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PRINCIPAL INVESTIGATOR: Linda Breeden, Ph.D.

CONTRACTING ORGANIZATION: Fred Hutchinson Cancer Research Center
Seattle, Washington  98104

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Several ANK repeat-containing proteins have been implicated in breast cancer and other tumor development. Our purpose has been to define the function of the ANK repeats using a combination of genetics and biochemistry. This involved exhaustively mutagenizing the ANK repeats of the yeast Swi6 protein in order to identify the critical residues within each repeat. The effects of these mutations upon DNA binding and transcriptional activity was determined. Partially defective mutants have been identified and used to search for interacting gene products using suppressor analysis. We have concluded that ANK repeats play a structural role. ANK repeats represent one way to stably fold a polypeptide chain, and this structure provides a scaffold upon which different residues can be displayed. It is most likely that the nonconserved residues within the repeats are responsible for the specific protein-protein interactions of each class of ANK repeat proteins. From this we conclude that the right path to understanding the functions of the oncogenic repeat proteins is to focus upon the nonconserved, surface residues within their ANK repeats and search for proteins which interact with them. This work and the available X-ray data provide a straightforward path to this goal.

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The Role of the ankyrin repeats in the Swi4/Swi6 transcription complex of budding yeast.

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INTRODUCTION

Over 100 proteins have been identified as containing ankyrin repeats. Several proteins have received particular interest because of potential roles for the ankyrin repeat motifs in tumor development. These include Bcl-3, int-3, TAN-1, and p16. The BCL-3 gene encodes a member of the I kappa B family of proteins and rearrangements involving the ankyrin repeat region BCL-3 have been identified in B-cell chronic lymphocytic leukemias [1]. Int-3, a mouse proto-oncogene, is a common insertion site for the Mouse Mammary Tumor Virus (MMTV). Activation of the int-3 ankyrin repeat sequences by MMTV produces poorly differentiated adenocarcinoma of the mammary and salivary glands in mice [2]. TAN-1, the human Notch homologue, was first described as a breakpoint region of t (7,9) gene rearrangements found in T-cell acute lymphoblastic leukemias. This rearrangement places the gene encoding the beta T-cell receptor adjacent to the ankyrin repeat motifs of TAN-1 [3]. p16 is the protein product of the MTS1 (multiple tumor suppressor-1) gene. It is composed of only a series of four ankyrin repeats and is one member of the cyclin dependent kinase inhibitor family of proteins. p16 can compete with cyclin D and displace the cyclin subunit from its kinase binding site, thereby inhibiting G1 to S progression through the cell cycle. Mutations in p16 have been noted in a variety of human tumors and tumor cell lines, including bladder and gastrointestinal neoplasms and mesotheliomas; in addition, mutations have been found in the germline of families predisposed to melanoma [4]. Ankyrin repeats were originally described by Breeden and Nasmyth [5] in the Swi6 transcription activator of the budding yeast, Saccharomyces cerevisiae. Swi6, with its two known protein binding partners, Swi4 and Mbp1, regulates gene expression during the G1 to S transition of the yeast cell cycle. All three of these proteins contain ankyrin repeats and the goal of this research was to understand the role of these repeats using a combination of genetics and biochemistry.

BODY

Our purpose has been to define the function of the ANK repeats. This involved exhaustively mutagenizing the ANK repeats of Swi6 to identify the critical residues within each repeat. The effects of these mutations upon DNA binding and transcriptional activity was determined. Partially defective mutants have been identified and used to search for interacting gene products using suppressor analysis. The results of these experiments has led us to the view that most of the ANK repeat domain has a structural role, and to consider alternate strategies for identifying surfaces of the ANK repeat motif which might bind to other proteins.

The technical objectives in our approved Statement of Work are to:

1. Generate monoclonal and polyclonal antibodies that recognize either Swi4, Swi6 or all ANK repeats and use them to screen for other ANK repeat-containing proteins.

2. Exhaustively mutagenize the ANK repeats of Swi4 and Swi6, and identify the crucial residues for their activity.

3. Develop a battery of genetic screens to identify mutations that cause loss or deregulation of known Swi4 and Swi6 functions.

4. Perform in vitro assays to correlate mutant phenotypes with known biochemical functions.
5. Identify second site suppressors, either within the Swi protein, or within associated proteins.

**TASK 1. Generation of antibodies to the Swi6 ANK domain.**

We have generated new polyclonal and monoclonal antibodies to the Swi6 protein. The polyclonals are very high quality and can be used at a dilution of 1:10,000 on westerns. These have been valuable reagents for isolating and analyzing swi6 mutants levels. The technical difficulties associated with obtaining large quantities of Swi4 protein have precluded the making of more Swi4 antibodies of any kind. Thus, we have focused our efforts upon understanding the ANK domain function of the Swi6 protein only. The Swi6 protein was purified as described [6] and the polyclonal serum was generated in rabbits with three subcutaneous injections of 100 micrograms of antigen in Freund's adjuvant following standard protocols [7]. Milligram quantities of Swi6 was also purified by Bernard Mai to facilitate chrystallization trials, which were unsuccessful.

Our monoclonal antibodies were generated by Dr. Elizabeth Wayner and Davis Creemer. Their respective reactivities with Swi6 on Elisa, Western and immunoprecipitations (IP) are summarized in Table 1. To generate these monoclonal cell lines, eight week old RBF/DnJ [or BALB/cByJ(8.12)5Bnr/J] mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were given multiple injections of Swi6 protein or ankyrin domain fragment (50-100 µg to immunize and 25 -50 µg to boost) emulsified in complete or incomplete Freund's adjuvant. Complete Freund's adjuvant was used only to immunize naive mice. All emulsifications were prepared as a ratio of 60% oil to 40% aqueous (PBS). Swi6 specific antibody titers in the polyclonal antiserum were determined by ELISA and Western blot. Mice were selected for fusion based on specific antibody titers to Swi6. Monoclonal antibodies to Swi6 were produced as previously described [8]. Briefly, spleen cells from the immunized mice were fused with NS-1/FOX-NY myeloma cells [9]. Viable heterokaryons were selected in RPMI 1640 medium supplemented with adenine/aminopterin/thymidine [10]. Cultures secreting antibody specific for Swi6 were identified by ELISA using an HRP-conjugated goat antibody (Roche Molecular Biochemicals, Indianapolis, IN) specific for mouse antibodies with an IgG-type heavy chain. Appropriate antibody cultures were further identified by Western blot. Monoclonal hybridoma cell lines secreting antibodies with the desired specificity were produced via two rounds of cloning via limiting dilution.

One obstacle to characterizing all of these monoclonal antibodies in more detail was the fact that the antibody titer is too low in culture supernatants to be useful. To get around this we have generated ascites fluid containing a subset of these antibodies. Large-scale mAb production was undertaken by ascites production using stable hybridoma cell lines injected into athymic nu/nu mice. Isotypes of resulting mAbs were determined by using Isostrip Mouse Monoclonal Antibody Isotyping Kits (Roche). There are 23 positive monoclonal cell lines in all, most of which have been generated with the isolated ANK domain. These are useful reagents for detecting Swi6 on Westerns, but they do not recognize any other ankyrin repeat containing proteins and thus are not recognizing epitopes common to other ANK domains. Thus they have not been useful in studying the structure or activities associated with ANK domains generally. However, these cell lines have been saved and will be useful for further studies of Swi6.
Table 1. Swi6 Ankyrin Repeat Domain Monoclonal Antibodies

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Swi6 (full length) Monoclonal Antibodies

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Table 1. The names and properties of the 23 monoclonal cell lines derived in this study are listed. A check mark indicates that supernatant and/or ascites fluid containing these antibodies react positively by ELISA, Western or immunoprecipitation (I.P.) assays against Swi6 as described in Appendices 1 and 2. Positions left unchecked indicate that this line has not been assayed for this activity.
TASK2. Define critical amino acids involved in ANK repeat function.

We have constructed a total of four libraries of \textit{SWI6} genes with randomly mutagenized ANK repeat regions. We have isolated about 30 different temperature sensitive (ts) and 110 unconditionally inactive (null) Swi6 ANK mutants. Since most of the null mutants appeared to have lost expression of Swi6, we concentrated our efforts on ts mutations, the majority of which expressed Swi6. All of the temperature sensitive mutants are listed in Appendix 1, Figure 2. This panel of mutations demonstrates that mutations occur throughout the region and are about as likely to occur in any of the four ANK repeats. There is some clustering at each end of the whole ANK domain (although this could be a PCR artifact). The only region lacking mutations is the non-structured part of the spacer. Thus, all four ANK repeats are critical for Swi6 function.

We have identified five critical residues: R344, G347, D375, A477 and N500 at which single substitutions are enough to compromise the activity of the whole protein. These are marked as solid ellipses in Figure 1. Most, if not all, of the mutants that we looked at were defective both in SCB driven transcription and in MCB driven transcription (see Task 3).

We have also performed a screen for mutations that will hyperactivate the ANK repeat. We used one of the libraries that was most heavily mutagenized. No ANK mutations with hyperactivating characteristics were found. However, on two occasions we identified rare recombination products that resulted in duplication of the \textit{HIS3} gene itself, which led to increased \textit{HIS3} expression from an \textit{MCB: HIS3} reporter. This suggested to us that our screening strategy (for elevated \textit{HIS3} expression using the growth advantage on aminotriazole) had worked. It is therefore likely that a hyperactivating mutation cannot occur in the ANK region, alternatively, that such mutation requires more than one amino acid substitution and therefore cannot be readily generated by point mutagenesis.

Surprisingly few of the mutations generated randomly were within the conserved core of the ANK repeats (shaded black or grey in Figure 1). To determine the extent to which the most conserved residues cores were contributing to Swi6 function, we performed site-directed mutagenesis of the core regions of each repeat, both singly and in combination. In each case the G-T-L core residues of the repeat were changed to alanines. We found that these mutants varied in function at 25 degrees, but all of these mutations conferred a temperature sensitive phenotype to Swi6. This indicates that the entire length of the ANK domain is critical for the stability of the Swi6 protein. Mutations in the first repeat had the least effect, then the second, third and the fourth increased in importance. The multiple mutants presented a more complex picture, in that many of the multiple mutants had more activity than the single fourth repeat mutant. The most surprising of these is the Swi6 mutant with all four repeats mutated. In this case, activity was much higher, about half that of wild type. This remains a mystery. Our working hypothesis is that the repeats are involved in some form of redundant negative control, such that mutation in a subset of the repeats doesn’t disrupt it, but mutation of all four repeats does. This data can be found in Appendix 1, Table 2.
Ankyrin/Swi6/Cdc10 repeat

Figure 1. Alignment of the ankyrin repeat regions of a family of yeast transcription factors.

These three *S. cerevisiae* and three *S. pombe* transcription factors all contain a centrally located ankyrin repeat domain that consists of four full ankyrin repeats and vestiges of a fifth that are tandemly arranged. The black boxes are situated at the most highly conserved residues within the ankyrin motif, known as the "core" region. Each of the ankyrin repeats within the transcription factors has these regions conserved to some degree. The light grey boxes denote residues that are conserved across more than one repeat within this family and the white boxes highlight residues that are conserved within only one repeat of the ankyrin domain.

○: Single residue substitutions at these sites confer a temperature-sensitive phenotype to Swi6;
○: Mutant with residue substitutions at these locations were isolated more than once.
The recent publication of the crystal structure of a p53 binding protein, 53BP2, which contains four ankyrin repeats [11], has enabled us to estimate the positions of the ankyrin repeat mutations of Swi6 that we have generated. First, the four ankyrin repeats of Swi6 and 53BP2 were aligned with 24 other ankyrin repeat domains using CLUSTAL-W [12]. This multiple alignment provided a reliable means of aligning the two proteins' ankyrin repeats despite their low sequence identity (21%). Then, the coordinates for the 53BP2 structure, generously provided by N. P. Pavletich, were used in conjunction with the MODELLER program [13] to generate and refine a model of the Swi6 ankyrin domain. This model was strikingly similar to the 53BP2 structure. It was validated by three independent methods (PROCHECK, VERIFY-3D, and ERRAT (see figure 7 legend, Appendix 1) and all three indicated a high degree of reliability. The structure that 53BP2 adopts, and the one that the modeled Swi6 conforms to, resembles an "L" in profile, with continuous, tightly packed α-helices perpendicular to and connected by short β-hairpins. A ribbon diagram of the Swi6 ankyrin model structure is provided in Appendix 1, Figure 7. The locations of the core residues and five single-point mutants are labeled. The regularity of this repeat structure is apparent in this view, as is the conserved positioning of the core residues in each repeat.

Four ankyrin repeats occur in tandem within the yeast transcription factor family, and the four full repeats have been modeled on the basis of the structure of another protein which also contains four repeats. N500, one of the residues shown to be critical for Swi6 function in this analysis, is adjacent and C-terminal to the four-repeat structure. This asparagine, and the glycine that follows three residues downstream, are the residues that would be expected to begin a fifth repeat. However, the residues that follow do not conform to the consensus sequence. The significance of this partial fifth repeat is unclear, but the fact that it maintains appropriate spacing and sequence conservation within this family suggests that it may be important in the overall domain structure. This is corroborated by the fact that point mutations in N500 have been found which disrupt Swi6 function. In addition, there is another ankyrin repeat containing protein in yeast, Yar1, which also ends its ankyrin repeat sequence in the same manner [14].

Among the PCR-generated ankyrin repeat mutants, the most common identified mutation, isolated 10 times from three independent screens, was the glycine G347 position and all were substitutions of glycine for aspartate (D). G347, as pictured in Appendix 1, figure 7, lies on the back surface of the modeled structure, in the β-hairpin connecting ankyrin repeats 1 and 2. As such, changes at that position are unlikely to destabilize the structure itself. Rather, the loss of function that this mutation causes suggests that this residue may be part of a surface on which there is a critical interaction with another protein, or with another domain of the Swi6 protein. This is also likely to be the case for the gain of function mutation, N330K, which does not confer a conformational change to the modeled Swi6 ankyrin repeat.

