

ORIGINAL ARTICLE

Genetic and functional analysis of the von Hippel-Lindau (*VHL*) tumour suppressor gene promoter

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J Med Genet 2002;**39**:463–472

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Revised version received
20 March 2002

Accepted for publication
21 March 2002

The *VHL* gatekeeper tumour suppressor gene is inactivated in the familial cancer syndrome von Hippel-Lindau disease and in most sporadic clear cell renal cell carcinomas. Recently the *VHL* gene product has been identified as a specific component of a SCF-like complex, which regulates proteolytic degradation of the hypoxia inducible transcription factors HIF-1 and HIF-2. pVHL is critical for normal development and mRNA expression studies suggest a role in nephrogenesis. Despite the importance of *VHL* in oncogenesis and development, little is known about the regulation of *VHL* expression. To investigate *VHL* promoter activity, we performed comparative sequence analysis of human, primate, and rodent 5' *VHL* sequences. We then proceeded to deletion analysis of regions showing significant evolutionary conservation between human and rat promoter sequences, and defined two positive and one negative regulatory regions. Analysis of specific putative transcription factor binding sites identified a functional Sp1 site, which was shown to be a regulatory element. Overlapping Sp1/AP2 sites were also identified and candidate E2F1 binding sites evaluated. Three binding sites for as yet unidentified transcription factors were mapped also. These investigations provide a basis for elucidating the regulation of *VHL* expression in development, the molecular pathology of epigenetic silencing of *VHL* in tumorigenesis, and suggest a possible link between Sp1, *VHL*, and nephrogenesis.

The isolation of the genes for rare familial cancer syndromes has often provided insights into the molecular pathology of sporadic cancers, and identified gatekeeper genes with a critical role in development. This is exemplified by the von Hippel-Lindau disease (*VHL*) tumour suppressor gene (TSG).¹ *VHL* disease is characterised by susceptibility to multiple tumour types including retinal and cerebellar haemangioblastoma, renal cell carcinoma (RCC), and pheochromocytoma.^{1–3} In addition, inactivation of the *VHL* TSG by somatic loss, mutation, and methylation occurs in the majority of sporadic clear cell RCC (the most common form of adult kidney cancer) and sporadic haemangioblastomas.^{4–7} The *VHL* TSG mRNA and protein are widely expressed during human and murine development.^{8–10} Further evidence implicating *VHL* in normal development is the observation that homozygous *vhl* mouse knockouts die in utero at 10.5 to 12.5 days of gestation.¹¹ Histopathological examination of the *vhl* ^{-/-} embryos suggested that death results from a failure of placental vasculogenesis.

The *VHL* TSG product is likely to have multiple and tissue specific functions.¹ However, a notable feature of *VHL* associated tumours is hypervascularity and expression of high levels of vascular endothelial growth factor (VEGF) and other hypoxia inducible mRNAs.^{12–15} Recently, compelling evidence has emerged to suggest that pVHL forms part of a multimeric complex with elongins B and C, CUL2, and RBX1 which targets specific proteins for ubiquitylation and proteosomal degradation (similar to the SCF complex in yeast).^{14–17} Furthermore, pVHL has been shown to regulate expression of the hypoxia inducible factors HIF-1 and HIF-2 (EPAS) by controlling the rate of ubiquitylation and proteolysis of the HIF-1 α and HIF-2 α subunits.^{18–20} HIF-1 and HIF-2 are heterodimeric basic helix-loop-helix transcription factors which activate a large number of hypoxia inducible target genes including *VEGF*, endothelin 1, erythropoietin, glucose transporters, and glycolytic enzymes.²¹ HIF-1 α deficient mice (homozygous null alleles) arrest development by E9.0 and die at E10.5 from cardiovascular malformations.

Promoter hypermethylation and silencing is an important mechanism of TSG inactivation in human cancer and ~15% of

sporadic RCC and tumours from *VHL* patients show de novo methylation of the *VHL* promoter.^{6, 22–23} An understanding of the regulation of *VHL* gene expression would provide a basis for investigating the role of pVHL and *VHL* target genes in human development and cell differentiation and in tumorigenesis. Previously, a minimal promoter region for the *VHL* TSG was defined and putative transcription factor binding sites in the 5' upstream region were suggested.²⁴

In the present study we have performed sequence comparisons between human, primate, and rodent 5' *VHL* sequences to identify evolutionarily conserved candidate regulatory regions. We tested their functional importance by deletion analysis in reporter assays. We also identified candidate transcription factor binding sites within the *VHL* promoter and investigated the functional significance of putative Sp1, AP2, and E2F1 sites.

MATERIALS AND METHODS

Evolutionary conservation

Ape promoter fragments were obtained from PCR amplification using three sets of primers based on the human sequence: Set 1: PF3 5'GAAATACAGTAACGAGTTGGCCTA and PR3: 5' ATACGGGCAGCAGCAGCGC; set 2: PF1 5'CCTAGGCAACAT-AGCG AGACTC and PR1 5' TAACGGAGGCGAGGCTAGG and set 3: PF2 5'ACTTTAT AAGCGTGATGATTGG and PR2 5' CAC-CATGCCCCGACTAATTA.

PCR conditions were as follows. Set 1: for chimp and gorilla, 96°C (five minutes) \times 1 (96°C (one minute), 63°C (one minute), 72°C (one minute)) \times 35, 72°C (10 minutes) \times 1; for baboon and macaque; 96°C (five minutes) \times 1 (96°C (one minute), 55°C (one minute), 72°C (one minute)) \times 40, 72°C (10 minutes) \times 1. Sets 2 and 3: for chimp and gorilla, 96°C (five minutes) \times 1 (96°C (one minute), 57°C (one minute), 72°C

Abbreviations: *VHL*, von Hippel-Lindau disease; TSG, tumour suppressor gene; RCC, renal cell carcinoma; VEGF, vascular endothelial growth factor; CR, conserved region

(one minute)) $\times 35$, 72°C (10 minutes) $\times 1$. Set 2 for baboon and macaque: 96°C (five minutes) $\times 1$ (96°C (one minute), 52°C (one minute), 72°C (one minute)) $\times 40$, 72°C (10 minutes) $\times 1$. Set 3 for baboon: 95°C (five minutes) $\times 1$ (95°C (one minute), 54°C (one minute), 72°C (one minute)) $\times 45$, 72°C (10 minutes) $\times 1$. Set 3 for macaque: 96°C (five minutes) $\times 1$ (96°C (one minute), 52°C (one minute), 72°C (one minute)) $\times 45$, 72°C (10 minutes) $\times 1$. The amplified fragments were sequenced on an ABI377 sequencer, and compared with the human *VHL* promoter sequence (Genbank AF010238), using the CLUSTALW (<http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html>), MULTALIN (<http://www.toulouse.inra.fr/multalin.html>), and Blast 2 against 1 (<http://www.ncbi.nlm.nih.gov/gorf/bl2.html>) alignment programs. In addition to this, rat (Genbank AF141022) and mouse (Genbank NM_009507) *vhl* promoter data were also included in this analysis.

VHL promoter sequence analysis

VHL promoter sequence (Genbank accession No AF010238, nucleotides 1-780) was analysed for putative transcription factor binding sites with MatInspector v2.2,²⁵ TFSEARCH v1.3,²⁶ and Yutaka Akiyama "TFSEARCH: Searching transcription factor binding sites" (<http://www.rwcp.or.jp/papia/>). The parameters allowing for comparison of a binding site with database were set as follows. MatInspector settings: matrix similarity 0.800 (maximum 1.00); TFSEARCH settings: threshold 80.0 points (maximum 100.0). To detect the second putative E2F1 binding site, the threshold was decreased to 79.0 points.

