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Cooperation at a distance between silencers and proto-silencers at the yeast *HML* locus

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Transcriptional repression at the silent yeast mating type loci is achieved through the formation of a particular nucleoprotein complex at specific *cis*-acting elements called silencers. This complex in turn appears to initiate the spreading of a histone binding protein complex into the surrounding chromatin, which restricts accessibility of the region to the transcription machinery. We have investigated long-range, cooperative effects between silencers by studying the repression of a reporter gene integrated at the *HML* locus flanked by various combinations of wild-type and mutated silencer sequences. Two silencers can cooperate over >4000 bp to repress transcription efficiently. More importantly, a single binding site for either the repressor activator protein 1 (Rap1), the autonomous replicating sequence (ARS) binding factor 1 (Abf1) or the origin recognition complex (ORC) can enhance the action of a distant silencer without acting as a silencer on its own. Functional cooperativity is demonstrated using a quantitative assay for repression, and varies with the affinity of the binding sites used. Since the repression mechanism is Sir dependent, the Rap1, ORC and/or Abf1 proteins bound to distant DNA elements may interact to create an interface of sufficiently high affinity such that Sir-containing complexes bind, nucleating the silent chromatin state.
Keywords: Abf1/DNA loops/ORC/Rap1/yeast mating type

Introduction

For many years *Drosophila* has been a paradigm for studying long-range interactions in chromatin-mediated gene regulation. Examples include the developmentally regulated expression of homeotic complexes (reviewed in Orlando and Paro, 1995), various *trans*-sensing effects between homologous chromosomes (reviewed in Tartof and Henikoff, 1991) and position–effect variegation (reviewed in Karpen, 1994). However, two examples of regulated chromatin domains in budding yeast recently have allowed extensive genetic analysis of related phenomena in this highly tractable eukaryotic system. These are the transcriptional repression at telomeres and at the silent mating type loci, *HML* and *HMR* (reviewed in Laurenson and Rine, 1992; Palladino and Gasser, 1994). A number of *trans*-acting factors appear to be common to both types of repression in yeast, suggesting similarities in their mechanism. These common factors include three out of the four silent information regulators (Sir2, Sir3, Sir4), an N-terminal acetylase (Aparicio *et al.*, 1991) and the repressor activator protein 1 (Rap1; Kurtz and Shore, 1991; Kyrion *et al.*, 1993), as well as N-terminal domains of the histones H3 and H4, implying involvement of chromatin structure at the nucleosomal level (Kayne *et al.*, 1988; Thompson *et al.*, 1994b). Other factors appear to be more specific for a particular repressed domain. For example, mutations in *SIR1* affect specifically mating type silencing (Aparicio *et al.*, 1991), while overexpression of the telomerase RNA gene suppresses only telomeric silencing (Singer and Gottschling, 1994).

The most likely model for the establishment of repression at the mating type loci and at telomeres is the spreading of a modified chromatin structure (less accessible or 'silent' chromatin) achieved through the binding of Sir3 and Sir4 to histones H3 and H4 (Hecht *et al.*, 1995). This apparent spreading of repression begins at 'initiation sites', which correspond to silencers at mating type loci, and to TG_{1-3} repeats at telomeres. Such sites appear to function by attracting and/or nucleating the binding of complexes containing Sir3 and Sir4 to chromatin. At telomeres, repression is mediated by Sir interaction with the C-terminal domain of Rap1, which itself is bound to multiple sites in the telomeric repeat (Kyrion *et al.*, 1993; Liu *et al.*, 1994). Other proteins, like Rif1 (Hardy *et al.*, 1992), also bind the Rap1 C-terminal domain and appear to compete for the Sir3–Sir4 interaction (Kyrion *et al.*, 1993; Buck and Shore, 1995). At *HML* and *HMR* the targeting of Sir proteins is achieved through interaction with two or three DNA binding proteins that recognize specific sequence elements within the silencer (reviewed in Laurenson and Rine, 1992), namely Rap1, the autonomous replicating sequence (ARS) binding factor 1 (Abf1) and an ARS consensus recognized by the origin recognition complex (ORC). These individual protein binding sites are referred to here as proto-silencers, in analogy to proto-enhancers, which have no enhancer function on their own, but which promote transcription in combination with one another (Fromental *et al.*, 1988).

There are four mating type silencers, with one termed E and one termed I (for essential and important, respectively) flanking each of the two *HM* loci. Each silencer contains an ARS consensus sequence and each is capable of supporting the initiation of DNA synthesis on a plasmid (Abraham *et al.*, 1984; Feldman *et al.*, 1984). In addition to the ARS consensus, each of the silencers at *HML*-E

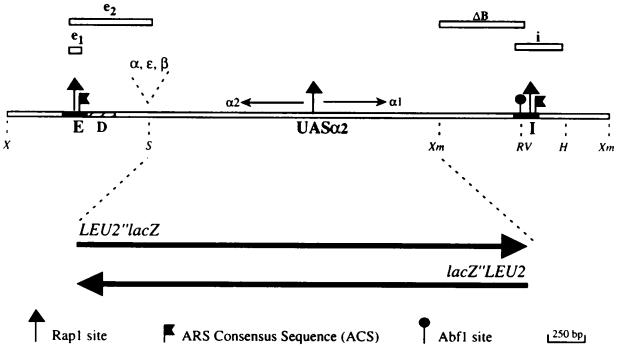

Fig. 1. The *LEU2"lacZ* reporter constructs used in this study. Top: shown is a diagram of the *HMLα* locus, indicating the positions of the *HML* alleles used in this work (see Materials and methods), important restriction enzyme sites, the upstream activating sequence (UASα2), the two *α1* and α2 mating type genes, the E and I silencers and the D element (hatched box). The minimal E and I silencers are shown as black boxes and their size (130 and 150 bp, respectively) and localization are according to Feldman *et al.* (1984). The symbols which represent the Rap1 sites, Abf1 sites and ARS consensus sequences (ACS) are shown at the bottom of the figure. Deletions used in this study are represented as open boxes above the *HML* diagram. The e_1 deletion corresponds to a substitution of 34 nucleotides by a *Bgl*II linker within E, inactivating both the Rap1 site and the ARS sequence (Mahoney *et al.*, 1991). The e_2 deletion extends e_1 until the *Spe*I site, removing the D element (see Materials and methods). ΔB corresponds to a deletion of the *HML Xmn*I–*Eco*RV 592 bp fragment carrying the Abf1 site of I. The i deletion, or $I^{\Delta 242}$ in Mahoney and Broach (1989), is a 280 bp deletion which removes the Abf1 site, the Rap1 site and the ARS sequence. Oligonucleotides corresponding to binding sites for ORC, Abf1 and Rap1 (named respectively α, β and ε, see Figure 5) were inserted at the junction between the *HML* DNA and the reporter gene at the *Spe*I site. (X) *Xba*I, (S) *Spe*I, (RV) *Eco*RV, (H) *Hin*dIII, (Xm) *Xmn*I. Bottom: the 3.4 kb *LEU2"lacZ* reporter gene DNA is inserted in place of a 2.1 kb *HML Spe*I–*Xmn*I DNA fragment.

