WT1 was originally identified as a Wilms' tumor suppressor gene, but it may have oncogenic potential in leukemia and in some solid tumors. WT1 is a transcription factor that has been implicated in the regulation of target genes related to apoptosis, genitourinary differentiation, and cell cycle progression. Because induction of WT1 leads indirectly to increased p21 expression in osteosarcoma cells, we investigated the possibility that other genes involved in the G1/S phase transition might also be WT1 targets. Cyclin E plays a crucial role in the cell cycle by activating cyclin-dependent kinase 2, which phosphorylates Rb, leading to progression from G1 into S phase. We identified several WT1 binding sites in the cyclin E promoter. We demonstrate that WT1 binds to these sites and that in transient transfection assays WT1 represses the cyclin E promoter. This activity is dependent on the presence of a binding site located downstream of the transcription start site. In intact cells, induction of WT1 expression down-regulates cyclin E protein levels. These results provide the first demonstration that WT1 can directly modulate the expression of a gene involved in cell cycle progression.

There are two classes of genes regulated by WT1. The first of these is composed of genes critical for the differentiation of the specific cell types that express WT1, in particular, the regulation of sex determination. For example, the Dax-1 promoter is dramatically up-regulated by the WT1 isoform lacking both exon 5 and the KTS insert (designated WT1(−/−)) and by the isoform that contains exon 5 but lacks the KTS insert (designated WT1(+/-)), whereas the isoforms containing the KTS insert (designated WT1(+/+)) and both exon 5 and the KTS insert (designated WT1(+/+)) have little effect (8). A similar pattern is seen in the regulation of the Mullerian inhibitory substance and SRY promoters by WT1 (9, 10). Each of these target genes is important in differentiation of the genitourinary system.

The second class of genes regulated by WT1 includes those involved in cell cycle regulation and apoptosis. The cyclin-dependent kinase inhibitor p21 is up-regulated when WT1(−/−) is induced, although there is no evidence that this is a direct effect of WT1 on the p21 promoter (11). Bcl-2 is also up-regulated by WT1(−/−), and this is a direct result of WT1 binding to sites in the bcl-2 promoter (12). These genes, despite serving more general roles in cellular function than Dax-1 and SRY, might also be important in terminal differentiation. The identification of p21 as a WT1-responsive gene led us to hypothesize that WT1 might also regulate the expression of other mediators of cell cycle progression. Here we present evidence that WT1 inhibits the expression of cyclin E and that the cyclin E promoter is a direct target of transcriptional regulation by WT1.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The generation of 32D cl3 transfectants stably expressing an inducible human WT1(−/−) cDNA under the control of the sheep metallothionein promoter is described elsewhere. These cells were grown in Iacove's modified Dulbecco's medium supplemented with 10% heat-inactivated fetal calf serum, 1 ng/ml interleukin 3 (R&D Systems, Minneapolis, MN), and 1 mg/ml Genetin (Invitrogen). NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum. Plasmids—pCB6WT1(−/−) contains the WT1(−/−) cDNA cloned downstream of the CMV immediate-early promoter, and pCB6WT1(+/-) contains the cDNA encoding WT1(+/-). Plasmid 2-3 (a kind gift from Dr. R. Weinberg, Whitehead Institute, Cambridge, MA) contains 2.2 kb of the human cyclin E promoter and first exon cloned into the multiple cloning site of pGL2basic, driving the expression of the cDNA for firefly luciferase (Promega, Madison, WI). The plasmid pRL-SV40 contains the cDNA for Renilla luciferase under the control of the SV40 early enhancer/promoter region.