Cell lines

A 293 kidney embryonal cell line and HeLa cervical cancer cell line were grown in DMEM media supplemented with 10% and 5% fetal calf serum, respectively, with penicillin (25 000 UI) and streptomycin (25 000 μg^{-1}).

Preparation of promoter constructs for luciferase assay

Our constructs for luciferase assay were prepared by PCR amplification from the Luc1 construct.²⁴ Forward primers had a *XhoI* site incorporated at the 5' end and reverse primers had a *HindIII* site. The constructs were prepared with the following primers: construct F1 with primers p1 and p4; F2 with p1 and p5; F3 with p1 and p6; F4 with p1 and p9WT; F4M with p1 and p9MT; F5 with p1 and p7; F6 with p2 and p4; F7 with p8WT and p4; F7M with p8MT and p4; F8 with p3 and p4. PCR conditions were 95°C for five minutes (95°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds) 40 \times ; 72°C for 10 minutes. The amplified fragments were ligated into the *XhoI/HindIII* sites of a pGL2-enhancer vector (Promega) cloned in *E coli* DH5 α strain and confirmed by sequencing on an ABI 377 analyser.

Primer sequences

p1: 5' GGGCTCGAGGCCTCCGGCCGGCTATTT; p2: 5' GGGCTC-GAGCTTACCGAGCGCGCGCG; p3: 5'GGGCTCGAGACTCG-GGAGCGCGCACGC; p4: 5' GGAAGCTTTCTCCGCCCTCCG-GGGCA; p5: 5' GGAAGCTTATTCCTCCGCGATCCAG; p6: 5' GGAAGCTTGCGGGTTCGGACGCGGGGC; p7: 5' GGAAGCTTGCTCGTGCGCGCTCCG; p8WT: 5' GGGCTCGAGACCGAGCGCGCGGAAGACTAC; p8MT: 5' GGGCTCGAGACCGAGCGCTACGAAGACTAC; p9WT: 5' GGAAGCTTCGGACGCGGGGCGGAGCTGC; p9MT: 5GGGAAGCTTCGGACGCGGTTCCGGACCTGC.

Transfection

Transfection was performed with Fugene 6 transfection reagent (Boehringer Mannheim), according to the manufacturer's instructions. A total of 5 μl of Fugene reagent with 2 μg of reporter plasmid and 0.2 μg of renilla plasmid were used per 35 mm culture dish with 5 $\times 10^5$ cells.

Luciferase assay

Luciferase assay was done using the Dual-Luciferase Reporter Assay System (Promega). The cells were collected 24 hours after transfection, lysed, and freeze thawed; 20 μl of cell extract was used per reaction. Luminescence was measured on an AutoLumat LB953 (EG&G Berthold). The results were corrected for differences in transfection efficiency by renilla luciferase activity. Four lysates from independent transfections were prepared for each plasmid. The experiment was repeated four times.

List of oligonucleotides used for probe preparation

Consensus sequences for Sp1, AP2, and Oct1 (Promega) and consensus sequence for E2F and mutated E2F (Santa Cruz Biotechnology) were purchased as double stranded probes: cSp1:5'ATTTCGATCGGGGCGGGGCGAGC; cAP2: 5'GATCGA ACTGACCGCCCGCGGCCCGT; cE2F: 5'ATTAAAGTTTCGCGCC CTTTCTCAA; cE2Fmut:5'ATTAAAGTTTCGATCCCTTCTCAA; cOct1: TGTCGAATGCAAATCACTAGAA.

The rest of the probes were prepared by annealing single stranded oligonucleotides, except probe 3 which was a restricted fragment (see below).

The mutation in Sp1 core sequence in probe 1Mut is identical to the mutated Sp1 consensus (Santa Cruz Biotechnology).

PROBE1: 5'ACGCAGTCCGCCCCGCGTCCGACCCGCGGAT; PROBE1MUT:5' ACGCAGTCCGaaCCGCGTCCGACCCGCGGAT; PROBE2:5'GGAATGCCCGGAGGGCGGAGAAGTGGG; PROBE4:5'CCGAGCGCGCGGAAGACTACGGAG; PROBE5: 5'GAGGATCCTTCTGCGCACGCGCAC; PROBE3: restricted *XhoI/SalI* fragment spanning nt -89 to -23 of the *VHL* promoter. Construct F5 was digested with restriction enzymes *XhoI/HindIII* and subsequently with *SalI* (present in VHL promoter sequence at nt -24). The *XhoI/SalI* fragment was gel purified with Qiaquick gel extraction kit and used as probe 3: (5'GCCTCCGGCCGGCTATTTCCGCGAGCGGTTCCATCCTCTACGAGCGCGCGG AAGACTACGGAGG).

Gel mobility shift assay

Pairs of complementary single stranded oligonucleotides (Gibco BRL) were annealed to generate double stranded probes (see below). The probes were end labelled with [γ -32P]ATP (Amersham, 3000 Ci/mmol) and 0.1- 0.9 ng of probes were used per binding reaction with either 10 μg of HeLa extract (Promega), or 1.4 μg AP2 extract (bacterial extract obtained from strain overexpressing human, recombinant AP2, Promega), or 1-3 μl of purified Sp1 protein (Promega). The binding conditions for Sp1 and AP2 were according to the Gel Shift Assay System (Promega). The binding conditions for E2F1 binding were as described previously.²⁷

In competition assays, we used 30, 60, and 90 fold excess of competitors. Sp1, AP2, and all the other competitors were purchased from Promega, except E2F consensus oligomer and E2F mutated oligomer (Santa Cruz Biotechnology). We preincubated extract for 10 minutes with competitors before adding the probe.

Antibodies anti-Sp1, anti-AP2, and anti-E2F1 were Trans Cruz supershift reagents (200 $\mu\text{g}/0.1\text{ml}$) (Santa Cruz Biotechnology). To detect an antibody supershift (or block of the DNA/protein complexes) 1, 2, or 3 μl of appropriate TransCruz antibody was added to binding reaction and incubated for 20 minutes at room temperature before adding the probe. DNA/protein complexes were resolved by electrophoresis through a non-denaturing, 4% polyacrylamide gel with glycerol in 0.5 \times TBE buffer at 100 V for about four hours at 4°C.

RESULTS

Sequence analysis

Evolutionary conservation of the *VHL* promoter and upstream sequence

To identify candidate regulatory regions within the *VHL* promoter, we searched for regions of sequence conservation in

the 732 nucleotides 5' to codon 1. Thus, we performed direct genomic sequencing of the *VHL* 5' region in chimpanzee (*Pan troglodytes*) (Accession No AF291824), gorilla (*Gorilla gorilla*) (Accession No AF291825), olive baboon (*Papio anubis*) (Accession No AF291826), and macaque (*Macaca fascicularis*) (Accession No AF291827). In addition, 5' sequence for the mouse (*Mus musculus*) and rat (*Rattus norvegicus*) *vhl* genes were obtained from the GenBank database (Accession Nos NM-009507 and AF141022 respectively).

