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A Novel Repressor, par-4, Modulates Transcription and Growth Suppression Functions of the Wilms' Tumor Suppressor WT1

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The tumor suppressor WT1 represses and activates transcription. The loss and/or imbalance of the dual transcriptional activity of WT1 may contribute to Wilms' tumor. In this study, we identified par-4 (for prostate apoptosis response) as a WT1-interacting protein that itself functions as a transcriptional repressor. par-4 contains a putative leucine zipper domain and is specifically upregulated during apoptosis of prostate cells (S. F. Sells, D. P. Wood, Jr., S. S. Joshi-Barve, S. Muthukkumar, R. J. Jacob, S. A. Crist, S. Humphreys, and V. M. Rangnekar, Cell Growth Differ. 5:457–466, 1994). The leucine repeat domain of par-4 was shown to interact with the zinc finger DNA binding domain of WT1. Immunoprecipitation-Western blot (immunoblot) analyses demonstrated in vivo WT1–par-4 interactions. par-4 was ubiquitously expressed, and the protein was found in both the nucleus and the cytoplasm. Functionally, par-4 inhibited transcription activated by WT1, but not by the related protein EGR1. Inhibition of WT1-mediated transcription was dependent on the domain of par-4 that mediates its physical association with WT1. In addition, par-4 augmented WT1-mediated repression, possibly by contributing an additional repression domain. Consistent with these results, par-4 functioned as a transcriptional repressor when brought to a promoter via a heterologous DNA binding domain. Significantly, par-4, but not a mutant unable to interact with WT1, rescued growth suppression caused by WT1. Thus, we identified a novel repressor that modulates transcription as well as growth suppression functions of WT1.

The Wilms' tumor candidate gene *WT1* is a tumor suppressor gene expressed in the developing kidney and in the adult urogenital system (reviewed in references 20, 47, and 48). Inactivation of WT1 has been correlated with the incidence of Wilms' tumor, a pediatric nephroblastoma, and Denys-Drash syndrome, which is characterized by severe genitourinary disorders and Wilms' tumor (7). The *WT1* gene is deleted or mutated in approximately 10% of sporadic Wilms' tumors and in nearly 100% of Denys-Drash patients (reviewed in references 7 and 19). In support of WT1 as a tumor suppressor, the WT1 protein has been shown to suppress cell growth in both Wilms' tumor cells and non-Wilms' tumor cells (21, 28, 32).

Four alternatively spliced WT1 mRNA isoforms (A, B, C, and D) encode zinc finger-containing proteins of 52 to 54 kDa (22). Compared with isoform A, isoforms B and D contain 17 extra amino acids, encoded by exon 5, that are inserted between the transactivation and DNA binding domains. Isoforms C and D contain 9 additional nucleotides encoding lysine, threonine, and serine residues inserted between zinc fingers 3 and 4 (22). Recently, different isoforms of WT1 have been suggested to play different biological roles (13, 29). The A and B isoforms of WT1 have been shown to bind the same consensus sequence that constitutes the recognition site for the early growth response family of transcription factors (46). Isoforms C and D recognize related but distinct DNA sequences (11, 46, 62), suggesting that they may regulate different sets of genes. Like many DNA-binding transcription factors, WT1 has been shown to repress and activate transcription depending on promoter and physiological contexts (33, 49, 61). WT1 negatively regulates many growth-related genes (10, 16, 33, 60, 63), some of which may be physiologically relevant target genes. Mutated *WT1* transcripts have been identified in Wilms' tumor samples, and some of the corresponding mutant proteins have been shown to be defective for the transcriptional repression activity (21, 41). Heterozygous WT1 mutations are also associated with the disease. These WT1 mutants were shown recently to function as dominant negatives that inhibit the transcriptional activation and repression functions of the wild-type WT1 allele (38, 49). The loss of WT1 transcriptional functions and/or an imbalance in its transcriptional repression and activation activities may lead to deregulated cell growth, which contributes to tumorigenesis.

One of the central questions regarding WT1 relates to the mechanisms that control its dual transcriptional activity. One model postulates that cellular proteins that interact with WT1 may influence its activity. It has been shown that WT1 physically interacts with another tumor suppressor protein, p53, which is thought to be required for the repression function of WT1 (36). The WT1-p53 interaction leads to the inhibition of p53-mediated apoptosis (35). However, it is likely that p53 is not the sole protein that modulates the transcriptional activities of WT1. Null mutations of WT1 in homozygous mice are embryonic lethal because of the failure in heart and kidney development (27), while most of the mice null for p53 are morphologically normal (8). In addition, analyses of Wilms' tumor samples revealed that the majority of Wilms' tumors do

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not contain p53 mutations (37), although p53 defects are associated with anaplastic Wilms' tumors (5).

In an attempt to systematically identify proteins that interact with WT1 and to determine the functional consequences of such interactions, we conducted a search for WT1-interacting proteins by using the yeast two-hybrid system. We identified one WT1-interacting protein that is the human homolog of the rat par-4 (prostate apoptosis response) protein. The rat *par-4* gene was isolated in a separate study aimed at identifying genes that are transcriptionally induced by apoptotic signals in the rat ventral prostate (54). *par-4* induction was apoptosis specific, as no change in par-4 expression was observed in response to growth stimulation, oxidative stress and necrosis, or growth arrest in prostate cells (54). The open reading frame of rat *par-4* predicts a protein of 332 amino acids, which contains a putative nuclear localization signal at the N terminus and a putative leucine repeat domain in the C-terminal region of the protein. par-4 has recently been suggested to sensitize cells to respond to apoptotic signals (52).

WT1 interacts with par-4 both in vivo and in vitro, and this interaction is mediated by the zinc fingers of WT1 and the leucine repeats of par-4. Through protein-protein interaction, par-4 inhibits transcriptional activation but enhances the transcriptional repression activity of WT1, suggesting that par-4 brings an additional repression domain to the promoter via its interactions with WT1. Consistent with this hypothesis, when fused to the GAL4 DNA binding domain, GAL4–par-4 repressed transcription of a reporter gene containing GAL4 binding sites. Biologically, par-4 partially rescues growth suppression induced by WT1 in a manner that is dependent on the leucine repeat domain that mediates its physical interaction with WT1.

MATERIALS AND METHODS

Plasmids. The pEGR$_3$TKCAT reporter plasmid contains three EGR1-WT1 binding sites inserted in tandem 5′ of the herpes simplex virus TK promoter in plasmid pBLCAT2 (49). pCMV-Par-4 was constructed by cloning full-length rat *par-4* into the *Eco*RI site of the pCB6$^+$ expression vector. pCMV-Par-4$_{1\text{-}267}$ was constructed by cloning the *Eco*RI-*Bgl*II-blunted fragment of rat *par-4* into pCB6$^+$ digested with *Eco*RI, and the DNA ends were filled in with Klenow fragment. pCMV-FLAG-par-4 was constructed by fusing full-length *par-4* to the FLAG moiety in the pBFT-4 vector (gift of X.-Y. Fu, Yale University). A *Sac*I-*Xho*I fragment containing the FLAG-par-4 coding sequence was subcloned into the *Hin*dIII site of the Rc/CMV expression vector to give rise to pCMV-FLAG-par-4. The RSV-WT1 (21) and EGR1 (42) expression plasmids were described previously, as were pGAL4-YY1, pGAL4TKCAT, pTKCAT, and pGEM7zf(+)-YY1 (57). pGAL4-WT1 was constructed by cloning full-length *WT1* cDNA in frame into the pG4 vector (30), which expresses the GAL4 (amino acids 1 to 147) DNA binding domain. pGAL4-par-4 was similarly constructed with full-length rat *par-4*. pGST-H2-73 and pGST-par-4 were constructed by fusing the H2-73 cDNA fragment obtained from the yeast two-hybrid screen and full-length rat *par-4*, respectively, in frame into the pGEX-2TK vector. GST-par-4$_{1\text{-}267}$ and GST-par-4$_{268\text{-}332}$ contain amino acids (aa) 1 to 267 and 268 to 332 fused in frame to the glutathione *S*-transferase (GST) moiety in the pGEX-2TK vector, respectively. The GST-WT1 plasmids were constructed by fusing cDNAs encoding amino acids 1 to 429 (GST-WT1), 1 to 180 (GST-WT1$_{1\text{-}180}$), 1 to 307 (GST-WT1$_{1\text{-}307}$), 307 to 429 (GST-WT1$_{307\text{-}429}$), and 181 to 429 (GST-WT1$_{181\text{-}429}$) in frame into pGEX-2TK.