and *HML*-I contains a Rap1 binding site, and *HML*-I also binds Abf1 (Buchman *et al.*, 1988; Hofmann *et al.*, 1989). The major silencer at *HMR*, *HMR*-E, contains high affinity sites for all three factors (Brand *et al.*, 1987). In the natural *HMR*-E silencer any two of the three sites is sufficient to promote silencing (Brand *et al.*, 1987), although in a synthetic silencer, created *de novo* from oligonucleotides, all three sites are essential (McNally and Rine, 1991). When *HML* is carried on a plasmid or is integrated at a centromere-proximal location, the presence of both silencers was found necessary for complete repression (Feldman *et al.*, 1984; Shei and Broach, 1995), while at its natural locus near the left telomere of chromosome III, either the E or the I silencer could function alone to repress the native α2 promoter (Mahoney and Broach, 1989). An as yet uncharacterized sequence adjacent to the *HML*-E silencer, called D, also seemed to participate in silencing of the α2 promoter (Mahoney *et al.*, 1991; see Figure 1). To date, the deletion studies defining important sites within *HML*-I are very limited and, apart from mapping the Abf1 site *in vitro* (Buchman *et al.*, 1988), it is not known which factors participate in *HML*-I function.

In vitro reconstitution using *HMLα* DNA and nuclear scaffold extracts showed that DNA loops can be formed between the two silencers and the promoter region. These were competed efficiently by an excess of Rap1 binding sites, consistent with the involvement of Rap1 (Hofmann *et al.*, 1989). Such observations suggest that long-range associations between silencers might contribute to the observed gene repression at this locus. In our current study, we investigated the functionality of these putative associations by measuring *HML* silencing in various combinations of silencers and proto-silencers. We demonstrate that the two silencers at *HML* do not work independently and that proto-silencers (individual protein binding sites) act as auxiliary elements to enhance the strength of a distant silencer. These silencing elements act in synergy over several kilobases, strongly suggesting that long-range interactions, even if transient, may be part of the silencing mechanism.

Results

In order to investigate the role of the *cis*-acting elements involved in *HML* silencing in a quantitative manner, we constructed a series of yeast strains in which the coding regions at *HML* are replaced by a minimal *LEU2* promoter (*LEU2'*) fused to the bacterial *'lacZ* reporter gene. This reporter construct is flanked by different combinations of wild-type and mutated E and I sequences (Figure 1) and the strains are otherwise fully isogenic. The degree of silencing was monitored by the β-galactosidase activity produced by the cells using a quantitative soluble assay, and was confirmed by the X-gal colour assay on filters (Breeden and Nasmyth, 1985).

A β-galactosidase assay for HML silencing

Cells carrying the *LEU2"lacZ* at the *HML* locus flanked by the natural *HML*-E and -I silencer sequences revealed very low β-galactosidase activity (0.07 Miller unit, Figure 2A). This was independent of the orientation of *LEU2"lacZ* with respect to the silencers. The assay for β-galactosidase activity on filters (X-gal assay) failed to detect any activity (i.e. all colonies are white, data not shown). The *lacZ* expression level is highly sensitive to known modifiers of mating type silencing: the loss of *SIR1* or *SIR3* by gene disruption increases β-galactosidase activity by 40- to 60-fold (3–4 Miller units), as does the overexpression of Sir4 or its C-terminal domain (pSir4 and pSir4C, Figure 2A). Intriguingly, an elevated dosage of either *SIR1* or *SIR3* by their presence on a multicopy plasmid also derepresses slightly (at most 6-fold in the soluble assay; see pSir1 and pSir3, Figure 2A). Consistent with the soluble β-galactosidase assay, these colonies are blue by the X-gal filter assay (data not shown). A slight derepression due to *SIR1* overexpression has been observed previously at telomeres (Chien *et al.*, 1993), but not at the mating type loci. These results confirm that *LEU2"lacZ* expression is repressed in a Sir-dependent manner when integrated at *HML*.

The advantage of the β-galactosidase activity assay is that it is linear over a large range of values and that the protein activity accurately reflects even small variations in gene transcription (Guarente and Mason, 1983). To demonstrate this, we use a strain that carries the reporter gene flanked by *HML*-E and -I, in which β-galactosidase expression is very low, and disrupt the genomic *HMR* locus. This reproducibly lowers expression levels (i.e. improves silencing) by 2-fold (Figure 2B). The 'normal' silenced level can be restored by introducing a low copy number (CEN-ARS) plasmid carrying the entire *HMR* region (pHMR), thereby restoring the normal dosage of

