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 a 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; Erkine 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 CiaI (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 Erkine 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; Erkine 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+/H11001 strains (– and +/H11001, respectively) bearing the indicated HMRE/HSP82 alleles. As a control, RNA was isolated from an HSP82+/H11001 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.
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![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.](image)

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<th>Allele</th>
<th>Non-induced (normalized to hsp82-201/sir4–)</th>
<th>Induced (20’) (normalized to hsp82-201/sir4–)</th>
<th>Recovery (15’) (normalized to hsp82-201/sir4–)</th>
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<td>1700±80 (n=6)</td>
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<td>1600±80 (n=6)</td>
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<tr>
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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 MluI 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,
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 (YAR1, 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
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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 metastable, 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

A

\[ hsp82-2001 \]

\[ \text{DNA} \quad \text{NHS} \quad \text{HS} \quad \text{Rec} \]

\[ \text{CTAG} \]

\[ \text{HSP82} \quad \text{ACTIN} \]

\[ 100 \quad 1700 \quad 225 \]

\[ 1 \quad 2 \quad 3 \quad 4 \]

\[ 5 \quad 6 \quad 7 \quad 8 \quad 9 \quad 10 \quad 11 \quad 12 \]

\[ 13 \quad 14 \quad 15 \quad 16 \quad 17 \quad 18 \quad 19 \quad 20 \quad 21 \quad 22 \quad 23 \quad 24 \quad 25 \quad 26 \]

\[ \text{DNase I} \]

\[ \text{TATA} \]

\[ \text{HAP2/3/5} \]

\[ \text{URS1} \]

\[ \text{HSE1} \]

\[ \text{HSE2} \]

\[ \text{HSE3} \]
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 AMPEX 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; Erkine 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 transcription 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 (Erkine 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.

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 affects 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 (Erkine 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,
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 HMLE 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 (Diffléy and Stillman, 1989). This would be consistent with observations that efficient silencing occurs most readily in specific nuclear compartments where silencing proteins are sequestered (Maiiet 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

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**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.

![Fig. 8. SIR repression alters occupancy of the HSP82 TATA box.](image-url)
the \textit{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 \textit{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 \textit{HMR} (Donze et al., 1999). Also consistent with this idea is the earlier finding that \textit{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 \textit{HSP82} to transcriptional repression may reflect special properties of HSF. This factor, virtually unique amongst \textit{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 \textit{trans}-activating target promoters \textit{in vivo}. Perhaps this ‘super-activating’ property of HSF allows it to induce \textit{HSP82} transcription even when the target promoter is bracketed by four silencers.

\textbf{\textit{SIR} catalyzes the formation of a distinctive chromatin structure at \textit{HSP82}}

Detailed analysis of the \textit{SIR}-dependent chromatin upstream of \textit{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 TFIIID or RNA polymerase (Oegenschlager 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 \textit{SIR} \textit{\textsuperscript{+}} nuclei, inconsistent with the structure being a nucleosome. A similar \textit{SIR}-dependent enhancement in MNase sensitivity has been observed within the UAS region at \textit{HML\textsubscript{\alpha}} (Weiss and Simpson, 1998).

Whatever these \textit{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 heterochromatin structure is likely to be the cause and not simply the consequence of genetic inactivation, since \textit{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).

\textbf{How does \textit{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, \textit{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 TFIIID or pol II holoenzyme complexes. Finally, \textit{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 \textit{Drosophila} and human (Rougvie and Lis, 1988; Brown et al., 1996); it is thus formally possible that heterochromatin silences \textit{HSP82} by causing the inappropriate pausing of RNA polymerase.

Our results are most consistent with \textit{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 \textit{SIR} \textit{\textsuperscript{+}} 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 \textit{HSP82} is subject to dominant \textit{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 KMnO\textsubscript{4} 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 \textit{HMRE/HSP82}. Indeed, epigenetic inheritance of transcriptional states has been seen at both \textit{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 shear
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 DNA conformation, groove-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 pA82.6 as template (Abraham et al., 1984) and TGCCTGAATTCGCAATATTAAAAACC and CCTAAAGCTCATAACTTGGACG as primers. The 5’ ends of these primers contain unique half sites for HMRE/HSP2 modules were separated by a 6 bp spacer. Following creation of the desired HMRE monomers and dimers, Cia1 linkers were appended and the resultant products cloned into the unique Cia1 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 hsp82 (+239) to Cia1 (+3116) HSP82 fragment cloned into pRS306 (URA3). Monomeric and dimeric HMRE modules were excised from Cia1-digested pGEM7Z-1 and pGEM7Z-2, respectively, blunt-ended by mung bean nuclease, and cloned into the unique Milu site of pES1000. The Milu site (+2342) lies 150 bp downstream of the HSP82 stop codon and –50 bp 3′ of the mapped transcription termination site (Farrellly 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 (hsp82A::CHIZ2, ch2, str4::HIS3) was transformed with Avnl-linearized pES101, pES102, pES201 and pES202 (Avnl is at position –914 of HSP82) creating the strains EAS101, EAS102, EAS201 and EAS202, respectively. URA4 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+ revertants were screened by PCR to identify clones bearing the desired hsp82 allele, where HMRE elements are separated by 3015 bp. To restore the SIR4+ phenotype, sir4+ strains were transformed with an episomal copy of SIR4 (pR368), backcrossed to an isogenic SIR4+ 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 sir4-A2::His3 allele, borne on plasmid pMM71 (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 EAS103 with the SIR4C expression vector pADH-SIR4C (2μ, LEU2) (Cockell et al., 1995), creating EAS1061. To render strains EAS101 and EAS2001 sir4+, 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 × 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 YPDA. 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 [100 mer spanning positions +2190 to +2289; coordinates 98686–98785 of ch. XVI (Saccharomyces Genome Database)] as probe. YAR1, ACT1, CIN2 and al hybridizations were conducted at 55°C using as probes antisense RNAs 2289 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 al-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 hsp82A strain (SLY103).

DMS in vivo footprinting

Cells were grown to 1–2 × 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 1. Yeast strains used

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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 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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References

control requiring sequences both 5' and 3' to the regulated region. 


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