Isolation of WT1-interacting proteins. *Escherichia coli* and *Saccharomyces cerevisiae* were manipulated essentially as previously described (4). EGY48 (*MAT*α *trp1 ura3 his3 LEU2*::pLexAop6-LEU2) was used as a host for all interaction experiments (64). Yeast plasmids were rescued into *E. coli* K-12 strain KC8 (*pyrF*::Tn*5 hsdR leuB600 trpC9830 lacD74 strA galK hisB436*) as previously described (64). The full-length WT1 isoform A cDNA was inserted into pEG202 at the *Eco*RI site, and the generated plasmid, pEG-WT1, was used as the bait. The oligonucleotide-primed HeLa cDNA yeast expression library, a generous gift from Russ Finley and Roger Brent (Massachusetts General Hospital), was screened essentially as previously described (64).

Fresh yeast colonies from His$^-$ Ura$^-$ Trp$^-$ plates (glucose or galactose as the energy source) were lifted onto nitrocellulose membranes and lysed by being submersed in liquid nitrogen for 1 min. The membranes were then placed on top of Whatman filter papers saturated with 3 ml of Z buffer (100 mM sodium phosphate [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 40 mM β-mercaptoethanol) containing 1 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. The membranes were incubated at room temperature, and the color of colonies was recorded through the course of 30 min.

Cells and transfection. HeLa and 293 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated calf serum (HeLa cells) or fetal calf serum (293 cells). Cells were transfected by the calcium phosphate precipitation method (57). The total amount of DNA was adjusted with plasmid pSP72 to be identical for each transfection. Cells were harvested 48 h after addition of the precipitate. All transfection assays were carried out with at least two independent DNA preparations and were repeated between three and five times. Human melanoma A375-C6 cells were cultured in RPMI 1640 medium supplemented with 10% serum and transfected as previously described (53). In cotransfection experiments, equal amounts of all plasmid DNAs were used. Transfectants were selected in culture medium supplemented with 300 μg of G418 sulfate (Life Technologies, Inc., Gaithersburg, Md.) per ml. To quantify the effects of WT1 and par-4 on the growth of A375-C6 cells, transfected cells were seeded at a density of 2,000 cells per 200 μl in 96-well plates and grown for 72 h. Thereafter, cells were pulsed with [^{3}H]thymidine for 8 h and subjected to [^{3}H]thymidine incorporation assays as previously described (53).

CAT assays. Whole-cell extracts were prepared from transfected cells. Chloramphenicol acetyltransferase (CAT) activity was assayed as previously described (57) and quantitated with a Beckman LS6500 scintillation counter. Proper amounts of cell extracts were used to measure CAT activities to ensure that the assays were performed within linear range.

Northern (RNA) blotting. Nitrocellulose filters containing approximately 2 μg of poly(A)$^+$ RNA per lane from 16 different adult human tissues (Clontech, Palo Alto, Calif.) were used for Northern analysis. Filters were prehybridized and hybridized in 50% deionized formamide–5× Denhardt's solution–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–0.5% sodium dodecyl sulfate (SDS)–100 μg of denatured salmon sperm DNA per ml at 42°C. Blots were hybridized with ^{32}P-labeled human par-4, WT1, or β-actin cDNA probes for 16 h at 42°C. The filters were washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 30 min at room temperature and twice in 0.2× SSC–0.1% SDS for 30 min at 65°C for 30 min.

Immunoprecipitation-Western blotting (immunoblotting) assays. 293 cells were cotransfected with 15 μg of pCMV-FLAG-par-4 together with either 15 μg of pRSV-WT1 or 15 μg of pRSV vector DNA. After 48 h, cells were lysed in a buffer containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.0), 0.25 M NaCl, 2.5 mM EDTA, 0.5 mM dithiothreitol, 10 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 2 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40 for 30 min on ice. Extracts were incubated with WT1 antibodies (C19; Santa Cruz Biotechnology Inc., Santa Cruz, Calif.) or normal rabbit serum (NRS) overnight, and immune complexes were collected with protein A-Sepharose beads at 4°C for 1 h. The beads were washed eight times with lysis buffer, and the proteins were eluted with Laemmli sample buffer. Proteins were separated by electrophoresis through a 10% polyacrylamide gel, transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, Mass.), and probed with 0.1 μg of monoclonal anti-FLAG antibody (Kodak, New Haven, Conn.) per ml. Blots were incubated with alkaline phosphatase-coupled goat anti-mouse antibody (Bio-Rad, Hercules, Calif.), and immunoreactive proteins were visualized with 5-bromo-4-chloro-3-indolyl phosphate–nitroblue tetrazolium (Boehringer Mannheim, Mannheim, Germany).

For the analysis of the interaction between endogenous WT1 and par-4, M15 cells (80% confluency) were lysed in ELB buffer (25 mM HEPES [pH 7.0], 250 mM NaCl, 2.5 mM EDTA, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 0.1% Nonidet P-40 containing 2 mM phenylmethylsulfonyl fluoride and 10 μg [each] of aprotinin and leupeptin per ml) for 30 min on ice. Lysates were clarified by centrifugation at 16,000 × *g* for 15 min at 4°C and incubated with a control monoclonal antibody (12CA5) or mouse anti-human par-4 monoclonal antibody (A10; Santa Cruz Biotechnology, Inc.). After lysates were rocked overnight, protein A-Sepharose beads were added and incubation continued for another 30 min at 4°C. Immunoprecipitates were washed four times in ELB buffer, denatured in SDS gel loading buffer, and fractionated on an SDS–12% polyacrylamide gel. Proteins were blotted onto nitrocellulose, and the filters were blocked with 5% nonfat milk in TBS-T (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20) for 2 h at room temperature. Primary antibody interaction was carried out by an overnight incubation of blots with a rabbit anti-WT-1 polyclonal antibody (C19; Santa Cruz Biotechnology, Inc.). After the binding of peroxidase-conjugated goat anti-rabbit immunoglobulin G F(ab′)$_2$ secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) for 1 h in Tris-buffered saline containing 1% nonfat milk, filters were washed in TBS-T and developed with the Supersignal CL-HRP substrate system (Pierce) according to the manufacturer's instructions.

GST-based assays of the WT1–par-4 interaction. All GST fusion proteins were purified as previously described (26), and the yield of each protein was determined by SDS-polyacrylamide gel electrophoresis (PAGE) analysis and Coomassie blue staining. GST proteins bound to glutathione-agarose beads (Sigma, St. Louis, Mo.) were washed twice in NET-50 (20 mM Tris [pH 8.0], 1 mM EDTA, 50 mM NaCl) for 15 min at room temperature and were incubated in 200 μl of binding buffer (25 mM HEPES [pH 7.5], 12.5 mM $MgCl_2$, 20% glycerol, 0.1% Nonidet P-40, 150 mM KCl, 1 mM dithiothreitol, 150 μg of bovine serum albumin per ml, 200 μg of ethidium bromide [EtBr] per ml) for 10 min at room

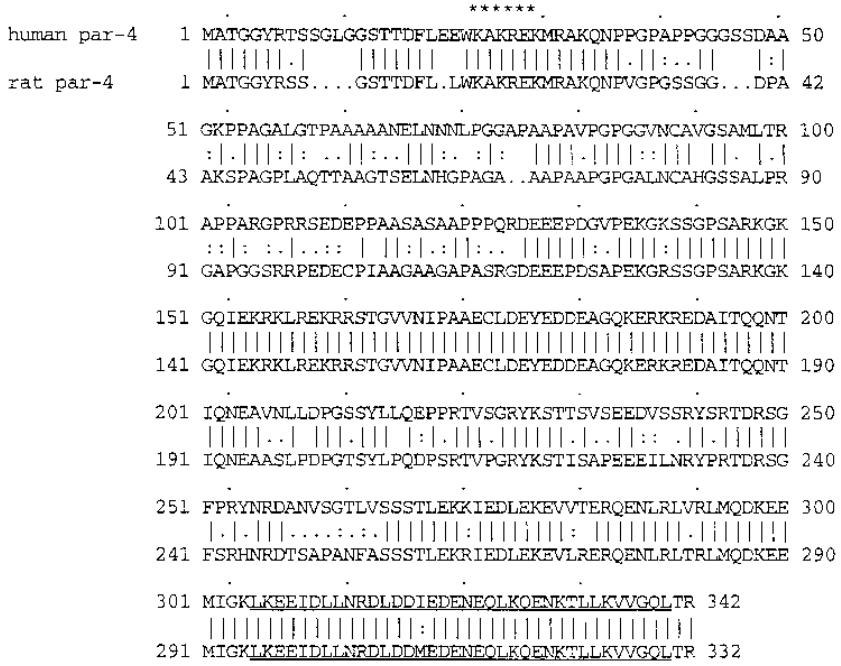


FIG. 1. Comparison of human and rat par-4 sequences. The deduced amino acid sequence of human par-4 was compared with that of the rat par-4 protein. The putative leucine zipper domain of human par-4 and rat par-4 is underlined, and the putative nuclear localization sequence is denoted by asterisks. The human par-4 sequence is 75% identical (vertical lines) and 84% similar (dots) to the rat par-4 sequence.

temperature. In vitro transcription-translation reaction mixtures (Promega, Madison, Wis.) containing [^{35}S]methionine (1,175 Ci/mmol) were programmed with pSP64-WT1 or pGEM7zf(+)-YY1, and 5 μl was incubated with immobilized GST fusion proteins for 1 h at room temperature as indicated. The beads were washed five times with 1 ml of washing buffer (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 200 μg of EtBr per ml). Bound proteins were eluted with Laemmli sample buffer and separated on an SDS–10% polyacrylamide gel soaked in the fluorographic solution Amplify (purchased from Amersham) and visualized by fluorography.

