Effects of Denys−Drash Syndrome Point Mutations on the DNA Binding Activity of the Wilms' Tumor Suppressor Protein WT1[†]

Franck Borel, Kathleen C. Barilla, Tatyana B. Hamilton, May Iskandar, and Paul J. Romaniuk*

Department of Biochemistry and Microbiology, P.O. Box 3055, University of Victoria, Victoria, British Columbia V8W 3P6, Canada

Received March 29, 1996; Revised Manuscript Received June 25, 1996[⊗]

ABSTRACT: A number of point mutations in the zinc finger domain of the Wilms' tumor suppressor protein WT1 have been isolated from the DNA of patients with Denys−Drash syndrome, an association of Wilms' tumor, nephropathy, and genital anomalies. To date, five different mutations that alter amino acids predicted to interact specifically with nucleotides in the target DNA sequence have been described. Two of these mutations are located in zinc finger 2 (R366H, R366C), and three are located in finger 3 (R394W, D396G, D396N). These five Denys−Drash mutations were introduced into WT1-ZFP, a recombinant polypeptide containing the zinc finger domain of WT1, and the effects of these mutations on DNA sequence specificity were determined using a selection, amplification, and binding (SAAB) assay. The SAAB assay was carried out using two different DNA templates, one with a randomized finger 2 subsite (GCG TGG **NNN** TGT) and one with a randomized finger 3 subsite (GCG **NNN** GCG TGT). A comparison of the DNA sequences selected by WT1-ZFP and by Denys−Drash mutants suggests that the point mutations reduce the sequence selectivity of the zinc finger protein. With the exception of the R394W mutant, the other Denys−Drash mutations selected one alternative sequence in addition to the wild-type DNA subsite sequence. The binding affinities of these proteins for their selected sequences were determined using a quantitative nitrocellulose filter binding assay. These results revealed that the wild-type WT1 binds with slightly higher affinity to sequences with GAG in the finger 2 subsite than sequences with the EGR-1 consensus GCG finger 2 subsite. With the exception of R394W, which appears to lack specific DNA binding activity, the Denys−Drash mutants bound to selected DNAs with 1.4−14-fold lower affinities than the wild-type WT1-ZFP. These results suggest that the clinical phenotype of Denys−Drash syndrome can be associated with a modest reduction in the DNA binding affinity of WT1.

Wilms' tumor is an embryonal malignancy of the kidney resulting from the continued proliferation of embryonic kidney blastemal cells. This tumor accounts for 85% of all childhood kidney cancer and affects approximately 1 in 10 000 children (Matsunaga, 1981; Young & Miller, 1975). A tumor suppressor gene for Wilms' tumor, *WT1*, has been identified at human chromosome 11p13 (Call et al., 1990; Gessler et al., 1990). The *WT1* gene spans 50 kb of the genomic DNA and contains 10 coding exons that produce 4 different WT1 mRNAs, reflecting the presence or absence of 2 alternatively spliced exons (Haber et al., 1991). *WT1* encodes a polypeptide that has several features characteristic of eukaryotic transcription factors. The polypeptide is composed of two functional domains: a proline/glutamine-rich amino terminus, essential for the function of WT1 in transcriptional regulation, and a carboxyl-terminal domain containing four consecutive zinc fingers, each encoded by a separate exon (Haber et al., 1991). The last three zinc fingers of WT1 exhibit a high degree of sequence homology with the three zinc fingers of the early growth response-1 protein (EGR-1, also called Zif 268) (Figure 1). EGR-1 contains

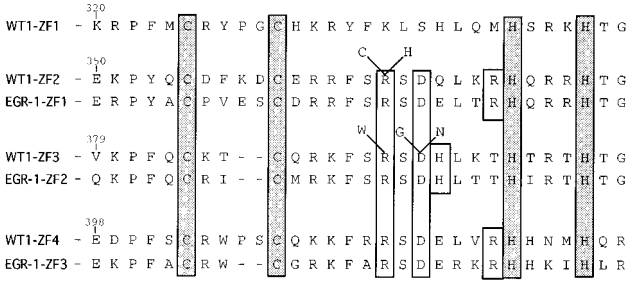

FIGURE 1: Comparison of the sequences of WT1 and EGR-1 zinc fingers. Residues involved in DNA binding and their counterparts in WT1 are enclosed in clear boxes. Shaded boxes indicate residues that coordinate to zinc. Finger 2 and 3 Denys−Drash missense mutations are also indicated.

three zinc fingers of the C_2H_2 class and activates transcription in a sequence-specific manner (Sukhatme et al., 1988). X-ray crystallographic analysis of a complex formed between the zinc finger domain of the EGR-1 protein and a consensus DNA sequence has demonstrated that each zinc finger interacts with a defined three base subsite within the 9 base pair sequence 5′ GCG TGG GCG 3′ (Pavletich & Pabo, 1991). Although WT1 is able to bind to this 9 base pair sequence (Rauscher et al., 1990), the high-affinity binding site for WT1 is the 12 base pair consensus sequence 5′ GCG TGG GCG (T/G)(A/G)(T/G) (Drummond et al., 1994; Hamilton et al., 1995).