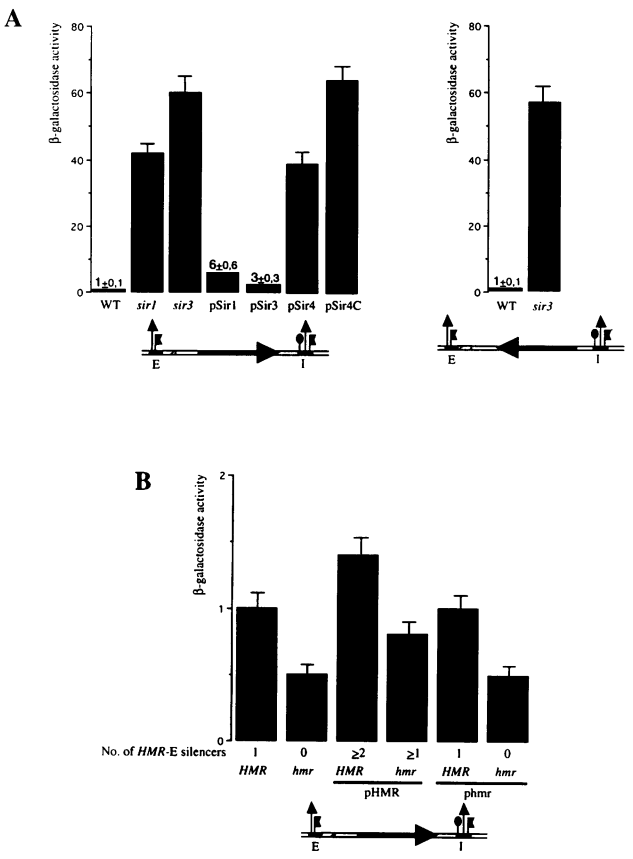

Fig. 2. *LEU2''lacZ* expression at the *HML* locus requires Sir proteins and is sensitive to the dosage of repressed domains. The relative β-galactosidase activities produced in yeast strains carrying a *LEU2''lacZ* reporter gene inserted between the E and I silencers at the *HML* locus are indicated in the bar graph. The structure of the *HML* region in the assayed strains is indicated below each diagram. The symbols are as in Figure 1. Strains with the reporter gene in either orientation according to the E and I silencers produced the same amount of β-galactosidase activity (0.07 Miller units, see Materials and methods) which was standardized as 1 (strain EG5, left diagram in A and B and strain EG33, right diagram in A). The error bars represent the standard deviation of the mean, calculated from the results of at least four independent trials; in most cases, the standard deviation did not exceed 10%. To avoid effects of leucine control, all strains were made LEU$^+$ by transformation and were grown in the absence of leucine.
(**A**) *LEU2''lacZ* repression depends on silencing factors. Strains lacking Sir proteins (*sir1*, *sir3*) or strains carrying multicopy plasmids carrying the genes encoding Sir1 (pSir1) or Sir3 (pSir3), Sir4 (pSir4) or the C-terminal third of Sir4 (pSir4C) are indicated. WT: wild-type for known silencing factors. Control strains carrying the vectors without inserted *SIR* genes produced the same amount of β-galactosidase as the corresponding WT strain (data not shown). For strains with a low level of expression, the actual numerical values are indicated. (**B**) The β-galactosidase activities according to the number of repressed *HMR*-E sequences in the cell. *hmr* indicates that the *HMR*-E region has been deleted (see Materials and methods). The number of *HMR*-E silencers present in each cell is indicated below each column, assuming that the copy number of the centromeric plasmid pHMR is one per cell. Beneath each column, the relevant strain characteristics and any additional plasmids in that strain are given.

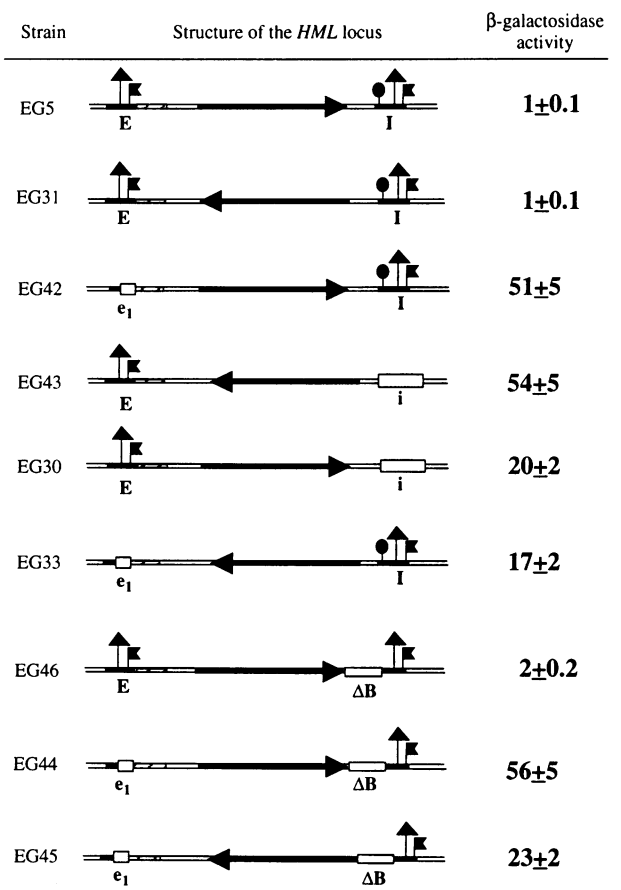

Fig. 3. Cooperative effects between two distantly located silencers. This figure includes the structure of various *LEU2''lacZ* insertions at *HML*, as well as the name of the strains to the left of the structure and the relative values of β-galactosidase produced in the corresponding strain to the right, standardized to the 0.07 Miller units found in EG5. All the strains derive from GA210 and contain a *LEU2* plasmid. The symbols are described in Figure 1 and the β-galactosidase activity measurements in Figure 2.

HMR. This suggests that a tight balance of factors is involved in repression, such that an increased dosage of one repressed locus alters silencing at another. Consistently, increasing the *HMR* copy number by introducing the CEN-based pHMR plasmid into wild-type cells, has the opposite effect and slightly disrupts the silencing of *LEU2''lacZ* at *HML* (Figure 2B). As a control, we have also introduced a derivative of pHMR carrying deletions of the *HMR*-E silencer (phmr, Figure 2B). These transformants show no modification of β-galactosidase expression, demonstrating that *HML* silencing can be affected significantly by adding or removing a single copy of a repressed, but not an active, *HMR* domain. This suggests that at least one factor that acts at both *HM* loci, for example Sir1 and/or the Sir3–Sir4 complex, is limiting within the cell. These highly reproducible shifts in β-galactosidase activity demonstrate the sensitive and quantitative character of our *lacZ* expression assay for monitoring levels of *HM* silencing.

How efficient is a single HML silencer at establishing repression?

We have deleted either the E or the I silencer from the *LEU2''lacZ* construct integrated at *HML*, to analyse the effect of a single silencer on gene expression (see Figure 1 for deletion boundaries). When a single silencer (either *HML*-E or *HML*-I) is located 4 kb away from the promoter, expression levels are similar to those measured in *sir3* cells, i.e. the gene is fully derepressed (compare strains EG5 with EG42 and EG31 with EG43 in Figure 3; and Figure 2A). When the single intact silencer is located directly upstream of the *LEU2'* promoter (within 0.5 kb),

derepression is partial (17- to 20-fold higher expression over the repressed state; compare strains EG5 with EG30 and EG31 with EG33, Figure 3). Similar results, i.e. strong repression with two silencers, weak repression with an upstream silencer and no repression with a downstream silencer, were also observed when *ADE2* was used instead of *LEU2″lacZ* (data not shown), showing that this phenomenon is not promoter dependent. These findings demonstrate first of all that E and I elements act as weak silencers on their own. That is, they partially repress a promoter in close proximity, but fail to act on a more distantly located promoter. Secondly, since the E and I elements together repress better than either element alone, even when promoter-proximal, the silencers must cooperate in this context to create an efficiently repressed domain. The efficiency of each individual silencer appears to be roughly equal, based on the level of β-galactosidase activity when each silencer is promoter-proximal.