The four other single mutations generated in this study that resulted in temperature-sensitive Swi6 proteins are all predicted to result in unfavorable interactions within the ankyrin repeat itself when modeled onto the structure. These findings help to validate the model and give us new insight into the key intramolecular interactions that occur in this structure. The core leucine (L) at position 474 changed to serine (S), and alanine (A) 477 changed to threonine (T) (Appendix 1, Figure 8A); both disrupt the tight hydrophobic packing that is predicted to occur within neighboring residues. In the case of histidine (H) 323 (Appendix 1, Figure 8B), which is replaced with arginine (R), the hydrogen bonds predicted to be formed by the histidine with aspartate (D) 348 are disrupted and the larger arginine side chain would be expected to perturb the local structure due to steric hindrance.
Finally, the space filling models depicted in panels A and B of Figure 9 (Appendix 1) clearly show the effect predicted by substitution of R344 for glycine (G). In the model, the loss of the bulky side chain of the arginine creates a cavity on the surface of the structure which exposes the hydrophobic side chains of L322 and L373. This cavity is large enough to make this region accessible to water and would be expected to destabilize the structure.

Subsequent to the publication of this work, the crystal structure of the Swi6 ANK domain was published [15]. These authors pointed out that their structure confirmed the structure we predicted with our model and the effects that these mutations would have.

**TASK 3. Analyzing ANK repeat mutants for loss of specific functions in vivo.**

Our screen for ANK repeat mutants was carried out with an HO: lacZ reporter group, which enabled us to assay Swi6 transcriptional activity with a simple colorimetric assay. We have since assayed these mutants using simplified promoter constructs. Specifically we have asked if these mutants show differential activity upon MCB versus SCB binding sites using specific reporter constructs which differ only in the DNA binding sites. Both sites are known to be bound by Swi6 [16-21], but it is unclear whether their binding would be affected similarly by these mutants. To obtain this data, we have compared lacZ levels produced from a set of reporter plasmids in yeast strains with different ts ANK mutations at nonpermissive temperature. The four reporter constructs we used had as a UAS (upstream activating sequences) in their promoters 1) a fragment of HO promoter, 2) a fragment of TAP1 promoter, 3) a synthetic trimer of SCB elements and 4) a synthetic trimer of MCB elements (TAP1 and HO fragments contained activating sequences that have MCB and SCB elements, respectively, in the native context). There was some variation between the different ts mutants as to the degree of defects in SCB versus MCB transcription, but all of them were defective at both types of promoter elements. Therefore, our results obtained so far suggest that the MCB and SCB transcription functions of Swi6 are not carried out by separate domains within the ANK repeat region.

To allow a direct comparison between SCB and MCB activation, we introduced a high-copy reporter construct carrying three tandem repeats of either MCB or SCB elements into the BY660 (ho, swi6::TRP1-197) strain carrying the mutant swi6 allele on a low-copy plasmid. This enabled us to compare SCB and MCB activation within an equivalent context. Figure 6 (Appendix 1) shows that most of the PCR-derived and site-directed mutants cause a more severe MCB transcriptional defect when compared with SCB transcriptional activity. The most notable differences are seen with swi6-1234, which has 44% activity on SCBs and 0% on MCBs, swi4-124, which has 40% SCB activity and 0% MCB activity, and swi6-421, which has 48% of the wild-type SCB activity compared with 14% of the wild-type MCB activity. In addition, there are a number of mutants (swi6-1000, -200, -123, -405, -417, and -424) that show comparably decreased levels of both SCB and MCB activity when measured from these analogous promoter constructs. The fact that the vast majority of ankyrin repeat mutants are not equivalently defective with respect to MCB and SCB activation indicates there are differential effects upon the Swi4-Swi6 and Mbp1-Swi6 transcription complexes. This is somewhat surprising since the C termini, and not the ankyrin repeats of these proteins, are sufficient for the interaction between Swi6 and Swi4 [6, 22] and between Swi6 and Mbp1 [23]. Interestingly, all of the mutants are more defective in MCB activation than in SCB activation, despite the fact that they were selected for their inability to act at the HO promoter, which is activated by SCB elements. This suggests that the Swi6-Mbp1 interaction or activity has a stronger dependence either upon the ankyrin domain itself or upon other residues that are brought into proper position by the ankyrin domain structure.
We have also analyzed the Swi6 ANK mutants for two other phenotypes conferred by swi6 deletion mutants: 1) the inability to recover from alpha factor [24], and 2) hydroxyurea sensitivity [25]. Interestingly, although the ANK mutants are highly defective in transcription, they appear to behave as the wild type in these two other assays. This is difficult to understand based upon current knowledge. It is possible that once global searches to identify all the Swi4/Swi6-dependent promoters are identified, candidate promoters that might be responsible for these phenotypes can be deduced and tested.

**TASK 4. Biochemical characterization of the ANK repeat SWI6 mutants and potential ANK interacting proteins with regard to their DNA binding activities.**

To test whether the inability of the Swi6 ankyrin mutant proteins to activate SCB transcription is due to a defect in the ability of Swi4-Swi6 complex to bind DNA, we compared the DNA binding activities of these mutant proteins to that of a wild-type Swi6 by band-shift analysis. Using whole cell extracts derived from the wild-type, swi6-405, and swi6-406 strains and a fragment of the HO promoter as the SCB DNA probe, we find that the wild-type complex migrates predominantly as a single lower complex (Appendix 1, Figure 3A). Extracts from the swi6-406 strain contain a complex that binds DNA about as efficiently as wild type, but the complex formed migrates more slowly (referred to as the upper complex). The swi6-405 extract produces both upper and lower complexes in approximately equal amounts. These differences in mobility could be due to differences in the protein composition of the complexes or to modification or conformational changes within the Swi4-Swi6 mutant complexes. The variations in steady state levels of Swi6 in the temperature-sensitive mutants compared with that in the wild type could also influence the amount and type of complexes that form.

To eliminate some of these complications, we have used in vitro translated Swi4 and Swi6 in the DNA binding reactions. All of the ankyrin repeat mutant plasmids, as well as the wild-type SWI6 plasmid, were transcribed and translated and found to yield comparable levels of Swi4 and Swi6 protein products (Appendix 1, Figure 3B and data not shown). DNA binding reactions using the in vitro translation products from swi6-406 and wild-type SWI6 plasmids were analyzed in parallel with those produced from whole cell extracts. The resulting band-shift patterns are qualitatively similar. Since the in vitro translated Swi4 and Swi6 were the only yeast proteins added to the reaction mixtures, it is unlikely that the tendency of the swi6-406 ankyrin repeat mutant to form upper complexes is due to binding of additional proteins to the DNA-protein complex. It is also unlikely that differences in protein modification are responsible, since the proteins are translated in a rabbit reticulocyte lysate system. The possibility that proteins in this lysate could bind or modify the yeast protein-DNA complex cannot be excluded, but to produce these results, the protein has to be specific for the Swi6 mutant complexes. The protein would also need to be present in both rabbit and yeast cells, because the band shift is identical whether the mutant protein is translated in vitro or harvested from yeast.

To observe the extent of variation in DNA binding activity, the collection of temperature-sensitive ankyrin repeat mutants of Swi6 were translated in vitro and surveyed for DNA binding activity at two temperatures. Most of the Swi6 mutants retained the ability to complex with Swi4 and bind SCB elements at the nonpermissive temperature. Frequently, however, the mobility of the ankyrin mutant complex was noticeably altered (Figure 4, Appendix 1) and migrated as an upper complex that could not be distinguished from that of swi6-406. The wild-type (SWI6+) band-shift pattern shows predominant lower band at 25 °C and a more dispersed pattern, including both upper and lower
complexes, at 37 °C. From the band-shift assay, the swi6 mutants can be grouped into four categories. The first group of mutants [swi6-406, -407 (G347D), and -401] all form the upper complex predominantly at both the permissive (25 °C) and nonpermissive (37 °C) temperatures. The second group of mutants [swi6-405 and swi6-410 (H323R), -422, -421, -424 (R344G), -402, and -417 (A477T)] form the upper complex at the nonpermissive temperature and both the upper and lower complex at the permissive temperature. The third phenotype is overall reduced binding, even at the permissive temperature, seen with mutants swi6-401, -417 and -422. The fourth group of mutants: swi6-409 (N500T), -415, -420, -423, and -418 (N500Y), show no binding defect at either temperature. Most of the mutants show altered binding characteristics with a propensity to form the upper complex, which is exacerbated by elevated temperatures. A minority have reduced overall DNA binding. Formation of the upper complex therefore correlates with the loss of transcriptional activation, which is also enhanced at high temperatures. Despite the considerable variability in the DNA binding complexes formed, the upper complex that most of the mutants display migrates at a very similar position in the band-shift gel. Since this is not likely to be due to changes in protein composition or modification states, we speculate that it may be the result of a global change in the conformational state of the Swi4-Swi6 complex which these mutations induce to varying degrees.

To measure the DNA binding defect associated with the core substitution mutants, we employed in vitro translation and band-shift assays as before, incubating the reaction mixtures at both 25 and 37 °C. As stated above, the wild-type Swi4-Swi6 complex exist as a combination of upper and lower complexes on SCB DNA, with a predominant lower complex at 25 °C. The core mutants all form the upper complex exclusively at both temperatures (Figure 5 of Appendix 1 shows the 25 °C experiment). The ability to form the upper band-shift complex did not localize to mutations within a particular core region. Thus, even more so than with the PCR-generated repeat mutants, we see a dramatic but uniform shift in mobility of the DNA binding complex with the core mutants of Swi6. This, and the temperature sensitivity of all these mutants, suggests that the conserved core residues of all four repeats are important for maintaining the native structure of Swi6 and/or Swi4-Swi6 complex.

**TASK5. A screen for high copy suppressors of ANK ts mutations.**

The two of temperature sensitive ANK mutants of SWI6 genes were used to identify high copy suppressors. These mutants were transformed with a 2µm-based yeast genomic library [26] and about 60,000 transformants were obtained for each. Detailed methods are available in Appendix 2. We recovered a total of 8 suppressor plasmids from swi6-406 and 19 from swi6-405 transformants. These are listed in Table 1, Appendix 2. Several suppressors were not pursued further because they activated ho::lacZ expression equally strongly in swi6-405 and swi6Δ cells and thus were completely independent of Swi6. Most of the suppressors suppressed the ho::lacZ expression defect to some extent in the absence of Swi6, but only one (c19) could suppress in the absence of Swi4. This requirement for Swi4 suggests that the majority of suppressors enhance Swi4-mediated activation, rather than by causing a general derepression of transcription. c15 showed no suppression of the ho::lacZ transcription defect in swi4 or swi6 deletion strains and thus was the best candidate for an allele-specific suppressor.

We obtained some sequence information for all of the suppressors and have searched genomic databases with these sequences to obtain full maps of the DNA inserts.
In each case more than one open reading frame was present on the insert. We have subcloned insert fragments to determine which of the open reading frames were encoding suppressors. The results of mapping and subcloning showed that previously cloned genes, _MSN1_ and _NHP6A_, were responsible for suppression phenotype of several of the clones. _c23_ appears to carry a different fragment of chromosome _XV_ and _c2_ carries a fragment from chromosome _XIII_. The open reading frames present on these fragments have not been characterized previously. We have determined which open reading frame of _c2_ is responsible for suppression. It is a new gene of unknown function (ORF YM8520.13c) that we called _PIB1_ (Partially Independent Bypass of Swi6). _PIB1_ has no close homologues in yeast or in higher eukaryotes, however it resembles several transcription factors. Interestingly, it bears some structural similarity to a _Drosophila_ transcription factor Dorsal, which is known to interact with the ANK repeat containing regulator Cactus [27]. We have disrupted _PIB1_ and found that it is not essential for yeast viability. Double mutants with disruption of _PIB1_ and either _SWI4_ or _SWI6_ are also viable. Also the steady state HO levels are unchanged in the _pib1Δ_, so this gene has not been pursued further.

Previously identified genes, _MSN1_ and _NHP6A_, were responsible for suppression phenotype of _c4_ and _c15_, respectively. _MSN1_ was originally cloned as a high copy suppressor of a temperature sensitive _SNF1_ kinase mutant for its ability to restore SUC2 expression [28]. It acts as a transcriptional activator when fused to _LexA_ and does not have any specific DNA binding activity [28]. _NHP6A_ has also been identified previously [29] and encodes an HMG1-like small protein, which binds DNA nonspecifically, and is capable of bending DNA [30]. RNA analysis showed that both _MSN1_ and _NHP6A_ exert their function at the mRNA level, rather than by affecting β galactosidase stability or activity.

To see if _MSN1_ and _NHP6A_ are normally involved in the transcription of the Swi4/Swi6-regulated promoters, we isolated mRNA from exponentially growing cultures of strains with or without _MSN1_, _NHP6A_ and _B_ gene products and compared the levels of _HO_ and _CLN1_ transcripts in these strains to the wild type strain by S1 protection (Figure 2, Appendix 2). The _msn1Δ_ strain expresses about three to five-fold less _HO_ transcript and the _nhp6abΔ_ strain shows a two-fold drop in _HO_ transcript compared to wild type. Interestingly, there is little or no effect of _msn1Δ_ on another Swi4/Swi6-regulated promoter, _CLN1_ (data not shown) but the _nhp6abΔ_ has a similar two-fold effect on _CLN1_ all methods are described in Appendix 2.

