Characterization of RNA Aptamer Binding by the Wilms' Tumor Suppressor Protein WT1[†]

Gary Zhai, Maya Iskandar, Kathleen Barilla, and Paul J. Romaniuk*

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

Received August 16, 2000; Revised Manuscript Received December 4, 2000

ABSTRACT: The interaction of the zinc finger protein WT1 with RNA aptamers has been investigated using a quantitative binding assay, and the results have been compared to those from a previous study of the DNA binding properties of this protein. A recombinant peptide containing the four zinc fingers of WT1 (WT1-ZFP) binds to representatives of three specific families of RNA aptamers with apparent dissociation constants ranging from 13.8 ± 1.1 to 87.4 ± 10.4 nM, somewhat higher than the dissociation constant of 4.12 ± 0.4 nM for binding to DNA. An isoform that contains an insertion of three amino acids between the third and fourth zinc fingers (WT1[+KTS]-ZFP) also binds to these RNAs with slightly reduced affinity (the apparent dissociation constants ranging from 22.8 to 69.8 nM) but does not bind to DNA. The equilibrium binding of WT1-ZFP to the highest-affinity RNA molecule was compared to the equilibrium binding to a consensus DNA molecule as a function of temperature, pH, monovalent salt concentration, and divalent salt concentration. The interaction of WT1-ZFP with both nucleic acids is an entropy-driven process. Binding of WT1-ZFP to RNA has a pH optimum that is narrower than that observed for binding to DNA. Binding of WT1-ZFP to DNA is optimal at 5 mM $MgCl_2$, while the highest affinity for RNA was observed in the absence of $MgCl_2$. Binding of WT1 to both nucleic acid ligands is sensitive to increasing monovalent salt concentration, with a greater effect observed for DNA than for RNA. Point mutations in the zinc fingers associated with Denys-Drash syndrome have dramatically different effects on the interaction of WT1-ZFP with DNA, but a consistent and modest effect on the interaction with RNA. The role of RNA sequence and secondary structure in the binding of WT1-ZFP was probed by site-directed mutagenesis. Results indicate that a hairpin loop is a critical structural feature required for protein binding, and that some consensus nucleotides can be substituted provided proper base pairing of the stem of the hairpin loop is maintained.

Wilms' tumor is a pediatric kidney malignancy that occurs with a frequency of 1 in 10000 (*1*, *2*). The *WT1* locus on chromosome 11p13 encodes a tumor suppressor protein that is inactivated in a subtype of Wilms' tumors (*3*, *4*). In normal cells, WT1 regulates gene networks during the development of the kidney and genitourinary systems (*5*). The protein has a nucleic acid binding domain consisting of four zinc fingers close to the carboxyl terminus and a regulatory domain rich in proline and glutamine residues located at the amino terminus (*6*, *7*).

Differential splicing of the primary *WT1* transcript yields four cDNA isoforms which differ in the presence or absence of either a 17-amino acid insertion in the regulatory domain of the protein, an in-frame insertion of three amino acids between the third and fourth zinc fingers, both insertions, or neither insertion (*3*, *8*). The protein isoforms that lack the three-amino acid insertion in the zinc finger domain are localized in the nucleus primarily in regions associated with active transcription (*9*). Consistent with this cellular localization, there is abundant evidence that these isoforms of the protein bind to specific sequences in DNA and regulate transcription from nearby promoters (*7*, *10*, *11*). In comparison, the protein isoforms that carry the three-amino acid insertion in the zinc finger domain are localized primarily in regions of the nucleus associated with the RNA splicing machinery (*9*). It has been demonstrated that the isoform of the WT1 zinc finger domain that includes the three-amino acid insertion between zinc fingers 3 and 4 binds preferentially to RNA molecules rather than to DNA molecules (*12*). Recent experiments based on the SELEX method of identifying high-affinity RNA ligands for biological molecules have shown that the isoform of the WT1 zinc finger domain that lacks the three-amino acid insertion in the zinc finger domain also has RNA binding activity with significant specificity and affinity (*13*). A direct biological role for RNA binding by this isomer of WT1 has not yet been demonstrated.

There is a growing body evidence which shows that zinc finger proteins are capable of binding to both DNA and RNA. The most investigated example is transcription factor IIIA (TFIIIA) from *Xenopus laevis*. This protein has nine zinc fingers and acts as a positive transcription factor in the expression of the 5S ribosomal RNA gene by binding to a 50 bp internal control region within the gene (*14*, *15*). In

[†] This work was supported by a grant from the Medical Research Council of Canada.

* To whom correspondence should be addressed. Telephone: (250) 721-7088. Fax: (250) 721-6227. E-mail: pjr@uvic.ca.

10.1021/bi001941r CCC: $20.00
Published on Web 01/26/2001

```
WT1 DNA: GCGTGGGCGTGT
  RNA20: GGGGCCACCAACGACAUUGACGAAUGCGUAAAUUGCUAGGUUGAUAUAAAUAGUGCCCAUGGAUCCGCGGGUGUCGGG
  RNA22: GGGGCCACCAACGACAUUGAUAUGGUGACCACCCCGGCGUUGAUAUAAAUAGUGCCCAUGGAUCCGCGGGUGUCGGG
  RNA38: GGGGCCACCAACGACAUUAUCACCCACCCCGAGCUGGCGUUGAUAUAAAUAGUGCCCAUGGAUCCGCGGGUGUCGGG
```

FIGURE 1: Nucleic acid ligands for the WT1 zinc finger peptides. The sequence of the top strand of the consensus double-stranded DNA binding site for WT1 is taken from ref *27*. The sequences of the three RNA ligands are taken from ref *13*, and each represents one of the three families of selected sequences. The consensus sequence for each family is shown in bold. The complete region that was randomized in the starting library is underlined, flanked by the fixed 5′ and 3′ sequences that also form part of the transcribed RNAs.

immature oocytes, TFIIIA also binds to 5S rRNA to form a 7S ribonucleoprotein particle that stores the RNA for use in ribosome assembly later in oogenesis (*16*). TFIIIA has a single nucleic acid binding site; it can bind to DNA or RNA but not both simultaneously. Zinc finger peptides can be derived from TFIIIA that bind specifically to either DNA or RNA (*17*, *18*); however, binding of the full-length protein to these ligands involves all nine zinc fingers. The binding of the protein to the two different nucleic acid targets is mediated by different features of the ligands: primarily base sequence in the case of DNA but primarily three-dimensional shape in the case of the 5S rRNA (*19*−*24*).

Our lab has previously carried out quantitative characterization of the DNA binding activity of the WT1 protein and the related zinc finger protein EGR1 (*25*). We have identified the contribution of individual base pairs in the DNA ligand and individual amino acids in the protein to the formation of a DNA−protein complex capable of regulating transcription (*25*−*27*). We report here on a quantitative analysis of the binding of the two zinc finger isoforms of the WT1 DNA binding domain, WT1-ZFP and WT1[+KTS]-ZFP, to specific RNA aptamers. The aptamer binding activity of WT1-ZFP was compared to the DNA binding activity of the protein, on the basis of assay conditions such as pH optima, monovalent and divalent salt concentrations, and temperature. The effects of specific point mutations in WT1-ZFP, first characterized in patients with Denys-Drash syndrome, on the DNA and RNA binding activities of the protein were compared. Site-directed mutations of a model RNA substrate for the WT1 form without KTS were used to probe the importance of RNA structure for the binding of WT1-ZFP to this aptamer.

