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Role of Chromatin Structure in *HMRE* Mediated Transcriptional Repression of the *HSP82* Heat Shock Gene

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We have examined the chromatin structure of the *HMRE/HSP82* and *HMRa/HSP82* alleles using three complementary approaches: DNase I chromatin footprinting, micrococcal nuclease (MNase) nucleosome-protected ladder assay, and an *in vivo E. coli dam* methylase accessibility assay. The footprinting results indicate that the promoter and silencer sequences are assembled into nucleoprotein complexes which exhibit no detectable change in structure, despite a 70-fold range in expression levels. In addition, the promoter region of the *HMRa/HSP82* allele is cleaved randomly by MNase in all cases, indicating the absence of canonical nucleosomes over this region irrespective of *SIR4* or heat-shock. Finally, no discernible difference in the accessibility of the *HMRE/HSP82* locus to *dam* methylase in *SIR4 vs. sir4* cells was seen, which again suggests that the chromatin structure of *HMRE/HSP82* allele is identical regardless of *SIR4*. Altogether, our results indicate that in contrast to other observations of the silent mating-type loci, no discernible structural alteration is detected at either *HMR/HSP82* allele regardless of *SIR* genetic background or transcriptional state of the gene.

Key words: Transcriptional repression, chromatin structure, DNase I footprinting

Eukaryotic cells present a number of cases in which the transcription state of a gene is affected by its position within the genome (20, 37). Repositioning a normally active gene near a heterochromatic region results in position effect variegation in *Drosophila* (6). Typically, the variegated phenotype presents as a patch of cells with the same phenotype surrounded by cells of a different phenotype. These patches represent clones of cells within which the spread of heterochromatin is sufficient to repress the gene causing the phenotype (6, 33). In the yeast *Saccharomyces cerevisiae*, position effect plays a critical role in determining the proper pattern of mating-type gene expression and in determination of cell type (10, 18, 19, 24). At *HML* and *HMR*, the mating-type genes are repressed by a mechanism that is region-specific: Simply transposing the mating-type genes away from either loci yields their activation, whereas inserting other RNA polymerase II- or RNA polymerase III-transcribed genes at either locus results in repression of the inserted gene (3, 21, 30). The genetic requirements for silencing at *HML* and *HMR* suggest the existence of a number of *trans*-acting factors (17, 23, 28, 29, 38) including products of the *SIR1-4* genes (28), as well as specific *cis*-active sites, or silencers, that may serve as a focal point on which *trans*-acting factors act (1, 3, 8, 21). Despite recent genetic and biochemical advances, the mechanism of the repression mediated by transcriptional silencers is far from clear.

Recent discoveries, however, suggest that silencing in yeast, like silencing in larger eukaryotes, results from the formation of particular chromatin structures that define a chromosomal domain. Although yeast does not possess heterochromatin at the cytological level, the connection between chromatin structure and yeast silencing comes from recessive mutations that affect the highly conserved amino-terminus of histone H4 and cause derepression of the silent loci (16, 17, 26). Thus, a particular histone H4-dependent chromatin structure is apparently

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required for silencing. Additionally, it has been known that the silent mating-type loci are refractory to a number of proteins that interact with DNA, including the HO endonuclease (25), enzymes involved in DNA repair (35, 36), and an ectopically expressed DNA methylase (7, 11, 14, 31). Thus, a *SIR*-dependent mechanism may make the silent mating-type loci less accessible to a variety of proteins that interact with DNA. Consistent with this notion are biochemical experiments evaluating nucleosome accessibility at particular genes (5). Using mercury-affinity chromatography, Chen *et al.* have found that histone H3 packaging the silent mating type cassette is less accessible to thiol reagents than those packaging actively transcribing gene (5). Recently, Braunstein et al. (4) have demonstrated that the silencing of mating type cassette is strictly associated with hypoacetylation of the ε-amino groups of lysines in the amino-terminal domains of three of the four core histones and this hypoacetylation was SIR-dependent. These studies suggest that silencing in yeast results from heterochromatin formation and argue that the silencing proteins participate in this formation.

In our lab, yeast strains have been created in which the function of an ectopic mating-type silencer could be examined. We found that this silencer element exerted *SIR4*-dependent repression of the *HSP82* heat shock gene. However, it repressed only basal, not induced, transcription. It rapidly lost its ability to repress the expression of *HSP82* following heat induction, and rapidly regained its function when cells were returned to nonstressful conditions. As these experiments indicated a striking flexibility in the regulation of the *HMRE/HSP82* allele, we wished to address more directly whether such changes in gene function were paralleled at the structural level. In this study, we have examined the chromatin structure of the *HMRE/HSP82* and *HMRa/HSP82* alleles using three complementary approaches: [1] DNase I chromatin footprinting; [2] micrococcal nuclease (MNase) nucleosome-protected ladder assay; and [3] an *in vivo E. coli* dam methylase accessibility assay. Our results indicate that in contrast to other observations of the silent mating-type loci (4, 5, 16, 17, 25, 26, 31, 35, 36), no discernible structural alteration is detected at either *HMR/HSP82* allele regardless of *SIR* genetic background or transcriptional state of the gene.