The Abf1 site at HML-I is redundant

Deletion analysis of the *HML*-E silencer has demonstrated a role for the Rap1 binding site, as well as mapping the functional ARS element (Mahoney *et al.*, 1991). To determine the role of Abf1 and other potential proto-silencers at *HML*-I, we have deleted the previously mapped Abf1 site, which is the only Abf1 site in this fragment (Buchman *et al.*, 1988; data not shown). This Abf1-deficient silencer (named ΔB) still has a perfect ARS consensus and a near-perfect Rap1 binding site adjacent to it (see Figure 4), and it is able to silence the promoter located at 4 kb from I, as long as the E silencer is present (compare strains EG30 and EG46, Figure 3). However, ΔB is inactive in the absence of E (strain EG44, Figure 3). This suggests that residual sites in the Abf1-less silencer are able to cooperate with E to achieve silencing of the *LEU2″lacZ* construct but, like the intact silencer, it is unable to silence a distant promoter on its own.

We next tested whether the ΔB silencer has activity on its own when it is proximal to the promoter, by placing it 5′ of the *LEU2″lacZ* promoter and deleting the *HML*-E silencer. In this case it silences the reporter gene partially, to the same degree as the full I silencer in the absence of E (compare strains EG33 and EG45, Figure 3). A weak repression was also observed when a single ΔB silencer was inserted upstream of *URA3* instead of *LEU2″lacZ*, showing that this effect is not promoter dependent (data not shown). These results show that the Abf1 site in *HML*-I is not essential for silencing and that there is a redundancy of binding sites here, as previously observed at *HMR*-E (Brand *et al.*, 1987). An examination of the sequence motifs in the residual silencer shows that the arrangement of Abf1 (labelled B), Rap1(labelled E) and ARS consenses (labelled A) at the *HML*-I silencer, are remarkably similar to the sites found at *HMR*-E, with respect to order, spacing and relative orientation (see Figure 4A).

Rap1 binds two sites within the HML-I silencer

Previous results suggesting the presence of a Rap1 site at *HML*-I used a 398 bp *Pvu*II–*Hin*dIII fragment and yeast nuclear scaffold extracts in a gel retardation assay (Hofmann *et al.*, 1989). To map the binding site relative to the functional ΔB deletion, and determine its affinity relative to the redundant Rap1 site in the *HMR*-E silencer (labelled E, Figure 4A), we have used affinity-purified, bacterially expressed Rap1 protein in gel retardation and footprinting assays. The Coomassie blue staining of the purified Rap1 preparation is shown in Figure 4C. Both the 158 bp *Pvu*II–*Eco*RV fragment that is removed from the truncated silencer, and the remaining 240 bp *Eco*RV–*Hin*dIII fragment, form specific complexes with Rap1 in a 1000-fold excess of non-specific competitor, while a specific oligonucleotide containing a telomeric Rap1 binding site efficiently competes for both interactions (Figure 4B). To determine the relative affinity of these sites in comparison with the Rap1 site at *HMR*-E, the three fragments were labelled to equivalent specific activities and complex formation was competed by a determined titration of oligonucleotide competitor (Figure 4B). Quantitation shows that the complex formed between Rap1 and the binding site at *HMR*-E is from 4- to 5-fold more stable than the complex formed between Rap1 and either the *Eco*RV–*Hin*dIII or the *Pvu*II–*Eco*RV *HML*-I fragment (Figure 4B).

Footprinting studies performed on the *HML*-I silencer using purified Rap1 reveal a Rap1-dependent DNase I footprint over nucleotides 199–211 (Figure 4D), as well as characteristic DNase I-hypersensitive sites 5′ of the consensus (see arrows). The footprint is lost in the presence of an oligonucleotide competitor (Figure 4D, lane 4). We also see hypersensitivity near the Rap1 consensus at bp 95–105, but very little protection by Rap1 binding, suggesting that this second site is of lower affinity (summarized in Figure 4A).

Deletion data (indicated above the *HML*-I sequence, Figure 4A) suggest that the Rap1 site located between the Abf1 site and the ARS consensus (bp 199–211) is physiologically relevant. It was shown recently that the *HMR*-E silencer, in most assays, is a more potent silencer than *HML*-I (Shei and Broach, 1995). In view of the overall similarity in their organization, this variation in silencer strength must reflect small differences in the spacing of sites, in flanking sequences, in the ability of Rap1 to bend the DNA as it binds, or simply the higher affinities of the individual sites for their respective factors. The mapping and deletion data underscore the important role that Rap1 sites play in *HML*-I silencer function, a role previously suggested by the sensitivity of *HML* silencing to mutations in the C-terminal domain of Rap1 (Liu *et al.*, 1994).

Rap1 binding reinforces silencer function at a distance

We next asked whether a single proto-silencer is also able to cooperate at a distance with a silencer to establish repression. A series of 44 bp oligonucleotides corresponding to binding sites for either Rap1, Abf1 or ORC (the ARS consensus sequence or ACS) were inserted near a deleted *HML*-E, 4 kb away from *HML*-I. The sequence of these oligonucleotides, named respectively ε, β and α, is given in Figure 5A.

Remarkably, in otherwise identical backgrounds, the insertion of a single Rap1 site in either orientation at 4 kb from the intact I silencer increases repression to approximatively the level obtained when both silencers are present (compare strains EG31, EG33, Figure 3 and

