Global alterations in chromatin accessibility associated with loss of SIN4 function

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ABSTRACT

Sin4p is a component of a mediator complex associated with the C-terminal domain of RNA polymerase II and SIN4 is required for proper regulation of several genes in yeast, including the HO endonuclease gene, glucose repressible genes and MAα cell-specific genes. Previous studies indicated that SIN4 may influence transcription through changes in the organization of chromatin. We have examined a specific chromatin structure associated with MAα cell-specific repression in sin4 MAα cells to determine if SIN4 is required for nucleosome positioning. Although the loss of SIN4 has no effect on nucleosome location, we find that the sensitivity of bulk chromatin from sin4 cells to micrococcal nuclease digestion is strikingly increased relative to chromatin from isogenic wild-type cells. The nuclelease hypersensitivity of chromatin from sin4 cells is not related to gross alterations in histone gene expression or to bulk increases in histone modification. Our experiments suggest that SIN4 directly or indirectly regulates a global aspect of chromatin accessibility, providing a molecular basis for phenotypic similarities between sin4 mutations and mutations in histones.

INTRODUCTION

Genetic analysis in yeast has revealed two opposing sets of global transcriptional regulators. The SWI/SNF genes encode positive regulators of diverse processes, including expression of the HO endonuclease, suppression of Ty or soloO insertion mutations and activation of glucose repressible genes (1). Conversely, the SPTISIN genes generally encode negative regulators (2). Mutations in the SPTISIN genes often suppress mutations in the positive SWI/SNF activators. Members of both families of regulators are thought to influence transcription through modulation of chromatin structure (2).

SIN4 is required for repression of the HO (3), IME1 (4) and MAα cell-specific genes (5). It is also required for full activation of genes such as CTS1 and HIS4 (3,6). Sin4p associates with Rgr1p in vivo (6,7) and mutations in either SIN4 or RGR1 exhibit similar phenotypes (4,6,8). The discovery that Sin4p and Rgr1p, together with Gal11p and p50, are associated with polymerase II mediator complexes (7) indicates that these proteins directly affect the function of basal transcription proteins in vivo. However, mutations in SIN4 or RGR1 exhibit pleiotropic phenotypes similar to mutation or depletion of histones, including activation of UASless promoters, suggesting a role (direct or indirect) for these gene products in the organization of chromatin (3,9). Mutations in SIN4 also partially suppress defects in transcriptional activation of ho–lacZ caused by mutations in the SWI2 gene, encoding an important subunit of the Swi/Snf chromatin remodeling complex, as well as other SWI genes (8,10,11). In addition, the superhelical density of plasmids is altered in sin4 (3) and rgr1 (9) cells and unique chromatin structures are associated with repression of several genes regulated by SIN4 and RGR1, including the GAL1–10 genes (12), PHO5 (13) and the MAα cell-specific genes (14,15).

To directly investigate whether mutations in SIN4 alter chromatin structure, we have compared nucleosome organization and stability in isogenic wild-type and sin4 cells. Our data indicate that SIN4 modulates a global aspect of chromatin accessibility.

MATERIALS AND METHODS

Yeast strains and media

Saccharomyces cerevisiae strains used in this study are listed in Table 1. DY150, DY151, DY1704 and DY2693 are isogenic strains in the W303 background (16). DY882 and DY1720 are isogenic strains in the YPH499/YPH500 (S288C) background (17). DY131 and DY1675 are isogenic strains in the K1107 background (18). Rich medium (YPD) and minimal medium (SD) supplemented with amino acids, uracil or adenine (as appropriate) were prepared as described (19).

RNA analysis

The methods for RNA blot hybridization and the ACT1 probe have been described previously (20). Hybridization probes were prepared from plasmids generously provided by Mary Ann Osley: the histone H2A probe was a 2.3 kb SacI fragment from plasmid YCp50-TRT1 (21) containing the HTA1 (and PRT1) gene. The histone H2B probe was a 1.6 kb BamHI–SacI fragment from plasmid YCp50–TRT1 containing the HTB1 gene. The histone H3 probe was a 2.1 kb HindIII–BamHI fragment from plasmid YCp50-H3-H4-copy2 containing the HHT2 gene. The histone H4 probe was a 2.3 kb SmaI–BamHI fragment from plasmid YCp50-H3-H4-copy1 containing the HHF1 gene.

