SIR repression of a yeast heat shock gene: UAS and TATA footprints persist within heterochromatin

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Previous work has suggested that products of the *Saccharomyces cerevisiae* Silent Information Regulator (*SIR*) genes form a complex with histones, nucleated by *cis*-acting silencers or telomeres, which represses transcription in a position-dependent but sequence-independent fashion. While it is generally thought that this Sir complex works through the establishment of heterochromatin, it is unclear how this structure blocks transcription while remaining fully permissive to other genetic processes such as recombination or integration. Here we examine the molecular determinants underlying the silencing of *HSP82*, a transcriptionally potent, stress-inducible gene. We find that *HSP82* is efficiently silenced in a *SIR*-dependent fashion, but only when *HMRE* mating-type silencers are configured both 5' and 3' of the gene. Accompanying dominant repression are novel wrapped chromatin structures within both core and upstream promoter regions. Strikingly, DNase I footprints mapping to the binding sites for heat shock factor (HSF) and TATA-binding protein (TBP) are strengthened and broadened, while groove-specific interactions, as detected by dimethyl sulfate, are diminished. Our data are consistent with a model for *SIR* repression whereby transcriptional activators gain access to their cognate sites but are rendered unproductive by a co-existing heterochromatic complex.

Keywords: heat shock factor/heterochromatin/mating-type silencers/TATA-binding protein/transcriptional silencing

Introduction

The expression state of a gene is influenced by its chromosomal position. Genes located within heterochromatin, the cytologically condensed form of chromatin, are susceptible to long-range, dominant repression, while genes located within euchromatin are not. Therefore, the location of a gene can dictate whether it is expressed or repressed. Such position-effect regulation is seen in eukaryotes as diverse as yeast, insects and mammals. In the budding yeast *Saccharomyces cerevisiae*, position effects have been identified at three distinct regions: the silent mating loci *HMR* and *HML*, the telomeres and the rDNA gene array. At each of these regions, repression is position dependent yet sequence independent. As such, pol II and pol III genes targeted to these regions are

transcriptionally silenced (Brand *et al.*, 1985; Schnell and Rine, 1986; Gottschling *et al.*, 1990; Smith and Boeke, 1997).

The *cis*-acting elements responsible for position-effect regulation are best understood for the *HM* loci. *HMR* and *HML* contain donor copies of the α and α genes, responsible for mating-type determination. The *HM* loci are maintained in a transcriptionally inactive state by flanking *cis*-acting elements, termed *E* and *I*, disposed to the left and right of each silent locus, respectively. These silencers each encompass a relatively short stretch of DNA (~150 bp) and are located ~1 kb from the genes they regulate. The most potent of these silencers, *HMRE*, is comprised of binding sites for three sequence-specific proteins: ORC (origin replication complex), RAP1 and ABF1 (reviewed in Loo and Rine, 1995). These proteins act by recruiting the Silent Information Regulatory (Sir) complex, composed of Sir2p, Sir3p and Sir4p (Rine and Herskowitz, 1987), which does not directly bind DNA. The same complex is also required for transcriptional repression at telomeres (Aparicio *et al.*, 1991). Recruitment of the Sir complex is mediated by Sir1p, which binds directly to both Orc1p and Sir4p (Triolo and Sternglanz, 1996), and when tethered to the *HMRE* silencer can bypass the requirement for ORC (Fox *et al.*, 1997). Interaction of Sir3p and Sir4p with RAP1 has also been demonstrated (Moretti *et al.*, 1994; Cockell *et al.*, 1995). Interestingly, repression of pol II genes targeted to the rDNA array requires only Sir2p (Smith and Boeke, 1997). In addition, the core histones H3 and H4 are required for silencing at both *HM* and telomeric loci (Kayne *et al.*, 1988; Aparicio *et al.*, 1991; Thompson *et al.*, 1994). Their N-termini engage in direct contacts with Sir3p and Sir4p, which may facilitate horizontal templating of the Sir complex (Hecht *et al.*, 1995).

How *SIR*-mediated heterochromatin elicits its repressive effects is unknown. An appealing notion, supported by several lines of evidence, is that this specialized chromatin structure sterically hinders the access of sequence-specific regulatory factors, as well as components of the pre-initiation complex (PIC) to promoter DNA, thereby impairing transcription. In support of this concept is evidence that *SIR* induces the local formation of a specialized chromatin structure, inaccessible to restriction endonucleases *in vitro* (Loo and Rine, 1994) and the site-specific HO endonuclease *in vivo* (Nasmyth, 1982; Weiss and Simpson, 1998). The *SIR*-dependent chromatin is also refractory to DNA repair enzymes (Terleth *et al.*, 1989) as well as ectopically expressed *dam* methyltransferase (Gottschling, 1992; Singh and Klar, 1992); however, the silenced chromatin at *HMR* and *HML* is fully permissive to homologous recombination that results in gene conversion of the *MAT* locus (Herskowitz *et al.*, 1992). Moreover, Ty retrotransposon integration at the *HM* loci is enhanced

by the Sir regulatory complex, as *HMRE* mutations that partially diminish silencing also partially reduce preferential Ty integration at these sites (Zou and Voytas, 1997). These latter observations raise the possibility that heterochromatin may act in a manner distinct from, or in addition to, strict steric interference.

In this study we investigate the *cis*-acting requirements for silencing a transcriptionally potent, stress-inducible gene, and provide a detailed analysis of the distinctive nucleoprotein complex accompanying the silenced state. We find that dominant silencing requires a minimum of two properly positioned *HMRE* silencers and is accompanied by the appearance of novel, *SIR*-dependent, wrapped chromatin structures within the core and upstream promoter regions. Strong, sequence-specific interactions mapping to regulatory sites—including those for the principal activators, heat shock factor (HSF) and TATA-binding protein (TBP)—are preserved; nonetheless, groove-specific interactions are altered. Our results are consistent with a model for *SIR* repression whereby activators gain access to their target binding sites but where these proteins, once DNA bound, are rendered unproductive by the co-existing heterochromatic complex.

Results

HSP82 is conditionally silenced by SIR irrespective of HMRE orientation or dosage

To explore the mechanistic basis for position-effect regulation, we used gene transplacement to target *HMRE* silencer elements to the *HSP82* chromosomal locus in an effort to bring it under *SIR* control. As *HSP82* regulatory elements and promoter chromatin structure have been extensively characterized (Szent-Gyorgyi *et al.*, 1987; Gross, 1995; Erkin *et al.*, 1999), the gene provides an ideal model system for such an investigation. In previous work we demonstrated that targeting an *HMRE* silencer upstream of *HSP82* resulted in moderate, *SIR*-dependent silencing under non-inducing conditions. This repression was conditional, in that it was rapidly overridden under inducing conditions (Lee and Gross, 1993). Our previous study used relatively large *HMRE* fragments (0.35 or 4.9 kb) containing multiple potential binding sites for ORC, RAP1 and ABF1 (DeBeer and Fox, 1999; Hurst and Rivier, 1999), which were installed opposite their native orientation. To test whether a minimal *HMRE* fragment—comprising single binding sites for ORC, RAP1 and ABF1—could silence *HSP82*, and whether altering its orientation or increasing its dosage might increase the efficiency of silencing, we constructed a new series of *HMRE/HSP82* alleles (illustrated in Figure 1).