**Transient Transfection Assays**—Transient transfection assays were performed using TransIT LT-1 (Fermentas, Madison, WI) following the manufacturer's protocol. NIH 3T3 cells were plated in 6-well plates at 105 cells/well. Each well was transfected with 2.5 μg of plasmid 2-3, 0.7 μg of either the WT1 construct or the pCB6 vector, and 0.1 μg of pRL-SV40 as an internal control. 32D cl3 cells were plated in 100-mm tissue culture plates at 2 × 105 cells/plate and were transfected with 10 μg of plasmid 2-3, 0.7 μg of either the WT1 construct or the pCB6 vector, and 0.1 μg of pRL-SV40 as an internal control.
Micrococcal nuclease digestion of chromatin

The promoter mutants were digested with micrococcal nuclease and the fragments were separated by gel electrophoresis and visualized by autoradiography. The results showed that WT1 expression was significantly reduced in all mutants compared to the control cells. The specific effects of WT1 on cyclin E expression were also confirmed by Western blotting and luciferase reporter assays.

**Results**

**WT1(+/−) Regulates Cyclin E Expression**—Among the proposed targets of WT1 is the cyclin-dependent kinase inhibitor p21 (11). Because p21 participates in the G1/S transition, we hypothesized that other regulators of this phase of the cell cycle might also be affected by WT1. To examine this possibility, we utilized a stably transfected cell line, designated 32DWT1.1, expressing an inducible human WT1(+/−) cDNA. The details of this cell line are described elsewhere, but briefly, the cell line was generated by transfecting 32D cl3 cells with a plasmid containing WT1 cDNA and selecting stable transformants in a mass culture. Treating 32DWT1.1 cells with 100 µM ZnCl2 overnight induced significant expression of the WT1(+/−) protein (Fig. 1). 32DWT1.1 cells and the control cell line 32DV4 (which was stably transfected with an empty metallothionein promoter-containing expression vector) were treated overnight with or without ZnCl2, and lysates were subjected to Western blotting with antibodies against a variety of proteins involved in cell cycle progression. In 32DWT1.1 cells, which express low levels of WT1(+/−) in the uninduced state, the basal level of p21WAF1/CIP1 was less than the basal level in 32DV4 cells, but induction of WT1(+/−) did not alter p21WAF1/CIP1 protein expression. This suggests that the ability of WT1 to induce p21 expression is cell type-specific. Induction of WT1(+/−) by ZnCl2 also did not affect p27Kip1 expression and had no effect on the expression of a number of other mediators of cell cycle progression, including Cdk2, Cdk3, Cdk4, cyclin D3, Cdc25a, JAK3 (Fig. 1), or cyclin D2 (data not shown). Under the same experimental conditions, however, the level of cyclin E protein in WT1(+/−)-expressing cells was reduced by almost 4-fold (Fig. 1).
WT1 Suppresses the Cyclin E Promoter—Next, we sought to determine whether the decrease in the level of cyclin E protein upon induction of WT1(−/−) expression occurs through direct transcriptional suppression of the cyclin E promoter by WT1 (−/−). A reporter plasmid containing firefly luciferase under the control of the human cyclin E promoter was transfected into NIH 3T3 cells. Cotransfection of WT1(−/−) under the control of the cytomegalovirus immediate-early promoter resulted in a 4-fold decrease in luciferase activity (Fig. 2B). There was some variability in the degree of suppression in different experiments, with a range of 2.5- to 5-fold suppression. In the same cells, the WT1(+/+) isoform was a much less efficient repressor of the cyclin E promoter, suppressing transcription by only 30% (Fig. 2B). A truncated form of WT1 lacking the carboxyl-terminal zinc finger region, which is required for DNA binding, had no effect on the cyclin E promoter in this assay (Fig. 2B). There was a clear dose-related suppression of the promoter by WT1(−/−), with decreasing luciferase activity as the ratio of WT1(−/−) to reporter plasmid increased (Fig. 2C). Because the ability of WT1 isoforms to modulate the activity of some promoters can vary by cell type, we wanted to determine whether the cyclin E promoter is also suppressed by WT1(−/−) in hematopoietic cells. Accordingly, we transiently transfected 32DWT1.1 cells with the cyclin E promoter-luciferase reporter construct and then treated the cells with ZnCl2 to induce the expression of the transfected WT1(−/−). Just as in the NIH 3T3 cells, the WT1(−/−) suppressed the cyclin E promoter in the 32DWT1.1 cells (Fig. 2D). No suppression was seen in the control 32DV4 cells transfected with the same construct and treated with ZnCl2. These findings suggest that WT1(−/−) efficiently and specifically down-regulates cyclin E levels, possibly by directly binding to the promoter and repressing transcription.

