The WT1 Protein Is a Negative Regulator of the Normal bcl-2 Allele in t(14;18) Lymphomas

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The translocated and normal bcl-2 alleles in the DHL-4 cell line with the t(14;18) translocation were separated by pulsed field electrophoresis. An in vivo footprint over a potential WT1 binding site in the bcl-2 5′-flanking sequence was identified on the normal silent allele. Electrophoretic mobility shift assays with the bcl-2 WT1 site demonstrated a single specific complex. UV cross-linking and Western analysis revealed that this gel shift complex contained WT1 protein. Deletion or mutation of the WT1 site resulted in an increase in activity of the bcl-2 promoter in DHL-4 cells. Cotransfection with a 3:1 ratio of a WT1 expression vector to the bcl-2 promoter construct led to a 3.0-fold repression of the bcl-2 promoter. Cotransfection with a WT1 expression vector and the bcl-2 promoter with the mutated WT1 site resulted in only 1.2-fold repression. We conclude that the WT1 site functions as a negative regulatory site for the normal silent bcl-2 allele in t(14;18) lymphomas. The WT1 site is not occupied on the translocated bcl-2 allele.

The bcl-2 gene was originally identified by its involvement in the t(14;18) translocation that is associated with human follicular lymphoma (1). The translocation of bcl-2 to the immunoglobulin heavy chain locus leads to deregulated expression of bcl-2, and high levels of bcl-2 mRNA are detected in cells with the t(14;18) translocation (2, 3). It has been shown that the expression of bcl-2 is entirely from the translocated allele and that the normal allele is silent (3). Although the mechanism of the deregulation of bcl-2 is unknown, regulatory elements of the immunoglobulin locus may play a role. It is also not known how the normal allele is silenced. The deregulated bcl-2 gene is believed to play a role in theogenesis of follicular lymphoma. Transgenic mice containing a bcl-2-immunoglobulin minigene show a polyclonal expansion of B cells with prolonged cell survival but no increase in cell cycling. Progression to high grade lymphomas is seen in these mice (4).

The major transcriptional promoter for bcl-2 in B cells, P1, is located 1386–1423 base pairs upstream of the translation start site (5). This is a TATA-less, GC-rich promoter that displays multiple start sites. A minor promoter, P2, utilized in some cell types, is located 13 kilobases downstream from the first one (5). Several negative regulatory sites have been described in the bcl-2 promoter region. One of these negative regulatory elements, is located upstream of the P2 promoter (6). The proteins that bind to this element have not been identified, although p53 was shown to mediate down-regulation of bcl-2 either directly or indirectly through a 195-base pair segment of this region (7). We have previously described three π1 binding sites, which are negative regulators of bcl-2 expression in pre-B cells (8). Normal pre-B cells express very little bcl-2, and extensive cell death by apoptosis occurs at this developmental stage. Levels of Bcl-2 protein are increased in mature B cells. We have found that the three π1 sites are not functional in mature B cells (8). Repression of the bcl-2 promoter by the WT1 protein in HeLa cells has been described recently (9). There are several potential WT1 binding sites in the bcl-2 promoter, and it is not clear whether all of them are functional.

We are studying in vivo protein binding to both the normal and translocated bcl-2 alleles in follicular lymphoma cells with a t(14;18) translocation. We identified an in vivo footprint at a CRE site in the 5′-flanking sequence of the translocated bcl-2 gene, and we demonstrated that CREB family proteins bind to this site in vitro and that the maximal increase in bcl-2 promoter expression mediated by the immunoglobulin heavy chain gene enhancers in transient transfection experiments was dependent on the CRE site (11). These results suggest that the CRE site functions as a positive regulatory element for the translocated bcl-2 allele in follicular lymphoma with the t(14;18) translocation.

We now describe an in vivo footprint over a potential WT1 site on the normal bcl-2 allele; this site is not occupied on the translocated allele. The putative Wilms′ tumor suppressor gene (wt1) encodes a zinc finger DNA binding protein that functions as a transcriptional repressor (12, 13). The WT1 protein binds to the target sequence GNGNNGGGN, which is also recognized by the zinc finger transcription factors EGR-1, EGR-2, and EGR-3. The gene for WT1 is mainly expressed in the developing kidney, testis, ovary, and spleen (14). Recent evidence suggests that the WT1 protein may play an important role in hematopoiesis, since it is expressed in immature hematopoietic cells and also in acute myeloid, acute lymphoblastic, and chronic

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1 The abbreviations used are: CRE, cAMP-responsive element; PCR, polymerase chain reaction; IGF-II, insulin-like growth factor II; EMSA, electrophoretic mobility shift assay.
WT1 Represses the Normal bcl-2 Gene

Experimental Procedures

Plasmid Constructs—The bcl-2-promoter-luciferase constructs have been described previously (10, 11). A construct with a mutated WT1 site has been described (19). The oligonucleotide sequence used for the PCR primer is CCCCTTCCT (mutated bases are in boldface and the WT1 site is underlined).

