Development 136, 2883-2892 (2009) doi:10.1242/dev.031773

# A highly conserved retinoic acid responsive element controls *wt1a* expression in the zebrafish pronephros

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The Wilms' tumor suppressor gene *Wt1* encodes a zinc-finger transcription factor that plays an essential role in organ development, most notably of the kidney. Despite its importance for organogenesis, knowledge of the regulation of *Wt1* expression is scarce. Here, we have used transgenesis in zebrafish harboring two *wt1* genes, *wt1a* and *wt1b*, in order to define regulatory elements that drive *wt1* expression in the kidney. Stable transgenic lines with approximately 30 kb of the upstream genomic regions of *wt1a* or *wt1b* almost exactly recapitulated endogenous expression of the *wt1* paralogs. In the case of *wt1b*, we have identified an enhancer that is located in the far upstream region that is necessary and sufficient for reporter gene expression in the pronephric glomeruli. Regarding *wt1a*, we could also identify an enhancer that is located approximately 4 kb upstream of the transcriptional start site that is required for expression in the intermediate mesoderm. Interestingly, this intermediate mesoderm enhancer is highly conserved between fish and mammals, is bound by members of the retinoic acid receptor family of transcription factors in gel shift experiments and mediates responsiveness to retinoic acid both in vivo and in cell culture. To our knowledge, this is the first functional demonstration of defined regulatory elements controlling *Wt1* expression in vivo. The identification of kidney-specific enhancer elements will help us to better understand the integration of extracellular signals into intracellular networks in nephrogenesis.

**KEY WORDS: Enhancer, Kidney development, Transcription factor, Transgenesis, Wilms' tumor, Zebrafish**

## INTRODUCTION

The Wilms' tumor suppressor gene *Wt1* encodes a zinc-finger transcription factor, which is a key regulator of kidney development. Inactivation of *WT1* in humans contributes to Wilms' tumor, a pediatric kidney cancer (Call et al., 1990; Gessler et al., 1990), as well as to other genitourinary malformations (reviewed by Rivera and Haber, 2005). A fundamental role for WT1 in the development of the urogenital system, as well as other organs, has also been demonstrated in mice. In this case, inactivation of *Wt1* causes organ anomalies affecting the kidneys, gonads, heart, spleen and retina, as well as the olfactory system (Herzer et al., 1999; Kreidberg et al., 1993; Wagner et al., 2002; Wagner et al., 2005).

In both its temporal and spatial pattern, *Wt1* expression is exquisitely regulated. In the mouse (and in humans), the highest expression levels are found in the developing kidney and the stromal cells of the gonads and spleen, as well as in the mesothelial cells lining the heart, diaphragm and peritoneum (Armstrong et al., 1993; Pritchard-Jones et al., 1990; Rackley et al., 1993). In the developing kidney, *Wt1* mRNA levels are low in the uninduced metanephrogenic mesenchyme and are absent in its reciprocally induced tissue, the ureter epithelium. *Wt1* expression rises significantly as the mesenchyme condenses. In the mature nephron, *Wt1* expression is confined to the podocytes, a highly specialized layer of epithelial cells that form the basis for blood filtration in the glomerulus.

In contrast to mammals, reptiles and amphibians (Kent et al., 1995), fish possess two *wt1* paralogs. In the case of zebrafish they are named *wt1a* and *wt1b*, and during development they are expressed in an overlapping, but not identical, spatial and temporal pattern (Bollig et al., 2006). With *wt1a* being expressed earlier than *wt1b*, the activity of both genes can be detected in the intermediate mesoderm and becomes gradually restricted to the developing glomeruli. Here, *wt1b* is expressed more laterally than *wt1a*. After the completion of pronephros differentiation, the expression of *wt1a* and *wt1b* is limited to the podocytes.

*wt1a* and *wt1b* also differ in terms of their function. Whereas the inactivation of *wt1a* leads to the failure of glomerular differentiation and morphogenesis, resulting in a rapidly expanding general body edema, the knockdown of *wt1b* is compatible with early glomerular development (Perner et al., 2007). After 48 hours, however, *wt1b* morphant embryos develop cysts in the region of the glomeruli and tubules and develop subsequent pericardial edema at 4 days post fertilization. Thus, *wt1a* plays an early role in pronephros development and is essential for the formation of glomerular structures, whereas *wt1b* functions at later stages of nephrogenesis.

A significant amount of work has been directed towards the elucidation of the cis and trans elements that regulate *Wt1* expression. The *Wt1* locus, however, is remarkably complex and our knowledge of its regulation is still fairly limited. In renal cells, for example, 24 kb of 5′ flanking sequence was not sufficient to drive *Wt1* expression. However, a yeast artificial chromosome (YAC) spanning 620 kb, harboring the murine *Wt1* gene flanked by 240 kb and 300 kb of 5′ sequence and 3′ sequence, respectively, was able to drive *Wt1* expression in the human renal 293 cell line to a level that is comparable to that of the endogenous gene (Scholz et al., 1997). In the course of this work, an element located ~15 kb upstream of the transcription start site was identified that included phylogenetically conserved sequence and was required, although not sufficient, for *Wt1* expression in 293 cells. In a study using transgenic mice, a 280 kb YAC construct containing the human *WT1* locus correctly reproduced the endogenous *Wt1* expression pattern (Moore et al., 1998).

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**Accepted 22 June 2009**

Two members of the paired-box transcription factors, Pax8 and Pax2, the zinc-finger protein Sp1 and members of the NF-κB/Rel family have been implicated in the regulation of *Wt1* expression (Cohen et al., 1997; Dehbi et al., 1996; Dehbi et al., 1998). *Pax2* itself is negatively regulated by WT1 (Ryan et al., 1995). However, all of these findings are based on transient overexpression and reporter gene assays in cell culture and their in vivo relevance has not been clarified.

In recent years, transgenesis in zebrafish has become a powerful tool to identify enhancers in the control regions of developmentally important genes (Muller et al., 1999; Woolfe et al., 2005). We have used this approach to identify elements that are responsible for the kidney-specific expression of zebrafish *wt1a* and *wt1b*. First, we generated stable transgenic GFP lines under the control of ~30 kb genomic fragments from the upstream regions of *wt1a* and *wt1b*. Notably, these lines recapitulated the endogenous expression of both *wt1* genes. By a combination of deletion analysis and stable, as well as transient, transgenesis we have identified a distal 336 bp upstream element in the *wt1b* regulatory region that is necessary and sufficient for *GFP* expression in pronephric glomeruli. In the upstream regulatory region of *wt1a*, we could also identify a 299 bp sequence, which was necessary for the proper expression in the area of the developing pronephros. This element, however, did not give rise to glomerular expression but was required for early expression in the intermediate mesoderm. Interestingly, this sequence was bound by members of the retinoic acid receptor family of transcription factors and mediated responsiveness to retinoic acid in vivo and in cell culture experiments.

## MATERIALS AND METHODS

### Fish maintenance

Zebrafish embryos were obtained from matings of wild-type fish of the TüAB strain that has been kept in laboratory stocks in Würzburg and Jena for many generations. Embryos were raised at 29°C and staged according to Kimmel et al. (Kimmel et al., 1995).

