Sin1p/Spt2p is a yeast chromatin protein that, when mutated or deleted, alters the transcription of a family of genes presumably by modulating local chromatin structure. In this study, we investigated the ability of different domains of this protein to bind four-way junction DNA (4WJDNA) since 4WJDNA can serve as a model for bent double helical DNA and for the crossed structure formed at the exit and entry of DNA to the nucleosomes. Sequence alignment of Sin1p/Spt2p homologues from 11 different yeast species showed conservation of several domains. We found that three domains of Sin1p/Spt2p fused to glutathione S-transferase can each bind independently in a structure-specific manner to 4WJDNA as measured in a gel mobility shift assay. A feature common to these domains is a cluster of positively charged amino acids. Modification of this cluster resulted in either abolishment of binding or a change in the binding properties. One of the domains tested clearly bound superhelical DNA, although it failed to induce bending in a circularization assay. Poly-L-lysine, which may be viewed as a cluster of positively charged amino acids, bound 4WJDNA as well. Phenotypic analysis showed that disruption of any of these domains resulted in suppression of a his4-912 allele, indicating that each domain has functional significance. We propose that Sin1p/Spt2p is likely to modulate local chromatin structure by binding two strands of double-stranded DNA at their crossover point.

Sin1p/Spt2p is a yeast chromatin non-histone protein. While the precise function of the protein is still unknown, it is known to function as a negative transcriptional regulator of a number of genes including SUC2 (1), INO1 (2), and SSA3 (3). Activity of the HO promoter, as measured from an HO promoter driving a lacZ gene (4), is also regulated by SIN1/SPT2. swi1, swi2, and swi3 mutants are unable to transcribe RNA from the HO promoter. However, in sin1/swi double mutants, transcription is restored. Mutants in SPT2 were first identified as suppressors of ty and δ insertions in the 5’ non-coding region of the HIS4 gene (5). As the negative regulation of these genes is overcome by the SWI/SNF chromatin remodeling complex, it was suggested that a function of SIN1p/SPT2p is to somehow maintain chromatin compaction at specific locations in the chromatin.

While Sin1p/Spt2p does not bind DNA in a sequence-specific way, it has been shown to bind double-stranded DNA (6). It is estimated that there are about 1200 (7) to 2000 (8) copies of the protein molecule per cell, indicating that it probably binds the chromatin at multiple locations. It was shown that in vitro the N terminus of Sin1p/Spt2p protein interacts with the tetrapeptide repeat domains of Cdc23p, a protein involved in chromosome segregation, whereas the C terminus of Sin1p/Spt2p binds proteins involved in transcriptional regulation (9, 10).

Sequence analysis shows that Sin1p/Spt2p contains two domains that have homology to HMG1 in higher eukaryotes as well as an acidic domain and a polar helical C-tail (6, 8) (see Fig. 1). It was shown that one of these domains (amino acids 100–162) was able to bind double-stranded DNA without substantial sequence specificity (6, 11). Some key features of HMG boxes, however, were not present in this sequence, and its similarity to HMG was disputed (12).

A detailed functional mapping of the molecule in the context of the SPT phenotype (5) was performed (8), identifying two functional regions. Amino acids 117–179 (overlapping the DNA-binding domain described above) contained a “dominance domain” that was required to be left intact in dominant spt2 mutants. In addition, deletion of the highly basic C terminus of Spt2p (amino acids 325–333) or a lessening of its charge resulted in the dominant suppressor phenotype.

A variety of proteins extending from prokaryotes to humans, including those containing HMG domains, can bind four-way junction DNA (4WJDNA) in a structure-specific way (13). In humans, for example, a cruciform-binding protein from the 14–3–3 family has been found (14, 15) as have its yeast counterparts Bmh1p and Bmh2p (16). These proteins have been implicated in initiation of replication (17). RAD51B, another human protein, binds 4WJDNA and has a function in homologous recombination repair (18). In other contexts, it is believed that the ability of proteins to bind 4WJDNA often reflects in vivo protein binding to bent DNA or to crossing strands of DNA (13).

It has been reported that in the absence of Mg2+ ions, 4WJDNA takes on an extended “open” conformation, while Mg2+ ions permit a more closed conformation, adopting more the shape of an X as the DNA undergoes partial folding by means of pairwise stacking of the double helical arms (19–23). Pohler et al. (24) carefully studied the interaction between HMG boxes and 4WJDNA. They found that a single HMG domain binds 4WJDNA exclusively in its extended open square conformation, which is predominant in solution lacking Mg2+ ions.

In this study, we investigated the nature of interactions between segments of Sin1p/SPT2p expressed as glutathione S-transferase (GST) fusions and 4WJDNA with the hope that these data will improve our understanding of how Sin1p/Spt2p binds chromatin in vivo.