[†] This work was supported by a grant from the National Cancer Institute of Canada with funds provided by the Canadian Cancer Society.

* Corresponding author. Telephone: (604) 721−7088. Fax: (604) 721-6227. E-mail: pjr@uvaix.uvic.ca.

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1996.

S0006-2960(96)00758-1 CCC: $12.00

Wilms' tumor also occurs in association with two disease syndromes with similar genital system malformation: WAGR syndrome (Miller et al., 1964) and Denys−Drash syndrome (Denys et al., 1967; Drash et al., 1970). The WAGR syndrome is an association of Wilms' tumor (W), aniridia (A), genitourinary malformation (G), and mental retardation (R). The *WT1* locus of several WAGR patients displays either short deletions leading to the production of a truncated protein (Baird et al., 1992a) or a point mutation resulting in a substitution of aspartic acid for glycine at position 201 which converts the WT1 protein from a transcriptional repressor to a transcriptional activator (Park et al., 1993).

The Denys−Drash syndrome (DDS) is a rare human developmental disorder in which severe urogenital aberrations result in progressive renal failure, male pseudohermaphroditism, and Wilms' tumor. No abnormalities of the X or Y chromosome and no large deletion of 11p13 have been detected in Denys−Drash patients, but a number of point mutations in the second and third zinc finger coding regions have been identified in WT1 DNA from tumor samples (Baird & Cowell, 1993; Baird et al., 1992b; Little et al., 1992, 1993; Nordenskjold et al., 1994; Ogawa et al., 1993; Sakai et al., 1993). These point mutations can be divided into three categories: nonsense mutations leading to the production of prematurely terminated protein; substitution mutations of amino acids involved in zinc coordination; and missense mutations altering amino acids which interact with the DNA target.

While the first two categories of point mutations will disrupt the zinc finger structure or produce truncated proteins and thus abolish the DNA binding activity of WT1, the third category may influence DNA binding specificity and/or affinity of WT1. In the second zinc finger, missense point mutations substitute either a histidine or a cysteine for an arginine residue (R366H, R366C) that is expected to interact with a guanine base in the target DNA according to the crystallographic data obtained for the EGR1−DNA complex (Pavletich & Pabo, 1991). Missense mutations in the third zinc finger result either in substitution of a tryptophan for an arginine residue (R394W) involved in interaction with DNA or in substitution of an asparagine or glycine residue for an aspartic acid residue (D396N, D396G) that is indirectly involved in DNA recognition.

To determine the effects of these missense mutations, we introduced each one into a recombinant polypeptide containing the zinc finger domain of WT1 (WT1-ZFP). Appropriately randomized DNA templates for finger 2 and finger 3 subsites were used to determine whether the Denys−Drash point mutations altered the DNA sequence specificity of WT1. The influence of these mutations on the binding affinity of WT1-ZFP for the wild-type DNA consensus sequence and novel cognate sequences was analyzed using a quantitative binding assay.

MATERIALS AND METHODS

Construction and Expression of Denys−Drash Mutations of WT1-ZFP. The WT1-ZFP Denys−Drash mutants (R366H, R366C, R394W, D396G, D396N) were constructed by site-directed mutagenesis (Nelson & Long, 1989). The plasmid pUCWT1ZFP, which contains the zinc finger domain of WT1 cloned into pUC19 (Hamilton et al., 1995), was used as a template, and the following mutagenic primers were used. The base alteration that introduces each mutation is underlined:

R366C: AGG TTT TCT TGT TCA GAG CAG

R366H: AGG TTT TCT CAT TCA GAG CAG

R394W: AGG TCC TCC TGG TCC GAC CAC

D396G: TCC CGG TCC GGC CAC CTG AAG

D396N: TCC CGG TCC AAC CAC CTG AAG

DNA sequencing was used to verify that only the desired mutation was introduced into the WT1-ZFP cDNA. Mutant cDNAs were cloned into the *Nde*I and *Bam*HI sites of the T7 expression vector pET16b (Studier & Moffatt, 1986; Studier et al., 1990). The resulting constructs encode zinc finger peptides with a histidine tag and a factor Xa cleavage site fused to the amino terminus and were used to transform *Escherichia coli* BL21(DE3) pLysS. Expression and purification of the zinc finger proteins were carried out as described previously (Hamilton et al., 1995). Fractional activity of protein preparations (typically 75−100%) was determined by a saturation DNA binding assay.