MATERIALS AND METHODS

Purification of Recombinant Zinc Finger Peptides. The synthetic genes used to generate recombinant peptides of the zinc finger domains of WT1 (WT1-ZFP and WT1[+KTS]-ZFP) and the purification of such peptides have been described elsewhere (*27*).

Construction of Templates for Enzymatic Synthesis of Radiolabeled RNA. Synthetic genes with T7 promoters used to generate radiolabeled RNA for use in quantitative binding assays were constructed by PCR. Genes encoding the short, model RNA molecules designated clone 20, clone 22, and clone 38 by Pelletier's group (*13*) were assembled from two large overlapping oligonucleotides that were first extended by a single-round PCR and then amplified with appropriate primers to generate a full-length gene. After PCR, each gene was cloned into the *Sma*I site of pUC19 and the sequence verified by DNA sequencing techniques. The sequence of each gene is exactly the same as originally described, with the addition of an *Rsa*I site at the 3′ end of the coding region to allow for the definition of a specific termination site for T7 transcription. Mutations of the clone 22 sequence were introduced by synthesizing the appropriate template oligonucleotides for the PCRs.

Enzymatic Synthesis of Radiolabeled RNA. Radioactively labeled RNA was synthesized in vitro using T7 RNA polymerase and template DNA that had been digested with *Rsa*I, using techniques described previously (*28*).

Nitrocellulose Filter Binding Assays. The binding affinities of zinc finger peptides for nucleic acid ligands were determined by a nitrocellulose filter binding assay that has been described elsewhere (*29*, *30*). The standard binding buffer used to measure RNA binding affinities contained 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 5 μM $ZnCl_2$, 5 μg/mL poly[d(I-C)], and 100 μg/mL bovine serum albumin. When the effects of altering binding conditions such as pH, divalent salt concentration, or monovalent salt concentration were being investigated, the binding buffer was altered accordingly. Thawed protein aliquots were diluted in binding buffer lacking the poly[d(I-C)]. Experimental data were fitted to the appropriate equation using Kaleidagraph version 3.0.8 software.

RESULTS

Equilibrium Binding of WT1 Zinc Finger Peptides to DNA and RNA

The equilibrium binding of the WT1 and WT1[+KTS] zinc finger peptides to a DNA molecule containing a WT1 consensus binding site, and to representatives of three classes of RNA aptamers (Figure 1), was assessed by a nitrocellulose filter binding assay. As the data in Figure 2A show, WT1-ZFP binds to the DNA containing a consensus binding site for WT1 with an apparent dissociation constant of 4.12 nM (Table 1). WT1[+KTS]-ZFP has no discernible affinity for this consensus DNA (Figure 2B), consistent with previous reports about its DNA binding properties (*6*, *11*, *31*).

In vitro selection experiments with a randomized library of RNA molecules resulted in the identification of three distinct classes of molecules with sequences specific for binding to a WT1 zinc finger peptide (*13*). Using the nitrocellulose filter binding assay, we have determined the apparent dissociation constants for representative molecules of each of the three classes of aptamers for both WT1-ZFP and WT1[+KTS]-ZFP. As the results in Figure 2A and Table 1 show, the three RNAs bind to WT1-ZFP with apparent dissociation constants that are 3.3−21 times higher than the dissociation constant for DNA. The K_d value of 13.8 nM for the RNA22 molecule measured with the nitrocellulose filter binding assay is considerably lower than the value of 700 nM estimated from a gel shift assay for the binding of a WT1 zinc finger peptide to this RNA (*13*). Such a

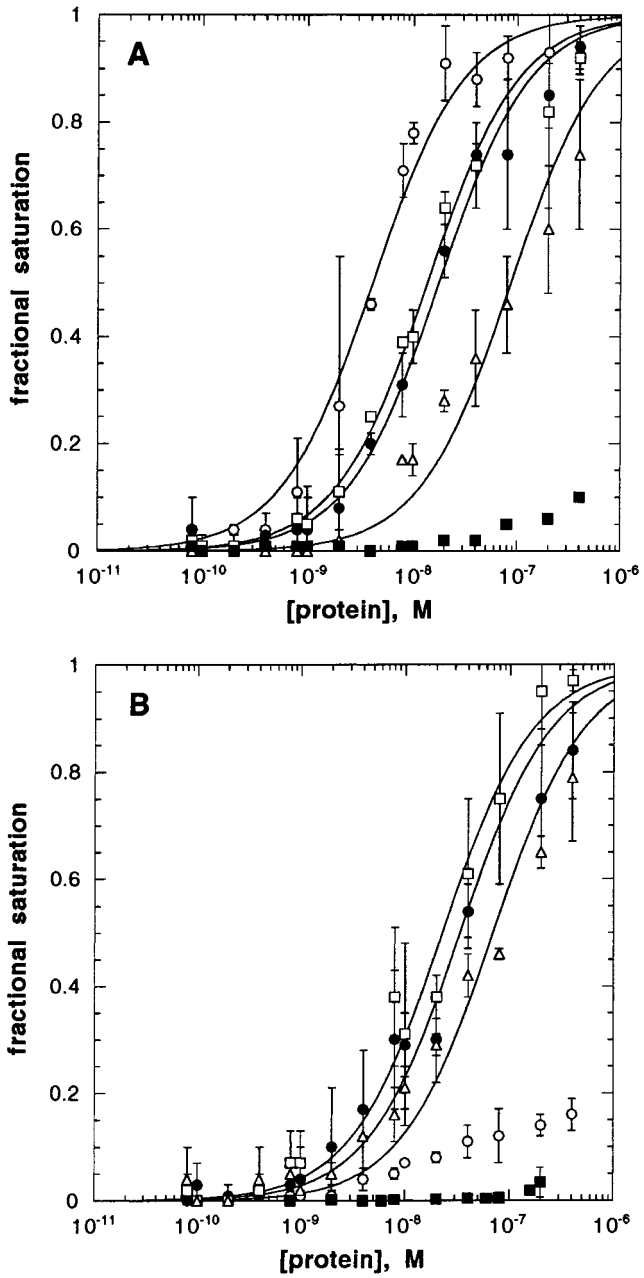

FIGURE 2: Equilibrium binding curves for binding of (A) WT1-ZFP and (B) WT1[+KTS]-ZFP to DNA and RNA. Affinities of consensus DNA (○), RNA22 (□), RNA38 (●), RNA20 (△), and 5S RNA (■) were measured using a nitrocellulose filter binding assay. Each line represents the best fit for the formation of a simple bimolecular complex (R values of 0.98−0.99). Each data point is the mean of three or more independent determinations, with the standard deviation indicated with error bars.

discrepancy is not unusual given that the nature of the zinc finger peptides and the assay conditions differ significantly in the two experiments. The data in Table 1 also show that the apparent dissociation constants measured in the filter binding assay reflect specific RNA binding; a nonspecific RNA of similar size (5S rRNA) has an apparent K_d for binding to WT1-ZFP that is much higher than the K_d values for the RNA aptamers.