To map the region of WT1 that interacts with par-4, various GST-WT1 fusion proteins were produced as described above. 293 cells transfected with 10 μg of pCMV-FLAG-par-4 were lysed in lysis buffer (25 mM HEPES [pH 7.0], 0.25 M NaCl, 2.5 mM EDTA, 0.5 mM dithiothreitol, 10 mg of leupeptin per ml, 1 mg of pepstatin A per ml, 2 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40), and approximately 1 μg of each fusion protein was incubated with 200 μg of whole-cell lysate. Beads were washed five times in 1 ml of washing buffer as described above, proteins were separated by SDS-PAGE, and bound FLAG–par-4 was visualized by Western blotting as described above.

Detection of par-4 and WT1 in nuclear and cytoplasmic fractions. 293 cells were cotransfected with 10 μg (each) of pCMV-FLAG-par-4 and pRSV-WT1, and cytoplasmic and nuclear fractions were prepared as previously described (9). Lysates were quantitated for protein by Bradford assay, and equal amounts of cytoplasmic and nuclear proteins were separated by SDS-PAGE. Subsequent Western analyses were performed with either an anti-FLAG monoclonal antibody (Kodak) or anti-WT1 antibody (Santa Cruz Biotechnology, Inc.).

Production of affinity-purified anti-par-4 polyclonal antibodies. Rabbits were immunized with human GST–par-4 as described previously (24). After three booster injections with GST–par-4, rabbit sera were tested for reactivity with par-4 by immunoprecipitation and Western blotting. Affinity-purified anti-par-4 antibodies were obtained by using immunoaffinity columns containing GST–par-4 and GST attached to cyanogen bromide-agarose beads (24).

Nucleotide sequence accession number. The nucleotide sequence of a full-length human par-4 cDNA has been deposited in the GenBank database under accession no. U63809.

RESULTS

Isolation of par-4 as a WT1-interacting protein. The yeast two-hybrid assay (14, 64) was used to identify proteins that interact with WT1. The A isoform of WT1 was fused to the DNA binding domain of LexA (LexA-WT1) and used as a bait. A specialized HeLa cell library (cDNAs fused to an acidic activation domain; gift of R. Brent) was used as a source for interacting proteins. Approximately 2×10^6 independent colonies were screened, and several candidate proteins that interacted with WT1 were identified. Sequence analysis revealed that one of the putative WT1-interacting proteins, H2-73, encodes a partial polypeptide that is 96% identical to the C terminus (aa 280 to 332) of rat par-4 (54). The interaction of this partial par-4 protein with WT1 is specific, as it did not interact with another zinc finger repressor and activator, YY1 (57), or the p85 subunit of PI3 kinase (58). With H2-73 cDNA as a probe, a full-length human *par-4* cDNA was isolated and its amino acid sequence was compared with that of rat par-4 (Fig. 1). The human and rat *par-4* sequences have 85% similarity throughout the entire coding region.

In vitro and in vivo interactions between par-4 and WT1. To confirm the WT1–H2-73 (par-4) interaction observed in yeast cells, GST fusion protein-based assays were carried out. As shown in Fig. 2A, in vitro-translated WT1 (isoform A) was specifically retained by GST–H2-73, but not by GST alone (lanes 3 and 4). As a specificity control, the zinc finger repressor and activator YY1 (15, 23, 40, 57) failed to interact with par-4 under the same assay conditions (Fig. 2A, lanes 5 and 6). To determine whether WT1 interacted with full-length rat par-4 and to identify domains within WT1 and par-4 that mediate the physical interaction, further in vitro binding experiments were performed. Full-length rat par-4 and the N (aa 1 to 267)- and C (aa 268 to 332)-terminal domains of rat par-4 were fused to GST. In vitro-translated WT1 bound both GST-par-4 and GST-par-$4_{268\text{-}332}$ (Fig. 2B, lanes 1 and 3) but failed to interact with GST-par-$4_{1\text{-}267}$ (Fig. 2B, lane 2). Therefore, aa 268 to 332 of par-4 are necessary and sufficient to mediate the physical interaction between par-4 and WT1. In addition, WT1 isoforms B, C, and D were found to bind par-4 in a manner similar to that of the WT1 A isoform (data not shown).

To map the region of WT1 that is involved in the physical interaction with par-4, par-4 was expressed in 293 cells, a hu-