Materials and Methods

Yeast strains

All *Saccharomyces cerevisiae* haploid strains constructed for this study are derivatives of W303-1B (*MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-1*), IV16-17A (*MATa, can1-100, his4, leu2-3,112, trp1-1, ura3-52, sir4-351*), and B-7056 (*MATa, leu2-3,112, ura3-52, cyc1 :: CYH2^s, cyc7-67, cyh2^r*). These strains were obtained from R. Rothstein, A. Rose/J. R. Broach, and M. Hampsey, respectively.

DNase I footprinting technique

Cells were cultivated at 30℃ and heat-shocked at 39℃. Following addition of sodium azide, cells were converted to spheroplasts at 30℃ using oxalyticase (OD_{600} of cells in 0.2% SDS solution was reduced to 10% of that of the original non-spheroplasted cells), and then subjected to hypotonic shock. To minimize protein exchange and degradation, spheroplast lysates were digested with DNase I (Worthington; DPRF grade) in the presence of 40 mM Hepes (pH 7.2), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 2 μg/ml pepstatin A, 0.5 μ/ml chymostatin, 2.0 μ/ml antipain, 7.0 μ/ml E-64, and 2 mM benzamidine at 3℃ for 80 min. Digestions were terminated through addition of EDTA to 10 mM, and DNA was deproteinized, restricted with *Eco*RI, and electrophoresed on a neutral 2% agarose gel, blotted and hybridized. Naked control DNA, restricted with *Eco*RI, was digested with DNase I at 37℃.

Micrococcal nuclease nucleosome-protected ladder analysis

Yeast cell cultures were split into two equal aliquots, one of which was maintained throughout at 30℃ and the other of which was heat-shocked at 39℃ as described above. Following addition of sodium azide, cells were converted to spheroplasts as above. Crude nuclei were purified according to Szent-Gyorgyi et al and gently suspended in digestion buffer (40 mM Hepes [pH 7.5], 1 mM $MgCl_2$, 1 mM $CaCl_2$). Suspended nuclei were divided into aliquots for varying degree of digestion, prewarmed at 37℃ for 5 min, and digested with MNase (50~400 units MNase/sample) for 10 min at 37℃. Digestions were terminated through the addition of one tenth volume of 10× TES (500 mM Tris-HCl, pH 8, 50 mM EDTA, 500 mM NaCl). DNA was deproteinized, size-fractionated on a neutral 1.8% agarose gel, blotted, and UV-crosslinked to nylon membrane. Immobilized DNA was hybridized and stringently washed using RNA probes homologous to the *HSP82* promoter region. To control for sample-to-sample variation in MNase digestion, *HSP82*-specific probe was eluted (by incubating the membrane in 0.05 N NaOH at 37℃ for 30 min) and rehybridized with a probe homologous to a region of intergenic chromatin on chromosome XIII. Nucleosome repeat length calculations were based on the mobility of end-labeled *Hae*III-φX174 DNA fragments electrophoresed in