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We found that a domain encompassing amino acids 100–162, previously suggested to have HMG-like structure, did not bind 4WJDNA in either its extended square conformation or in its more closed X conformation, while another HMG-like domain (amino acids 1–96) was able to bind both four-way junction DNA conformations. Surprisingly an acidic domain (amino acids 224–304) was found to bind 4WJDNA only in its extended conformation in a structure-specific manner as do genuine HMG boxes. Additionally the polar C-tail domain (amino acids 303–333) was found to bind 4WJDNA in both conformations. Substitution of several of the positively charged amino acids with alanine led to the disappearance of the binding. As each of these domains that bind 4WJDNA contains a cluster of positively charged amino acids, we propose that this cluster may be involved in the binding of the peptides to the 4WJDNA. Protein binding to 4WJDNA often implicates the ability of the protein to bind a crossed structure of DNA in vivo such as at the entrance and exit of DNA from the nucleosome. Our data suggest that several domains of Sin1p/Spt2p secure crossing DNA in the chromatin. This binding could result in increased chromatin compaction or a localized change in the ability of particular DNA sequences to bind regulatory proteins.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Bacterial Strains**—The expression vectors used are derivatives of pGEX-3X (Amersham Biosciences) that have portions of derivatives of pGEX-3X (Amersham Biosciences) that have portions of the 4WJDNA. These plasmids were generated by substituting an appropriate DNA fragment into pGEX-3X between the HindIII and XhoI sites using the HindIII and XhoI sites of the 4WJDNA as cloning sites.

**PCR Mutagenesis of the Acidic Domain of SIN1**—Mutagenesis of W267R mutation. PCR of sections 2 of 4 yielded the complete acidic domain with the K275A mutation. The PCR was carried out for 16 cycles of 95 °C for 1 min (denaturation), 43 °C for 45 s (annealing), and 72 °C for 1 min (extension). Next a PCR mixture containing primers 27209 and 26238, dNTPs, Taq polymerase buffer, and Taq polymerase was added to each tube, and 45 cycles of PCR were performed. This resulted in amplification of the entire acidic domain of SIN1/SPT2. Following amplification, the DNA fragment was digested with BamHI and EcoRI and ligated into similarly digested pGEX-3X as described above.

Delete of the acidic domain for functional assays was performed similarly using pLL10 (8) as template and with the first PCR using primers ADLEFT and ADLEFTMID and the second reaction using primers ADRIGHTMID and ADRIGHT. Following the amplifications, the PCR fragment was digested with HindIII and ligated into similarly digested pLL10. The precise deletion was confirmed by DNA sequencing. Mutations W267R and K275A were introduced into the acidic domain using the same PCR mutagenesis technique and primers 1517994 and 1517992.

**Gel Mobility Shift Assay—4WJDNA, 3WJDNA, and duplex oligonucleotides (both having the same sequence as the arms of the 4WJDNA) were made for experiments as described previously (27).** One of the oligonucleotides was end radiolabeled using [γ-32P]ATP. The formation of the 4WJDNA was verified by digestion with AluI and EcoRI, each of which have digestion sites in an arm of the 4WJDNA (data not shown). Binding reactions were performed in 20 μl containing 10 fmol of 32P-labeled 4WJDNA, 10 mM Tris-HCl, pH 7.5, 25 mM NaCl, 10 mM MgCl2, 5 mM MgCl2, and 0.2 mg/ml herring sperm DNA. Aliquots of the recombinant protein were incubated with 4WJDNA for 45 min at room temperature. Reactions not containing Mg2+ ions were made isonic with the above reaction mixture by substitution of an appropriate amount of Na+ ions. Reaction mixtures were electrophoresed on a 6.5% polyacrylamide gel at 5 V/cm for 3–3.5 h in Tris borate/EDTA buffer adjusted to pH 7.3. The gel was dried and autoradiographed. Prior to applying the samples to the gel, it was extensively prerun. Each experiment was repeated two to three times with recombinant protein samples from independently prepared batches.

**Protein Binding to Superoiled DNA—Increasing concentrations of peptide were mixed with plasmid pBR322 (a mixture of supercoiled, nicked, and linearized (with HindIII) DNA) in 15 μl of 50 mM Tris-Cl, pH 7.5, 20 mM NaCl, 1 mM EDTA, 0.1% Triton X-100 for 40 min at room temperature as described previously (28).** The reaction mixtures were then electrophoresed through 2% agarose in TAE buffer (40 mM Tris acetate, pH 7.5, 1 mM EDTA) at 8 V/cm. Following the electrophoresis the gel was stained with ethidium bromide and photographed.

**Functional Analysis of Sin1p/Spt2p Acidic Domain (Amino Acids 224–304) in Holoprotein—The Lefebvre and Smith (8) assay system was used to monitor the ability of Sin1p/Spt2p to suppress the his4-9123 mutation containing SIN1/SPT2 wild type, sin1Δspt2Δ with a deleted acidic domain (sin1Δ/spt2Δ 224–304), and sin1Δspt2Δ mutated in both W267R and K275A.**

**RESULTS**

Recently, as a result of sequencing of multiple fungal genomes, it has become possible to align putative amino acid sequences of homologous genes and to deduce from the alignment evolutionarily conserved regions of the gene that are presumably important for the function of the protein (29, 30).
We have, therefore, aligned *Saccharomyces cerevisiae* Sin1p/Spt2p homologues from *Saccharomyces mikatae*, *Saccharomyces kudriavzevii*, *Saccharomyces bayanus*, and *Saccharomyces castellii* (30); *Saccharomyces paradoxus* and *S. mikatae* (29); *Candida glabrata*, *Yarrowia lipolytica*, *Kluyveromyces lactis*, and *Debaryomyces hansenii* (52); and *Candida albicans* (Stanford Genome Technology Center) using the PILEUP algorithm included in the Genetics Computer Group (GCG) package of programs.