The three RNA aptamers were derived via an in vitro selection experiment that used a WT1 zinc finger peptide lacking the three-amino acid insertion between fingers 3 and 4 (*13*). We measured the affinity of these aptamers for WT1-[+KTS]-ZFP (Figure 2B). Previous reports have indicated that the WT1[+KTS] isoform of the full-length protein is associated with the RNA splicing machinery in the nucleus, has RNA binding activity, and is associated in the cell with ribonucleoprotein particles (*9*, *12*, *32*, *33*). As the results in Figure 2B and Table 1 show, WT1[+KTS]-ZFP binds specifically to the three RNA aptamers but not to consensus DNA or 5S rRNA. For two of the RNA aptamers, the affinity of WT1[+KTS]-ZFP is approximately 2 times lower than the affinity of WT1-ZFP for these RNAs. This RNA binding activity was unexpected because a different WT1[+KTS] zinc finger peptide did not bind to RNA22 in a qualitative gel shift assay (*13*). It would appear that the construct we have used to prepare WT1[+KTS]-ZFP results in a peptide that is able to form specific RNA−protein complexes with the RNA aptamers.

Table 1: Affinity of WT1 Zinc Finger Peptides for DNA and RNA[a]

nucleic acid	WT1-ZFP K_d (nM)	WT1[+KTS]-ZFP K_d (nM)
TGT DNA	4.12 ± 0.39	>1000[b]
RNA20	87.4 ± 10.4	69.8 ± 8.4
RNA22	13.8 ± 1.1	22.8 ± 2.3
RNA38	17.8 ± 1.4	33.7 ± 4.4
5S rRNA	>1000[b]	>1000[b]

[a] Apparent dissociation constants determined by the nitrocellulose filter binding assay. Each value reported represents the mean of three or more independent determinations with the associated standard deviations. [b] The binding affinity was too low to be determined accurately.

Comparison of the DNA and RNA Binding Properties of the WT1 Zinc Finger Peptide

pH Dependence of DNA and RNA Binding. It has previously been demonstrated that the binding of WT1-ZFP to a consensus DNA molecule occurs over a broad pH optimum from 5.5 to 9.0 (*25*). This broad pH optimum indicates that titratable groups on either the protein or DNA are not essential to their interaction. In comparison, the binding of WT1-ZFP to RNA22 occurs optimally in the range of pH 7.0−8.5, with a significant decrease in RNA binding affinity observed at pH <7.0 (Figure 3). This result suggests that one or more titratable groups on either the protein or RNA make significant contributions to this RNA−protein interaction.

Mg^{2+} Dependence of DNA and RNA Binding. As indicated in Figure 4, the binding of WT1-ZFP to a consensus DNA molecule has a broad optimum for Mg^{2+} in the binding buffer. In comparison, optimal binding of WT1-ZFP to RNA22 occurs when Mg^{2+} is omitted from the binding buffer, the level decreasing steadily as the concentration of Mg^{2+} increases (Figure 4).

Monovalent Salt Dependence of DNA and RNA Binding. The contribution of ionic protein−DNA bonds to the binding free energy of the WT1-ZFP−DNA interaction has been reported previously (*25*). Analysis of the salt dependence of K_a is based upon an ion displacement model (*34*), where the number of ions released in the formation of a protein−DNA complex can be obtained using the equation $\ln(K_{obsd}) = \ln K^\circ - Z\psi \ln[M^+] - Z \ln\{0.5[1 + (1 + 4K^{Mg}_{obsd}[Mg^{2+}])^{0.5}]\}$, where K° is the apparent K_a at 1 M salt, Z is the number of cations and anions released upon complex formation, ψ is the fractional counterion bound per phosphate in the DNA

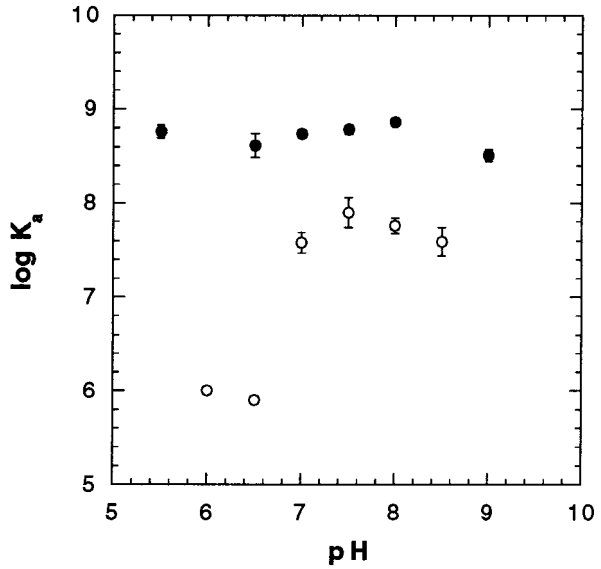

FIGURE 3: pH dependence of the binding of WT1-ZFP to DNA (●) and RNA22 (○). Each data point is the mean of three or more independent determinations, with the standard deviation indicated with error bars. Data for DNA binding are taken from ref *25*.

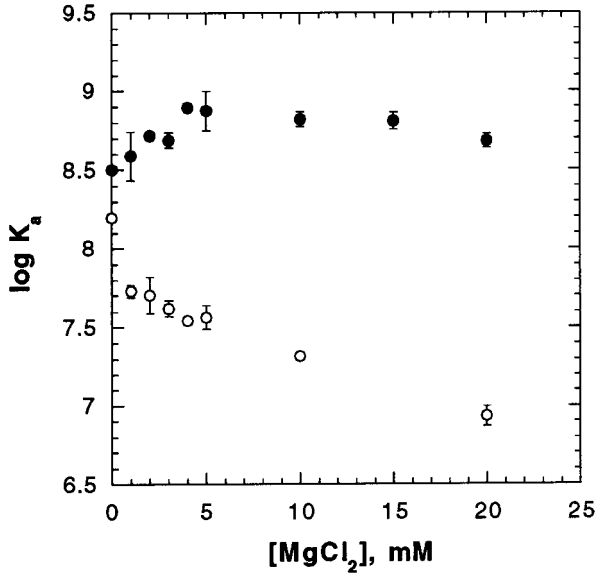

FIGURE 4: Effect of magnesium ion concentration on the binding of WT1-ZFP to DNA (●) and RNA22 (○). Each data point is the mean of three or more independent determinations, with the standard deviation indicated with error bars. Data for DNA binding are taken from ref *25*.

(assumed to be 0.88 for double-stranded DNA), and K^{Mg}_{obsd} is the observed binding constant for the Mg^{2+}−DNA interaction. The data in Figure 5 were fitted to the above equation assuming the Mg^{2+} binding to the WT1 consensus DNA is adequately represented by Mg^{2+} binding to T7 DNA. The data indicated that there are 9.2 ± 1.8 ions released in the formation of the WT1−DNA complex. Application of this model to RNA−protein interactions is less straightforward because it requires several untested assumptions. For example, the binding of counterions to phosphates and the binding constant of Mg^{2+} may be influenced by RNA structure and therefore cannot adequately be described by data for ribopolymers. In addition, the effects of changing monovalent salt concentration on RNA structure are not accommodated by the theoretical model. Therefore, the number of ion pairs determined from the analysis (3.8 ± 0.8) can only be regarded as a very rough estimate. The data in Figure 5 for the monovalent salt dependence of RNA22 binding by WT1-ZFP are consistent with the conclusion that RNA binding is significantly less dependent upon ionic interactions than DNA binding.