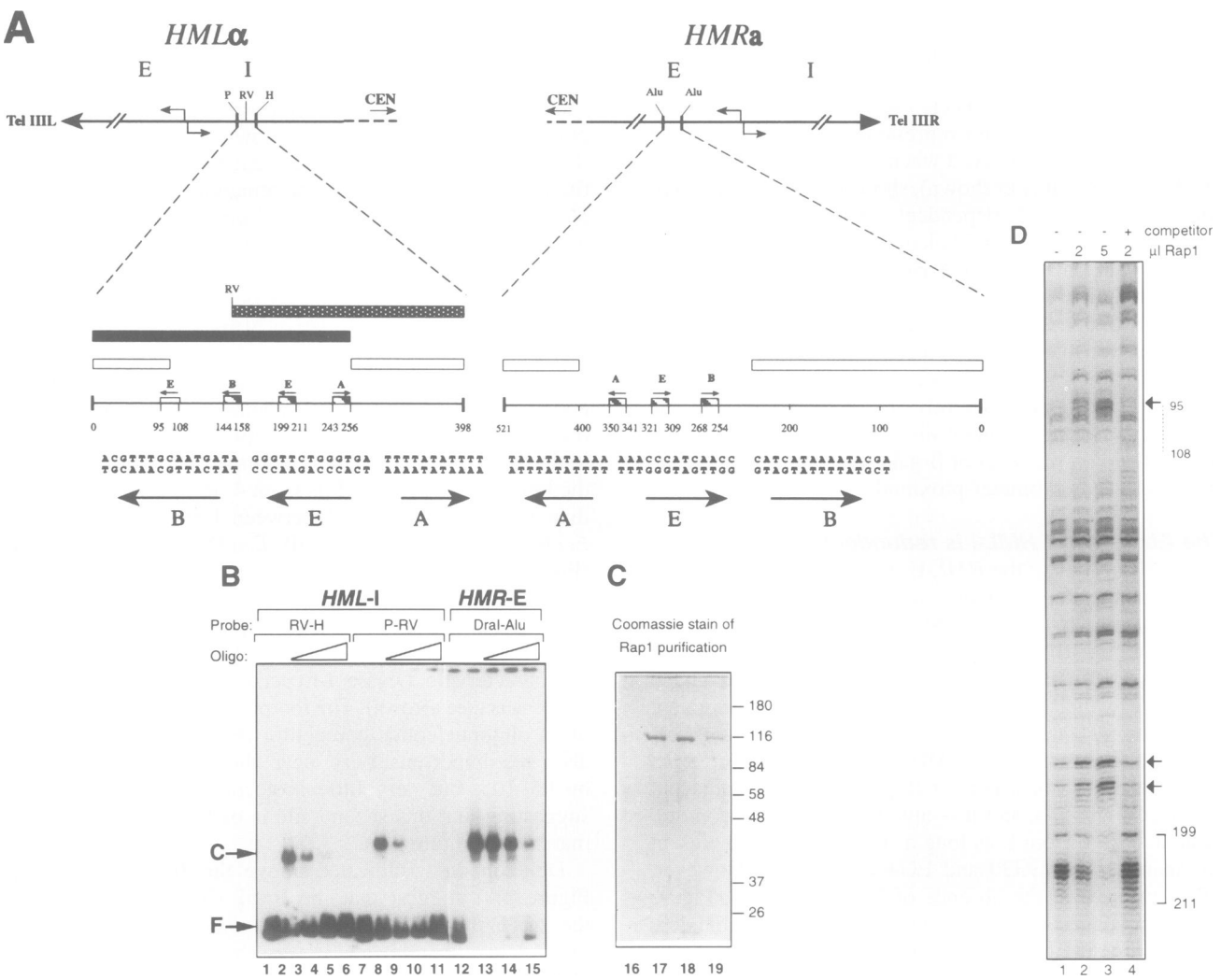

Fig. 4. The minimal *HML*-I silencer has an ARS consensus and binding sites for Rap1 and Abf1. (**A**) A comparison of the two silencers *HML*-I and *HMR*-E, situated near the telomere of either the left or the right end of the yeast chromosome III, respectively. The top sketch shows the organization of the silent mating type loci on chromosome III. The 398 bp *Pvu*II–*Hin*dIII fragment containing I silencer activity from *HML*α (left side) and the 521 bp *Alu*I–*Alu*I E silencer from the *HMR* locus are enlarged in detail. The open bars indicate sequences that are either not necessary or not sufficient to confer repression (Feldman *et al.*, 1984; Brand *et al.*, 1987), while the black box indicates the minimal functional *HML*-I silencer (Feldman *et al.*, 1984). The stippled box indicates the ΔB silencer fragment (*Eco*RV–*Hin*dIII) which we show here to confer significant repression on the *LEU2*′*lacZ* reporter. Within the necessary region of *HML*-I we indicate the perfect or near perfect consenses for Rap1 (labelled E; see consensus in Figure 5 and Gilson and Gasser, 1995), the binding site for Abf1 (labelled B, based a footprint and the consensus TATCATN5ACGA, Buchman *et al.*, 1988) or the ARS consensus (labelled A, representing A/TTTTAC/T A/GTTTA/T). The open box labelled E at *HML*-I is a slightly less perfect Rap1 consensus (10/13), but which is not essential for full silencer activity. The binding sites indicated at *HMR*-E are based on Brand *et al.* (1987), and the sites for the three factors are in nearly identical orientation and spacing to that found at *HML*-I, in relation to the repressed promoters. Below the map, the actual sequences of the relevant sites are given. At *HML*-I, these are bp144–158 for the Abf1 or B site; bp 199–211 for the Rap1 or E site; bp 243–256, for the ACS or A site; and at *HMR*-E, bp 350–341 for the ACS or A site; bp 321–309 for the Rap1 or E site; and bp 268–254 for the Abf1 or B site. Numbering is from the telomere proximal side towards the centromere, based on the Chr. III sequence in the EMBL data base. *In vitro* footprint assays confirm that Rap1 binds the two indicated sites at *HML*-I. RV, *Eco*RV; H, *Hin*dIII; P, *Pvu*II; and Alu, *Alu*I. (**B**) Gel retardation assays were performed using *E.coli*-expressed and affinity-purified Rap1 protein. The three end-labelled probes used for the assay correspond to the 240 bp *Eco*RV–*Hin*dIII fragment (lanes 1–5) and the 158 bp *Pvu*II–*Eco*RV fragment (lanes 6–10) of the *HML*-I silencer, and the 134 bp *Dra*I–*Alu*I fragment of the *HMR*-E silencer (lanes 11–15). Equivalent moles of each probe were incubated with the same molar excess of Rap1 protein, 0.2 µg of non-specific competitor, and either no specific competitor (lanes 2, 7 and 12) or with 25 ng (lanes 3, 8 and 13), 50 ng (lanes 4, 9 and 14) or 100 ng (lanes 5, 10 and 15) of the 003–004 oligonucleotide containing a telomeric Rap1 site (see Materials and methods). Lanes 1, 6 and 11 contain probes alone, in the absence of Rap1. Protein–DNA complexes were separated on an 0.8% agarose gel (see Materials and methods). 'C' = Rap1–DNA complex; 'F' = free probe. Quantitation shows that the Rap1–*HMR*-E complex is 4.3-fold more stable than the complex formed with the *Eco*RV–*Hin*dIII fragment and 4.5-fold more stable than the complex formed with the *Pvu*II–*Eco*RV fragment. (**C**) A Coomassie blue-stained SDS gel shows the peak elution fractions (lanes 17 and 18) and one fraction before and after these (lanes 16 and 19) from the affinity purification of bacterially expressed Rap1 protein. Molecular weight markers are indicated at the left in kDa. Rap1 is estimated to be 95% pure by this criterion and no major DNA binding contaminant could be detected. (**D**) DNase I footprint with purified Rap1 protein on the 398 nt (*Pvu*II–*Hin*dIII) *HML*-I silencer fragment. Rap1 protein binding to the I-silencer fragment and DNase I digestions were performed as described in Materials and methods. The 398 nt I-silencer fragment (10 ng each) was incubated without (lane 1), with 2 µl (~24 ng, lanes 2 and 4) and with 5 µl (~60 ng, lane 3) of Rap1 protein. Nuclease digestions were performed with 0.1 µg/ml DNase I (lanes 1–4). Digestion products were PCR amplified using the end-labeled oligonucleotide, SG105, and analysed on an 8% polyacrylamide–7 M urea sequencing gel together with dideoxy sequencing products of the 398 nt I-silencer fragment (not shown). The site of Rap1 binding is indicated by a bracket along the autoradiogram, the slightly less perfect Rap1 consensus by a dotted line. The numbers correspond to I-silencer sequences of putative Rap1 binding sites as in (A). Arrows along the autoradiogram indicate DNase I-hypersensitive sites.

