Zinc finger proteins of the Cys_2 His_2 class represent a large group of DNA-binding proteins. A major subfamily of those proteins, the Krüppel-associated box (KRAB) domain-containing Cys_2 His_2-zinc finger proteins, have been described as potent transcriptional repressors. So far, however, no DNA-binding sites for KRAB domain-containing zinc finger proteins have been isolated. Using a polymerase chain reaction-based selection strategy with double- and single-stranded DNA, we failed to reveal a binding site for Kid-1, one member of KRAB-zinc finger proteins. Binding of Kid-1 both to single- and homoduplex double-stranded DNA was negligible. We now present evidence that Kid-1 binds to heteroduplex DNA. Similar to Kid-1, the non-KRAB-zinc finger protein WT1 also bound avidly to heteroduplex DNA (both the −KTS and +KTS splice variant of WT1), whereas the POU domain protein Oct-6, the e1s domain protein Ets-1 and the RING finger of BRCA-1 did not bind to heteroduplex DNA. Binding of WT1 to heteroduplex DNA was markedly reduced in naturally occurring mutants. The recognition of certain DNA structures by transcriptional repressor proteins may therefore represent a more common phenomenon than previously thought.

Dna-binding proteins are essential for the utilization of genetic information. They take part in the replication of the genome, in transcription of DNA into mRNA and in the repair of the genome. In these processes the interaction with DNA takes place in a variety of ways. Interaction with sequence-specific DNA is a common and well established phenomenon. There are many examples of proteins binding to a specific sequence of double-stranded DNA. In contrast, sequence-specific interaction with single-stranded DNA seems to occur more rarely. An example for such an interaction would be YB-1, a member of the Y box family of transcription factors, which binds to a region of single-stranded DNA spanning the X box of the major histocompatibility class II DNA promoter (1).

Interaction independent of a specific sequence is another possibility, but so far only a few sequence-independent, structural motifs have been described as binding sites. Poly(ADP-ribose) polymerase and DNA-dependent protein kinase are essential elements of the cellular response to DNA damage (for reviews see, Refs. 2 and 3). Whereas poly(ADP-ribose) polymerase binds to DNA containing single-strand breaks, DNP-PK binds to double-strand DNA breaks, and both do so independently of the DNA sequence. In the case of single-strand DNA breaks, the DNA molecule assumes a V-shaped conformation, which is recognized by poly(ADP-ribose) polymerase. Other examples of protein/DNA interaction based on structural characteristics of the DNA molecule can be found in the recognition of cruciform DNA by the high-mobility group protein HMG-1 (4), the tumor suppressor protein p53 (5), and the transcriptional regulator complex SWI-SNF (6).

Transcription factors as a subclass of DNA-binding proteins play an important role during growth and differentiation of cells and organs. Several families of transcription factors have been described, one of which is the family of zinc finger proteins (for recent reviews, see Refs. 7 and 8). So far, however, binding sites have been characterized for only a subset of zinc finger proteins. “Classical” zinc finger proteins like Sp1, Egr-1, and WT1 have been shown to recognize sequence-specific double-stranded DNA (e.g. Ref. 9). One large subfamily of zinc finger proteins is characterized by the presence of a highly conserved motif, the so-called KRAB-A domain or FBP (10, 11), which has been identified as a potent transcriptional repressor domain (12-15). Because the KRAB-A domain is present in approximately one-third of the large number of Cys_2 His_2-zinc finger proteins (10) (there are several hundred genes coding for those proteins in the mammalian genome (16)), it represents a very important paradigm of transcriptional repression. The identification of the KRAB-A domain as a transcriptional repressor motif was soon followed by the cloning of proteins that interact with the KRAB-A domain (17-19), but up to this point no DNA-binding site for a KRAB-zinc finger protein could be identified. Only in the case of the human KRAB-zinc finger protein ZNF74 has an affinity for RNA homopolymers been described (20).

Kid-1 is a 66-kDa protein with 13 Cys_2 His_2-zinc fingers at its COOH terminus. The zinc fingers are clustered in two groups of four and nine zinc fingers and are separated by a 32-amino acid spacer (21). The Kid-1 protein also contains a KRAB-A domain at its NH2 terminus. Here we demonstrate binding of the zinc finger region of Kid-1 to heteroduplex DNA. Another transcriptional repressor protein, the Wilms’ tumor protein WT1, also binds to heteroduplex DNA. This function is impaired in naturally occurring mutants of WT1.

MATERIALS AND METHODS

Expression and Purification of Proteins in Escherichia coli—In the case of Kid-1 and WT1, recombinant plasmids encoding fusion proteins with glutathione S-transferase were constructed using the plasmid pGEX-KG (22). Details are available from the authors upon request.
The recombiant proteins were purified from bacterial extracts according to standard protocols (23).