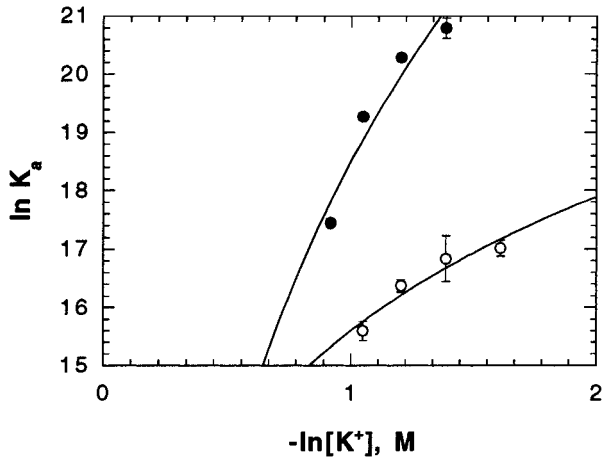

FIGURE 5: Dependence of the binding of WT1-ZFP to DNA (●) and RNA22 (○) on monovalent salt concentration. Each data point is the mean of three or more independent determinations, with the standard deviation indicated with error bars. Data for DNA binding are taken from ref *25*. The experimental data were fit to eq 5 of ref *34* by a regression method that varied the number of ion pairs and the apparent K_a at 1 M salt. The solid lines represent the best least-squares fit for the experiments, yielding values of 9.2 ± 1.8 ion pairs for DNA (R = 0.96) and 3.8 ± 0.8 ion pairs for RNA22 (R = 0.96). See the text for a discussion of the limitations in applying this analysis to RNA−protein interactions.

Temperature Dependence of DNA and RNA Binding. The temperature dependencies of the affinities of WT1-ZFP for consensus WT1 DNA and the RNA22 aptamer are shown in Figure 6. For both nucleic acid binding activities, the affinity of the protein increases with increasing temperature. At 22 °C, the WT1-ZFP−DNA interaction has a $\Delta G°$ value of −12.1 kcal mol^{-1}, an unfavorable $\Delta H°$ value of 6.6 kcal mol^{-1}, and a favorable $\Delta S°$ value of 63.3 cal mol^{-1} deg^{-1}. The thermodynamic parameters for the WT1-ZFP−RNA22 interaction are similar: $\Delta G° = -10.6$ kcal mol^{-1}, $\Delta H° =$ 2.9 kcal mol^{-1}, and $\Delta S° = 45.8$ cal mol^{-1} deg^{-1}. Thus, formation of the DNA and RNA complexes with WT1-ZFP is driven by entropy.

Effect of Denys-Drash Syndrome Point Mutations on the DNA and RNA Binding Properties of the WT1 Zinc Finger Peptide

Some patients with Denys-Drash syndrome have missense mutations in the zinc finger domain of the WT1 gene. These mutations target key amino acids in fingers 2 and 3 that are involved in forming sequence-specific contacts with base pairs in the WT1 consensus binding site in DNA (*35*). The effects of these mutations on the DNA binding affinity of WT1-ZFP range from a 2.5-fold decrease to a >100-fold decrease (*26*). In comparison, the data in Table 2 show that the effects of these mutations on the binding of WT1-ZFP to RNA22 are relatively benign, decreasing RNA binding activity by 3.3−5-fold.

WT1 Binding Determinants in RNA

The three classes of RNAs selected in vitro from a random library of RNA molecules by a WT1 zinc finger peptide share

Table 2: Affinity of Denys-Drash Mutants of WT1-ZFP for DNA and RNA[a]

protein	DNA[b]	RNA22	protein	DNA[b]	RNA22
wild-type	1.000	1.000	R394W	<0.01[c]	0.20 ± 0.03
R366H	0.26 ± 0.02	0.19 ± 0.05	D396G	0.38 ± 0.03	0.30 ± 0.04
R366C	0.07 ± 0.01	0.21 ± 0.07	D396N	0.12 ± 0.01	0.28 ± 0.12

[a] Apparent dissociation constants determined by the nitrocellulose filter binding assay. Each value reported represents the mean of three or more independent determinations with the associated standard deviations. Relative affinites were determined by dividing the apparent K_a for the mutant protein by the apparent K_a for the wild-type protein determined in parallel. The errors for relative affinities are given by the expression $\sigma = [(\sigma_1/M_1)^2 + (\sigma_2/M_2)^2]^{1/2} \times M_1/M_2$, where M_1 and M_2 are the association constants for the wild-type and mutant proteins, respectively, and the σ values are the corresponding standard deviations for these determinations. [b] Data taken from Borel et al. (*26*). [c] The binding affinity was too low to be determined accurately.

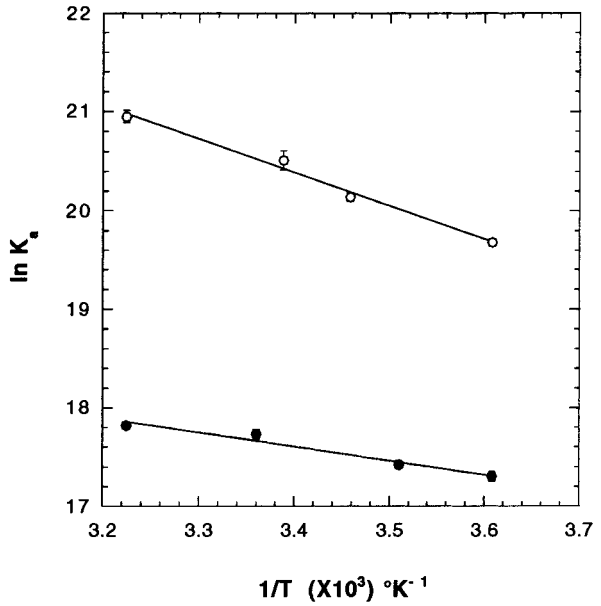

FIGURE 6: Temperature dependence of the binding of WT1-ZFP to DNA (●) and RNA22 (○). The van't Hoff plot was used to calculate the enthalpies of these interactions from the slope of the lines ($R = 0.99$ for DNA and 0.98 for RNA22) using the equation $d[\ln(K_a)]/d(1/T) = -\Delta H°/R$. Each data point is the mean of three or more independent determinations, with the standard deviation indicated with error bars. Data for DNA binding are taken from ref *25*.

no obvious similarity in terms of either sequence or putative structural motifs (*13*). This observation lead Pelletier and co-workers to conclude that RNA binding by the WT1 zinc finger peptide is directed by the sequence but not the structure of the RNA. The importance of the selected sequences in RNA22 and RNA38 was demonstrated by the deleterious effects of point mutants within the selected sequences.

We used the mfold program (*36*, *37*) to assess the secondary structure potential of all members of the three classes of selected RNA aptamers. For two of these classes, represented by RNA20 and RNA38, respectively, the selected sequences were presented in a variety of secondary structural elements in the lowest-free energy forms resulting from the mfold program (data not shown). In comparison, the members of the class represented by RNA22 were consistently folded into a common structure. As shown in Figure 7, the selected sequence forms part of a hairpin loop.

