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PAX8-mediated activation of the *wt1* tumor suppressor gene

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The developing renal system has long been exploited to study the regulation of gene expression during mesenchymal–epithelial transitions. Several transcription factors, including WT1 and PAX8, are expressed early in nephrogenesis and play a key role in this process. The expression of PAX8 occurs in the induced mesenchyme of the developing kidney prior to the upregulation of WT1 levels in the same cells. In this report, we assessed whether the *Pax-8* gene product resides upstream of *wt1* in a common regulatory pathway. Transfection studies, as well as gel-shift assays, indicate that PAX8 transactivates *wt1* through elements within a 38 bp conserved motif, present in human and murine promoters. Two PAX8 isoforms, generated by alternative splicing at the C-terminus and previously thought to lack transactivation potential, were found to be capable of activating *wt1* expression. We also demonstrate that the endogenous *wt1* promoter can be upregulated by exogenously supplied PAX8, suggesting that a function of PAX8 during mesenchymal–epithelial cell transition in renal development is to induce *wt1* gene expression.

Keywords: kidney development/*Pax-8*/Wilms tumor/*wt1*

Introduction

Mammalian kidney development involves the reciprocal inductive interaction between the Wolffian duct-derived ureteric bud and the metanephrogenic mesenchyme (for a review see Paterson and Dressler, 1994). This event results in increased cellular proliferation and differentiation in the mesenchyme and ureteric bud. Genetic lesions which deregulate this process result in renal agenesis, kidney malformations and malignancies. Wilms tumors (WTs) have long been thought to be a paradigm for the study of fetal kidney development gone awry because this malignancy is derived from embryonic tissue that persists beyond fetal development. This pediatric cancer affects ~1/10 000 children and exhibits a broad range of differentiation, classically consisting of three cell types: blastemal, epithelial and stromal (Beckwith *et al.*, 1990). The tumor suppressor gene, *wt1*, is involved in the initiation of this disease (Park *et al.*, 1993) and plays a paramount role in normal urogenital development (Pelletier *et al.*, 1991a; Kriedberg *et al.*, 1993).

The *wt1* gene encodes a transcription factor belonging to the early growth response family of Cys_2–His_2 zinc finger proteins and is mutated in 5–15% of sporadic WTs (Call *et al.*, 1990; Gessler *et al.*, 1990; Little *et al.*, 1992; Coppes *et al.*, 1993; Varanasi *et al.*, 1994). The pre-mRNA is alternatively spliced at two coding exons to produce four WT1 polypeptide isoforms containing four zinc fingers (Haber *et al.*, 1991). The first alternatively spliced exon inserts or removes 17 amino acids, and is capable of mediating repression when fused to a heterologous DNA binding domain (Wang *et al.*, 1995). The second alternative splice site inserts or removes three amino acids (KTS), between zinc fingers III and IV, and alters the DNA binding specificity of the protein (Rauscher *et al.*, 1990). Additional diversity is provided by an alternative translation initiation event upstream of, and in-frame with, the *wt1* initiator ATG, producing isoforms with unique N-termini (Bruening and Pelletier, 1996). All these isoforms localize to separate but overlapping regions in the nucleus, suggesting possible functional differences (Larrson *et al.*, 1995). The WT1 isoforms recognize the GC-rich motif, 5′-GCGGGGGCG-3′, as well as a $(TCC)_n$ motif, albeit with different affinities, and can affect the expression of a number of genes involved in the regulation of cell proliferation or differentiation (for a review see Rauscher, 1993). These include insulin-like growth factor (IGF)-II (Drummond *et al.*, 1992), IGF-I receptor (Werner *et al.*, 1993), platelet-derived growth factor A-chain (Gashler *et al.*, 1992; Wang *et al.*, 1992), colony stimulating factor-1 (Harrington *et al.*, 1993), transforming growth factor-β1 (Dey *et al.*, 1994), retinoic acid receptor-α (Goodyer *et al.*, 1995), Pax-2 (Ryan *et al.*, 1995), c-*myb* (McCann *et al.*, 1995), epidermal growth factor receptor (Englert *et al.*, 1995) and the *wt1* gene itself (Rupprecht *et al.*, 1994). The *wt1* gene product mediates both transcriptional repression (Drummond *et al.*, 1992; Harrington *et al.*, 1993; Dey *et al.*, 1994; Rupprecht *et al.*, 1994; Goodyer *et al.*, 1995) and activation (Wang *et al.*, 1992), depending on the architecture of the promoter under study and the cell lines in which the transfection assays are performed.

The *wt1* promoter has three transcription initiation sites and is a member of the GC-rich, TATA-less and CCAAT-less class of RNA polymerase II genes (Pelletier *et al.*, 1991b; Hofmann *et al.*, 1993). Elements responsible for regulating the tissue-specific expression of *wt1* are not known, although Sp1 and GATA-1 have been shown to positively modulate *wt1* expression (Hofmann *et al.*, 1993; Wu *et al.*, 1995). In addition, a cell type-specific enhancer has been identified within the 3′ end of the human *wt1* gene (Fraizer *et al.*, 1994). Both the 3′ enhancer element and GATA-1 factor have been implicated in regulating *wt1* only during hematopoiesis, and are not functional in the developing urogenital system where expression of the *wt1* gene product is spatially and temporally regulated (Pritchard-Jones *et al.*, 1990; Pelletier *et al.*, 1991b). WT1

is present in tissues of mesodermal origin that undergo a mesenchymal–epithelial transition, such as the fetal gonads and kidneys. Immunohistochemical studies have demonstrated that the WT1 protein is nuclear and present in all tissues expressing *wt1* mRNA (Mundlos *et al.*, 1993). The *wt1* gene is expressed in the induced renal mesenchyme, as well as in differentiating epithelial structures such as S-shaped bodies and the glomerular podocytes of the fetal kidney (Pritchard-Jones *et al.*, 1990). WT1 plays an essential role in differentiation of the urogenital system, as evidenced by *wt1*-null mice in which this system fails to develop (Kriedberg *et al.*, 1993), as well as by aberrant differentiation of this system in many children with germline *wt1* lesions (Pelletier *et al.*, 1991a; Bruening and Pelletier, 1994).