### Vector construction

Generation of the construct pBS-wt1b-GFP containing the 25.9 kb *Xho*I/*Apa*I fragment from the genomic region of zebrafish *wt1b* has been described (Perner et al., 2007). For the construction of pBS-wt1a-GFP, a 35.1 kb *Apa*I/*Apa*I fragment lying 5′ of the *wt1a* gene was excised from PAC clone BUSMP706E04239Q9 of the zebrafish genomic P1 artificial chromosome (PAC) library (Amemiya and Zon, 1999) and ligated into the *Apa*I site of the vector pBS-eGFP (Perner et al., 2007).

The vector pBS-wt1a-6.8kb-GFP was constructed by the insertion of the 6.8 kb *Xba*I/*Apa*I fragment lying upstream of the *wt1a* gene into the vector pBS-eGFP. Subsequently, a 100 bp region was deleted using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) and primers 5′-CAGTGACTTCTGCTGACATGACAGGTAGGACCAGATCTCAG-CTCATC-3′ and 5′-GATGAGCTGAGATCTGGTCCTACCTGTCAT-GTCAGCAGAAGTCACTG-3′, resulting in the vector pBS-wt1a-6.8kb-delta-GFP.

For transient transgenesis we employed several wt1b-GFP deletion vectors (vectors 1-14), which were all generated using standard cloning procedures. Deletion vectors 2-4 were constructed by the digestion of pBS-wt1b-GFP with the restriction enzymes indicated in the corresponding figures and the subsequent re-ligation of the remaining vector. For deletion vectors 5-8, genomic fragments were amplified by PCR and inserted between the *Nde*I and *Cla*I sites of vector 4. Deletions in vectors 9-12 were introduced by means of the QuikChange XL Site-Directed Mutagenesis Kit using vector 4 as a template. The vector pBS-wt1b-coreProm (vector 14) was generated by the excision of a 23.6 kb fragment from the vector pBS-wt1b-GFP using *Xba*I and the re-ligation of the remaining DNA fragment. An *Nde*I site in the vector pBS-wt1b-coreProm, which lies 1.5 kb upstream of eGFP, was used for the insertion of the 336 bp *wt1b* enhancer region, resulting in vector 13 (pBS-wt1b-coreProm-wt1bEnh). The sequences of the used primers and of all cloned fragments are available upon request.

The luciferase reporter vector pGL3-WT1-Enh was constructed by the insertion of the 287 bp human *WT1* enhancer region into the vector pGL3-Promoter (Promega) via *Kpn*I and *Sac*I sites. The enhancer region was amplified with primers 5′-CTAGGGTACCTCTCTCGTGGTTTTTCT-TTTCC-3′ and 5′-CTAGGAGCTCGGCACTGCCTCTATTATTATACCG-3′ using human genomic DNA as a template. Deletions of the conserved elements 1-3 were introduced by means of the QuikChange XL Site-Directed Mutagenesis Kit.

### Generation of transgenic zebrafish

Generation of transgenic zebrafish was performed essentially as described (Perner et al., 2007; Thermes et al., 2002). Briefly, the plasmids were co-injected with I-*Sce*I meganuclease in 1-cell stage zebrafish embryos. The injection solution contained 150 ng/μl plasmid DNA, 0.3-0.4 U/μl I-*Sce*I meganuclease (Roche), 5 mM $MgCl_2$, 0.1% Phenol Red (Sigma) and 4 mM Tris-HCl pH 7.5.

For the isolation of transgenic founder fish, 50-100 embryos injected with a given plasmid were raised to maturity and intercrossed. Offspring were analyzed for *GFP* expression using a fluorescence stereomicroscope (SteREO Lumar.V12, Zeiss). GFP-positive embryos of individual founder fish were used to raise at least three stable transgenic lines per construct.

### Fluorescence microscopy

Zebrafish embryos treated with 0.003% 1-phenyl-2-thiourea (PTU) and 0.016% tricaine were embedded in 1% low melting point agarose with their dorsal side facing the bottom of a μ-Dish (ibidi GmbH, Munich, Germany) or in 3% methyl cellulose. Subsequently, embryos were imaged using an inverted fluorescence microscope (Axiovert 135 TV, Zeiss) or a fluorescence stereomicroscope (SteREO Lumar.V12, Zeiss). Overlays of transmission and fluorescence images were generated using the AxioVision software (Zeiss).

### Sequence alignments

Alignments of long genomic sequences (4-150 kb) were performed with the mVISTA program (Brudno et al., 2003; Frazer et al., 2004). FASTA sequences lying upstream of *Wt1* in different vertebrate genomes (*wt1a* and *wt1b* in fish genomes) were retrieved using either the Ensembl (Flicek et al., 2008) or the UCSC (Kent et al., 2002) genome browser. The sequences correspond to the following regions in the UCSC genome browser (August 2008): human chromosome 11: 32,412,822-32,420,821; mouse chromosome 2: 104,959,600-104,967,599; *Xenopus* scaffold 399: 895,538-907,235 (for *wt1* exon 1 this sequence was complemented by cDNA clone, accession number BC168476); stickleback chromosome 2: 12,809,391-12,813,390; zebrafish chromosome 25: 15,039,784-15,044,783. For an alignment of the conserved region upstream of *Wt1* at the nucleotide level the ClustalW program (Larkin et al., 2007) was used. For the prediction of transcription factor binding sites the programs MatInspector and PROMO were employed.

### Histology

In situ hybridization on paraffin sections of adult zebrafish kidney was performed essentially as described (Leimeister et al., 1998). The probe for *wt1a* is described in Bollig et al. (Bollig et al., 2006). The *wt1b* probe was transcribed from a 944 bp cDNA fragment including exons 1-5 of *wt1b*, which was amplified with primers 5′-CATTCTGCTTCAGCAGAC-GCCTTC-3′ and 5′-CTCACTGCTGTCTGATGATGATT-3′. For GFP immunohistochemistry, paraffin sections were first incubated with rabbit anti-GFP IgG fraction (Invitrogen) and, after PBS washing, with biotinylated goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories). Antigen detection was carried out using the VECTASTAIN ABC Kit and the DAB Substrate Kit for peroxidase (Vector Laboratories). Finally, sections were counterstained with Hematoxylin.

For zebrafish embryos, whole-mount immunohistochemistry was performed before sectioning and counterstaining with DAPI. Embryos were fixed in 4% paraformaldehyde and permeabilized in acetone. After blocking, embryos were incubated with anti-GFP antibody followed by secondary antibody conjugated with Alexa Fluor 488 (Invitrogen).

### Electrophoretic mobility shift assay

Proteins for electrophoretic mobility shift assay (EMSA) analysis were prepared by a coupled in vitro transcription/translation reaction using the TNT system (Promega). Constructs pSG5mRARβ and pSG5hRXRα were used as templates, each harboring the full-length cDNA of the respective gene and an upstream T7 promoter. Both plasmids were kindly provided by A. Baniahmad (Jena University Hospital, Jena, Germany). Plasmid Rc/CMV (Invitrogen) was used as a negative control. For probe generation, primers 5′-TTCCCCATTTTTTCCCCTGTTG-3′ and 5′-TGGCCAGAGCAGATACGTAG-3′ and the template plasmid pGL3-WT1-Enh were used to amplify a 152 bp fragment from the human *WT1* enhancer. The mutant competitor fragment was generated using the same primers and pGL3-WT1-Enh-Δ1. For the zebrafish probe (144 bp), primers 5′-TTTCCTCGCTCTCCTCTTCCC-3′ and 5′-TGGCTAAGCAGAGGCAGCTC-3′ and the template plasmid pBS-wt1a-6.8kb-GFP were employed. For generation of the mutant zebrafish fragment, a template harboring the Δ1 deletion in the background of the zebrafish enhancer was employed. Labeling of the probes, sample preparation and gel electrophoresis using a 12% polyacrylamide gel were performed as described (Wilhelm and Englert, 2002). For competition experiments, a 500-fold molar excess of unlabeled fragment was used. For supershift experiments, 1 μg of polyclonal anti-RARβ (C-19, Santa Cruz) or a control antiserum (anti-HA, Santa Cruz) were pre-incubated for 10 minutes at room temperature before addition of the probe.