These results prompted us to test key features of this proposed secondary structure for RNA22 by changing sequences adjacent to the selected sequence. In the binding of proteins to hairpin structures in RNA, loop size and the stability of the base-paired stem are critical. To test the potential function of loop size in the binding of WT1-ZFP to RNA22, we created a series of mutations. The size of the loop was decreased in one mutant by deletion of four nucleotides from the center of the loop (Figure 8B). In another mutant, loop size was decreased by increasing the complementarity of the nucleotides at the start and end of the loop (Figure 8D). In addition, we attempted to increase the size of the loop by inserting four nucleotides into the center, although the mfold program predicts that this insertion will convert the hairpin loop into an internal loop (Figure 8C). As the results in Figure 8 show, mutations that alter the loop size of RNA22 without changing the selected sequence reduce WT1-ZFP binding affinity by 30−50-fold.

The importance of the base-paired stem of the hairpin structure was investigated using several mutants. The first mutant disrupted the base pair stem by changing the sequence of nucleotides on the 5′ side of the stem. These nucleotides were part of the fixed sequence in the original library of random RNA molecules. The effect of this mutation on the structure of the RNA is shown in Figure 9A, where it is predicted by the mfold program to significantly change the overall structure from that found in wild-type RNA22. This mutation decreases the level of binding of the WT1 zinc finger peptide by more than 50-fold, even though the entire selected sequence is still present in the RNA. In RNA22 and other members of this class of selected RNAs, the common selected sequence occurs immediately after the 5′ fixed sequence and involves 9 of the 20 random sites in the original library. In the mutant RNA22 shown in Figure 9B, we have "swapped" the position of the selected sequence with the rest of the "random" part of the molecule. Although the predicted secondary structure of this construct is quite different from that of the wild-type RNA22, it includes a hairpin loop with the selected sequence on the 3′ side. The nature of the base pairing in the stem is similar, but not identical, to that of the wild-type RNA molecule. This mutant RNA22 molecule binds WT1-ZFP with an only 5-fold decrease in affinity. Finally, we constructed a mutant form of RNA22 in which the sequence on both sides of the stem was altered but the base pairing remained intact. In this mutant, only the four selected nucleotides in the loop remain (Figure 9C). This mutant exhibits a reduction of 9-fold in WT1 binding affinity, much less of a deleterious effect than that observed for mutations that disrupted stem pairing or altered the size of the loop.

For RNA binding proteins such as the R17 phage coat protein, all of the binding determinants are encompassed within a single hairpin structure found in a much larger RNA molecule (*38*). To determine whether the hairpin structure found in RNA22 contains all of the binding determinants for WT1-ZFP, we constructed the truncation mutant shown

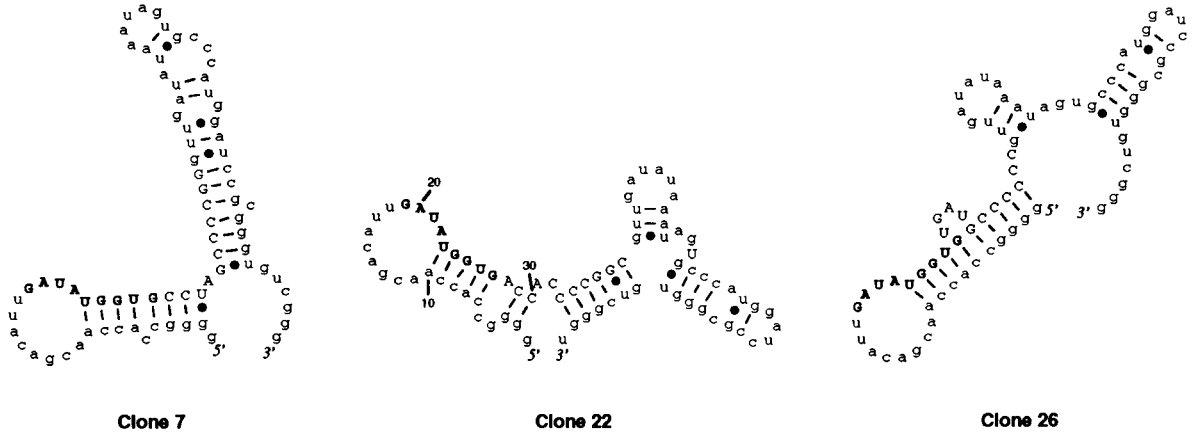

FIGURE 7: Lowest-free energy structures predicted for three members of the A family of RNA aptamers (*13*), as determined by the mfold program (*36*, *37*). The consensus sequence (GAUAUGGUG) that defines this family of aptamers is indicated in uppercase bold letters. The remaining nucleotides indicated by uppercase letters are derived from the random region of the SELEX template, but are not common to all members of this family. All other nucleotides (shown in lowercase letters) are encoded by the 5′ and 3′ fixed sequences of the SELEX template.

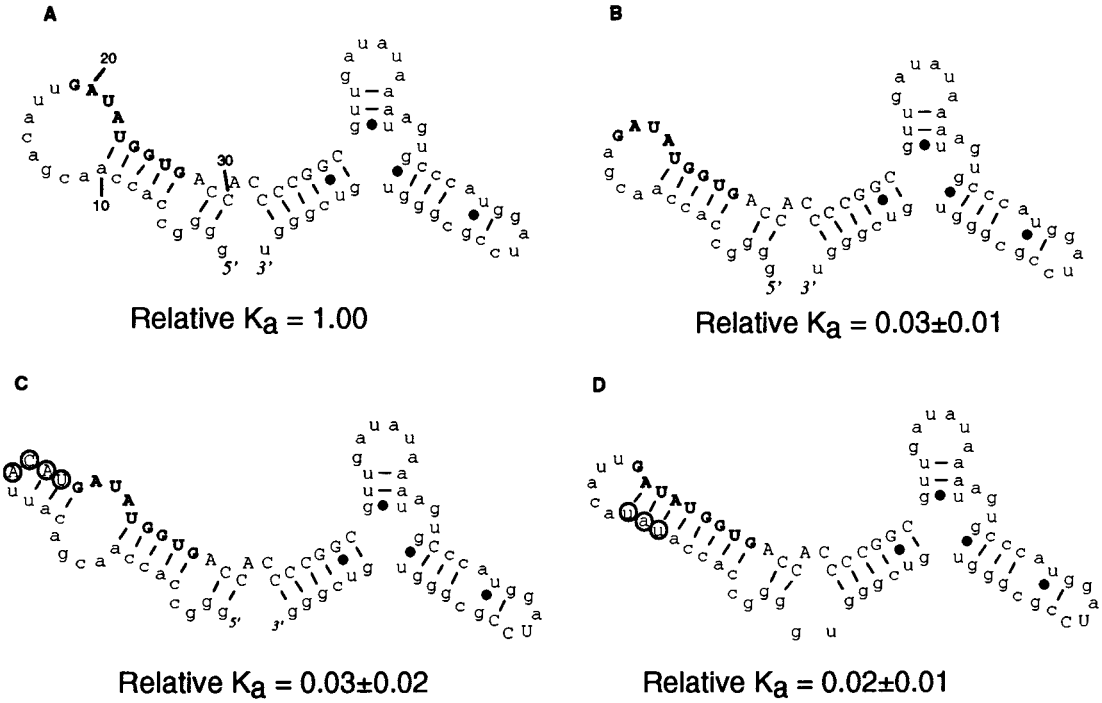

FIGURE 8: Relative binding affinities of RNA22 mutants with altered size of the hairpin loop. Binding affinities relative to that of the wild-type RNA22 are indicated below each structure and are the means ± standard deviation from three or more independent determinations. Altered nucleotides are enclosed with circles. (A) Wild-type RNA22. (B) Deletion of the four nucleotides (CAUU) preceding the consensus sequence. (C) Insertion of four nucleotides (ACAU) immediately before the consensus sequence. (D) The first three nucleotides at the 5′ side of the loop have been substituted to be complementary to the final three nucleotides of the loop.

in Figure 9D. This RNA mutant bound WT1-ZFP with a 12-fold lower affinity than the full-length RNA22 molecule.