Pax-8 and *Pax-2* belong to a gene family that shares a common protein coding domain, the paired-box, first described in the *Drosophila* segmentation genes *paired* and *gooseberry* (Bopp *et al.*, 1986). Both gene products have been implicated in normal renal development. *Pax-2* is expressed during early nephrogenesis in both the induced metanephric blastema and the inducing ureteric bud, and then abruptly downregulated. Ryan *et al.* (1995) have demonstrated that WT1 can repress *Pax-2* expression, and we have demonstrated recently that PAX2 can transactivate the *wt1* promoter *in vitro* and *in vivo*, suggesting the existence of a regulatory circuit between these two genes (Dehbi *et al.*, 1996). The constitutive expression of *Pax-2* in transgenic mice results in kidney abnormalities of the glomerular and tubular epithelium, implying that the temporal regulation of this gene product is essential for proper renal development (Dressler *et al.*, 1993). *Pax-8* is expressed earlier than the *wt1* gene in the developing kidney, predominantly in induced tissue (i.e. mesenchymal condensations and S-shaped bodies), and is abruptly downregulated after reciprocal induction by the ureter (Poleev *et al.*, 1992). *Pax-8* and *Pax-2* are expressed in a high percentage of WTs (Dressler and Douglass, 1992; Poleev *et al.*, 1992; Tagge *et al.*, 1994; Eccles *et al.*, 1995), consistent with the embryonic origin of these tumors. In this study, we have addressed whether PAX8 can act as a potential upstream regulator of *wt1*. Our data indicate that *Pax-8* can activate the *wt1* promoter. We speculate that this induction is important for normal renal development.

Results

Identification of a conserved motif in human and murine promoters

Three transcription initiation sites define the immediate proximal murine and human *wt1* promoter sequences (Pelletier *et al.*, 1991b; Hofmann *et al.*, 1993). Sequence comparison of the human and murine promoters revealed the presence of a 38 bp conserved region (CR) between positions –71 and –33, relative to the first transcription start site of the murine promoter (Figure 1A). Of 38 base pairs of the CR, 37 are identical in both the human and murine promoters. Present within the CR are binding sites for Sp1, as well as a core motif recognized by paired-domain proteins (Dressler and Douglass, 1992; Zannini *et al.*, 1992; Figure 1A).

PAX8 modulates wt1-dependent reporter activity

The presence of core *Pax* binding sites within the *wt1* promoter prompted us to investigate the possibility that *Pax-8* could regulate *wt1* expression, because PAX8 levels peak before *wt1* expression is upregulated in the developing kidney (Paterson and Dressler, 1994). To this end, we cloned a 3.1 kbp genomic fragment harboring the *wt1* promoter upstream of the bacterial chloramphenicol acetyl transferase (CAT) gene (*Sal*I–CAT) and generated a series of promoter deletion mutants (Figure 1B). Expression of the *Pax-8* gene is developmentally regulated and alternatively spliced to generate at least six different isoforms (Kozmik *et al.*, 1993). The most abundant of these, *Pax-8a*, was used in a series of transfection studies performed in cells which express little (293) or no (K562 and NIH 3T3) *Pax-8* mRNA. When cytomegalovirus (CMV)/*Pax-8a* and *Sal*I–CAT were introduced into 293 (Figure 2A) or NIH 3T3 (Figure 2C) cells, CAT activity was reproducibly enhanced 4- to 7-fold. As a negative control, cells co-transfected with the reporter pCAT–Enh (which lacks *wt1* upstream regulatory elements) failed to demonstrate activation by PAX8a (Figure 2A–C).

In the kidney 293 cell line, co-transfection of CMV/*Pax-8a* with reporters containing the CR element consistently produced a 4- to 7-fold activation (Figure 2A). Removal of the CR element (*Bgl*II^{E}–CAT) generated a reporter construct no longer capable of responding to PAX8a. Similar results were obtained in K562 cells, an erythroleukemic cell line which expresses low levels of *wt1* (Call *et al.*, 1990). Expression of the two deletion mutants, *Spe*I–CAT and *Eco*RI–CAT, is stimulated 7- to 8-fold in the presence of PAX8a, compared with levels obtained with CMV/Δ (Figure 2B). The reporter *Bgl*II^{I}–CAT, harboring the 38 bp CR element, is transactivated ~4-fold in K562 cells when co-transfected with CMV/*Pax-8a* (Figure 2B). This is in contrast to the reporter plasmid lacking the CR, *Bgl*II^{E}–CAT, which fails to respond to PAX8a (Figure 2B). We conclude that the CR within the *wt1* promoter is capable of mediating transactivation by PAX8a in 293 and K562 cells.

These results were also reproduced in a cell that does not express endogenous *wt1* mRNA, NIH 3T3 cells. Heterologous reporter constructs were still responsive to PAX8 in NIH 3T3 cells, although the degree of transcriptional activation was not as pronounced as in K562 or 293 cells. Deletion mutants *Sal*I–CAT, *Spe*I–CAT and *Eco*RI–CAT were reproducibly upregulated (~3- to 4-fold) upon the inclusion of CMV/*Pax-8a* in the transfection mixture (Figure 2C). The deletion mutant *Bgl*II^{I}–CAT showed a 4-fold stimulation in response to PAX8a (Figure 2C). Removal of the 38 bp CR (*Bgl*II^{E}–CAT) abolished this response, demonstrating the requirement for a DNA motif within the CR for PAX8a-mediated activation (Figure 2C).

Activation of the wt1 promoter by different PAX8 isoforms

A number of PAX8 isoforms have been described and characterized for their transactivation properties (Kozmik *et al.*, 1993). All PAX8 isoforms retain the paired domain as their DNA binding motif. The most abundant isoform in the kidney is PAX8a, which contains a serine/threonine/tyrosine-rich C-terminus, whereas two less abundant iso-