Nuclear extracts from P19 cells were prepared as described (Schreiber et al., 1989). Supershift experiments were performed with 5 μg of nuclear extract and an aliquot of monoclonal anti-RARα (9α9A6; a gift from H. Gronemeyer, IGBMC, Illkirch, France), control monoclonal antibody (anti-GST, Dianova), 0.5 μg of polyclonal anti-RARβ or control antiserum.

### Luciferase reporter assay

Murine P19 cells ($1\times10^6$) were transfected using the Nucleofector Kit V (Amaxa) according to the manufacturer's instructions, with 1 μg of the respective luciferase reporter vector and 50 ng phRL-Tk (Renilla) control plasmid (Promega). Subsequently, cells were incubated for 4 days with 1 μM all-trans retinoic acid or 0.1% ethanol. Firefly and Renilla luciferase activity were measured using the Dual-Luciferase Assay Kit (Promega).

## RESULTS

### The *Wt1* genomic regions of human and zebrafish show partial synteny

The similarity in *Wt1* expression patterns in mammals, frogs and fish during early kidney development suggests that this expression is based on similar regulatory mechanisms that have been conserved through evolution. In a study employing transgenic mice generated using human genomic fragments (Moore et al., 1998), it was shown that regulatory elements driving *WT1* expression during kidney development are located 5′ of the *WT1* gene. We have therefore assumed that these elements lie in the intergenic region between *WT1* and the upstream neighboring gene *EIF3M* (Fig. 1A).

When compared to the *WT1* region in humans, both zebrafish regions show partial synteny. Interestingly, only *ccdc73* can be found 5′ of *wt1a* (on chromosome 25) and only *eif3m* (also known as *ga17* or *pcid1*) can be found 5′ of *wt1b* (on chromosome 18), although the respective mammalian counterparts (*CCDC73* and *EIF3M*) lie close together and even overlap in their 3′ regions (Fig. 1A). The distance of these genes to *wt1* is much smaller in zebrafish (~30 kb) compared with humans (~50 kb), which facilitates the search for regulatory elements upstream of *wt1a/b*.

We cloned the intergenic regions between the zebrafish *wt1* paralogs and the respective 5′ neighboring genes and fused both fragments to a *GFP* reporter gene, yielding the constructs *wt1a*::*GFP* and *wt1b*::*GFP*. In the case of *wt1a*, this fragment includes the last two exons of the *ccdc73* gene, a short putative gene with unknown function, and part of *wt1a* exon 1 (Fig. 1B). Regarding *wt1b*, the genomic fragment encompasses almost the entire region between *eif3m* and *wt1b*, including part of exon 1 of the latter. The generation of *wt1b*::*GFP* transgenic fish has already been published (Perner et al., 2007).

### Reporter gene expression in transgenic animals recapitulates expression of *wt1* paralogs

All three *wt1a*::*GFP* lines showed a similar and consistent *GFP* expression pattern, which was restricted to the pronephric region (see below) and the dorsal hindbrain (see Fig. S1 in the supplementary material). The *wt1b*::*GFP* lines also showed expression in the pronephros and in several other embryonic organs [e.g. the pancreas, gut, eyes and epicardium, described in detail by Perner et al. (Perner et al., 2007)].

The pronephric expression of *wt1a* and *wt1b* in zebrafish is well documented (Bollig et al., 2006; Drummond et al., 1998; Serluca and Fishman, 2001; Wingert et al., 2007). *wt1a* starts to be expressed as bilateral stripes in the intermediate mesoderm 11 hours post fertilization (hpf) at the 3-somite stage, whereas *wt1b* expression in the pronephric field appears later (14 hpf) and is restricted to a smaller area (see schematic representation in Fig. 2). GFP fluorescence in the transgenic lines recapitulated this pattern with a

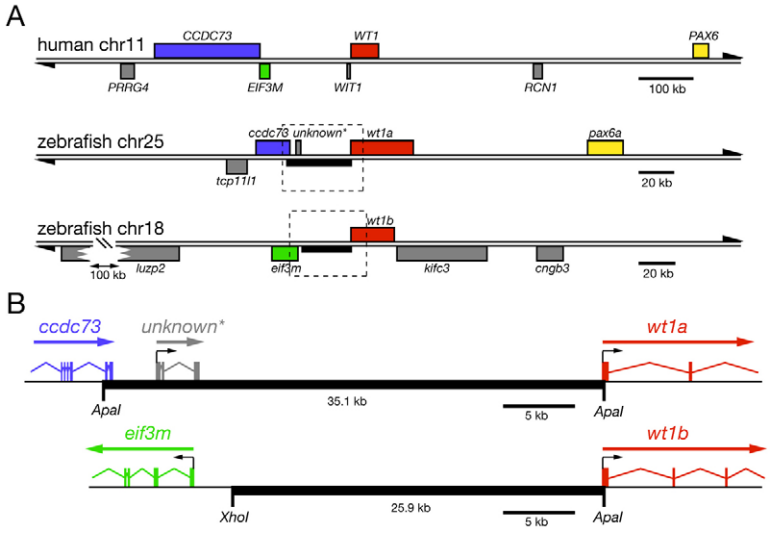


**Fig. 1. Structure of *Wt1* genomic regions in human and zebrafish.** (**A**) The genomic regions surrounding *WT1* on human chromosome 11 and *wt1a* and *wt1b* on zebrafish chromosomes 25 and 18, respectively, are shown (modified from the Ensembl genome browser, release 49). Colored boxes represent genes that are syntenic between the human *WT1* and the zebrafish *wt1a* and *wt1b* loci. Gray boxes represent non-syntenic genes. Genes above the double lines are transcribed in the same direction as *Wt1*, genes below in the opposite direction. (**B**) Higher magnifications of the areas indicated by dashed rectangles in A, illustrating the fragments used for the generation of transgenic fish lines. The 5′-to-3′ orientation of the genes is indicated by arrows. The gene marked by an asterisk is predicted by the Ensembl genome browser (gene *A2BG01*). The function of this gene is not known and no orthologs in other vertebrates were predicted. Black vertical bars indicate restriction enzyme sites.