Blast 2 against 1 analysis of the primate sequence shows that the promoter is generally well conserved across the primate species (fig 1). Thus, nucleotide identity within the 106 bp minimal promoter region²⁴ and throughout the whole 5' sequence analysed was 99% and 98% respectively in chimpanzee, 97% and 96% respectively in the gorilla, and 95% and 93% in baboon. The exception was macaque which showed much lower nucleotide identity, 50% in the minimal promoter region and 45% throughout all of the 5' sequence analysed, reflecting the fact that *Macaca fascicularis* diverged at an earlier stage than the other primates analysed.²⁸

The close similarities between human and primate 5' *VHL* sequences did not allow discrete regions of evolutionarily conserved sequences to be identified. Therefore, we extended our analysis to include comparisons to rodent 5' *vhl* sequences. Several multiple sequence alignment packages were used to align the human, ape and rodent sequences: ClustalW,²⁹ Multalin,³⁰ and Blast 2 against 1.³¹ To identify discrete areas of conservation, we considered that a conserved region should be >20 nt in length and show >60% nucleotide identity between human and rat. With these criteria, four regions were identified (fig 1). Sequence conservation over 100 million years of evolution suggests that these regions (*CRs*) are of functional significance. To test this hypothesis we investigated three of these regions, *CR2* and *CR3* (which are contained within the minimal promoter) and *CR4* (which is downstream of the transcription start site), by deletion analysis and reporter gene assays.

In silico identification of candidate transcription factor binding sites

To identify potential transcription factor binding sites within the *VHL* promoter, we performed an analysis with the MatInspector v 2.2 and TFSEARCH version 1.3 programs,²⁶ focusing on the *VHL* minimal promoter region and the four evolutionarily conserved regions. Predicted transcription factor binding sites were: Conserved Region 1 (*CR1*), GATA2, GATA3, NF-1, and Barbie box; *CR2*, cRel and NFkappa; *CR3*, Sp1, Egr, AhR/Ar, GATA2, AP2, and AP4; and *CR4*, cRel, NFkappa, Sp1, MZF1, and AP2. Two further regions, located outside the regions of conservation, were also analysed (fig 2); a region within the minimal promoter between *CR2* and *CR3* (nt -60 to -12) contained putative sites for E2F and GATA-2, and the region upstream of the minimal promoter (between nt -114 and -91) contained potential GATA-2, HSF, AhR/Ar, USF, MYC/MAX, N-myc, and E2F sites. Most of these putative transcription factor binding sites were conserved in the rodent 5' *vhl* sequences.

Deletion analysis of evolutionarily conserved regions within the *VHL* promoter

To investigate the functional importance of the conserved regions identified, we prepared a set of *VHL* promoter deletions which were analysed by a luciferase reporter assay in human kidney embryonal cell line 293 and HeLa cells. To perform detailed deletion analysis, we narrowed our region of interest to a 176 bp region between nucleotides -89 to +87 (fig 2), corresponding to construct F1 in fig 3. This region is better conserved than the region upstream of nt -89 and therefore very likely to span important regulatory elements for the *VHL* promoter. The results are presented in fig 3 and are

expressed relative to a control construct Luc1 already described.²⁴ The F1 construct had 30% of Luc1 activity in 293 cells and 26% in HeLa cells. Deletion of 19 nucleotides from the 3' end of F1 (corresponding to *CR4*) resulted in only slight decrease of promoter activity to 25% or 18% depending on the cell line (see F2 in fig 3). Deletion of a further 45 nucleotides (nt +68 to +23) further reduced promoter activity (fig 3). Intriguingly, deletion of a further six nucleotides (nt +23 to +17) resulted in increase of promoter activity to 62% or 28% in 293 and HeLa cells respectively, suggesting the presence of a negative regulatory element within this region (fig 3, constructs F3 and F4). Deletion of the next 15 nucleotides (construct F5) (nt +17 to +2) markedly reduced *VHL* promoter activity to 7% in 293 cells and to 6% in HeLa cells. The deleted region contained most of *CR3*.

Deletion of *CR2* (nt -89 and -49) was assessed with F6 and F7 constructs, but did not have any significant effect on *VHL* promoter activity. However, deletion of a further 20 nucleotides (construct F8) resulted in a significant reduction in promoter activity to 12% or 6% indicating the presence of an important positive regulatory element within this region (nt -49 to -19) (fig 3).

In summary, deletion analysis of the *VHL* promoter between nt -89 and +87 indicated the presence of positive regulatory regions between nt -49 to -19 and +2 to +17 respectively, and of a negative element between nt +17 and +87 (fig 3).

Characterisation of candidate *VHL* promoter transcription factor binding sites

To investigate the candidacy of putative transcription factor binding sites within the *VHL* promoter region we undertook functional analysis at the following sites.

Candidate Sp1 binding site at nt +1 to +11.

We investigated this putative Sp1 binding site which is located just downstream of the main transcription start, for its ability to bind Sp1 in in vitro electrophoretic mobility shift assays (EMSA) (fig 4). A pair of complementary oligomers spanning the putative Sp1 site (probe 1, nt -5 to +27) were tested for ability to bind specifically Sp1 from HeLa cell nuclear extract and to bind purified Sp1 protein. Incubation of probe 1 with HeLa extract resulted in appearance of a band corresponding to a DNA/protein complex (fig 4A, lane 6), which specifically disappeared in competition assay after preincubation of HeLa extract with Sp1 consensus oligomer (cSp1) (fig 4A, lane 7), but not with an unrelated oligomer Oct1 (fig 4A, lane 8) or other non-specific oligomers (data not shown). Furthermore, probe 1 competed with Sp1 consensus oligomer (cSp1) for Sp1 binding (fig 4A, lane 3). Although computer analysis had predicted an overlapping AP2 site in this region (at nt +6 to +17), an AP2 consensus oligomer (cAP2) did not compete with probe 1 (data not shown). Mutation of two nucleotides (CC→AA) (see Methods) in probe 1 abolished the ability of the mutated probe 1 to bind Sp1 from HeLa extract (fig 4A, lanes 10-12) (an identical mutation in cSp1 had similar effects).

To investigate further potential Sp1 and AP2 binding sites in probe 1 we performed supershift assays (fig 4B). Preincubation of HeLa extract with increasing concentrations of anti-Sp1 antibodies before binding probe 1 resulted in disappearance of the DNA/protein complex (fig 4B, lanes 3-5) but anti-AP2 antibodies had no effect (fig 4B, lanes 6-8). Finally we examined the ability of probe 1 to bind purified Sp1 protein. Incubation of probe 1 with increasing concentrations of purified Sp1 protein (fig 4C, lanes 6-8) showed binding to probe 1 similar to that seen with cSp1 (fig 4C, lanes 2-4).