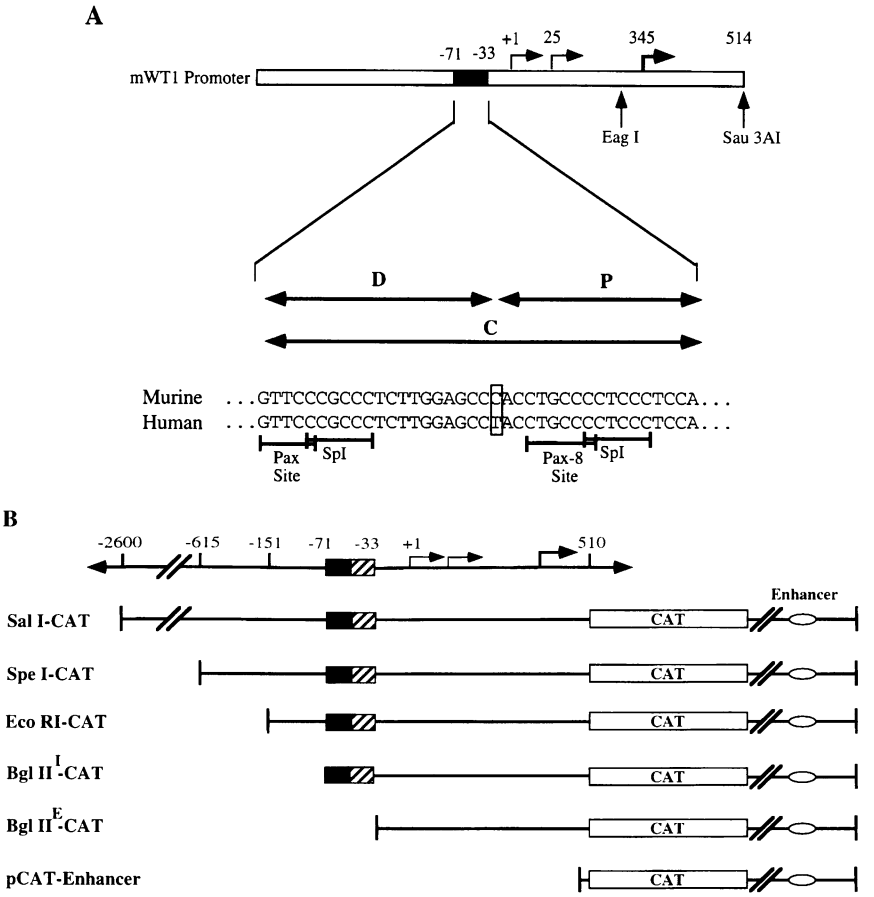

Fig. 1. Schematic representation of the murine *wt1* promoter and *wt1* promoter deletions. (**A**) The relative positions of the transcription initiation start sites are shown with right-angled arrows. The thickness of the arrow indicates the relative strengths of the transcription initiation sites. The unique CR element is represented by a blackened box. Within the CR are Sp1 and Pax binding sites. A sequence comparison between the human and murine CR motif is shown, with the single nucleotide difference between the two species highlighted in a box. For functional studies presented here, the CR element (referred to as C) was also subdivided into two regions, D and P, both of which contain Sp1 and Pax binding sites. (**B**) Schematic representation of the WT1–CAT reporter constructs used in this study. Upstream deletions within the *wt1* promoter were generated as described in the text and fused to the CAT reporter gene as shown. The 38 bp CR has been divided into two regions: a distal part (D) denoted by a blackened box, and a proximal part (P) denoted by a hatched box. The sites of transcription initiation are represented by right-angled arrows. The CAT coding region is denoted by a box, whereas the SV40 enhancer element is denoted by an oval.

forms, PAX8c and PAX8d, are alternatively spliced and produce proteins in which the C-terminus is translated in a different, proline-rich reading frame (Figure 3A). Although PAX8a can stimulate transcription from a heterologous reporter containing paired-domain recognition sequences, PAX8c and PAX8d cannot (Kozmik *et al.*, 1993).

Therefore we undertook a series of transfection experiments to determine if the PAX8c or PAX8d products could affect expression from the *wt1* promoter. In our system, both PAX8c and PAX8d were capable of stimulating *wt1* promoter activity (Figure 3B; see *Eco*RI–CAT and *Bgl*II^{I}–CAT). This transactivation potential was dependent on the presence of the CR element, because *Bgl*II^{E}–CAT failed to respond to CMV/*Pax-8a*, CMV/*Pax-8c* or CMV/*Pax-8d*, whereas *Bgl*II^{I}–CAT did respond. These results ascribe a novel function to the PAX8c and PAX8d isoforms and indicate that they are competent for mediating transactivation.

The CR element can mediate PAX8 but not PAX5 responsiveness in a heterologous context

To analyze the structural requirements for *Pax-8* responsiveness within the CR element, we inserted multiple copies of this motif upstream of the minimal human β globin promoter, pβ–CAT, to produce pC-β–CAT (Figure 4A). In addition, multiple copies of the D as well as P subfragments of the CR were introduced into the same reporter vector (Figure 4A). Mutations which abolished the putative PAX sites in either D or P subfragments were also generated (pΔD-β–CAT and pμP-β–CAT). A deletion in the CR site which does not affect the core PAX or Sp1 binding sites was also tested (pΔC-β–CAT) (see legend to Figure 4). Expression from the reporter pC-β–CAT was stimulated ~8-fold by CMV/*Pax-8a* compared with pβ–CAT (Figure 4B). A stimulatory effect was also observed on pΔC-β–CAT, where an 8 bp deletion adjacent to the PAX binding site has been created. This mutation does not affect PAX8a DNA binding and serves to demonstrate that the CR element can function in an orientation-independent fashion to mediate *Pax* responsiveness. PAX8a was found to transactivate the β-globin promoter through both P and D subfragments of the CR element (pD-β–CAT and pP-β–CAT). PAX8a activation requires the integrity of a *Pax* core binding site because a point mutation or a deletion in the *Pax* element within the P (pμP-β–CAT) or D (pΔD-β–CAT) fragment, respectively (Figure 4A), prevented transactivation. The ability of