Cell Lines and Transient Transfection Assays—DHL-4 cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. This cell line has a t(14;18) translocation. The cells were washed with RPMI and resuspended at 3 × 10^5 cells/ml in RPMI medium containing 25 μg/ml streptomycin and 100 μg/ml neomycin (23, 24). After electrophoresis of the DNA, one lane of the gel was transferred to a nitrocellulose filter; a probe for JH or Jκ was labeled with [32P]dCTP and Klenow polymerase. Binding conditions then subjected to amplification by ligation-mediated PCR essentially as described by Mueller and Wold (28), Pfeifer et al. (29), and Garrity and Wold (30). Sequenase was used for first strand synthesis, and Taq DNA polymerase was used for PCR. Conditions used for amplification were 95 °C for 2 min, 61 °C for 2 min, and 76 °C for 3 min. After 20–22 cycles of PCR, samples were hybridized with end-labeled primers (primer#5 of each primer set) and amplified by one more cycle of PCR. The reaction mixes were resolved in a 6% polyacrylamide denaturing gel. The primers used for PCR were purified on Applied Biosystems oligonucleotide purification cartridges. The common linkers used were GCCGGGAGATCTGAATTC and GAATTCAGATC. The primers for the coding strand were TTCCGCTGAGCCCGGGGC, GCGCCCGCCAGGAG, and GCGGGGCCAGCGAGAG. The noncoding strand primers were GAGGTTTACGACGAAACCG, TCGGGCCTGAGCAGATGAGAA, and CGGCGCTGATCGAGAAGAAGAAGAAG.

Quantitation of footprints was performed as described previously (23) with ImageQuant software version 4.15 (Molecular Dynamics). Percent protection values of below 20% were considered too low and were not interpreted as footprints.

Electrophoretic Mobility Shift Assay (EMSA)—The double-stranded oligonucleotides used for EMSA of the WT1 region are shown below (21).

WT1 Represses the Normal bcl-2 Gene
FIG. 3. Denaturing SDS-polyacrylamide gel and Western analysis of the EMSA complexes formed with DHL-4 nuclear extracts and the bcl-2 WT1 site. A, denaturing SDS-polyacrylamide gel analysis of the UV cross-linked EMSA complexes. Lane 1 contains protein from the corresponding region of the EMSA with the mutated bcl-2 WT1 site (no EMSA complex was visible). Lane 2 contains protein from the specific EMSA complex in Fig. 2. The migration of the molecular mass markers is shown on the left. B, Western blot analysis of the cross-linked EMSA complexes using a WT1 antibody that recognizes the C terminus of WT1. The lanes are labeled as described above for panel A. The molecular masses of the proteins in lane 2 are 63 and 50 kDa. C, Western blot analysis of the noncross-linked EMSA complexes using a WT1 antibody. The lanes are labeled as described in panel A. The molecular mass of the protein in lane 2 is 50 kDa. D, Western blot analysis of the cross-linked EMSA complexes using a WT1 antibody that recognizes the N terminus of WT1. The lanes are labeled as described above for panel A. The molecular masses of the proteins in lane 2 are 63 and 50 kDa.

Samples were incubated in the presence or absence of excess competitor oligonucleotides for 15 min at room temperature. Electrophoresis was performed at 30 mA at 4 °C in a 0.5 × Tris borate-EDTA 5% polyacrylamide gel. For the supershifts, 1 μg of an antibody specific for a region near the N terminus of WT1 (180 from Santa Cruz Biotechnology) or an antibody specific for a region near the C terminus of WT1 (C-19 from Santa Cruz Biotechnology) was used in EMSA with the conditions above.

UV Cross-linking and SDS-Polyacrylamide Gel Electrophoresis—EMSA was performed as described above. UV cross-linking was performed as described previously (32) with a short-wavelength UV light box at 4 °C for 60 min. An autoradiograph of the gel was used to locate the EMSA complexes. Regions of the gel containing the complexes were cut out, and the individual complexes were eluted at room temperature overnight in 50 mM Tris-HCl (pH 7.9), 0.1% sodium dodecyl sulfate (SDS), 0.1 mM EDTA, 5 mM dithiothreitol, 150 mM NaCl, and 0.1 mg/ml bovine serum albumin. The eluted protein was precipitated with acetone, washed with ethanol, and dried. After resuspension in Laemmli loading buffer, SDS-polyacrylamide gel electrophoresis was performed. The 32P-labeled proteins were visualized by autoradiography. The Amersham ECL kit was used for Western analysis. The WT1 antibodies, 180 and C-19, are described above.