To see whether there is a direct interaction between Nhp6A and Swi6, we immunoprecipitated Swi4 or Swi6 proteins out of wild type extracts carrying HA-Nhp6A and then immunoblotted with anti HA antibodies to detect HA-Nhp6A. Despite the fact that this HA-tagged Nhp6A is functional and can suppress the _swi6-405_ transcription defect (Figure 1b, Appendix 2), there was no indication that HA-Nhp6A coprecipitates either with Swi4 or Swi6 under the same conditions that we use to detect Swi4/Swi6 association [6].

We then prepared fusions of Nhp6A and Msn1 proteins with Gst. The _GST-NHP6A_ and _GST-MSN1_ fusions were put under the control of _GAL1-10_ promoter and expressed in a wild type strain. Because of their toxicity when overproduced, we purified these Gst fusions from cells grown in raffinose and then induced by galactose addition for only 3-4 hours. Though we could purify the fusion proteins from these cells under low stringency conditions, there was no detectable Swi6 copurifying with either of them. We also purified these Gst fusion proteins from _E. coli_ on glutathione beads and then incubated the fusion-bound beads with recombinant Swi6 [6], or _in vitro_ translated Swi4/Swi6 complex [31]. Even under these conditions, we could not detect interaction between Swi4, or Swi6, and the fusion proteins.

_MSN1_ has been originally cloned as a high copy suppressor of a temperature sensitive _SNF1_ kinase mutant [32]. It has activator properties and has an extremely weak
and nonspecific DNA binding activity. MSNI has been also selected by other groups as FUP1[11] and PHD2, an activator of pseudohyphal growth [6] as well as a protein capable of activating mating specific genes when overproduced [33]. Therefore, it is likely that MSNI is a non-DNA binding transcription activator, or coactivator, that is utilized by several unrelated promoters. Strains deleted for MSNI grow slower than the wild type suggesting that MSNI is involved in the regulation of genes important for viability. We found that in msn1 deletion strain the transcription of HO is reduced to 20-30% of the wild type. Thus, MSNI is required for maximal expression of HO.

NHP6A has also been identified before [34, #238] and encodes an HMG1-like small protein, which binds DNA nonspecifically, and is capable of looping DNA [20]. NHP6A has a close homologue in yeast, NHP6B, which encodes a similar protein with potentially overlapping activity [34]. Strains with deletions in both NHP6A and NHP6B have multiple phenotypes, including dramatically reduced growth rates. Both NHP6 proteins have long been implicated in facilitating gene expression by inducing favorable chromatin conformations on the promoters, however, this is some of the first direct evidence that nhp6A-6B deletion strains impair transcription.
KEY RESEARCH ACCOMPLISHMENTS

- Some portion of the ANK domain of Swi6 is accessible to antibodies in the native state. Swi6 is an antigenic protein, as are the ANK repeats in isolation. The fact that at least one monoclonal antibody generated against the ANK domain can be used to immunoprecipitate Swi6 from yeast extracts suggests that some portion of the ANK domain is accessible in the native state. This was corroborated by our modeling studies and verified in the crystal structure of Swi6.

- There are at least four functional ANK repeats in Swi6. The fourth repeat of Swi6 is the most critical for Swi6 function, but the conserved core of each repeat is important for Swi6-mediated activation of HO: lacZ transcription.

- Many residues within the ANK domain are critical for function. Mutants across the spectrum of severity have been isolated from this screen. We have focussed upon temperature sensitive ones, but many non-conditional null mutants have also been isolated. It is clear that the ANK domain of Swi6 contains a large number of residues that are critical for its function.

- Mutations that cause loss of Swi6 function at high temperature are found throughout the ANK domain. There is some clustering of mutations at each end of the ANK domain, but each ANK repeat is important. The only region lacking mutations is the non-structured part of the spacer.

- We have identified some of the critical residues: R344, G347, D375, A477 and N500, whose substitution is enough to compromise the activity of the whole region. All but one of these (G347) compromise the structural integrity of ANK domain.

- G347 is a surface residue critical for Swi6 function. This mutation also causes a dramatic conformation change in the Swi4/Swi6 complex, so we expect that G347 resides within a domain of contact between Swi4 and Swi6, or an intramolecular interface.

- Most if not all of the mutants that we looked at were defective both in SCB- and MCB-driven transcription, indicating that the two activities are not separable by mutation in the ANK domain.

- Hyperactivating mutations cannot occur in ANK region, or such a phenotype requires more than a couple of amino acid substitutions and therefore cannot be generated by this mutagenesis procedure.

- Site-directed mutations within the conserved cores of the ANK repeats all confer a temperature sensitive phenotype to Swi6. This indicates that the entire length of the ANK domain is critical for the stability of the Swi6 protein. Mutations in the first repeat had the least effect, then the second, third and the fourth increased in importance.
• The enhanced activity of multiple repeat mutants suggest the possibility that the repeats are critical for some form of negative control over Swi6 activity.

• **We have identified three genes that can suppress ts ANK defects of SWI6.** *PIBI* is a new gene, with homology to some other transcription factors, but no known function. The other two: *NHP6A* and *MSN1*, are previously identified genes. We have established that two of these genes are required for maximal *H0* expression. *NHP6A* facilitates Swi4/Swi6 complex formation on DNA, but we have no evidence of direct interaction between Swi6 and any of these proteins. Thus, we conclude that they act indirectly to facilitate Swi6-dependent transcription.

• Most of the ANK mutants show a coordinated loss of both SCB and MCB-activation. However, there is a more severe defect in MCB transcriptional activity than in SCB transcriptional activity in several mutants. This indicates that these two activities are not carried out by different repeats within the ANK domain, and that MCB activity is more dependent upon the structural integrity of the Swi6 protein.

• There is a surprising range of effects of these ANK mutants upon DNA binding. Some mutants are extremely defective in DNA binding. Others show a predominant upper complex compared to the wild type cells. Since these assays are carried out with only Swi4 and Swi6 proteins produced from reticulocyte lysates, it is unlikely that the reduced mobility of these upper complexes is due to the addition of another protein in the complex. Rather, we conclude that it is most likely that the ANK mutations are causing a fairly radical change in the conformation of the complex.
REPORTABLE OUTCOMES

Bibliography of manuscripts and meeting abstracts


Breeden, L., J. Partridge, J. Sidorova, C. McInerny, and G. Mikesell Transcriptional control of the early events in the budding yeast cell cycle. ICN-UCLA symposium 1996, Taos, New Mexico


Cell lines (See Table 1 in Final Report)

20 monoclonal cell lines derived from injection of the Swi6 ANK domain

3 monoclonal cell lines derived from injection of the full length Swi6 protein

Employment and Research opportunities

Dr. Sandra Ewaskow was awarded a 1 year fellowship from the College of American Pathologists Foundation (1995).

Dr. Sandra Ewaskow was awarded a five year Physician Scientist Award (K11) from the National Institutes of Health for 12/95 to 1/00

Dr. Julia Sidorova was awarded a three year Leukemia Society of America Senior Fellowship for 7/99 to 6/02
CONCLUSION

Within the four ankyrin repeats in this family of transcription factors, three levels of conservation are observed. There are "core" residues, which are identical, as well as residues with chemical similarities that are conserved throughout all four of the repeats. Second, there are specific residues which are shared by repeats 1 and 4, making them much more similar to each other than the other repeats in these or any other set of ankyrin repeat proteins. Third, there are residues which are conserved within the individual repeats of all of the family members, but which differ widely between repeats. This study provides evidence that the core residues of all four of the repeats are important, but whether this is due to a structural requirement for a four-repeat domain or whether these repeats actually have different roles is unclear. The fact that the mutations in the core residues all result in temperature sensitivity and all cause what appears to be a dramatic conformational change in the Swi4-Swi6 DNA binding complex is evidence that the role of these residues may be structural.

Modeling studies of our single-point mutants also show that all but one of these mutations are likely to disrupt the structure of the ankyrin repeat domain. The one exception is G347D, which may define a surface on which there is a critical interaction. This interaction is most likely to be between Swi6 and Swi4, or with another part of Swi6, as this mutant also causes a dramatic shift in the ternary complex between Swi4, Swi6, and DNA on band-shift gels. Our transcript measurements show that all of the ankyrin repeat mutants, which were initially selected for defects in SCB-driven transcription in the context of the native HO promoter, have an even more severe defect in MCB-driven transcription. This greater dependence upon the ankyrin repeat domain for Mbp1-Swi6 than that for Swi4-Swi6-dependent transcription indicates differences in the contacts within the two complexes. These residues may be identifiable with more exhaustive genetic screens.

Ankyrin repeats were initially identified as statistically significant homologies between the repeats of Swi6 and its nearest S. pombe relative, Cdc10. Ankyrin repeats were also found in the Notch protein of Drosophila and the Caenorhabditis elegans lin-12 protein, both of which are also highly related [5]. Since then, ankyrin repeats have been identified in more than 100 proteins with highly diverse functions [35]. The sequence consensus has been relaxed considerably over that time, and the discovery of new members of the family is aided as much by the fact that they are nearly always present as tandem repeats as by their sequence similarities.

The function of the ankyrin repeat has been investigated in many different systems, and several key protein-protein interactions have been shown to depend on them [36-40]. However, the repeats do not appear to interact with each other, nor has any other "signature" sequence been identified that is diagnostic of an ankyrin repeat-interacting protein. This, coupled with the fact that the proteins in which these ankyrin repeats are found have highly diverse functions and are located in a myriad of different cellular compartments (spider venoms, membrane transport proteins, and transcription factors), suggests that these repeats do not have a common function or binding partner. Rather, they should be viewed as structural units, which confer a particular type of protein fold. Gorina and Paveletich [11] have noted that ankyrin repeats form a novel L-shaped structure. Our study provides evidence that the conserved residues within each repeat are required to produce this structure, and as such, they provide a scaffold upon which different residues can be displayed. It is most likely that the nonconserved residues, which are unique to the different classes of ankyrin repeat-containing proteins, are responsible for specific interactions with other proteins and for providing the biological specificity and function to
the ankyrin repeat proteins. The conserved residues, which are the defining feature of an ankyrin repeat, may play a strictly conformational function. Thus, the ankyrin repeat may be more appropriately viewed as a novel type of protein fold which provides a stable structure with surfaces that can be tailored for many different macromolecular interactions. Our data indicate that the ankyrin repeats of Swi6 are critical for the thermostability of Swi6 and for maintaining the proper conformation of the ternary complex between Swi4, Swi6, and DNA. It is still possible that the unique faces of the Swi6 ankyrin repeat domain form a binding site for another protein that has not yet been identified. However, it is more likely that these repeats provide a rigid structure that holds the Swi4-Swi6 complex in a precise and functional spatial arrangement with respect to the DNA.

ANK repeats were first found in the Swi6 transcription factor of Saccharomyces cerevisiae and since then were identified in many proteins of eukaryotes and prokaryotes. These repeats are thought to serve as protein association domains. In Swi6, ANK repeats affect DNA binding of both the Swi4/Swi6 and Mbp1/Swi6 complexes. We have described generation of random mutations within the ANK repeats of Swi6 that render the protein temperature sensitive in its ability to activate HO transcription. Two of these SWI6 mutants were used in a screen for high copy suppressors of this phenotype. We found that MSNI, which encodes a transcriptional activator, and NHP6A, which encodes an HMG-like protein, are able to suppress defective Swi6 function. Both of these gene products are involved in HO transcription, and Nhp6A may also be involved in CLN1 transcription. Moreover, since overexpression of NHP6A can suppress caffeine sensitivity of one of the SWI6 ANK mutants, swi6-405, other SWI6-dependent genes may also be affected by Nhp6A. We hypothesize that Nhp6A and Msn1 modulate Swi6-dependent gene transcription indirectly, through effects on chromatin structure or other transcription factors, since we have not been able to demonstrate that either Msn1 or Nhp6A interact with the Swi4/Swi6 complex.

**SO WHAT?**

At the time that this research was proposed there was a real possibility and, indeed, an expectation that ANK repeats carried out a common function, that probably had to do with protein-protein interaction. These biases were guided by the discoveries of other short repeated motifs that, like SH2 domains, that were recognizable by their "signature" sequence and conferred very specific binding to another conserved class of proteins. It was our hope that if the same was true with ANK repeats, we could use the simple genetic system of yeast to identify the class of binding partner that ANK repeats associated with. Since a considerable number of defects in ANK repeat proteins had been correlated with tumor development, it was apparent that understanding the role of these repeats in molecular detail could provide new insight into oncogenesis. It was on this basis that we began this research.

Our results have shown that the conserved residues within the ANK repeat play a critical structural role in Swi6. This work and the results with other ANK repeat proteins compel a different view of these repeats, as structural units that confer a particular type of protein fold. Now, it is clear from the X-ray structures of four different types of ANK repeat proteins that their overall structure is very similar. Each of these proteins interacts with other proteins, but different surfaces of the ANK domain are engaged in these interactions. Thus, the ANK repeats represent one way to fold a polypeptide chain into a stable structure, and this structure provides a scaffold upon which different residues can be displayed. It is most likely that the nonconserved residues within the repeats are responsible for the specific protein-protein interactions of each class of ANK repeat proteins.

This new view leads me to reject my original hypothesis, that understanding the function of the Swi6 ANK repeats would lead to insight into the function of oncogenic
ANK repeat proteins. More importantly, perhaps, it also suggests that the right path to their understanding is to focus upon the nonconserved, surface residues within these oncogenic repeat proteins and search for proteins which interact with them.