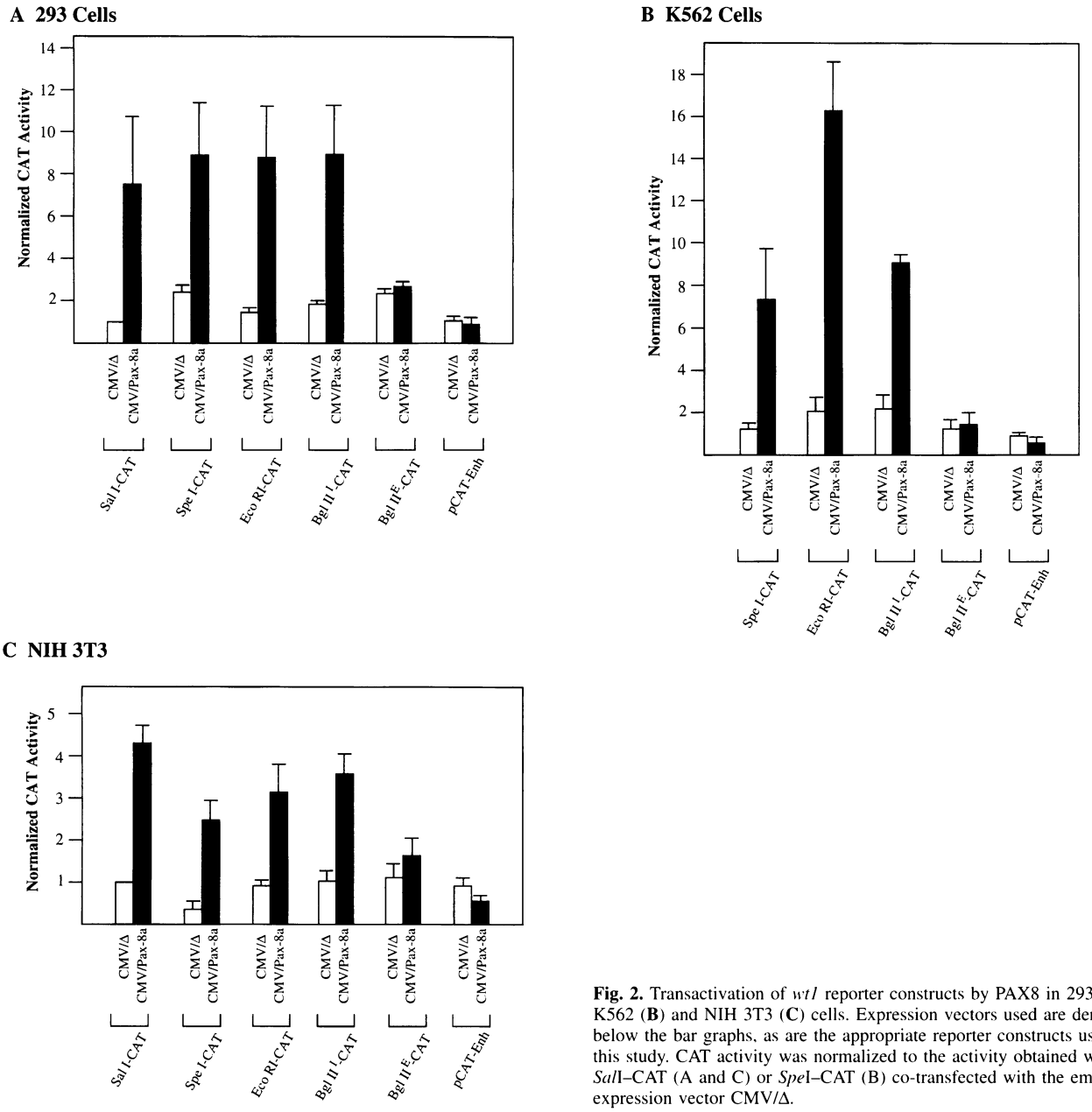

Fig. 2. Transactivation of *wt1* reporter constructs by PAX8 in 293 (**A**), K562 (**B**) and NIH 3T3 (**C**) cells. Expression vectors used are denoted below the bar graphs, as are the appropriate reporter constructs used in this study. CAT activity was normalized to the activity obtained with *Sal*I–CAT (A and C) or *Spe*I–CAT (B) co-transfected with the empty expression vector CMV/Δ.

PAX8a to transactivate pC-β–CAT and pP-β–CAT has been reproduced in the kidney cell line, COS-1, as well as in NIH 3T3 cells, indicating that these results are not cell specific (M.Dehbi, unpublished data). Our results indicate that two sites within the CR motif (one within D and one within P) are responsible for mediating PAX8a responsiveness.

Previous experiments from our laboratory have demonstrated that PAX2 can also mediate transactivation of the *wt1* reporter, and that this effect is mediated only through the D element (Dehbi *et al.*, 1996). Because substantial sequence and structural homology exists among PAX2, 5 and 8, we wished to assess whether PAX5 could also activate the *wt1* promoter. Figure 5 demonstrates that PAX5 did not significantly transactivate the reporters pΔ1-β–CAT or pP-β–CAT.

Binding of PAX8a to the CR element

To complement the above functional studies, we assessed the potential of PAX8a to interact directly with the 38 bp CR. To this end, D and P segments were used as probes in a series of electrophoretic mobility shift assays (EMSA) using PAX8a produced by *in vitro* translation. The PAX8a protein has been shown previously to bind the promoters and activate the transcription of three cell-specific genes: thyroglobulin (Tg; Zannini *et al.*, 1992), thyroperoxidase (Francis-Lang *et al.*, 1992) and the gene encoding neural cell adhesion molecule, N-CAM (Holst *et al.*, 1994). This effect is mediated through an interaction of PAX8a with a core motif consisting of 5′-TGGCC-3′ or 5′-GTCC-3′. Results shown in Figure 6 demonstrate that oligonucleotides containing the D and P subsites of the CR form two complexes with PAX8a (indicated by an arrowhead and an arrow). One of the complexes (indicated by an arrow) formed on oligonucleotides D and P could be competed with a 100-fold molar excess of D (compare lane 2 with lane 1) or P (compare lane 6 with lane 5), but not by oligonucleotides containing mutations within the PAX binding site (M.D. or M.P.) (compare lanes 4 and 8 with

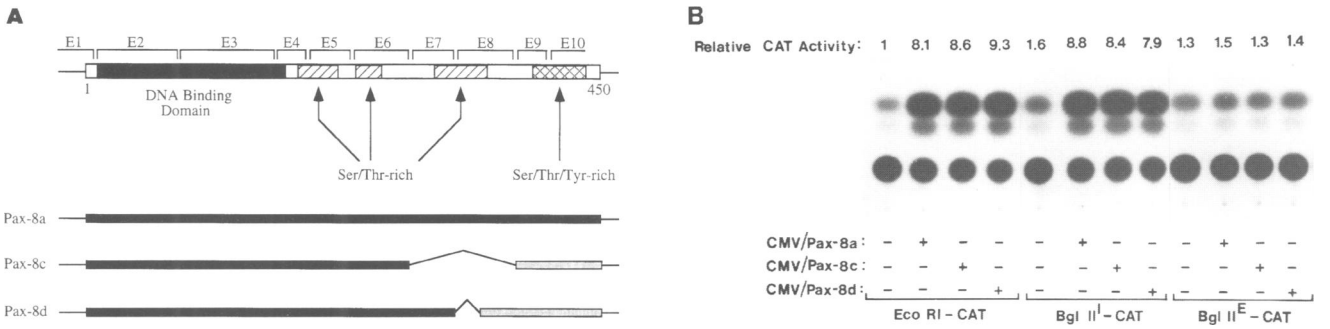

Fig. 3. Transactivation of *wt1* by three PAX8 isoforms. (**A**) Schematic diagram illustrating human *Pax-8* splice variants. The exon–intron structure of the *Pax-8* gene is schematically shown. The paired domain is represented by a chequered box, the serine/threonine-rich regions are indicated by hatched boxes, and the serine/threonine/tyrosine-rich domain is indicated by a cross-hatched box. The proline-rich region is denoted by a shaded box. (**B**) Transactivation of *wt1* promoter reporter constructs by *Pax-8* isoforms in K562 cells. The expression plasmids used in the transactivation studies are indicated below the panel. The relative CAT activity is denoted above each CAT reaction and is standardized relative to the activity obtained with the reporter vector *Eco*RI–CAT. Kinetic analyses indicated that the observed CAT activity was in the linear response range. The bottom spot represents unacetylated chloramphenicol and the top two spots are acetylated forms of chloramphenicol.

lanes 1 and 5, respectively), indicating that the formation of this complex is specific. In addition, the mutant oligonucleotides M.P. and M.D. used in this assay are not capable of forming specific complexes with PAX8a (M.Dehbi, data not shown). An oligonucleotide harboring a PAX8 binding site present in the Tg promoter can also competitively inhibit formation of the PAX8–DNA complex (compare lanes 3 and 7 with lanes 1 and 5, respectively). The inability to competitively inhibit formation of the second complex (indicated by an arrowhead) indicates that this complex is non-specific. These results demonstrate that PAX8 interacts with both D and P fragments of the CR element and are consistent with the ability of PAX8 to transactivate *wt1* expression through these sites (Figures 2–4).

