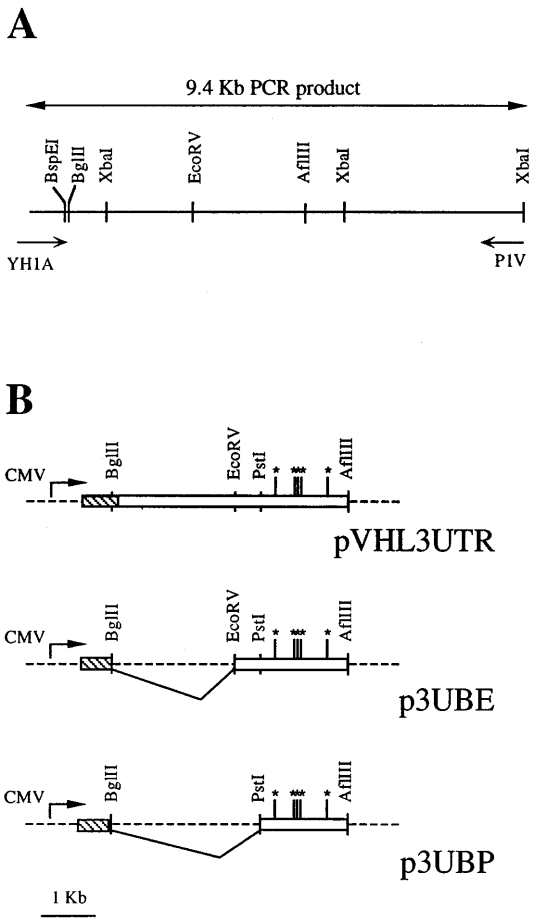


**Fig. 1 A, B** DNA fragment and construct maps. **A** Partial restriction map of the 9.4-kb PCR product derived from extra-long PCR reaction using the phage P1 191 as template. Primer YH1A sits in the 3′ end of intron 2, and primer P1V sits in the cloning site of the P1 vector. **B** von Hippel-Lindau (VHL) gene expression constructs used for transfection of UMRC6 cells, and subsequent Northern analysis. The VHL sequence begins at the first ATG, the coding region is *hatched*. Putative ATTAAA poly(A) sites are marked by an *asterisk*, deletions are indicated by a *dip* below the *dotted line*

move 2.43- and 2.96-kb fragments, respectively (Fig. 1). Depending on which of the poly(A) signals was active, pUBE could be expected to yield messages of 897, 1281, 1337, 1417, or 1917 pb, whereas pUBP could be expected to yield messages of 1420, 1804, 1860, 1940, or 2380 bp. After transfection into UMRC6 cells, G418 selection, and colony expansion, RNA was again harvested from pooled colonies, run, blotted, and probed with an exon 1 DNA fragment. The transfected p3UBE construct yielded a strong band in the 1.4-kb range, while p3UBP yielded a strong band in the 2-kb range (Fig. 2 A, lanes 2 and 3), demonstrating that a functional poly(A) signal was present, and most probably encoded by either the second, third, or fourth ATTAAA sequences. The authenticity of these signals is demonstrated by using an exon 3 probe located in the coding sequence just downstream from the *Bgl*II site (Fig. 2 B).

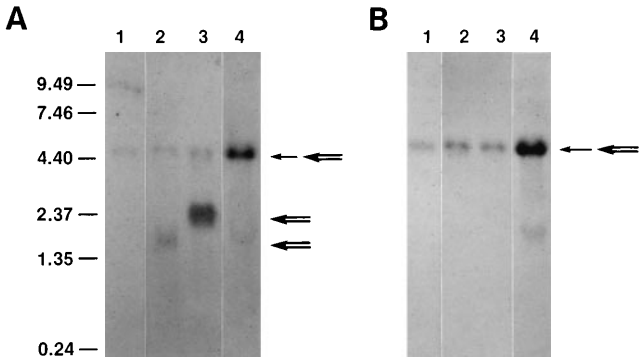


**Fig. 2 A, B** Northern blot analysis of VHL expression constructs. **A** Total RNA (10 μg) was isolated from pooled colonies transfected with different expression constructs, separated on a denaturing gel and probed with a $^{32}$P-labeled exon 1 VHL fragment. *Lane 1* nontransfected UMRC6 cells; *2* UMRC6 cells transfected with p3 UBP; *3* UMRC6 cells transfected with p3UBE; and *4* UMRC6 cells transfected with pVHL3UTR. **B** same as in **A**, but with an exon 3 probe located in the coding sequence downstream of the *Bgl*II site and absent in the deletion constructs