REFERENCES


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Linda Breeden, Ph.D., principle investigator
Julia Sidorova, Ph.D., postdoctoral fellow
Sandra Ewaskow, M.D., medical fellow
Bernard Mai, postdoctoral fellow
Elizabeth Wayner, Ph.D, Staff Scientist
Davis Creemer, technician
Jennifer Flannery, student lab aide
James Emerson, student lab aide
Chris Mandingo, undergraduate summer research
Paul Riley, student lab aide
Katrina, Smith, student lab aide
Natalie Steele, undergraduate summer research
Mathew Tremper, student lab aide
Jessica Viles, student lab aide
Poonam Lata, student lab aide
APPENDICES

APPENDIX 1


APPENDIX 2

Mutation and Modeling Analysis of the
*Saccharomyces cerevisiae* Swi6
Ankyrin Repeats

Sandra P. Ewaskow, Julia M. Sidorova, Jörg Hendle,
J. Craig Emery, Deborah E. Lycan, Kam Y. J. Zhang, and
Linda L. Breeden

Division of Basic Sciences, Fred Hutchinson Cancer Research Center,
Seattle, Washington 98109, and Department of Biology, Lewis and
Clark College, Portland, Oregon 97219

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Mutation and Modeling Analysis of the *Saccharomyces cerevisiae* Swi6 Ankyrin Repeats†

Sandra P. Ewaskow,‡ Julia M. Sidorova,‡ Jörg Hendle,‡ J. Craig Emery,† Deborah E. Lycan,§ Kam Y. J. Zhang,‡ and Linda L. Breeden*‡

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, and Department of Biology, Lewis and Clark College, Portland, Oregon 97219

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ABSTRACT: The Swi4–Swi6 family of transcription factors confers G1/S specific transcription in budding and fission yeast. These proteins contain four ankyrin repeats, which are present in a large number of functionally diverse proteins and have been shown to be important for protein–protein interaction. However, no specific sequence has been identified that is diagnostic of an ankyrin repeat-interacting protein. To determine the function of the ankyrin repeats of Swi6, we generated both random and site-directed mutations within the ankyrin repeat domain of Swi6 and assayed the transcriptional function of these mutant *swi6* alleles. We found six single mutations, scattered within the first and the fourth repeats, that generate a temperature-sensitive Swi6 protein. In addition, we found that alanine substitutions for the most conserved residues in each repeat were highly deleterious and also confer temperature sensitivity. Most of these *swi6* alleles are able to form ternary complexes with Swi4 and DNA, but these complexes display reduced mobility in band-shift gels, suggesting a dramatic conformational change. We have modeled the ankyrin repeats of Swi6 using the coordinates derived for 53BP2 and find that, despite its low level of sequence conservation, these modeling studies and our mutation data are consistent with Swi6 having a structure very similar to that of 53BP2. Moreover, all but one of our single mutants and all of the site-directed mutants disrupt critical structural features of the predicted folding pattern of these repeats. We conclude that the ankyrin repeats play a major structural role in Swi6. Ankyrin repeats are unlikely to have inherent protein or DNA binding properties. However, they form a characteristic and stable structure with surfaces that may be tailored for many different macromolecular interactions.

The ankyrin–Swi6–Cdc10 repeat was first identified in a family of yeast transcription factors (1, 2). Included within this family are the *Schizosaccharomyces pombe* transcription factors Cdc10, Res1, and Res2 and the *Saccharomyces cerevisiae* transcription factors Swi6, Swi4, and Mbp1 (Figure 1). Members of these groups associate with one another through their C termini (3, 4). The target genes for these transcription factors are expressed at the G1/S transition in yeast and include DNA synthesis genes (5, 6), *HO* endonuclease (7), and the G1 cyclins (8, 9) that are required for progression through the yeast mitotic cell cycle. Swi6 is one of the transcription factors that regulates gene expression during G1/S, either as a complex with Mbp1, with which it binds MCB [MluI cell cycle box (ACCGTTG)] DNA elements (5, 6, 10), or in combination with Swi4, with which it binds SCB [SWI4/6-dependent cell cycle box (CAGGAAAA)] elements (11–13) as well as MCB-like elements (14). Swi6 is not known to bind DNA directly; rather, both Mbp1 and Swi4 confer the DNA binding ability to the complex through their N termini.

A common central motif consisting of four ankyrin repeats is present among all the known G1/S specific yeast transcription factors. Ankyrin repeats typically occur as four or more continuous copies of a 33-amino acid sequence characterized by the consensus sequence ----t-G-o-LHφAφ----tt-xφφx—LX—t---- (Figure 1), where t indicates a residue frequently found in turns, x a polar residue, φ a hydrophobic residue, o a serine or threonine residue, and capital letters indicate highly conserved amino acids (15). A further defining feature of the yeast transcription factor ankyrin repeats is the central core region, denoted by the consensus sequence G-T-L (Figure 1). Ankyrin repeats are present in a large number of functionally diverse proteins and have traditionally been considered sites of protein–protein interaction. Circular dichroism and NMR studies of the tumor suppressor protein p16INK4A, which consists of four ankyrin repeats, have confirmed the predominantly α-helical nature of the ankyrin repeat region (16). The crystal structure of the p53 core domain bound to 53BP2 reveals that a single ankyrin repeat structurally consists of one β-hairpin and two α-helices (17).

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‡ To whom correspondence should be addressed: Fred Hutchinson Cancer Research Center, Division of Basic Sciences, A-2, 1100 Fairview Ave. N., Seattle, WA 98109-1024. Phone: (206) 667-4484. Fax: (206) 667-6526. E-mail: lbreeden@fhcrc.org.

§ Fred Hutchinson Cancer Research Center.

‖ Present address: EMBL Hamburg Outstation, c/o DESY, Bldg. 25A, Notkestrasse 85, D-22603 Hamburg, Germany.

¶ Lewis and Clark College.

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Ankyrin/Swi6/Cdc10 repeat

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EXPERIMENTAL PROCEDURES

PCR Mutagenesis of the Swi6 Ankyrin Repeats. Oligonucleotide primers BL58 (2.5 𝜇M) (5’CCTGTAGATGAGCATGG3’) and BL202 (2.5 𝜇M) (5’CCAAATCCCCAGGCTCT3’) were used to perform mutagenic PCR in the presence of 5.0 mM deoxynucleotide triphosphate and either 0.5 mM (screen 1), 0.25 mM (screen 2), or 0.1 mM (screen 2) manganese chloride to facilitate nucleotide misincorporation (22). A Swi6 CEN URA3+ plasmid pBD1378 (0.05 𝜇M) (23) was the double-stranded DNA template. To produce single-stranded templates from the double-stranded mutagenized PCR product, 25 rounds of PCR were performed using kinased primer BL202 (2.5 𝜇M) and 1 𝜇L of the double-stranded mutagenized PCR library product in a 50 𝜇L reaction volume. The resulting single-stranded mutagenized product was then annealed to a single-stranded SW6 DNA template produced in the Δu+Δn+ Escherichia coli strain (24). The heteroduplex plasmids were then introduced into a Δu+Δn+ E. coli strain to propagate, generating the high (0.5 mM MnCl2), middle (0.25 mM MnCl2), and least (0.1 mM MnCl2) mutagenized libraries. We isolated the mutagenized plasmids from E. coli using the alkaline plasmid prep procedure (25) and then introduced the plasmids into the swi6 deletion strain BY600 (MATa, his3Δ1, leu2Δ1, ura3Δ1, trp1Δ1, can1Δ100, metα) (MATa, his3Δ1, leu2Δ1, ura3Δ1, trp1Δ1, can1Δ100, metα) using the lithium acetate method of transformation (26) and isolated those mutants that grew on uracil-deficient agar. Strains bearing the mutant swi6 alleles were constructed by targeted integration (27) of the BY600 strain with linearized pRS305 plasmids carrying the HndIII–SmaI fragments of swi6. Both rich (YPD) and minimal, selective (YC) growth conditions were as described previously (28).

Generation of Site-Directed Mutants. Mutant plasmids pBD1819 and pBD1822 were generated from a SacI digest of pBD1028 and pBD1029, respectively, followed by ligation of the 1.5 kilobase fragment into the SacI-digested pRS316. The remainder of the site-directed mutants were generated through substitution of the indicated core residues using oligonucleotide-directed mutagenesis of a single-stranded SW6 template (pBD1378) (24). The first ankyrin core region was mutated using primer Bd1035 (mutant base pairs are underlined) (5’CTGTATGAGATGGACAAAATGCGTCTGCAGTTGCGGTTGCGTGCTCATCTAC3’), the second using primer BL86 (5’GTCTTATCAGCGTCCGTGATTTGACCC3’), the third using primer BL87 (5’GTATGAGATGGACAAAATGCGTCTGCAGTTGCGGTTGCGTGCTCATCTAC3’), and the fourth using primer Bd1036 (5’CTGTGCAATGGGTTGACAAAGCATGCGATTTGACCC3’). Identification of the Temperature-Sensitive Mutant Plasmids. BY600 (swi6Δ::TRP1−197, his3Δ1) was transformed with the PCR-generated libraries and grown on YC-ura plates at 25 °C, then replica plated, and grown at both 25 and 37 °C. The colonies were transferred to nitrocellulose filters and assayed for β-galactosidase activity as described (21). The his3Δ1 reporter construct for the blue-white screen has been described previously (21). Transformers that were blue at 25 °C and white at 37 °C were selected, as well as cells bearing null alleles, which were white at both 25 and 37 °C. Mutant plasmids were then isolated from the yeast strain and reintroduced into the BY600 (swi6Δ::TRP1−197) strain to confirm the dependence of the temperature-sensitive blue-white phenotype on the plasmid-borne swi6 gene.

To identify mutant plasmids that had significant defects in Swi6 protein stability, extracts were prepared from the temperature-sensitive swi6 mutants after incubation at 37 °C for 10–12 h. The differences in Swi6 levels are pronounced in some cases and could be contributing to the defective phenotype of ankyrin repeat mutants. Thus, the temperature-sensitive mutants that were selected for further characterization were those which maintain near wild-type levels of Swi6 protein and are likely defective due to a loss of function at high temperatures.

Generation of Yeast Strains. Yeast strains BY2214 through BY2223 were generated by ligating the 2.8 kilobase HindIII- and SmaI-digested swi6 fragment from the mutagenized URA3+ pRS316 construct into the LEU+ pRS305 vector (29). The pRS305 vector was then linearized by digesting with HpaI (or NarI for BY2220, -2214, -2215, -2216, and -2217) and translocated into the chromosomal leu2 locus of the BY600 (swi6Δ) strain using targeted integration as described previously (27). Integrated mutants were placed under LEU selection, and transplacement was confirmed with both PCR and sequencing.

Immunoblotting. Yeast cell cultures were grown overnight at 30 °C in selective media to an OD600 of <0.5, then diluted to an OD600 of 0.2, and split and grown at either 25 or 37 °C for up to 10–12 h (OD600 of 0.4–0.5). Protein extracts were made by lysing the cells with glass beads under hypotonic conditions in extract buffer [100 mM Tris (pH 8.0), 20% glycerol, 0.1% Triton X-100, and 1 mM EDTA] in the presence of β-mercaptoethanol (10 mM) and protease inhibitors [PMSF (1 mM), leupeptin (1 μg/mL), and pepstatin A (1 μg/mL)]. The protein concentration of each extract was measured using the Bradford assay (30), and 100–200 μg of protein from each sample was boiled in SDS buffer [62 mM Tris-HCl (pH 6.8), 10% glycerol, 5 mM β-mercaptoethanol, and 3% SDS] and then run on a 10% polyacrylamide gel for 2.5–3 h. After semidry transfer of the protein to a nitrocellulose filter (MSI), the filter was blocked with TBST buffer [50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.05% Tween 20] containing 5% nonfat dry milk for 2 h. The filter was then probed with a rabbit polyclonal Swi6 antibody (1:700) and a goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (1:4000). The protein bands were detected using the ECL Western blotting detection system from Amersham.

Band Shifts. The plasmid pBD972 was used for in vitro translation of Swi6. It contains a 700 bp CITE (31, 32) and myc tag sequence insertion of the SWI4 ATG codon and was constructed by inserting the SWI4 gene into pBD939, which is a pBKS+ vector with the CITE and myc tag sequence insertions between the ApaI and EcoRI restriction sites. To generate the wild-type Swi6 in vitro translation construct, pBD972 was used as a template for primers BL139 (5’CTCGAGTCTCCAGTTGAGGCGCC’ and the M13 reverse primer to generate a 700 bp fragment containing the CITE sequence. After the PCR product was cloned into the pCRII vector (Invitrogen) (pBD2158), the CITE fragment was released by XhoI digestion and substituted for the 300 bp XhoI fragment of pBD1378, generating the wild-type Swi6 CITE construct, pBD2090. Mutant allele swi6Δ−405 (pBD2091) and swi6Δ−406 (pBD2094) CITE constructs were
showing positions of residue substitutions. (A) Eleven PCR-generated temperature-sensitive mutants were isolated from the
0.5 mM MnCl₂ library after a single round of screening. (B) The second group of mutants (screen 2) was isolated from both the 0.25 and the 0.1 mM MnCl₂ libraries. Only one of the mutations was isolated from this screen. It was isolated four times as a single mutation and had been previously identified as a temperature-sensitive mutation in a parent plasmid that had been previously identified in either screen 1 or 2. The residues in parentheses indicate the additional mutations originally identified in the parent plasmid (described in the text). (C) Mutants swi6-420, -421, -422, and -423 and R344G substitutions (screen 3). One R344G substitution was isolated from this screen. The arrows at the top of the figure indicate single-residue substitutions that confer a temperature-sensitive mutation in the Swi6 protein sequence.
FIGURE 3: Band-shift comparison of SWI6+ and mutant alleles using both in vitro translated proteins and whole cell extracts. (A) This band-shift gel shows comparable band-shift patterns with regard to upper and lower complex formation between a whole cell extract derived from a wild-type strain (lane 4) and in vitro translated Swi4 and Swi6 protein products (lane 1). A 130 bp fragment of the HO promoter, -503 to -374, was used as the DNA probe for the band-shift experiment which was performed at 25 °C. Lanes 1–3 contain proteins that were translated in vitro. Lanes 4 and 5 contain proteins expressed in whole cell extracts. Lane 1: Swi4 (pBD972) and Swi6 (pBD2090). Lane 2: Swi4 and swi6–405 (pBD971) (mutant protein showing both the upper and lower complex at 25 °C). Lane 3: Swi4 and swi6–406 (pBD2094) (mutant protein showing a predominantly upper complex). Lane 4: wild type [BY600 transformed with SWI6+ (pBD1378)]. Lane 5: swi6–406 (pBD2046) expressed on a low-copy plasmid in the swi6Δ strain, BY600. (B) Both Swi4 and Swi6 are produced at comparable levels after in vitro translation (described in Experimental Procedures) at 30 °C. Lane 1: SWI6+ (pBD2090). Lane 2: swi6–405 (pBD2091). Lane 3: swi6–406 (pBD2094). All other Swi6 mutants show similar expression levels in this reaction.

parallel with those produced from whole cell extracts. The resulting band-shift patterns are qualitatively similar [compare lanes 1 and 3 of Figure 3A (in vitro translation products) to lanes 4 and 5 (whole cell extracts)]. Since in vitro translated Swi4 and Swi6 were the only yeast proteins added to the reaction mixtures in lanes 1–3, it is unlikely that the tendency of the swi6–406 ankyrin repeat mutant to form upper complexes is due to binding of additional proteins to the DNA–protein complex. It is also unlikely that differences in protein modification are responsible, since the proteins are translated in a rabbit reticulocyte lysate system. The possibility that proteins in this lysate could bind or modify the yeast protein–DNA complex cannot be excluded, but to produce these results, the protein has to be specific for the swi6 mutant complexes. The protein would also need to be present in both rabbit and yeast cells, because the band shift is identical whether the mutant protein is translated in vitro or harvested from yeast.