Activation of the endogenous wt1 mRNA by PAX8a

Our results strongly suggest that *wt1* expression is positively modulated by the product of the *Pax-8* gene. To address this issue, we examined the ability of PAX8a to upregulate expression of the *wt1* gene in a cell line in which the *wt1* promoter is active. For these studies, we chose the K562 cell line which expresses low but detectable levels of *wt1* mRNA (Call *et al.*, 1990). We introduced a *Pax-8a* expression vector into K562 cells and directly examined the levels of *wt1* RNA by Northern blotting. As demonstrated in Figure 7A, the levels of *wt1* RNA are enhanced significantly in cells transfected with CMV/*Pax-8a* relative to those having received an empty expression vector (compare lane 2 with lane 1). Equal amounts of RNA were loaded in each lane, as indicated by ethidium bromide staining of the samples (Figure 7B). These results directly demonstrate that *wt1* expression can be upregulated *in vivo* by the product of the *Pax-8* gene.

Discussion

The Pax family of transcription factors plays an important role in development, as attested by the number of murine and human developmental abnormalities with mutations in Pax genes (Gruss and Walther, 1992). In addition, overexpression of Pax genes leads to transformation in cell culture dependent on the presence of a functional paired domain (Maulbecker and Gruss, 1993), and a chromosomal translocation of *Pax-3* has been implicated in the etiology of rhabdomyosarcoma (Galili *et al.*, 1992; Barr *et al.*, 1993; Shapiro *et al.*, 1993).

The expression profile of the *Pax-8* gene in the developing kidney and WTs suggests that it is required for the early mesenchymal–epithelial cell transition. *Pax-8* expression becomes maximal during renal vesicle formation, a point at which the transcription of *wt1* is shifted from low to relatively high levels. This expression profile is consistent with the results presented here, indicating that the products of the *Pax-8* gene transactivate *wt1* expression (Figure 8). Subsequent to the extinction of *Pax-8* expression, *wt1* levels begin declining, probably the result of a regulatory loop whereby WT1 protein inhibits the transcription of its own promoter (Rupprecht *et al.*, 1994). We have demonstrated recently that *Pax-2* is also capable of transactivating the *wt1* promoter (Dehbi *et al.*, 1996). *Pax-2* expression is induced in the condensing mesenchyme. When *Pax-2* and *Pax-8* expression becomes maximal, *wt1* levels begin increasing (Figure 8). The expression profile of *Pax-2*, *Pax-8* and *wt1* are thus consistent with our results, indicating the transactivation of *wt1* by both paired-domain proteins.

PAX8a has been shown to bind to and regulate the Tg (Zannini *et al.*, 1992), thyroperoxidase (Francis-Lang *et al.*, 1992) and N-CAM (Holst *et al.*, 1994) promoters. In this report, we demonstrate that *Pax-8* also regulates the *wt1* gene, by interacting directly with motifs within the D and P subfragments of the CR element. Gel-shift assays using mutant oligonucleotides derived from a sequence within the *Drosophila even-skipped* promoter, called e5, led to the identification of the pentanucleotide 5′-GTTCC-3′ as the DNA binding core motif recognized by the paired domain (Chalepakis *et al.*, 1991). Such a site is present in the D subfragment of CR. However, despite a high degree of conservation among the paired domains of the Pax members, sequences that bind various paired boxes often appear unrelated to one another or do not contain the 5′-GTTCC-3′ motif (Adams *et al.*, 1992; Zannini *et al.*, 1992; Czerny *et al.*, 1993, Epstein *et al.*, 1994). A site related to the Tg *Pax-8* binding site (5′-CTGCCCAGTCAAGTGTTCTTGAA-3′) is present within the P subfragment (5′-CTGCCC-3′) and probably mediates