As can be seen in Fig. 1, Sin1p/Spt2p is conserved across the species, although the conservation is not uniform throughout the molecule. About 20 amino acids centered at about amino acid 60 and another 20 amino acids centered at about amino acid 145 are conserved. Each of these sequences is centered around the hypothetical HMG domains as suggested by LeFebvre and Smith (8).

Particularly conserved is the C-terminal quarter of the molecule that includes the acidic domain and the highly basic C terminus. These data suggest that these domains play an important role in the function of the molecule.

**Interaction between the “HMG-like Boxes” in Sin1p/Spt2p and Four-way Junction DNA**—As mentioned above, sequence analysis indicated that Sin1p/Spt2p contains two regions showing sequence similarity with HMG boxes (1, 2): box 1 (amino acids 26–88) and box 2 (amino acids 98–159) (Fig. 1) (8).

As binding to 4WJDNA is a characteristic of HMG box domains, we asked whether these domains could bind four-way junction DNA *in vitro*. For this purpose, we used two GST-Sin1p fusion proteins containing amino acids 1–96 and 100–162, engineered as described previously (10, 25, 26), each with a GST tag at the N terminus. Recombinant peptides are shown in Fig. 2. 4WJDNA was radiolabeled and incubated with increasing concentrations of GST-Sin1p (amino acids 1–96) and GST-Sin1p (amino acids 100–162) as described under “Experimental Procedures” and in Fig. 3. Binding reactions were performed both in the presence of 10 mM Mg$^{2+}$ ions and in its absence. As illustrated in Fig. 3a, the 4WJDNA can take on either an X conformation or a more open crosslike conformation dependent on the presence or absence of Mg$^{2+}$ ions (24).

As seen in Fig. 3b, amino acids 1–96 of GST-Sin1p containing putative HMG box 1 were able to bind 4WJDNA both in the X conformation and in the open form. The X conformation of 4WJDNA was retarded only slightly by this peptide even at relatively high concentrations (32 $\mu$M) of peptide. In contrast, the 4WJDNA was increasingly retarded as higher concentrations of protein were added to the reaction in the absence of...
Mg\(^{2+}\) ions, much like the binding of the globular domain of histone H5 to 4WJDNA (31). At the higher concentrations, the band on the gel became indiscrete, suggesting that the DNA–protein complex was an aggregate without defined structure.

A GST-Sin1p peptide (amino acids 100–162) containing HMG box 2 was unable to bind 4WJDNA under any conditions tested (Fig. 3c). At concentrations above 12 \(\mu\)M protein, a portion of the 4WJDNA does not enter the gel regardless of the presence of Mg\(^{2+}\) ions.

\(\text{Mg}^{2+}\) ions, much like the binding of the globular domain of histone H5 to 4WJDNA (31). At the higher concentrations, the band on the gel became indiscrete, suggesting that the DNA–protein complex was an aggregate without defined structure.

A GST-Sin1p peptide (amino acids 100–162) containing HMG box 2 was unable to bind 4WJDNA under any conditions tested (Fig. 3c). At concentrations above 12 \(\mu\)M protein, the peptide appeared to form an insoluble DNA-protein complex that did not enter the polyacrylamide gel. This was true for 4WJDNA either in its open or in its X conformation. GST alone was unable to bind 4WJDNA with or without Mg\(^{2+}\) ions at any concentration of protein tested (data not shown).

An Acidic Domain, GST-Sin1p-(224–304), Binds 4WJDNA in the Open Conformation—As shown in Fig. 1, two additional domains were distinguished by sequence analysis. One, amino acids 224–304, is particularly acidic. We investigated the ability of this domain to bind 4WJDNA in an experiment similar to that described above. Surprisingly, despite the strong acidic nature of this domain, it bound 4WJDNA quite efficiently as can be seen in Fig. 4a.

A complex was first formed at about 3 \(\mu\)M protein, and then, starting at 9 \(\mu\)M, a second slower migrating complex was observed. Concentrations of this peptide needed for binding were about the same as published values for the HMG1 B-domain when tagged with maltose-binding protein (24). An apparent \(K_D\) of 19 \(\mu\)M was measured where \(K_D\) approximates the protein

\(\text{FIG. 2.}\) Recombinant GST-Sin1p fragments separated by 10% SDS-PAGE. White asterisks indicate full-length peptide. Lane 1, GST alone; lane 2, GST-Sin1p(1–96); lane 3, GST-Sin1p(100–165); lane M, marker (sizes in kDa); lane 4, W267A mutant of GST-Sin1p-(224–304); lane 5, wild type GST-Sin1p-(224–304); lane 6, K275A,W267A mutant of GST-Sin1p-(224–304); lane 7, K325A mutant of GST-Sin1p-(303–333); lane 8, wild type GST-Sin1p-(303–333); lane 9, R326A mutant of GST-Sin1p-(303–333); lane 10, K325A,R326A,R327A,R328A mutant of GST-Sin1p-(303–333).