These findings showed the ability of the putative Sp1 site to bind specifically to Sp1 in EMSA assays. As this site is contained within a region which strongly influences *VHL* promoter activity (fig 3), we wished to investigate the significance

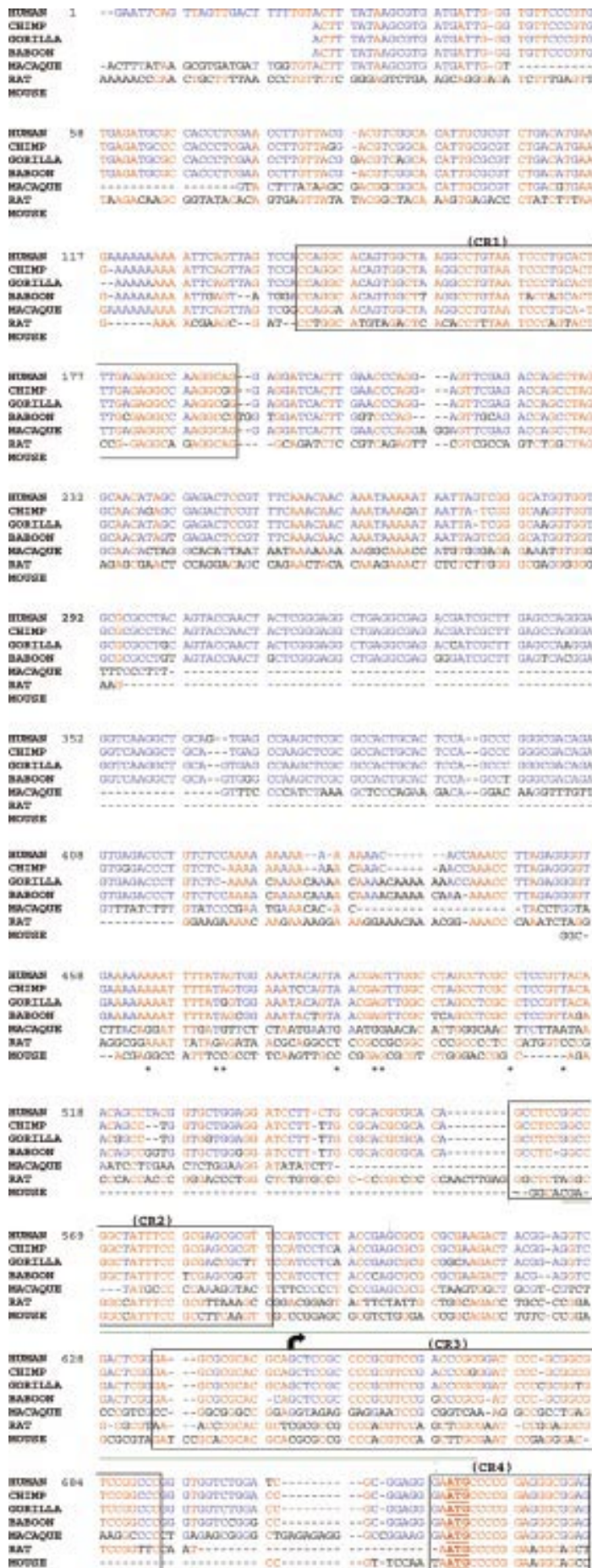


Figure 1 Multiple sequence alignment of human, primate, and rodent *VHL* 5' sequences (ClustalW). Blue = conservation of nucleotide between human and primate. Red = conservation of nucleotide between human and rodent. Blank = sequence not obtainable owing to primer design constraints. Dashed line = sequence missing. Boxed areas CR1 to CR4 = regions of conservation between human and rat. Underlined in green, minimal promoter, bent arrow = the main transcription start.

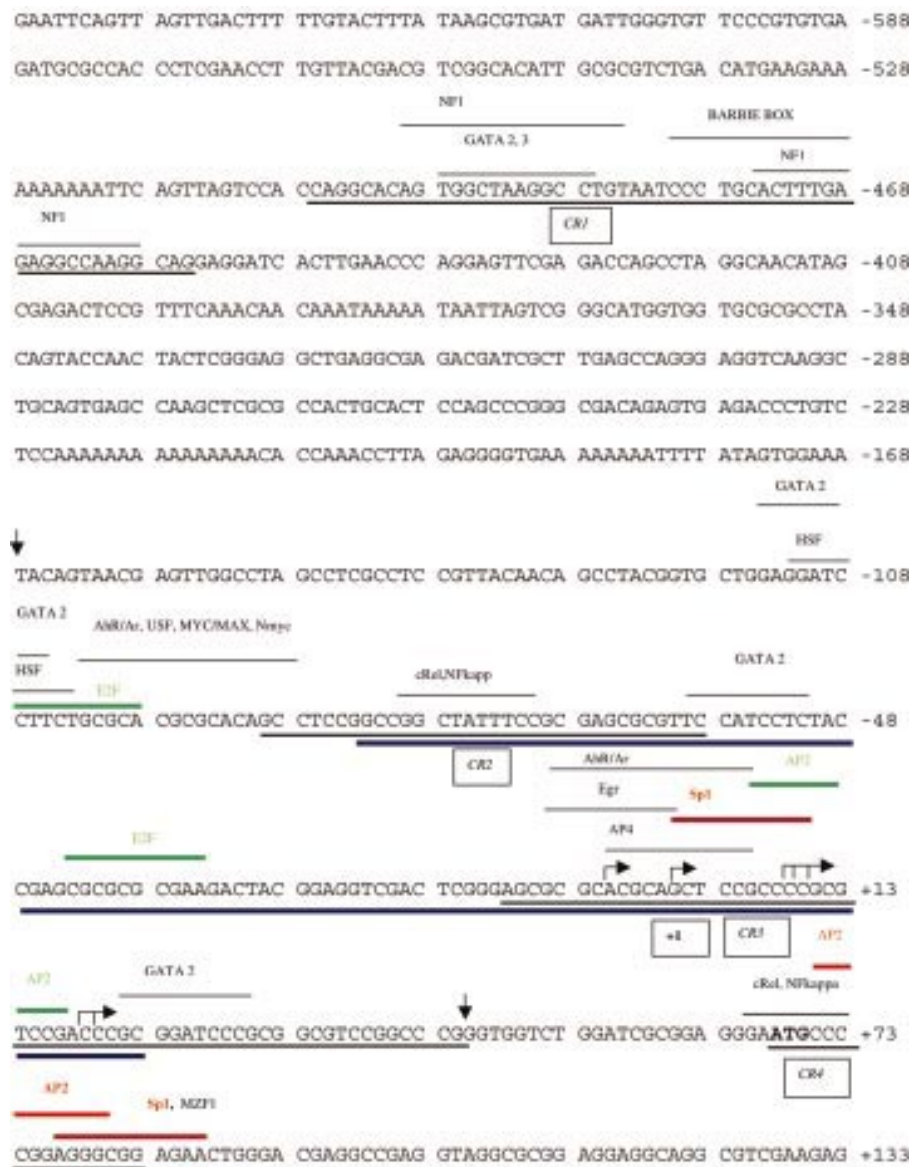


Figure 2 *VHL* promoter sequence. Underlined with a bold black line and labelled with boxes, CR1 to CR4 are the regions of conservation between human and rat. Above *VHL* promoter sequences are the predicted putative binding sites for transcription factors. The candidate sites are shown only in either the regions of conservation or in the regions experimentally analysed in this paper. Binding sites for factors analysed and confirmed are shown in red, analysed and not confirmed in green. Minimal promoter (as defined by Kuzmin *et al.*²⁴) is underlined in dark blue. Bent arrows: transcription starts. ATG: start codon. Vertical arrows mark the borders of a fragment which showed regulation by E2F1.

of the Sp1 site for regulation of *VHL* promoter activity. Thus, we evaluated the activity of a reporter construct with a CC→AA Sp1 site mutation previously shown to abolish Sp1 binding (fig 4A, lanes 10-12). Mutation of the Sp1 site (F4M at fig 3) profoundly decreased promoter activity (to 9% in 293 and to 10% in HeLa cells compared to wild type construct F4). The decrease in promoter activity caused by mutation of the Sp1 site is similar to the decrease caused by deleting the region (fig 3); therefore the Sp1 site (nt +1 to +11) seems to have an important activatory role for the *VHL* promoter.