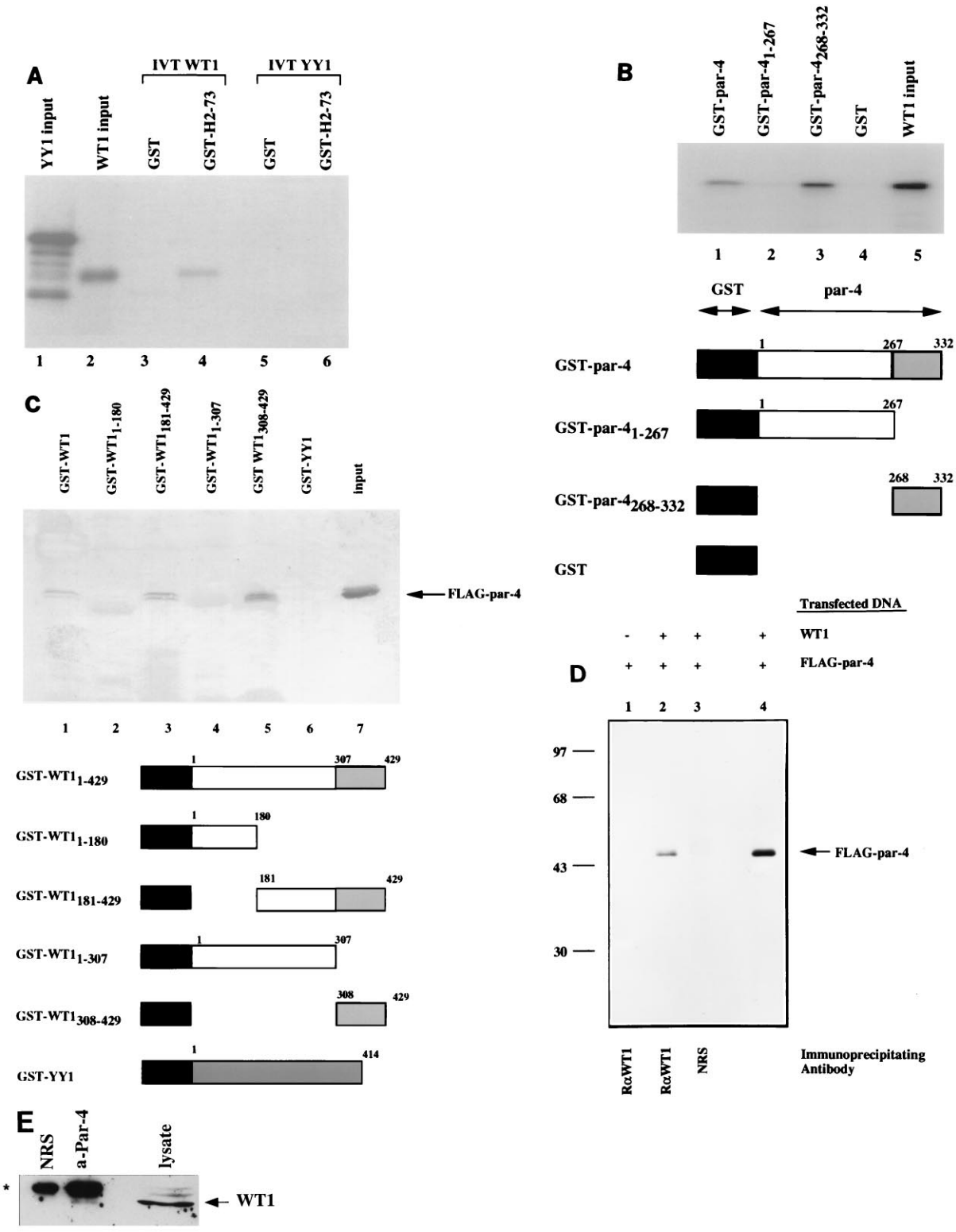


FIG. 2. Physical interaction between par-4 and WT1. (A) WT1 and H2-73 specifically interact in vitro. In vitro-transcribed and -translated (IVT) ^{35}S-labeled WT1 and YY1 were incubated with 1 μg of either GST–H2-73 (lanes 4 and 6) or GST alone (lanes 3 and 5) coupled to glutathione-agarose beads. The input lanes (lanes 1 and 2) were loaded with one-fifth the amount of WT1 and YY1 used in the binding reaction mixtures. (B) WT1 binds to the leucine repeats of par-4. In vitro-transcribed and -translated ^{35}S-labeled WT1 was incubated with 1 μg of either GST-par-4 (lane 1), GST-par-4$_{1-267}$ (lane 2), GST-par-4$_{268-332}$ (lane 3), or GST alone (lane 4) coupled to glutathione-agarose beads. The input lane (lane 5) was loaded with one-fifth the amount of WT1 used in the binding reaction mixtures. Schematic representations of the GST fusion proteins used in this experiment are shown. Black boxes, GST; white boxes, par-4$_{1-267}$; shaded boxes, par-4$_{268-332}$. (C) par-4 binds the zinc fingers of WT1. 293 cells were transfected with 10 μg of pCMV-FLAGpar-4, and whole-cell lysates were prepared. Approximately 200 μg of lysate was incubated with either GST-WT1 (lane 1), GST-WT1$_{1-180}$ (lane 2), GST-WT1$_{181-429}$ (lane 3), GST-WT1$_{1-307}$ (lane 4), GST-WT1$_{308-429}$ (lane 5), or GST-YY1 (lane 6) coupled to glutathione-agarose beads. Beads were washed, and bound proteins were separated by SDS-PAGE, transferred to a nitrocellulose-nylon membrane, and

man embryonic kidney cell line (18), as a fusion protein with a FLAG epitope attached to the amino terminus (FLAG–par-4). Whole-cell lysates were incubated with purified GST-WT1, GST-$WT1_{1-180}$, GST-$WT1_{181-429}$, GST-$WT1_{1-307}$, GST-$WT1_{308-429}$, and GST-YY1. Bound FLAG–par-4 was detected by Western blotting using a monoclonal anti-FLAG antibody (Kodak). As shown in Fig. 2C, FLAG–par-4 was captured specifically by full-length WT1, $WT1_{181-429}$, and $WT1_{308-429}$ (lanes 1, 3, and 5) but not by $WT1_{1-180}$ or $WT1_{1-307}$ (lanes 2 and 4). These results suggest that aa 308 to 429 of WT1 are necessary and sufficient to interact with par-4. This region of WT1 is composed of four zinc fingers that constitute the DNA binding domain of WT1 (6, 17, 46). Since FLAG–par-4 did not bind GST-YY1, which also contains zinc fingers at its C terminus (Fig. 2C, lane 6), these findings indicate that par-4 binds specifically to the zinc finger domain of WT1, not to zinc finger structures in general. Finally, two-hybrid analysis also identified the zinc finger domain of WT1 as the par-4-interacting domain in yeast cells (data not shown), consistent with the results obtained from the GST fusion protein-based assays described above.

The WT1–par-4 interaction was further examined in mammalian cells by coimmunoprecipitation assays. FLAG–par-4 was cotransfected with WT1 or pRSV vector into 293 cells. Cells were lysed, and WT1 was immunoprecipitated with anti-WT1 polyclonal antibodies with subsequent Western blotting using anti-FLAG antibodies to detect the presence of FLAG–par-4. As shown in Fig. 2D, FLAG–par-4 was specifically coimmunoprecipitated by anti-WT1 antibodies (lane 2) but not by preimmune serum (lane 3). Coimmunoprecipitation of par-4 was not due to antibody cross-reactivity, as par-4 was absent among proteins immunoprecipitated by anti-WT1 antibodies from 293 cells that lacked transfected WT1 (Fig. 2D, lane 1).

We further examined the endogenous WT1–par-4 interaction in the M15 mouse mesonephric cell line without overexpression of either protein. Both WT1 and par-4 proteins are expressed in M15 cells (29) (see Fig. 4B). par-4 was immunoprecipitated from M15 whole-cell lysates, and the immunoprecipitate was analyzed for the presence of WT1 by Western blotting using anti-WT1 antibodies. As shown in Fig. 2E, WT1 was coimmunoprecipitated with an anti-par-4 monoclonal antibody (lane 2) but not NRS (lane 1). This result, combined with the WT1–par-4 interaction in 293 cells and yeast two-hybrid and GST binding data, strongly suggests that WT1 and par-4 interact with one another both in vivo and in vitro.

WT1 and par-4 tissue and cellular expression. WT1 has limited expression in adult tissues. Previously, WT1 mRNA was shown to be present in the kidney, ovary, testis, heart, diaphragm, peritoneum, and uterus of the adult human, rat, and mouse (2, 39, 44, 47, 56). We analyzed adult human poly(A)$^+$ mRNAs from a variety of tissues for WT1 expression by Northern blot analysis. A 3.6-kb WT1 mRNA species was strongly expressed in the testis and ovary and weakly expressed in the heart and kidney (Fig. 3). Marginal expression of WT1 was also found in the prostate and colon (Fig. 3). The presence of two transcripts in the testis is similar to that observed in mice by Pelletier et al. (43). The blots were stripped and reprobed with radiolabeled human par-4 cDNA. Three major species, designated 1, 2, and 3, of approximately 7.3, 5.0 and 2.1 kb, respectively, were detected (Fig. 3). The 2.1-kb species was expressed in all tissues, with very weak expression in skeletal muscles and strong expression in the testis and ovary. The 5.0-kb species was not detected in the brain, skeletal muscles, the spleen, or peripheral blood lymphocytes, while the 7.3-kb species was not present in the brain, skeletal muscles, the spleen, the thymus, or the prostate. The par-4 mRNA species designated 1, 2, and 3 were also detected by par-4 cDNA probes consisting only of the most 5′ or 3′ sequences (data not shown). As a control for the amount of RNA present on the blot, the blot was reprobed with radiolabeled human β-actin cDNA (Fig. 3). It is unclear whether the three RNA species detected by the par-4 probe represent alternatively spliced isoforms, differentially processed nuclear precursors, or closely related family members of par-4. Nevertheless, these data indicate that par-4 transcripts are ubiquitously expressed.

Then the subcellular localization of par-4 was examined. FLAG–par-4 and WT1 were expressed in 293 cells. Cells were lysed, fractionated into cytoplasmic and nuclear preparations, and probed with anti-FLAG and anti-WT1 antibodies. As shown in Fig. 4A, while WT1 was found exclusively in the nuclear fraction (lanes 3 and 4), FLAG–par-4 was detected in both cytoplasmic and nuclear fractions, with greater than 50% of par-4 found in the nuclear fraction (lanes 1 and 2). As a further control for the quality of the fractionation procedure, a cytoplasmic protein, $p70^{s6k}$ (51), was detected only in the cytoplasmic fraction (Fig. 4A, lanes 5 and 6), suggesting that there was minimal cross-contamination. The distribution of the native par-4 protein in M15 mouse mesonephric cells, in which WT1 is expressed (29), was also determined. As shown in Fig. 4B, par-4 was again found in both nuclear and cytoplasmic fractions by using affinity-purified anti-par-4 polyclonal antibodies (Santa Cruz Biotechnology, Inc.). Immunofluorescence analysis of M15 cells with affinity-purified anti-par-4 polyclonal antibodies detected par-4 in the nucleus as well as diffuse staining in the cytoplasm (data not shown). Taken together, fractionation-Western blotting experiments and immunofluorescence staining identified par-4 in both the nucleus and the cytoplasm. The nuclear localization of par-4 is consistent with its role as a transcriptional regulator.

