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Autoregulation of the human WT1 gene promoter

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Abstract

The human Wilms tumour suppressor gene, WT1, encodes a zinc-finger protein which can function as a transcriptional activator or suppressor. This study reports the analysis of the human WT1 gene promoter, and demonstrates that high levels of WT1 expression lead to autosuppression of the WT1 promoter. Deletion analyses of the promoter region implicate sequences 5′ and 3′ of the transcriptional start site as being crucial in WT1 autosuppression. Loss or alteration of this function of WT1 may be important in tumourigenesis.

Key words: Wilms tumour gene; Promoter regulation

1. Introduction

Wilms tumour is a paediatric renal malignancy affecting 1/10,000 children, representing the most common abdominal solid tumour of childhood [1]. Cytogenetic analyses of the WAGR syndrome (Wilms tumour, anirridia, genitourinary malformation and mental retardation) identified a region on chromosome 11p13 as one of the loci of genes involved in these diseases [2,3]. Subsequently, several groups cloned a Wilms tumour gene, WT1 [4–6], which has been shown to undergo large homozygous deletions as well as small intergenic deletions and point mutations [5–11]. These studies strongly implicate loss of WT1 function as an important factor in tumour development, and identify WT1 as a putative tumour suppressor gene.

The pattern of WT1 gene expression in the condensing mesenchyme, renal vesicles and glomerular epithelium of developing kidney suggest that WT1 plays a role in kidney differentiation [12]. The structure of the WT1 gene product shows a glutamine/proline-rich negatively charged amino-terminus characteristic of transcription-regulating regions of transcription factors, and a carboxy-terminal region containing four zinc finger domains involved in DNA binding. The WT1 protein is expressed as four isoforms arising from two alternative splice sites (I and II) in the gene [13,14]. Splice II occurs within the zinc finger domain, inserting or omitting three amino-acids (KTS) between zinc fingers 3 and 4. The WT1 protein without KTS (WT1−KTS) specifically binds to the EGR (early growth response) consensus sequence, whereas WT1+KTS does not [15]. By binding to the EGR sites in the promoter regions of genes such as IGFII and PDGF-A, WT1 acts as a transcriptional repressor [16–19]. More recent studies have shown that WT1 can also activate transcription and bind a second DNA sequence motif comprising TCC repeats [20,21]. Thus WT1 can negatively or positively regulate transcription.

The human WT1 gene promoter region has recently been characterized [22,23]. In view of the observations that tight and specific control of the WT1 gene is important in normal kidney development, we have sought to define the basal WT1 promoter unit, and to examine whether the WT1 protein has the potential for self-regulation. As only about 10% of Wilms tumours show mutations of the coding region (reviewed in [24]), aberrant self-regulation may be a mechanism by which quantitative changes in WT1 contribute to WT1 dysfunction and tumour development.

2. Materials and methods

2.1. Cloning of WT1 cDNA and genomic clones

The WT1 cDNA and WT1 promoter region were cloned from a human foetal kidney cDNA library (Clontech) and a human B-cell genomic library (λSha2001, kindly supplied by T.H. Rabbitts, MRC, Cambridge), respectively. For each library, Plaque screen filters (Du Pont) were prepared in situ from 1×10^6 phage [25]. Filters were hybridized in 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardts solution, 0.5% SDS and 100 μg/ml salmon sperm DNA at 65°C. Washing was performed at high stringency (0.1 × SSC, 0.5% SDS, 65°C). For the cDNA library, a partial WT1 cDNA obtained by PCR amplification was used as a probe. The DNA sequence of a full-length cDNA isolated from the cDNA library was determined by the dideoxy chain termination method [26], and a 700 bp fragment from the 5′ terminus of the cDNA was used for probing the genomic library. Probes were radiolabelled with [α-^{32}P]dCTP (Amersham) according to the random primer method [27].

The full-length WT1 cDNA was subcloned into pMEP4 (Invitrogen) to give the WT1 eukaryotic expression construct pMWT. The promoter region of the WT1 gene was subcloned as a series of deletion mutants in pGL2-E (Promega) (see Fig. 2). DNA manipulations were according to standard methods [28].

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Abbreviations: IGFII, insulin-like growth factor II; PDGF-A, platelet-derived growth factor A; SSC, standard saline citrate; DMEM, Dulbecco's modification of Eagle's medium; bp, base-pairs; kb, kilobases.