\(\text{FIG. 3.}\) Gel mobility shift assay of Sin1p/Spt2p HMG boxes binding to 4WJDNA. a, 4WJDNA structure is dependent on the presence of Mg\(^{2+}\) ions. b, recombinant GST-Sin1p-(1–96) was added to radiolabeled 4WJDNA in increasing concentrations of protein in the presence and the absence of Mg\(^{2+}\) ions. c, same as b except that recombinant GST-Sin1p-(100–162) was used. At concentrations higher than 12 \(\mu\)M protein, a portion of the 4WJDNA does not enter the gel regardless of the presence of Mg\(^{2+}\).

\(\text{FIG. 4.}\) Gel mobility shift assay of GST-Sin1p-(224–304) binding to 4WJDNA. a, recombinant GST-Sin1p-(224–304) was added to radiolabeled 4WJDNA in increasing concentrations of peptide in the presence or absence of Mg\(^{2+}\). b, same as a except that the recombinant protein was a W267R mutant. c, same as a except that the recombinant protein was a W267A mutant. d, same as b except that the recombinant protein included an additional K275A mutation. e, titration of GST-Sin1p-(224–304)-4WJDNA complex with unlabeled 4WJDNA. GST-Sin1p-(224–304) (21 \(\mu\)M final concentration) at a constant protein concentration was added to 0.5 nM labeled 4WJDNA. In the left lane SDS was added to 0.1% following incubation with protein. The second lane is without added protein, and the following lanes include increasing amounts of unlabeled 4WJDNA in the absence of Mg\(^{2+}\).
disruption of an It was shown that a W45R mutation in HMG1 A domain might have a rigid structure as well. might affect its binding to 4WJDNA. an analogous mutation in GST-Sin1p-(224–304), W267R, however, and in contrast to the wild type peptide, it was also ability to bind 4WJDNA in the open conformation. In addition, was explained by the formation of a rigid structure composed of two α-helical arms that specifically bind the 4WJDNA in its open conformation. The angle of about 80° between the two helical arms of HMG1 domain B is defined by a number of conserved, predominantly aromatic residues (Phe14, Phe17, Trp45, Lys53, and Tyr56) (33). While the amino acid composition of Sin1p-(224–304) is substantially different from HMG domain B of HMG1, careful examination of the Sin1p-(224–304) sequence shows that residues (Phe240, Trp267, Lys275, and Tyr279) are nearly in the same conserved positions (Fig. 1, marked with stars) as in HMG1 domain B, suggesting that the Sin1p-(224–304) peptide might have a rigid structure as well. It was shown that a W45R mutation in HMG1 A domain reduced the ability of this domain to bind 4WJDNA because of disruption of an α-helix (34, 35). We, therefore, first asked how an analogous mutation in GST-Sin1p-(224–304), W267R, might affect its binding to 4WJDNA.

As can be seen in Fig. 4b, this mutant peptide retained its ability to bind 4WJDNA in the open conformation. In addition, however, and in contrast to the wild type peptide, it was also able to bind 4WJDNA in the presence of Mg2+ ions in which the X form of the 4WJDNA predominates.

We next asked how absence of charge at this position in a W267A mutation would affect 4WJDNA binding. As can be seen in Fig. 4c, the results were very similar to those for W267R with both the open and X conformations of the 4WJDNA being bound by the peptide. When we tested a mutant peptide having a double replacement in this assay, W267R,K275A, similar results were obtained both with and without Mg2+ ions (Fig. 4d).

For each of the GST-Sin1p-(224–304) constructs, increasing concentrations of peptide resulted in the appearance of two retarded bands with the more rapidly migrating band decreasing in intensity as the slower migrating band increased in intensity. These data likely indicate that two molecules of the GST-Sin1p-(224–304) peptide were able to bind a single 4WJDNA at the higher protein concentrations. The open conformation 4WJDNA was first bound by the peptide at about 3 μM for wild type, at 10 μM for the W267R mutant, at 9 μM for the W267A mutant, and at 10 μM for the W267R,K275A double mutant. $K_D$ values of 19, 28, 23, and 36 μM, respectively, were noted for each of the peptides. Thus, it appears that the binding of the peptide to 4WJDNA is weakened in each of the mutant peptides although not to a very large extent.

To better investigate the stoichiometry of binding, we performed the gel mobility shift assay using a constant concentration of protein (21 μM) and increased the concentration of unlabeled 4WJDNA from 0 to 485 nM 4WJDNA. When SDS to 0.1% was added to the reaction following binding, the complex completely dissociated, indicating that the retardation in the gel is due to a complex between the protein and the 4WJDNA. Addition of increasing amounts of unlabeled 4WJDNA to the reaction seen in Fig. 4c resulted in a shift of the protein complex from the least mobile band to the most mobile band as expected.