Selection, Amplification, and Binding (SAAB) Assay. The SAAB assay was carried out according to a published method (Blackwell & Weintraub, 1990), with minor modifications. The 67 base pair template oligonucleotides contained a central region including the WT1 consensus sequence randomized in either the finger 2 or the finger 3 recognition sites (5′ TAAT GCG TGG **NNN** TGT CCTAA 3′ and 5′ TAAT GCG **NNN** GCG TGT CCTAA 3′, respectively) flanked by *Eco*RI (5′) and *Bam*HI (3′) recognition sequences and the M13 forward universal primer (FUP) sequence (5′) and a sequence complementary to the M13 reverse universal primer (RUP, 3′).

The template was labeled for the initial round of selection by primer extension using 10 pmol of the oligonucleotide annealed to 80 pmol of RUP in 1× Klenow buffer (New England Biolabs), and extended in 0.2 mM dGTP, 0.2 mM dCTP, 0.2 mM dTTP, 20 μCi of [α-^{32}P]-dATP, and 9 units of Klenow (New England Biolabs) with incubation at room temperature for 45 min. The resulting double-stranded DNA containing a mixture of 64 possible sequences in the randomized region was purified on a 12% nondenaturing polyacrylamide gel, and eluted overnight at room temperature in 250 μL of 0.6 M ammonium acetate, 1 mM EDTA, and 0.1% SDS.

Labeled DNA (200 000 cpm) was incubated with the proteins as described by Hamilton et al. (1995), except the incubation mixture used Tris rather than HEPES to buffer the solution. The reactions were loaded onto a 5% nondenaturing polyacrylamide gel which had been preelectrophoresed for 20 min at 250 V at 4 °C. Migration on the gel was monitored by loading one lane with 5 μL of 0.1% bromophenol blue, 0.1% xylene cyanol, 25% glycerol. The gel was run in 0.3 × TBE at 250 V at 4 °C until the bromophenol blue had migrated 12 cm. The gel was then subjected to autoradiography for 2−4 h at 4 °C, the band corresponding to the lowest protein concentration at which a protein−DNA complex was visible was excised, and the DNA was eluted as described above. The eluted DNA was

Table 1: Finger 2 Subsite Sequences Selected from a Randomized Template by Wild-Type WT1-ZFP and the Finger 2 Point Mutants

	protein				protein		
subsite sequence	WT1-ZFP	R366H	R366C	subsite sequence	WT1-ZFP	R366H	R366C
CAC	−	1	−	GGC	−	2	−
GAA	−	1	−	GGG	3	−	1
GAC	−	2	1	GGT	−	−	1
GAG	38	13	19	GTA	−	−	2
GAT	3	23	3	GTG	1	3	5
GCA	−	2	7	GTT	1	1	2
GCC	1	−	1	TAA	−	−	1
GCG	2	1	2	TAG	−	1	−
GCT	−	−	4	TCC	1	−	−
GGA	−	−	2				

Table 2: Frequencies of Each Nucleotide Selected at the Finger 2 Subsite Positions by WT1-ZFP and the Finger 2 Point Mutants

	base 7				base 8				base 9			
protein:	none	WT1	R366H	R366C	none	WT1	R366H	R366C	none	WT1	R366H	R366C
A	29	0	0	0	25	82	82	47	17	0	6	24
C	19	0	2	0	25	8	6	28	17	4	10	4
G	21	98	96	98	19	6	4	8	29	88	36	53
T	31	2	2	2	17	4	8	18	37	8	48	20

amplified using FUP and RUP as primers and radioactively labeled, and a new round of selection was begun using lower protein concentrations.

Four rounds of selection were sufficient to isolate the highest affinity binding sites for each protein from the pool of random DNA sequences. The lowest protein concentrations at which a protein−DNA complex could be observed in round 4 were as follows: WT1-ZFP, 0.5 nM; R366H, 1.25 nM; R366C, 7.5 nM; R394W, 100 nM; D396N, 12.5 nM; D396G, 12.5 nM. The final PCR products of round 4 were digested with *Eco*RI/*Bam*HI and cloned into pUC19. The original randomized oligonucleotides were converted to a double-stranded form with cold dNTPs, and also subject to PCR amplification, restriction endonuclease digestion, and ligation into pUC19. A sample of each ligation was used to transform *E. coli* strain JM109. Plasmid DNA was isolated from at least 50 colonies of each transformation and sequenced using either the Sequenase kit (Amersham) or an automated sequencer and dye primer chemistry (Applied Biosystems).