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Extracts were prepared from sin4 or wild-type cells bearing the indicated reporter plasmids (22) and β-galactosidase assays were performed as described (19).

Isolation and analysis of chromatin

Three different methods of chromatin preparation were used in these experiments and in all cases chromatin from sin4 cells exhibited increased nuclease sensitivity relative to wild-type cells. Chromatin for analysis of the histone genes was prepared after micrococcal nuclease (MNase) digestion of NP-40 permeabilized spheroplasts basically as described by Kent et al. (23). To compare rates of nuclease digestion, wild-type or sin4 cells were grown to equal densities (4 x 10^7 cells/ml) and then nuclei were prepared from each as previously described (15). In the ‘mixing’ experiment shown in Figure 3, nuclei were prepared from wild-type cells, sin4 cells or an equal mixture of these cells. MNase digestion and purification of the DNA were as previously described (15). Indirect end-labeling analysis and isolation of TALS chromatin was performed as described (14). After MNase digestion, DNA was purified and digested with EcoRV and subjected to Southern blot analysis.

Southern blots

DNA samples were resolved by electrophoresis in 1.2% agarose gels and were prepared for transfer as described (24). DNA was transferred by posi blot (Stratagene) onto GeneScreen+ (NEN) and then UV crosslinked to the membrane. Prehybridization, hybridization and washes were performed as described (25). Probes included an EcoRV–HindIII fragment of the TALS plasmid for the indirect end-labeling experiment in Figure 4; a HindIII fragment (for H4) and a BamHI–HindIII (for H3) fragment from plasmid YCp50-H3-H4-copy2 and a PCR-generated fragment containing sequences between 1535 and 1800 bp of HMLα for the blots shown in Figure 6; an EcoRI–HpaI fragment of the rRNA repeat for the blot shown in Figure 7B.

Analysis of plasmid linking number

The SUP4-o tRNA plasmid M3264 consists of a 0.6 kb PstI–EcoRI fragment with ARS1 and a 0.4 kb XhoI–PstI fragment with the Sup4-o tRNA gene cloned into the Bluescript KS+ vector. Two-dimensional electrophoresis of DNA in the presence of chloroquine was performed as described previously (3). Topoisomers of M3264 were detected in the Southern blot using a 32P-labeled ARS1 fragment.

Preparation of yeast histones

Yeast histones were isolated as described previously (26–28). Histones were resolved by SDS–PAGE or acid urea–PAGE as described (29,30).

RESULTS

Increased nuclease sensitivity is a global property of sin4 chromatin

MNase is commonly used to probe chromatin structure because this enzyme preferentially cleaves linker DNA located between nucleosomes. Partial digestion of chromatin with this nuclease yields a ‘ladder’ of oligonucleosome sized fragments. Examination of bulk chromatin isolated from isogenic wild-type or sin4 cells after digestion with MNase reveals a striking and global increase in nuclease sensitivity in the chromatin isolated from sin4 cells (Fig. 1A) relative to wild-type cells. For example, MNase ‘ladders’ are evident in chromatin from sin4 cells upon digestion with 12.5–25 U/ml MNase. Indeed, at every level of enzyme used, the chromatin from sin4 cells exhibits greater digestion than the wild-type chromatin. Importantly, the increased digestion observed in the absence of Sin4 is not due to decreased recovery of
Figure 2. An increased rate of MNase digestion is observed in sin4 chromatin. SIN4 (+) and sin4 (−) chromatin was digested with a single concentration (50 U/ml) of MNase for increasing lengths of time. (A) Agarose gel resolution of MNase digestion products. Note that nucleosomal ladders are evident in the sin4 chromatin after only 2 min digestion, but are not evident in the wild-type chromatin until 5 min digestion. All samples were resolved on the same gel. (B) Scans of bands from the the wild-type 15 min digest and the sin4 10 min digest. Sizes of fragments calculated relative to marker DNA (not shown) are indicated underneath each peak.