**Oligonucleotides**—The binding site selections were carried out using the random oligonucleotide 5'-CTT GAT CCT AAG ATT CCC TG-N$_{45}$ AGG GCC AAA GCT GAA TTA CT-3', the random oligonucleotide 5'-CTT GAT CCT AAG ATT CCC TG-N$_{59}$ AGG CTC AAA GCT GAA TTA CT-3', and the PCR primers 5'-CTT GAT CCT AAG ATT CCC TG-TGT CGA C-N$_{52}$ GTC GAC CCC TTT TAG TGA GGG TT-3' and the PCR primers 5'-CTT GAT CCT AAG ATT CCC TG-TGT CGA C-N$_{52}$ GTC GAC CCC TTT TAG TGA GGG TT-3'.

To further demonstrate the specificity of the interaction be-

cluster and a 15-bp random oligonucleotide, with the nine-zinc finger cluster and a 35-bp random oligonucleotide, and finally with all 13 zinc fingers of Kid-1 and a 75-bp random oligonucleotide did not result in the isolation of a binding site for any of the zinc finger constructs. We therefore also initiated a binding site selection with single-stranded DNA and the 13-zinc finger construct of Kid-1. Although we noticed a weak but consistent shift throughout the selection process, we were unable to define a consensus sequence. The observation of a weak shift even with nonselected single-stranded DNA prompted us to try a gel shift with heteroduplex DNA. Heteroduplex DNA occurs as a result of "bubble formation" during transcription of DNA into RNA, when the double-stranded DNA is melted so that RNA polymerase II can read the template strand, and may therefore serve as a natural substrate for transcriptional repressor proteins. A heteroduplex DNA with 20 paired nucleotides at the 5'- and 3'-end and a bubble of 25 nucleotides in the middle was designed randomly, consisting of single-stranded oligonucleotides ss1 and ss2. Care was taken that the nucleotides facing each other in the bubble consisted of pyrimidine-purine and purine-purine base pairs, to avoid non-Watson/Crick base pairs. Such a heteroduplex DNA was shifted efficiently by a fusion protein between the 13 zinc fingers of Kid-1 and glutathione S-transferase (GST), whereas either single-stranded oligonucleotide was only barely shifted (Fig. 1A). When the 13-zinc finger construct was incubated with homoduplex DNA consisting of the single-stranded oligonucleotides ss2 used in the heteroduplex DNA, and another fully complementary single-stranded oligonucleotide, no shift could be detected (Fig. 1B). A heteroduplex DNA with the same paired sequences at either end but only a bubble of 7 nucleotides in the middle could not be shifted as efficiently as the heteroduplex DNA with a bubble of 25 nucleotides (Fig. 1B). Glutathione S-transferase could not shift any of the DNA molecules just described (Fig. 1, A and B).
Kid-1 and WT1 Bind to Heteroduplex DNA

Fig. 2. A, the gel shift between radiolabeled heteroduplex DNA and the 13 zinc fingers of Kid-1 can be disrupted by an excess of unlabeled heteroduplex DNA, but not homoduplex or single-stranded DNA. Gel shift experiments were performed with radiolabeled heteroduplex DNA ss1/ss2 (25-nucleotide bubble) and the fusion protein between GST and the 13-zinc finger domain of Kid-1. When the binding between radiolabeled heteroduplex DNA and the 13 zinc fingers of Kid-1 was competed with a 5-, 25-, and 125-fold molar excess of unlabeled heteroduplex DNA (het25, lanes 4–6), homoduplex DNA (hom, lanes 7–9), and single-stranded oligonucleotide ss1 (lanes 10–12), only an excess of heteroduplex DNA had an effect. B, an intact zinc finger structure is important for the binding of Kid-1 to heteroduplex DNA with a 25-nucleotide bubble. Preincubation of the fusion protein between GST and the 13 zinc fingers of Kid-1 with a combination of 10 mM EDTA and 0.1 mM 1,10-phenanthroline resulted in the complete abrogation of the shift (lane 4). Treatment with ethanol, the solute for 1,10-phenanthroline, had no effect (lane 5). F, free DNA; B, bound DNA; ø, no protein added; G, GST; 1–13, fusion protein between GST and the 13 zinc fingers of Kid-1; C, no pretreatment; E/P, pretreatment with EDTA and 1,10-phenanthroline; EtOH, pretreatment with Ethanol.

The Transcriptional Repressor Protein WT1 Also Binds to Heteroduplex DNA, but Oct-6, Ets-1, and the RING Finger of BRCA-1 Do Not—Kid-1 belongs to the zinc finger class of transcription factors, and we therefore wanted to know whether other zinc finger proteins and members of other classes of transcription factors also bound to heteroduplex DNA. The POU domain protein Oct-6 (25) and the ets domain protein Ets-1 (26) bind to homoduplex DNA sequences of 8 nucleotides and 6 nucleotides in length, respectively. Neither protein, however, bound to heteroduplex DNA when examined in a gel shift assay (Fig. 4). The specificity of the shift by the Kid-1 zinc fingers was further demonstrated by the inability of another zinc finger structure, the RING finger domain of BRCA-1, to bind to heteroduplex DNA (data not shown).