Because WT1 has been shown to act as a dimer (15), we investigated the possibility that the WT1(+/+) isoform could interfere with the ability of the WT1(−/−) isoform to repress the cyclin E promoter in this assay. NIH 3T3 cells were therefore transfected with the cyclin E reporter plasmid and WT1 (−/−), WT1(+/-), or a combination of the two isoforms in two
different ratios. WT1(−/−) again efficiently repressed the cyclin E promoter, whereas WT1(+/+), was much less effective. Remarkably, the degree of promoter repression was less with increasing ratio of WT1(+/+) to WT1(−/−) (Fig. 2E). This is unlikely to represent competitive DNA binding, because WT1(+/+) has only weak affinity for WT1(−/−) binding sites. More likely, this reflects the ability of WT1(+/+) to heterodimerize with WT1(−/−) and, thereby, inhibit DNA binding by the WT1(−/−) isoform.

**WT1(−/−) Binds to the Putative Binding Sites in the Cyclin E Promoter**—There are three potential binding sites in the cyclin E promoter for this isoform of WT1 (Fig. 2A); two sites are 5′ of the transcription start site, and one is within exon 1 (although 5′ of the translation start site), an arrangement seen in other promoters, such as the insulin-like growth factor I receptor, that are repressed by WT1(−/−). To confirm that WT1(−/−) binds to the putative binding sites identified in the cyclin E promoter, we performed electrophoretic mobility shift assays. Nuclear extracts were made from 32DV4 cells and from stably transfected cells treated overnight with ZnCl₂ to induce a high level of WT1(+/−) expression. The presence of WT1 protein in the extracts from the transfected cells was confirmed by Western blotting (Fig. 3A). Nuclear extract from the control cells contains an activity that binds to and retards the migration of a radiolabeled oligonucleotide containing WT1 binding site 1 (Fig. 3B). Nuclear extracts from WT(+/−)-expressing cells also contain DNA binding activity that recognizes this oligonucleotide. In contrast to the control cells, nuclear extracts from the WT1-expressing cells generate two distinct species in this assay. One of these species comigrated with the complex derived from the 32DV4 cells, and the second complex migrated more rapidly (Fig. 3B). Inclusion of an excess of unlabeled oligonucleotide in the reaction blocked the formation of both complexes. Strikingly, inclusion of an excess of an unlabeled oligonucleotide containing two point mutations that abrogate WT1 binding specifically eliminated the formation of the more slowly migrating complex, with no effect on the more rapidly migrating complex. These findings demonstrate that the slower migrating species represents a nonspecific protein-oligonucleotide complex, whereas the more rapidly migrating complex represents WT1(−/−) binding to the labeled oligonucleotide.

Another identical result was seen with a radiolabeled oligonucleotide containing WT1 binding site 2. A single species was found in the nuclear extract from the control cells, whereas two species, one of which migrated more rapidly than the species from the control cells, were found in the extracts from the WT1-expressing cells. Both species were inhibited by an excess of unlabeled oligonucleotide, but only the more slowly migrating species was inhibited by an oligonucleotide containing a mutation that abrogates binding by WT1 (Fig. 3C).

**Electrophoretic mobility shift assay** was also carried out using these nuclear extracts and an oligonucleotide containing the third putative WT1 binding site, this one located in the 5′ untranslated portion of the mRNA. Unlike the other two sites, there was no activity in the nuclear extract from the control 32DV4 cells capable of retarding the migration of the oligonucleotide containing binding site 3 (Fig. 3D). An activity capable of binding this oligonucleotide was present in the nuclear extract from WT1(−/−)-expressing cells (Fig. 3D). This activity was inhibited by an excess of unlabeled oligonucleotide but not by an excess of unlabeled oligonucleotide containing a mutation that abrogates binding by WT1. Thus, this sequence is also bound by WT1(−/−), but unlike the other two binding sites, it is not bound by an activity present in the untransfected cells.