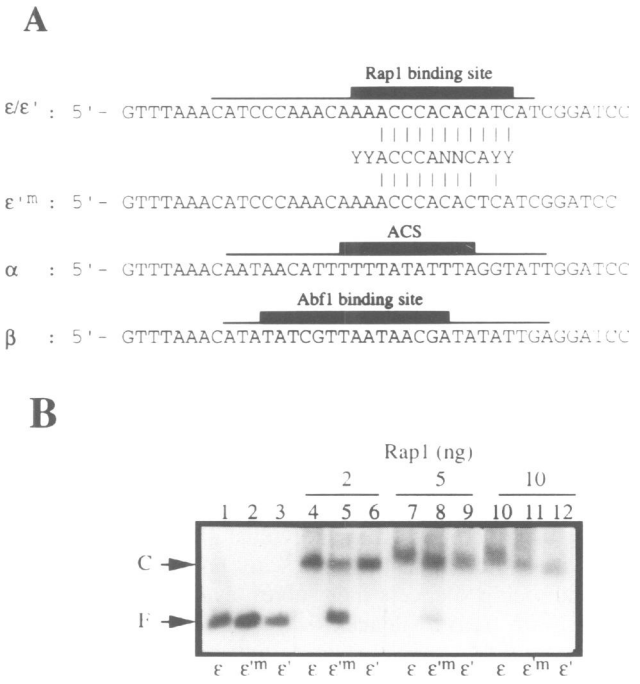

Fig. 5. Sequence of the oligonucleotides corresponding to the individual proto-silencers and demonstration of Rap1 binding. (**A**) The sequences of the oligonucleotides used for integration in the *HML* reporter constructs are presented. The black boxes over the sequences correspond to the putative binding site. ε indicates the Rap1 binding site oligonucleotide (ε′ indicates that it is inserted in the opposite orientation at *HML*, see text and Figure 6). A 13 nt consensus for Rap1 binding (Buchman *et al.*, 1988) is indicated below the sequence of ε. The matches with this consensus are indicated by bars. The indicated sequence in ε corresponds to that of UASα (Giesman *et al.*, 1991) with one modification at the 8th position of the consensus (G→C) which is known to preserve the binding capacity of Rap1 to this site (Vignais *et al.*, 1990). The mutated form of this oligonucleotide (ε'^{m}) is indicated below the Rap1 consensus and contains a single base pair deletion of the 11th position of the consensus. The oligonucleotide α contains a perfect ARS consensus sequence and the indicated flanking region is identical to the flanking sequence of the ACS in *HMR*-E. The oligonucleotide β contains a perfect Abf1 consensus, and the line above the sequence corresponds to flanking sequence identical to that used in the synthetic silencer (McNally and Rine, 1991). (**B**) Gel retardation experiments with three probes corresponding to a 600 nt fragment containing ε in either orientation (ε and ε′) and the mutated Rap1 site oligonucleotide (ε'^{m}). These probes were incubated with 0 (lanes 1–3), 2 ng (lanes 4–6), 5 ng (lanes 7–9) and 10 ng (lanes 10–12) of purified Rap1 protein and 1 μg of poly d(I–C) non-specific competitor, prior to analysis on an 0.8% agarose gel (see Materials and methods). The name of the probe is given below the gel. 'C' indicates the position of the Rap1–DNA complex and 'F' the position of the free DNA.