When targeted ~700 bp upstream of the heat shock gene, a minimal *HMRE* element, oriented ORC-site-proximal (*hsp82-102*; see Figure 1), reduces *HSP82* transcript levels by 30% under non-heat-shock conditions (Figure 2, lane 2 versus 1). This silencing is conditional, as it is lost upon heat shock (Figure 2, lane 4 versus 3; see also Figure 3) but it is re-established within 15 min of recovery (Figure 2, lane 6 versus 5). Our ability to assay the re-establishment of transcriptional repression is facilitated by the short half-life of *HSP82* RNA (<1.7 min at 30°C; Lee and Gross, 1993), permitting rapid changes in transcription rates to be monitored. Notably, the repression

seen is *SIR* dependent, as insertion of the *HMRE* fragment in a *sir4* background has no effect on *HSP82* expression (Figure 2, compare '–' lanes with corresponding *HSP82*⁺ lanes). Thus, the minimal silencer exerts a partial, conditional position effect upon *HSP82*.

It is possible that the relatively weak silencing observed with this and the constructs studied previously is attributable to their unnatural orientation with respect to the promoter (see, for example, Shei and Broach, 1995). To test this, we constructed an *hsp82* allele with the *HMRE* silencer installed at the same site but in its native orientation (*hsp82-101*). However, as shown in Figure 2 (lanes 7–12), inverting the *HMRE* silencer had no effect: as above, *SIR* only partially represses non-induced expression levels; this repression is fully overridden by heat shock. A summary of multiple independent experiments is provided in Figure 3.

To investigate whether silencer activity at *HSP82* was additive, we constructed *HMRE/HSP82* strains in which tandem *HMRE* modules separated by a 6 bp spacer were installed ~700 bp upstream of the *HSP82* coding region. Additive effects might be seen if, as suggested by a number of previous studies, silencers act to nucleate the assembly of a Sir complex whose constituent proteins are in limiting concentration within the nucleus (Renauld *et al.*, 1993; Maillet *et al.*, 1996; Marcand *et al.*, 1996). However, the level of *SIR* repression mediated by dimeric constructs is similar to that seen with the monomeric constructs (Figure 3, compare *hsp82-201* with *hsp82-101*). Moreover, as above, this is true irrespective of *HMRE* orientation. Taken together with previous findings showing equivalent repression mediated by *HMRE* elements positioned either 0.75 or 2.7 kb upstream of *HSP82* (Lee and Gross, 1993), we conclude that *SIR* repression of the heat shock gene is independent of orientation, distance or dosage of 5' silencers.

YAR1 is fully and unconditionally silenced by a single, ectopic HMRE element

The inability of *HMRE*, even in its natural orientation, to repress *HSP82* significantly (Figure 2, lanes 7–12), contrasts markedly with its efficiency in silencing genes at either *HMR* or *MAT* (Brand *et al.*, 1985; Shei and Broach, 1995). To test whether conditional silencing reflects an attribute of the heat shock promoter (such as its intrinsic strength), rather than an inability of *HMRE* to function efficiently at this locus (95 kb from the left telomere of chromosome XVI), we measured transcript levels of *YAR1*, a divergently transcribed gene located adjacent and centromere-proximal to *HSP82* (see Figure 1). The principal *YAR1* start site maps ~50 bp downstream of the *HMRE* insertion site (Lycan *et al.*, 1996). Nonetheless, *HMRE per se* has little effect on *YAR1* transcription (Figure 2, compare lanes 1, 7 and 13). However, in a *SIR*⁺ context, quantitative silencing of *YAR1* is seen independently of *HMRE* orientation or dosage (e.g. Figure 2, lanes 2 and 8). Thus, at this locus, *SIR* can fully and unconditionally silence one gene while being simultaneously overridden in the opposite direction by another.

Dominant repression of HSP82 can be established by bracketing the gene with silencers

The foregoing results argue against a simple model in which the dosage of *cis*-acting silencers is proportional to

Fig. 1. Physical map of the *HSP82* locus and location of *HMRE* insertions. The heat shock gene is 95 kb distant and oriented towards the left telomere of chromosome XVI; it is flanked by *YAR1* upstream and *CIN2* downstream. *HMRE* elements (arrows; see inset) were targeted to unique restriction sites within the locus, at either *Clal* (position -673, where +1 is the transcription start site) or *MluI* (position +2342, ~50 bp 3' of the transcription termination point), or both, in the orientations indicated. Stably positioned nucleosomes are indicated by continuous ovals, less precisely positioned nucleosomes by broken ovals, 'split' nucleosomes by half ovals, and DNase I hypersensitive, nucleosome-disrupted regions by horizontal lines (adapted from Erkin et al., 1995). Constitutively occupied sites within the *HSP82* promoter are indicated by solid rectangles, inducibly bound sites by open rectangles (Gross et al., 1990; Giardina and Lis, 1995; Erkin et al., 1999).

Fig. 2. Position-effect regulation of *HSP82* by an upstream silencer is weak and conditional irrespective of its orientation, while *YAR1* is fully and unconditionally silenced by the same element. Total cellular RNA was isolated from isogenic *sir4⁻* and *SIR⁺* strains ('-' and '+', respectively) bearing the indicated *HMRE/HSP82* alleles. As a control, RNA was isolated from an *HSP82⁺* strain (SLY101; lanes 13–15). Samples were isolated from non-shocked cultures (NHS), following heat shock at 39°C (HS), or following return to 30°C (Rec). *HSP82* and *YAR1* transcript levels were quantitated by PhosphorImager and internally normalized to those of *ACT1*.

Fig. 3. Polar *HMRE/HSP82* alleles: summary of Northern analyses. *HSP82* transcript levels of isogenic *sir4⁻* and *SIR⁺* strains ('-' and '+', respectively) bearing the alleles indicated are graphically illustrated (values are means ± SEM; they are normalized to non-heat-shocked *hsp82-201/sir4⁻*, whose transcript level was assigned a value of 100). *n*, number of independent RNA isolations. Note that these data were derived from strains bearing either chromosomal or episomal copies of *SIR4* (see Table I); no difference in expression levels was seen.

the extent of *SIR* repression. However, it is possible that functional cooperativity between *HMRE* elements is more effectively achieved by positioning them 5' and 3' of the promoter, permitting looping or other topological interactions to take place between them (Hofmann *et al.*, 1989). To investigate this possibility, we constructed a strain whose *hsp82* allele is bracketed by two *HMRE* silencers, one 5' of the promoter and the other 3' of the transcription termination point, thereby positioning them ~3 kb apart (*hsp82-1001*; Figure 1). The ability of this strain to silence *HSP82* was analyzed by Northern blotting as above. In marked contrast to *hsp82* alleles bearing two 5' silencers (Figure 3), the bracketed allele is strongly silenced: 11-fold under non-inducing conditions, 2-fold following heat shock and 20-fold following a 15 min recovery from heat shock (Figure 4). As expected, the effect of bracketing is independent of silencer orientation (Figure 4, compare *hsp82-1001* with *hsp82-1002*). However, in contrast to the polar constructs discussed above, silencing is enhanced by increasing *HMRE* dosage. In strains bearing either the *hsp82-2001* or *hsp82-2002* allele, non-induced and recovery transcript levels are virtually eliminated (down 50- to 100-fold), while induced transcript levels are also significantly repressed (4-fold).