The Acidic Domain GST-Sin1p-(224–304) Binds Supercoiled DNA—4WJDNA can serve as a model for DNA as it enters and exits the nucleosome where two DNA helices pass one over the other. In supercoiled DNA, DNA helices necessarily pass one over the other. Thus supercoiled DNA has been used to assess the ability of linker histone to bind two intersecting strands of DNA (36–38). We, therefore, decided to test whether supercoiled DNA would specifically bind GST-Sin1p-(224–304) and would compete with its binding to 4WJDNA. We repeated the gel mobility shift assay in the presence of increasing concentrations of supercoiled plasmid pBR322. As can be seen in Fig. 5a, at about 50-fold molar excess of plasmid to 4WJDNA, the supercoiled plasmid was able to begin to compete with the 4WJDNA, reducing the proportion of 4WJDNA molecules bound to the protein. In another experiment (Fig. 5b), a mixture of supercoiled, nicked, and linear plasmid pBR322 DNA was mixed with recombinant peptide and electrophoresed through an agarose gel in TAE buffer. At about 500-fold peptide:supercoiled DNA (molar ratios), supercoiled DNA began to be retarded, while the nicked and linear DNA was unaffected (Fig. 5). GST alone did not affect the mobility of any of the species of DNA. These data suggest that the acidic domain of Sin1p/Spt2p is able to bind two strands of DNA where they cross although not at high affinity.

**The C-Terminal Domain GST-Sin1p-(303–333) Is Also Able to Bind 4WJDNA—**The C-terminal domain, amino acids 303–333, is known to contain functional activity since a change in amino acid 314 from glutamic acid to lysine eliminates sin1 suppression of swi1 mutants (4, 9). Furthermore a series of mutations in this domain caused phenotypic changes in the SPT phenotype (8). Comparison of the Sin1p/Spt2p homologues (Fig. 1) shows that this domain is particularly well conserved, and that, where amino acid replacements took place, they were almost all conservative. We found that a GST fusion to this peptide also had the ability to bind 4WJDNA. Binding to the X conformation of 4WJDNA was first detected at 1.5 μM protein concentration at which there is half-maximal shift of probe (32).

Interestingly GST-Sin1p-(224–304) was unable to bind 4WJDNA in its X conformation even at the highest concentration of protein tried (34 μM). These results were reminiscent of published results with HMG1 domain B (24). The differential binding of HMG1 domain B to the open versus X conformation was explained by the formation of a rigid structure composed of two α-helical arms that specifically bind the 4WJDNA in its open conformation. The angle of about 80° between the two helical arms of HMG1 domain B is defined by a number of conserved, predominantly aromatic residues (Phe14, Phe17, Trp267, Lys275, and Tyr279) (33). While the amino acid composition of Sin1p-(224–304) is substantially different from HMG domain B of HMG1, careful examination of the Sin1p-(224–304) sequence shows that residues (Phe240, Trp267, Lys275, and Tyr279) are nearly in the same conserved positions (Fig. 1, marked with stars) as in HMG1 domain B, suggesting that the Sin1p-(224–304) peptide might have a rigid structure as well. It was shown that a W45R mutation in HMG1 A domain reduced the ability of this domain to bind 4WJDNA because of disruption of an α-helix (34, 35). We, therefore, first asked how an analogous mutation in GST-Sin1p-(224–304), W267R, might affect its binding to 4WJDNA.

As can be seen in Fig. 4b, this mutant peptide retained its ability to bind 4WJDNA in the open conformation. In addition, however, and in contrast to the wild type peptide, it was also able to bind 4WJDNA in the presence of Mg2+ ions in which the X form of the 4WJDNA predominates.

We next asked how absence of charge at this position in a W267A mutation would affect 4WJDNA binding. As can be seen in Fig. 4c, the results were very similar to those for W267R with both the open and X conformations of the 4WJDNA being bound by the peptide. When we tested a mutant peptide having a double replacement in this assay, W267R,K275A, similar results were obtained both with and without Mg2+ ions (Fig. 4d).

For each of the GST-Sin1p-(224–304) constructs, increasing concentrations of peptide resulted in the appearance of two retarded bands with the more rapidly migrating band decreasing in intensity as the slower migrating band increased in intensity. These data likely indicate that two molecules of the GST-Sin1p-(224–304) peptide were able to bind a single 4WJDNA at the higher protein concentrations. The open conformation 4WJDNA was first bound by the peptide at about 3 μM for wild type, at 10 μM for the W267R mutant, at 9 μM for the W267A mutant, and at 10 μM for the W267R,K275A double mutant. $K_D$ values of 19, 28, 23, and 36 μM, respectively, were noted for each of the peptides. Thus, it appears that the binding of the peptide to 4WJDNA is weakened in each of the mutant peptides although not to a very large extent.

To better investigate the stoichiometry of binding, we performed the gel mobility shift assay using a constant concentration of protein (21 μM) and increased the concentration of unlabeled 4WJDNA from 0 to 485 nM 4WJDNA. When SDS to 0.1% was added to the reaction following binding, the complex completely dissociated, indicating that the retardation in the gel is due to a complex between the protein and the 4WJDNA. Addition of increasing amounts of unlabeled 4WJDNA to the reaction seen in Fig. 4c resulted in a shift of the protein complex from the least mobile band to the most mobile band as expected.