Candidate Sp1/AP2 binding site at nt +72 to +87

Next we investigated a second predicted Sp1 site (position +77 to +87), which overlapped with a putative AP2 site (position +72 to +80). This site is located downstream of the transcription start site, adjacent to the first pVHL translation initiation site at nucleotide +69 (there is a second translation initiation site downstream).^{32,33} A pair of complementary oligomers were synthesised (probe 2, nt +65 to +92) to cover

this site and cSp1 and cAP2 probes were used as controls. We tested probe 2 for its ability to bind Sp1 and AP2 using HeLa nuclear extract and AP2 extract (Methods). The DNA/protein complex was apparent with probe 2 except when the AP2 extract was preincubated with cAP2 as a competitor (fig 5A, lane 8) and unlabelled probe 2 competed successfully with cAP2 for AP2 binding (fig 5A, lane 4). We then investigated binding to probe 2 by HeLa nuclear extract (fig 5B) and found that this produced a novel double band, suggesting the formation of two DNA/protein complexes (fig 5B, lane 1). In competition assay, cAP2 competed specifically for binding of both complexes (fig 5B, lane 2), but cSp1 competed for binding of the upper complex only (fig 5B, lane 3). Both bands disappeared in the presence of both cAP2 and cSp1 (fig 5B, lane 4). These findings were consistent with probe 2 binding both AP2 and Sp1 in the HeLa nuclear extract.

We further tested the ability of probe 2 to bind specifically to Sp1 and AP2 by supershift experiments. After establishing antibody specificity (fig 5C, lanes 3-8), we investigated the

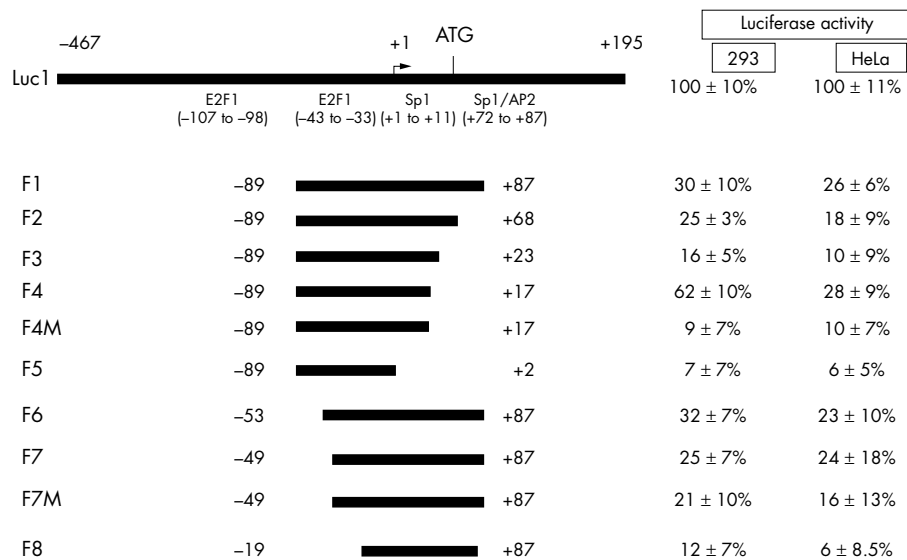


Figure 3 Luciferase reporter assay in 293 and HeLa cells. Luc1 = Luc1 *VHL* promoter construct. Other *VHL* promoter constructs are black bars, F1 to F8. The numbers at the sides of the bars are positions of the first and the last nucleotide of the construct. Binding sites for transcription factors studied in this paper are labelled below Luc1 construct together with coordinates (in brackets) indicating their position as predicted by MatInspector. ATG = start codon, bent arrow = the main transcription start. Luciferase activity is mean value (SD), relative to Luc1 activity in 293 cells and to Luc1 activity in HeLa cells (100% Luc1 activity in 293 cells = 1 324 814 light units, but in HeLa cells 100% = 880 302 units). The results are based on four repetitions.

effect on probe 2-HeLa extract complexes. After preincubation of HeLa extract with anti-AP2 antibodies, only the faster complex (C₂) was supershifted (fig 5C, lanes 10-12), whereas the slower complex (C₁) disappeared on preincubation with anti-Sp1 antibody (fig 5C, lanes 13-15). Preincubation of HeLa

extract with both antibodies prevented the formation of both complexes (fig 5C, lane 16). This Sp1/AP2 site is contained within the 19 nucleotides deleted from construct F1 to produce F2 (fig 3); however, this deletion resulted in only slight alteration in promoter activity.