Downstream *HMRE* elements silence *HSP82* inefficiently

The preceding experiments indicate that monomeric or dimeric *HMRE* sequences integrated 3' of the *HSP82*

coding region, in combination with identical sequences integrated at the 5' end, impose dominant *SIR* repression. One interpretation, as discussed above, is that 5' and 3' silencers functionally cooperate to mediate this effect. Alternatively, it is possible that the potent silencing seen is due exclusively to the presence of the 3' silencer(s). This might be the case if propagation of the *Sir* complex along chromatin requires contiguous nucleosomes (Hecht *et al.*, 1995), absent within the promoter but present within the transcription unit of *HSP82* (Szent-Gyorgyi *et al.*, 1987; see Figure 1). To test the repressive activity of 3' silencers alone, we constructed alleles bearing single or dimeric copies of *HMRE* installed at the +2342 *Mlu*I site (termed *hsp82-301* and *hsp82-401*, respectively; Figure 1). While virtually complete, *SIR*-dependent silencing of the neighboring *CIN2* gene was seen at each allele, there was no discernable silencing of *HSP82* (data not shown). We conclude that the dominant silencing characteristic of the bracketed alleles requires both 5' and 3' silencers.

***SIR* repression is overridden in a progressive, not quantum, fashion**

The foregoing experiments indicate that when subjected to maximally inducing conditions, the *HSP82* promoter partially overrides *SIR* repression, even when bracketed by silencers (Figure 4). However, *HSP82* can be induced to submaximal levels that correlate with the intensity of the applied stress (E.A.Sekinger and D.S.Gross,

Fig. 4. Bracketed *HMRE/HSP82* alleles: summary of expression data. Methodology, symbols and abbreviations are as in Figure 3. Samples were normalized to non-heat-shocked *hsp82-2001/sir4⁻*.

unpublished observations). Thus, it is formally possible that silencing remains near-absolute until a threshold temperature is reached, with transcription detectable only once this threshold is breached. However, we have found that an incremental increase in the severity of heat shock is accompanied by a corresponding decrease in *SIR*-mediated repression (data not shown). Diminished repression is unlikely to reflect thermolability of the *SIR* complex for two reasons. First, *HMRE*-mediated silencing of non-stress-responsive genes (*YARI*, *CIN2*, **a1**) is not affected by heat shock (Figure 2 and data not shown). Secondly, a similar inverse relationship between fold repression of *HSP82* and severity of stress is elicited by an inhibitor of oxidative phosphorylation, 2,4-dinitrophenol (DNP; data not shown). We conclude that there is no threshold stress level; rather, the extent to which *HSP82* overrides *SIR* repression correlates with the severity of the stress.

SIR* repression is accompanied by the de novo assembly of a specialized chromatin structure at *HSP82

Previous work has shown that *SIR* repression at other loci is accompanied by a heterochromatic-like structure inaccessible to DNA repair and modifying enzymes (Terleth *et al.*, 1989; Gottschling, 1992; Singh and Klar, 1992) and endonucleases (Nasmyth, 1982; Loo and Rine, 1994; Weiss and Simpson, 1998). To investigate whether dominant *SIR* repression of *HSP82* is likewise accompanied by a novel chromatin structure, we performed

a nucleotide resolution DNase I footprinting analysis. Spheroplast lysates isolated from *SIR⁺* and *sir4⁻* strains were digested with DNase I and the *HSP82*-specific pattern analyzed by amplified primer extension (AMPEX). Such an analysis reveals the presence of a distinctive, *SIR*-dependent chromatin structure at *hsp82-2001*, evident under all three expression states and extending over the entire upstream regulatory region (Figure 5). In the absence of *SIR*, chromatin-specific protections are primarily restricted to HSEs 1–3 and TATA (Figure 5, open bars, compare lanes 7–12 with lanes 5 and 6). In the presence of *SIR*, protection over HSE1 is retained and is in fact extended ~70 bases downstream, encompassing both URS1 and HAP2/3/5 sites (Figure 5, solid bars). Punctuating the cleavage profile of each strand is a prominent DNase I hypersensitive site flanking HSE1 (Figure 5, arrows). These hypersensitive sites, coupled with the intervening protection, are consistent with the sequence-specific binding of HSF (McDaniel *et al.*, 1989; Gross *et al.*, 1990), see below. Additional chromatin-specific protections map to the core promoter, where the TATA-associated upper-strand footprint appears extended to at least position –50 in the *SIR⁺* strain (Figure 5B, solid bar, lanes 13–18).

There are at least two other striking attributes revealed by DNase I. First, a series of nine chromatin-specific cleavages spaced at either 10–12 or 20–25 nt intervals, and spanning ~150 bp of the core promoter and 5' end of the transcription unit (dots), is evident in the lower-strand analysis (Figure 5A). Such a pattern may indicate the

presence of a wrapped structure. A similar, although more regular pattern of cleavages is seen in genetically inactivated *hsp82* promoter mutants lacking the high-affinity HSF binding site, HSE1. These mutants exhibit a near-perfect 10–11 nt cleavage periodicity spanning –120 to +28, possibly reflecting the presence of a rotationally phased nucleosome (Gross *et al.*, 1993). Secondly, the *SIR*-dependent chromatin structure appears to be meta-stable, since it is cleaved quite differently at high levels of digestion (Figure 5A, lanes 18, 22 and 26). The lower-strand hypersensitive site and accompanying footprints are lost; in their place is a ladder of uniformly spaced cleavages ~10 nt apart, mapping principally to the core promoter. Thus, DNase I genomic footprinting reveals the

presence of a novel, *SIR*-dependent structure compatible with sequence-specific DNA binding proteins.