DISCUSSION

The primary transcript of the WT1 gene undergoes alternative splicing that generates four protein isoforms (*3*, *8*). One of the alternative splicing events results in the inclusion or exclusion of 17 amino acids in the amino half of the protein encoded by exon 5. The second alternative splicing event results in the inclusion or exclusion of three amino acids (with or without KTS) between the third and fourth zinc fingers encoded by alternative 5′ donor splice sites at the end of exon 9. The WT1 protein isoforms with the three-amino acid insertion are localized primarily to structures in the nucleus associated with RNA processing (*9*). Consistent with this observation, there is evidence that the WT1[+KTS] protein associates with splicing complexes and harbors RNA binding activity (*12*, *32*, *33*). The isoforms of the WT1 protein lacking the three-amino acid insertion are primarily associated with the region of the nucleus where active transcription takes place (*9*). These isoforms of the protein bind DNA in a sequence-specific fashion and regulate transcription from promoters with the appropriate binding sites (*7*, *10*, *11*). Association of the WT1[−KTS] isoforms with transcription complexes does not rule out a role involving RNA binding. RNA aptamers that bind specifically to a zinc finger peptide of WT1[−KTS] have recently been isolated (*13*). Thus, the WT1[−KTS] isoform is a zinc finger

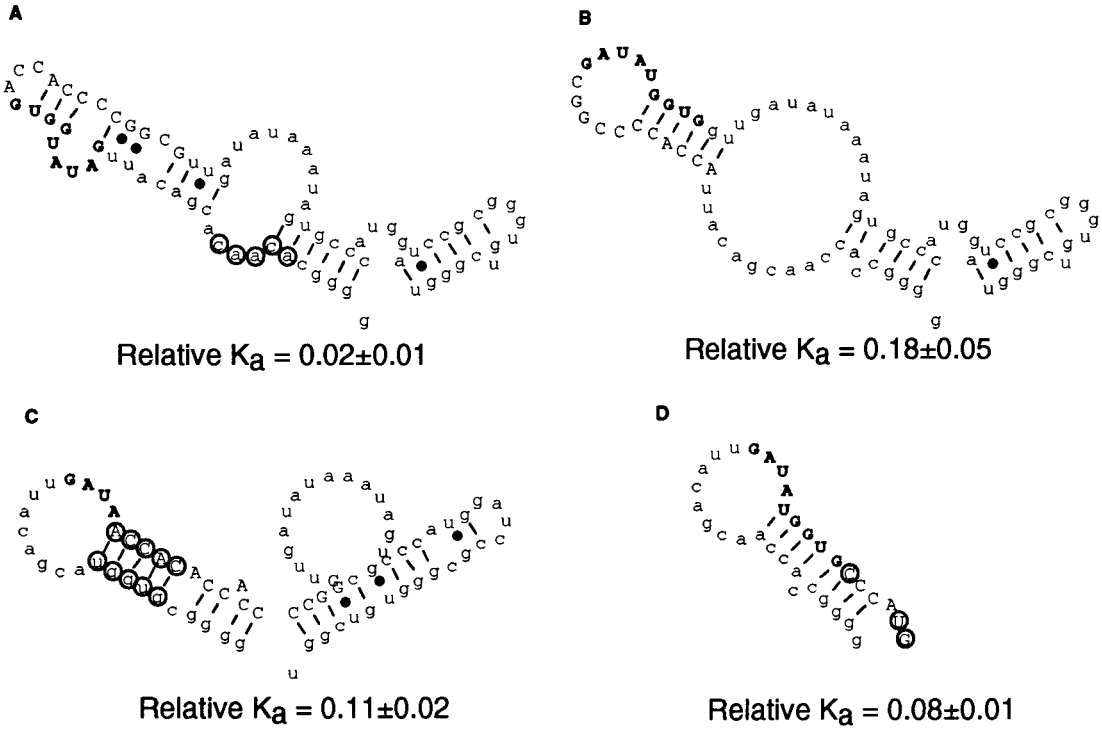

FIGURE 9: Relative binding affinities of RNA22 mutants with altered base pairing. Binding affinities relative to that of wild-type RNA22 are indicated below each structure and are the means ± standard deviation from three or more independent determinations. Altered nucleotides are enclosed with circles. (A) The sequence of nucleotides 6−10 has been changed to disrupt the base pairing of the hairpin loop−stem structure. (B). The position of the consensus nucleotides has been exchanged with the position of the rest of the random sequence. (C) The sequence of the base pairs closing the hairpin loop (nucleotides 6−10 and 23−27) has been exchanged to maintain base pairing, but with an altered sequence of base pairs. (D) The RNA22 molecule has been truncated after nucleotide 30.

protein with sequence-specific DNA and RNA binding activities. A biological role for the RNA binding activity of this isoform of WT1 has not yet been demonstrated.

Transcription factor IIIA was the first zinc finger protein to be described that had the ability to bind specifically to both DNA and RNA. We have previously used site-directed mutagenesis of nucleic acid binding targets combined with quantitative equilibrium binding assays to compare the DNA and RNA binding activities of TFIIIA (*18*−*24*, *28*−*30*). In this report, we applied the same techniques to characterize the interaction of WT1-ZFP with RNA aptamers. The results of this analysis are compared with the previously characterized DNA binding properties of this isoform (*25*).

In the original study of RNA binding by a WT1 zinc finger peptide, three families of RNA aptamers were derived by in vitro selection from a random RNA library (*13*). Equilibrium binding of WT1-ZFP to a representative of each of these RNA families and a consensus DNA molecule was assessed using a quantitative nitrocellulose filter binding assay. The results show that WT1-ZFP binds to the RNAs with affinities that are 3.3−21 times lower than the affinity for the consensus DNA molecule. The specificity of the RNA binding activity is demonstrated by the fact that 5S rRNA is a very poor ligand for binding to WT1-ZFP. The ratio of specific to nonspecific RNA binding affinity is at least 2 orders of magnitude, similar to that observed for the RNA binding activity of TFIIIA (*29*).

Although the three RNA aptamers were selected by a WT1 zinc finger peptide (*13*), a zinc finger peptide with the KTS insertion binds to these RNAs with only slightly reduced affinity and identical sequence specificity. The WT1[+KTS] zinc finger peptide fails to bind to the consensus DNA binding site that is bound by WT1-ZFP with high affinity.