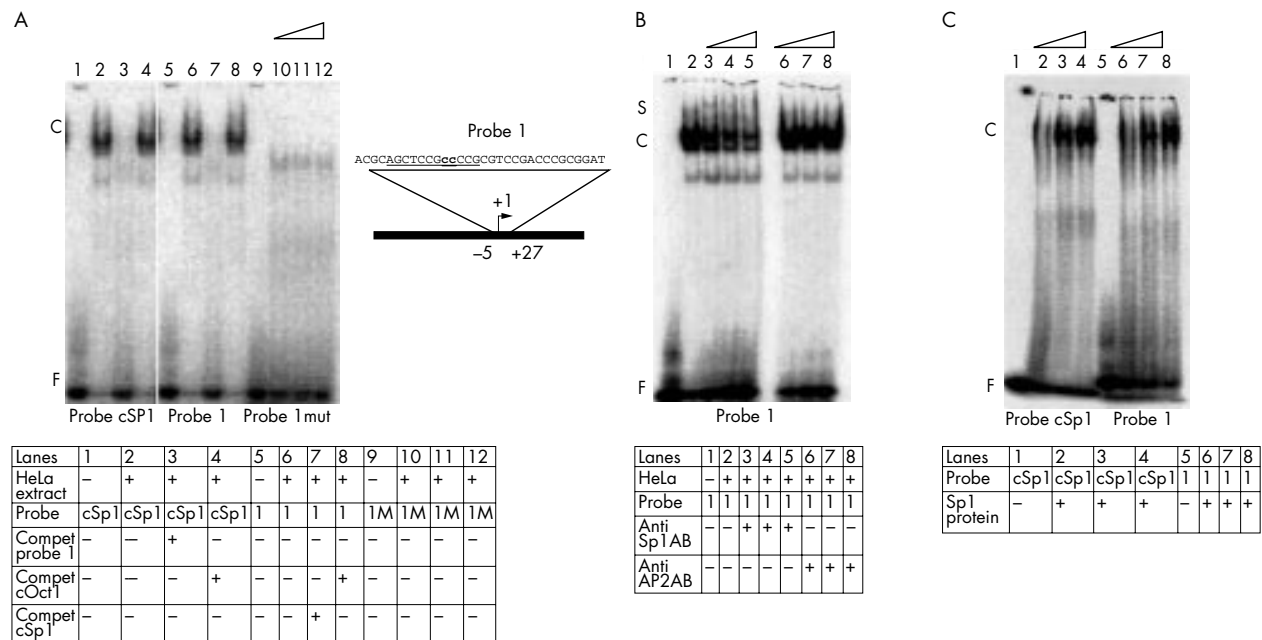


Figure 4 Gel mobility shift assay, binding of Sp1 factor to probe 1. (A) Competition assay and mutational analysis. Lanes 1-4, specific binding of Sp1 from HeLa extract by Sp1 consensus oligomer: 1, no protein, 2-4, HeLa extract (10 µg/reaction), 3, competitor probe 1 (30-fold excess), 4, competitor cOct1 oligomer (30-fold excess). Lanes 5-8, specific binding of Sp1 from HeLa extract by probe 1: lane 5, no extract, 6-8, HeLa extract (10 µg/reaction), 7, competitor cSp1 (30 fold excess), 8, competitor cOct1 (30 fold excess). Lanes 9-12: lack of binding of Sp1 from HeLa extract by mutated probe 1, lane 9, no protein, 10-12 increasing concentrations of HeLa extract (10, 15, 20 µg). To the right of the figure the sequence of probe 1 and its location within the promoter region is shown. Underlined sequence, Sp1 site (+1 to +11) as predicted by MatInspector; lower case, nucleotides mutated in probe 1 mut. (B) Supershift assay. Lane 1, no extract, lanes 2-8, HeLa extract (10 µg/reaction), lanes 3-5 increasing concentration of anti Sp1 antibodies (2, 4, 6 µg), lanes 6-8 increasing concentrations of anti AP2 antibodies (2, 4, 6 µg/reaction). (C) Binding of purified Sp1 protein. Lanes 1-4, Sp1 consensus sequence (cSp1); lanes 5-8, probe 1. Lanes 1 and 5, no protein, 2-4 and 6-8 increasing concentrations of purified Sp1 (1, 2, 3 µl). Below each gel, a table indicating components of each binding reaction is presented. F, free DNA, C, DNA/protein complex, S, supershift.

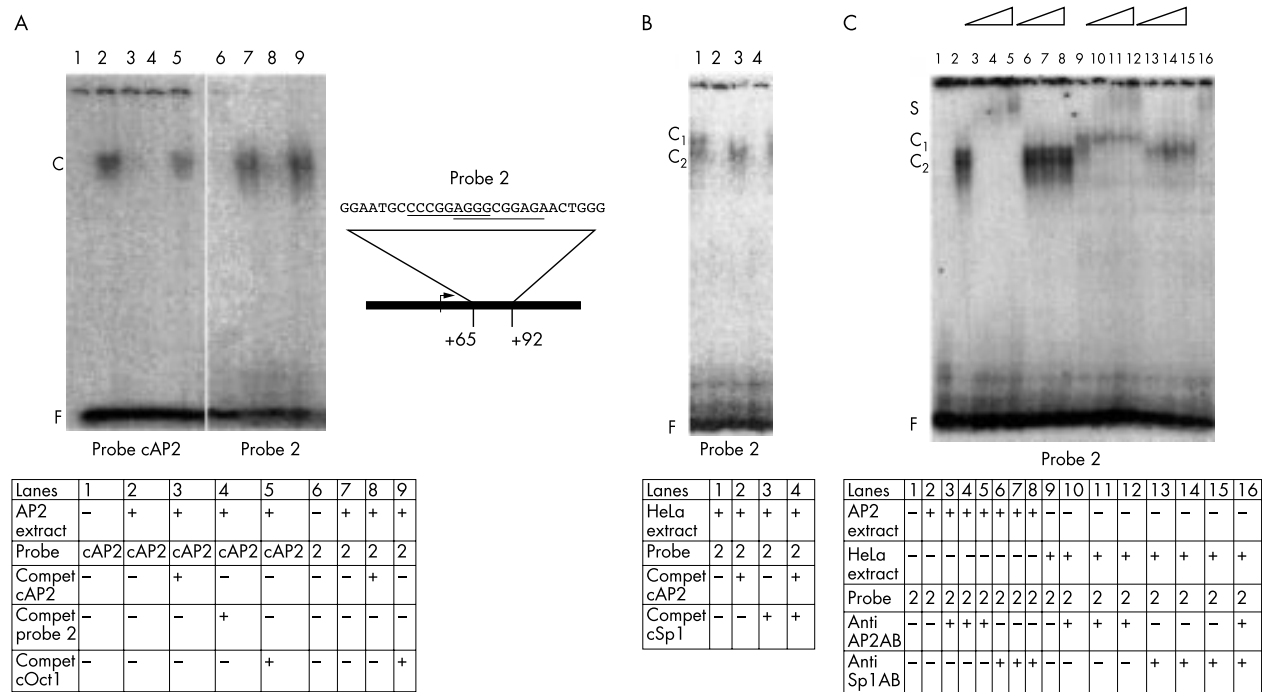


Figure 5 Gel mobility shift assay; binding of Sp1 and AP2 transcription factors to probe 2. (A) Competition assay. Lanes 1-5: AP2 binding by AP2 consensus sequence. Lane 1, no AP2 extract, lanes 2-5, AP2 extract (1.4 µg/reaction), lane 3, cAP2 as a competitor (30-fold excess), lane 4, cold probe 2 as a competitor (30-fold excess), lane 5, cOct1 as a competitor (30-fold excess), lanes 6-9, AP2 binding by probe 2, lane 6, no AP2 extract, lane 7-9 AP2 extract (1.4 µg/reaction), lane 8, cAP2 competitor (30-fold excess), lane 9, cOct1 as a competitor (30-fold excess). To the right of the figure the sequence of probe 2 and its location within the promoter region is shown. Underlined sequence shows Sp1 site (nt +77 to +87) and AP2 site (nt +72 to 80) as predicted by MatInspector. (B) Binding of Sp1 by probe 2. Lanes 1-4 HeLa extract (10 µg/reaction), lane 2, cAP2 oligomer as a competitor (30-fold excess), lane 3, cSp1 oligomer as competitor (30-fold excess), lane 4, both cAP2 and cSp1 competitors (30-fold excess). (C) Supershift assay. Lanes 1-8 probe 2 and AP2 extract (1.4 µg/reaction), lane 1 no extract, lanes 2-8 AP2 extract, lanes 3-5 increasing concentrations of anti-AP2 antibodies (2, 4, 6 µg), lanes 6-8 increasing concentration of anti-Sp1 antibodies (2, 4, 6 µg), lanes 9-16 probe 2 and HeLa extract (10 µg/reaction), lanes 10-12 increasing concentrations of anti-AP2 antibodies (2, 4, 6 µg/reaction), lanes 13-15 increasing concentrations of anti-Sp1 antibodies (2, 4, 6 µg/reaction), lane 16 both anti-Sp1 and anti-AP2 antibodies (2 µg/reaction). F: free probe, C₁, C₂: DNA/protein complexes. S: supershift. Below each gel is a table indicating components of each binding reaction.

Candidate E2F1 binding site at nt -33 to -43.

The deletion analysis of the minimal promoter described above suggested a functionally important region between nucleotides -49 and -19 (see F7 and F8, fig 3), although conservation was less than for flanking sequences. Computer analysis suggested the presence of an E2F1 binding site within this region. Fujita *et al*³⁴ reported that the *VHL* promoter is activated by E2F1, so we investigated further the putative E2F1 site by EMSA. Probe 3 (nt -89 to -23) was prepared (Methods) to cover this site and binding of HeLa extract to probe 3 resulted in appearance of two main complexes, C₁ and C₂ (fig 6A, B). Both complexes were specific for this fragment (fig 6A, lanes 8 and 9). However, neither of the complexes was affected by preincubation of HeLa extract with E2F consensus sequence (fig 6A, lanes 3 and 4), nor by preincubation with anti-E2F1 antibodies (fig 6B, lanes 3-5), suggesting that E2F1 is not present in either of the two complexes (under the same conditions we obtained specific binding of E2F1 to cE2F oligomer, which was proven by competition assay and anti-E2F1 antibody supershift, data not shown).