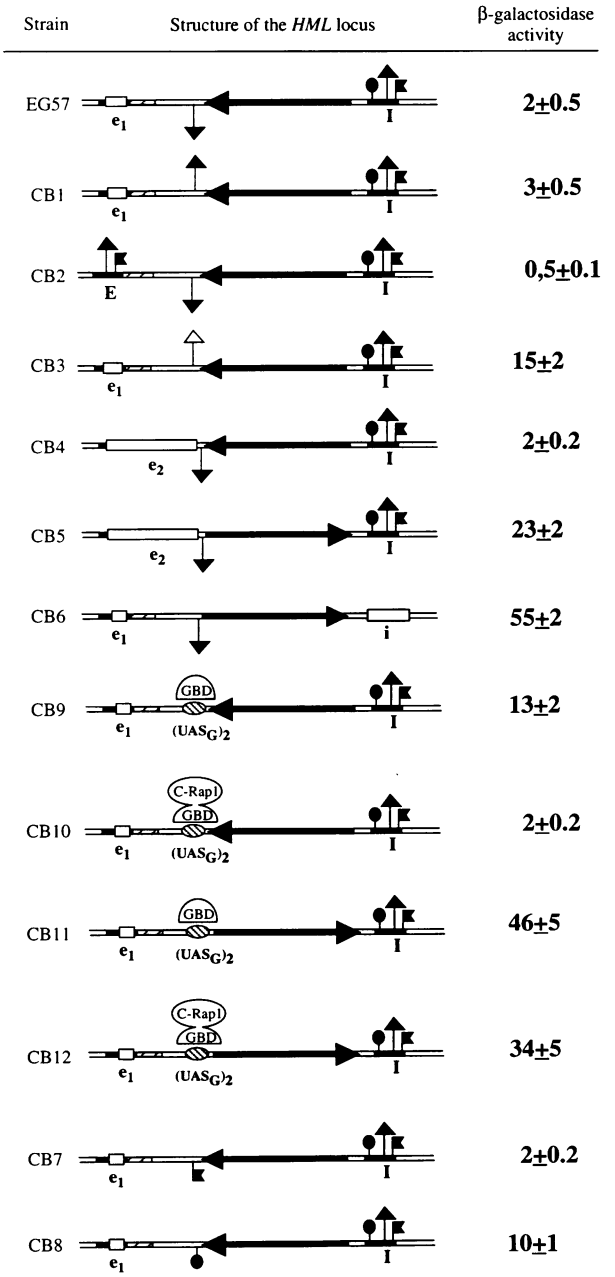

Fig. 6. Cooperative effects between I and individual silencer elements. This figure includes the structure of various *LEU2″lacZ* insertions at *HML*, as well as the name of the strains to the left of the structure and the relative values of β-galactosidase produced in the corresponding strain to the right. The symbols are described in Figure 1 and the β-galactosidase activity measurements are as previously described, always standardized to the 0.07 Miller units obtained with EG5 and EG31. In every experiment, the EG5 control is performed in parallel and values are reproducibly 0.07 ± 0.01. The symbols corresponding to proto-silencers are below the line when the orientation is different from the orientation in the natural *HML*-E silencer. The triangle which symbolizes a Rap1 binding site is empty in the case of ε'^{m} oligonucleotide insertion, which corresponds to a mutated form of a Rap1 site (see Figure 5). All the strains derived from GA210 and are LEU^{+}. The strains with a dimer of Gal4 binding sites [$(UAS_G)_2$] carry a disrupted chromosomal *GAL4* gene (*gal4:::LEU2*). CB9 and CB11 contain a low copy number plasmid expressing the Gal4 DNA binding domain (GBD) and CB10 and CB12 contain a low copy number plasmid expressing a hybrid protein between GBD and aa 653–827 of Rap1.

EG57, CB1, Figure 6). These results suggest that a single Rap1 site is able to cooperate with a silencer at a distance to establish repression. An alternative explanation is that the additional Rap1 site converts a previously cryptic proto-element near the E silencer, into a bona fide silencer. This explanation can be ruled out by placing the Rap1 binding site in the same position, but in the absence of the I silencer (strain CB6, Figure 6). Under these conditions the reporter gene is fully derepressed, indicating that the insertion of a single Rap1 binding site neither reveals a cryptic silencer, nor acts as a silencer on its own.

The insertion of an additional Rap1 site next to an intact E silencer can improve silencing another 2-fold

over the normal repressed level (a drop from 1 to 0.5 in β-galactosidase activity; strain CB2, Figure 6), indicating that a redundancy of elements can improve silencing. This argues for a dosage- or concentration-dependent mechanism for Rap1 action, much like that proposed for telomere-proximal silencing (Renauld *et al.*, 1993), rather than a catalytic or on/off switch.

In order to investigate the nature of this apparent cooperation between a proto-silencer (a single Rap1 site) and the I silencer, we further characterized the effect of the Rap1 site insertion on *LEU2″lacZ* expression. First, we showed that the improvement in silencing provided by a Rap1 site is dependent upon the Sir proteins: a disruption of either the *sir1* or *sir3* gene results in a total derepression, while overexpression of *SIR1* partially derepresses, as observed in Figure 2 (data not shown). Secondly, we show that the D region, which remains in the previous constructs after deletion of E, does not act in synergy with the Rap1 site, nor is it necessary for Rap1-enhanced silencing. A total deletion of both *HML*-E and D does not alter the improvement in silencing provided by the Rap1 site insertion (compare strains EG57 and CB4, Figure 6). Finally, insertion of a mutated version of the Rap1 site (called ε'^{m}, Figure 5A), which exhibits a lowered affinity for Rap1, as visualized by band-shift assays with increasing amounts of purified Rap1 (Figure 5B), has much less effect on silencing (compare strains CB1 and CB3, Figure 6). This is consistent with our proposal that the binding of Rap1, and not only the presence of the consensus sequence, is important for the observed cooperativity.

In most of the constructs discussed above, the promoter is located near the intact silencer and the Rap1 site is inserted at the 3′ end of the gene. When the *LEU2″lacZ* reporter is in the opposite orientation, i.e. 4 kb from the intact I silencer, the insertion of a Rap1 site near the promoter has a more modest effect on silencing (compare strains CB4 and CB5, Figure 6), showing again that individual Rap1 sites themselves cannot initiate silencing. Indeed, a promoter-proximal Rap1 site in the absence of I is unable to repress transcription (strain CB6, Figure 6). Such a construct is reminiscent of the promoter of the native α2 gene, in which a Rap1 site located immediately upstream of the TATA box acts as a transactivator. Although the single Rap1 site cannot silence on its own, it can cooperate at a distance to enhance the effect of a nearby silencer, suggesting that the promoters of targeted genes can indeed influence the establishment or maintenance of Sir-mediated repression.

Does the bound Rap1 molecule itself participate in the long-range cooperative effect with I ? To answer this, the Rap1 site was replaced by two binding sites for Gal4 [$(UAS_G)_2$], in our host strain which is *gal4*. In the absence of E, the expression of a hybrid protein containing the DNA binding domain of Gal4 (GBD) and the silencing domain of Rap1 (aa 653–827) reinforces silencing when the reporter promoter is located near I (strain CB10, Figure 6), while GBD alone did not (strain CB9, Figure 6). The presence of GBD–Rap1 has only a modest effect on silencing when the reporter gene's promoter is located near $(UAS_G)_2$ and away from I (strains CB11 and CB12, Figure 6). These results demonstrate that a targeted silencing domain of Rap1, like a single Rap1 binding site, is able to reinforce the action of a distantly located *HML*-I silencer, although it has no silencer activity on its own.

Individual ACS or Abf1 binding sites can also cooperate to promote silencing

Is Rap1 the only proto-silencer element able to cooperate at a distance to enhance silencer function? Similarly sized oligonucleotides containing either an individual Abf1 binding site or the ARS consensus (β or α, respectively), were introduced at the 3′ end of the reporter gene, 4 kb from the intact I silencer, in strains deleted for the *HML*-E silencer region. The insertion of an ARS consensus sequence reinforces silencing induced by the I silencer with the same efficiency as the inserted Rap1 binding site (compare strains EG33, Figure 3 and EG57 and CB7, Figure 6). The insertion of an Abf1 site increases repression reproducibly, but to a more modest level (compare strains EG33, Figure 3 and CB8, Figure 6). Overall, these findings allow us to propose that individual proto-silencer sites can act as auxiliary elements to enhance repression, without being silencers themselves, even when located as far as 4 kb from a silencer.