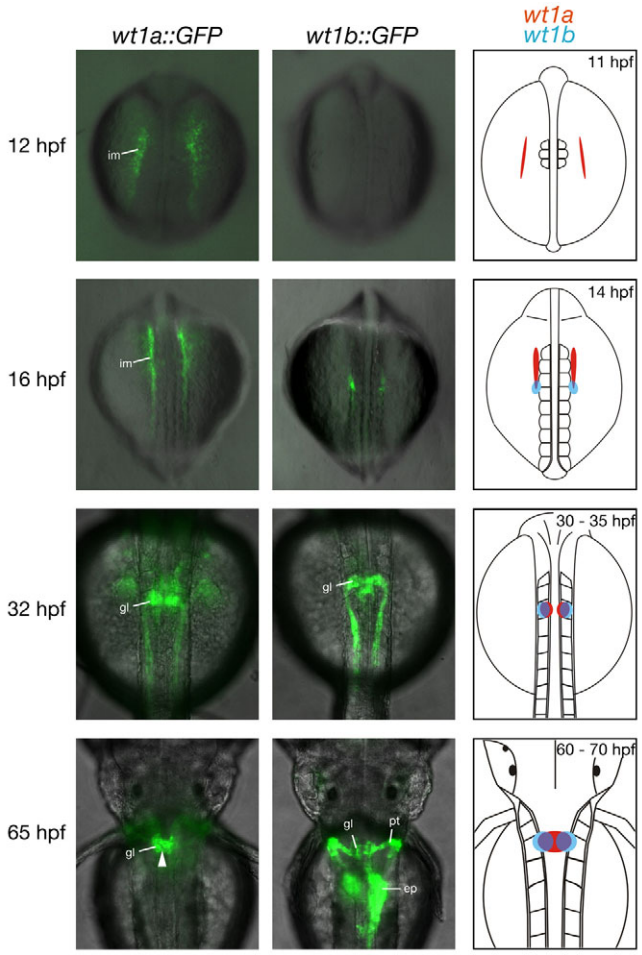


**Fig. 2. Reporter gene expression in transgenic zebrafish embryos recapitulates expression of *wt1* paralogs.** Transgenic *wt1a*::*GFP* (left) and *wt1b*::*GFP* (middle) embryos were imaged using a fluorescence microscope. Shown are overlays of dorsal transmission and fluorescence images at the indicated stages. Arrowhead in the bottom left panel marks fusion of the GFP signal at the midline. The schematic representation of *wt1a* and *wt1b* expression on the right is based on published data (Bollig et al., 2006; Drummond et al., 1998; Serluca and Fishman, 2001; Wingert et al., 2007) and is confined to expression in the pronephric region; *wt1a* expression is shown in red, *wt1b* expression in blue. ep, exocrine pancreas; gl, glomerulus; hpf, hours post fertilization; im, intermediate mesoderm; pt, pronephric tubule.

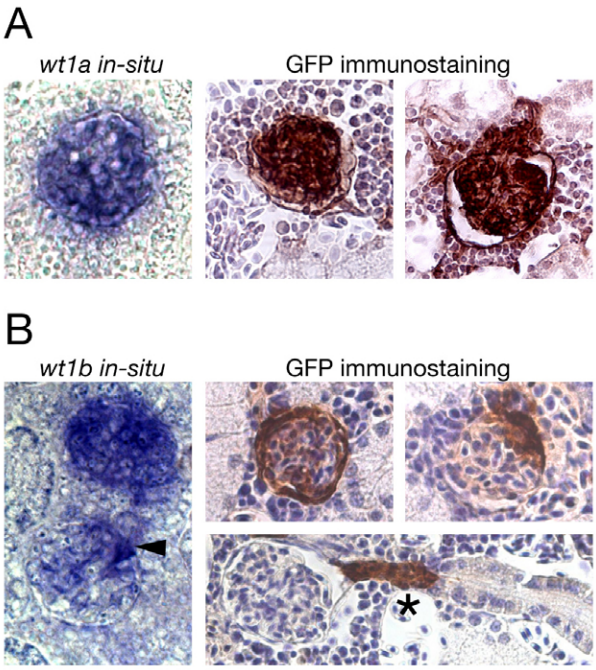


**Fig. 3. *GFP* expression in the mesonephros of adult transgenic zebrafish recapitulates expression of *wt1* paralogs.** (**A**,**B**) In situ hybridization for *wt1a* (A) and *wt1b* (B) on sections of wild-type mesonephros (left) and GFP immunostainings on sections of *wt1a*::*GFP* line 1 (A) and *wt1b*::*GFP* line 1 (B) mesonephros (right) are shown. The kidneys were taken from 4- to 6-month old wild-type and transgenic zebrafish. In the immunostainings, cell nuclei are stained blue (Hematoxylin counterstaining) and GFP-positive cells are brown. Arrowhead marks a glomerulus in which only a subset of cells is labeled, asterisk denotes a GFP-positive neck region.

delay of 1 to 2 hours (Fig. 2). This is probably caused by the translation of *GFP* mRNA into protein and the accumulation of GFP protein to detectable levels. At later stages (>24 hpf), *wt1a* and *wt1b* expression is restricted to a small area ventral of the third somite where the glomeruli are developing. In the functional pronephric glomeruli (>40 hpf), *wt1a* expression fields are fused in the midline, whereas *wt1b* is expressed more laterally in the glomeruli. In line with this pattern, a fusion of GFP fluorescence in the glomerular region could be seen only in *wt1a*::*GFP* embryos. The only difference between the pronephric expression of the *wt1* paralogs and the *GFP* reporter gene in the respective lines was the appearance of *GFP* expression in the pronephric tubules of *wt1b*::*GFP* embryos.

In adult zebrafish the clearance function is accomplished by the mesonephros. From serial histological sections, we estimated the number of nephrons in the mesonephros to be 50-100 (data not shown). We compared the expression of the *wt1* paralogs (by in situ hybridization) with the expression of *GFP* (by immunostaining) in the respective transgenic lines (Fig. 3). The expression of *wt1a* and *GFP* in *wt1a*::*GFP* transgenic zebrafish was found throughout entire glomeruli.

The *wt1b* expression profile in the mesonephros is highly variable. Some glomeruli were stained completely (Fig. 3B) and others possessed *wt1b* mRNA only in a subset of cells. This variability was reflected by the *GFP* expression pattern, with complete but also with partial staining of the glomeruli. Only the appearance of *GFP* expression in the neck region of the tubule differed from the *wt1b* expression pattern. This is highly reminiscent to the situation in the embryonic kidney of *wt1b*::*GFP* fish (Fig. 2) where *GFP*, but not *wt1b*, is expressed in the pronephric tubule.

In conclusion, both in the embryonic pronephric kidney and in the mesonephros, transgene-driven *GFP* expression recapitulates the endogenous expression of the *wt1* genes.

## A 336 bp element in the *wt1b* upstream region is necessary and sufficient for reporter gene expression in pronephric glomeruli

Using different bioinformatics tools we could not find any significant conservation of the region 5′ of *wt1b* compared to regions upstream of *Wt1* in other vertebrates. We therefore decided to systematically narrow down the region(s) upstream of *wt1b* that is/are important for expression in the pronephros by generating

several wt1b-GFP deletion constructs and using transient transgenesis (Fig. 4A). Using this strategy, we could localize two adjacent regions of 170 bp that were indispensable for glomerular expression (construct numbers 10 and 11) and that together represent a 336 bp region necessary for reporter gene expression in the pronephros. We then wanted to test whether the identified element was also sufficient to drive glomerular expression. To this end, we inserted the 336 bp element close to the *wt1b* transcription start site. Using the resulting construct (construct number 13), we did indeed find expression in the glomerulus, but not when the 336 bp enhancer region was absent (construct number 14).