We were concerned that such patterns of digestion might reflect increased activity of endogenous endo- or exonucleases in the sin4 chromatin preparations, rather than a change in chromatin structure. Therefore, we incubated circular or linearized plasmid DNA with our chromatin preparations under the same conditions used for our chromatin analysis (omitting the addition of exogenous nuclease). The plasmid DNA was then purified and analyzed by Southern blot (Fig. 1B). Neither the wild-type nor the sin4 chromatin contained significant endogenous endo- or exonuclease activity, as evidenced by the persistence of uncut and linear DNA in both extracts (Fig. 1B).

We confirmed the increased nuclease sensitivity of the sin4 chromatin by comparing the rate of digestion of chromatin isolated from wild-type and sin4 cells (Fig. 2A). In this experiment, wild-type and sin4 cells were grown to equal cell densities and isolated nuclei were then digested with a single concentration (50 U/ml) of MNase. Digestion was halted at various times by the addition of EDTA. Nucleosome ladders were clearly evident after only 2 min digestion of the sin4 chromatin (Fig. 2A). Comparable digestion of the isogenic wild-type chromatin was not observed for at least 5 min, indicating that the sin4 chromatin was digested at least twice as quickly as the wild-type chromatin. As expected, the differential rates of digestion became less apparent with increasing time, as both types of chromatin neared more complete digestion. We also examined the size and repeat length of bands from samples exhibiting approximately equal degrees of digestion (Fig. 2B). The profile of digestion of the wild-type sample that was digested for 15 min (lower curve, Fig. 2B) is almost identical to that of the sin4 sample digested for 10 min (upper curve, Fig. 2B), as indicated by the overlay scans of these two lanes. Mononucleosome-sized bands migrated the same distance in each sample, as did dinucleosome sized bands, etc. The mononucleosome size calculated (187 bp) for the mononucleosome sized band may be an overestimate, because in both samples these bands migrated faster than the smallest marker DNA fragment on the gel (not shown). The calculated sizes of the dinucleosome, trinucleosome and tetranucleosome sized bands indicate an average mono-
nucleosome size of 163 ± 1.5 bp in both wild-type and sin4 chromatin. An identical repeat length was calculated using bands generated in the wild-type sample that was digested for 10 min and the sin4 sample that was digested for 5 min (data not shown).

To further test whether the increased nuclease sensitivity of the sin4 chromatin actually reflects a property of the structure of this chromatin, rather than some other difference between wild-type and sin4 cells, chromatin was prepared from a mixture of the two cell types (Fig. 3). Wild-type cells containing a plasmid with a TRP1 marker (pRS424) were mixed with an equal number of sin4 cells containing a highly similar plasmid (pRS426) carrying a URA3 marker (17). Nuclei were prepared from each cell type alone, as well as from the mixture, digested with MNase and the purified DNA was then probed either with TRP1- or URA3-specific sequences. The pattern of digestion of the TRP1 chromatin from wild-type cells was extremely similar in the mixture as in the wild-type chromatin alone (Fig. 3, TRP1), indicating that addition of the sin4 cells did not influence digestion of the wild-type chromatin. Similarly, digestion of the sin4 chromatin in the mixture was very much like that of the sin4 chromatin alone (Fig. 3, URA3). The same number of mono- and oligonucleosome sized fragments is apparent in both preparations. As expected, the degree of digestion of bulk chromatin (data not shown) and URA3 plasmid chromatin from the sin4 cells was increased relative to the wild-type chromatin. These data indicate that the difference in nuclease sensitivity observed in these cells is not due to a gross inhibition of MNase in the wild-type chromatin but to selective activation of MNase in the sin4 chromatin.