To observe the extent of variation in DNA binding activity, the collection of temperature-sensitive ankyrin repeat mutants of Swi6 were translated in vitro and surveyed for DNA binding activity at two temperatures. Most of the Swi6 mutants retained the ability to complex with Swi4 and bind SCB elements at the nonpermissive temperature. Frequently, however, the mobility of the ankyrin mutant complex was noticeably altered (Figure 4) and migrated as an upper complex that could not be distinguished from that of swi6–406. The wild-type (SWI6+) band-shift pattern shows the predominant lower band at 25 °C and a more dispersed pattern, including both upper and lower complexes, at 37 °C. From the band-shift assay, the swi6 mutants can be grouped into four categories. The first group of mutants [swi6–406, –407 (G347D), and –401] all form the upper complex predominantly at both the permissive (25 °C) and nonpermissive (37 °C) temperatures. The second group of mutants [swi6–405 and swi6–410 (H3233R), –422, –421, –424 (R344G), –402, and –417 (A477T)] form the upper complex at the nonpermissive temperature and both the upper and lower complex at the permissive temperature. The third phenotype is overall reduced binding, even at the permissive temperature, seen with mutants swi6–401, –417, and –422. The fourth group of mutants [swi6–409 (N500T), –415, –420, –423, and –418 (N500Y)] show no binding defect at either temperature. Most of the mutants show altered binding characteristics with a propensity to form the upper complex, which is exacerbated by elevated temperatures. A minority have reduced overall DNA binding. Formation of the upper complex therefore correlates with the loss of transcriptional activation, which is also enhanced at high temperatures. Despite the considerable variability in the DNA binding complexes formed, the upper complex that most of the mutants display migrates at a very similar position in the band-shift gel. Since this is not likely to be due to changes in protein composition or modification states,
we speculate that it may be the result of a global change in the conformational state of the Swi4—Swi6 complex which these mutations induce to varying degrees.

Effects of Alanine Substitutions in Core Residues of the Ankyrin Repeats of Swi6. To assess the importance and function of the individual ankyrin repeats, and because the significance of the middle repeats was still a question, we created mutations at equivalent sites in each of the four repeats. These mutations, substitution of alanines for each of the core residues (G-T-L in repeat 1 and 4, G-S-L in repeat 2, and N-T-L in repeat 3), eliminated the three most conserved residues in the repeats. In so doing, we hoped to completely disrupt the function of the conserved residues in each repeat in a similar manner. In addition, we created various combinations of the individual core region substitutions to determine whether any of these combinations would have additive effects. Immunoblots were performed on these strains as described earlier. Each of these site-directed mutants produces a detectable Swi6 protein product at 37 °C, although, like those of the PCR-generated mutants, the
protein levels tend to be lower than that of the wild-type control. One surprising observation is that the mutants containing alanine substitutions within multiple cores attain a higher level of Swi6 protein than those mutants in which a single core region is alanine substituted (data not shown).

Using an integrated ho:lacZ reporter construct, we performed β-galactosidase assays to determine the transcriptional activity of these site-directed mutants. All of the core mutants displayed a temperature-sensitive phenotype in our most sensitive X-gal filter assay (21) of ho:lacZ expression (data not shown), and these results were confirmed by quantitative assays (Table 2). The four mutants that contain only one alanine-substituted core were all defective in ho:lacZ expression at the nonpermissive temperature. In the context of the native HO promoter, the fourth repeat appears to be the most critical for HO, or SCB-driven, transcription, showing severely defective transcriptional activity at both permissive and nonpermissive temperatures. There is no suggestion that any one of these repeats performs a function that is redundant. In addition, the fact that all of these mutants are temperature-sensitive suggests that the conserved residues within each repeat play critical roles in stabilizing the native structure of the Swi6 protein.

Another surprising property, revealed by combining these core substitution mutants, is that the swi6−1234 allele, which contains mutations within the core residues of all four ankyrin repeats and was expected to be the most defective, has the highest level of β-galactosidase activity when compared with the other site-directed mutants (70% at 25 °C and 21% at 37 °C) using the ho:lacZ reporter construct. In contrast, substitutions within only the fourth core produce a highly defective protein, with no discernible β-galactosidase activity at the same temperatures. Apparently, substitutions within the three additional core regions produce a stabilizing or compensatory effect. The addition of substitutions in the first, second, and third core regions in varying combinations with the fourth also appears to increase activity compared to that seen with the single fourth core substitution at the permissive temperature, but not to the same extent as swi6−1234. One plausible explanation of this high level of activity could be that the ankyrin repeats are targets of negative regulation. In that case, loss of all four repeats would eliminate all targets of this repressive activity and would result in increased ho:lacZ expression. We cannot exclude this possibility, but we have monitored ho:lacZ transcription through the cell cycle in the swi6−1234 strain and have found it to be cell cycle-regulated (data not shown). This indicates that negative regulation during the cell cycle still persists.

To measure the DNA binding defect associated with the core substitution mutants, we employed in vitro translation and band-shift assays as before, incubating the reaction mixtures at both 25 and 37 °C. As stated above, the wild-type Swi4–Swi6 complex exists as a combination of upper and lower complexes on SCB DNA, with a predominant lower complex at 25 °C. The core mutants all form the upper complex exclusively at both temperatures (Figure 5, 25 °C experiment shown). The ability to form the upper band-shift complex did not localize to mutations within a particular core region. Thus, even more so than with the PCR-generated repeat mutants, we see a dramatic but uniform shift in mobility of the DNA binding complex in the core mutants of Swi6. This, and the temperature sensitivity of all these mutants, suggests that the conserved core residues of all four repeats are important for maintaining the native structure of Swi6 and/or the Swi4–Swi6 complex.

Differential Effects upon SCB- versus MCB-Directed Transcription. We originally isolated the PCR-generated mutants because they were defective in transcription from the predominantly SCB-driven ho:lacZ promoter, so it was
Figure 6: ONPG assay results for SCB and MCB reporter constructs. Using three tandem SCB (CACGAAAA) or MCB (ACGCCT) DNA binding elements driving the LacZ reporter, we measured the transcriptional activity (details in Experimental Procedures) of selected temperature-sensitive mutants. The activity is reported as a percent of the wild-type activity. The wild-type SW6 gene and each of the mutant alleles were on a low-copy plasmid in the presence of the reporter construct, which was expressed from a 2 μM plasmid.

of interest to measure the activities of these mutant proteins in MCB-driven transcription, which is activated by Swi6 bound to another partner, Mbp1 (5, 6, 10). To allow a direct comparison between SCB and MCB activation, we introduced a high-copy reporter construct carrying three tandem repeats of either MCB or SCB elements into the BY660 (ho, swi6::TRP1–197) strain carrying the mutant swi6 allele on a low-copy plasmid (Figure 6). This enabled us to compare SCB and MCB activation within an equivalent context. Figure 6 shows that most of the PCR-derived and site-directed mutants cause a more severe MCB transcriptional defect when compared with SCB transcriptional activity. The most notable differences are seen with swi6–1234, which has 44% activity on SCBs and 0% on MCBs, swi4–124, which has 40% SCB activity and 0% MCB activity, and swi6–421, which has 48% of the wild-type SCB activity compared with 14% of the wild-type MCB activity. In addition, there are a number of mutants (swi6–1000, -200, -123, -405, -417, and -424) that show comparably decreased levels of both SCB and MCB activity when measured from these analogous promoter constructs. The fact that the vast majority of ankyrin repeat mutants are not equivalently defective with respect to MCB and SCB activation indicates there are differential effects upon the Swi4–Swi6 and Mbp1–Swi6 transcription complexes. This is somewhat surprising since the C termini, and not the ankyrin repeats of these proteins, are sufficient for the interaction between Swi6 and Swi4 (3, 4) and between Swi6 and Mbp1 (9). Interestingly, all of the mutants are more defective in MCB activation than in SCB activation, despite the fact that they were selected for their inability to act at the HO promoter, which is activated by SCB elements. This suggests that the Swi6–Mbp1 interaction or activity has a stronger dependence either upon the ankyrin domain itself or upon other residues that are brought into proper position by the ankyrin domain structure.

Modeling Studies of the Ankyrin Repeat Mutations. The recent publication of the crystal structure of a p53 binding protein, 53BP2, which contains four ankyrin repeats (17), has enabled us to estimate the positions of the ankyrin repeat mutations of Swi6 that we have generated. First, the four ankyrin repeats of Swi6 and 53BP2 were aligned with 24 other ankyrin repeat domains using CLUSTAL-W (36). This multiple alignment provided a reliable means of aligning the two proteins' ankyrin repeats despite their low sequence identity (21%). Then, the coordinates for the 53BP2 structure, generously provided by N. P. Pavletich, were used in conjunction with the MODELLER program (37) to generate and refine a model of the Swi6 ankyrin domain. This model was strikingly similar to the 53BP2 structure. It was validated by three independent methods [PROCHECK, VERIFY-3D, and ERRAT (see the Figure 7 legend)], and all three indicated a high degree of reliability. The structure that 53BP2 adopts, and the one that the modeled Swi6 conforms to, resembles an "L" in profile, with continuous, tightly packed α-helices perpendicular to and connected by short β-hairpins. A ribbon diagram of the Swi6 ankyrin model structure is provided in Figure 7. The locations of the core residues and five single-point mutants are labeled. The regularity of this repeat structure is apparent in this view, as is the conserved positioning of the core residues in each repeat.

Alanine substitutions were made in the three most conserved core residues of the Swi6 ankyrin repeats in an effort to completely eliminate whatever function this repeating and conserved structure might have. These substitutions clearly disrupt Swi6 function, but because there are three changes in the structure in each repeat, it is not possible to be certain which of these substitutions is the most disruptive. However, sequence comparison with other ankyrin repeat-containing proteins, as well as inspection of the model of the Swi6 structure, suggests that the third substitution within the cores, which substitutes alanine for leucine at positions L322, L355, L392, and L474, is the most disruptive. First, while the glycine and threonine are conserved within the Swi6 family, these specific residues are not conserved in the ankyrin repeat family as a whole. In contrast, the leucine is highly conserved among the hundreds of ankyrin repeats that have been identified (15). In addition, inspection of the
Swi6 model shows that these leucines are part of a hydrophobic core which is likely to play a critical role in stabilizing the four α-helix bundles that are the predominant feature of the Swi6 ankyrin domain. The first of these three core leucines, L322, lies at the interface of four α-helices and is spaced appropriately to form tight hydrophobic interactions with three other hydrophobic residues in the adjacent helices (V334, V338, and L373). Leucine is often found to be a preferred residue in leucine zippers, coiled coils, and other structures in which α-helices are tightly packed (38), and in ankyrin repeats, they may also play a critical role in stabilizing the helix bundles.

Four ankyrin repeats occur in tandem within the yeast transcription factor family, and the four full repeats have been modeled on the basis of the structure of another protein which also contains four repeats. N500, one of the residues shown to be critical for Swi6 function in this analysis, is adjacent and C-terminal to the four-repeat structure. This asparagine, and the glycine that follows these residues downstream, are the residues that would be expected to begin a fifth repeat. However, the residues that follow do not conform to the consensus sequence. The significance of this partial fifth repeat is unclear, but the fact that it maintains appropriate spacing and sequence conservation within this family suggests that it may be important in the overall domain structure. This is corroborated by the fact that point mutations in N500 have been found which disrupt Swi6 function. In addition, there is another ankyrin repeat containing protein in yeast, Yar1, which also ends its ankyrin repeat sequence in the same manner (39).

Among the PCR-generated ankyrin repeat mutants, the most commonly identified mutation, isolated 10 times from three independent screens, was at the glycine G347 position and all were substitutions of glycine for aspartate (D). G347, as pictured in Figure 7, lies on the back surface of the modeled structure, in the β-hairpin connecting ankyrin...
repeats 1 and 2. As such, changes at that position are unlikely to destabilize the structure itself. Rather, the loss of function that this mutation causes suggests that this residue may be part of a surface on which there is a critical interaction with another protein, or with another domain of the Swi6 protein. This is also likely to be the case for the gain of function mutation, N330K, which does not confer a conformational change to the modeled Swi6 ankyrin repeat.