To confirm the results from transient transgenesis we generated stable transgenic lines with the two latter constructs. Two lines harboring the wt1b-coreProm transgene (construct number 14) displayed only weak and diffuse GFP expression (Fig. 4B, left panel). By contrast, injection of the wt1b-coreProm-wt1bEnh construct (construct number 13) gave rise to five transgenic lines all showing a strong GFP signal anterior and ventral to the third somite (Fig. 4B, right panel), indicating expression in the pronephric glomeruli.

Taken together, we identified a 336 bp enhancer region located 21 kb 5′ of *wt1b* exon 1, which is necessary and sufficient for *wt1b* expression in the pronephric glomeruli of zebrafish. We did not find any similarities between this sequence (see Fig. S2 in the supplementary material) and the zebrafish *wt1a* locus or *wt1* loci in other fish genomes.

## A conserved region upstream of *wt1a* drives reporter gene expression in the intermediate mesoderm

In contrast to the situation for *wt1b*, we found a conserved region 4.2 kb upstream of the *wt1a* gene when we aligned the genomic region 5′ of *wt1a* with regions lying upstream of *Wt1* in human, mouse and frog, and upstream of *wt1a* in stickleback (Fig. 5A). The distance of the conserved region to the first exon of *Wt1* varies from 2.1 kb (stickleback) to 10.9 kb (*Xenopus*). The conserved region could be detected also in several other vertebrates, such as rat, opossum, platypus, lizard, medaka and pufferfish (data not shown), and harbors three highly conserved elements (cons 1-3 in Fig. 5B) with 12, 16 and 10 identical nucleotides, respectively. The first conserved element (cons 1, 5′-AGTTGA-(N)$_5$-GGGTCA-3′) is predicted as an RXR heterodimer binding site. For the other two elements (cons 2 and 3), several transcription factor binding sites were suggested, however, the significance of these predictions is low due to the shortness and the frequent occurrence of the sites.

To test the hypothesis that the conserved region upstream of *wt1a* acts as a regulatory element in vivo, we generated *GFP* reporter fish with two further constructs (6.8 kb and 6.8 kb delta, Fig. 6A). In the first construct, ~28 kb is deleted from the 5′ end of the vector wt1a-GFP (which is 35 kb), and the conserved region is still present. In the second construct, a 100 bp region including the first two conserved elements is deleted within the 6.8 kb fragment (indicated in Fig. 5B). In the offspring of all founder fish harboring the 6.8 kb construct, *GFP* was expressed in bilateral stripes in the intermediate mesoderm (Fig. 6B), ventrolateral to the somites. Compared with *wt1a*::*GFP* embryos (35 kb fragment) this early expression in the intermediate mesoderm was expanded anteriorly, as well as posteriorly. At 42 hpf, GFP fluorescence could be detected outside of the glomerular region of *wt1a-6.8kb*::*GFP* embryos, whereas in the glomeruli (adjacent to the third somite) *GFP* expression was low. This indicates that the deleted 5′ region harbors elements that are necessary for both restricted *wt1a* expression in the intermediate mesoderm and expression at later stages in the glomeruli.

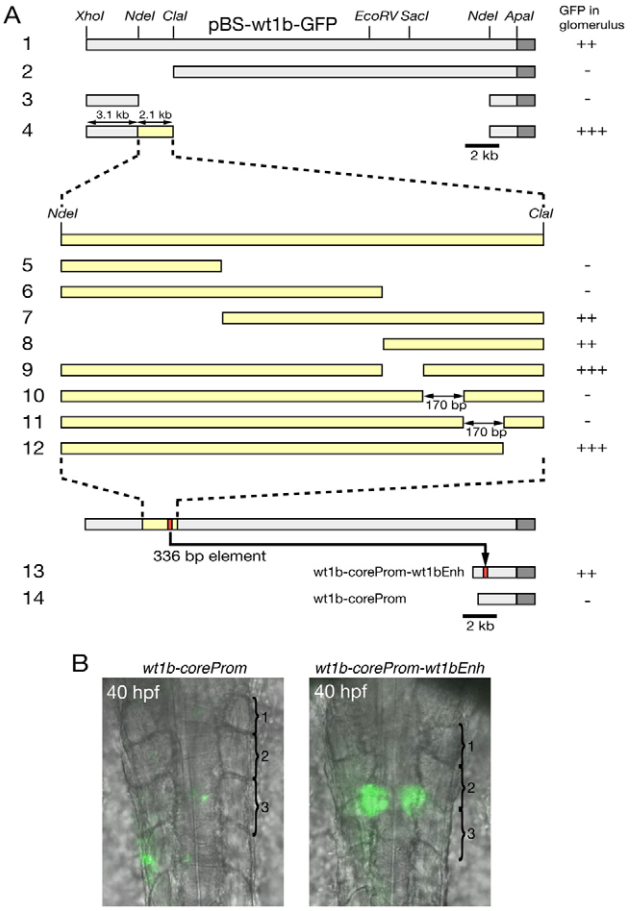


**Fig. 4. A 336 bp regulatory region upstream of *wt1b* is necessary and sufficient for expression in the pronephric glomeruli.** (**A**) Deletion constructs were generated using plasmid pBS-wt1b-GFP (row 1). Construct numbers are indicated on the left. Dark gray bars represent the *GFP* gene, yellow bars represent the 2.1 kb region between *Nde*I and *Cla*I sites or subfragments thereof (shown in higher magnification in rows 5-12). At least 40 embryos were investigated for *GFP* expression in the glomeruli, which are located ventral of the third somite, 24 hours after injection of the respective reporter construct. For quantification, the number of embryos with *GFP* expression in the glomerulus was divided by the total number of *GFP*-positive embryos (*GFP* expression anywhere in the embryo). +++, >40%; ++, 20-40%; –, <5%. (**B**) Overlay of brightfield transmission and fluorescence images of stable *wt1b-coreProm* and *wt1b-coreProm-wt1bEnh* transgenic embryos (dorsal view). The first three somites are numbered and are marked by parentheses.

Deletion of the conserved 100 bp fragment in the transgene, to give *wt1a-6.8kb-delta*::*GFP* embryos, completely abrogated early *GFP* expression in the intermediate mesoderm (see Fig. S3 in the supplementary material). Altogether, our data strongly indicate that the conserved region upstream of *wt1a* is indispensable for early *wt1a* expression in the intermediate mesoderm.