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Higher concentrations of protein in the gel mobility shift assay resulted in replacement of one retarded band by another having lower mobility. At concentrations as high as 150 μM, no protein aggregation was observed. Gel mobility shift assays with the 4WJDNA in the open square conformation (without Mg²⁺) showed a similar pattern (Fig. 6a), but insoluble aggregates began to be observed at protein concentrations above 11 μM (data not shown).

Examination of the polar C-tail of the Sin1p/Spt2p sequence showed that it contains a cluster of six positively charged amino acids (KRRRKK). As the center of the 4WJDNA contains a very high density of negative charge (31), we thought it likely that this cluster participates in the binding of this peptide to 4WJDNA. In addition, as can be seen in Fig. 1, the positive charge at these positions (marked with stars) is almost completely retained in the amino acid replacements that took place during evolution. We, therefore, substituted these positively charged amino acids (K325,R326,R327,A328) with alanine. These substitutions led to the disappearance of binding (Fig. 6b) in the 4WJDNA binding assay. Substitution of just Lys325 or Arg326 with alanine were each sufficient to abolish binding to 4WJDNA.

Binding of Domains GST-Sin1p-(224–304) and GST-Sin1p-(303–333) with Four-way Junction DNA Is Not Competed by Double-stranded DNA—In each of the gel mobility shift assays with 4WJDNA described above, a large excess of herring sperm DNA (0.2 mg/ml) was included in the reaction. In control experiments, a large excess of double-stranded DNA having the same sequence as two arms of the 4WJDNA had no effect on the mobility of 4WJDNA complex with any of the peptides. This double-stranded DNA was generated by hybridizing oligonucleotides 1 and 6 (27). In experiments shown in Fig. 7, a and c, GST-Sin1p-(224–304) and GST-Sin1p-(303–333) were challenged with up to a 1000-fold excess of the duplex oligonucleotide, which was first incubated with protein before the addition of labeled 4WJDNA. As can be seen in the figure, this preincubation did not change the binding of the protein to the 4WJDNA. When labeled 4WJDNA was added first and competing duplex DNA was added second, the same results were obtained (not shown). As duplex DNA of the same sequence did not compete for the 4WJDNA, the binding of these peptides to 4WJDNA is structure- and not sequence-specific. Similar results were obtained for 4WJDNA interactions with GST-Sin1p-(1–96) and with a duplex oligonucleotide generated from oligonucleotides 3 and 5 (data not shown).

In another experiment, we bound GST-Sin1p-(224–304) to labeled 4WJDNA and titrated the binding with unlabeled 3WJDNA (triads) (Fig. 7b). These results also suggest a structure specific interaction between the peptide and the DNA.
Poly-l-lysine Binding to 4WJDNA—Finally we asked whether a large cluster of positively charged amino acids in the form of poly-l-lysine could bind 4WJDNA and if so how it would affect the mobility of the DNA. For this purpose, poly-l-lysine of molecular mass 9600 daltons was added to radiolabeled 4WJDNA in increasing concentrations of poly-l-lysine as was done for the Sin1p/Spt2p peptides. Our results in Fig. 8 show that a single complex was formed at the lowest concentration of poly-l-lysine (0.15 μM). This complex migrated a bit more rapidly than the free 4WJDNA. The mobility of the complex was unaffected by the presence of Mg2+ or by an excess (3000-fold) of duplex oligonucleotide having the same sequence as two arms of the 4WJDNA (not shown).

Functional Analysis of the Acidic Domain (Amino Acids 224–304)—Previous investigators have done a functional mapping of Sin1p/Spt2p (8). Their work showed that mutations or deletions of the C terminus (amino acids 325–333) resulted in a dominant suppression of the his4-912 allele and that expression of this phenotype required the presence of amino acids 117–179. We used their experimental system to ask whether we could identify a functional role for the acidic domain of Sin1p/Spt2p in the context of the holoprotein. To that end we constructed a yeast plasmid expressing Sin1p/Spt2p lacking the acidic domain, transformed yeast carrying the his4-912 allele and either the SLL5 (Sin1p/Spt2Δ) or the SLL7 (Sin1p/Spt2Δ::URA3) gene, and tested them for growth on a plate lacking histidine. In this assay, only cells in which the δ insertion is suppressed grow on His+ plates. Our results (Fig. 9) clearly showed that both SLL5 and SLL7 transformants expressing Sin1p/Spt2Δ lacking the acidic domain grew on the His+ plates but that SLL5 transformants grew on these plates. These results indicate that the acidic domain is required for normal function of Sin1p/Spt2p and that its absence causes functional dominance over the wild type.

SLL7 transformed with a mutant plasmid encoding Sin1p/Spt2p carrying point mutations W267R and K275A grew well on His+ plates, while SLL5 transformed with this plasmid did not grow on those plates (Fig. 9). These data indicate that mutations in these two amino acids are sufficient to cause a non-dominant spt2 suppressor phenotype.