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2.2. Cell culture and transfections

293 cells were maintained in DMEM (Gibco BRL) supplemented with 10% foetal calf serum and 2 mM L-glutamine. Cell line T5A1 is derived from stable transfection of the 293 cell line with the inducible WT1 expression vector pMWT which contains a full-length WT1 cDNA containing splice I but not splice II (WT1–KTS). Stable transfection was achieved by electroporation (200 V, 960 μF) of 2 × 10^6 293 cells grown to 50% confluence with 10 μg of pMWT. Clonal selection of T5A1 cells was carried out using culture medium further supplemented with 100 μg/ml hygromycin B (Boehringer-Mannheim). Thereafter, T5A1 cells were maintained in media containing hygromycin.

For transient transfections, 40 μg of each promoter construct was transfected into 2 × 10^6 cells by electroporation, after which cells from each electroporation were split equally into two plates. For each electroporation, cadmium chloride was added to 1 μM to one of the duplicate plate sets, yielding samples 293^+ and $T5A1^+$. Cells were incubated at 37°C for 40 h and subsequently lysed and analysed for luciferase activity according to the manufacturer's protocol (Promega). Plasmid pGL2-E was used as the negative control, and pGL2-C, which contains the SV40 promoter, was used as a positive control. Assays were performed at least three times, and transfection efficiencies were monitored by co-transfecting 10 μg of pSV-βgal.

2.3. Northern and Western blot analyses

For Northern blots, total cellular RNA was isolated by guanidine thiocyanate extraction [28] and 10 μg electrophoresed on a 1% agarose-37% formaldehyde RNA gel. RNA was then transferred onto Hybond-N^+ membranes (Amersham) in 20 × SSC, and probed with a WT1 cDNA probe. Hybridization and washing conditions were as outlined in section 2.1.

Cellular proteins for Western blot analysis were prepared by lysing 1 × 10^6 cells in 50 μl gel loading buffer (125 mM Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, 10% 2-mercaptoethanol, Bromophenol blue). Samples were boiled for 3 min, electrophoresed on 10% SDS-polyacrylamide gels [29] and electroblotted onto an Immobilon-P membrane (Millipore). After blocking with low-fat dry milk (5% w/v in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl), the membrane was incubated overnight with human WT1 monoclonal antibody C-19 (Santa Cruz Biotechnology). The membrane was washed in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% v/v Tween-20, and antibody binding was visualized with peroxidase-conjugated swine anti-rabbit secondary antibody (Dako) and ECL reagents (Amersham).

3. Results and discussion

Deletion constructs from the 5′ flanking region of the WT1 gene were assayed for promoter activity in 293 and T5A1 cells. Maximal promoter activity was apparent in sequences extending from −450 to +200, relative to the major transcriptional start site [23]. The basal activity of pEN-7 (−450 to +200), pEN-SH (−1102 to +200) and pEN-1.1 (−1102 to +44) was not significantly different. Sequences 5′ to nucleotide −450 displayed no promoter activity, as shown by plasmid pEN-d5 (see Fig. 2). Plasmids pEN-2 to -6 exhibit a reduced level of promoter activity (approximately 20–50% of pEN-SH activity).

Cellular systems expressing different levels of WT1 were designed in order to examine the effect of WT1 expression on transiently transfected WT1 promoter–luciferase gene constructs (see Fig. 1). The luciferase assay results summarized in Fig. 2 clearly demonstrate that expression of high levels of WT1 lead to repression of the WT1 promoter activity of plasmids pEN-SH, pEN-7 and pEN-6. The activities of these plasmids is curtailed by approximately 40–50% in induced T5A1 cells ($T5A1^+$) compared to uninduced T5A1 cells and 293 cells. Northern and Western blot analysis (see Fig. 1) shows that uninduced T5A1 cells express elevated levels of WT1 mRNA and protein relative to untransfected 293 cells. This is presumably due to 'leaky' metallothionein promoter activity. Interestingly, this level of WT1 expression does not appear to be sufficient for repression of WT1 promoter activity. The promoter activities of pEN-1.1 (spanning nucleotides −1102 to +44), pEN-2 (nucleotides −148 to +10), pEN-3 (nucleotides +4 to +200) and pEN-4 (nucleotides −144 to +200), were not affected by WT1 expression, despite containing 2–4 EGR/WT1 binding sites (see Fig. 2).