## Retinoic acid acts via the conserved region upstream of *wt1a*

Two pieces of evidence suggested that the conserved region upstream of *wt1a* could be a target for retinoic acid (RA) signaling. First, a conserved element within this region is predicted to act as an RXR heterodimer binding site (Fig. 5B, cons 1). Second, it was

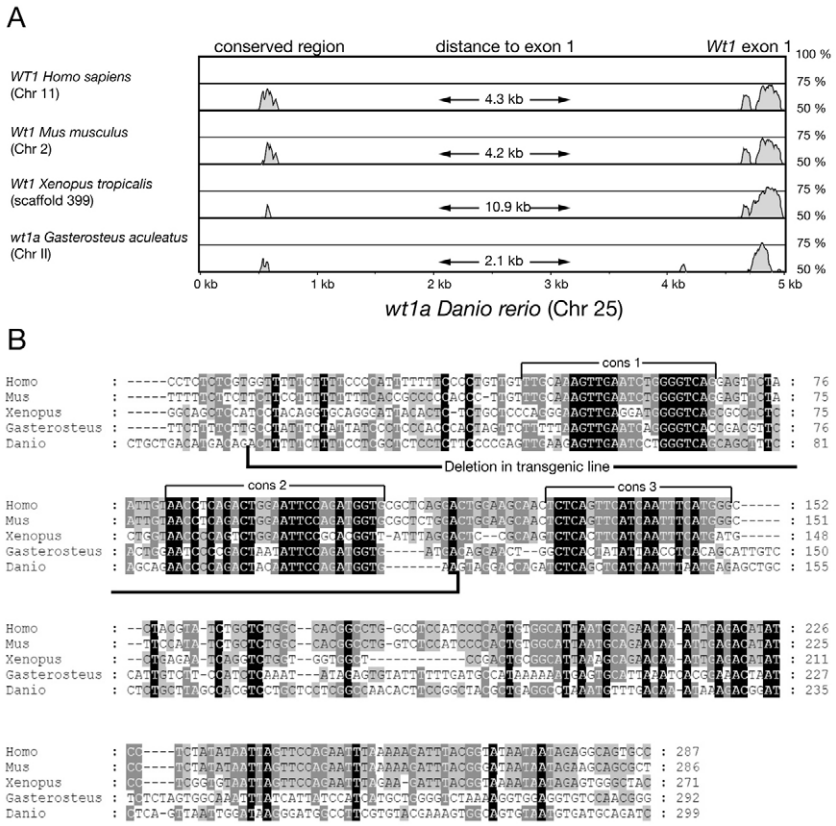


**Fig. 5. Alignment of *Wt1* genomic regions reveals a highly conserved element upstream of zebrafish *wt1a*.** (**A**) Genomic sequences upstream of *Wt1* from human (*Homo sapiens*, 8 kb), mouse (*Mus musculus*, 8 kb), frog (*Xenopus tropicalis*, 12 kb) and stickleback (*Gasterosteus aculeatus*, *wt1a*, 4 kb) were aligned to a 5 kb genomic zebrafish (*Danio rerio*) sequence upstream of *wt1a*. The different sequence lengths (4-12 kb) are due to different distances of the conserved region to the first exon (2.1-10.9 kb). The calculation window was 50 bp with a minimum conservation identity of 60%. (**B**) The conserved regions shown in A from human, mouse, frog, stickleback and zebrafish were aligned at the nucleotide level. For graphical representation the GeneDoc software (www.nrbsc.org/gfx/genedoc/) was used. Nucleotides that are identical in all species are shaded in black and those that are identical in four or three species are shaded in dark and light gray, respectively. The three conserved elements (cons 1-3) are indicated.

shown recently that treatment of zebrafish embryos with all-trans retinoic acid (atRA) leads to a strong increase of *wt1a* mRNA levels in the intermediate mesoderm (Wingert et al., 2007). In order to test whether the conserved element upstream of *wt1a* indeed mediates RA responsiveness, we treated zebrafish embryos from different transgenic lines with 1 μM atRA from 12 hpf to 24 hpf (Fig. 7). In atRA-treated *wt1a*::*GFP* embryos (35 kb fragment) a strong increase in and also an expansion of *GFP* expression was detectable, whereas in *wt1b*::*GFP* embryos reporter gene expression decreased slightly after RA treatment. This indicates the presence of RA responsive element(s) in the region upstream of *wt1a*. The strongest increase of GFP fluorescence was observed in atRA-treated embryos harboring the truncated *wt1a* transgene (*wt1a-6.8kb*::*GFP*). With this construct, ectopic *GFP* expression was found in the brain and in the tail, probably due to a loss of inhibitory elements. By contrast, responsiveness of the reporter gene to RA was completely lost in all three *wt1a-6.8kb-delta*::*GFP* lines that lack the 100 bp fragment within the conserved region. In conclusion, we provide evidence that the conserved region upstream of *wt1a* is responsive to RA in vivo.

## The *Wt1* enhancer is bound by RAR/RXR complexes in vitro and is activated in RA-treated P19 embryonic carcinoma cells

Retinoic acid acts by binding to heterodimers of one of the family members of the retinoic acid receptor (RAR) α/β/γ transcription factors with the partner protein retinoic X receptor (RXR). We therefore examined binding of RAR/RXR proteins to the *Wt1* enhancer by EMSA analysis. The zebrafish and the human *Wt1* enhancers encompassing all three conserved elements were bound by complexes of RARβ and RXRα but not by either of the proteins alone (Fig. 8A). The binding could be competed with an excess of unlabeled wild-type probe but not with a fragment that harbored a deletion of the conserved region 1. No gel shift could be observed when the latter was used as labeled probe (data not shown). This shows that members of the RAR/RXR family preferentially bind to conserved region 1. Pre-incubation of the extracts with RARβ antiserum, but not with a control antiserum, resulted in immunodepletion of the DNA-protein complexes. Of note, the zebrafish *wt1a* enhancer was efficiently bound by a complex of the mammalian RARβ and RXRα proteins.

We then used extracts from P19 mouse embryonic carcinoma cells and performed supershift experiments using the human *WT1* enhancer and antibodies against RARβ and RARα. We chose P19 cells because it has been shown that *Wt1* expression is strongly induced in these cells after differentiation with RA (Scharnhorst et al., 1997). After 24 hours of treatment with RA, the extracts showed a supershift with the RARβ as well as with the RARα antibody (Fig. 8B). In the absence of RA, a supershift was only obtained with the RARα antibody. This could be explained by the presence of different levels of the two transcription factors in P19 cells. Moreover, RA treatment of P19 cells seemed to enhance RARβ and RARα levels and/or increased their binding affinity. These data show that the endogenous proteins of the RAR family can also bind to the *WT1* enhancer.

In order to test the function of the conserved region upstream of human *WT1*, we performed luciferase reporter assays in P19 cells. The human conserved region (287 bp) was cloned in front of the SV40 promoter (pGL3-WT1-Enh, Fig. 8C). In addition, constructs were generated in which the elements that show the highest conservation among vertebrates were deleted (Δ1-3). No significant difference in luciferase activity was seen in cells transfected with the different constructs after treatment with the vehicle alone (Fig.