RESULTS

An in Vivo Footprint Is Located over a WT1 Site—The translocated and normal bcl-2 alleles from DHL-4 cells were separated by pulsed field electrophoresis as described previously (11). Ligation-mediated PCR was performed on each one. With primer sets that cover a region of the bcl-2 5′-flanking region, we found a footprint located on the normal bcl-2 allele that was not present on the translocated bcl-2 allele (Fig. 1). Two guanine residues were protected on the coding strand, and six guanine residues demonstrated protection on the noncoding strand. The protected sequence is similar to the WT1 consensus sequence. Although there are several potential WT1 sites in the bcl-2 promoter, we found protection of only one of these sites.

WT1 Protein in DHL-4 Cells Binds to the bcl-2 WT1 Site in Vitro—A double-stranded oligonucleotide that encompassed the protected sequence was used in EMSA. Two complexes were formed with the bcl-2 WT1 oligonucleotide (Fig. 2A, lane 1). Competition with excess cold oligonucleotide demonstrated that only the more rapidly migrating complex was specific (Fig. 2A, lanes 2 and 3). A 100-fold molar excess of the mutated WT1 oligonucleotide did not compete against the specific complex (Fig. 2A, lane 5). The mutated WT1 oligonucleotide did not form the specific complex (Fig. 2A, lanes 6–10).

Cross-competition with the IGF-II P3 B1 site was performed in EMSA to determine whether the same protein bound to the bcl-2 WT1 site and the IGF-II P3 B1 site. As shown in Fig. 2B (lanes 1–3), the IGF-II site competed with the bcl-2 WT1 site for protein binding. The bcl-2 WT1 site also competed with the labeled IGF-II site for protein binding (Fig. 2B, lanes 4–6).

Supershift experiments were performed with antibodies that recognize either a region near the N terminus of the WT1 protein or a region near the C terminus of WT1. As shown in Fig. 2B (lane 8), the specific EMSA complex was decreased in intensity in the presence of the antibody against the N terminus of WT1, and a supershifted complex was visible. The addition of the antibody against the C terminus of WT1 caused a reduction in the intensity of the specific complex, but no supershifted complex was visible (Fig. 2B, lane 9). It is possible that this antibody interferes with efficient binding of WT1 to DNA since the DNA binding domain is located near the C terminus.

To further characterize the proteins that bind to the bcl-2 WT1 site, UV cross-linking followed by denaturing polyacrylamide gel electrophoresis was performed with the specific EMSA complex (Fig. 3A). UV cross-linking yielded a protein of 63 kDa. Correction for bound oligonucleotide predicted a protein of 50 kDa (Fig. 3A, lane 2).

Western analysis was performed on the UV cross-linked protein with an antibody against a region near the C terminus of WT1. As shown in Fig. 3B (lane 2), the protein present in this EMSA complex reacted with an antibody against WT1. In addition, a reactive protein of 50 kDa was also seen. Most likely this was uncross-linked protein that was present in the EMSA complex. To obtain a more accurate estimate of the molecular mass of the protein in this complex, Western analysis without UV cross-linking was performed. A protein of molecular mass of 50 kDa was observed (Fig. 3C, lane 2). When the Western analysis of the UV cross-linked protein was performed with an antibody against a region near the N terminus of WT1, the same protein bands were seen (Fig. 3, compare D, lane 2, with B, lane 2). No cross-linked protein or protein reactive with either WT1 antibody was observed when the corresponding region of the gel from EMSA with the mutated WT1 site was analyzed (Fig. 3, A–D, lane 1). From these results, one can conclude that WT1 does not simply comigrate with the EMSA complex during electrophoresis through the nondenaturing gel.

Deletion or Mutation of the WT1 Site Leads to an Increase in bcl-2 Promoter Activity—To determine whether the WT1 site in the bcl-2 5′-region had any functional activity, transient transfection experiments were performed. The constructs for the transient transfection experiments are illustrated in Fig. 4A. As shown in Fig. 4B, deletion of the bcl-2 WT1 site resulted in an increase in activity of approximately 2.1-fold. The activity of these deletion constructs of the bcl-2 was low because the positive regulatory region had been deleted. To study the function of the WT1 site in the context of the full-length promoter,
a mutation was constructed in this site. Mutation of the WT1 site resulted in a 2.6-fold increase in promoter activity compared with the full-length wild-type promoter (Fig. 4).