Comparison of pEN-1.1 with pEN-SH implicates an exonic region of 157 bp between the *SmaI* site at position +38 and the *PstI* site at position +195 (numbering according to [23]) as being crucial for the autorepressive

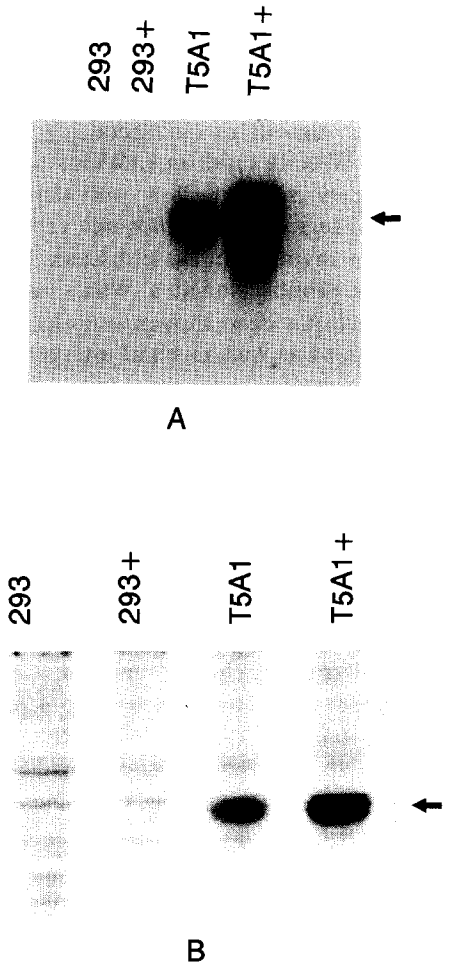

Fig. 1. Expression of WT1 mRNA (A) and WT1 protein (B). Expression of the WT1 cDNA cloned in pMWT is driven by the metallothionein promoter and was induced by addition of 1 μM cadmium chloride to the medium (lanes 293^+ and $T5A1^+$). Northern hybridization with a WT1 cDNA probe (A) shows the increased mRNA expression in T5A1 and $T5A1^+$ cells relative to the 293 cells. Western blotting analysis (B) demonstrates the parallel increase in WT1 protein induction.