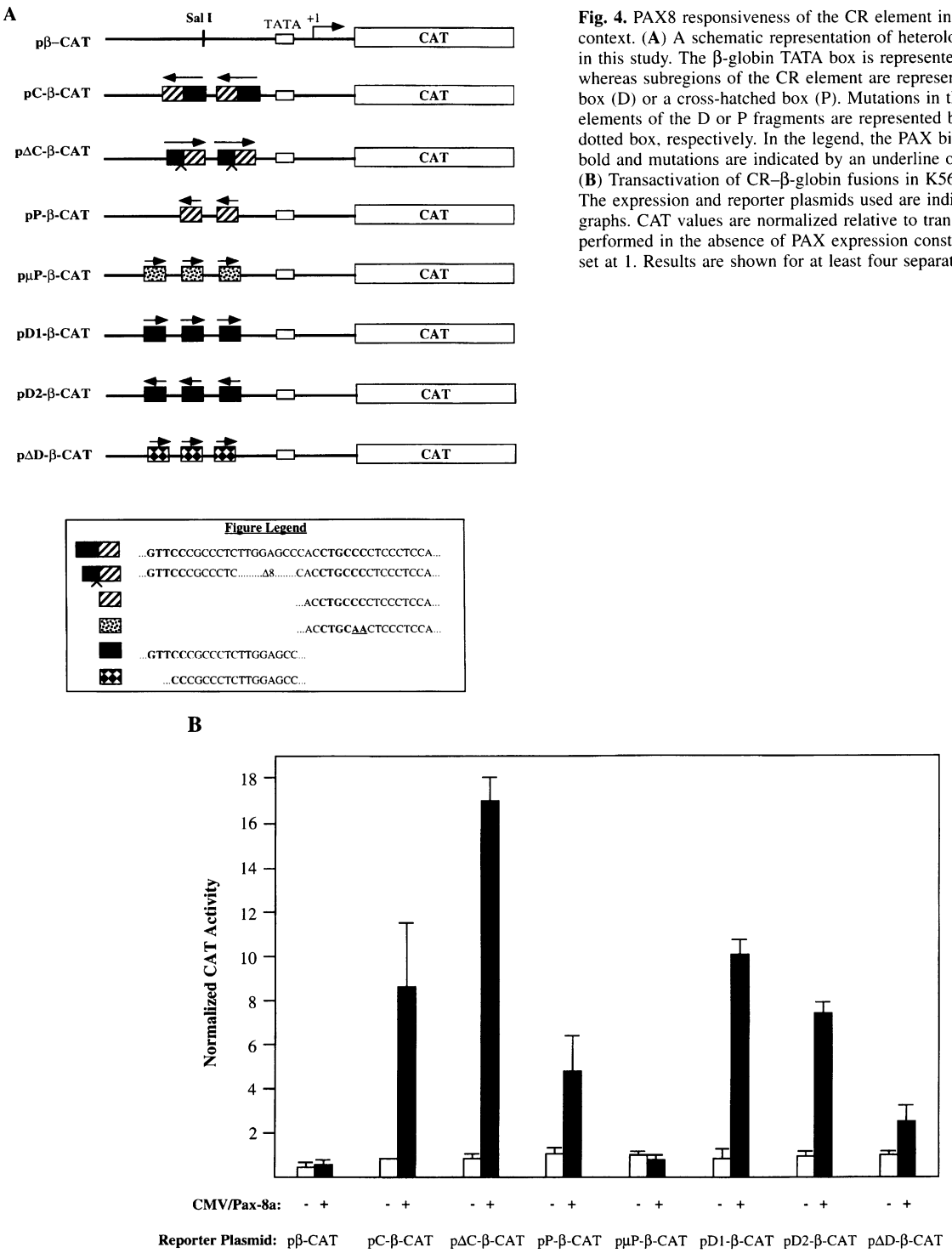

Fig. 4. PAX8 responsiveness of the CR element in a heterologous context. (**A**) A schematic representation of heterologous fusions used in this study. The β-globin TATA box is represented by an open box, whereas subregions of the CR element are represented by a blackened box (D) or a cross-hatched box (P). Mutations in the Pax recognition elements of the D or P fragments are represented by a chequered or a dotted box, respectively. In the legend, the PAX binding sites are in bold and mutations are indicated by an underline or by a Δ symbol. (**B**) Transactivation of CR–β-globin fusions in K562 cells by PAX8. The expression and reporter plasmids used are indicated below the bar graphs. CAT values are normalized relative to transfections of pβ–CAT performed in the absence of PAX expression constructs, which were set at 1. Results are shown for at least four separate experiments.

Pax-8 responsiveness. Although core Pax motifs exist in both D and P subfragments (Figure 1), we have not characterized the complete nature of the binding sites, which in some cases can span 20 bp (Czerny *et al.*, 1993; Epstein *et al.*, 1994).

The expression of *Pax-8* and *Pax-2* in WTs has been analyzed extensively (Dressler and Douglass, 1992; Poleev *et al.*, 1992; Tagge *et al.*, 1994; Eccles *et al.*, 1995). Like *wt1*, both Pax genes are predominantly expressed in epithelial structures (Dressler and Douglass, 1992; Eccles *et al.*, 1995) and weakly in blastemal components of the tumor (Eccles *et al.*, 1995). Although a majority of tumors express all three genes (*Pax-2*, *Pax-8* and *wt1*), there are clear examples of tumors and cell lines where *Pax-8* is not expressed but *wt1* is (Kozmik *et al.*, 1993; Tagge *et al.*, 1994) or vice versa (Dressler and Douglass, 1992). Our results suggest that *wt1* expression in WTs could be abrogated by mutations in *Pax-8* or in the CR element. We have failed to detect *Pax-8* or CR mutations in 75 WTs by PCR–single-strand conformational polymorphism (M.Ghahremani, M.Discenza and J.Pelletier, data not shown). However, we have yet to characterize the *Pax-8*

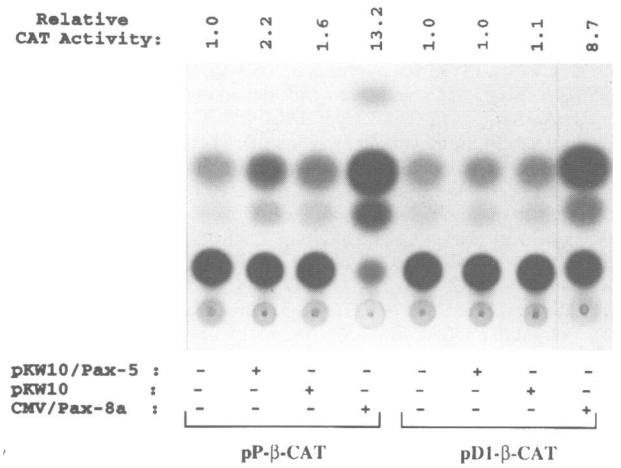

Fig. 5. Effect of PAX5 on the expression of the heterologous reporters pD1-β–CAT or pP-β–CAT. Expression and reporter vectors used are denoted below the panel. The relative CAT activity is denoted above each CAT reaction and is standardized relative to the activity obtained by the reporter vectors. The bottom spot represents unacetylated chloramphenicol and the top two spots are acetylated forms of chloramphenicol.

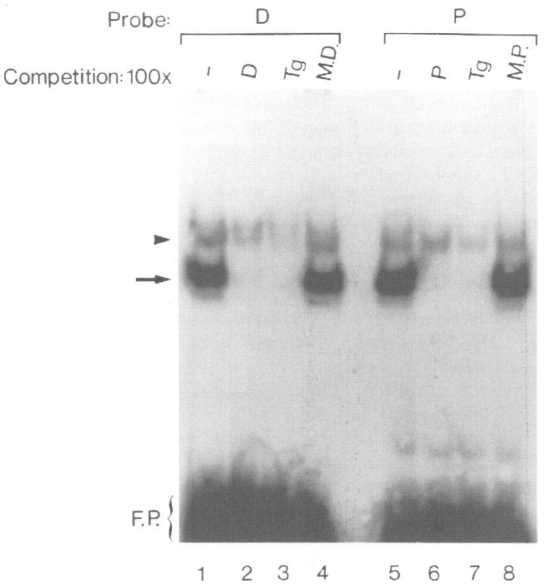

Fig. 6. EMSA demonstrating direct binding of PAX8 to elements within the 38 bp CR sequence. The behavior of PAX8–DNA complexes in competitive situations. The specific PAX8–DNA complex is indicated by an arrow; the non-specific complex is denoted by an arrowhead. The identity of probe and competitor used is indicated above the panel. The sequence of each probe and competitor used is as follows (Pax binding sites are in bold and mutated nucleotides are in italic): D, 5′-TCGA**GTTCC**CGCC-CTCTTGGAGCC-3′; M.D., 5′-TCGAG*G*TCCCGCCCTCTTGG-AGCCCAC-3′; P, 5′-TCGACAC**CTGCCC**CTCCCTCCA-3′; M.P., 5′-TCGACACCTGC*AA*CTCCCTCCA-3′; Tg, 5′-TCGAC-ACTGCCCAGTCAAGTGTTCTTGA-3′. F.P. indicates free probe.

mRNA expression profile in our tumor collection to identify those samples in which *Pax-8* is expressed but *wt1* is not. Alternatively, redundancy may exist in this system to maintain *wt1* expression in the absence of *Pax-8* expression.

Although PAX5 is not expressed in the developing renal system, it is present during the early stages of B cell differentiation (Barberis *et al.*, 1990; Adams *et al.*, 1992).