To determine whether the *SIR*-dependent chromatin structure was also recognized by micrococcal nuclease (MNase), which recognizes distinct attributes of DNA structure (Gross and Garrard, 1988), we performed MNase genomic footprinting (Figure 6). The most prominent finding is a ladder of chromatin-specific cleavages between –112 and –225 (Figure 6, bracketed), which exhibits a distinctive 10 nt periodicity on both strands (stars); this ladder may even extend to –260 (dots), thereby spanning 148 bp. The structure detected by MNase appears to be an intrinsic feature of the UAS region since a majority of the cut sites are also seen in the *sir4*[–] strain, yet are greatly accentuated in

the *SIR*⁺ background. Interestingly, this cleavage ladder, which may reflect the presence of a wrapped structure over the upstream promoter, is far less prominent in the heat-shocked *SIR*⁺ strain (Figure 6, compare lane 11 with either 10 or 12; and lane 23 with either 22 or 24), more closely resembling the cleavage profile of the *sir4*⁻ strains. Also notable are broad regions of *SIR*-dependent protection on the upper strand mapping to the intergenic region between

HSP82 and *YAR1* (Figure 6, solid bars), the significance of which is unclear. In contrast, the prominent regions of MNase protection evident within the core promoter and coding region of the recovered *sir4*⁻ strain (Figure 6, lane 8) suggest the presence of translationally positioned nucleosomes (dotted ovals). While such nucleosomes may accompany the transcriptional down-regulation seen during recovery from heat shock, it is notable that no evidence for

Fig. 5. A novel, *SIR*-dependent chromatin structure is formed over the *HSP82* promoter as revealed by DNase I genomic footprinting. Isogenic *SIR*⁺ and *sir4*⁻ strains bearing the *hsp82* alleles indicated were grown to early log phase in rich medium at 30°C, heat shocked for 20 min at 39°C, then downshifted to 30°C for 15 min. Spheroplast lysates were isolated from appropriate aliquots [NHS, HS and Rec (N, H and R), respectively], digested with DNase I, genomic DNA extracted, and lower- and upper-strand cleavage profiles revealed by AMPLEX using primers -342→-315 and +26→-11, respectively. (A) Lower-strand analysis. (B) Upper-strand analysis. Note that all DNA samples are derived from *hsp82*-2001 strains except lanes 19 and 20 (*hsp82*-1001), and lanes 21 and 22 (*hsp82*-201) of (B). Dots, *SIR*-specific cut sites; arrows, location of DNase I hypersensitive sites marking the upstream and downstream flanks of HSE1; open bars, sequence-specific protection in a *sir4*⁻ background; solid bars, sequence-specific protection in an *SIR*⁺ background. C, T, A and G, dideoxy sequence ladders (sequence of the footprinted strand is shown). DNA (D), naked genomic DNA digested with DNase I and processed identically to chromatin samples. Depicted below each lane is an RNA analysis of the same sample. *HSP82* transcript levels relative to those of *ACT1*, quantitated by PhosphorImager, are indicated. Note that three different gels are depicted in (B): lanes 1–12 and 23–24 are derived from gel 1; lanes 13–18 are from gel 2; and lanes 19–22 are from gel 3.

Fig. 6. MNase genomic footprinting reveals the presence of a novel, *SIR*-dependent chromatin structure upstream of *HSP82*. Isogenic *SIR*⁺ and *sir4*⁻ strains bearing the double bracketed *HMRE/HSP82* allele (*hsp82-2001*) were cultivated under non-shocked (N), heat shocked (H) or recovery (R) conditions, and nuclei isolated. Nuclei were digested with MNase, genomic DNA purified, and strand-specific footprints revealed by AMPEX as in Figure 5. As a control, deproteinized genomic DNA (D) was digested with MNase and analyzed similarly. Bottom panel, Northern analysis of RNA isolated from the same cultures used for nuclear isolation. Stars, cleavages novel to (or strongly enhanced in) *SIR*⁺ nuclei spaced at an ~10 nt periodicity; these span the UAS region of each strand (brackets). Dots, cleavages extending the region of 10 nt cutting periodicity (not chromatin specific). Ovals, inferred positions of nucleosomes in recovered *sir4*⁻ samples. Filled bars, regions of *SIR*-dependent protection. T, TATA box; H, HAP2/3/5 consensus sequence; U, URS1; 1, 2, 3, HSEs 1–3.

discrete ~160 nt regions of protection can be found in the *SIR*⁺ samples despite far lower transcript levels (Figure 6, compare lane 8 with either 10 or 12). Finally, strong MNase protection is seen at position -183 on the lower strand (Figure 6, lanes 10–12), a site of intense DNase I cleavage under all three transcription states (Figure 5A). Thus, the MNase analysis detects structures distinct from those recognized by DNase I, yet both argue for the presence of

a specialized, *SIR*-dependent chromatin structure upstream of *HSP82*.

The novel chromatin structure is unique to dominantly silenced alleles

Is the distinctive chromatin structure seen at the double bracketed *hsp82-2001* allele functionally linked to dominant silencing, or is it also present at partially and condition-

ally silenced *HMRE/HSP82* alleles? To address this question, we digested nuclei purified from strains carrying either the *hsp82-201* or *hsp82-1001* allele with DNase I, and analyzed the resultant cleavage profile by AMPEX as above. Both of these alleles bear two silencers, yet only one is efficiently repressed (Figures 3 and 4). Therefore, if chromatin is the cause and not the consequence of this transcriptional silencing, the signature features of the DNase I genomic footprint—the hypersensitive site at position –155 and the extended UAS and core promoter footprints—will be seen at *hsp82-1001* but not at *hsp82-201*. Such an expectation is dramatically confirmed, as shown in Figure 5B (compare lanes 19 and 20 with lanes 21 and 22). Interestingly, the cleavage profile of the polar construct (Figure 5B, lanes 21 and 22) is novel, and resembles neither that of the *sir4⁻* control (lanes 7–12) nor that of the *SIR*-repressed bracketed alleles (lanes 13–20).

***SIR* alters but does not obviate methylation**

protection at heat shock and TATA elements in vivo

DNase I and MNase genomic footprinting suggest that stable *SIR* repression of *HSP82* is associated with novel chromatin structures localized to both upstream and core promoter regions. The DNase I analysis additionally suggests that sequence-specific interactions are preserved in an *SIR⁺* context and may in fact be strengthened and broadened, particularly in alleles subject to robust silencing. To investigate the effect of the *SIR* repressive complex on protein–DNA interactions in more detail, we conducted dimethyl sulfate (DMS) *in vivo* footprinting. Cells bearing an *hsp82* allele bracketed with single silencers were reacted with DMS during the final 2 min of cultivation, and genomic DNA was isolated and subjected to AMPEX. As illustrated in Figure 7, HSE1 is strongly occupied in a *sir4⁻* background, even under non-inducing conditions (indicated by protection of guanines at –161, –162 and –174; lane 6). This is consistent with previous work demonstrating that *S.cerevisiae* HSF binds to high-affinity sites constitutively (Jakobsen and Pelham, 1988; Gross *et al.*, 1990; Erkin *et al.*, 1995). Furthermore, the low-affinity heat shock elements 2 and 3 are inducibly occupied (indicated by hyper-reactivity of –210G) (Figure 7, lane 7). Recovery from heat shock has little effect on HSF–HSE interactions despite the substantial reduction in transcript levels (Figure 7, lane 8), although diminished interaction at the HAP2/3/5 site (indicated by reduced reactivity of –109A) is apparent. These results closely resemble those obtained with the wild-type allele in this genetic background (Erkin *et al.*, 1999), indicating that in the absence of a functional *SIR* complex, occupancy of regulatory sites is unaffected by the presence of silencers.