To map more precisely the binding sites for the factors present in these complexes, we performed a competition assay with oligonucleotide spanning region -48 to -24, which we called probe 4. One complex (C₁) disappeared after preincubation of HeLa extract with cold probe 4 (fig 6B, lanes 1 and 2) suggesting that the binding site for this unknown transcription factor (designated VHL-TF1) present in C₂ maps between nt -48 and -24. As the C₁ complex was not affected by competition with probe 4, a further transcription factor (designated VHL-TF2) must account for this complex.

Although the deletion of nucleotides -49 to -19 which removes the predicted E2F1 binding site resulted in a significant reduction in promoter activity (constructs F7 and F8, fig 3), this reduction might be accounted for by loss of other transcription factor binding sites. To test this we prepared a construct (F7M) with a mutated (CG→AT) E2F1 binding site and found that the F7M construct showed only a slight reduction in promoter activity compared to the control sequence (construct F7). Thus, the difference between F7 and F8 activities is more likely to be accounted for by the as yet unidentified factors VHL-TF1 and/or VHL-TF2 than by E2F1.

Candidate E2F1 site at nt -107 to -98

In view of the report of E2F1 activation of a *VHL* promoter fragment (nt -167 to +45), we proceeded to study the second best candidate E2F1 site at nt -107 to -98. Probe 5 (nt -114 to -91) spanning this site was prepared and tested by EMSA assay as described before. Binding of HeLa extract by probe 5 resulted in appearance of a specific complex (fig 7). However, the complex did not disappear after preincubation with cE2F (fig 7, lanes 4-6) and unlabelled probe 5 used in a competition experiment was unable to compete with cE2F oligomer for E2F1 binding (data not shown). Preincubation of HeLa extract with anti-E2F-1 antibodies did not result in supershifting of this complex (fig 7, lanes 10-12) suggesting that the complex contained a further, as yet unidentified factor (VHL-TF3).

DISCUSSION

We performed a series of genetic and functional studies to investigate the *VHL* gene promoter. Despite the importance of

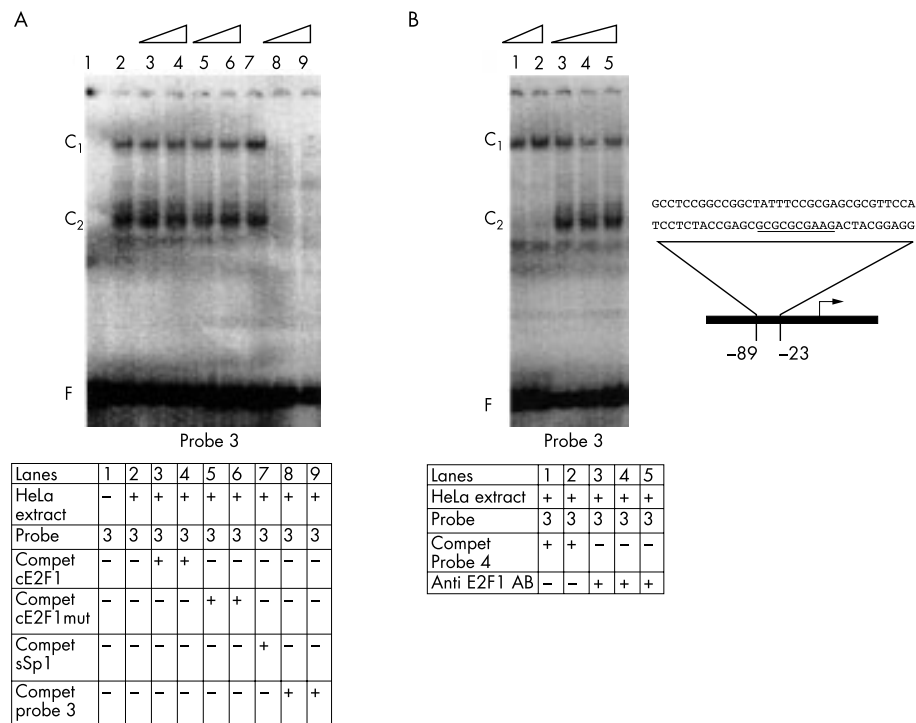


Figure 6 Gel mobility shift assay; binding of HeLa extract to putative E2F1 sites. (A) Binding of HeLa extract to probe 3. Lanes: 1 no extract, 2-9 HeLa extract (10 μ g/reaction), 3, 4 cE2F as a competitor (60- and 90-fold excess), 5, 6 competitor cE2F mut (60- and 90-fold excess), 7 unrelated cSp1 oligomer as a competitor (60-fold excess), 8, 9 cold probe 3 as a competitor (60- and 90-fold excess). (B) Binding of HeLa extract to probe 3. Lanes 1-5 HeLa extract (10 μ g/reaction), 1, 2 cold probe 4 as a competitor (60- and 90-fold excess), lanes 3-5 increasing concentrations of anti-E2F1 antibody (2, 4, 6 μ g). To the right of the figures, the sequence of the probe and its location within the promoter is shown. Underlined sequence shows E2F1 site (nt -33 to -43) as predicted by MatInspector. F, free probe. C₁, C₂, DNA-protein complexes. Below each gel is a table indicating components of each binding reaction.

pVHL in cancer biology and its relevance for development and regulation of angiogenesis, there are only two previous studies of the *VHL* promoter. Kuzmin *et al*²⁴ mapped a minimal *VHL* promoter by deletion analysis, but putative transcription factor binding sites were not investigated. Recently, evidence was reported for E2F1 activation of the *VHL* promoter, although an E2F1 binding site was not identified.³⁴ We attempted initially to identify candidate regulatory regions by defining regions of evolutionary conservation, and then proceeded to investigate these regions and specific putative transcription factor binding sites by EMSA and promoter activity assays. Evolutionary conservational analysis was most informative for comparisons between human and macaque and rodent sequences. Thus, the macaque showed only 45% nucleotide identity with human sequence across the whole promoter and 50% within the minimal promoter region. Comparison of human and rodent 5' sequences identified four conserved regions (CRs) where nucleotide identity was above 65% (fig 1). In silico analysis of the CRs showed the presence of many candidate transcription factor binding sites (fig 2).