par-4 inhibits WT1-mediated transcriptional activation. To determine the functional consequences of the WT1–par-4 interaction, transfection experiments were carried out to analyze the effects of par-4 on both transcriptional activation and repression activities of WT1. WT1 A and B isoforms have previously been shown to activate a reporter construct containing three WT1-EGR1 binding sites ($pEGR_3TKCAT$ [49]). As shown in Fig. 5A, cotransfected WT1 activated this reporter approximately 12-fold in 293 cells (lane 2), similar to the results reported previously (49). While addition of the pCMV

probed with an anti-FLAG monoclonal antibody. The position of FLAG–par-4 is indicated by an arrow on the right. The input lane (lane 7) was loaded with approximately 40 μg of lysate. Schematic representations of the GST fusion proteins used in this experiment are shown. Black boxes, GST; white boxes, WT1; shaded boxes, YY1. (D) WT1 and par-4 interact in 293 cells. FLAG–par-4 was transfected into 293 cells with (+) or without (−) WT1. Immunoprecipitations were performed with anti-WT1 (RαWT1) or preimmune rabbit serum (NRS). The immunoprecipitates were analyzed for the presence of FLAG–par-4 by Western blotting with an anti-FLAG monoclonal antibody. The positions of molecular mass markers (in kilodaltons) are indicated on the left, and the position of FLAG–par-4 is indicated by an arrow on the right. (E) Interaction of endogenous WT1 and par-4 in M15 mouse mesonephric cells. M15 whole-cell extracts were subjected to immunoprecipitation with either NRS or affinity-purified anti-par-4 (a-Par-4) antibodies. The immunoprecipitated proteins were analyzed by Western blotting using anti-WT1 antibodies. The position of the WT1 protein is indicated by an arrow on the right. The asterisk on the left indicates the position of immunoglobulin H that cross-reacted with the secondary antibodies. Lane 1, NRS; lane 2, anti-par-4 antibodies; lane 3, input M15 cell extract (lysate).

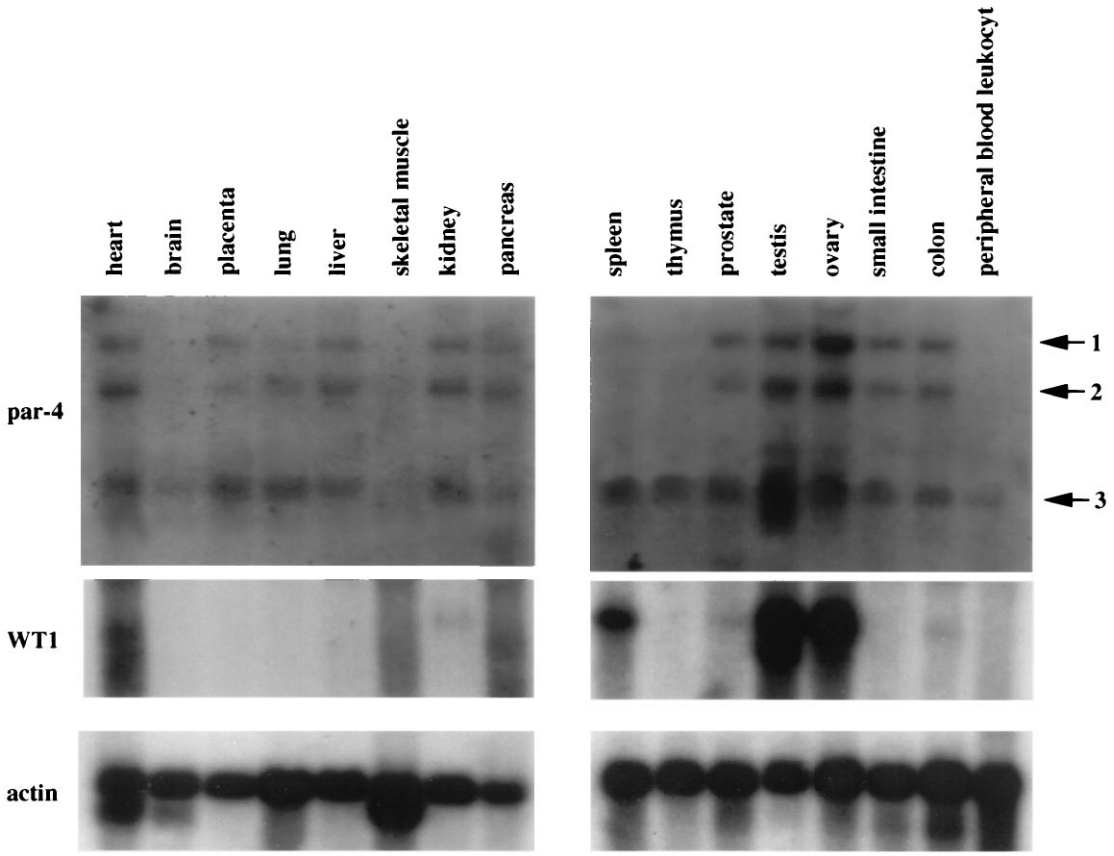


FIG. 3. Tissue expression of par-4 and WT1 mRNAs. Approximately 2 μg of poly(A)$^+$ RNAs from various human tissues (Clontech) was used for Northern blot analyses. The tissue origins of the RNA samples are indicated above lanes. The blots were probed with ^{32}P-labeled par-4 (upper panels), WT1 (middle panels), or β-actin (actin; lower panels) cDNAs. The positions of three major bands (designated 1, 2, and 3) of approximately 7.3, 5.0, and 2.1 kb detected with the par-4 probe are indicated by arrows on the right. The β-actin probe was used as a control for the amount of mRNA loaded onto the gel.

vector had no effect on WT1-induced activation of the pEGR$_3$TKCAT reporter (Fig. 5A, lanes 10 and 11), increasing amounts of pCMV-Par-4 resulted in a dose-dependent decrease in WT1-mediated transcriptional activation (Fig. 5A, lanes 6 through 9). par-4 was found not to affect the basal activity of the pEGR$_3$TKCAT reporter (Fig. 5A, lane 4). The specificity of the WT1–par-4 interaction was demonstrated by examining the effects of par-4 on transcription mediated by EGR1, which shares with WT1 DNA recognition sites (46). As shown in Fig. 5B, EGR1 activated the same reporter, pEGR$_3$TKCAT, in 293 cells (lane 2). However, cotransfection of pCMV-Par-4 or pCMV had little effect on EGR1-induced transcriptional activation (Fig. 5B, lanes 4 through 7).

To rule out the possibility that the observed inhibition of WT1-induced transcriptional activation by par-4 is due to the effects of par-4 on the expression of the cotransfected WT1 plasmid, the levels of WT1 in the presence and absence of transfected par-4 were compared. As shown in Fig. 5C, transfection of 1 to 15 μg of pCMV-Par-4 resulted in an increase in par-4, as judged by Western blotting using anti-par-4 antibodies. However, the increase in the expression of transfected par-4 had little effect on the expression level of transfected WT1 protein (Fig. 5C). Thus, the decrease in WT1-mediated transcriptional activation caused by par-4 is not due to a reduction in the WT1 protein level.

To determine whether the physical interaction between WT1 and par-4 underlies their functional interaction, the ability of a par-4 mutant to modulate the transcriptional activity of WT1 was examined. This mutant (pCMV-Par-4$_{1\text{-}267}$) lacks the putative leucine repeat domain and is unable to physically interact with WT1 (Fig. 2B). As shown in Fig. 5D, par-4$_{1\text{-}267}$ had little effect on WT1-mediated transcriptional activation of the pEGR$_3$TKCAT reporter (lane 8). Thus, the ability of par-4 to inhibit WT1-mediated transcriptional activation is correlated with its ability to physically interact with WT1. Taken together, these results suggest that par-4 specifically inhibits WT1-induced transcriptional activation through its physical association with WT1.

par-4 augments WT1-mediated transcriptional repression. WT1 is capable of activating and repressing transcription. When fused to the DNA binding domain of GAL4, GAL4-WT1 efficiently repressed the target plasmid pGAL4TKCAT (Fig. 6, lane 2), as previously reported (30, 34). Cotransfection of pCMV-Par-4 resulted in further repression of the reporter (Fig. 6, lane 3). Since the pCMV vector caused a slight reduction in CAT activity (Fig. 6, lane 4), the net contribution of par-4 to the enhanced WT1-mediated repression was calculated to be approximately threefold. This effect of par-4 on the repression function of WT1 was specific, as par-4 did not augment the ability of GAL4-YY1 to repress transcription (Fig. 6, lanes 5 through 7). These results suggested that in addition to inhibiting the activation function of WT1, par-4 specifically enhanced its repressor activity.