In the presence of the repressive chromatin structure discussed above, major groove interactions at HSE1 are virtually abolished under non-inducing conditions, except for a single protection (–174G) (Figure 7, compare lanes 9 and 10). Interactions at HSEs1–3 and HAP2/3/5 are strengthened, however, upon heat shock (Figure 7, lane 11). Following shift to recovery conditions, interactions at HSEs 2 and 3 and HAP2/3/5 are once again undetectable (Figure 7, lane 12). Thus, *SIR* repression diminishes groove-specific interactions at activator binding sites under non-inducing and recovery conditions, yet such interactions are restored upon heat shock.

Fig. 7. *SIR* repression alters occupancy of the heat shock UAS as revealed by DMS *in vivo* footprinting. Isogenic *SIR⁺* and *sir4⁻* strains bearing *hsp82-1001* were cultivated under non-shocked (N), heat shocked (H) or recovery (R) conditions, and intact cells were incubated in the presence of DMS for 2 min at either 30 (N, R) or 39°C (H). Genomic DNA was purified and subjected to AMPEX using the upper-strand complementary primer +26→–11. Naked genomic DNA (D) was methylated and analyzed in a similar fashion. C, T, A, G, upper-strand sequencing ladders. Dots, purine residues evincing *SIR*-specific protection. Depicted below each lane is an RNA analysis of the same sample.

A central role of activators is to recruit components of the PIC to the core promoter as the initial step in transcriptional activation (Roeder, 1996; Ptashne and Gann, 1997). Indeed, we have previously shown that a critical role for HSF in regulating *HSP82* is to stabilize the TBP–TATA interaction (Gross *et al.*, 1993). It was therefore of interest to ask whether the *SIR*-dependent chromatin structure also affected TBP binding to the TATA box. To address this, we once again used DMS *in vivo* footprinting coupled with AMPEX, exploiting the sensitivity of this method for detecting the minor groove-binding protein, TBP (Erkin *et al.*, 1996). Densitometric scans from such an analysis are shown in Figure 8. These reveal that *SIR* somewhat reduces the extent of minor groove protection within the TATAAA sequence under non-heat-shock and recovery conditions, but not under inducing conditions. Thus, the effect of *SIR* on TBP binding to TATA parallels its effect on HSF binding to HSE1: sequence-specific interactions are altered but not abolished.

Discussion

HMRE silencing of HSP82: a central role for spacing, a variable role for dosage and no role for orientation

In this study we have identified the *cis*-acting requirements for efficient silencing of a robustly transcribed,

Fig. 8. *SIR* repression alters occupancy of the *HSP82* TATA box. *SIR*⁺ and *sir4*⁻ strains bearing the *hsp82-1001* allele were cultivated under the conditions indicated and treated with DMS at the appropriate temperature. DNA was extracted and upper-strand-specific methylation sites detected by AMPEX using primer +26→-11. Densitometric scans are shown. Scans were normalized with respect to -70 G (virtually identical results were obtained when normalization was to -95 G). DNA, deproteinized genomic DNA.

stress-inducible gene. We did this by targeting *HMRE* mating-type silencers to the native locus of *HSP82* and measuring *SIR*-dependent alterations in the gene's expression level. The effect of altering orientation, dosage and spacing of silencers was tested. Two parameters, sufficient *HMRE* dosage and appropriate spacing, were found to be absolutely necessary for efficient silencing. A single *HMRE* element, irrespective of its orientation or site of integration, could elicit only a partial, conditional position effect. In striking contrast, *hsp82* alleles flanked 5' and 3' with silencers were subject to dominant, unconditional silencing. The bracketing configuration was critical, since merely increasing *HMRE* dosage was of little consequence.

It is possible that functional cooperation between *HMRE* elements derives solely from their relative spacing. In the weakly repressed *HMRE/HSP82* alleles *hsp82-201* and *hsp82-202*, individual *HMRE* elements are spaced only 6 bp apart. In their strongly silenced counterparts (*hsp82-1001* and *hsp82-1002*), the two silencers are spaced ~3 kb apart, thereby mimicking the natural arrangement of silencers at the *HM* loci. Thus, broad spacing of two or more silencers may be required for dominant *SIR* repression. In this respect, it is interesting that tandem *HMRE* silencers installed adjacent to the *MAT* locus and spaced at 3 kb intervals efficiently repressed the mating-type genes contained within, whereas a single silencer could not (Shei and Broach, 1995). Likewise, strong cooperativity was seen between distantly spaced silencers in repressing *LEU2/lacZ* installed at *HML* (Boscheron *et al.*, 1996). Therefore, functional cooperation between silencers is likely to be dependent upon the presence of sufficient spacing between them. What might account for this spacing requirement? As Sir proteins appear to spread physically from individual silencers over the silenced locus (Hecht *et al.*, 1995, 1996), broad spacing might facilitate physical interaction/functional cooperation between silencers by permitting the formation of DNA loops (or other higher order structures) (Hofmann *et al.*,

1989). Alternatively, the 3–4 kb of DNA interposed between silencers at the natural *HM* loci, and in examples of efficient ectopic silencing, may reflect corresponding intervals of attachment sites at the nuclear envelope (Andrulis *et al.*, 1998) or nuclear matrix (Diffley and Stillman, 1989). This would be consistent with observations that efficient silencing occurs most readily in specific nuclear compartments where silencing proteins are sequestered (Maillet *et al.*, 1996; Marcand *et al.*, 1996; Andrulis *et al.*, 1998).

It is notable that the two genes flanking *HSP82*, *YAR1* and *CIN2*, are fully repressed by a single ectopic silencer. Therefore, the local concentration of Sir proteins at this locus in single silencer strains is sufficient for repressing less potently activated genes. Also noteworthy is the efficiency with which a single *HMRE* element, irrespective of its orientation, silences *YAR1*. This contrasts with the orientation dependence of *HMRE* at *MAT* (Shei and Broach, 1995) but is consistent with the ability of *HMRE* to impose bidirectional silencing at *HMR* (Brand *et al.*, 1985; Donze *et al.*, 1999). These observations suggest that silencing at the *YAR1-HSP82-CIN2* locus adheres to the same rules as at the *HM* loci. Additional evidence for this comes from overexpression of Sir4C, the dominant-negative C-terminal domain of Sir4p (Marshall *et al.*, 1987; Cockell *et al.*, 1995). Silencing of *hsp82-1001* is abolished, concomitant with derepression of the *HM* loci (data not shown). A similar phenotype is observed in a *sir4-42* background (data not shown), presumably due to sequestration of Sir proteins in the nucleolus (Kennedy *et al.*, 1995).

HSF: insulator to the spread of heterochromatin?