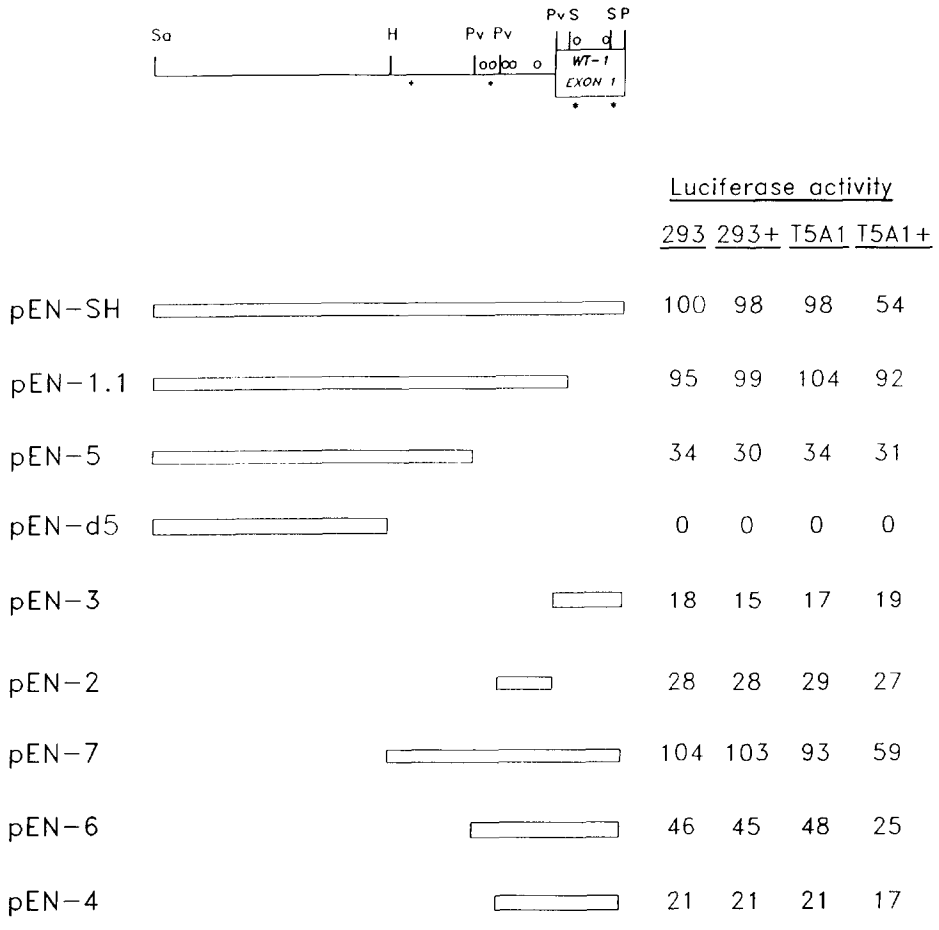

Fig. 2. Analysis of WT1 promoter activity in cell lines 293 and T5A1. A 1.3 kb *Sac*I–*Pst*I fragment encompassing 1.1 kb of the 5′-flanking region and 200 bp of exon 1 was subcloned in the luciferase reporter plasmid pGL2-E (Promega) to give plasmid pEN-SH. The 5′ region of the WT1 gene is illustrated with circles marking potential EGR/WT1 DNA binding sites and asterisks marking the GC boxes (Sp1) binding sites identified by DNA sequencing [23]. Restriction sites shown are *Sac*I (Sa), *Hin*dIII (H), *Pvu*II (Pv), *Sma*I (S) and *Pst*I (P). The flanking region cloned in pEN-SH and deletion constructs derived from it are shown diagramatically. Luciferase activities are expressed as percentages of the basal pEN-SH activity in 293 cells.

effect of WT1. The DNA sequence of this region contains 2 GC boxes which may be involved in the transcriptional activation of the WT1 promoter by Sp1 which has recently been demonstrated. These Sp1 sites also lie immediately proximal to the 2 EGR/WT1 consensus binding sites in this exonic region [23]. A similar binding site juxtaposition has been shown in the murine adenosine deaminase gene promoter [30] where expression is regulated co-ordinately by Sp1 (activator) and EGR (repressor). The apparent threshold effect in our studies of the WT1 promoter, where repression does not occur in the uninduced T5A1 cells despite their elevated WT1 expression status relative to 293 cells, but does occur in induced T5A1 ($T5A1^+$) cells, suggests that competition at the EGR/WT1–Sp1 binding loci may be responsible for WT1-mediated autorepression. However, the promoter activity of plasmid pEN-3, which is likely to be mediated by Sp1, is not affected by high levels of WT1 expression. The threshold effect may therefore reflect the need for WT1 binding to multiple sites up- and downstream of the transcriptional start site in order for repression to occur.

Plasmids pEN-2 and pEN-1.1 retain 3 and 5 of the consensus EGR/WT1 binding sites upstream of the transcriptional start site, respectively, but neither exhibits the WT1-mediated repression apparent with pEN-SH, pEN-7, or pEN-6, emphasizing the requirement for sequences 3′ of the transcriptional start site. However, pEN-4, which retains the exonic region and 3 upstream EGR/WT1 binding sites, does not display repression, indicating that the exonic region is necessary but not sufficient for repression to occur. Plasmid pEN-6, which extends 74 bp further upstream relative to pEN-4, does exhibit WT1-mediated repression. This data identifies 5′ sequences also necessary for efficient repression. The additional sequences in pEN-6 relative to pEN-4 restore additional EGR/WT1 consensus binding sites in the 5′ region (see Fig. 2).

The data presented demonstrate that the human WT1 gene promoter is subject to autosuppression in cells expressing high levels of the WT1−KTS isoform of the WT1 protein, and that specific sequences either side of the transcriptional start site are critical for WT1-mediated autosuppression. Constructs containing sequences solely from either 5′ or 3′ of the transcriptional start site do not exhibit the high level of repression apparent with promoter constructs spanning 5′ and 3′ sequences. These findings, together with the high levels of WT1 expression necessary for repression to occur, suggest that WT1 binding at multiple 5′ and 3′ loci is required for destabilization of the transcriptional preinitiation complex. The transcriptional suppression of the human PDGF-A gene has recently been shown to require 5′ and 3′ binding sites relative to the transcriptional initiation site [20].

During the preparation of this manuscript, negative autoregulation of the mouse WT1 promoter was also reported [31], all isoforms of the WT1 protein exhibiting transcriptional suppressor activity. Deletions of the region immediately downstream of the transcriptional start site were shown to affect a similar loss of repression, mediated by a different WT1 isoform (WT1+KTS) from the one we have studied (WT1−KTS). However, 5′ deletions were not shown to affect repression mediated by the WT1+KTS isoform. This may be due to the different WT1 splice forms used and/or functional variations between the human and mouse promoter sequences. We note that upstream deletions in the mouse promoter lead to a higher basal promoter activity whereas similar deletions curtail activity of the human promoter.

Our studies on the human WT1 promoter, together with those on the mouse promoter [31], illustrate the complexity of factors influencing the WT1 promoter. In view of the importance of controlled WT1 expression in normal kidney development [12], disruption of WT1 autoregulation by promoter and/or coding sequence alterations may play a key role in tumourigenesis. It will therefore be of interest to examine Wilms tumours for mutations in this region. Such studies are now underway.

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