par-4 is a novel transcriptional repressor. The fact that

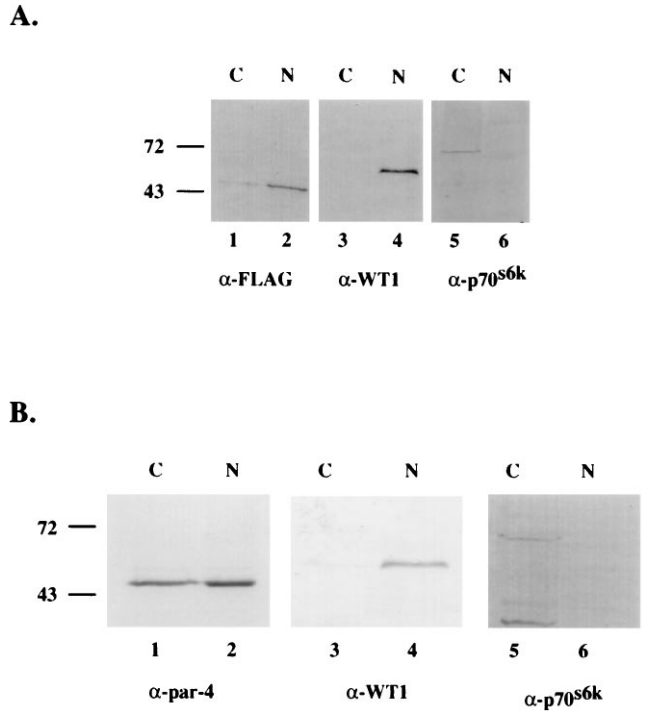


FIG. 4. Subcellular expression of par-4 and WT1. Nuclear (N) and cytoplasmic (C) lysates were prepared from 293 cells cotransfected with 10 μg of pCMV-FLAG-par-4 and 10 μg of pRSV-WT1 (A) and from the M15 mouse mesonephric cell line (B). Proteins were separated by SDS-PAGE and detected by Western blotting using anti-FLAG, anti-WT1, and anti-$p70^{s6k}$ antibodies (A) and affinity-purified anti-par-4 polyclonal antibody and anti-WT1 and anti-$p70^{s6k}$ antibodies (B). The positions of molecular mass markers (in kilodaltons) are indicated on the left.

par-4 not only inhibited the activation function but also enhanced the repression function of WT1 suggested that par-4 itself is a transcriptional repressor. To test this hypothesis, full-length par-4 was fused to the GAL4 DNA binding domain and the fusion protein (GAL4–par-4) was analyzed for its transcriptional activity. As shown in Fig. 7, while the GAL4 DNA binding domain alone had no effect, GAL4–par-4 repressed CAT expression directed by the pGAL4TKCAT reporter plasmid in a dose-responsive manner (lanes 5 through 9). This repression was dependent on the GAL4 sites, as GAL4–par-4 had no effect on pTKCAT that lacks the GAL4 binding sites (Fig. 7, lane 11). These results demonstrated that par-4 is capable of repressing transcription when brought to a promoter via a heterologous DNA binding domain.

par-4 partially rescues WT1-induced growth suppression in a human melanoma cell line. To determine the biological consequences of the WT1–par-4 interaction, the ability of WT1 and par-4, either alone or together, to regulate cell growth was analyzed in A375-C6 cells, a melanoma cell line (12). Cells were transfected separately with the pCMV vector, pCMV-WT1, pCMV-Par-4, or pCMV-Par-4_{1-267} or cotransfected with pCMV-WT1 plus pCMV-Par-4 or pCMV-Par-4_{1-267}. The number of G418-resistant colonies obtained with plasmid pCMV-WT1 was about 40% of that seen with vector DNA, pCMV-Par-4, or pCMV-Par-4_{1-267} alone, suggesting that WT1 caused growth suppression of melanoma cells. Interestingly, cotransfection with pCMV-Par-4, but not with pCMV-Par-4_{1-267}, restored the colony number to about 90% of that seen with the vector, suggesting that par-4 is capable of rescuing cells from growth suppression by WT1 and that the WT1-interacting domain of par-4 is necessary for this effect. This potential biological interaction between WT1 and par-4 in A375-C6 cells was also studied by [^{3}H]thymidine incorporation. Data for two different transfectant cell lines (L1 and L2) expressing the indicated plasmids are shown in Fig. 8. Cells transfected with vector DNA, pCMV-Par-4, or pCMV-par-4_{1-267} alone in 72 h of culture showed similar proliferation potentials. Consistent with the results of colony assays, cells transfected with pCMV-WT1 showed approximately 40% [^{3}H]thymidine incorporation compared with that of cells transfected with the vector DNA alone, indicating growth suppression by WT1. In contrast, cells transfected with WT1 and par-4 together showed about 75% thymidine incorporation compared with that of vector DNA alone. This represents an increase of about 35% in thymidine intake compared with that of cells transfected with WT1 alone, suggesting that par-4 partially rescued growth suppression caused by WT1. Significantly, the par-4 mutant (CMV–Par-4_{1-267}) that lacks the WT1-interacting domain had no effect on the ability of WT1 to suppress cell growth. Taken together, these results suggest that WT1 inhibits the growth of A375-C6 cells and that the ability of par-4 to overcome growth suppression by WT1 is dependent on the physical interaction between these two proteins.

DISCUSSION

The following conclusions have been drawn from this study: (i) par-4 is a WT1-interacting protein that modulates the transcriptional activities of WT1 via physical interactions; (ii) par-4 overcomes growth suppression caused by WT1 in melanoma cells, possibly as a result of the ability of par-4 to modulate the transcriptional activity of WT1; and (iii) par-4 is a novel transcriptional repressor. The WT1–par-4 physical interaction was supported by multiple independent protein-protein interaction assays that demonstrated the WT1–par-4 association both in vitro (GST assay) and in vivo (M15, 293, and yeast cells). The significance of the WT1–par-4 interaction was substantiated by the finding that par-4 regulates the transcription function as well as the growth suppression function of WT1 in a manner that is dependent on the WT1-interacting domain of par-4 (Fig. 5, 6, and 8). Transcriptionally, par-4 specifically inhibited WT1-mediated transcriptional activation but enhanced the ability of WT1 to repress transcription (Fig. 5 and 6). When analyzed as a GAL4 fusion protein, par-4 potently repressed transcription (Fig. 7). These results suggest a model in which the novel repressor par-4 physically interacts with WT1, bringing an additional repression domain to the promoter. As a result, par-4 inhibits WT1 activation but potentiates WT1 repression. One biological consequence of this interaction is that par-4 rescued cells whose growth was suppressed by WT1 (Fig. 8).

Physical and functional interactions between WT1 and par-4. By using the yeast two-hybrid approach, a clone, H2-73, that encoded a polypeptide capable of specifically interacting with WT1 was obtained. A search of the database revealed 96% identity between H2-73 and the rat par-4 protein, indicating that H2-73 was a partial clone of the human homolog of rat par-4. Subsequent GST-based assays confirmed the WT1–par-4 interaction observed in yeast cells (Fig. 2A through C). This interaction was also recapitulated when par-4 and WT1 were coexpressed in 293 cells, a human embryonic kidney cell line. Significantly, the interaction between endogenous WT1 and par-4 without overexpression can also be detected in mouse mesonephric cells (Fig. 2E). As judged by the amounts of coprecipitated proteins, only a small percentage of either protein is found in the complex.