## Sequence analysis of the VHL 3′UTR

Approximately two-thirds of eukaryotic genes contain GT- or T-rich elements immediately downstream of poly(A) sites; these are thought to modulate the efficiency of poly(A) signal usage (Wahle and Keller 1992). The three possible poly(A) signals, identified here that would yield transcripts of appropriate length, all contain GT-rich sequences slightly downstream from each of the ATTAAA sites, again suggesting that any or all three of these sites could be functional in 3′ end formation. The second poly(A) site at nt +4237 is followed at a distance of +20 nt by the sequence GTTTGTTTTCATTT, the third site at nt +4292 is followed at a distance of +40 nt by the sequence GTTTTTTTTGTTTTGTTTTGTTTTT, and the fourth poly(A) site is followed at a distance of +19 nt by the sequence GGGTGTTGTGTGGTGTTTT. Another 3′UTR control element with a consensus sequence in this region is the adenylation control element (ACE), or cytoplasmic polyadenylation element (CPE, in *Xenopus*), TTTTTAT or TTTTAAT (Wormington 1993, for review). The latter variation is found at nt +4367, just upstream of the fourth ATTAAA sequence. In addition, the ACE/CPE consensus sequence also appears in the beginning of the 3′UTR at transcript nt +1066, +1366, +1935, and downstream from the fourth ATTAAA poly(A) site at nt +4550.

We searched the available DNA sequence databases for sequences with similarities to the VHL 3′UTR using the Wisconsin Genetics Computer Group program FASTA (Wisconsin Package, Version 8, September, 1994) and BLAST (Altschul et al. 1990). The most striking observations we made were an approximately 300-bp region conserved in the human, mouse, and rat VHL 3′UTRs, and a preponderance of *Alu* repeats distributed throughout the UTR. Thirty bases downstream from the coding region begins a 319-bp region, which shares a 68% identity with the rat VHL 3′UTR from rat nt 694–nt 997 (accession no.

U 14 746; Duan et al. 1995). The same region shares a 335-bp region with the mouse VHL 3′UTR, which is 66.6% identical from mouse nt 713–nt 1032 (accession no. S76 748; Gao et al. 1995). Together these three sequences share a 313-bp overlap, which is 60% identical. An analysis using the Wisconsin GCG program BESTFIT of the human and rat 3′UTRs shows that this region of similarity extends another 320 bp, for a total of 638 bp beginning (human VHL sequence +737–1542 including a 73-bp gap) with a total identity of 68%. Although there is strong similarity between the mouse and rat sequences in this region, the shared similarity of all three sequences drops to 50%. The only other area of similarity is a 30-nt stretch from the rat poly(A) signal, with 80% identity to the human sequence, centered over the fourth ATTAAA site.

The degree of conservation seen in all three species suggested the possibility that this region may play a regulatory role, and we decided to examine VHL families in which no mutation had been detected in the known sequence. Thirteen families were selected that have been clinically well characterized, and previously examined for VHL mutations by Southern blot and single-strand conformation polymorphism (SSCP) analysis of the first two exons and the coding region of the third (including splice junctions). Direct sequencing was performed on the first 300-bp region, which was the most highly conserved in all three species (nt +737–1051) in the 3′UTR, on these 13 families, as well as on six additional normal individuals from the Centre d'Etude du Polymorphisme Humain (CEPH, Paris). Aside from a previously reported polymorphism at nt +1001 (Payne et al. 1994; nt 1149 according to the sequence numbers quoted therein), only one base change was detected, a C-T transition at nt +833 just outside of the conserved area in a nonaffected CEPH individual (data not shown). This new polymorphism has yet to be characterized in the general population. No VHL putative germline mutations in the examined individuals were detected in this region.