**The WT1 Binding Site in Exon 1 Is Necessary for Cyclin E Promoter Repression**—Having demonstrated that WT1(−/−) can bind to each of the putative binding sites in the cyclin E promoter, we wanted to determine the relative importance of...
each of these sites for the regulation of the promoter. We therefore generated deletion mutants of the cyclin E promoter lacking each binding site alone and in combination. Luciferase reporter constructs containing these deletion mutants were cotransfected into NIH 3T3 cells with the plasmid encoding WT1(−/−). Similar to what was seen with the full-length promoter, deletion mutants lacking site 1, site 2, or both of these sites, were strongly repressed by WT1(−/−). In contrast, deletion mutants lacking site 3 alone, or in combination with the other binding sites, were no longer repressed by this isoform of WT1 (Fig. 4). Although the basal activity of the constructs lacking binding site 3 was significantly less than the other constructs (which might reflect an effect on either RNA stability or on the basal rate of transcription), there was still sufficient activity present compared with the promoterless pGL2basic (Fig. 4) that an effect of WT1(−/−) could have been observed. This finding suggests that transcriptional repression of the cyclin E promoter requires the presence of a WT1 binding site downstream of the transcription start site.

**DISCUSSION**

Although WT1 was originally identified as a tumor suppressor gene, recent literature supports the view that it may have oncogenic potential in acute leukemia and in breast cancer (16, 17). The protein product of the WT1 gene has transcriptional regulatory activity. A number of target genes have been identified that might be important for neoplasia. Because induction of WT1 expression leads to up-regulation of p21, we examined the promoters of other genes that function in the G1/S transition for the presence of the well defined consensus DNA binding sequence for the WT1(−/−) isoform. We discovered that the promoter for cyclin E, another key regulator of cell cycle progression, contains three potential WT1(−/−) binding sites. Cyclin E functions by binding to and activating Cdk2 (18, 19). The cyclin E-Cdk2 complex is a critical promoter of initiation of the S phase of the cell cycle.

We have demonstrated here that WT1(−/−) binds to all three consensus binding sites in the cyclin E promoter, negatively regulates the promoter in transient transfection assays, and in intact cells, down-regulates cyclin E protein levels. These findings support the idea that cyclin E is a direct target of the transcriptional regulatory activity of WT1. This conclusion is strengthened by our finding that deleting the binding site from the 5′ untranslated region of exon 1 eliminates the ability of WT1 to repress the promoter in transient transfection assays. Deletion of the region surrounding this site clearly decreases the basal level of promoter activity substantially; nevertheless, there is still significant residual activity, which WT1(−/−) does not suppress.

Our finding that WT1(−/−) directly suppresses cyclin E complements the published observation that induction of WT1(−/−) in Saos2 cells up-regulates the expression of p21WAF1/CIP1 (11). Those studies were limited in that no evidence, such as promoter reporter assays or electrophoretic mobility shift assays, was presented to support the conclusion that WT1(−/−) directly affects the p21 promoter, leaving open the possibility that some other WT1 target is a p21 transcriptional activator. The present report, therefore, contains the first demonstration that the WT1(−/−) isoform is a direct transcriptional regulator of a key gene involved in cell cycle progression.

Interestingly, in our 32D cl3 cells stably transfected with an inducible WT1(−/−) construct, we saw no evidence that induction of WT1(−/−) alters p21 expression (Fig. 1). There are two possible explanations for this observation. One possibility is that WT1 is not the direct transcriptional activator of p21. In this case, the WT1 target that mediates the up-regulation of p21 may not be expressed in hematopoietic cells. Alternatively, this may represent a cell type-specific effect of WT1 on the p21 promoter. There is precedence for this with bcl-2. In transient transfection assays, WT1(−/−) activates the bcl-2 promoter in Saos2 and CV-1 cells but represses the identical reporter construct in HeLa cells (12).