We conclude that the changes in MNase sensitivity observed here and above reflect alterations in chromatin structure, rather than alterations in endogenous or exogenously added nuclease activities.
influences some global aspect of nucleosome accessibility without altering nucleosome locations.

Histone gene expression is unchanged by loss of SIN4

The pleiotropic effects of sin4 mutations on transcription and chromatin structure might be explained by alterations in histone gene expression. For example, sin4 mutations suppress loss of HIS4 expression caused by insertion of a δ element of the Ty1 transposon into the HIS4 promoter. Mutations in other genes which cause a Spt− phenotype, such as SPT10 and SPT21, reduce expression of certain histone genes (36). Interestingly, deletion of one copy of the genes encoding histone H2A and H2B (Δhta1−htb1) has previously been reported to cause local alterations in MNase digestion patterns of specific genes (37), in contrast to the global affects on digestion we observe in the absence of SIN4.

To investigate whether loss of SIN4 also affects histone gene expression, we examined histone mRNA levels in wild-type and sin4 cells (Fig. 5A). No obvious differences were observed in the steady-state levels of mRNA from any of the histone genes in these cells, indicating that SIN4 does not grossly alter expression of these genes. This conclusion is further supported by measurement of expression of histone H2A1 or H2B2 promoter–β galactosidase reporter constructs (Fig. 5B). Although the level of expression from these two promoters differed, both were expressed equally well in wild-type and sin4 cells.

Altered transcription is not required for hypersensitivity of sin4 chromatin

The hypersensitivity of chromatin from sin4 cells could result from a global activation of transcription. Conversely, this hypersensitivity could reflect an altered chromatin structure that plays a causal role in gene activation. To distinguish these possibilities, we examined the nuclease sensitivity of chromatin from loci which did not exhibit a change in transcription in sin4 cells relative to wild-type cells.

As shown above, histone gene expression is unaffected by SIN4 loss (Fig. 5). We analyzed MNase digestion patterns of chromatin
Figure 7. A sin4 mutation also affects the chromatin structure of genes transcribed by RNA polymerases III and I. (A) SIN4 influences the supercoil density of a plasmid lacking a polymerase II transcription unit, but containing a polymerase III transcribed gene. Plasmid M3264, containing the SUP4-o tRNA gene transcribed by polymerase III and the ARS1 origin of DNA replication, was transformed into strains DY151 (SIN4, ade2-1) and DY1704 (sin4, ade2-1), selecting for adenine prototrophy. The ade2-1 allele contains a nonsense mutation that is suppressed by the SUP4-o tRNA. DNA was isolated from logarithmically growing cells and electrophoresed in two-dimensional chloroquine–agarose gels. Shown is a Southern blot probed with ARS1-specific sequences. The directions of the first and second dimensions are indicated. (B) Chromatin isolated from Sin4(+) and sin4(–) cells was digested with increasing levels of MNase as shown and then probed with rDNA-specific sequences. As for polymerase II transcribed genes, the chromatin from the sin4 cells exhibited an increased sensitivity to MNase digestion.

Chromatin structure of RNA polymerase I and III transcription units are also altered in sin4 cells