Searching the databases for other homologous sequences yielded many matches with *Alu* repeat elements. To precisely map the number and location of these elements we utilized the GCG program BESTFIT and the Blur8 *Alu* repeat, which is a commonly used fragment in *Alu* detection hybridization experiments (accession no. J00 091; Rubin et al. 1980). Eight *Alu* repeat sequences were identified within the VHL 3′UTR and three more within the region 2 kb immediately downstream from the probable poly(A) signals (Fig. 3). Of the 11 repeats, were homologous to the Blur8 *Alu* sequence, all of which had an identity of greater than 81%. One *Alu* sequence identified by the FASTA search was not detected by homology to the Blur8 repeat, and most probably represents another *Alu* subfamily. Seven of the *Alu* repeats have regions of homology greater than 250 bp, while the remaining four are truncated sequences ranging from 124 bp to 190 bp. The first, fifth, and tenth repeats lie in the reverse orientation, such that the fourth and fifth *Alu* sequences are arranged head to head, and can yield an almost perfect 700-bp stem and loop tertiary structure.

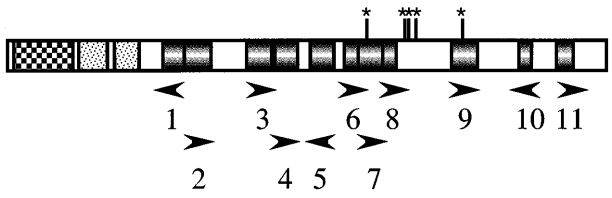


**Fig. 3** Summary of the VHL coding, 3′-untranslated, and downstream regions. The diagram begins as the major transcription start site, the coding region is *checkered*, *triangles* above the region mark splice junctions. The conserved human-mouse-rat regions of the 3′UTR are *stippled*. *Asterisks* mark the five putative ATTAAA poly(A) signals. The *Alu* repeat sequences, designated by gradients, are arbitrarily numbered 1-11, the *arrowheads* indicate orientations, only the second *Alu* repeat is not part of the Blur8 family. *Alu* 1, nt +1646–1898; *Alu* 2, nt +1882–2158; *Alu* 3, nt +2548–28099; *Alu* 4, nt +2830–3098; *Alu* 5, nt +3217–3480; *Alu* 6, nt +3598–3772; *Alu* 7, nt +3732–3999; *Alu* 8, nt +3960–4151; *Alu* 9, nt +4750–5015; *Alu* 10, nt +5475–5599; and *Alu* 11, nt +5876–6022

## Discussion

We have isolated the VHL 3′UTR from genomic DNA sequences carried on a P1 phage isolate previously shown to contain the entire VHL transcribed region (Kuzmin et al. 1994; Latif et al. 1993). The full VHL 3′UTR sequence had appeared 'unclonable' from lambda cDNA or cosmid libraries, but the strategy employed here allowed for the stable propagation of full-length clones. We constructed a VHL minigene composed of the coding region and 3′UTR, by ligating this isolated region with the previously cloned (group 7) VHL partial cDNA containing the VHL coding sequence, on an expression vector lacking an endogenous poly(A) signal. When this construct was transfected into cultured renal carcinoma cells, it yielded transcripts of identical size to the endogenous VHL message, indicating that all the signals necessary for proper 3′ end formation and polyadenylation were contained on the isolated DNA fragment.

Although sequencing of the entire fragment did not reveal the highly conserved AATAAA poly(A) signal, a variant, ATTAAA, was identified at five closely spaced locations. This sequence has been shown to act as an efficient substrate for polyadenylation, and it appears as a natural poly(A) signal in 12% of vertebrate mRNAs (Wickens 1990). By examining Northern blot analysis of transfected VHL deletion mutants, we were able to exclude the first and fifth ATTAAA sequences as being the probable poly(A) signals. However, the second, third, and fourth ATTAAA sequences identified in the VHL 3′UTR lay within a 150-bp region, and each of which could conceivably generate the 4.4- to 4.6-kb transcript observed here. In addition, each of these hexamers is followed by GT- or T-rich sequences, elements believed to modulate the efficiency of poly(A) sites (Guo and Sherman 1995). Although any of these three ATTAAA sites may be responsible for in vivo poly(A) activity, the fourth site aligned to a 30-bp stretch centered over the AATAAA poly(A) site of the rat VHL 3′UTR in a BESTFIT analysis, and represents the most probable site.