Cyclin E and p21 are both key regulators of progression from G1 into S phase. Cyclin E is a positive regulator of this process, binding to and activating cyclin-dependent kinase 2 (Cdk2).
Activated Cdk2 phosphorylates Rb, which is necessary for progression from G1 into the S phase of the cell cycle (19). One of the roles of p21, on the other hand, is to inhibit the activity of Cdk2 (20). There have been other reports in the literature suggesting that the G1/S phase transition is regulated, at least in part, by WT1 (21). Evidence that WT1(−/−) up-regulates an inhibitor of G1/S progression (p21) and down-regulates a promoter of cell cycle progression (cyclin E) strongly suggests that inhibition of this point of the cell cycle is a critical role for WT1.

How does the finding that WT1(−/−) is a negative regulator of cell cycle progression fit with a putative oncogenic role? WT1 is overexpressed in the majority of cases of acute leukemia (16), and mutant forms of WT1 encoding nonsense mutations predicted to encode truncated proteins lacking the DNA binding domain have been cloned from the leukemic blasts of several patients (13). Similar mutations have been identified in patients with Denys-Drash syndrome, and these mutations have dominant negative activity in vitro (8). WT1 functions as a dimer, and both homo- and heterodimers can form (15). In leukemias that overexpress wild type WT1, the most abundant isoform is WT1(+/+), which not only suppresses the cyclin E promoter poorly as a homodimer but also inhibits the ability of WT1(−/−) to repress this promoter (presumably through heterodimerization). Overexpression of this isoform could "derepress" cyclin E transcription, resulting in inappropriate overexpression of this target. In cases of acute myeloid leukemia involving a truncated WT1, this dominant negative form of the molecule should also inhibit the activity of full-length WT1(−/−) toward target promoters such as cyclin E through heterodimer formation. In fact, cyclin E is reported to be overexpressed in the majority of cases of acute myeloid leukemia (22), suggesting that this may be a major mechanism contributing to leukemogenesis. Furthermore, we have found that induction of WT1(−/−) in the stably transfected cell line described in this paper potentiates granulocyte-colony stimulating factor-mediated differentiation, whereas another group reported that constitutive expression of WT1(+/+) in 32D cl3 cells interferes with differentiation (23).

The regulation of cyclin E protein levels has recently been a prominent focus of cancer researchers. Cyclin E levels vary with the cell cycle, with low levels present in early G1, rising through G1 to peak at the G1/S transition, falling rapidly during early S phase, and remaining low through G2, M, and early G1 phases. This precise regulation of the protein is achieved by balancing transcription and proteasome-dependent degradation. Dysregulation of cyclin E is associated with premature entry into S phase, genomic instability, and tumorigenesis. An F-box protein, which mediates association with ubiquitin ligase, specific for cyclin E has recently been described (24–26), and mutations in the gene encoding this protein, which would lead to elevated cyclin E levels, have been implicated in breast and ovarian cancer (25, 26). Our discovery that WT1(−/−) is a transcriptional repressor of cyclin E suggests a complementary mechanism by which cyclin E might be aberrantly overexpressed in tumors through derepression of the cyclin E promoter by either overexpression of the WT1(+/+) isoform or by expression of a dominant-negative WT1 mutant. Experiments to further define the interactions between the different isoforms of WT1 in regulating the cyclin E promoter are ongoing. WT1 has been implicated in a variety of solid tumors other than Wilms’ tumor. For example, WT1, predominantly the (+/+) isoform, is overexpressed in a significant proportion of breast cancers (17). The ability of this isoform to derepress the cyclin E promoter might be a contributory factor in mammary carcinogenesis. Whether cyclin E derepression is involved in other WT1-associated tumors such as malignant mesothelioma remains to be determined.