The four other single mutations generated in this study that resulted in temperature-sensitive Swi6 proteins are all predicted to result in unfavorable interactions within the ankyrin repeat itself when modeled onto the structure. These findings help to validate the model and give us new insight into the key intramolecular interactions that occur in this structure. The core leucine (L) at position 474 changed to serine (S), and alanine (A) 477 changed to threonine (T) (Figure 8A); both disrupt the tight hydrophobic packing that is predicted to occur within neighboring residues. In the case of histidine (H) 323 (Figure 8B), which is replaced with arginine (R), the hydrogen bonds predicted to be formed by the histidine with aspartate (D) 348 are disrupted and the larger arginine side chain would be expected to perturb the local structure due to steric hindrance. Finally, the space filling models depicted in panels A and B of Figure 9 clearly show the effect predicted by substitution of R344 for glycine (G). In the model, the loss of the bulky side chain of the arginine creates a cavity on the surface of the structure which exposes the hydrophobic side chains of L322 and L373. This cavity is large enough to make this region accessible to water and would be expected to destabilize the structure.

DISCUSSION

Within the four ankyrin repeats in this family of transcription factors, three levels of conservation are observed. There are "core" residues, which are identical, as well as residues with chemical similarities that are conserved throughout all four of the repeats. Second, there are specific residues which are shared by repeats 1 and 4, making them much more similar to each other than the other repeats in these or any other set of ankyrin repeat proteins. Third, there are residues which are conserved within the individual repeats of all of the family members, but which differ widely between repeats. This study provides evidence that the core residues of all four of the repeats are important, but whether this is due to a structural requirement for a four-repeat domain or whether these repeats actually have different roles is unclear. The fact that the mutations in the core residues all result in temperature sensitivity and all cause what appears to be a dramatic conformational change in the Swi4-Swi6 DNA binding complex is evidence that the role of these residues may be structural.

Modeling studies of our single-point mutants also show that all but one of these mutations are likely to disrupt the structure of the ankyrin repeat domain. The one exception is G347D, which may define a surface on which there is a critical interaction. This interaction is most likely to be between Swi6 and Swi4, or with another part of Swi6, as this mutant also causes a dramatic shift in the ternary complex between Swi4, Swi6, and DNA on band-shift gels. Our transcript measurements show that all of the ankyrin repeat mutants, which were initially selected for defects in
SCB-driven transcription in the context of the native HO promoter, have an even more severe defect in MCB-driven transcription. This greater dependence upon the ankyrin repeat domain for Mbp1—Swi6 than that for Swi4—Swi6-dependent transcription indicates differences in the contacts within the two complexes. These residues may be identifiable with more exhaustive genetic screens.

Ankyrin repeats were initially identified as statistically significant homologies between the repeats of Swi6 and its nearest S. pombe relative, Cdc10. Ankyrin repeats were also found in the Notch protein of Drosophila and the Caenorhabditis elegans lin-12 protein, both of which are also highly related (1). Since then, ankyrin repeats have been identified in more than 100 proteins with highly diverse functions (15). The sequence consensus has been relaxed considerably over that time, and the discovery of new members of the family is aided as much by the fact that they are nearly always present as tandem repeats as by their sequence similarities.

The function of the ankyrin repeat has been investigated in many different systems, and several key protein—protein interactions have been shown to depend on them (40–44). However, the repeats do not appear to interact with each other, nor has any other “signature” sequence been identified that is diagnostic of an ankyrin repeat-interacting protein. This, coupled with the fact that the proteins in which these ankyrin repeats are found have highly diverse functions and are located in a myriad of different cellular compartments (spider venoms, membrane transport proteins, and transcription factors), suggests that these repeats do not have a common function or binding partner. Rather, they should be viewed as structural units, which confer a particular type of protein fold. Gorina and Pavletich (17) have noted that ankyrin repeats form a novel L-shaped structure. Our study provides evidence that the conserved residues within each repeat are required to produce this structure, and as such, they provide a scaffold upon which different residues can be displayed. It is most likely that the nonconserved residues, which are unique to the different classes of ankyrin repeat-containing proteins, are responsible for specific interactions with other proteins and for providing the biological specificity and function to the ankyrin repeat proteins. The conserved residues, which are the defining feature of an ankyrin repeat, may play a strictly conformational function. Thus, the ankyrin repeat may be more appropriately viewed as a novel type of protein fold which provides a stable structure with surfaces that can be tailored for many different macromolecular interactions. Our data indicate that the ankyrin repeats of Swi6 are critical for the thermostability of Swi6 and for maintaining the proper conformation of the ternary complex between Swi4, Swi6, and DNA. It is still possible that the unique faces of the Swi6 ankyrin repeat domain form a binding site for another protein that has not yet been identified. However, it is more likely that these repeats provide a rigid structure that holds the Swi4—Swi6 complex in a precise and functional spatial arrangement with respect to the DNA.

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The MSNI and NHP6A Genes Suppress SWI6 Defects in Saccharomyces cerevisiae

Julia Sidorova and Linda Breeden
Fred Hutchinson Cancer Research Center, Seattle, Washington 98109
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ABSTRACT

Ankyrin (ANK) repeats were first found in the Swi6 transcription factor of Saccharomyces cerevisiae and since then were identified in many proteins of eukaryotes and prokaryotes. These repeats are thought to serve as protein association domains. In Swi6, ANK repeats affect DNA binding of both the Swi4/Swi6 and Mbp1/Swi6 complexes. We have previously described generation of random mutations within the ANK repeats of Swi6 that render the protein temperature sensitive in its ability to activate HO transcription. Two of these SWI6 mutants were used in a screen for high copy suppressors of this phenotype. We found that MSNI, which encodes a transcriptional activator, and NHP6A, which encodes an HMG-like protein, are able to suppress defective Swi6 function. Both of these gene products are involved in HO transcription, and Nhp6A may be also involved in CLN1 transcription. Moreover, because overexpression of NHP6A can suppress caffeine sensitivity of one of the SWI6 ANK mutations, swi6-105, other SWI6-dependent genes may also be affected by Nhp6A. We hypothesize that Nhp6A and Msn1 modulate Swi6-dependent gene transcription indirectly, through effects on chromatin structure or other transcription factors, because we have not been able to demonstrate that either Msn1 or Nhp6A interact with the Swi4/Swi6 complex.

THE Swi6 protein of Saccharomyces cerevisiae is involved in the regulation of dozens of genes that are transcribed at the G1/S transition of the cell cycle. These include the genes encoding the HO endonuclease, G1 cyclins (Nasmith and D'Urso 1991; Tollefson et al. 1991; D'Urso et al. 1992; Measday et al. 1994), and many of the genes involved in DNA replication (McIntosh et al. 1988; Lowndes et al. 1991, 1992; D'Urso et al. 1992). Swi6 associates with at least two DNA-binding factors, Swi4 and Mbp1. These associations are mediated by the C-terminal domains of the proteins (Andrews and Moore 1992b; Primmer et al. 1992; Koch et al. 1993; Sidorova and Breeden 1993), and the N termini of Swi4 and Mbp1 confer the DNA-binding specificity to these complexes. The Swi4/Swi6 complex binds to SCB (CAGGAAA) elements in CLN2, HO, and PCL1 (PHO85 CgCIn) and MCB-like elements of CLN1 (ANDREWS and Moore 1992a; Primmer et al. 1992; Partridge et al. 1997). The Mbp1/Swi6 complex binds to MCB (ACCGGTnA) elements (McIntosh et al. 1991; Lowndes et al. 1992; Koch et al. 1993).

Swi4, Mbp1, Swi6, and the S. pombe homologues Res1, Res2, and Cdc10 contain four ankyrin (ANK) repeats. These repeats are degenerate 33 amino acid motifs that are found in tandem in many different proteins in both eukaryotes and prokaryotes (Bork 1993). In several cases ANK repeats have been shown to be involved in protein-protein association (Thompson et al. 1991; Henkel et al. 1992; Lambert and Bennett 1993; Schröder et al. 1994). The Swi6 ANK repeats are critical for its function, but their role is unclear. Our modeling analysis of Swi6 ANK repeats (Ewasko et al. 1998) suggests that they may represent a new protein fold that supports active conformation of Swi6 complexes and/or properly displays protein association interfaces.

We have generated numerous ANK repeat mutations in Swi6 that render a transcriptionally inactive protein (Sidorova and Breeden 1993; Ewasko et al. 1998). At the biochemical level, these mutations can lead to a reduced DNA binding, but most also cause a significant shift in the mobility of Swi4/Swi6 complexes in mobility shift gels (Ewasko et al. 1998). The latter suggests the possibility that the DNA-bound Swi4/Swi6 complex can undergo a significant conformational change. Furthermore, it is possible that there are accessory proteins that may modulate this change to affect transcriptional activity of the Swi4/Swi6 complex.

In this work we used two of the temperature-sensitive ANK alleles of SWI6 in a high copy suppressor screen to find proteins needed for the full activity of the Swi4/Swi6 complex. We report the results of this screen and the further analysis of two of these high copy suppressors, NHP6A and MSNI.

MATERIALS AND METHODS

Strains and plasmids: The BY600 MATa swi6::TRP1 ade his3::LacZ uRA3 his3::LEU2 ade2::LacZ ura3::His3 leu2-3,112 trp1-1 can1-100 met2 and BY606 MATa swi6::LEU2 ade2::LacZ ura3::His3 leu2-3,112 trp1-1 can1-100 met2 strains are described in Sidorova and Breeden (1993). The BY1998 strain MATa his3Δ200 ura3-52 by2-801...
The plasmid pSBW16 (pBD1378) was described previously (Sidorova et al., 1995), pSW16-406 (pBD2046) and pSW16-405 (pBD2051) are analogs to pBD1378, but contain mutated alleles of SW16. Positions of these and other mutations in the SW16 alleles used in this study are listed in Table 2. Strains BY1954 swi6A LEU2:swi6-405, BY1956 swi6A LEU2:swi6-406, and analogous strains bearing other ANK mutations were constructed by integrative transformation of BY600 strain (Sidorova and Breeden 1993) with linearized pRS305 plasmids carrying SW6 DNA (HenIII to Smal fragments). High copy suppressor subclones were generated in pRS426 or pZC122 2µa vector backgrounds. Subcloned suppressors were sequenced with M13 universal or reverse primers and library clones borne on YEp24 vector were sequenced with primer BL1465 'ACTAGCGGATCAGG3'. The plasmid pBD2068 was a gift from M. Snyder and contains an HA-tagged NHp6A on the YEp352 vector (Costigan et al. 1994). The plasmids pDK267 and pRZ268 were prepared by D. Kolodrubetz. pDK267 contains NHp6B sequence flanked by 700 bp of genomic sequence on either side, cloned into EcoRI-HindIII-digested YEp352. pRZ268 carries a 1.8-kb EcoRI-PstI fragment containing the NHp6A gene and flanking sequences cloned into YEp352.

Growth conditions: All rich (YPD) and minimal (YP) media and growth conditions were as described previously (Breeden and Mikesell 1991). Temperature-sensitive ANK mutant strains were cultivated at 30° and shifted to 37° for 8–12 hr when grown in liquid media. When grown on plates, they were incubated at 37° for the whole period of growth.

DNA, RNA, and protein analysis: FACS analysis of yeast cells was done as described in (Heicham and Roberts 1996) and data were analyzed using CellQuest software. Procedures for RNA isolation and S1 protection were performed as described previously (Breeden and Mikesell 1991). Protein extract preparation, immunoprecipitation, and Western blotting were done as described before (Sidorova and Breeden 1993; Sidorova et al. 1995).

In vitro transcription and translation: The plasmid pBD972 was used for in vitro translation of Swi4 (Ewaskow et al. 1998). pBD972 was added to a TNT rabbit reticulocyte lysate coupled transcription translation system (Promega, Madison, WI) along with 20–50 µg of the recombinant Swi6 purified from Schizosaccharomyces cerevisiae (Sidorova and Breeden 1993). Reactions were carried out according to manufacturer's recommendations with cold amino acids. Reaction products were added directly to HO promoter DNA-binding reactions or loaded onto SDS PAGE.

GST fusion and purification from yeast or bacterial cells: To construct GST fusions, MSN1 and NHp6A were generated by polymerase chain reaction (PCR) from pM4 (pBD2050) and pN5 (pBD2055), respectively, using M13 reverse primer and BL1385 'GGATCCATGTCACCCCGCAAG3' primer for NHp6A, and M13 reverse and BL1357 'GGGATCCATGTCGGGCAAG3' primers for MSN1. PCR fragments were cloned into pGRII (Invitrogen, Carlsbad, CA) generating pBD2056 and pBD2059, respectively. For construction of the GST-NHP6A fusion, the 2.9-kb BamH1 fragment with the 2.5-kb BamH1 digest with the 2.9-kb EcoRV fragment of pBD2057, containing a portion of the URA3 gene and GAL-GST-NHP6A, into EcoRV-cut pBD2055; the resulting construct is called pBD2063.

For the GST-MSN1 fusions (pBD2061 and 2062), first the 1.5-kb SnaI fragment from pBD2049 was substituted for the ScaI fragment in pBD2058. Then the 1.4-kb BamH1 fragment of the resulting pBD2060 containing MSN1 was cloned into BamHI-cut pBD1905, to give rise to pBD2061, or into BamHI-cut pGex2T (Pharmacia), to give rise to pBD2062.