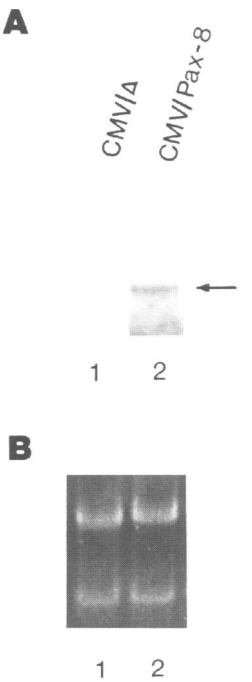

Fig. 7. Activation of the endogenous *wt1* promoter by PAX8. (**A**) Northern blot analysis of RNA isolated from transfected K562 cells. RNA was isolated from K562 cells transiently transfected with CMV/Δ (lane 1) or CMV/*Pax-8a* (lane 2). Following electrophoresis in a 1.2% agarose/37% formaldehyde gel, the RNA was transferred to Nytran and probed using the human WT1 cDNA (Call *et al.*, 1990). The arrow indicates the position of the 3.1 kb WT1 mRNA species. The blot was washed up to a stringency of 0.1× SSC/0.1% SDS at 55°C for 60 min and exposed against X-Omat (Kodak) film at –70°C for 24 h. Note that on this relatively short exposure, the endogenous *wt1* mRNA is not detectable in the RNA samples isolated from CMV/Δ transfected cells (negative control). (**B**) Ethidium bromide staining of samples blotted in (A) presented as a control for loading.

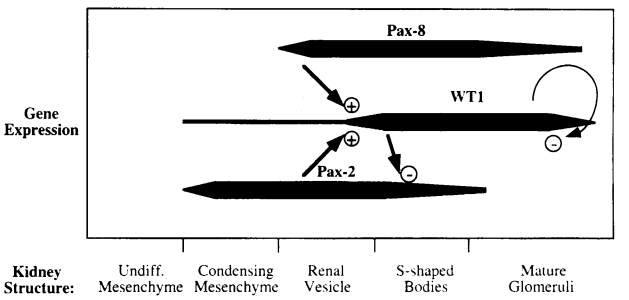

Fig. 8. Schematic diagram demonstrating the temporal expression profiles of *Pax-2*, *Pax-8* and *wt1* during nephrogenesis. Increased expression is denoted by a thickening bar. Arrows link regulatory factors with their downstream targets and signify repression (–) or activation (+). Although WT1 represses *Pax-2* expression, sustained *Pax-8* expression may be sufficient to maintain high WT1 levels.

Interestingly, *wt1* cDNA clones have been isolated from pre-B cell cDNA libraries, suggesting that the expression of these two genes may overlap (Call *et al.*, 1990). Our results suggest that unlike PAX2 (Dehbi *et al.*, 1996) or PAX8 (this manuscript), PAX5 cannot transactivate *wt1* expression through the CR element (Figure 5).

All PAX8 isoforms characterized to date contain a common DNA binding domain but have different C-terminal domains because of alternative splicing. The PAX8c and PAX8d isoforms have a unique proline-rich region, generated by translation in a different reading frame

(Figure 3A). Previous transient transfection experiments using heterologous promoter constructs with *Pax-8c* or *Pax-8d* expression vectors failed to reveal any transactivation function of these isoforms (Kozmik *et al.*, 1993; Poleev *et al.*, 1995). In this report, we demonstrate that these isoforms are capable of stimulating expression from the *wt1* promoter, revealing that PAX8c and PAX8d can indeed activate gene expression (Figure 3B). In our hands, these two isoforms were not capable of mediating transactivation from the heterologous report, pC-β–CAT (M.Dehbi, data not shown), suggesting that the mechanism of transactivation by these isoforms may be dependent on promoter architecture. Consistent with this hypothesis is the recent finding that several members of the PAX family (including PAX2 and PAX8) can repress p53 expression (Stuart *et al.*, 1995). Should this pathway be functional in WTs, one would expect to find low levels of p53 in the majority of these cancers. However, the opposite is true: p53 levels are elevated in the majority of WTs and this pathway may not exist or be abrogated in this particular cancer type.

Our results do not exclude the possibility that *Pax-8* activation of *wt1 in vivo* occurs through additional sites present within the *wt1* promoter. However, we have identified at least two functional sites within the *wt1* transcriptional regulatory region through which the *Pax-8* gene products can activate transcription. It is interesting to note that the two Pax core motifs overlap with Sp1 sites in both D and P subsites. These particular sites are competent for Sp1 binding, as demonstrated previously by DNase I footprinting (Hofmann *et al.*, 1993). This raises the interesting possibility that the binding of either paired box proteins to D or P is mutually exclusive with Sp1 binding. Alternatively, specific interactions between PAX8 and Sp1 may be required for the efficient stimulation of transcription by PAX8. We have now demonstrated that *Pax-8* is also part of a cascade involved in regulating *wt1* expression. Elucidating the regulation of the *wt1* gene in normal renal development will serve to increase our understanding of how this process is usurped during the initiation of malignancy in WTs.

Materials and methods

Cell lines

NIH 3T3 and COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM), and 293 cells were maintained in α-MEM supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin and streptomycin. The erythroleukemic cell line, K562, was maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, penicillin and streptomycin.

Plasmid constructs

The CAT reporter plasmids used in this study (illustrated in Figure 1A) contained the murine *wt1* promoter (Pelletier *et al.*, 1991b) fused to the CAT reporter gene. The chimeric vector, pSK/WT1-5, contains a 3.1 kbp promoter fragment in the *Bam*HI site of pSKII$^+$ (Stratagene) and was used to generate promoter deletions. The pCAT–Enhancer plasmid (Promega) contains the CAT reporter gene and an SV40 enhancer, but lacks a promoter. A *Bam*HI site in pCAT–Enhancer was generated by cloning a *Bam*HI linker into the unique *Xba*I site (after Klenow repairing) upstream of the CAT gene. To generate *Sal*I–CAT, pSK/WT1-5 was digested with *Sal*I and *Bam*HI and the promoter fragment transferred into the *Sal*I–*Bam*HI sites of pCAT–Enhancer. The five deletions mutants, *Spe*I–CAT and *Eco*RI–CAT, were made by digesting *Sal*I CAT with *Spe*I or *Eco*RI. Because an additional site exists upstream of the WT1 promoter, the resulting vectors were simply religated. The deletion mutants *Bgl*II^{I}–CAT and *Bgl*II^{E}–CAT were generated using PCR with either of the following oligonucleotides: S^{I}, 5′-GAAGATCTTCGTTCCCGCCCTCTTGGAGC-3′; or S^{E}, 5′-GAAGATCTTCCCCCACCCTTCTGATTAC-3′, in conjunction with the antisense oligonucleotide AS 5′-GAAGATCTTCGATCGCGGCGAGGAGGC-3′ (which lies 552 bp downstream of the conserved 38 bp motif). The resulting products were digested with *Bgl*II and inserted into the *Bam*HI site of pCAT–Enhancer. The authenticity of the promoter sequence was confirmed by sequencing.