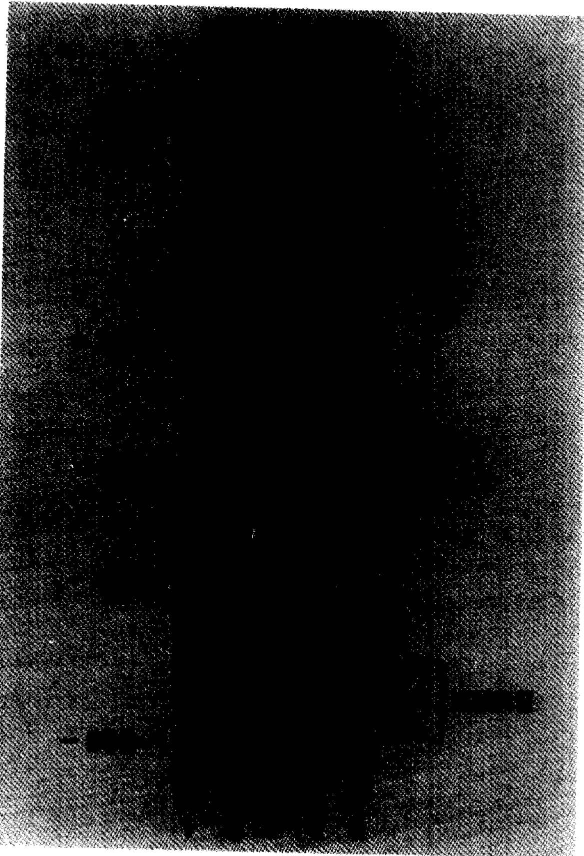

Fig. 1. DNase I chromatin footprints of the *HMRE/HSP82* alleles under transcriptionally inducing and non-inducing conditions. Spheroplast lysates, generated from YEp213-transformed (lanes 1, 4) or pKAN59-transformed (lanes 2, 5) SLY105 cells, non-heat-shocked (NHS) and 11 minutes heat-shocked (HS) as indicated, were digested for 80 minutes at 3℃ with 5×10^{-5} units DNase I/μg DNA. The DNA was purified, cleaved with *Eco*RI, electrophoresed on a 2% agarose gel, capillary blotted to GeneScreen, and indirectly end-labeled. Naked genomic DNA (lane 3), purified from strain SLY 105, was digested for 10 minutes at 37℃ with 1×10^{-5} U DNase I/μg DNA and processed similarly. The positions of landmark *HSP 82* restriction fragments are indicated on left; the locations and identity of protected sequences relative to the naked DNA control are provided on right. Also indicated is the location of the *HSP82* transcriptional unit (arrow), mapped with respect to the underlying DNA sequence. The strong protection at position −600 (dot), seen under all four states, has not yet been analyzed at nucleotide resolution but maps to a consensus ABF1 site (13/13 match between −600 and −588). It is also seen in the $HSP82^{+}$ allele (Fig. 2).

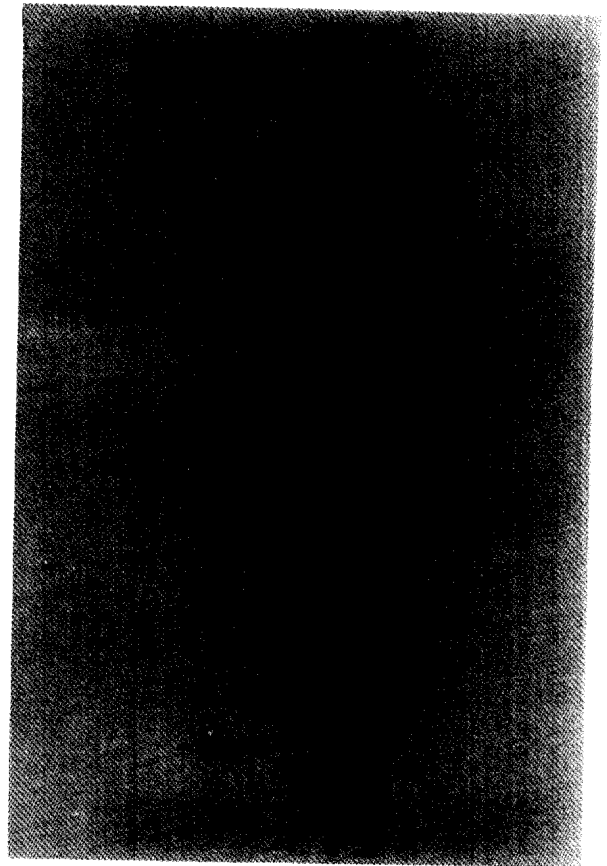

Fig. 2. DNase I chromatin footprints of the $HSP82^{+}$ allele under transcriptionally inducing and non-inducing conditions. Spheroplast lysates, generated from control (−) or 11 minute heat-shocked (+) W303-1B cells (lanes 1, 3 respectively), were digested with DNase I and processed as above. Deproteinized genomic DNA (lane 2), isolated from strain W303-1B, was digested with DNase I and processed as in Fig. 1. Sequence coordinates (left) and symbols (right) are as in Fig. 1.

parallel.

Results

Protein/DNA interactions within the regulatory region of *HMRE/HSP82* remain unchanged irrespective of *SIR4* or heat shock

Previous studies have shown that the promoter region of *HSP82* is organized into a constitutive, non-nucleosomal DNase I hypersensitive site in chromatin (4, 12). Similarly, *HMRE* is localized within a region of chromatin that is hypersensitive to cleavage by DNase I in both SIR^{+} and sir^{-} genetic backgrounds (25). To ask whether alterations in chromatin structure accompany changes in *HMRE/HSP82* function, we mapped sites of protein/DNA interaction within the upstream regulatory region of the *HMRE/HSP82* allele in both control and heat-shocked cells using the spheroplast lysate technique (12). Briefly, spheroplasts were obtained from both control and heat-shocked $SIR4^{+}$ and $sir4^{-}$ cells, lysed in a hypotonic buffer containing divalent cations, and mildly digested with DNase I. Purified DNA was then restricted with *Eco*RI, electrophoresed, blotted, and indirectly end-labeled. The results, depicted in Fig. 1, indicate that the promoter and silencer sequences are assembled into nucleoprotein complexes which exhibit no detectable change in structure, despite a 70-fold range in expression