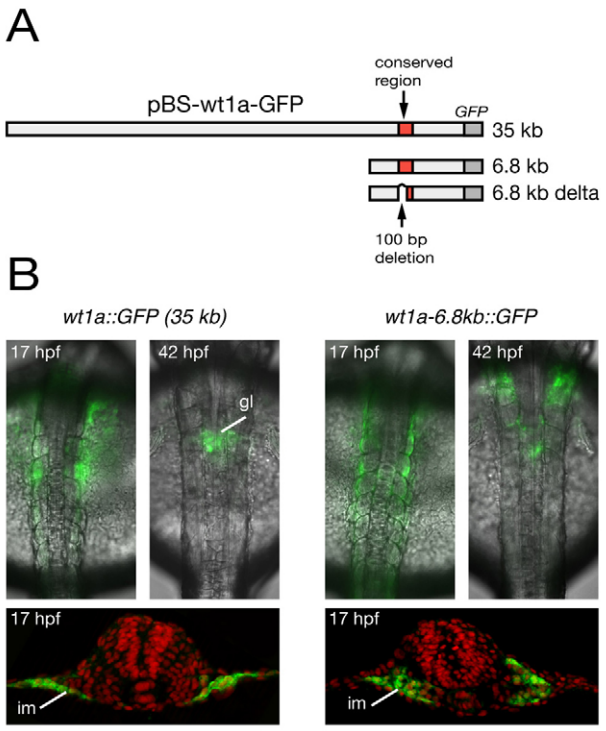


**Fig. 6. The conserved region upstream of *wt1a* is required for its expression in the intermediate mesoderm.** (**A**) Schematic representation of *wt1a* reporter constructs. The upper row represents the plasmid pBS-wt1a-GFP, which was used for the generation of *wt1a*::*GFP* transgenic zebrafish. In addition, two truncated wt1a-GFP plasmids are shown containing a 6.8 kb genomic fragment (middle row) and the same fragment lacking 100 bp within the conserved region (lower row). A detailed illustration of the deleted 100 bp region is shown in Fig. 5B. (**B**) Overlay of brightfield transmission and fluorescence images from *wt1a*::*GFP* (left) and *wt1a-6.8kb*::*GFP* (right) transgenic embryos at 17 and 42 hpf (top). For detailed analysis, embryos at 17 hpf were stained with anti-GFP antibody (green) and were sectioned (bottom). Counterstaining with DAPI is shown in false color (red). gl, glomeruli; im, intermediate mesoderm.

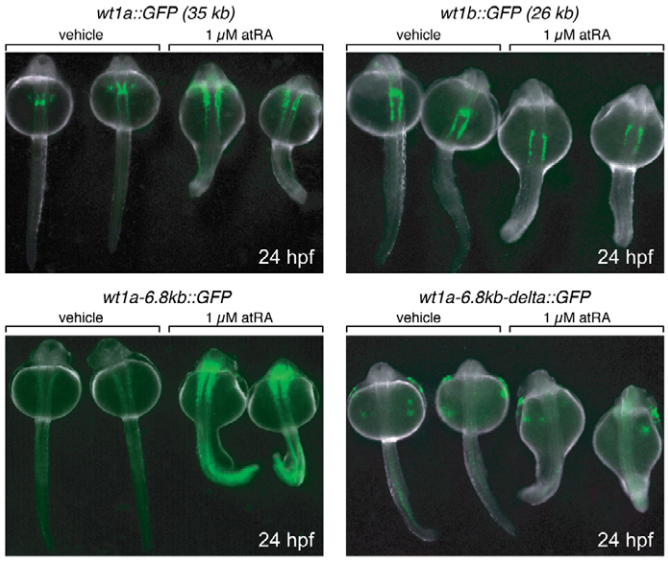


**Fig. 7. Retinoic acid acts via the conserved region upstream of *wt1a*.** Embryos of the indicated transgenic lines were treated either with 1 μM all-trans retinoic acid (atRA) or 0.1% DMSO (vehicle) from 12 hpf (6-somite stage) to 24 hpf. Subsequently, embryos were embedded in 3% methyl cellulose and imaged with a fluorescence stereomicroscope (dorsal view). RA- and vehicle-treated embryos of each line were captured together. Note that the weak fluorescence signal of the vehicle-treated *wt1a-6.8kb*::*GFP* embryos is due to the short exposure time used for recording the image.

8D). However, in cells treated with RA the *WT1* enhancer region mediated a threefold increase in luciferase activity compared with pGL3-transfected cells. In line with the EMSA analysis, deletion of the first conserved element led to a total abrogation of this effect, whereas the enhancer regions harboring either the second or third deletion were still partially responsive to RA. This demonstrates that the conserved enhancer upstream of *Wt1* is involved in the induction of *Wt1* expression by RA both in vivo in zebrafish and in mammalian cells, thus underscoring the function of this element.

## DISCUSSION

In this study, we took advantage of the more compact *wt1* loci (compared with human and mouse) and efficient transgenesis in zebrafish. We identified two genomic regions that are important for the expression of the *wt1* paralogs in the developing zebrafish pronephros. The first 336 bp element lies ~21 kb upstream of the *wt1b* gene and drives glomerular expression, whereas the second 299 bp element lies 4.2 kb upstream of the *wt1a* gene, is responsive to retinoic acid (RA) and is necessary for early expression in the intermediate mesoderm.

### The enhancer element upstream of *wt1b* drives glomerular expression but seems not to be conserved among vertebrates

Based on the similarities of the *Wt1* expression pattern in different vertebrates, including mammals, reptiles, amphibians and fish (Bollig et al., 2006; Carroll and Vize, 1996; Kent et al., 1995), we assumed that the mechanisms driving the glomerular expression of *Wt1* are conserved between mammals and fish, and involve a set of orthologous transcription factors. Surprisingly, we could not find any sequence conservation of the identified 336 bp *wt1b* enhancer region in mammals and not even in other fish species such as medaka, fugu and tetraodon.

There is a growing list of studies demonstrating that the regulatory function of enhancer elements is maintained over long evolutionary distances despite the remarkable turnover of transcription factor binding sites. For instance, it was shown that regulatory sequences from the human *RET* locus are capable of driving *ret*-specific expression in zebrafish, even though no sequence similarity between human and zebrafish regulatory elements was detected (Fisher et al., 2006). Along the same line, enhancer elements from the *even skipped* locus, which are functionally conserved between scavenger flies (Sepsidae) and vinegar flies (Drosophilidae), do not show sequence conservation (Hare et al., 2008). Based on these studies, we assume that elements exist in other vertebrates, which are functionally equivalent to the *wt1b* enhancer region that drives expression in the developing pronephric glomerulus. However, alterations within transcription factor binding sites such as base substitutions might obscure the identification of homologous elements by sequence comparison.