It is interesting that *YAR1* is only repressed by 5' silencers and *CIN2* by 3' silencers. This is despite the fact that *HMRE* has previously been shown to repress genes ~3 kb away, at both *HMR* and *HSP82* (Brand *et al.*, 1985; Lee and Gross, 1993). One possibility is that sequences within

the *HSP82* promoter itself block the spread of silenced chromatin. Consistent with this notion, clustered RAP1 sites were found to act as boundary elements, preventing the horizontal spread of silencing emanating from the *HMLE* silencer (Bi and Broach, 1999). Likewise, a small DNA fragment encompassing a tRNA gene serves as a boundary element to the spread of heterochromatin from *HMR* (Donze *et al.*, 1999). Also consistent with this idea is the earlier finding that *SIR* repression is not observed on the centromeric side of an actively transcribed telomeric gene (Renauld *et al.*, 1993). We predict, although have not tested, that the constitutive HSF–HSE1 complex plays a key role in insulating the spread of heterochromatin at this locus.

The resilience of *HSP82* to transcriptional repression may reflect special properties of HSF. This factor, virtually unique amongst *S.cerevisiae* activators, can bypass the requirement for a number of key PIC components—including TAF17, Srb4, Srb6, Kin28 and the C-terminal domain of the large subunit of pol II (Apone *et al.*, 1998; Lee and Lis, 1998; McNeil *et al.*, 1998; Moqtaderi *et al.*, 1998)—in *trans*-activating target promoters *in vivo*. Perhaps this ‘super-activating’ property of HSF allows it to induce *HSP82* transcription even when the target promoter is bracketed by four silencers.

SIR* catalyzes the formation of a distinctive chromatin structure at *HSP82

Detailed analysis of the *SIR*-dependent chromatin upstream of *HSP82* reveals the presence of novel structures mapping to both core and upstream promoter regions. These structures appear to be wrapped, based on the presence of 10 nt cleavage ladders. The structure within the core promoter could be a rotationally phased nucleosome, based both on the breadth of the DNase I cleavage ladder (~150 bp) and the pattern of cleavage, in which cutting at certain sites is suppressed (Lutter, 1978). However, other properties, such as metastability of the structure and the fact that only one strand shows periodic cleavage, are uncharacteristic of a nucleosome, so it is possible that the cleavage ladder instead reflects the presence of a wrapped PIC component such as TFIID or RNA polymerase (Oelgeschlager *et al.*, 1996; Robert *et al.*, 1998). The apparent wrapped structure centered over the UAS is also unusual. While a 10 nt MNase cleavage period is evident on both strands and spans ~148 bp, there is no corresponding ladder in the DNase I digest, typically diagnostic of a rotationally phased nucleosome. Also, accessibility to MNase within the UAS is significantly enhanced in *SIR*⁺ nuclei, inconsistent with the structure being a nucleosome. A similar *SIR*-dependent enhancement in MNase sensitivity has been observed within the UAS region at *HMLα* (Weiss and Simpson, 1998).

Whatever these *SIR*-dependent structures might be, it is striking that they are detected only at the dominantly silenced alleles. Bracketed silencers strongly repress transcription; likewise, bracketed alleles are uniquely packaged in the distinctive chromatin structure. This heterochromatic structure is likely to be the cause and not simply the consequence of genetic inactivation, since *hsp82* alleles inactivated by a 2 bp mutation in the TATA box show little, if any, alteration in promoter chromatin structure (Lee and Garrard, 1992).

How does *SIR*-induced heterochromatin silence transcription?

Theoretically, the Sir protein complex could negatively regulate promoter activity through a number of distinct mechanisms. It could act by sterically hindering access of activators to their cognate sites within the promoter. Consistent with this, *SIR*-regulated chromatin is generally less accessible to exogenous and endogenous DNA binding proteins, as discussed above. A second possibility is that the DNA binding capability of activators is preserved, yet the subsequent recruitment of PIC components is impaired, possibly through the Sir complex interfering with the function of activation domains or by impairing access of either the TFIID or pol II holoenzyme complexes. Finally, *SIR* may be permissive to both activator binding and PIC assembly, yet exert its inhibitory effect at the level of promoter clearance or polymerase elongation. The presence of paused RNA polymerase has been inferred for a number of euchromatic genes, including heat shock genes of both *Drosophila* and human (Rougvie and Lis, 1988; Brown *et al.*, 1996); it is thus formally possible that heterochromatin silences *HSP82* by causing the inappropriate pausing of RNA polymerase.

Our results are most consistent with *SIR* acting primarily, although not necessarily exclusively, at a point downstream of activator and TBP binding. We base this conclusion on the surprising finding that DNase I protection mapping to promoter regulatory elements, including binding sites for HSF and TBP, is not diminished and may even be broadened in *SIR*⁺ strains. Moreover, DMS methylation protection patterns at HSE1 and TATA, while altered, are not abolished. Thus, it is possible that HSF and TBP remain bound to the promoter even when *HSP82* is subject to dominant *SIR* repression.

Alternatively, these footprints might be due to heterochromatin-specific proteins and not to the bona fide activators. Previous studies of mitotically repressed mammalian genes have shown a similar pattern of reduced DMS reactivity at activator binding sites coupled with retention of either promoter-associated DNase I hypersensitivity or KMnO₄ hyperreactivity (Martinez-Balbas *et al.*, 1995; Michelotti *et al.*, 1997). These altered footprints have been interpreted as representing heterochromatin-specific proteins acting as ‘molecular bookmarks’, preserving such promoters for subsequent activation (Michelotti *et al.*, 1997).

Another possibility is that two or more epigenetic states exist at *HMRE/HSP82*. Indeed, epigenetic inheritance of transcriptional states has been seen at both *HM* and telomeric loci (Pillus and Rine, 1989; Gottschling *et al.*, 1990). In this view, one state, found in a relatively small fraction of the population, would be characterized by the presence of DNA-bound activator and transcription initiation complexes. The other state, found in the majority of cells, would be characterized by the presence of a repressive Sir complex. These epigenetic states might be established stochastically sometime following replication and be stably maintained, or they might exist in a dynamic equilibrium. In either case, the genomic footprints would represent a composite picture of two (largely independent) states.

Additional experimentation will be required to distinguish between these alternatives. Nonetheless, the sheer

rapidity of both activation and re-repression at silencer-flanked *hsp82* alleles argues against the necessity for passage through S phase (during which time alternative chromatin structures could be assembled), previously shown to be obligatory for the onset of *SIR* repression at the *HM* loci (Miller and Nasmyth, 1984). Rather, it indicates that activators and repressors either co-exist at the *SIR*-repressed promoter or are able to rapidly exchange between promoter-bound and promoter-free states. It is possible that HSF and TBP gain access to the DNA prior to maturation of the heterochromatin. The subsequent formation of the *SIR*-dependent nucleoprotein complex, readily detected by both DNase I and MNase, would, by this thinking, render such regulatory proteins inactive, 'imprisoning' them on the surface of the DNA. In support of this idea, groove-specific interactions at HSEs 1–3 are perturbed to the same degree in a weakly repressed, single-silencer strain as in one bearing a strongly repressed, silencer-flanked gene (data not shown). Thus, the hyper-repressed state can not be entirely explained by altered sequence-specific interactions at the UAS; a subsequent event must contribute to dominant silencing. Further studies will be required to unravel the detailed mechanisms by which this heterochromatic structure silences the heat shock gene and how HSF, once activated, overrides it.