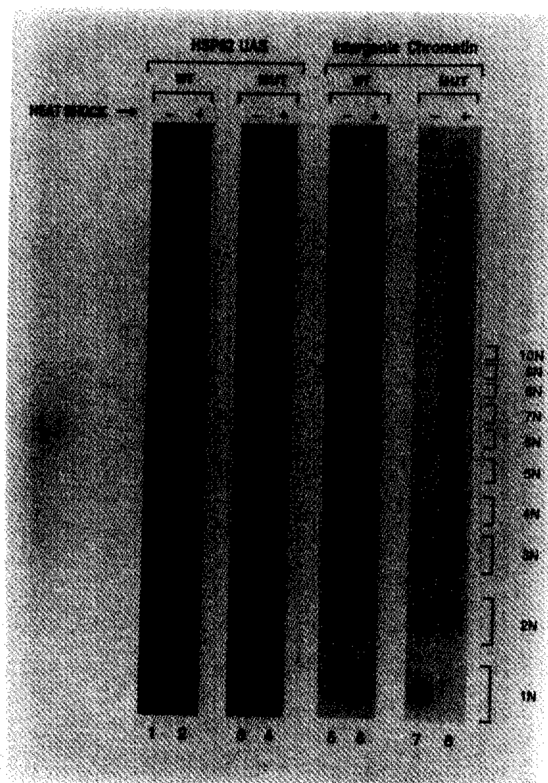

Fig. 3. MNase nucleosome-protected ladder assay of *HSP82*$^+$ and *hsp82-△HSE1* alleles. Nuclei were isolated from wild-type cells (WT) and HSE1 deletion mutant cells (strain *hsp82-△HSE1*, MUT) that were non heat-shocked (−) and heat shocked (+). The nuclei were digested with MNase and genomic DNA was isolated, electrophoresed, and transferred to a nylon membrane. The blot was hybridized with a probe specific for the promoter of *HSP82*. As a control, the *HSP82*-specific probe was eluted and the membrane was re-hybridized with a probe specific for a region of intergenic chromatin (IC) on chromosome XIII known to be packaged in canonical nucleosomes (38).

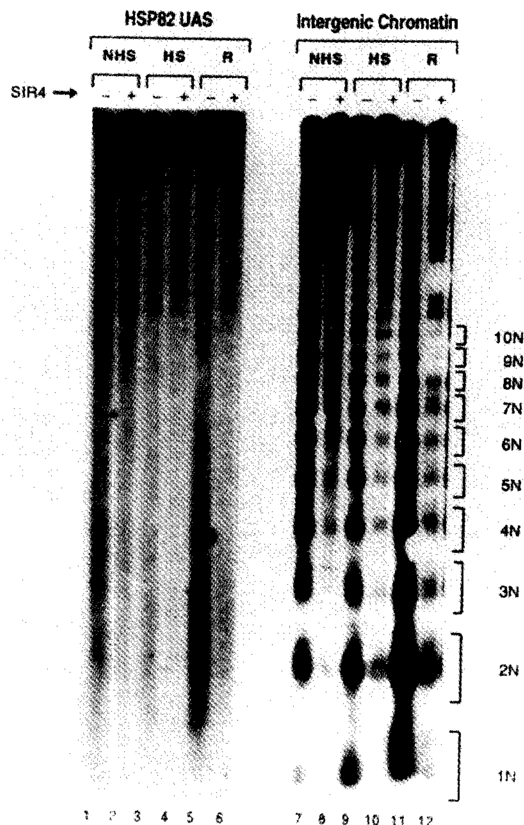

Fig. 4. MNase nucleosome-protected ladder assay of the *HMRa/HSP82* allele. Nuclei were isolated from SLY107 cells with *SIR4*$^+$ and *sir4* genetic backgrounds, control (NHS), heat shocked at 39℃ for 20 min (HS), and those heat-shocked at 39℃ for 20 min and then downshifted to 30℃ for 15 min (recovery [R]). The nuclei were digested with MNase, genomic DNA was isolated, electrophoresed, and transferred to a nylon membrane. The blot was hybridized with a probe specific for the promoter of *HSP82*. As a control, the *HSP82*-specific probe was eluted and the membrane was re-hybridized with a probe specific for a region of intergenic chromatin (IC) on chromosome XIII known to be packaged in canonical nucleosomes (38).