Since Sin4p associates with the RNA polymerase II holoenzyme complex (7), we wondered whether the effects of sin4 mutations on chromatin are restricted to polymerase II transcription units. To address this question, we examined the properties of chromatin associated with the SUP4-o tRNA gene, which is transcribed by RNA polymerase III. This gene was inserted into the plasmid Bluescript KS+, which lacks any known polymerase II transcription units, together with an ARS (autonomously replicating sequence) element. The SUP4-o tRNA suppresses ochre mutations such as that found in the ade2-1 allele and thus yeast strains containing this plasmid can be selected for adenine prototrophy. The linking number of the SUP4-o tRNA plasmid was determined after transformation into wild-type and sin4 cells by two-dimensional electrophoresis on agarose gels containing chloroquine. Each nucleosome induces a single superhelical turn in closed circular DNA. Determination of superhelical density gives a relative measure of nucleosome content. The two-dimensional gel resolves topoisomer as an arc, with linking number increasing in a clockwise direction. The average distribution of topoisomers in the sin4 mutant is clearly shifted clockwise relative to the distribution from wild-type cells (Fig. 7A), associated with individual histone genes by Southern blot to determine whether SIN4 affects the structure of these loci. As for TALS and bulk chromatin, we observed increased digestion of histone gene chromatin in the absence of SIN4. No digestion of chromosomal H4 (HHF2) DNA was observed in the absence of added nuclease (Fig. 6, 0) and addition of MNase generated regularly spaced, nucleosome ‘ladders’ in both the wild-type and sin4 chromatin. However, these ladders were evident at much lower concentrations of nuclelease in the chromatin from sin4 cells. A similar increased sensitivity to nuclease was observed for chromatin associated with the HHT2 gene, which encodes histone H3 (Fig. 6B). Again, smaller digestion products were evident in the sin4 chromatin at lower concentrations of nuclease than in the wild-type chromatin and larger molecular weight fragments were depleted more readily. Chromatin associated with independent H2A and H2B loci also exhibited hypersensitivity to MNase digestion in the sin4 cells (data not shown).

We also examined the nuclease sensitivity of a gene which is not transcribed in wild-type or sin4 cells. Silencing of the HML mating locus is unaffected in sin4 MATa cells (data not shown), but loss of SIN4 results in hypersensitivity of HML chromatin to nuclease digestion (Fig. 6C), as for all other chromatin examined in these cells. Together with the above results, our data indicate that transcription is not required for increased nuclease sensitivity of sin4 chromatin.
consistent with a change in chromatin structure, as previously reported for plasmids containing polymerase II transcription units. The 35S precursor of rRNA is transcribed by RNA polymerase I from the cluster of tandemly repeated 9 kb rDNA genes on chromosome XII. To determine whether a sin4 mutation affected the chromatin structure of these RNA polymerase I transcription units, we examined MNase sensitivity of the rDNA genes. Chromatin isolated from SIN4 and sin4 strains was digested with MNase and a Southern blot was probed with rDNA-specific sequences. The rDNA chromatin from both SIN4 and sin4 cells exhibited a much less regular and more smeared pattern of digestion than seen for the polymerase II genes examined above, perhaps reflecting the very high transcriptional activity of these genes. However, like the polymerase II genes above, the rDNA chromatin from sin4 cells exhibited increased sensitivity to MNase digestion relative to the chromatin from SIN4 cells (Fig. 7B). We conclude that a sin4 mutation has global effects on chromatin structure which are not limited to polymerase II transcribed genes.

**Histone modifications are not grossly changed in sin4 cells**

Post-translational modification of histones, particularly acetylation of lysine residues, is often correlated with increased nucleosome sensitivity and transcriptional activation. To determine if the increased nucleosome sensitivity of sin4 chromatin is associated with increased histone modification, histones were isolated from wild-type and sin4 cells. Differentially charged histone isoforms were first separated by acid urea–PAGE and then subjected to SDS–PAGE. Histones were identified in the second dimension gels by mobility (Fig. 8) and Western blot analysis (data not shown). The pattern of histone modification resolved in this analysis was unaltered by the loss of SIN4. For example, the same three isoforms of H4 are apparent in both sin4 and wild-type chromatin. Similar profiles of H2B, H2A and H3 isoforms are also observed in both chromatin preparations. We confirmed the absence of differences in acetylation levels in immunoblotting experiments using antibodies specific for acetylated forms of H4 or H3 (data not shown). The increased accessibility of sin4 chromatin to nuclease cannot be explained by bulk histone hyperacetylation.

**DISCUSSION**

Previous studies indicated that changes in gene expression might be related to changes in chromatin structure resulting from mutations in SIN4, as indicated by changes in linking number of nucleosomal plasmids (3,6,9). Our current studies have further defined these changes as a global increase in nucleosome sensitivity, reflecting an increased accessibility of chromatin in sin4 cells.