To purify GST fusions from E. coli, pBD2064 and pBD2062 were transformed into DH5α cells and the resulting strain were treated according to Pharmacia Biotech Gene Fusion System protocols. Bacterial cultures were grown to OD 0.6, and fusion protein expression was induced by 0.1 mM isopropyl thiogalactoside for 2 hr. Cells were then harvested, sonicated, centrifuged, and extracts were incubated with glutathione Sepharose 4B beads (Sigma, St. Louis) for 30 min at 4°. To determine if the GST fusions were capable of interacting with Swi6, these glutathione beads with fusion proteins immobilized on them were incubated with recombinant Swi6 or in vitro-translated Swi4/Swi6 complex, washed, boiled, and loaded onto SDS PAGE.

To obtain GST fusions from yeast, pBD2057, pBD2063, and pBD2061 were transformed into W303-la strain. The resulting strains were grown in selective media with raffinose overnight and then expression of the fusions was induced by galactose for 3–4 hr. Cells were harvested, and protein extracts were prepared as described before (Sidorova et al. 1995) and incubated for 1 hr with glutathione beads in GST buffer containing protease inhibitors (100 mM Tris HCl pH 8.0, 100 mM NaCl, 0.5% NP40 with 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml pepstatin A). Beads were then washed in three or four changes of GST buffer. To elute fusion proteins from the beads, the beads were resuspended in 50 µl of glutathione buffer, prepared according to the Pharmacia Biotech protocol. Fusions were eluted for 15 min at room temperature. To determine if Swi6 copurified with any of the GST fusions from yeast, glutathione eluates were loaded onto SDS PAGE, and Western blots were performed with Swi6 antibodies. Alternatively, extracts of yeast cells expressing fusion proteins were subjected to immunoprecipitation with Swi6 or Swi4 antibodies. Immunoprecipitates were loaded onto SDS PAGE, Western blotted, and probed with GST antibodies (Santa Cruz).

Thrombin cleavage of the Nhp6A from the Gst-Nhp6A fusion, bound to glutathione beads, was performed according to Pharmacia Biotech protocols. Glutathione beads were mixed with 58 µl of PBS and 2 µl of thrombin solution (1 unit/µl thrombin in PBS), incubated overnight at room temperature, and centrifuged. Supernatants were used directly for DNA-binding reactions.

Gel retardation: Gel retardation analysis was performed exactly as described (Sidorova and Breeden 1993; Ewaskow et al. 1998). When the in vitro-translated Swi4/Swi6 complex was bound to DNA, little (0.2–0.5 µg) or no nonspecific competitor dl-dC was added. The binding pattern was the same, regardless of whether dl-dC was present in the reaction or not. Thrombin or glutathione eluates of the Gst-Nhp6A fusion were directly added to DNA-binding reactions with HO promoter fragment. No dl-dC competitor was used in these reactions since Nhp6A is a nonspecific DNA binder (Paul and Johnson 1995) and can be competed from HO DNA by dl-dC (J. Sidorova, unpublished results).

RESULTS

Screen for high copy suppressors of temperature-sensitive hox: lacZ expression phenotype of swi6-405 and
### Table 1

**High copy suppressors of SWI6 ankyrin repeat mutants**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Suppressor</th>
<th>suppression of hox::lacZ expression in strains with the following mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>swi6-406</td>
</tr>
<tr>
<td>A. Suppressors isolated from swi6-406 strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c1 (5)</td>
<td>SW16</td>
<td>+</td>
</tr>
<tr>
<td>c2a (1)</td>
<td>ND⁴</td>
<td>+</td>
</tr>
<tr>
<td>c4 (1)</td>
<td>MSNI</td>
<td>+</td>
</tr>
<tr>
<td>c6 (1)</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>B. Suppressors isolated from swi6-405 strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c1 (1), c14 (2)</td>
<td>SW16</td>
<td>+</td>
</tr>
<tr>
<td>c2 (1)</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>c4 (8), c5 (1), c9 (1), c12 (1)</td>
<td>MSNI</td>
<td>+</td>
</tr>
<tr>
<td>c15 (1)</td>
<td>NHF6A</td>
<td>+</td>
</tr>
<tr>
<td>c19 (1)</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>c23 (1)</td>
<td>ND</td>
<td>+</td>
</tr>
</tbody>
</table>

The strains indicated above were transformed with vectors or with vectors bearing suppressing genes, grown on plates at 37°C, and screened for β-galactosidase activity with X-gal assays. Numbers in parentheses by the clone names indicate the number of times this clone was isolated.

*Not applicable.

⁴ Not determined.

**swi6-406 mutants:** We have previously carried out random mutagenesis of the ANK repeat-encoding region of the SWI6 gene (Ewaskow et al. 1998). Two of these mutants, swi6-405 (N330T, N500Y) and swi6-406 (T326I, T402S), were used in this study. Both mutants express Swi6 protein at the nonpermissive temperature; however, the level of Swi6 is reduced as compared to the wild-type protein level (Figure 2A). There is no hox::lacZ activity detected in swi6Δ strains expressing Swi6-405 or Swi6-406 from the CEN plasmid pRS316 at 37°C but at 30 and 25°C, they confer partial activity as judged by cell morphology and hox::lacZ transcript levels (data not shown and see below). In band shift assays, Swi4/Swi6-405 complex is less active in binding to SGB elements than the wild-type complex, and Swi4/Swi6-406 complex has an altered mobility (Ewaskow et al. 1998).

The mutated SWI6 genes were integrated at the LEU2 locus of the swi6Δ hox::lacZ strain, giving rise to strains BY1954 (swi6-405) and BY1956 (swi6-406). These strains were transformed with a 2μ-based yeast genomic library (Carlson and Böttstein 1982) and about 60,000 transformants were obtained for each. Colonies were grown at 30°C for the first 2 days upon transformation and then incubated at 37°C overnight. Colonies were transferred to nitrocellulose filters and assayed for β-galactosidase activity using the X-gal filter assay (Breeden and Nasmith 1985). Transformants that developed blue color above the background were selected. Library plasmids were isolated out of these cells, retransformed into BY1954 or BY1956 strains, and reassayed to confirm suppression. They were also transformed into BY000 swi6Δ hox::lacZ strain to determine if candidate suppressors were able to bypass the Swi6 function.

We recovered a total of 8 suppressor plasmids from swi6-406 and 19 from swi6-405 transformants. Using restriction digestion and PCR, we identified four different suppressors for swi6-406 and six for swi6-405. These are listed in Table 1. The SWI6 gene was isolated five times in the screen with swi6-406 and three times with swi6-405. Two suppressors (c2a and c6) were not pursued further because they activated hox::lacZ expression equally strongly in swi6-405 and swi6Δ cells and thus were completely independent of Swi6. Most of the suppressors (c2, c4, c5, c9, c12, c19, and c23) suppressed the hox::lacZ expression defect to some extent in the absence of Swi6, but only one (c19) could suppress in the absence of Swi4. This requirement for Swi4 suggests that the majority of suppressors enhance Swi4-mediated activation, rather than cause a general derepression of transcription. c15 showed no suppression of the hox::lacZ transcription defect in swi4 or swi6 deletion strains and thus was the best candidate for an allele-specific suppressor.

Sequence information was obtained for all the Swi4- and Swi6-dependent suppressors. c4, c5, c9, and c12 carry overlapping fragments from chromosome XV, and c23 has a nonoverlapping fragment from the same chromosome. c15 carries a fragment from chromosome XVI. Finally, c2 has a fragment from chromosome XIII. In each case more than one open reading frame was present on the insert. In this article we describe identification and further analysis of the genes responsible for suppression by c15 and c4.

We have subcloned fragments of c4 and c15 into pRS426 and transformed the resulting constructs into swi6-406 or swi6-405 to determine which of the open
reading frames encode suppressors. The results of mapping and subcloning are summarized in Figure 1. Previously identified genes, MSN1 and NHP6A, were responsible for the suppression phenotypes of c4 and c15, respectively. MSN1 was originally cloned as a high copy suppressor of a temperature-sensitive SNF1 kinase mutant for its ability to restore SUC2 expression (ESTRUCH and CARLSON 1990). It acts as a transcriptional activator when fused to LexA and does not have any specific DNA-binding activity (ESTRUCH and CARLSON 1990). NHP6A has also been identified previously (KOLDRUBETZ and BURGUM 1990) and encodes an HMG1-like small protein, which binds DNA nonspecifically, and is capable of bending DNA (PAUL and JOHNSON 1995).

**MSN1 and NHP6A suppress the HO transcription defect of SWI6 ANK mutants**: Since NHP6A (c15) was incapable of bypassing Swi6 function, we sought to determine if NHP6A suppression was specific to the swi6-405 allele of SWI6. The NHP6A gene on pRS426 was transformed into strains with different mutant alleles of SWI6, and X-gal filter assays were performed. All these mutants express Swi6 at nonpermissive temperature (37°C; EWASKOW et al. 1998; and data not shown). swi6-Δ21 encodes a nonconditional and highly defective Swi6 protein (SIDOROVA and BREEDEN 1993). As seen in Table 2, the temperature-sensitive hox: lacZ expression phenotype of many of the ANK mutants, including swi6-406, could be suppressed by elevated levels of NHP6A. The one exception was swi6-401, which is the most defective mutant of the set tested. In addition, NHP6A could not suppress the hox: lacZ expression defect of swi6-Δ21, which carries a deletion of the putative leucine zipper in SWI6. Thus, NHP6A displays allele-specific suppression. It enhances transcription by some temperature-labile Swi6 proteins and has no detectable suppressing activity with others. This could indicate a direct interaction between Nhp6A and Swi6, which is disrupted by only a subset of the Swi6 mutants. However, because Nhp6A suppresses all but the most defective alleles of SWI6, this could also be explained if there is a threshold

**Figure 1.** MSN1 and NHP6A suppress SWI6 mutants. Shown are the maps of suppressors c4 and c15 and the subclones generated to identify the open reading frames responsible for suppression. Triangles within boxes show direction of transcription of the genes. The names of the subclones generated in this study are listed on the right; M stands for MSN1 and N for NHP6A. The 1-kb segment below designates the scale. The subclones of c4 and c15 were transformed into BY1956 swi6-406 and BY1954 swi6-405, respectively, streaked onto selective media plates, and grown at 37°C. Then X-gal filter assays were performed. BY1954 and BY1956 transformed with pRS426 or pZUC12 served as negative controls for these assays. The development of blue color above the negative control level was scored as suppression.
for detection of suppression and some mutants fall below this threshold.

β-Galactosidase assays show that suppression by the 2μ plasmid-borne NHP6A or MSNI of the hac: lacZ expression defect in swi6-405 or swi6-406 cells at 37°C is low but well above background. For example, β-galactosidase activities for swi6-406 transformed with MSNI (pM2) or NHP6A (pN5) are 22 and 15 units, respectively, compared to 5 units for the vector-transformed control. To see if this increased expression occurred at the transcription level, we analyzed levels of hac: lacZ mRNA in these strains by S1 protection. Both at 25 and 30°C, the hac: lacZ mRNA level was noticeably higher in the swi6-405 and swi6-406 strains transformed with high copy NHP6A and MSNI plasmids, respectively, as compared to the same strains carrying the vector alone (Figure 2, B and C). At 37°C, hac: lacZ mRNA level in these strains was too low to be reproducibly quantitated by S1 protection even in the presence of suppressors (data not shown).

A similar result was obtained when MSNI plasmid was transformed into swi6-405 (Figure 2D). These data show that both MSNI and NHP6A exert their function at the mRNA level, rather than by affecting β-galactosidase stability or activity.

Nhp6A has a close homologue, Nhp6B, which has a set of properties indistinguishable from Nhp6A. The two proteins may have overlapping functions, because only deletion of both genes has a discernible phenotype (Costigan et al. 1994). However, NHP6B was not among the suppressors that we isolated. Thus, we tested NHP6B directly for suppression of SWI6 ANK mutations. When expressed from a high copy vector (pDK267), NHP6B also suppresses swi6-405. It is a weaker suppressor than NHP6A (pDK268, Figure 2B), but this difference may be due to the lower levels of NHP6B expression, which could also explain why different NHP6A-expressing plasmids suppress the hac: lacZ mRNA transcription defect to slightly different degrees (Figure 2B).

Swi6-405 and Swi6-406 are maintained at lower levels than the wild-type protein, so one indirect mechanism of suppression by NHP6A and NHP6B could be that of increasing expression of swi6-405, swi6-406, or the SWI4 gene. To test this possibility, we looked at SWI4 transcription in the swi6-405 strains with or without the elevated level of Nhp6A and found no difference in SWI4 mRNA levels (data not shown). We were not able to test if the SWI4 protein levels were affected, because available antibodies do not detect endogenous levels of this protein. However, because Nhp6A and Nhp6B are generally considered to be involved in DNA metabolism rather than in protein stability, the fact that SWI4 transcript levels are not affected by Nhp6A overproduction makes it likely that the protein levels are also unaltered. We also measured the levels of Swi6-405 protein at 37°C in cells transformed with vector alone or with NHP6A- or NHP6B-expressing plasmids and found that the mutant Swi6 accumulated to the same level in all strains tested (Figure 2A). Thus, the suppression by Nhp6A or Nhp6B proteins cannot be attributed to the increase in SWI4 or SWI6 expression.