Further GST binding analyses identified the domains of

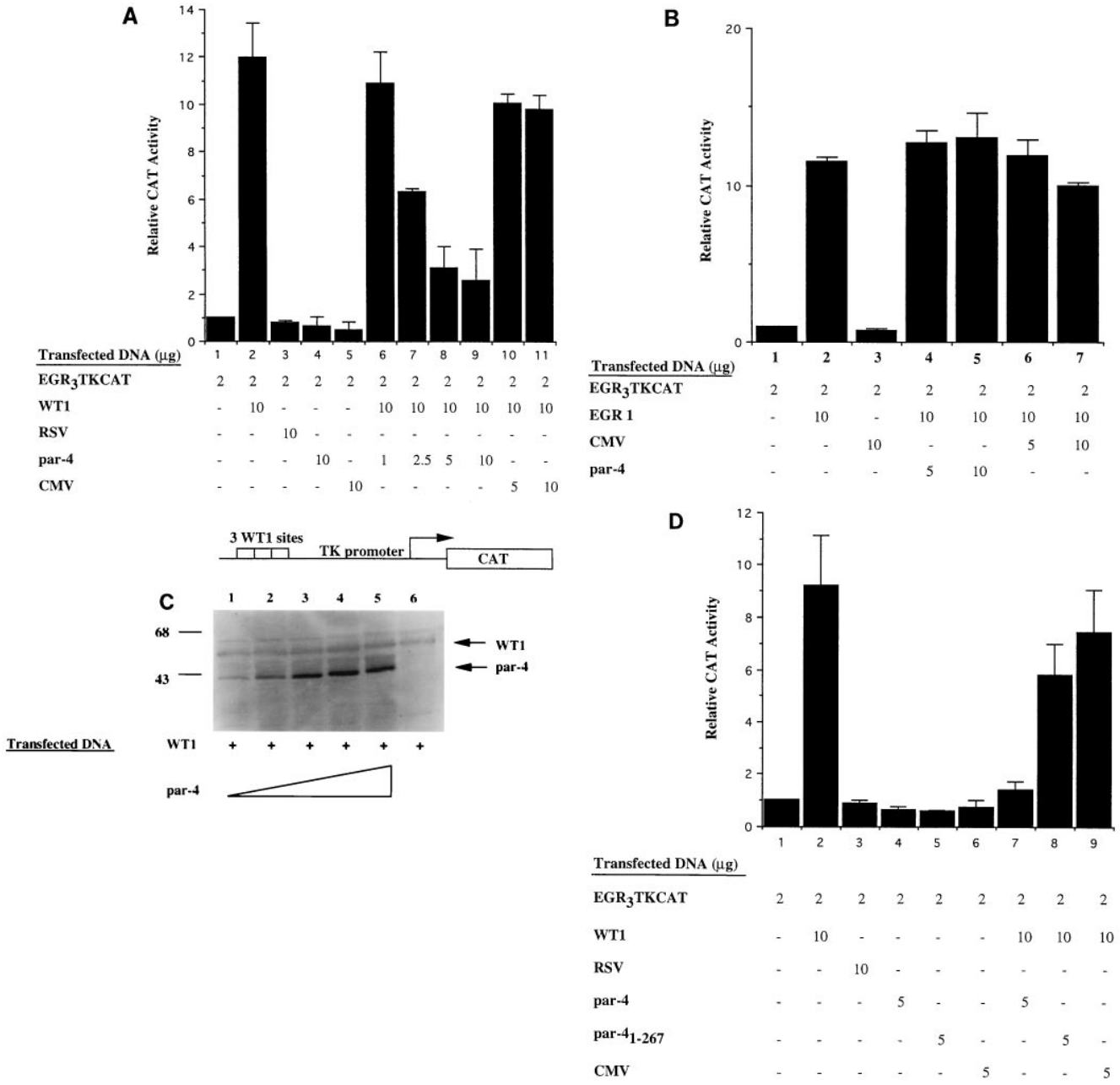
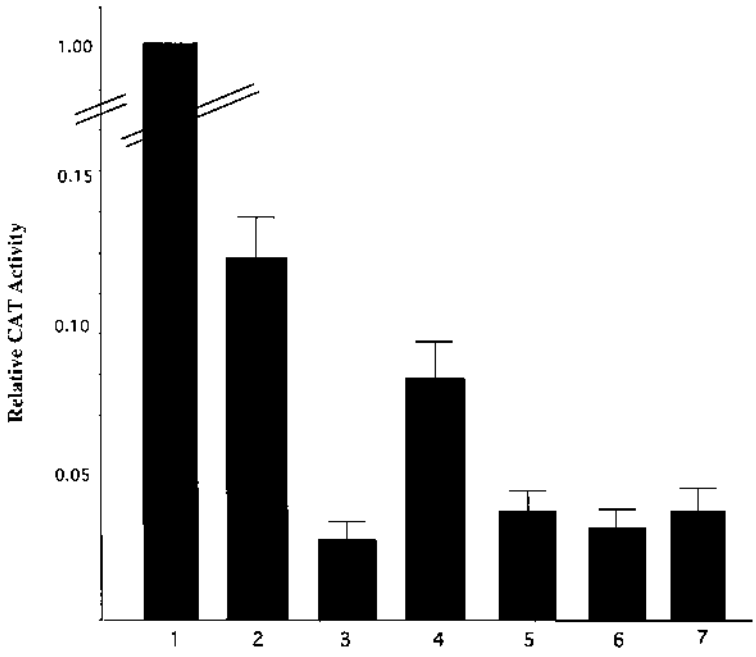


FIG. 5. par-4 specifically modulates the transcriptional activity of WT1. Data for all CAT assays are the means ± standard deviations of three independent transfections and CAT assays. (A) par-4 inhibits WT1-mediated transcriptional activation. A reporter plasmid containing three WT1-EGR1 binding sites was cotransfected with pRSV-WT1 and increasing amounts of pCMV-par-4 expression plasmid (lanes 6 through 9) or with the expression plasmid alone (lanes 10 and 11). The amounts of transfected plasmids are indicated below lanes. (B) par-4 does not affect EGR1-mediated transcriptional activation. The same reporter plasmid used in panel A was cotransfected with pCMV-EGR1 and increasing amounts of pCMV-Par-4 expression plasmid (lanes 4 and 5) or with the expression plasmid alone (lanes 6 and 7). The amounts of transfected plasmids are indicated below lanes. (C) Cotransfection of par-4 does not change WT1 expression levels. Cells were transfected with (+) 10 μg of pRSV-WT1 and 1 to 15 μg of pCMV-Par-4 (lanes 1 through 5). Whole-cell lysates were prepared, and WT1 and par-4 were detected by Western blotting. The positions of molecular mass markers (in kilodaltons) are shown on the left. (D) A mutant par-4 protein lacking the WT1-interacting domain does not affect WT1-mediated transcriptional activation. The same reporter plasmid used in panel A was cotransfected with WT1 and expression plasmids encoding full-length par-4 (lane 7) or par-4 lacking the WT1-interacting domain (lane 8) or with vector plasmid alone (lane 9). The amounts of transfected plasmids are indicated below the lanes.

WT1 and par-4 that were involved in their physical interactions. The interaction of WT1 was mediated by the four C_2H_2-type zinc fingers located at its C terminus (Fig. 2C). This interaction was clearly not due to the general zinc finger structure but was specific to the zinc fingers of WT1, as YY1, another C_2H_2-type zinc finger protein, did not interact with par-4. The idea that zinc fingers, in addition to binding DNA and RNA, may mediate protein-protein interaction has been demonstrated in other experimental systems. For instance, the zinc fingers of YY1 have been shown to be involved in physical interactions with Sp1 (31, 55), the transcriptional cofactor p300 (30), and bZIP-containing proteins such as CREB (15a, 65). The zinc fingers of Sp1 have been shown to interact with the GATA factors (45).



Transfected DNA (μg)							
GAL4TKCAT	10	10	10	10	10	10	10
GAL4-WT1		5	5	5			
GAL4-YY1					5	5	5
CMV-par4			5			5	
CMV	10			5			5

FIG. 6. par-4 augments WT1-mediated transcriptional repression. A reporter plasmid containing five GAL4 DNA binding sites (pGAL4TKCAT) was cotransfected with the different combinations of plasmids indicated, with the amounts of transfected plasmids also shown. Data for all CAT assays are the means ± standard deviations of three independent transfections and CAT assays.

The finding that the zinc fingers of WT1 mediate its interaction with par-4 is reminiscent of the WT1-p53 interaction reported previously (36). In these studies, the zinc fingers of WT1 were shown to interact with p53; this p53-WT1 interaction plays a role in WT1-mediated transcriptional repression (36). More recently, WT1 was shown to inhibit the ability of p53 to induce apoptosis (35). It thus appears that WT1 may interact with a number of cellular proteins via its zinc finger domain. The biochemical relationships among these three proteins, WT1, p53, and par-4, are currently unknown. It is possible that p53 and par-4 compete for binding to WT1 since they both bind the zinc fingers of WT1. Alternatively, they may bind to the same WT1 molecule simultaneously. Our preliminary data suggest that under certain conditions, p53 and par-4 are able to bind the same WT1 molecule (51a). However, a definitive answer to this question requires detailed characterizations of the binding sites on WT1 for p53 and par-4, accompanied by studies of the possible biological consequences of this putative three-way interaction.

Previously, the transcriptional repression and activation domains of WT1 have been mapped to regions outside the zinc finger domain. A transcriptional cofactor of WT1 is expected to interact with these defined transcriptional domains. Since the WT1–par-4 interaction does not occur within the established transcriptional domains of WT1, par-4 may not function as a classical cofactor for WT1-mediated transcription. Rather, par-4 may serve a critical modulatory role, i.e., its interaction with WT1 may contribute to the determination of whether WT1 functions as an activator or repressor. Significantly, this presumed modulatory role of par-4 was supported by the observation that overexpression of par-4 overcame growth suppression induced by WT1 (Fig. 8). In this regard, it is informative to consider the zinc finger-containing transcription factors GATA1 and GATA2, which are believed to play an important role in the development of erythroid cells, megakaryocytes, and mast cells. In cell culture, the zinc fingers of GATA1 alone can rescue GATA1-deficient embryonic stem cells (5a). The C-terminal zinc fingers of these GATA factors are also sufficient to induce megakaryocytic differentiation without the requirement of any known transcriptional domains (59).