levels. In particular, the DNase I cleavage pattern reveals the presence of several internal footprints within broad regions of hypersensitivity relative to the naked DNA control (Figure 1, lanes 1, 2, 4, and 5 vs. 3). Protected sequences map to the TATA box and principal heat shock element (HSE1) of *HSP82*, as well as to each of the three functionally defined domains of the *HMRE* silencer: the ARS core consensus site A, the RAP1 binding site E, and the ABF1 binding site B (Figure 1). Therefore, the *HMRE/HSP82* allele is organized into a chromatin structure poised for transcriptional activation, much as seen in the wild-type allele (Fig. 2). We conclude that *HMRE*, while clearly functional and occupied by sequence-specific DNA binding proteins, has no detectable effect on the DNase I cleavage profile of the *HSP 82* promoter.

The promoter region of *HMRa/HSP82* is devoid of canonical nucleosomes irrespective of SIR4 or heat-shock

To test the possibility that *HSP82* promoter, normally nucleosome-free, is assembled into a more nucleosomal-like structure in the *HMRa/HSP82* allele, we employed the micrococcal nuclease (MNase) nucleosome-protected ladder assay. This assay has previously provided clear evidence for the *de novo* presence of nucleosomes in the promoter region of an *HSP82* promoter mutant lacking the high-affinity heat-shock factor (HSF) binding site, HSE1 (13). As shown in Fig. 3, discrete mono- and dinucleosomal-length bands are seen upon promoter-

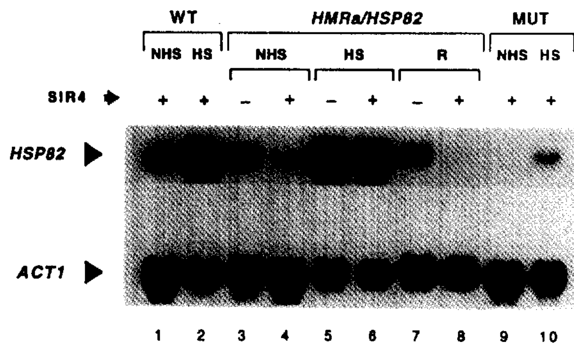

Fig. 5. Northern analysis of *HSP82*⁺, *HMRa/HSP82*, and *hsp82-△HSE1* cells. Total RNAs isolated from early log cultures of *HSP82*⁺, *HMRa/HSP82*, and *hsp82-△HSE1* cells prior to (NHS) and following (HS) a 20 minute, 30℃ to 39℃ temperature shift were electrophoretically separated and transferred to nylon membrane (Magnagraph, MSI). The membrane was sequentially hybridized with probes for *HSP82* and *ACT1*. *HMRa/HSP82* cells were additionally subjected to a 15 min down-shift protocol (R) as described before.