Nitrocellulose Filter Binding Assay. The binding affinities of wild-type and mutant WT1 zinc finger peptides for the selected DNAs were measured using a nitrocellulose filter binding assay (Romaniuk, 1990). Plasmids containing selected target DNA sequences were digested with *Eco*RI and *Hind*III, and the double-stranded DNAs were labeled with [α^{32}-P]dATP (Sambrook et al., 1989). The 63 base pair DNA restriction fragment was purified by nondenaturing gel electrophoresis and eluted as described above. Labeled DNA fragments (10 000 cpm) were incubated with serial dilutions of protein ranging from 0.01 nM to 400 nM. Typically, 1 assay included 15−20 protein concentrations (5 per order of magnitude). The DNA−protein mixtures were incubated in 200 μL of TMK buffer (Romaniuk, 1990) containing 5 μM $ZnCl_2$ and 5 μg/mL poly[d(IC)] at 22 °C for 30 min, and then an aliquot was removed for filtration. Experimental data (at least six measurements per data point) were fitted to the appropriate equation using Kaleidagraph version 3.0 software. In each case, correlation coefficients were greater than 0.99.

RESULTS

Isolation of DNA Binding Subsites for WT1-ZFP and for Denys−Drash Mutants. A selection, amplification, and binding assay (SAAB) was used to determine if Denys−Drash mutants and WT1-ZFP recognized different DNA target sequences. For each mutant, four rounds of SAAB analysis were carried out in order to select high-affinity binding sites. A selection procedure with WT1-ZFP was performed in parallel to serve as a control. According to the paradigm of a 3 base pair subsite recognition per zinc finger observed for WT1 and EGR family proteins, we used the randomized DNA pools 5′-GCG TGG **NNN** TGT-3′ and 5′-GCG **NNN** GCG TGT-3′ for finger 2 and finger 3 subsite selection, respectively. While this approach will allow the determination of the direct effects of each mutation on sequence specificity for the appropriate subsite, it will not identify any indirect effects the mutation may have on recognition of neighboring subsite sequences by the other zinc fingers. After the final round of selection, individual PCR products were cloned and sequenced. The sequences of about 50 randomly chosen clones from the finger 2 SAAB experiment are shown in Table 1, and the frequencies of the 4 possible nucleotides at each of the 3 randomized positions are given in Table 2. It is of interest to note that for finger 2 the EGR-1 consensus sequences G(C/G)G are very poorly selected by the wild-type WT1-ZFP (only 10% of all selected subsites), the preferred DNA binding subsite appearing to be GAG. Mutant R366C has a low stringency for the identities of base pairs 8 and 9, while R366H has a low stringency for the identity of base pair 9 (Table 2). Despite this fact, R366C and WT1-ZFP share the same preference for the GAG subsite whereas R366H displays a preference for the GAT subsite.

The sequences of about 50 randomly chosen clones from the finger 3 SAAB experiment are shown in Table 3, and the frequencies of the 4 possible nucleotides at each of the 3 randomized positions are given in Table 4. A comparison of the data in Table 3 with those in Table 1 shows that a smaller number of triplet subsites were selected during finger 3 SAAB assays than were selected by finger 2, suggesting

Table 3: Finger 3 Subsite Sequences Selected from a Randomized Template by Wild-Type WT1-ZFP and the Finger 3 Point Mutants

	proteins					proteins			
subsite sequence	WT1-ZFP	D396G	D396N	R394W	subsite sequence	WT1-ZFP	D396G	D396N	R394W
CGG	–	–	1	–	TAG	3	–	–	5
GAG	–	–	–	1	TAT	–	–	–	6
GGG	17	23	5	6	TGG	32	27	45	12
GGT	–	–	–	1	TGT	–	–	–	22
GTG	1	–	–	–	TTT	–	–	–	1

Table 4: Frequencies of Each Nucleotide Selected at the Finger 3 Subsite Positions by WT1-ZFP and the Finger 3 Point Mutants

	base 4					base 5					base 6				
protein:	none	WT1	D396G	D396N	R394W	none	WT1	D396G	D396N	R394W	none	WT1	D396G	D396N	R394W
A	19	0	0	0	0	20	6	0	0	22	7	0	0	0	0
C	22	0	0	2	0	27	0	0	0	0	18	0	0	0	0
G	30	34	46	10	15	27	92	100	100	76	35	100	100	100	44
T	29	66	54	88	85	26	2	0	0	2	40	0	0	0	56

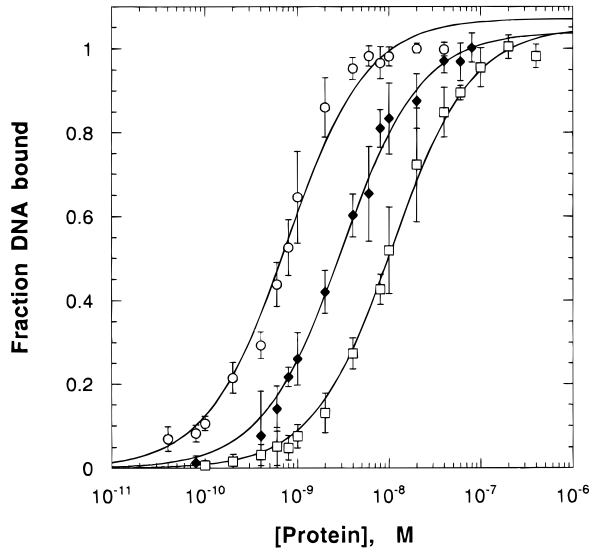

FIGURE 2: Equilibrium binding of the WT1 element GCG TGG GAG TGT to WT1-ZFP (open circles), R366H (closed diamonds), and R366C (open squares). Data points represent the mean of six or more determinations, and error bars indicate the standard deviations. Curves represent the best fit of the data to a simple bimolecular equilibrium.