Materials and methods

Gene constructions

Targeting of *HMRE* silencer elements to the *HSP82* locus was done using derivatives of the transplacement vector p103, a Yip5 derivative (*URA3*) bearing the *HSP82* *EcoRI* fragment spanning –1300 to +1601 (where +1 corresponds to the principal transcription start site) (Lee and Gross, 1993). A minimal *HMRE* element, containing the core ABF1, RAP1 and ORC binding sites, was amplified by PCR using pJA82.6 as template (Abraham *et al.*, 1984) and TGGCTTAAATCGCAATT-TAATACC and CCTAAGCTCATAAATTGGACG as primers. The 5' ends of these primers contain unique half sites for *SphI* and *StuI*, which were used in the construction of *HMRE* dimers, and also facilitate identification of the desired ligation products (head-to-tail). Using this strategy, individual *HMRE* modules were separated by a 6 bp spacer. Following creation of the desired *HMRE* monomers and dimers, *Clal* linkers were appended and the resultant products cloned into the unique *Clal* site of p103, located 673 bp upstream of *HSP82*. pES101 and pES102 bear single *HMRE* elements (in native and inverse orientation, respectively), while pES201 and pES202 contain the corresponding *HMRE* dimers.

To construct *hsp82* alleles flanked at the 3' end by *HMRE* modules, a second integrating vector, pES1000, was made. pES1000 contains the *BglIII* (+239) to *Clal* (+3116) *HSP82* fragment cloned into pRS306 (*URA3*). Monomeric and dimeric *HMRE* modules were excised from *Clal*-digested pGEM7Z-1 and pGEM7Z-2, respectively, blunt-ended by mung bean nuclease, and cloned into the unique *MluI* site of pES1000. The *MluI* site (+2342) lies 150 bp downstream of the *HSP82* stop codon and ~50 bp 3' of the mapped transcription termination site (Farrelly and Finkelstein, 1984). The resultant constructs were termed pES1001 (*HSP82* 3'-flanked with an *HMRE* monomer) and pES2001 (*HSP82* 3'-flanked with an *HMRE* dimer). All six *HMRE/HSP82* constructs were confirmed by DNA sequencing.

Strain constructions

To construct strains bearing upstream silencers, SLY103 (*hsp82Δ::CYH2^s, cyh2^s, sir4Δ::HIS3*) was transformed with *AvaI*-linearized pES101, pES102, pES201 and pES202 (*AvaI* is at position –914 of *HSP82*) creating the strains EAS101, EAS102, EAS201 and EAS202, respectively. *URA⁺* transformants were selected, *URA⁻* recombinants counterselected, and cycloheximide resistant clones screened as previously described (Lee and Gross, 1993).

To construct strains bearing a single 3' *HMRE* module, EAS101 and EAS102 were transformed with *EcoRI*-linearized pES1001, producing

EAS1001 and EAS1002, respectively. Similarly, to construct strains bearing 3' *HMRE* dimers, EAS201 and EAS202 were transformed with pES2001 resulting in the strains EAS2001 and EAS2002, respectively. Transformants were selected and counterselected as above. *FOA^r* revertants were screened by PCR to identify clones bearing the desired *hsp82* allele, where *HMRE* elements are separated by 3015 bp. To restore the *SIR⁺* phenotype, *sir4⁻* strains were transformed with an episomal copy of *SIR4* (pJR368), backcrossed to an isogenic *SIR⁺* strain (P2a), sporulated, and tetrads dissected. Spores were screened for histidine auxotrophy (*SIR4⁺*), mating type, and presence of the *HMRE/HSP82* allele (the latter via PCR). Strains EAS311 and EAS411, bearing 3' *HMRE* element(s) only, were constructed by transforming SLY101 (*HSP82⁺*) with *EcoRI*-linearized pES2001 (monomer) or pES2002 (dimer), respectively, and uracil prototrophs selected and counterselected as above. Desired recombinants were screened via PCR. In addition, these strains were rendered *sir4⁻* by replacing the *SIR4* gene with the *sir4Δ-2::HIS3* allele, borne on plasmid pMM7.1 (a gift of A.B.Rose and J.R.Broach, Princeton University) producing EAS301 (monomer) and EAS302 (dimer). To permit overexpression of *SIR4C* in an *SIR4⁺* background, we transformed strain EAS1031 with the *SIR4C* expression vector pADH-SIR4C (2μ; *LEU2*) (Cockell *et al.*, 1995), creating EAS1061. To render strains EAS101 and EAS2001 *sir4Δ-2*, we transformed them with LP793 (CEN; *LEU2*) (Kennedy *et al.*, 1995). (pADH-SIR4C and LP793 were generously provided by J.Lowell and L.Pillus, University of California, San Diego). Strains are listed in Table I; allele configurations are depicted in Figure 1.

Cultivation and heat shock/recovery conditions

Yeast strains were cultivated at 30°C in either rich medium (YPD) supplemented with 0.04 mg/ml adenine or synthetic complete medium lacking uracil (SDC-ura). Heat shock induction was achieved by adding an equal volume of pre-warmed medium (53°C) to an early log culture ($1-2 \times 10^7$ cells/ml), resulting in an instantaneous 9°C increase in temperature to 39°C. Following a 20 min heat shock, cultures were returned to non-inducing conditions (30°C) via addition of 0.8 vol of 4°C medium (recovery from heat shock). All aliquots removed for expression and structural assays were metabolically frozen by immersion in ice and the simultaneous addition of sodium azide to a final concentration of 20 mM as previously described (Lee and Gross, 1993).

Chemical induction

Chemical induction was accomplished via the addition of either 2 mM DNP, a mitochondrial uncoupler or 8% ethanol. Cultures were grown to 2×10^7 cells/ml in 40 ml of YPD. Following removal of a 10 ml control aliquot, an equal volume of medium containing either 4 mM DNP or 16% ethanol was added. Incubations were allowed to proceed for 10, 20, 30, 40 and 60 min. At each time point a 10 ml aliquot was removed and cellular RNA isolated.

Northern analysis

Total cell RNA was isolated from 10 ml aliquots of non-heat-shocked (NHS), heat-shocked (HS) and recovery (Rec) cultures, and processed as previously described (Erkine *et al.*, 1999). *HSP82* hybridization was performed at 45°C using a synthetic oligonucleotide [100mer spanning positions +2190 to +2289; coordinates 98686–98785 of ch. XVI (Saccharomyces Genome Database)] as probe. *YAR1*, *ACT1*, *CIN2* and *a1* hybridizations were conducted at 55°C using as probes antisense RNAs of 249 nt (ch. XVI, coordinates 99736–99985), 1434 nt (ch. VI, coordinates 53261–54695), 176 nt (ch. XVI, coordinates 95886–96061) and 486 nt (ch. III, coordinates 292556–293043) lengths, respectively. Following each round of hybridization, the blot was exposed to a Phosphor Screen (Molecular Dynamics) and hybridization signals were quantified using ImageQuant software. *HSP82*-, *YAR1*-, *CIN2*- and *a1*-specific signals were normalized to those of *ACT1*. To optimize quantitation of scarce *HSP82* transcripts, background was set equal to the signal present in RNA isolated from an *hsp82Δ* strain (SLY103).