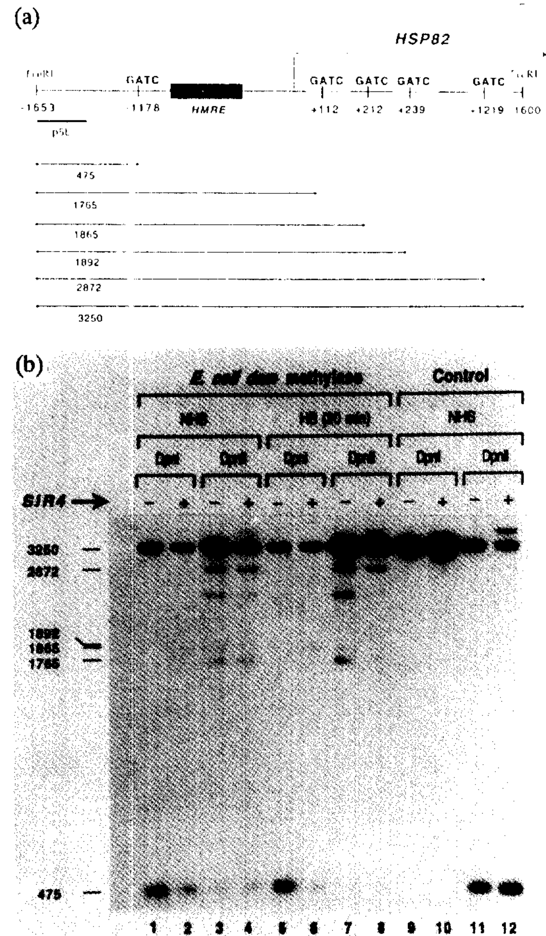

Fig. 6. *In vivo E. coli dam* methylase accessibility assay of the *HMRE/HSP82* chromosomal locus. A. Strategy of E. coli dam methylase accessibility assay. Top: Diagram of the structure of the *HMRE/HSP82* allele. Indicated are *Eco*RI restriction sites, GATC sites present upstream and coding region of *HSP82*, and the probe (5L) used in hybridization. Bottom: Expected restriction fragment sizes for *Eco*RI, *Dpn*I, *Dpn*II digestion. B. Southern blot analysis of *Eco*RI-restricted genomic DNA isolated from *HMRE/HSP82* cells. Exponentially growing cultures of SLY105 and SLY106 transformed with pMFH1 (*E. coli dam* methylase-containing plasmid) and pRS 425 (control 2μ LEU2 plasmid) were divided into two and one part of the cultures was subjected to 20 min 30 to 39℃ heat-shock. The genomic DNA was prepared from yeast cells according to Holm *et al.* (35). After an overnight digestion with 2.5 fold excess of *Eco*RI, genomic DNA was purified with IAC extraction, precipitated in ethanol, and dissolved in 1× TE. DNA was aliquoted for *Dpn*I and *Dpn*II digestion. After digestion for 5 hours with 2.5 fold excess of the *Dpn*I and *Dpn*II, genomic DNA was purified again. The digests were resolved by electrophoresis in a 1.2% agarose gel. After blotting to nylon membrane (GeneScreen, NEN) according to Southern (36), hybridization with p5L probe (spanning −1653 *Eco*RI site to −1403 *Hin*dIII site) was performed using rotating hybridizer (Polytech Products, Somerville, Mass), followed by high stringency washes and autoradiography at −70℃ with an intensifying screen. Lanes 1~8, pMFH1-transformed *HMRE/HSP82* strains SLY105 (lanes 1, 3, 5, 7) and SLY106 (lanes 2, 4, 6, 8). Lanes 8~12, pRS425-transformed SLY105 (lanes 9, 11) and SLY106 (lanes 10, 12).

specific hybridization of MNase digested chromatin DNA isolated from the *hsp82-△HSE1* strain (lanes 3 and 4). This is in striking contrast to the near-random MNase cleavage pattern of the wild-type *HSP82* promoter (lanes 1 and 2, Fig. 3). As a control, the HSP82-specific probe was eluted and the membrane was re-hybridized with a probe specific for a region of intergenic chromatin (IC) on chromosome XIII known to be packaged in canonical nucleosomes (2). All samples were found to be cleaved in typical nucleosomal arrays (Fig. 3, lanes 5-8).

To address whether nucleosomes similarly assemble over the promoter region of the *HMRa/HSP82* allele, nuclei were isolated from SLY107 cells with *SIR4*⁺ and *sir4*⁻ genetic backgrounds (conferred by the presence of either the SIR4-containing CEN plasmid, pJR368, or vector alone, YCp50). These cells were maintained at 30℃ (NHS), heat-shocked at 39℃ for 20 min (HS), or heat-shocked for 20 min followed by a 39℃ to 30℃ downshift for 15 min (R). The nuclei were digested with MNase, genomic DNA was isolated, electrophoresed, and transferred a nylon membrane. The blot was hybridized with a probe specific for the promoter of *HSP82*. As clearly seen in Fig. 4, the promoter region of the *HMRa/HSP82* allele is cleaved randomly by MNase in all cases, indicating the absence of canonical nucleosomes over this region irrespective of *SIR4* or heat-shock (lanes 1-6). Integrity of NHS, HS, and R samples was confirmed by re-hybridization of the blot with the IC-specific probe (Fig. 4, lanes 7-12). This result is consistent with the DNase I assay and indicates that there are no discernible structural differences in the promoter of *HMRa/HSP 82* despite the striking differences in transcription seen