that WT1-ZFP finger 2 recognition may be less stringent and tolerate a higher degree of sequence diversity compared with finger 3. The finger 3 SAAB experiments performed with WT1-ZFP and the mutants D396G and D396N gave very similar results: the three proteins select the same EGR-1 consensus high-affinity binding site (T/G)GG. However, substitution of the aspartate with an asparagine does appear to push the preference for a T rather than G at the first position of the subsite (Table 3). On the other hand, the R394W mutant peptide had much weaker DNA binding affinity and selected a larger variety of sequences, the most frequently selected subsites including the TGG consensus and the non consensus sequence TGT. It appears that the R394W mutation alters specificity for the middle and last base pairs of the finger 3 subsite (Table 4).

Determination of the Binding Constants. (A) Binding of WT1-ZFP. A nitrocellulose filter binding assay (Romaniuk, 1990) was used to investigate the binding affinity of the different proteins for the selected DNA target. Typical binding curves are shown in Figure 2. The apparent dissociation constant (K_d) can be determined from these data assuming a simple bimolecular interaction. All the K_d values measured are given in Table 5 for the finger 2 mutant proteins and Table 6 for the finger 3 proteins. The results of the filter binding assays demonstrate that the interaction of finger 2 with DNA is more efficient when the subsite GAG is present. WT1-ZFP binds to the GAG element with a K_d of 0.77 nM, whereas its affinity for the DNA subsites GCG and GAT is respectively 1.54 nM and 1.93 nM. Since the K_d values for GCG and GAT are almost equivalent and the affinity for GAG is 2 times greater, the high-affinity WT1-ZFP finger 2 subsite is GAG and not the G(G/C)G EGR-1 consensus sequence. The results of the binding assays performed with the selected finger 3 element indicate that WT1-ZFP binds to the TGG element with a K_d of 1.54 nM, but has approximately a 2-fold lower affinity for the GGG element ($K_d = 3.56$ nM). The most striking result is observed with the TGT sequence. In that case, we were not able to determine the affinity since a G to T change in the last position of the finger 3 subsite abolished binding to WT1-ZFP (data not shown).

(B) Effect of DDS Mutations on DNA Binding. Among the five mutants studied, only the mutation R394W resulted in a protein which lacks specific DNA binding activity, even with the most frequently selected TGT finger 3 subsite (data not shown). In spite of the apparent selection against certain bases, the binding observed during the SAAB experiments was probably due to unspecific protein/DNA interactions because of the relatively high protein concentration ($\geq$100 nM) used in the last round of selection. However, the four other mutants were still able to bind in a specific manner to the selected DNA target, but for each of the WT1 elements tested, the mutants always displayed a lower binding affinity than WT1-ZFP.

The experiments carried out with the finger 2 mutants reveal that the R366C mutation affects the DNA binding capacity of the protein to a greater extent than the R366H mutation (Table 5). In comparison to WT1-ZFP, the mutations result in decreases in affinity of 3.9- and 14-fold with the GAG element, and decreases in affinity of 1.4- and 7.2-fold with the GAT element for R366H and R366C, respectively. One should note that R366H is the only polypeptide to have a slightly higher affinity for the DNA element GAT versus GAG. This result suggests that substitution of a histidine for the arginine at position 366 could partially compensate for the effect of a guanine to

Table 5: Binding Affinities of Wild-Type and Finger 2 Mutant WT1-ZFP for Selected DNA Sequences

	GCG-TGG-**GAG**-TGT		GCG-TGG-**GAT**-TGT		
WT1-ZFP protein	K_d (nM)[a]	relative affinity[b]	K_d (nM)[a]	relative affinity[b]	selectivity[c]
wild type	0.77 ± 0.07	1	1.93 ± 0.08	1	2.50
R366H	2.99 ± 0.19	0.26	2.62 ± 0.17	0.71	0.88
R366C	10.8 ± 0.57	0.07	14.1 ± 0.91	0.14	1.30

[a] Dissociation constants are expressed as the mean with standard deviations of a minimum of six independent determinations. [b] Ratio of K_d(wild-type WT1-ZFP)/K_d(mutant WT1-ZFP). [c] Ratio of K_d(**GAT** subsite)/K_d(**GAG** subsite).