The Wilms’ tumor protein WT1 also belongs to the family of Cys2His2-zinc finger proteins, it contains four zinc fingers at its COOH terminus. Several naturally occurring splice variants of WT1 have been described with different DNA binding specificities (for a recent review, see Reddy and Licht (27)). A binding site selection with the splice variant lacking the tripeptide KTS between the third and fourth zinc finger of WT1 resulted in the isolation of a binding site that closely resembles that of the immediate-early zinc finger protein Egr-1 (28). WT1, which does not belong to the subfamily of KRAB-zinc finger proteins, has been found to act as a transcriptional repressor in transient transfection experiments (e.g., Refs. 29–31). When a fusion protein between GST and all four zinc fingers of WT1 was incubated with the Egr-1-binding site, a strong gel shift could be detected only with the splice variant lacking the tripeptide KTS between the third and fourth zinc finger, but not with the WT1–KTS form (Fig. 4). Surprisingly, both WT1–KTS and WT1+KTS bound strongly to heteroduplex DNA consisting of single-stranded oligonucleotides ss1 and ss2 (Fig. 4). Similar to Kid-1, WT1–KTS and WT1+KTS also bound strongly to the second large heteroduplex consisting of oligonucleotides ss3 and ss4 (data not shown). The specificity of those gel shifts was demonstrated by competition experiments. In the case of the WT1–KTS splice variant, successful competition was carried out with an excess of both unlabeled heteroduplex DNA and Egr-1-binding site (Fig. 5). Without competition, the gel shift of the heteroduplex DNA by WT1+KTS could only be competed by an excess of unlabeled heteroduplex DNA, but not Egr-1-binding site (Fig. 5C). Binding of WT1–KTS and WT1+KTS to heteroduplex DNA consisting of one of the oligonucleotides used in the heteroduplex DNA and a fully complementary oligonucleotide was very weak (data not shown).
The ets domain protein Ets-1 and the POU domain protein Oct-6 do not bind to heteroduplex DNA, but WT1 does. Heteroduplex DNA ss1/ss2 with a 25-nucleotide bubble (het25) was incubated with GST, with the 13 zinc fingers of Kid-1, with the ets domain proteins Ets-1, with the POU domain protein Oct-6, and with WT1–KTS and WT1–KTS. The 13 zinc fingers of Kid-1 only bind to the heteroduplex DNA but not to any of the other binding sites (lanes 9–12). Ets-1 (lanes 13 and 14) and Oct-6 (lanes 15 and 16) only bind to their respective binding site but not to heteroduplex DNA. WT1–KTS binds to both the Egr-1/WT1-binding site and heteroduplex DNA (lanes 17 and 18), whereas WT1+KTS binds to heteroduplex DNA only (lanes 19 and 20). F, free DNA; B, bound DNA; ø, no protein added; G, GST, 1–13, fusion protein between GST and the 13 zinc fingers of Kid-1; Ets-1, chicken Ets-1 protein; Oct-6, murine Oct-6 protein; –, KTS, fusion protein between GST and the four zinc fingers of WT1 without the KTS tripeptide; + KTS, fusion protein between GST and the four zinc fingers of WT1 with the KTS tripeptide; het25, heteroduplex oligonucleotide with a 25-nucleotide bubble; O_{Egr}, oligonucleotide with an Egr-binding site; O_{Oct}, oligonucleotide with octamer motif; O_{E/O/W}, oligonucleotide with an Egr-lWT1 binding site.

Kid-1 and WT1 Bind to Heteroduplex DNA

To define the structural requirements for the binding of WT1 to heteroduplex DNA, we made use of naturally occurring WT1 mutants. Both a WT1+KTS mutant harboring a deletion of zinc finger 3 (32) as well as a mutant protein with a missense mutation in zinc finger 3 found in a patient with Denys-Drash syndrome (R394W) showed decreased binding to heteroduplex DNA (Fig. 5D). This suggests that zinc finger 3 is involved in this interaction.

The results described above demonstrate for the first time the binding of a KRAB-zinc finger protein to DNA. Surprisingly, it is DNA structure that is recognized by Kid-1, because two unrelated heteroduplex DNAs with bubbles of 25 nucleotides are shifted with the same efficiency. Together with the finding that another transcriptional repressor protein, WT1, also binds to heteroduplex DNA without obvious sequence specificity, this represents an important new paradigm. Although this particular DNA structure, heteroduplex DNA, resembles the “transcription bubble,” which can be found when RNA polymerase II is transcribing a gene, it appears unlikely that the transcription bubble serves as the natural recognition site. Kid-1 bound only inefficiently to heteroduplex DNA with a 7-nucleotide bubble, a size that approximates the region of the transcription bubble not covered by RNA polymerase II. Further experiments are needed to clarify where those recognition sites are located in the nucleus and how they are recognized in addition to their structure.

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