To ascertain whether WT1 was the active transcription factor, cotransfection experiments were performed. Transfection of a WT1 expression vector with the full-length bcl-2 promoter resulted in a 1.4-fold decrease in activity at a 1:1 ratio and a 3.0-fold decrease at a 3:1 ratio of WT1 to bcl-2 construct (Fig. 5A). The majority of this repression was mediated through the single WT1 site because mutation of this site in the bcl-2 promoter resulted in a reduced level of repression of the promoter. At a 3:1 ratio, the activity was decreased by 1.2-fold (Fig. 5B).

The Activity of the WT1 Site Is Markedly Reduced in the Presence of the Immunoglobulin Enhancers—Addition of enhancer regions 3′ of the murine immunoglobulin gene to the bcl-2 promoter-luciferase construct (diagrammed in Fig. 6A) resulted in a 20-fold increase in activity (Fig. 6B). We wished to determine whether mutation of the WT1 site would lead to a further increase in activity in the presence of the immunoglobulin enhancers. The WT1 site was mutated, and transient transfection experiments were performed in DHL-4 cells. As shown in Fig. 6B, there was no significant change in the bcl-2 promoter activity when the WT1 site was mutated in the presence of the immunoglobulin enhancers.

We also examined the effect of cotransfection of a WT1 expression vector on the bcl-2 promoter activity in the presence of the immunoglobulin enhancers. Even at a ratio of 3:1 WT1 expression vector to the bcl-2-luciferase construct, there was no significant change (1.07-fold decrease) in the bcl-2 promoter activity (Fig. 6C). These results suggest that the immunoglobulin enhancers modify the activity or accessibility of the WT1 site in the bcl-2 promoter.

**DISCUSSION**

*In vivo* footprinting has been used to identify a region in the bcl-2 promoter that is occupied on the silent normal allele but
WT1 Represses the Normal bcl-2 Gene

negative regulatory activity and because cotransfection with an
EGR1 expression vector had no effect on bcl-2 promoter activity. However, it is possible that EGR1 can also bind to this site in vivo and influence bcl-2 promoter activity.

Hewitt et al. (9) described repression of the bcl-2 promoter by WT1 in cotransfection experiments in HeLa cells. Because there are several potential WT1 sites in the bcl-2 promoter, it was not clear which site or sites mediated this repression. We found an in vivo footprint over a single WT1 site on the normal bcl-2 allele. Our studies with cotransfection of WT1 and mutation of this site suggest that, at least in DHL-4 cells, this is the major functional site.

In transient transfection experiments, we showed that the WT1 site in the bcl-2 promoter demonstrated little activity in the presence of the immunoglobulin heavy chain gene enhancers. Mutation of this site did not lead to an increase in bcl-2 promoter activity. In cotransfection experiments with a WT1 expression vector, we were not able to demonstrate significant repression of the bcl-2 promoter in the presence of the immunoglobulin enhancers. These findings and the results of the in vivo footprinting studies suggest that the presence of the immunoglobulin enhancers prevents repression of the bcl-2 promoter by WT1. It is possible that the activity of the WT1 protein is influenced by the immunoglobulin enhancers; for example, the activators bound to the enhancers may be able to overcome the repression function of WT1.

It is interesting to note that two tumor suppressor gene products, WT1 and p53, have been shown to negatively regulate the bcl-2 promoter. These genes are frequently deleted or mutated in malignancies, and this may lead to increased bcl-2 expression and an increased resistance to apoptosis. WT1 protein is expressed in the DHL-4 cell line, and it participates in the repression of the normal bcl-2 allele, while it has no effect on the translocated allele.

We have shown that the CRE site in the bcl-2 promoter functions as a positive regulatory element in transfection studies (10), and we believe that it is involved in the expression of the translocated bcl-2 allele in t(14;18) lymphomas. This site is occupied on the translocated bcl-2 allele and not on the normal allele, as indicated by in vivo footprinting (11).

It is likely that the deregulation of the translocated bcl-2 allele is a consequence of interactions between the bcl-2 promoter region and regulatory elements of the immunoglobulin locus. Previously we have demonstrated that several of the immunoglobulin heavy chain enhancers increase bcl-2 promoter activity and that the maximal increase is dependent on an intact bcl-2 CRE site (11). We now show that a WT1 site is occupied on the normal silent bcl-2 allele and that it functions as a negative regulatory site. This site appears to have little activity in the presence of the immunoglobulin enhancers. We are currently investigating the interactions between the transcription factors that bind the immunoglobulin enhancers and the transcription factors that bind to the translocated bcl-2 promoter in t(14;18) lymphoma cells.

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2
WT1 Represses the Normal bcl-2 Gene

19614

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