MSNI and NHP6A are involved in HO transcription:
To determine if MSNI and NHP6A are normally in-

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### Table 2: NHP6A is an allele-specific suppressor of SWI6

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Allele</th>
<th>Mutation</th>
<th>ANK repeat affected</th>
<th>Suppression by NHP6A</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY1954</td>
<td>swi6-405</td>
<td>N330T, N500Y</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>BY1956</td>
<td>swi6-406</td>
<td>T326I, T402S</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>BY1957</td>
<td>swi6-402</td>
<td>A329T, K339R</td>
<td>1</td>
<td>±</td>
</tr>
<tr>
<td>BY1958</td>
<td>swi6-401</td>
<td>I395T, K357E</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>BY1959</td>
<td>swi6-A1</td>
<td>N469I, A494G</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T326I, I328L</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L453S, K501R</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G547D</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>BY1693</td>
<td>swi6-Δ21</td>
<td>D(E585-L606)</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>BY2223</td>
<td>swi6-4</td>
<td>G470A, T472A, L474A</td>
<td>4</td>
<td>+</td>
</tr>
</tbody>
</table>

The original residue, its position, and the substituting residue describe mutations (for example N330T is glutamine at position 330 in the Swi6 protein changed to threonine). The subdivision of the ANK region into four full copies of the repeats is as in BREDEN (1996). The spacer region is a stretch of amino acids between the third and fourth repeats of Swi6 that is not conserved among the members of the Swi4/Swi6 family (BREDEN 1996). Plasmid pN5-bearing NHP6A gene was transformed into the strains carrying indicated mutant SWI6 alleles. These transformants were then grown at 37°C (or at 50°C, in the case of swi6-Δ21) on plates and subjected to X-gal assays. Strains transformed by pRS426 vector served as controls for these assays.
involved in the transcription of the Swi4/Swi6-regulated promoters, we isolated mRNA from exponentially growing cultures of strains with or without MSN1 or NHP6A and NHP6B gene products and compared the levels of HO and CLN1 transcripts in these strains to the wild-type strain by S1 protection (Figure 3). The msn1Δ strain expresses about three- to fivefold less HO transcript and the nhp6Δ strain shows a twofold drop in HO transcript compared to wild type. Interestingly, there is little or no effect of msn1Δ on another Swi4/Swi6-regulated promoter, CLN1 (data not shown), but the nhp6Δ strain has a similar twofold effect on CLN1 (Figure 3, B and C).

**NHP6A and NHP6B genes can suppress the caffeine sensitivity of the swi6-405 allele:** The NHP6A gene has been implicated as a downstream target of the Slt2/Mpk1 MAP kinase pathway that leads from Fkc1 and is involved in growth control and cell morphogenesis (Costigan et al. 1994), in part because overexpression of NHP6A or NHP6B suppresses several Slt2 pathway defects, including the caffeine sensitivity of slt2 mutants. Because swi6Δ mutants are also sensitive to caffeine (Igual et al. 1996), we examined whether the SWI6 ANK mutant swi6-405 is sensitive to caffeine, and, if so, whether this defect can be suppressed by overexpression of NHP6A or NHP6B. The swi6-405 strain cannot grow at 37°C on plates containing 4.5 mM caffeine, whereas the wild-type cells were capable of growing on these plates (data not shown). However, when NHP6A and NHP6B genes expressed from high copy plasmids were transformed into the swi6-405 strain, they restored the ability of these cells to grow on plates containing up to 5.5 mM caffeine (Figure 4A).

Nhp6A probably affects Swi4/Swi6 DNA binding indirectly: To see whether there is a direct interaction be-
between Nhp6A and Swi6, we immunoprecipitated Swi4 or Swi6 proteins out of wild-type extracts carrying HA-Nhp6A and then immunoblotted with anti-HA antibodies to detect HA-Nhp6A. Despite the fact that this HA-tagged Nhp6A is functional and can suppress the swi6-405 transcription defect (Figure 2B), there was no indication that HA-Nhp6A coprecipitates either with Swi4 or Swi6 under the same conditions that we use to detect Swi4/Swi6 association (Sidorova and Breeden 1993).

We then prepared fusions of Nhp6A and Msn1 proteins with Gst. The GST-Nhp6A and GST-Msn1 fusions were put under the control of GAL1-10 promoter and expressed in a wild-type strain. When plated under conditions which select for the plasmid and cause overexpression of the fusion, neither one of these strains was able to form colonies (Figure 4B), indicating that overexpression of either Msn1 or Nhp6A as Gst fusions is lethal. Liquid cultures of the same strains were grown in raffinose and then incubated with galactose for up to 8 hr. FACS profiles of these cultures did not show accumulation of cells in any single compartment of the cell cycle; thus overproduction of Gst-Msn1 or Gst-Nhp6A did not lead to a specific cell cycle arrest (data not shown).

Because of their toxicity, we purified these Gst fusions from cells grown in raffinose and then induced by galactose addition for only 3–4 hr. Though we could purify the fusion proteins from these cells under low-stringency conditions, there was no detectable Swi6 copurifying with either of them, as we judged by probing the fusion protein isolates with Swi6 antibodies on Western blots. We also purified these Gst fusion proteins from E. coli on glutathione beads and then incubated the fusion-bound beads with recombinant Swi6 (Sidorova and Breeden 1993) or in vitro-translated Swi4/Swi6 complex (Ewaskow et al. 1998). Even under these conditions, we could not detect interaction between Swi4/Swi6 on one side and the fusion proteins on the other (data not shown).

Nhp6A may not directly associate with Swi6, yet it could facilitate the binding of the Swi4/Swi6 complex to SCBs by inducing a favorable bend in DNA. Thus, we tested the involvement of Nhp6A in the Swi6 complex formation on DNA. Band shift analysis was carried out with Swi4/Swi6 complexes obtained by in vitro translation of Swi4 in the presence of recombinant Swi6. These complexes appeared to be identical to the complex observed in whole cell extracts, in that they migrated with the same mobility, contained both Swi4 and Swi6 proteins, as judged by their supershifting by Swi4 and Swi6 antibodies, and were specific to SCB elements of the HO promoter fragment (Figure 5A and data not shown). The purified Gst-Nhp6A fusion was first tested in gel retardation assays with the HO promoter fragment. The Gst-Nhp6A fusion, but not Gst alone, was able to nonspecifically bind DNA (Figure 5B, lane 8).
We also cleaved the Gst moiety off the Gst-Nhp6A fusion with thrombin and tested the released Nhp6A in gel retardation assays. As anticipated, thrombin cleavage of Gst-Nhp6A released a DNA-binding component that forms a much smaller complex on DNA (Figure 5B, lane 15). However, both Gst-Nhp6A and Nhp6A were able to bind DNA and both could form a series of bands indicating that multiple Nhp6A molecules bound simultaneously to one DNA molecule. Next, the in vitro-translated Swi4/Swi6 complex was mixed together with varying amounts of Gst-Nhp6A or Nhp6A and was added to the HO promoter fragment (Figure 5B, lanes 5-7 and 12-14). These reactions were compared to the ones in which Swi4/Swi6 complex was mixed with Gst only (lanes 1-3), thrombin cleavage mixture only (lanes 9-11), and Gst-Nhp6A or Nhp6A mixed with the rabbit reticulocyte lysate (lanes 8 and 15). The amount of the DNA-bound Swi4/Swi6 complex was unaffected by the addition of Gst-Nhp6A (compare lanes 1-3 with 5-7) and slightly reduced upon the addition of Nhp6A (compare lanes 9-11 and 12-14). These results indicate that there is no cooperation between Nhp6A and Swi4/Swi6 in binding to DNA under the conditions of this assay. It is also worth noting that there appeared to be a negative effect on the amount of DNA-bound Gst-Nhp6A and Nhp6A if the Swi4/Swi6 complex was present in the reaction (compare lanes 7 to 8 and 14 to 15). Because the HODNA was in excess in the reaction, and the Swi4/Swi6 complex was at subsaturating level, this cannot be attributed simply to competition for binding sites.

**DISCUSSION**

In this article we describe a screen for genes that are able to suppress ANK defects of Swi6 when expressed at high copy levels. Two of these genes, MSNI and NHP6A, were further characterized. Both NHP6A and MSNI are weak suppressors of swi6 ANK mutant alleles. Both of the gene products are required for maximal expression of HO, as mutations in these genes lead to a two- and threefold drop in HO mRNA levels, respectively. However, neither protein appears to directly associate with Swi6.

MSNI (also named FUP1, PHD2, MSS10) has been isolated multiple times as a gene which, when overproduced, improves iron uptake (Eide and Guarente 1992), enhances pseudohyphal growth (Gimeno and Fink 1994) at least in part through activation of the MUC1 gene (Lambrechts et al. 1996a), ectopically activates FUS1 transcription (Ramer et al. 1992), and dere-
presses glucoamylase genes (Lambechts et al. 1996b). In all of the screens where they could be measured, including ours, increased levels of MSN1 led to activation of gene expression. It is therefore likely that Msn1 is an activator protein. Consistent with this, Estruch and Carlson (1990) have shown that, when fused to LexA, Msn1 functions as a transcriptional activator. Msn1 does not have strong DNA-binding activity of its own, suggesting that it may bind to the protein component of transcription complexes. The spectrum of action of MSN1 seems to be rather broad and now includes HO, FUS1, SUC2, MUC1, and STA1-3 genes. The fact that Gst-Msn1 overexpression is toxic to cells suggests that MSN1 may have other targets that are critical for growth and viability, and their deregulation is deleterious for the cells. Alternatively, Gst-Msn1 could titrate out essential components of transcriptional apparatus or interfere with chromosome mechanics.

Interestingly, PHD1, a gene coding for a protein with homology to Swi4, was isolated in the same screen for enhancers of pseudohypalle growth in which MSN1 was identified (as PHD2) (Gimeno and Fink 1994). It is plausible that Phd1 and Msn1 may cooperate in activation of genes required for pseudohypallere formation in a manner similar to the way Msn1 and Swi4 cooperate to activate HO. The fact that the Msn1-mediated enhancement of his::lacZ transcription absolutely requires Swi4 is consistent with this possibility. However, this cooperation is unlikely to be mediated through stable interaction with Swi4/Swi6 complexes because Msn1
suppression of HO transcription can occur in the absence of Swi6 and does not occur at a second Swi4/Swi6-regulated gene, CLN1. In addition, we cannot detect physical association between Msn1 and either Swi4 or Swi6. Perhaps it is more likely that Msn1 activates transcription through other site(s) within the HO promoter, or it may assist the basic transcriptional machinery as a cofactor. Alternatively, it may facilitate formation of an active chromatin configuration within the HO promoter.

A different picture emerges in the case of NHP6A (KoLODRUBETZ and BURGUM 1990), which is the other suppressor of SWI6 mutations that we have characterized. Nhp6A and its close homologue Nhp6B bear homology to the higher eukaryotic HMG1 protein family, which has been implicated in DNA replication and transcription (ButLER et al. 1985; TREMETNICK and MOLLOY 1988; SINGH and DIXON 1990; GE and ROEDER 1994; STELZER et al. 1994; SEYKIND et al. 1995). In vitro, Nhp6A and Nhp6B have been shown to bind DNA nonspecifically but with relatively high affinity. The proteins wrap DNA in a way that introduces negative supercoils (PAULL and JOHNSON 1995). Recently Nhp6A and Nhp6B were shown to be required for inducible transcription of several messenger RNAs in vivo, and to facilitate formation of Tbp complexes with the TATA regions of promoters in vivo (PAULL et al. 1996). Our results indicate that the two Swi4/Swi6-regulated promoters, HO and CLN1, are also influenced by Nhp6A and Nhp6B proteins. Elevated levels of Nhp6A or Nhp6B increase het/loc2 transcription in strains carrying defective but not null alleles of SWI6. Moreover, strains deleted for both NHP6A and NHP6B have low HO and CLN1 mRNA levels that can be rescued by transformation of NHP6A on a high copy plasmid into these strains. This suggests that Nhp6A may cooperate with Swi4/Swi6 in transcriptional activation. However, if this is the case, the interaction is probably unstable because we have not been able to demonstrate any association between Nhp6A and Swi6, nor have we been able to detect any positive effect of Nhp6A upon the ability of the Swi4/Swi6 complex to bind DNA in vitro. It is equally plausible that NHP6A and NHP6B affect transcription indirectly by increasing expression of an unidentified protein that enhances Swi4/Swi6 activity or that NHP6A and NHP6B affect the chromatin conformation of the promoters. Such an activity would be undetectable in vitro with bandshift assays, which use naked DNA.

Massive overproduction of Nhp6A is deleterious to the cell (ESPINER et al. 1995 and data not shown), but does not lead to the cell cycle stage-specific arrest. The DNA-binding function of this Gst-Nhp6A fusion is intact, so its toxicity may be attributed to increased binding to genomic DNA, which might cause general inhibition or inappropriate activation of transcription. Recently the NHP6A gene was isolated as a high copy suppressor of slk1(bck1) defects and implicated as a downstream component of the Slt2/Mpk1 MAP kinase pathway that leads from Pkc1 and is involved in growth control and cell morphogenesis (COSTIGAN et al. 1994). nhp6A/B deletion strains were shown to share many phenotypes with pck1, slk1(bck1) and slt2(mpk1) deletion strains. The exact function of Nhp6 proteins in the Slt2 pathway has not been elucidated, but it has been proposed that they participate in Slt2-responsive transcription. We have found that Nhp6A and Nhp6B affect expression of HO and CLN1, which are controlled by the Swi4/Swi6 complex. This complex has also been implicated in transcriptional regulation of some of the Slt2-responsive genes (IGUAL et al. 1996). Moreover, Swi6 can be phosphorylated by Slt2 in vitro, and its phosphorylation state correlates with Slt2 activity in vivo (MADDEN et al. 1997). Consistent with this, swi6 deletion strains have been shown to be more sensitive to caffeine than wild type, and they share this phenotype with slt2 mutants (IGUAL et al. 1996). We have found that the swi6-405 mutant is also sensitive to caffeine and that this phenotype can be suppressed by Nhp6A or Nhp6B expressed from a high copy plasmid (Figure 4A). All these findings are consistent with the notion that Nhp6A and Nhp6B proteins may be utilized in a similar fashion both at "classic" Swi4/Swi6-dependent promoters, HO and CLN1, as well as at the promoters that may be both Slt2 dependent and Swi4/Swi6 dependent.

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LITERATURE CITED


Suppressors of Swi6 Transcription Factor


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