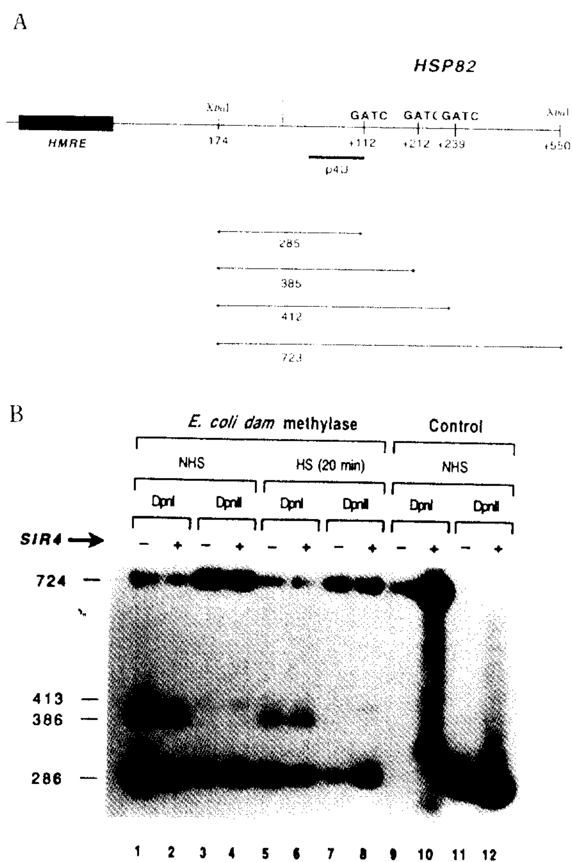

Fig. 7. *In vivo E. coli dam* methylase accessibility assay of the *HMRE/HSP82* transcriptional unit. A. Strategy of *E. coli dam* methylase accessibility assay. Top: Diagram of the structure of the *HMRE/HSP82* allele. Indicated are *Xba*I restriction sites, GATC sites present in the coding region of *HSP82*, and the probe (4U) used in hybridization. Bottom: Expected restriction fragment sizes for *Xba*I, *Dpn*I, *Dpn*II digestion. B. Southern blot analysis of *Xba*I-restricted genomic DNA isolated from *HMRE/HSP82*. Exponentially growing cultures of SLY105 and SLY106 transformed with pMFH1 (*E. coli dam* methylase containing plasmid) and pRS425 (control plasmid) were divided into two and one part of the cultures was subjected to 20 min 30 to 39°C heat-shock. The genomic DNA was prepared from yeast cells according to Holm *et al.* (35). After an overnight digestion with 10 fold excess of *Dpn*I and *Dpn*II digestion, genomic DNA was purified and digested with 10 fold excess of *Xba*I. After digestion for 5 hours, genomic DNA was purified again. The digests were resolved by electrophoresis in 1.75% agarose gel. After blotting to nylon membrane (GeneScreen, NEN) according to Southern (36), hybridization with p4U probe was performed using rotating hybridizer (Polytech Products, Somerville, Mass), followed by high stringency washes and autoradiography at −70°C with an intensifying screen.

in the same samples (more than 100-fold difference between heat-shocked and 15 min recovery samples; Fig. 5, compare lane 6 vs. 8). We surmise that the mechanism of the silencer-mediated repression at the *HMRa/HSP82* allele differs significantly from the chromatin-mediated repression seen at the *hsp82-△HSE1* allele, which also shows 100-fold reduction in *HSP82* transcript levels compared to wild-type (Fig. 5, compare lanes 9, 10 with 1, 2).

In vivo accessibility of *E. coli dam* methylase to the upstream and coding regions of *HMRE/HSP82* is identical irrespective of *SIR4* or heat-shock

Higher eukaryotes display methylation of cytosine residues in the CpG sequences in DNA (9), whereas *Saccharomyces cerevisiae* lacks this modification (27). Thus in yeast, mechanisms of gene expression and repression must operate independently of DNA methylation. Taking advantage of this phenomenon, we have exploited the yeast system by expressing a foreign DNA methylase to monitor changes in chromatin structure directly *in vivo*. Recent studies with *S. cerevisiae* have found that transcriptionally active loci are methylated more efficiently in their promoter and coding regions than are inactive genes (31). Thus, methylation of DNA in chromatin by *dam* methylase can be used as a novel and powerful probe for studying alterations in chromatin structure. This *in vivo* approach is complementary to the DNase I chromatin footprinting and MNase nucleosome mapping techniques, since each of the latter requires the breakage of cells to permit access of nuclei to the exogenous nucleases.

Four GATC sites exist within the coding region of *HSP82* (+112, +212, +239, and +1219) and one GATC site is present upstream of the transcription start site (−1178). To determine the *in vivo* accessibility of these GATC sites, the *HMRE/HSP82* strains SLY105 (*sir4*⁻) and SLY106 (*SIR4*⁺) were transformed with either the high-copy number (2μ plasmid pMFH1 (containing the *E. coli dam* methylase gene) or pRS425 (control plasmid). The genomic DNA was isolated from yeast cells according to Holm *et al.* (15) and digested with *Eco*RI and subsequently cleaved with *Dpn*I and *Dpn*II. DpnI only cleaves a fully methylated GATC site, whereas DpnII only cleaves a fully unmethylated GATC site. The digests were resolved by electrophoresis in 1.2% agarose gel and blotted to a nylon membrane (GeneScreen, NEN) according to Southern (32). The membrane was hybridized with a far-upstream probe. As depicted in Fig. 6.A, *in vivo* accessibility of the *HMRE/HSP82* allele can be monitored by examining the fragments generated by DpnI digestion. If *E. coli dam* methylase can access the DNA in the chromatin, *Eco*RI-*Dpn*I digestion of genomic DNA will generate fragments less than 3.25 kb in length. Fig. 6.B clearly demonstrates such fragments indicating dam methylase access to DNA (lanes 1, 2 and 5, 6 vs. lanes 9, 10). Dissapointingly, no discernible difference in the accessibility of the *HMRE/HSP82* locus to *dam* methylase in *SIR4* vs. *sir4* cells was seen (compare

lane 1 vs. 2 and 5 vs. 6). This result again suggests that the chromatin structure of *HMRE/HSP82* allele is identical regardless of *SIR4*.