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REFERENCES

1. Call, K. M., Glesser, T., Ito, C. Y., Buckler, A. J., Pelletier, J., Haber, D. A., Rase, E. A., Kral, A., Yeger, H., and Lewis, W. H. (1990) Cell 60, 509–520
2. Gessler, M., Poustka, A., Cavenee, W., Neve, R. L., Orkin, S. H., and Bruns, G. A. P. (1990) Nature 343, 774–778
3. Haber, D. A., Sohn, R. B., Buckler, A. J., Pelletier, J., Call, K. M., and Housman, D. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9618–9622
4. Bickmore, W. A., Oghe, K., Little, M. H., Seawright, A., van Heyningen, V., and Hastie, N. D. (1992) Science 255, 235–237
5. Wang, Z. Y., Qiu, Q. Q., Huang, J., Guerrieri, M., and Deuel, T. F. (1995) Oncogene 10, 415–422
6. Larsson, S. H., Charleux, J. P., Miyagawa, K., Engelkamp, D., Rasoulzadeh, M., Ross, A., Cuzzin, F., van Heyningen, V., and Hastie, N. D. (1995) Cell 81, 391–401
7. Davies, R. C., Calvico, C., Bratt, E., Larsson, S. H., Lamond, A. I., and Hastie, N. D. (1998) Genes Dev. 12, 3217–3225
8. Kim, J., Pravitt, D., Bardeesy, N., Torban, E., Vicarne, C., Goodyer, P., Zabel, B., and Pelletier, J. (1999) Mol. Cell. Biol. 19, 2289–2299
9. Hossain, A., and Saunders, G. F. (2001) J. Biol. Chem. 276, 16817–16823
10. Nachtigal, M. W., Hirokawa, Y., Enyeart-VanHouten, D. L., Flanagan, J. N., Hammer, G. D., and Ingraham, H. A. (1998) Cell 93, 445–454
11. Engert, C., Maheshwaran, S., Garvin, A. J., Kreidberg, J., and Haber, D. A. (1997) Cancer Res. 57, 1429–1434
12. Mays, M. W., Wang, C. Y., Drouin, S. S., Madrid, L. V., Marshall, A. F., Reed, J. C., Weissman, B. E., and Baldwin, A. S. (1999) EMBO J. 18, 3990–4003
13. King-Underwood, L., and Pritchard-Jones, K. (1999) Blood 91, 2961–2968
14. Schreiber, E., Matthias, P., Muller, M. M., and Schaffner, W. (1989) Nature 341, 6419
15. Reddy, J. C., Morris, J. C., Wang, J., English, M. A., Haber, D. A., Shi, Y., and Licht, J. D. (1995) J. Biol. Chem. 270, 10878–10884
16. Inoue, K., Sugiyama, H., Ogawa, H., Nakagami, T., Miwa, H., Kita, K., Hirooka, A., Masaoka, T., and Nasu, K. (1994) Blood 84, 3071–3079
17. Loeb, D. M., Evron, E., Patel, C. B., Sharma, P. M., Niranjan, B., Buluwela, L., Weitzman, S. A., Korz, D., and Sukumar, S. (2001) Cancer Res. 61, 921–925
18. Ohstobo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. (1995) Mol. Cell. Biol. 15, 2803–2804
19. Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J. W., Elledge, S., Nishimoto, T., Morgan, D. O., Franza, B. R., and Roberts, J. M. (1992) Science 257, 1689–1694
20. Ball, K. L. (1997) Prog. Cell Cycle Res. 3, 125–134
21. Kudoh, T., Ishidate, T., Moriyama, M., Toyoshima, K., and Akiyama, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4517–4521
22. Isida, H., Towatari, M., Tanimoto, M., Morishita, Y., Kodera, Y., and Saito, H. (1997) Blood 90, 3707–3713
23. Inoue, K., Tamaki, H., Ogawa, H., Oka, Y., Soma, T., Tatekawa, T., Oji, Y., Tsuboi, A., Kim, E. H., Kawakami, M., Akiyama, T., Kishimoto, T., and Sugiyama, H. (1998) Blood 91, 2969–2976
24. Koepf, D. M., Schafer, L. K., Ye, X., Keyomarsi, K., Chu, C., Harper, J. W., and Elledge, S. J. (2001) Science 294, 173–177
25. Strehlmaier, H., Spruck, C. H., Kaiser, P., Won, K. A., Sangfelt, O., and Reed, S. I. (2001) Nature 413, 316–322
26. Moberg, K. H., Bell, D. W., Wahrer, D. C., Haber, D. A., and Hariharan, I. K. (2001) Nature 413, 311–316
Cyclin E Is a Target of WT1 Transcriptional Repression
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