The domain within par-4 that mediates its interaction with WT1 has also been identified. The C-terminal leucine repeats of par-4 were necessary and sufficient to mediate the WT1–par-4 physical interaction (Fig. 2B). Thus, the par-4–WT1 interaction occurs via the leucine zipper domain of par-4 and the zinc finger region of WT1. It has been recently shown that the interaction between the ATF/CREB family of bZIP transcription factors and the zinc finger protein YY1 is also mediated by leucine zipper-zinc finger interactions (65). The leucine repeat structure has been found in many transcription factors, including Jun, Fos, and C/EBP (reviewed in reference 25). In addition to leucine repeats, these transcription factors contain an adjacent subdomain composed of a consensus sequence of

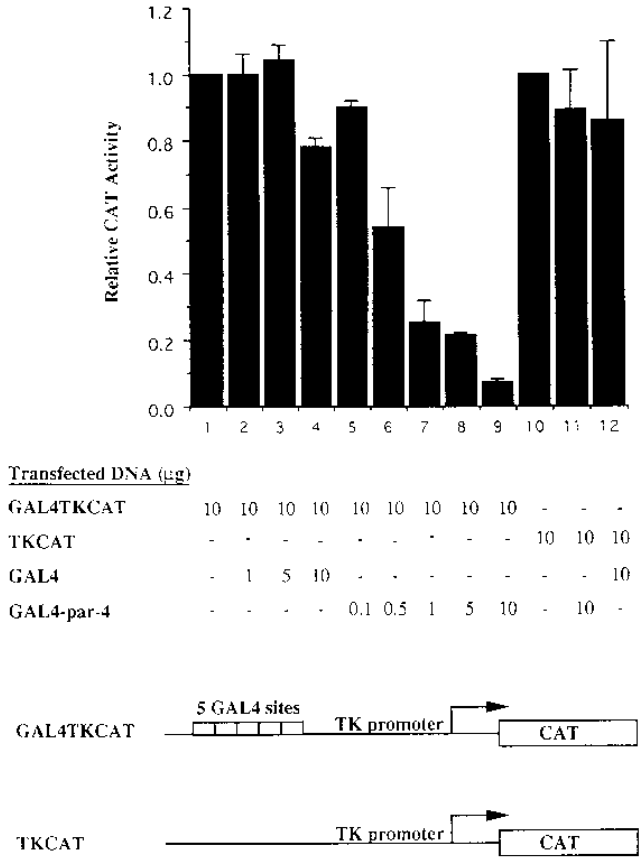


FIG. 7. par-4 is a transcriptional repressor. CAT reporter plasmids with (GAL4TKCAT) and without (TKCAT) GAL4 DNA binding sites were cotransfected with GAL4–par-4 (lanes 5 through 9 and 11) or GAL4 (lanes 2 through 4 and 12). The amounts of transfected plasmids are indicated. Data are the means ± standard deviations of three independent transfections and CAT assays.

predominantly basic amino acids known as the basic region (45). The leucine repeats are essential for the formation of homo- and heterodimeric complexes, while the basic region is responsible for the DNA binding properties of these proteins (25). As expected, par-4 is capable of homo-oligomerization and the C-terminal 56 aa containing the leucine repeats are necessary and sufficient for oligomerization (25a). However, upon inspection of the par-4 sequence, a consensus basic region is not obvious. While par-4 does not seem to contain an immediately adjacent basic domain, there are stretches of basic residues farther 5′ to the first leucine. The ability of par-4 to bind DNA is currently under investigation.

By Northern analysis, par-4 was found to be ubiquitously expressed. Consequently, par-4 and WT1 mRNA were found to be expressed in some of the same adult tissues (Fig. 3). Although circumstantial, this result is consistent with the possibility that the WT1–par-4 interaction is physiologically important. Indeed, physical interactions between the two proteins can be detected in mouse mesonephric cells, and the biological significance of the WT1–par-4 interaction is underscored by the finding that par-4, but not a mutant defective for binding WT1, partially rescued growth suppression caused by WT1.

par-4 as a modulator of WT1. The mechanisms that control the activation and repression functions of WT1 have yet to be fully elucidated. The number as well as the position of the WT1 binding sites with respect to the TATA box may affect the ability of WT1 to either activate or repress the transcription of reporter plasmids (61). The presence or absence of functionally active p53 may also affect the transcriptional activity of WT1 (36). We have demonstrated here that par-4 can modulate the transcriptional activities of WT1 via physical interactions. By using a reporter containing WT1 binding sites that has been shown previously to be specifically activated by WT1 (49), overexpression of par-4 resulted in a specific dose-dependent decrease in WT1-mediated activation (Fig. 5A). This effect of par-4 was likely to be mediated by its physical interaction with WT1, as the mutant par-4 lacking the WT1-interacting domain failed to inhibit WT1-induced transcription. Importantly, the effect of par-4 was specific to WT1, since transcription induced by EGR1, which recognizes the same DNA site as does WT1, was unaffected by overexpression of par-4 (Fig. 5B). Furthermore, par-4 was shown to augment the transcriptional repression directed by GAL4-WT1 (Fig. 6).

A number of possibilities may explain how par-4 inhibits activation mediated by WT1. First, par-4 may interfere with the ability of WT1 to bind its recognition sequences. However, the addition of either in vitro-translated or bacterially purified par-4 proteins had no effect on the DNA binding ability of WT1 in electrophoretic mobility shift assays (25a). Second, the ability of par-4 to inhibit WT1 activation may be due to the possibility that par-4 itself is a repressor. By bringing an additional repression domain to the promoter via its interactions with WT1, par-4 may counteract transcriptional activation by WT1. This hypothesis also takes into consideration the observation that par-4 augments the repression function of WT1. Indeed, when assayed as a GAL4 fusion protein, par-4 was found to be a potent transcriptional repressor (Fig. 7). The finding that par-4 is capable of repressing transcription when targeted to promoters, together with the rest of the data presented here, favors the latter hypothesis.

The fact that par-4 partially rescued growth suppression of melanoma cells caused by WT1 strengthens the importance of the WT1–par-4 interaction. Transcriptionally, par-4 inhibits the activation function but potentiates the repression function of WT1. It is unclear at present whether the biological effects of par-4 on WT1 are correlated with the ability of par-4 to

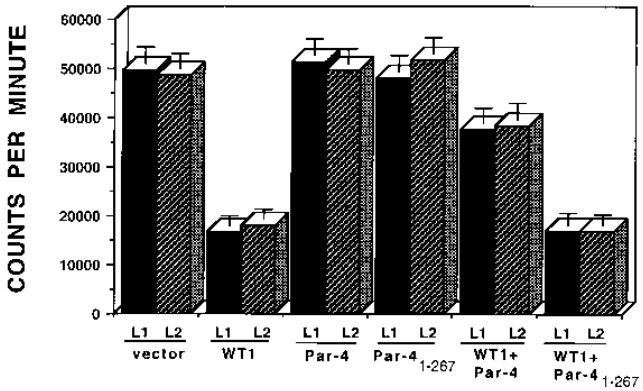


FIG. 8. par-4 overcomes WT1-induced growth suppression of A375-C6 cells. A375-C6 cells were transfected separately with the pCMV vector, pCMV-WT1 (WT1), pCMV-Par-4 (Par-4), or pCMV-Par-4_{1-267} (Par-4_{1-267}) or cotransfected with pCMV-WT1 plus pCMV-Par-4 (WT1 + Par-4) or pCMV-WT1 plus pCMV-Par-4_{1-267} (WT1 + Par-4_{1-267}). Stably transfected clones were selected with G418 sulfate, and pools of transfected clones were maintained as cell lines L1 and L2. The transfected cells were seeded in 96-well plates and cultured for 72 h. Thereafter, cells were pulsed with [^{3}H]thymidine for 8 h and the incorporation of [^{3}H]thymidine was determined. Three separate experiments were performed. Data are the means (in counts per minute) of 12 different observations; error bars indicate standard deviations.

influence the activation or repression function of WT1 or both. Both activation and repression functions of WT1 have been implicated in its tumor suppressor functions (21, 41, 49).

In addition to WT1 on 11p13, chromosomes 11p15.5 (50) and 16q (6a, 37a) have been implicated in Wilms' tumorigenesis (50). Recently, deletion of chromosome 16 and duplication of chromosome 12 have been found in certain Wilms' tumor patients (3). A number of other genetic loci have also been suggested to contribute to the disease (1). These findings may explain the low-level frequency of WT1 mutations (approximately 10%) in Wilms' tumors (7, 19) and suggest the involvement of novel gene products. It is conceivable that mutations or abnormal expression of proteins, such as par-4, that modulate both the transcriptional and growth regulatory functions of WT1 could also lead to aberrant expression of certain growth-regulatory proteins and thus contribute to Wilms' tumor formation.

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