Reporter plasmids carrying the heterologous human β-globin gene promoter with the upstream conserved motif (C, 5′-TCGAGTTCCCGCCCTCTTGGAGCCCACCTGCCCCTCCCTCCA-3′) sites, or derivatives of this site (D, 5′-TCGAGTTCCCGCCCTCTTGGAGCC-3′; P, 5′-TCGACACCTGCCCCTCCCTCCA-3′) are illustrated in Figure 4A. The plasmid pβ–CAT was constructed by inserting a double-strand oligonucleotide, consisting of a TATA box and the initiation start site of the human β-globin gene (5′-TCGAGTATAAAAGGTGAGGTAGGATCAGTTGCTCCTCACAC-3′), into the unique *Xho*I site of the pJFCAT1 expression vector (Fridovich-Keil *et al.*, 1991). Multiple copies of C, D or P were inserted into the unique *Sal*I site of plasmid pβ–CAT and designated pC-β-CAT, pD-β–CAT and pP-β–CAT, respectively. A vector containing an 8 bp deletion adjacent to the first Sp1 site in subfragment D was generated using the following oligonucleotide: ΔC, 5′-TCGAGTTCCCGCCCTCCACCTGCCCCTCCCTCCA-3′; the resulting vector was designated pΔC-β–CAT. A similar strategy was used to generate mutant constructs with Pax binding sites in D (oligo ΔD, 5′-TCGACCCGCCCTCTTGGAGCC-3′; vector pΔD-β–CAT) and in P (oligo M.P., 5′-TCGACACCTGC**AA**CTCCCTCCA-3′; vector pμP-β–CAT), where the modified nucleotides are in bold. All clones were sequenced to ensure the absence of undesired nucleotide changes. The expression vectors encoding *Pax-8a* (CMV/*Pax-8a*; kindly provided by Dr P.Gruss, Max-Planck Institute, Germany) and *Pax-8c* and *Pax-8d* (kindly provided by Dr M.Busslinger, IMP, Austria) have been described previously (Plachov *et al.*, 1991; Kozmik *et al.*, 1993). For *in vitro* transcriptions, the *Pax-8* cDNA was isolated from the CMV-based expression vector by digesting with *Bam*HI–*Xba*I and recloning the insert into the *Bam*HI–*Xba*I sites of pKSII$^+$ (Stratagene).

Transfection and CAT assays

NIH 3T3 and 293 cells were seeded 1 day prior to transfection at a density of 5×10^5 cells/100 mm plate. At 24 h later, cells were transfected by the calcium phosphate method with 10 μg of the reporter plasmid, and either 10 μg of the *Pax-8* expression vector or the empty expression vector CMV/Δ. As a positive control to monitor the transfection efficiency, cells were co-transfected with 3 μg pRSV/β-gal. K562 cells were transfected by electroporation, as described previously (Weitzman *et al.*, 1993). Briefly, cells were collected by centrifugation, washed twice with serum and antibiotic-free RPMI 1640 medium (incomplete RPMI). Cells were resuspended at 2×10^7 cells/ml in incomplete RPMI medium and 0.5 ml of this suspension were mixed with 50 μl DNA and placed into a 4 mm electroporation cuvette. The DNA (10 μg CAT reporter plasmid, 10 μg CMV/Δ or CMV/*Pax-8* and 3 μg RSV/β-gal) was dried under vacuum and resuspended in 50 μl incomplete RPMI medium. Cells and DNA were incubated for 5 min at room temperature and then electroporated at 960 μF, 280 V using the Bio-Rad Gene Pulser. After electroporation, cells were incubated for 10 min at 4°C, transferred to 15 ml complete RPMI 1640 medium and incubated at 37°C. At 36–44 h post-transfection, cells were harvested and assayed for β-galactosidase and CAT activity (Gorman, 1985). Following thin-layer chromatography analysis, regions containing ^{14}C-acetylated chloramphenicol, as well as unacetylated chloramphenicol, were isolated from the plates and quantitated in a liquid scintillation counter.

RNA isolation and Northern analysis

Total cytoplasmic RNA was isolated from K562 cells transiently transfected with CMV/*Pax-8a* or CMV/Δ (negative control) by the high-salt precipitation method described by Auffray and Rougeon (1980). 10 μg total RNA were electrophoresed through a 1% agarose–formaldehyde gel and transferred to Nytran membrane (Schleicher & Schuell). Hybridizations were carried out using the human WT1 cDNA as a probe (Call *et al.*, 1990), which had been labeled with [α-^{32}P]dCTP by the random priming method (Feinberg and Vogelstein, 1983). Ethidium bromide staining of RNA samples was used to standardize for possible differences in RNA sample loading.

In vitro transcriptions and translations

For *in vitro* transcriptions and translations, pKS/*Pax-8a* was linearized with *Xba*I and transcribed using T3 RNA polymerase (Pharmacia). After

synthesis, the RNA was subjected to two phenol–chloroform extractions, a G50 Sephadex purification and ethanol precipitation. After centrifugation, the RNA was resuspended in 20 µl H_2O and 5 µl (~1 µg) were used for *in vitro* translation using a rabbit reticulocyte lysate (Promega) at a concentration of ~10 µg/ml.

EMSA

An EMSA with *Pax-8* protein was performed essentially as described by Zannini *et al.* (1992). Briefly, 3 µl *in vitro*-translated protein were incubated for 25 min at room temperature with 20 mM Tris–$HCl_{8.0}$, 75 mM KCl, 1 mM dithiothreitol, 10 µg bovine serum albumin, 12% glycerol, 2 µg poly(dI–dC) and 100 ng Bluescript DNA ($pKSII^+$) as a non-specific competitor, in a total volume of 15 µl in the presence of 0.5 ng (20 000 c.p.m.) end-labeled oligonucleotide probe. Probes were labeled by filling in the ends with the Klenow fragment of DNA polymerase I using [α-^{32}P]dCTP. For competition experiments, reaction mixtures were preincubated for 15 min with an excess of unlabeled oligonucleotides prior to the addition of radiolabeled probes. The mutant oligonucleotides used were oligo M.D. (5′-TCGA***GGTCCC***GCCCT-CTTGGAGCCCAC-3′) and oligo M.P. (5′-TCGACACC***TGCAA***CT-CCCTCCA-3′), where the modified nucleotides are in bold and the core Pax sites are in italic. Samples were then electrophoresed through a 4% polyacrylamide gel in 0.5× TBE buffer at 140 V for 2.5 h at 4°C. After electrophoresis, the gel was dried and subjected to autoradiography. The positive control oligonucleotide harboring a *Pax-8* binding site used in this study was derived from the Tg promoter (5′-TCGACACTGCCCA-GTCAAGTGTTCTTGA-3′; Zannini *et al.*, 1992).

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