Table 6: Binding Affinities of Wild-Type and Finger 3 Mutant WT1-ZFP for Selected DNA Sequences

	GCG-**TGG**-GCG-TGT		GCG-**GGG**-GCG-TGT		
WT1-ZFP protein	K_d (nM)[a]	relative affinity[b]	K_d (nM)[a]	relative affinity[b]	selectivity[c]
wild type	1.54 ± 0.12	1	3.56 ± 0.34	1	2.33
D396G	4.11 ± 0.29	0.37	5.51 ± 0.29	0.63	1.33
D396N	12.4 ± 0.80	0.12	26.2 ± 2.44	0.14	2.13

[a] Dissociation constants are expressed as the mean with standard deviations of a minimum of six independent determinations. [b] Ratio of K_d(wild-type WT1-ZFP)/K_d(mutant WT1-ZFP). [c] Ratio of K_d(**GGG** subsite)/K_d(**TGG** subsite).

thymine change in the target DNA sequence. In terms of sequence selectivity, both substitutions at R366 significantly reduce the selectivity ratio, with the largest effect observed for the R366H mutation (Table 5).

With the finger 3 mutants, the stronger decrease in binding affinity is observed for the mutation D396N, being 8-fold for the TGG element and 7.4 fold for the GGG element compared to WT1-ZFP (Table 6). A smaller but similar effect is produced by the mutation D396G, where the affinities for the TGG and GGG elements are decreased 2.7-fold and 1.6-fold, respectively. Of the two substitutions at D396, only the glycine mutation reduces the sequence selectivity of the zinc finger domain significantly (Table 6).

DISCUSSION

Since more than 95% of Denys−Drash patients display various point mutations within the zinc finger domain of WT1 (Coppes et al., 1993), we have undertaken this work to elucidate the effect of some of these mutations on the DNA binding capacity of the WT1 protein. The point mutations analyzed here are those altering amino acids predicted, by analogy with EGR-1, to be involved in specific contacts with the DNA.

Our experiments indicate that zinc finger 2 of WT1 had a higher affinity for the subsite GAG over GCG and GAT. This apparent specificity for the central adenine cannot be explained on the basis of the EGR-1/DNA interaction. To account for this specificity, a third interaction between a glutamine residue (Q369) in WT1, not found in EGR-1 (Figure 1), and the central adenine of the triplet was proposed (Nakagama et al., 1995). This type of contact has been described previously for the interaction of the *Drosophila* chorion transcription factors I and II (Gogos et al., 1992) and may be a more general contact in protein−DNA interactions (Seeman et al., 1976). Since the frequency of selection obtained for this adenine is similar to the frequencies observed for all the other nucleotides predicted to make specific contacts with WT1-ZFP (G5, G6, G7, G9), we believe that the proposed interaction is highly probable. This suggests that the high-affinity recognition sequence of WT1 is related but not identical to the EGR-1 recognition sequence.

Crystallographic analysis of the EGR-1/DNA complex revealed that the first base pair of the EGR-1 finger 2 subsite (and by analogy of WT1 finger 3) is not involved in forming a specific interaction with the protein (Pavletich & Pabo, 1991). Histidine 397 is coplanar with the guanine in the second position of the triplet and is stacked against the thymine of the first base pair. This stacking limits the conformational flexibility of the histidine and thus enhances the specificity of the hydrogen bond formed between this histidine and the guanine situated at the second position in the DNA subsite. When this thymine is replaced by a guanine, we observed a 2 fold decrease in the apparent affinity of the DNA for WT1-ZFP. Our selection results agree with the observation that a cytosine at this position results in an even more pronounced decrease in affinity for WT1 (Nakagama et al., 1995). These results suggest that the histidine−thymine stacking interaction is required at the first position of the finger 3 subsite for high-affinity binding.

Replacement of the guanine in the third position of the finger 3 subsite by a thymine completely abolished binding by WT1-ZFP. Previous experiments have shown that converting the EGR-1 motif GCG GGG GCG to GCG GGT GCG (Rauscher et al., 1990) or the 10 base pair WT1 element GCG TGG GAG T to GCG TGA GAG T (Nakagama et al., 1995) results in a total loss of binding by WT1. In comparison, substitution of the thymine in the third position of the finger 2 subsite with a guanine did not produce such a drastic effect, resulting in a modest 2.5 fold decrease in the binding affinity of WT1-ZFP (Table 5). This result is remarkable since the protein/DNA contacts at these two guanines are made in exactly in the same way in the EGR-1/DNA complex (Pavletich & Pabo, 1991). Previous binding site selection experiments for the finger 1 subsite of WT1 indicated that a certain degree of sequence diversity could be tolerated (Drummond et al., 1994; Hamilton et al., 1995). When one considers the results of all the SAAB analyses carried out with WT1, only the guanine in position 6 of the WT1 recognition sequence is selected with a frequency of 100%, suggesting that this position forms the energetically most important contact with WT1. Consistent with this conclusion is the observation that the mutant R394W, in which the arginine residue interacting with this guanine is replaced, loses its DNA binding capacity. Thus, this guanine

base is one of the major identity elements of the WT1 recognition sequence.