As this strategy did not clearly resolve the coding region of *HSP82*, an additional assay was employed. Genomic DNA was prepared as before and initially digested with *Dpn*I or *Dpn*II, followed by digestion with *Xba*I. The digestions were resolved on a 1.75% agarose gel and blotted as above. The membrane was hybridized with a probe specific for the *HSP82* transcription unit (p4U). If *E. coli dam* methylase can access the DNA in the chromatin, *Xba*I-*Dpn*I digestion of genomic DNA will generate fragments less than 724 bp (Fig. 7A). Again we found a similar accessibility of *dam* methylase to chromatin in both *SIR4* and *sir4* genetic backgrounds (compare lanes 1 vs. 2 and 5 vs. 6 of Fig. 7B). Moreover, 15-fold higher expression of *HSP82* by heat-shock has no discernible effect on accessibility of *dam* methylase (compare lane 2 vs. 6). Thus, this result coupled with the DNaseI and MNase experiments discussed above suggest that at the *HMR/HSP82* allele, silencer-mediated repression is not mediated by substantial alterations in chromatin structure.

Discussion

A number of observations suggest that modification of local chromatin structure is involved in silencing at both *HMR* and *HML*. First, deletion of the highly conserved N-terminus of histone H4 causes derepression of both silent mating-type loci (17). Second, genetic evidence suggests that SIR3 protein directly interacts with the N-terminus of H4 (16), thereby implicating a role for interactions between non-histone and histone proteins in silencing at the *HM* loci. Third, chromatin structural analysis of the three loci containing mating-type information reveals that only in the genetically active *MAT* locus (or at *HM* loci in a sir^- background) is there a DNase I hypersensitive site corresponding to the HO endonuclease cleavage site; *SIR* causes the specific disappearance of such a site at *HML and HMR* (25). Fourth, a correlation between silencing of the mating-type loci and hypoacetylation of the e-amino groups of lysines in the amino-terminal domains of three of the four core histones has been recently demonstrated (4).

In contrast, we have presented three independent lines of evidence suggesting that silencing at *HMR/HSP82* may be either lost or regained in the absence of a substantial alteration of chromatin structure. First, DNase I footprinting analysis of the *HMRE/HSP82* allele has indicated the presence of constitutive protein/DNA interactions at all three functional domains of the *HMRE* silencer in both *SIR4* and *sir4* genetic backgrounds. Both elements normally occupied by sequence-specific DNA binding proteins within the wild-type *HSP82* promoter, HSE1 and TATA (34, 12), are likewise occupied in the silencer allele irrespective of *SIR* or heat shock, suggesting concurrent occupancy of silencer and promoter binding sites. Second, the MNase nucleosome-protected ladder assay indicates that there are no discernible structural differences in the promoter of *HMRa/HSP82* despite the striking differences in transcriptional states (more than 100-fold difference between heat-shocked and 15 min recovery samples; compare lane 4 vs. 6 of Fig. 4 and the corresponding lanes 6 vs. 8 of Fig. 5). This result indicates that the promoter region of the *HMRa/HSP82* allele is devoid of canonical nucleosomes independent of either *SIR4* or heat shock. Third, *in vivo E. coli dam* methylation reveals no discernible difference in the degree of DpnI digestion of the *HMRE/HSP82* genetic locus in genomic DNAs isolated from *SIR4* and *sir4* cells, suggesting that *E. coli dam* methylase has equal accessibility to chromatin regardless of *SIR4*. These results are consistent with the remarkable flexibility in transcriptional regulation at the *HMR/HSP82* alleles. Specifically, rapid re-establishment of repression at the *HMR/HSP82* alleles appears to occur in the absence of significant chromatin remodelling. This situation would thus appear to contrast with the one prevailing at the *HM* loci, where complete inactivation of mating-type information required nearly 10 hours following shift of a sir3ts mutant to the permissive temperature (22). However, in the experiments with the $sir3^{ts}$ mutant, a functional *SIR* complex needed to be re-established *de novo*, a process requiring passage through S-phase. In our experiments, the *SIR* complex, while functionally neutralized, likely retains its structural integrity during heat shock since the ectopic a1 gene remains silenced under conditions that strongly induce *HSP82*. We suggest that following a return to non-stressful conditions, the preexisting *SIR* complex dominates and the basal level of *HSP82* transcription is silenced immediately.

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