**DISCUSSION**

Sin1p/Spt2p is a chromatin protein whose activity may be dependent on its ability to modulate higher order chromatin structure. To investigate the nature of the interaction of Sin1p/Spt2p with DNA, we asked whether this protein could bind 4WJDNA and superhelical DNA in vitro. Proteins able to bind 4WJDNA in vitro have been shown to have the ability to bind bent DNA and/or to preferentially interact with DNA supercoils (13). They appear to primarily be architectural proteins, although some have very specific functional roles as well. In this study, we found that several domains of Sin1p/Spt2p are able to bind 4WJDNA in vitro, suggesting that Sin1p/Spt2p could be involved in maintenance of compacted DNA structure.

The Sin1p/Spt2p molecule was divided into four domains based on primary sequence analysis and conservation of these domains across evolutionary time (Fig. 1). Two domains (amino acids 28–88 and 98–159, respectively) were suggested to be homologous to HMG1 (6, 8). We produced two GST fusion peptides composed of amino acids 1–96 and 100–162, respectively, based on these suggestions. Our experiments showed that the 1–96 peptide could bind 4WJDNA in both the X and open conformations, while the 100–162 peptide could not bind 4WJDNA under any conditions. It is interesting to note that Landsman (12) contended that the second is unlikely to be a genuine HMG domain. These experiments do not preclude the binding of this domain to DNA in vivo where it could be stabilized by other domains of the protein and by other factors in the nucleus.

Residues 224–304 of Sin1p/Spt2p are particularly rich in acidic amino acids. The isoelectric point of this domain was calculated to be 3.78. Notwithstanding this fact, this domain bound 4WJDNA avidly in a structure-specific manner but only in the extended, open conformation of the DNA. As we increased the concentration of protein in the assay, discrete bands were seen with the faster migrating band first increasing in intensity and then decreasing in intensity as a second, slower migrating band became more intense. These data suggested that two molecules of the acidic domain might bind a single 4WJDNA molecule at the higher concentrations of protein. This conclusion is supported by the data in Fig. 4e that show that when the concentration of peptide is held constant and the amount of 4WJDNA is increased, the binding is shifted from the slower to the faster migrating band. The C-terminal 31 amino acids are substantially (50%) basic, and they too bind 4WJDNA in both the open and X conformations as did the 1–96 peptide.

Each of the three domains that bind 4WJDNA contain clusters of positively charged amino acids (Fig. 1). We suggest that these clusters of positive charge may offset the high density of phosphate charge at the center of 4WJDNA (31), initiating an interaction with the 4WJDNA, which may then be followed by other more specific protein-DNA interactions. Basic amino acids in histone H1 and HMG-I(Y) and HMG1 interact with phosphate groups at the center of 4WJDNA (24, 39, 40).

The binding of GST-Sin1p(224–304) with 4WJDNA was reminiscent of gel mobility shift assays done with domain B of
HMG1 (24, 33) as both proteins bound only the open, extended conformation of 4WJDNA. This Sin1p/Spt2p peptide has a cluster of positively charged amino acids (RGKRR). Alignment of peptides containing HMG-boxes (33) shows conservation of lysine and arginine residues in the region that is implicated in 4WJDNA binding. This region is conserved in the Sin1p/Spt2p homologues as well (Fig. 1).

In the case of HMG1, the protein structure was determined using NMR spectroscopy (33). Mutational analysis showed that particular amino acids are responsible for maintaining two α-helices in the protein at an angle of about 80° (35). These amino acids in HMG1 (Phe17, Trp45, Lys53, and Tyr56) have possible analogues in Sin1p/Spt2p (Phe240, Trp267, Lys377, and Tyr379) that we speculated could be important in determining the structure of Sin1p/Spt2p. We found that substitution of either tryptophan with arginine at position 267 or lysine with alanine at position 275 resulted in the ability of the peptide to bind the X conformation of 4WJDNA. This suggests that the mutations may have disrupted a rigid structure in the Sin1p/Spt2p peptide, allowing the more flexible peptide to bind to the X conformation of the 4WJDNA. The rigidity of the native protein could play a role in its binding to DNA in the chromatin.

As mentioned above, we noticed that all the domains that bound the 4WJDNA contained significant clusters of positively charged amino acids. Examination of the amino acid sequences of other proteins known to bind 4WJDNA supports the notion that the cluster is important for DNA binding. For example, there is a cluster of positively charged amino acids in the C terminus of endonuclease 1 of bacteriophage T7 (RLRKKGK) (41) and a cluster in the center of the second zinc-binding domain of human papillomavirus oncoprotein E6 (KKQR) (42), each of which, when mutated, abolishes the binding of the protein to 4WJDNA. RusA, an E. coli Holliday junction nuclease, contains a cluster of arginines from amino acids 66 to 69 followed by an aspartic acid. This protein binds 4WJDNA, but when the aspartic acid is replaced by an asparagine, binding to the 4WJDNA becomes more stable (43). Mammalian HMG1 box A contains the sequence KKPRGKM. A single point mutation replacing the arginine with glycine reduces the affinity of this protein for 4WJDNA at least 10-fold (34).

To examine whether the cluster of positively charged amino acids is responsible for the binding of the C-terminal domain of Sin1p/Spt2p to 4WJDNA, we substituted alanines for amino acids 325KRRR328. As can be seen in Fig. 6b, these substitutions resulted in the inability of the peptide to bind 4WJDNA. Importantly, however, the substitution of amino acid Lys325 or Arg326 alone with alanine had the same effect.