The DNA binding properties of certain Denys−Drash mutants of WT1, including R394W, D396G, and D396N, have been investigated previously (Little et al., 1995; Pelletier et al., 1991). Under the conditions of the gel shift assays employed, neither the full-length WT1 protein prepared by *in vitro* translation, nor a fusion protein containing the zinc finger domain was able to bind to a variety of WT1 elements. In the present study, we have demonstrated that the zinc finger domains of four point mutants of WT1 isolated from Denys−Drash patients (D396G, D396N, R366H, R366C) have specific DNA binding activities that can be quantified using a nitrocellulose filter binding assay.

Extrapolating from crystallographic data for the EGR-1/DNA complex (Pavletich & Pabo, 1991), R366 of WT1 should form two specific hydrogen bonds with the last guanine (G9) of the finger 2 DNA subsite. The decrease in the selection specificity observed for base pair 9 indicates that in the wild-type WT1-ZFP/DNA complex both of these interactions do occur and are disrupted by the R366H and R366C mutations. We also observed that the mutant R366C induced a loss of selection specificity for position 8, whereas R366H displayed a selection pattern similar to WT1-ZFP for this position. Apparently the cysteine residue alters the local conformation of the recognition α helix of the second zinc finger enough to weaken or prevent the proposed interaction between Q369 and the middle adenine of the triplet (Nakagama et al., 1995), leading to a stronger effect on the DNA binding specificity and affinity of R366C compared to R366H. Another interesting consequence of the R366 mutations is the observed decrease in DNA sequence selectivity (Table 5). While wild-type WT1-ZFP has a 2.5-fold higher affinity for a consensus WT1 binding site with the GAG finger 2 subsite vs the GAT finger 2 subsite, both of the R366 mutants have roughly equal affinities for these two binding sites.

The aspartic acid replaced in the finger 3 mutants D396G and D396N is not directly in contact with the target DNA, but it has an important structural role: through two hydrogen bonds, it stabilizes the long side chain of R394 and enhances the specificity of the R394 hydrogen bonding contact to the guanine at position 6 (Pavletich & Pabo, 1991). As described above, the interaction of R394 with the guanine is essential for the binding of WT1 to its target DNA sequence; thus, the mutations D396N and D396G probably weaken this interaction but do not prevent it. It is interesting that the decrease in binding affinity is greater for the D396N mutant, in which there is only a relatively minor modification to the amino acid side chain, than it is for the mutant D396G. In the case of the D396G mutation, the amino acid side chain is completely removed, allowing free motion of the long side chain of R394. Without stabilization, the arginine may become less accessible and the probability of contact between R394 and the guanine at position 6 is decreased. In the D396N mutant, the buttressing contacts between the asparagine side chain and the arginine side chain may alter the orientation of the latter to the DNA, altering the contacts between the arginine and the base pairs.

This detailed characterization of the effects of Denys−Drash point mutations on the DNA binding activity of WT1 has provided some interesting clues to the biological consequences of these mutations. The results of the SAAB assays demonstrate that this collection of mutants does not give rise to a single, unified change in DNA sequence specificity. This result rules out the possibility that Denys−Drash syndrome is the result of a change in the network of genes regulated by WT1. These results do demonstrate that even modest changes in the DNA binding affinity of WT1 (less than 3-fold down in the case of D396G) can result in the wide range of biological consequences that lead to the onset of Denys−Drash syndrome. This observation suggests that the concentration of WT1 in the nucleus is so tightly regulated that even a 4-fold reduction in DNA binding affinity has significant biological consequences.

Finally, this work has provided important insight into the role of amino acids in the recognition α helix of zinc finger domains. It has demonstrated that replacement of a DNA-contacting arginine with other amino acids can influence the affinity, DNA sequence specificity, and DNA sequence selectivity of a zinc finger protein. It has also demonstrated how an amino acid involved in buttressing the side chain of a DNA-contacting residue contributes to these aspects of the DNA binding properties of a zinc finger protein.

REFERENCES

Baird, P. N., & Cowell, J. K. (1993) *Hum. Mol. Genet. 2*, 2193−2194.

Baird, P. N., Groves, N., Haber, D. A., Housman, D. E., & Cowell, J. K. (1992a) *Oncogene 7*, 2141−2149.

Baird, P. N., Santos, A., Groves, N., Jadresic, L., & Cowell, J. K. (1992b) *Hum. Mol. Genet. 1*, 301−305.