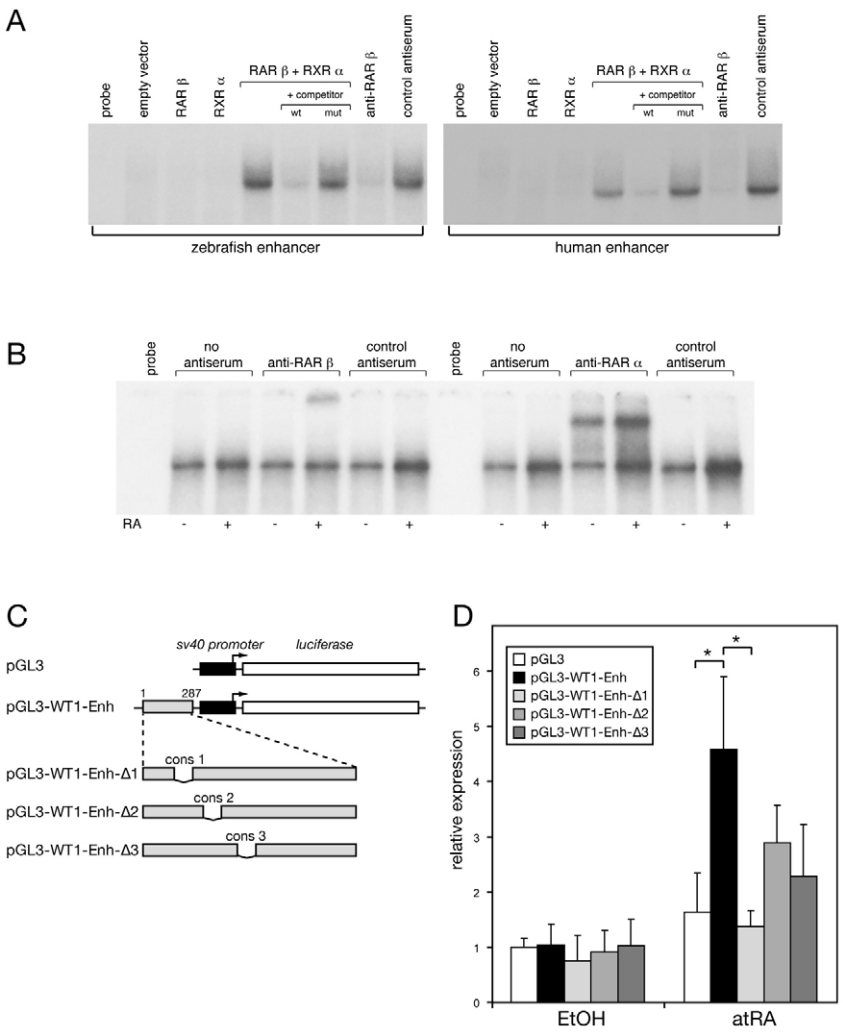


**Fig. 8. Characterization of the retinoic-acid-responsive *Wt1* enhancer.** (**A**) Electrophoretic mobility shift assay (EMSA) using in vitro transcription/translation (TNT) products of RARβ and RXRα on the zebrafish (left) and the human (right) enhancer. TNT extract programmed with the empty Rc/CMV vector was used as a negative control. Mutant fragments for competition experiments harbored a deletion of conserved region 1 (cons 1) in the background of the zebrafish or human enhancer. For supershifts, an antibody against RARβ or control antiserum was used. (**B**) Supershift assays using the human *WT1* enhancer fragment as the probe. Nuclear extracts were prepared from P19 cells treated with RA (+) or ethanol (–) for 24 hours. Analysis was performed using antibodies specific for RARβ and RARα or respective control antibodies. The different mobility of the RARβ and RARα supershift complexes is most likely to be explained by the antibodies used (monoclonal versus polyclonal antiserum). Note that pre-incubation of the extracts with RARβ antiserum resulted in either immunodepletion (A) or supershift (B) of the DNA-protein complexes, depending on the type of extract. (**C**) Schematic luciferase reporter constructs. The sequence of the inserted human *WT1* enhancer region (287 bp) and of the deleted conserved elements (cons 1-3) is shown in Fig. 5B. (**D**) P19 cells were transfected with the constructs shown in A and grown for 4 days in the presence of 1 μM all-trans retinoic acid (atRA) or vehicle (0.1% ethanol). Subsequently, cells were lysed and luciferase activity was measured. *Luciferase* expression in vehicle-treated cells transfected with pGL3 vector was set to one. The figure shows one representative experiment. Data represent the means and standard deviations of triplicates. Asterisks indicate statistically significant differences, **P*<0.05, Student's *t* test. mut, mutant; wt, wild type.

## The enhancer region upstream of *wt1a* is highly conserved among vertebrates and mediates responsiveness to retinoic acid

We have identified a 299 bp region ~4.2 kb upstream of the *wt1a* gene in zebrafish that is conserved in other fish species, amphibians, reptiles and mammals. According to the criteria set by Bejerano et al. (Bejerano et al., 2004), the respective region upstream of human *WT1* can almost be regarded to be an 'ultraconserved' element, with an identity of 97.8% over 226 bp to the orthologous regions in mouse and rat. Interestingly, it has been reported that 'ultraconserved' regions are often located around genes that are involved in the regulation of development, including a large set of transcription factor genes (Bejerano et al., 2004; Woolfe et al., 2005). Many of these conserved regions have been shown to function as tissue-specific enhancers in transgenic zebrafish (Woolfe et al., 2005) and in transgenic mice (Pennacchio et al., 2006).

It is notable that the genes *wt1a* and *wt1b* themselves show very high sequence homology (Bollig et al., 2006), whereas the respective regulatory regions are vastly different. This is in line with the notion that after gene duplications the control region sequences of the duplicates change much faster than the sequences within the genes, and that subfunctionalization occurs at the regulatory level rather than at the level of the respective coding region (Kleinjan et al., 2008).

Retinoic acid (RA) has been shown to act as a morphogen in embryonic development (Ross et al., 2000). In this process, its local concentration is regulated by both synthesizing (Raldh family) and metabolizing (cytochrome P450) enzymes (Ross et al., 2000). RA controls gene expression at the transcriptional level, functioning as a ligand for heterodimeric nuclear receptors (RXR and RAR) that typically bind to direct repeats of hexamer sequences separated by one to five nucleotides (Germain et al., 2006).

Within the *wt1a* enhancer we found three stretches of nucleotides that are conserved between mammals and fish. All investigated sequences harbored the motif AGTTGA-$(N)_5$-GGGTCA, predicted as a nuclear receptor binding site, within the first conserved element. Deletion of a region including the first two conserved elements caused a total loss of intermediate mesoderm expression and responsiveness to RA in transgenic fish in vivo. Consistently, an in-vitro-translated RAR/RXR complex bound to the human as well as the zebrafish *Wt1* enhancer element in vitro. This binding was abrogated upon deletion of conserved region 1. In addition, we could show by supershift experiments that endogenous RARα and RARβ from P19 cells also bind to the human *WT1* enhancer. Finally, in reporter gene assays using P19 cells, deletion of either one of the three elements in the context of the human enhancer region led to a significant reduction of RA-mediated activation, whereby deletion of the first element had the most dramatic effect.

In zebrafish, the inhibition of RA signaling results in a loss of the glomeruli and proximal parts of the tubules (Wingert et al., 2007). It has been shown in this study by Wingert et al. that RA-deficient embryos failed to express *wt1a* in the intermediate mesoderm, whereas RA-treated embryos showed increased *wt1a* expression. This indicates that *wt1a* expression in the intermediate mesoderm is driven by endogenous RA and that in RA-deficient embryos the lack of Wt1a contributes at least in part to the observed phenotype. The authors of this study proposed a model in which the adjacent paraxial mesoderm is the source for RA.

Our data suggest that the induction of *wt1a* by RA is mediated by the conserved enhancer that we have identified. It is tempting to speculate that this is a common mechanism in all vertebrates, as both the conserved region and early *Wt1* expression in the intermediate mesoderm (Armstrong et al., 1993; Kent et al., 1995) can be found in several vertebrates.

**Acknowledgements**

We thank Jürgen Klattig, Helmut Pospiech, Eric Rivera-Milla and Christoph Winkler for critically reading and improving this manuscript, as well as Klaus Huse and Karol Szafranski for stimulating discussions. We also thank Hinrich Gronemeyer for the anti-RAR antiserum; Aria Baniahmad for providing the RAR and RXR plasmids; and Ulrike Gausmann for help in bioinformatics. We are grateful to Dagmar Kruspe for technical support and to Florian Perner for help with the art work. This work was supported by grant En280/7-1 from the Deutsche Forschungsgemeinschaft to C.E.

**Supplementary material**

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/17/2883/DC1

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