While the sequence AATAAA (or in the case of VHL, ATTAAA) plays a role in nuclear polyadenylation and cleavage, the sequences TTTTTAT or TTTTAAT often play metabolic roles as signals in cytoplasmic deadenylation and readenylation. This 3′UTR control element, called the CPE, or ACE, has been shown to effect changes in the length of certain maternal transcript poly(A) tails during development, which has in turn, been correlated with changes in the translational activity of these mRNAs. Among the messages shown to follow this mode of translational control are c-mos, cyclins A1, B1, and B2 and cdk2 (Vassalli and Stutz 1995). Although the VHL mRNA has been shown to be ubiquitously expressed in all human tissues (Latif et al. 1993), as a regulator of cell growth it would be expected to be tightly regulated. The appearance of CPE/ACE consensus sequences in the 3′UTR suggest this as a possible mechanism.

The human, rat, and mouse VHL gene coding sequences are highly conserved in the large majority of the coding region. However the human 3′UTR, which is 3.6 kb, is significantly larger than the 2-kb mouse UTR, and the 2.1-kb rat UTR. The human 3′UTR begins with a 635-bp region that is 50–60% conserved with the rodent 3′UTR sequences, but is followed by a region with a very high concentration of *Alu* repeat sequences. These repeats appear to account for most of the difference in the transcript size. The interspecies conservation of the untranslated region following the coding sequences suggests a possible metabolic role that may affect VHL gene expression. Although this region has previously been examined by SSCP or heteroduplex analysis of both germline VHL families and somatic clear cell carcinomas without finding putative mutations (Crossey et al. 1994; Shuin et al. 1994), we directly sequenced the first 313 bp of this region (which shows a higher percentage of identity) in 13 families in which no mutation had yet been documented. One base change was detected in a nonaffected individual just outside of a conserved portion of this region indicating a new polymorphic site. No putative mutations were revealed in affected individuals. A more thorough mutational analysis of the entire untranslated region is needed to help clarify this point.

Interspersed repetitive sequences have traditionally been dismissed as nonfunctional; however, more evidence is accumulating that suggests that these sequences can play a role in primate gene expression and neoplastic transformation. *Alu* repeat sequences are estimated to account for 5% of human genomic DNA, and are found in 5% of fully spliced cDNAs, usually in the 3′UTR (Yulug et al. 1995). The region of the VHL gene that includes the 3′UTR contains 11 *Alu* repeat elements in a 4.5-kb DNA segment. This may partially explain the difficulties encountered while screening for full-length VHL cDNAs and in cDNA walking. Recently, a nuclear protein was purified that specifically binds to *Alu* DNA repeats (Chesnokov and Schmid 1995), and a major *Alu* subfamily consensus sequence was shown to contain a functional retinoic acid response element (Vansant and Reynolds 1995). The Wilms' tumor gene, WT1, was found to be regulated by a 460-bp transcriptional silencer that contains a full-length *Alu* repeat (Hewitt et al. 1995), and an *Alu*-mediated recombination event has been implicated in a founding mutation in the MLH1 gene of hereditary colon cancer (Nystrom-Lahti et al. 1995). At present no evidence exists that suggests a VHL-related functional role for these repeats. However, the high concentration of *Alu* sequences identified in this region (1 per 400 bp), may indicate a mechanism for different types of deletions, duplications, and inversions based on *Alu*-mediated recombination, both in germline and somatic tissues.

**Acknowledgements** We would like to thank Dr. L. Schmidt, Dr. F. Chen, L. Geil, and M.L. Orcutt for technical advice and assistance throughout this project. We acknowledge the National Cancer Institute for allocation of computing time and staff support at the Frederick Biomedical Supercomputer Center of the Frederick Cancer Research and Development Center. BLAST searching was done at NCBI using the BLAST network service. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products or organizations imply endorsement by the U.S. Government.

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