Blackwell, T. K., & Weintraub, H. (1990) *Science 250*, 1104−1110.

Call, K. M., Glaser, T., Ito, C. Y., Buckler, A. J., Pelletier, J., Haber, D. A., Rose, E. A., Kral, A., Yeger, H., Lewis, W. H., Jones, C., & Housman, D. E. (1990) *Cell 60*, 509−520.

Coppes, M. J., Campbell, C. E., & Williams, B. R. G. (1993) *FASEB J. 7*, 886−895.

Denys, P., Malvaux, P., Van Den Berghe, H., Tanghe, W., & Proesmans, W. (1967) *Fr. Pediatr. 24*, 729−739.

Drash, A., Sherman, F., Hartman, W. H., & Blizzard, R. M. (1970) *J. Pediatr. 76*, 585−593.

Drummond, I. A., Rupprecht, H. D., Rohwernutter, P., Lopezguisa, J. M., Madden, S. L., Rauscher, F. J., & Sukhatme, V. P. (1994) *Mol. Cell. Biol. 14*, 3800−3809.

Gessler, M., Poustka, A., Cavenee, W., Neve, R. L., Orkin, S. H., & Bruns, G. A. P. (1990) *Nature 343*, 774−778.

Gogos, J. A., Hsu, T., Bolton, J., & Kafatos, F. C. (1992) *Science 257*, 1951−1955.

Haber, D. A., Sohn, R. L., Buckler, A. J., Pelletier, J., Call, K. M., & Housman, D. E. (1991) *Proc. Natl. Acad. Sci. USA 88*, 9618−9622.

Hamilton, T. B., Barilla, K. C., & Romaniuk, P. J. (1995) *Nucleic Acids Res. 23*, 277−284.

Little, M. H., Prosser, J., Condie, A., Smith, P. J., van Heyningen, V., & Hastie, N. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4791−4795.

Little, M. H., Williamson, K. A., Mannens, M., Kelsey, A., Gosden, C., Hastie, N. D., & van Heyningen, V. (1993) *Hum. Mol. Genet. 2*, 259−264.

Little, M., Holmes, G., Bickmore, W., van Heyningen, V., Hastie, N., & Wainwright, B. (1995) *Hum. Mol. Genet. 4*, 351−358.

Matsunaga, E. (1981) *Hum. Genet. 57*, 231−246.

Miller, R. W., Fraumeni, J. F. J., & Manning, M. D. (1964) *N. Engl. J. Med. 270*, 922−927.

Nakagama, H., Heinrich, G., Pelletier, J., & Housman, D. E. (1995) *Mol. Cell. Biol. 15*, 1489−1498.

Nelson, R. M., & Long, G. L. (1989) *Anal. Biochem. 180*, 147−151.

Nordenskjold, A., Friedman, E., & Anvret, M. (1994) *Hum. Genet. 93*, 115−120.

Ogawa, O., Eccles, M. R., Yun, K., Mueller, R. F., Holdaway, M. D. D., & Reeve, A. E. (1993) *Hum. Mol. Genet. 2*, 203−204.
Park, S., Tomlinson, G., Nisen, P., & Haber, D. (1993) *Cancer Res. 53*, 4757−4760.
Pavletich, N. P., & Pabo, C. O. (1991) *Science 252*, 809−817.
Pelletier, J., Bruening, W., Kashtan, C. E., Mauer, S. M., Manivel, J. C., Striegel, J. E., Houghton, D. C., Junien, C., Habib, R., Fouser, L., Fine, R. N., Silverman, B. L., Haber, D. A., & Housman, D. (1991) *Cell 67*, 437−447.
Rauscher, F. J., Morris, J. F., Tournay, O. E., Cook, D. M., & Curran, T. (1990) *Science 250*, 1259−1262.
Romaniuk, P. J. (1990) *J. Biol. Chem. 265*, 17593−17600.
Sakai, A., Tadokoro, K., Yanagisawa, H., Nagafuchi, S., Hoshikawa, N., Suzuki, T., Kohsaka, T., Hasegawa, T., Nakahori, Y., & Yamada, M. (1993) *Hum. Mol. Genet. 2*, 1969−1970.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Seeman, N. C., Rosenberg, J. M., & Rich, A. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 804−808.
Studier, F. W., & Moffatt, B. A. (1986) *J. Mol. Biol. 189*, 113−130.
Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
Sukhatme, V. P., Cao, X. M., Chang, L. C., Tsai-Morris, C. H., Stamenkovich, D., Ferreira, P. C., Cohen, D. R., Edwards, S. A., Shows, T. B., & Curran, T. (1988) *Cell 53*, 37−43.
Young, J. L., Jr., & Miller, R. W. (1975) *J. Pediatr. 86*, 254−258.

BI960758O