Although we do not yet know the structural basis of this increased accessibility, our findings help to explain other phenotypes reported for sin4 cells. UASless promoters, for example, are activated in the absence of SIN4 (3). These promoters are thought to be repressed by chromatin in wild-type cells, since mutation of genes encoding histones H2A, H2B and H3 or depletion of histone H4 relieves this repression (38,39). The increased nucleosome sensitivity of chromatin we observe in sin4 cells may reflect a more open structure which mimics that resulting from histone mutation, allowing activation of UASless promoters. Similarly, mutations in histone genes (2) or resulting from histone mutation, allowing activation of UASless promoters. Similarly, mutations in histone genes(2) or SIN4 (3,9) restore activity to promoters disrupted by Ty insertions. This Spt phenotype is again consistent with a loosening of a repressive chromatin structure (2). Finally, sin4 mutations partially suppress transcriptional defects at the ho–lucZ and SUC2 loci caused by mutations in the SWZ2 gene (6,9,40), which encodes a central component of the Swi/Snf chromatin remodeling machine (2). The increased chromatin accessibility generated in the absence of SIN4 may obviate the need for Swi/Snf function.

Several conditions could lead to the increased nucleosome sensitivity we observe in the absence of SIN4. Obvious possibilities, including depletion of histones or bulk increases in histone acetylation, however, were not observed. Our experiments, of course, do not rule out altered usage of specific acetylation sites (or other modifications) in particular histones in the absence of SIN4. Interestingly, we did not observe an increased susceptibility of sin4 chromatin to digestion by DNase I, which might indicate that histone:DNA contacts are not grossly altered in the absence of SIN4. A sin4 mutation does cause changes in the supercoiling density of plasmids (3,9; Fig. 7), suggesting that the sin4 mutation may change the number of nucleosomes loaded onto plasmid chromatin or increase loss of nucleosomes from the plasmid. SIN4 might also influence some aspect of higher order chromatin folding, either through direct interactions with chromatin or through regulation of another factor which affects chromatin accessibility (see below). Indeed, previous observations indicate that the actions of SIN4 in transcriptional repression are context dependent. For example, sin4 mutations can suppress defects in the swi5 activator to allow expression of ho–lucZ reporter genes but cannot suppress the swi5 defect at the native HO locus (Jiang and Stillman, unpublished observations). Similarly, a sin4 mutation causes derepression of a PHOS–lucZ reporter, but not of the native PHO5 gene (Horz, personal communication). Moreover, SIN4 negatively regulates PHO5 transposed into the URA3 locus, but does not negatively regulate PHO5 in its natural
chromosomal location (41). These position effects could reflect a role for SIN4 function in the organization of higher order structures.

Interestingly, the loss of repression of the MATα2 cell-specific genes observed in sin4 cells (5) is consistent with the increased chromatin accessibility we observe. Even though nucleosomes are positioned properly in the absence of SIN4, alterations in nucleosome stability or in nucleosome–nucleosome contacts would provide a more open environment conducive to transcription.

Sin4p is associated with the RNA polymerase II holoenzyme as part of a mediator complex (7). A putative subcomplex consisting of Sin4p, Rgr1p, Gal11p and an undefined 50 kDa peptide (p50) co-purifies with the mediator, raising the possibility that Sin4p plays a direct role in transcriptional regulation. This idea is consistent with previous findings that chimeric proteins consisting of Sin4p or Rgr1p fused to the bacterial lexA DNA binding domain are able to activate lexA operator-containing reporters (3; unpublished observations). Additionally, sin4 mutants are defective for the activation of specific genes, including MATα2, CTS1 and HNF4 (3,6). Given the association of Sin4p and the mediator complex with polymerase II (7), it seems likely that SIN4 participates in chromatin organization indirectly, through regulation of the expression of a chromatin modifying activity or a non-histone structural component of chromatin. Identification of such a factor or definition of direct interactions between Sin4p and chromatin may yield further insights into the connections between chromatin structure and the regulation of gene expression.

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