The effects of altering assay conditions such as pH, temperature, and monovalent and divalent salt concentration can provide additional insight into subtle differences in the nucleic acid binding activities of a protein. Such an approach has been utilized to differentiate the DNA and RNA binding activities of TFIIIA (*29*, *30*), the RNA binding activities of the related zinc finger proteins TFIIIA and p43 (*39*), and the DNA binding activities of the related zinc finger proteins WT1 and EGR1 (*25*). A comparison of the DNA and RNA binding properties of WT1-ZFP was undertaken using RNA22 as a high-affinity ligand and comparing the results to previously published data on the DNA binding properties of this peptide. There are clearly differences in the dependence of the two nucleic acid binding activities upon pH, divalent salt concentration, and monovalent salt concentration. Titratable groups do not play a role in DNA binding by WT1-ZFP, but protonation of one or more groups at pH <7.0 does significantly decrease the level of RNA binding interaction. DNA binding by the peptide reaches an optimum at a Mg^{2+} concentration of 5 mM, while the level of RNA binding is reduced by the presence of Mg^{2+} in the buffer. Such a reduction by Mg^{2+} in the RNA binding activity of a protein has also been observed for the zinc finger protein p43 (*39*). Analysis of the monovalent salt dependence of DNA and RNA binding by WT1-ZFP suggests that more ions are released upon formation of the DNA−protein complex than are released upon formation of the RNA−protein complex.

The DNA and RNA binding properties of WT1-ZFP have the same dependence on the temperature of the assay. The thermodynamic parameters derived from an analysis of the temperature dependence of the binding affinity indicate that formation of both DNA−protein and RNA−protein complexes is driven by entropy. Such a phenomenon was also observed for the RNA binding activity of the p43 zinc finger protein (*39*). Entropy-driven binding of proteins to nucleic acids likely involves the formation of hydrophobic interactions and the release of counterions and ordered water molecules from the individual components as a consequence of binding.

Substitution mutations in the zinc fingers of the WT1 gene have been isolated from patients with Denys-Drash syndrome (*40*) and have significant effects on the DNA binding activity and biological function of the protein (*41*, *42*). We have previously shown that these mutations can reduce the level of binding of WT1-ZFP to a consensus DNA sequence by as little as 3.3-fold or by more than 100-fold (*26*). When the effects of these same mutations on RNA binding by WT1-ZFP were measured, the largest reduction that was observed was 5.5-fold. Indeed, three of the five mutations have very similar and relatively small effects on both DNA and RNA binding by WT1 (Table 2). The R366C and R394W mutations both had a significantly greater effect on DNA binding by WT1-ZFP than on RNA binding by this protein. In the crystal structure of the related zinc finger protein EGR1 bound to consensus DNA, these two arginine residues form critical hydrogen bonds to guanine bases in the DNA. By analogy, these arginine residues in WT1-ZFP would be expected to perform the same function in DNA binding. However, our results would suggest that these two arginine residues do not form critical hydrogen bonds to functional groups on RNA22.

Binding of RNA to proteins involves the presentation of specific functional groups on the RNA in a unique three-dimensional context. The three classes of RNA species selected by a WT1 zinc finger peptide from a library of random sequences do not have a common sequence motif and are unlikely to have a common three-dimensional structure (*13*). We used computational methods to predict the lowest-free energy forms of the selected RNA sequences. Members of the A family of selected RNAs consistently folded into a structure that put the consensus sequence on the 3′ side of a hairpin stem−loop structure. Common structures were not predicted for members of the other two families of selected RNAs. Since Bardeesy et al. demonstrated by site-directed mutagenesis that the consensus sequence within RNA22 is required for protein binding (*13*), we turned our attention to the role of the putative structure of RNA22 in the binding of WT1-ZFP. Mutations in the nonconsensus region of RNA22 that altered the size of the hairpin loop containing part of the consensus sequence reduced the affinity for binding dramatically, from 30- to 50-fold. A similar effect was observed for a mutation that disrupted the base pairing of the stem structure without altering the sequence or placement of the consensus sequence in RNA22. The structure of this mutant RNA was predicted to be significantly rearranged compared to the wild-type RNA22.

Three mutations of RNA22 had only a modest impact on WT1-ZFP binding. These included one mutant that swapped the position of the consensus sequence with the rest of the random part of the original RNA. This mutant RNA is predicted to fold into a structure that has a hairpin loop containing the consensus sequence, which appears to mimic (but not duplicate) the wild-type structural context and thus reduces WT1-ZFP binding affinity by 5.5-fold. This reduction in affinity may reflect the difference in the size of the hairpin loop in the mutant RNA and the wild-type RNA, or may result from a shortening of the stem closing the loop.

The second mutant with a modest effect on WT1-ZFP binding affinity is one where the base pairs of the stem are "flipped", which in essence replaces five of the consensus nucleotides but maintains the RNA structure. This mutant RNA binds WT1-ZFP with 8-fold lower affinity than wild-type RNA22. This reduction in affinity may result from the loss of a minor sequence-specific contact formed to the base pairs of the stem that is disrupted by the mutation. Alternatively, the reduction in RNA binding affinity may arise because of the change in the size of the internal loop within the stem.

We also constructed a truncated version of RNA22 that contained only a hairpin loop with the consensus sequence. This RNA binds WT1-ZFP, but with a 12-fold reduced affinity compared to full-length RNA22. This reduction in affinity may result from an overall decreased stability of the folded RNA (predicted $\Delta G = -11.2$ kcal/mol compared to $\Delta G = -28.4$ kcal/mol for RNA22). Stem stability is greatly enhanced by the presence of "dangling" bases at the 5′ or 3′ end (*43*, *44*). The reduction in affinity may also reflect a change in the conformation of the base-paired stem in the truncated RNA, where a bulged nucleotide separates the final two base pairs formed by the consensus sequence and a small internal loop found in the wild-type RNA is absent from the truncated RNA. Studies of other RNA−protein interactions have demonstrated the importance of bulged nucleotides and internal loops in the binding of proteins to RNA (*23*, *28*).

The computational structure derived for RNA22 has provided a useful guideline for the generation of rationally based site-directed mutants of the aptamer, and provided a context for analyzing the effects of those mutations on WT1-ZFP binding. In most cases, the effects that the mutations of RNA22 have on WT1 binding are consistent with the predicted changes in the structure of the aptamer. However, in the absence of independent data on the solution structure of RNA22, the interpretation provided by reference to this structure is only one of a number of possible interpretations. Detailed analysis of the wild-type and mutant RNA22 molecules using chemical and enzymatic probes would offer additional information about the effects that the site-directed mutations had on the structure of the RNA that might support the predicted structures, or refute those structures. Nevertheless, the mutational analysis we have conducted clearly demonstrates that nucleotides outside the consensus sequence of RNA22 are critical for the binding of WT1-ZFP.

Like TFIIIA, WT1 is an example of a zinc finger protein which is able to bind specifically to DNA and RNA. For both zinc finger proteins, the overall structure of the RNA ligand is a major determinant of binding affinity. In the case of TFIIIA, both nucleic acid binding activities have biological significance. At this time, only the biological relevance of the DNA binding activity of WT1 is known. Cellular RNA targets for WT1 have not been identified, and the question

of whether there is a biological role for RNA binding by this protein has not been addressed yet. Site-directed mutagenesis of TFIIIA has resulted in mutant proteins that lack either DNA binding activity, RNA binding activity, or both binding activities (*45*). Like that of TFIIIA, the DNA and RNA binding activities of WT1 appear to be distinct. Thus, a similar approach to studying the structure−function relationships of the zinc finger domain of WT1 may result in the identification of separate mutations of each nucleic acid binding activity that will be useful in elucidating the biological role of RNA binding by this protein.