Lefebvre and Smith (8) performed a functional mutational analysis of Sin1p/Spt2p. They assayed for suppression of his4-912Δ in a wild type strain with respect to SIN1/SPT2 (SLL5) and a sin1p/spt2Δ::URA3 strain (SLL7) by transforming with a yeast plasmid expressing various sin1p/spt2 mutants. Their experiments identified the functional importance of the Sin1p/Spt2p C-terminal domain (amino acids 325–333) as replacement of these amino acids with non-basic amino acids led to a dominant suppressor allele. They also found that a single substitution of K325S had a partial suppressor phenotype. In addition, they found another domain overlapping with HMG box 2 that is required for the dominant suppressor phenotype to be expressed. In this study we extended Lefebvre and Smith’s work, using their system, to investigate the functional requirement of the acidic domain (amino acids 224–304). In our deletion construct, only these amino acids were deleted, while the remainder of the molecule including the basic C terminus was left intact. An additional mutant containing only the W267R and K275A point mutations was constructed as well.

Our results clearly showed that the acidic domain, like the basic C terminus, has functional significance as either its deletion or inclusion of two point mutations resulted in the suppressor phenotype. Similar to two different point mutations in the C terminus (8), the deletion of the acidic domain caused dominance of the mutant over the wild type (growth of SLL5 transformants on His- plates).

As poly-L-lysine can be viewed as a cluster of positively charged amino acids, it may be instructive in the context of this study to consider its structural interaction with DNA. Poly-L-lysine interactions with double-stranded DNA have been studied previously (44–47). While there has not been a crystal structure of a poly-L-lysine-double-stranded DNA complex, x-ray analysis of fibers of this complex has suggested that poly-L-lysine is positioned in the minor groove of the DNA in an elongated form (45). The suggested structure is consistent with the negatively charged phosphate groups in both chains being neutralized by lysine side groups alternatively directed to each chain. Our results (Fig. 8) showed that binding of poly-L-lysine to the 4WJDNA caused an increase in mobility of the 4WJDNA in a polyacrylamide gel. We interpret this result by postulating that interaction with poly-L-lysine may result in a more compact structure of 4WJDNA due to a decrease in the electrostatic repulsion of the arms. We propose, therefore, that the clusters of positively charged amino acids in Sin1p/Spt2p and in other proteins able to bind 4WJDNA may be responsible for decreasing the angle between arms of the junction, making the structure more compact.

Since protein binding to 4WJDNA is seen as an indication of the ability of a protein to bind bent DNA or to bind the crossing of helices of DNA (13), we used a DNA circularization assay to determine whether the acidic domain of Sin1p/Spt2p (amino acids 224–304) could induce a bend in DNA. Using a protocol that was successfully used for HMG1 (48), we attempted to circularize an 87-bp fragment of DNA (data not shown). While 87 bp are too short to circularize on their own, protein that induces bends in the DNA is expected to allow circularization. We were unable to achieve circularization at any protein concentration we tried. In contrast, the acidic domain was able to bind supercoiled DNA (Fig. 5) in which DNA helices necessarily cross. We, therefore, conclude that it is unlikely that the acidic domain of Sin1p/Spt2p is involved in bending of DNA but rather that it stabilizes crossing helices of DNA much as linker histone does at the entrance and exit of the DNA from the nucleosome.

Our results show that Sin1p/Spt2p may interact with DNA by association with multiple domains of the protein. The acidic domain clearly binds 4WJDNA in a structure-specific manner, showing very discrete bands in the gel mobility shift assay. The binding of domains at both the N terminus and C terminus to 4WJDNA was less discrete for unknown reasons. It is possible that the binding of the complete molecule might start at the conserved acidic domain and then be stabilized by the two other domains. We speculate that the Sin1p/Spt2p molecule may bind the DNA at particular locations in the chromatin, inducing a change in structure that could further influence the binding of other proteins to the DNA. Alternatively the specificity of Sin1p/Spt2p binding to the DNA could be a consequence of the binding of other proteins that modify the local DNA structure, making the minor groove more readily accessible to Sin1p/Spt2p binding.

Pérez-Martín and Johnson (49) studied the interaction of domains of Sin1p/Spt2p with portions of the SWI/SNF complex. Their results indicate that the C-terminal half of the protein can interact directly or indirectly with at least three components of the SWI/SNF complex and that the N terminus of
Sin1p/Spt2p can compete with this interaction. Their data suggested that there could be a conformational change of Sin1p/Spt2p that would allow or prevent its binding to Swi1p. Presumably these conformational changes could also affect, or be affected by, binding of Sin1p/Spt2p domains to DNA. The two HMG box domains of HMG1 have been shown to be capable of manipulating DNA that results in its looping and compaction. The acidic C-tail of HMG1 down-regulates these effects by modulation of its DNA binding properties (50). It will be interesting if the similarities between Sin1p/Spt2p and HMG1 extend to the abilities of the molecule to loop and compact DNA in chromatin. Further experiments will be needed to test these hypotheses.

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