DMS in vivo footprinting

Cells were grown to $1-2 \times 10^7$ cells/ml in 210 ml of YPD at 30°C. Following removal of a 10 ml aliquot for RNA isolation (NHS), a portion of the culture was reacted with either 0.1 or 0.2% DMS at 30°C for 2 min. The balance was diluted with an equal volume of 53°C medium and incubated at 39°C for 20 min, and an aliquot was removed for RNA isolation (HS) and another for DMS methylation at 39°C. The final 1/3 of the culture was mixed with 0.8 vol of 4°C medium and incubated at 30°C for 15 min. At that point a third RNA sample (Rec) was removed and the remainder reacted with DMS at 30°C. DMS

Table I. Yeast strains used

Strain	Genotype	Source
SLY101	<i>MATα ade⁻ can1-100 his3-11,15 leu2-3,112 trp 1-1 ura3 cyh2^r</i>	Lee and Gross (1993)
SLY103	SLY101; <i>hsp82Δ::CYH2^s sir4Δ-2::HIS3</i>	Lee and Gross (1993)
SLY105	<i>MATα can1 his4 leu2-3,112 trp1-1 ura3-52 sir4-351 HMRE/HSP82</i>	Lee and Gross (1993)
SLY106	<i>MATα can1 leu2-3,112 trp1-1 ura3 HMRE/HSP82</i>	Lee and Gross (1993)
P2a	SLY101; <i>MATα hsp82-P2</i>	C.B.Venturi
EAS101	SLY101; <i>sir4Δ-2::HIS3 hsp82-101</i>	this study
EAS111	SLY101; <i>hsp82-101</i>	this study
EAS121	EAS101; pRS316 (<i>CEN-URA3</i>)	this study
EAS131	EAS101; pJR368 (<i>SIR4-CEN-URA3</i>)	this study
EAS141	EAS101; pRS315 (<i>CEN-LEU2</i>)	this study
EAS151	EAS101; pLP793 (<i>sir4-42-CEN-LEU2</i>)	this study
EAS102	SLY101; <i>sir4Δ-2::HIS3 hsp82-102</i>	this study
EAS112	SLY101; <i>hsp82-102</i>	this study
EAS122	EAS102; pRS316 (<i>CEN-URA3</i>)	this study
EAS132	EAS102; pJR368 (<i>SIR4-CEN-URA3</i>)	this study
EAS201	SLY101; <i>sir4Δ-2::HIS3 hsp82-201</i>	this study
EAS211	SLY101; <i>hsp82-201</i>	this study
EAS221	EAS201; pRS316 (<i>CEN-URA3</i>)	this study
EAS231	EAS201; pJR368 (<i>SIR4-CEN-URA3</i>)	this study
EAS202	SLY101; <i>sir4Δ-2::HIS3 hsp82-202</i>	this study
EAS212	SLY101; <i>hsp82-202</i>	this study
EAS221	EAS202; pRS316 (<i>CEN-URA3</i>)	this study
EAS232	EAS202; pJR368 (<i>SIR4-CEN-URA3</i>)	this study
EAS301	SLY101; <i>sir4Δ-2::HIS3 hsp82-301</i>	this study
EAS311	SLY101; <i>hsp82-301</i>	this study
EAS401	SLY101; <i>sir4Δ-2::HIS3 hsp82-401</i>	this study
EAS411	SLY101; <i>hsp82-401</i>	this study
EAS1001	SLY101; <i>sir4Δ-2::HIS3 hsp82-1001</i>	this study
EAS1011	SLY101; <i>hsp82-1001</i>	this study
EAS1021	EAS1001; pRS316 (<i>CEN-URA3</i>)	this study
EAS1031	EAS1001; pJR368 (<i>SIR4-CEN-URA3</i>)	this study
EAS1051	EAS1001; pRS316 (<i>CEN-URA3</i>) pRS315 (<i>CEN-LEU2</i>)	this study
EAS1061	EAS1001; pJR368 (<i>SIR4-CEN-URA3</i>) pADH-SIR4C (<i>SIR4C-2μ-LEU2</i>)	this study
EAS1002	SLY101; <i>sir4Δ-2::HIS3 hsp82-1002</i>	this study
EAS1022	EAS1002; pRS316 (<i>CEN-URA3</i>)	this study
EAS1032	EAS1002; pJR368 (<i>SIR4-CEN-URA3</i>) <i>hsp82-1002</i>	this study
EAS2001	SLY101; <i>sir4Δ-2::HIS3 hsp82-2001</i>	this study
EAS2011	SLY101; <i>hsp82-2001</i>	this study
EAS2021	EAS2001; pRS316 (<i>CEN-URA3</i>)	this study
EAS2031	EAS2001; pJR368 (<i>SIR4-CEN-URA3</i>)	this study
EAS2041	EAS2001; pRS315 (<i>CEN-LEU2</i>)	this study
EAS2051	EAS2001; pLP793 (<i>sir4-42-CEN-LEU2</i>)	this study
EAS2002	SLY101; <i>sir4Δ-2::HIS3 hsp82-2002</i>	this study
EAS2022	EAS2002; pRS316 (<i>CEN-URA3</i>)	this study
EAS2032	EAS2002; pJR368 (<i>SIR4-CEN-URA3</i>) <i>hsp82-2002</i>	this study

reactions were quenched through the addition of an equal volume of stop buffer (0.2 M 2-mercaptoethanol, 40 mM sodium azide, 0.2 M EDTA) and chilled immediately. Genomic DNA was then isolated and analyzed as previously described (Erkine *et al.*, 1996). Sites of methylation were detected by AMPEX using a lower-strand primer spanning coordinates +26→-11. Reaction products were electrophoresed on an 8% sequencing gel, exposed to a Phosphor Screen and analyzed with ImageQuant software.

Nuclease digestion of chromatin

Cells were cultivated in 450 ml of YPD and subjected to essentially the same experimental conditions as above. Following NHS, HS and Rec incubations, samples were metabolically poisoned with 20 mM sodium azide and immediately chilled. Aliquots (10 ml) were then removed for RNA analysis. Cells were harvested and spheroplast lysates isolated and digested with DNase I (Sigma #D7291) essentially as described (Erkine *et al.*, 1995). Naked genomic DNA was isolated and digested with DNase I also as before. For MNase digestion, cells were cultivated as above, and nuclei isolated and digested with MNase (Pharmacia Biotech #27-0584-01) as described elsewhere (E.A.Sekinger, in preparation).

HSP82-specific cleavages were detected by AMPEX using both upper-strand (-342→-315) and lower-strand (+26→-11) primers as described above.

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