REFERENCES

1. Matsunaga, E. (1981) *Hum. Genet. 57*, 231−246.
2. Young, J. L. J., and Miller, R. W. (1975) *J. Pediatr. 86*, 254−258.
3. Gessler, M., Poustka, A., Cavenee, W., Neve, R. L., Orkin, S. H., and Bruns, G. A. P. (1990) *Nature 343*, 774−778.
4. 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., and Housman, D. E. (1990) *Cell 60*, 509−520.
5. Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D., van Heyningen, V., and Hastie, N. (1990) *Nature 346*, 194−197.
6. Rauscher, F. J., Morris, J. F., Tournay, O. E., Cook, D. M., and Curran, T. (1990) *Science 250*, 1259−1262.
7. Madden, S. L., Cook, D. M., Morris, J. F., Gashler, A., Sukhatme, V. P., and Rauscher, F. J. (1991) *Science 253*, 1550−1553.
8. Haber, D. A., Sohn, R. L., Buckler, A. J., Pelletier, J., Call, K. M., and Housman, D. E. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9618−9622.
9. Larsson, S. H., Charlieu, J.-P., Miyagawa, K., Engelkamp, D., Rassoulzadegan, M., Ross, A., Cuzin, F., van Heyningen, V., and Hastie, N. D. (1995) *Cell 81*, 391−401.
10. Nakagama, H., Heinrich, G., Pelletier, J., and Housman, D. E. (1995) *Mol. Cell. Biol. 15*, 1489−1498.
11. Drummond, I. A., Rupprecht, H. D., Rohwernutter, P., Lopezguisa, J. M., Madden, S. L., Rauscher, F. J., and Sukhatme, V. P. (1994) *Mol. Cell. Biol. 14*, 3800−3809.
12. Caricasole, A., Duarte, A., Larsson, S. H., Hastie, N. D., Little, M., Holmes, G., Todorov, I., and Ward, A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 7562−7566.
13. Bardeesy, N., and Pelletier, J. (1998) *Nucleic Acids Res. 26*, 1784−1792.
14. Bogenhagen, D. F., Sakonju, S., and Brown, D. D. (1980) *Cell 19*, 27−35.
15. Miller, J., McLachlan, A. D., and Klug, A. (1985) *EMBO J. 4*, 1609−1614.
16. Picard, B., and Wegnez, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 241−245.
17. Clemens, K. R., Wolf, V., Mcbryant, S. J., Zhang, P. H., Liao, X. B., Wright, P. E., and Gottesfeld, J. M. (1993) *Science 260*, 530−533.
18. McBryant, S. J., Veldhoen, N., Gedulin, B., Leresche, A., Foster, M. P., Wright, P. E., Romaniuk, P. J., and Gottesfeld, J. M. (1995) *J. Mol. Biol. 248*, 44−57.
19. You, Q., Veldhoen, N., Baudin, F., and Romaniuk, P. J. (1991) *Biochemistry 30*, 2495−2500.
20. You, Q., and Romaniuk, P. J. (1990) *Nucleic Acids Res. 18*, 5055−5062.
21. Romaniuk, P. J. (1989) *Biochemistry 28*, 1388−1395.
22. Veldhoen, N., You, Q. M., Setzer, D. R., and Romaniuk, P. J. (1994) *Biochemistry 33*, 7568−7575.
23. Baudin, F., Romaniuk, P. J., Romby, P., Brunel, C., Westhof, E., Ehresmann, B., and Ehresmann, C. (1991) *J. Mol. Biol. 218*, 69−81.
24. Baudin, F., and Romaniuk, P. J. (1989) *Nucleic Acids Res. 17*, 2043−2056.
25. Hamilton, T., Borel, F., and Romaniuk, P. J. (1998) *Biochemistry 37*, 2051−2058.
26. Borel, F., Barilla, K. C., Hamilton, T. B., Iskandar, M., and Romaniuk, P. J. (1996) *Biochemistry 35*, 12070−12076.
27. Hamilton, T. B., Barilla, K. C., and Romaniuk, P. J. (1995) *Nucleic Acids Res. 23*, 277−284.
28. Romaniuk, P. J., de Stevenson, I. L., and Wong, H.-H. A. (1987) *Nucleic Acids Res. 15*, 2737−2755.
29. Romaniuk, P. J. (1985) *Nucleic Acids Res. 13*, 5369−5387.
30. Romaniuk, P. J. (1990) *J. Biol. Chem. 265*, 17593−17600.
31. Bickmore, W. A., Oghene, K., Little, M. H., Seawright, A., Vanheyningen, V., and Hastie, N. D. (1992) *Science 257*, 235−237.
32. Davies, R. C., Calvio, C., Bratt, E., Larsson, S. H., Lamond, A. I., and Hastie, N. D. (1998) *Genes Dev. 12*, 3217−3225.
33. Ladomery, M. R., Slight, J., McGhee, S., and Hastie, N. D. (1999) *J. Biol. Chem. 274*, 36520−36526.
34. Record, M. T., Jr., Lohman, T. M., and de Haseth, P. (1976) *J. Mol. Biol. 107*, 145−158.
35. Pavletich, N. P., and Pabo, C. O. (1991) *Science 252*, 809−817.
36. Mathews, D. H., Sabina, J., Zuker, M., and Turner, D. H. (1999) *J. Mol. Biol. 288*, 911−940.
37. Zuker, M., Mathews, D. H., and Turner, D. H. (1999) in *RNA Biochemistry and Biotechnology* (Barciszewski, J., and Clark, B. F. C., Eds.) pp 11−43, NATO ASI Series, Kluwer Academic Publishers, Dordrecht, The Netherlands.
38. Romaniuk, P. J., Lowary, P., Wu, H.-N., Stormo, G., and Uhlenbeck, O. C. (1987) *Biochemistry 26*, 1563−1568.
39. Zang, W. Q., and Romaniuk, P. J. (1995) *J. Mol. Biol. 245*, 549−558.
40. Coppes, M. J., Campbell, C. E., and Williams, B. R. G. (1993) *FASEB J. 7*, 886−895.
41. Little, M., Holmes, G., Bickmore, W., Vanheyningen, V., Hastie, N., and Wainwright, B. (1995) *Hum. Mol. Genet. 4*, 351−358.
42. 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., and Housman, D. (1991) *Cell 67*, 437−447.
43. Romaniuk, P. J., Hughes, D. W., Grégoire, R. J., Neilson, T., and Bell, R. A. (1978) *J. Am. Chem. Soc. 100*, 3971−3972.
44. Freier, S. M., Alkema, D., Sinclair, A., Neilson, T., and Turner, D. H. (1985) *Biochemistry 24*, 4533−4539.
45. Zang, W. Q., Veldhoen, N., and Romaniuk, P. J. (1995) *Biochemistry 34*, 15545−15552.

BI001941R