Analysis of a range of promoter deletions in reporter gene assays identified several functionally important regulatory regions in both 293 and HeLa cell lines; two regulatory regions positively affecting *VHL* promoter activity were found. Thus, deletion of nucleotides +2 to +17 dramatically reduced *VHL* promoter activity and deletion of the region between nucleotides -49 to -19 also significantly reduced activity. In addition, we identified a negative regulatory region downstream of nucleotide +17 (removal of which resulted in several fold increase of promoter activity). A Sp1 transcription factor binding site was shown at nt +1 to +11. The profound effect on promoter activity of mutating this Sp1 site suggested it accounted for the important positive regulatory element in this region. Although the transcription start site lies in this region, and it could be postulated that the decrease in

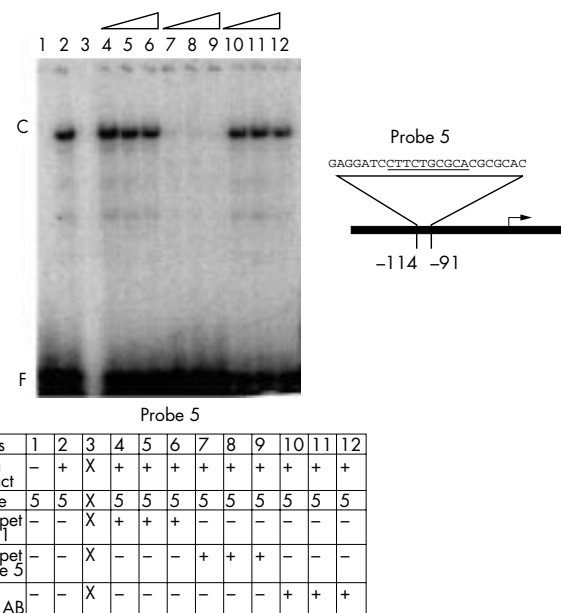


Figure 7 Binding of HeLa extract to probe 5. Lanes: 1 no extract, 2, 4-12 HeLa extract (10 μ g), 3 free lane, 4-6 cE2F as a competitor (30-, 60-, 90-fold excess), 7-9 cold probe 5 as a competitor (30-, 60-, 90-fold excess), 10-12 increasing concentrations of anti-E2F1 antibody (2, 4, 6 μ g). To the right of the figure is shown the sequence of the probe and its location within the promoter. Underlined sequence shows E2F1 site (nt -107 to -98) as predicted by MatInspector. F, free probe, C, DNA-protein complexes. Below the gel a table indicating the components of each binding reaction is presented.

promoter activity may be related to disruption of transcript initiation, the fact that a point mutation disrupting the Sp1 site decreases promoter activity almost as much as deletion of this region suggests an important role for Sp1. It has also been suggested that Sp1 may be required to anchor the basal initiation complex to the promoter through protein-protein interaction.³⁵ The significance of this Sp1 site for *VHL* promoter activity in the context of full length promoter fragment has not yet been determined, but as shown by deletion analysis,²⁴ other important regulatory sites are located both upstream and downstream of the studied region.

We also investigated putative overlapping Sp1 and AP2 sites (Sp1: nt +77 to +87, and AP-2: nt +72 to +80) downstream of the transcription initiation site. Although EMSA assays showed that both Sp1 and AP2 bound this site *in vitro*, deletion of this site only slightly reduced promoter activity. Thus, the functional significance of this site is uncertain as it could be more significant *in vivo* and in the context of the whole promoter. Two more candidate Sp1 sites were predicted 5' to the *VHL* minimal promoter (at nt -147 to -138 and -361 to -369) and more Sp1 and AP2 sites were predicted within exon 1.

Sp1 is a zinc finger transcription factor, which binds to GC rich sequences known as "GC boxes",^{36, 37} which have been identified in 5' regions of many genes.³⁸ Sp1 sites are reported often to regulate initiation of transcription in TATA-less promoters (the *VHL* promoter is TATA-less).³⁹⁻⁴¹ Although generally considered to be ubiquitous, Sp1 has also been implicated in regulation of tissue specific gene expression.⁴²⁻⁴⁴ It was reported that Sp1 is a critical regulator of the Wilms tumour suppressor gene, *WT1*, and that Sp1 expression is temporally and spatially regulated during nephrogenesis.⁴⁵ Thus, Sp1 has been implicated in renal development. As *VHL* mRNA fetal expression patterns are consistent with a role in nephrogenesis,⁹ we speculate that Sp1, *WT1*, and *VHL* may have interrelated roles in nephrogenesis. Although AP2 expression has also been implicated in nephrogenesis,⁴⁶ we were unable to show a significant effect of AP2 on *VHL* promoter activity.

In addition to identifying evolutionarily conserved regions, we also investigated a poorly conserved but functionally significant region between nt -49 and -19 within the minimal promoter. This region illustrates that functionally significant elements are not necessarily conserved, indeed such elements may have evolved as a result of differing functions for pVHL in humans and lower orders. Computer analysis had predicted a putative E2F1 binding site as the highest scoring response element in this region. This coupled with the report that the *VHL* promoter was activated by E2F1 in reporter assays in *Drosophila* cells³⁴ led us to investigate this site for evidence of E2F1 binding activity. The *VHL* promoter fragment studied by Fujita *et al*³⁴ corresponded to nucleotides -167 and +45 (fig 2) and the same putative E2F1 binding element as predicted in our analysis was suggested as the most likely E2F1 responsive element. Although when we investigated a 66 nt restriction fragment (probe 3) which spanned this site for ability to bind E2F1 in an EMSA assay we found evidence for two specific DNA/protein complexes, competition and supershift assays did not show the presence of E2F1 in either complex. Furthermore, mutation of the putative E2F1 binding site did not significantly alter *VHL* promoter activity in the reporter assay. However, as yet unidentified factors VHL-TF1 and/or VHL-TF2 (which were shown to bind to this region by EMSA) may account for the observed decrease in promoter activity seen when nt -49 to -19 are deleted. Computer analysis did not suggest likely candidates for the identity of VHL-TF1. The likely candidates for VHL-TF2 include cRel, NFkappa, and GATA2.

As the *VHL* promoter fragment studied *in vivo* in *Drosophila* cells by Fujita *et al*³⁴ extended beyond the minimal promoter, we analysed the whole region from nt -167 to +47 for the

presence of further candidate E2F binding sites. The second best prediction was for a site 5' to the minimal promoter region between nt -107 and -98. Although, probe 5 which spanned this region did form a DNA-protein complex in EMSA assay, this protein was not E2F1. Possible candidates for this transcription factor (VHL-TF3) include GATA2, HSF1, HSF2, USF, MYC/MAX, and Nmyc.

The failure to find a functioning E2F1 binding site might indicate that E2F binds cooperatively to the *VHL* promoter and requires the presence of other, lower homology E2F1 sites *in cis* (such sites are present at nt -67 to -75 and at nt -5 to -12).

E2F1 is the first cloned member of E2F family consisting of five members (E2F1-5).^{47, 48} Through its interaction with retinoblastoma protein, E2F is implicated in the regulation of the genes involved in transformation, cell growth, and DNA replication.⁴⁹ Binding of E2F1 to the *VHL* promoter might suggest that *VHL* promoter activity was cell cycle dependent; however, evidence for cell cycle dependent variations of *VHL* transcription have not been reported.⁵⁰

In summary we have identified a significant positive regulatory element for *VHL* promoter activity as an Sp1 binding site at nt +1 and +11, and mapped another positive regulatory region element between nt -49 and -19. This latter region was shown specifically to bind as yet unidentified factors, VHL-TF1 and VHL-TF2, which were confirmed not to be E2F-1. Upstream of the *VHL* minimal promoter we identified a region (nt -114 and -91) capable of specific binding of a factor which we called VHL-TF3. Although a second best candidate E2F-1 site spans this region, VHL-TF3 was confirmed not to be E2F-1.

These studies have provided a basis for determining the mechanisms of regulation of *VHL* expression and an intriguing link between Sp1, VHL, and nephrogenesis. Further studies are required to characterise VHL-TF1, -TF2 and -TF3 but our investigations also provide data for investigating the *VHL* promoter silencing in tumourigenesis.

ACKNOWLEDGEMENTS

We thank the Association for International Cancer Research (AICR), Cancer Research (UK), and the Medical Research Council for financial support. This work was funded in part by DHHS NO1-CO-12400.

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