Discussion

Yeast mating type silencers are *cis*-acting sequences which initiate the propagation of a repressed state of chromatin ('silent chromatin') at the two *HM* loci. They represent the juxtaposition of protein binding sites (here referred to as proto-silencers) which cooperate with one another over a short distance (usually <100 bp) in order to create a functional unit. These proto-silencers include binding sites for Rap1 and Abf1, as well as an ARS element recognized by the ORC, a six-polypeptide factor required for the initiation of DNA synthesis (Bell *et al.*, 1993; Foss *et al.*, 1993). All three of these factors have many binding sites throughout the yeast genome, but at most of these sites the factors, even when they occur in pairs, do not confer Sir-dependent repression of adjacent genes. Thus the function of these individual proto-silencers is clearly context dependent. Deletion studies at *HMR* and *HML* have revealed two silencers at each locus, E and I, located to either side of the repressed gene (Abraham *et al.*, 1984; Feldman *et al.*, 1984). They have also shown that *HMR*-E, *HML*-E and *HML*-I are each sufficient to confer silencing on their own on the α2 or α1 promoters at the native *HM* loci near the ends of chromosome III, while *HMR*-I is not (Mahoney and Broach, 1989; reviewed in Laurenson and Rine, 1992). We have tried to address the question of why partially or fully functional silencers should be duplicated in regions flanking repressed promoters or even in the promoter itself. Is it purely a question of redundancy, or do these sites have a role in *HM* repression?

Using a highly sensitive β-galactosidase assay and a promoter that has neither a Rap1 nor an Abf1 site, we are able to demonstrate in a quantitative manner two aspects of silencer function. First, silencers are able to cooperate at a distance to enhance the establishment of a repressed domain. Second, proto-silencers, which do not have silencer function on their own, are able to cooperate with a silencer at a distance of 4 kb to establish an efficiently repressed domain. As a corollary to this, Rap1 or Abf1

sites in the promoters of targeted genes are predicted to function as silencer auxiliary elements to enhance the efficiency of repression. This is particularly relevant since factors involved in the establishment of repression, such as Rap1 and Abf1, are also transcriptional activators required for gene expression (Shore, 1994; Gilson and Gasser, 1995).

In the case of Rap1, the factor itself and not simply the binding sequence mediates both the activation and repression functions since: (i) a mutated Rap1 site which poorly binds Rap1 alone is unable to cooperate fully with a distantly located silencer (Figures 5 and 6); (ii) the C-terminal domain of Rap1 alone, targeted through a Gal4 binding site, is able to reinforce the action of a distant silencer (Figure 6); (iii) Rap1 is known to be the only DNA binding factor necessary for the UASα-dependent transactivation (Giesman *et al.*, 1991); (iv) mutations in Rap1 have demonstrated a role in silencing (Liu *et al.*, 1994); and (v) *in vivo* footprinting experiments visualized a footprint consistent with Rap1 protein bound at UASα, in both the repressed and the derepressed state (M.Tsai-Pflugfelder and S.M.Gasser, in preparation). Thus, at least in the case of *HML*α, the transcriptional inactivation apparently does not entail the exclusion of all factors from the promoter.

Proto-silencers are equivalent to transcription-promoting elements

Superficially, the requirement for both E and I silencers for full repression of the *LEU2''lacZ* construct appears to contradict results of Mahoney and Broach (1989), who did not observe derepression upon removal of either the E or I silencer at *HML*α. Most of their published constructs, however, preserve the α2 promoter, which itself contains a functional Rap1 site. Indeed, consistent with our analyses, the removal of one silencer at *HML* is not expected to derepress transcription, because the Rap1 site in UASα should be able to act as a proto-silencer to enhance repression (see above, Giesman *et al.*, 1991). In other reported cases, the α2 region was replaced by the **a1** region, which contains an Abf1 site (McBroom and Sadowski, 1994). Abf1 is also able to reinforce the strength of a silencer located at a distance. Thus, even though Sir-dependent silencing is a chromatin-dependent process thought to spread from the silencer in a manner independent of the targeted gene, it seems that the presence or absence of a proto-silencer in the targeted promoter can influence the efficiency of repression. It is not known whether other transcription factor sites can play the same role. The binding site for the transcriptional activator PPR1 at a telomere-proximal *URA3* gene, however, clearly has the opposite effect, since the efficiency of telomeric silencing drops in the presence of PPR1 (Renauld *et al.*, 1993).

Intriguingly, the efficiency with which the introduction of an *HMR*-E silencer represses the transcriptionally active *MAT* locus also appears to depend on the promoter being repressed. Shei and Broach (1995) observed that introduction of the *HMR*-E silencer represses the *MAT***a** promoter less efficiently than that of *MAT*α; for the *MAT***a1** promoter, a 'conditional' silencing was observed that varied with growth conditions, while the inserted silencer repressed the *MAT*α2 promoter under all growth conditions. As described above, the α2 promoter contains a Rap1 binding site, while the **a1** promoter does not (Giesman *et al.*, 1991). Consistent with these observations, our studies show that an individual Rap1 site functions at a distance to make a full silencer construct more efficient, while Abf1 does so less efficiently. We do not interpret this long-range effect of a Rap1 site on a distal silencer as transformation of the Rap1 site into a bona fide silencer, but rather as an increase in the efficiency of the distal silencer, since the Rap1 site, even in the *HML* context, has little or no silencing activity on its own.

Previous analysis of *cis*-acting sequences involved in *HMR* silencing led to the conclusion that the minimal silencer is composed of any combination of two of the three proto-silencers (Brand *et al.*, 1987; Kimmerly *et al.*, 1988). Our analysis would suggest that such combinations, as illustrated by the *HML*-E silencer or an Abf1-less allele of *HML*-I, indeed act as silencers, but very poorly. To be fully active they need to be combined with at least one of the three proto-silencers. This auxiliary element can be part of the silencer, as in *HMR*-E, or at a distance (at least up to 4 kb). The original redundancy studies of the *HMR*-E element were performed in the presence of *HMR*-I, which contains an Abf1 site and an ARS element, both of which might cooperate with the mutated versions of *HMR*-E. Indeed, a synthetic *HMR*-E sequence behaves as an effective silencer only in the presence of *HMR*-I (McNally and Rine, 1991; Fox *et al.*, 1994, 1995) and lacks much of the redundancy observed in the wild-type *HMR*-E silencer (McNally and Rine, 1991). The weakness of silencers containing 'two out of three' proto-elements that we observe with the *LEU2''lacZ* reporter helps to explain why promoters containing a Rap1 site adjacent to an Abf1 site (e.g. promoters at *PGK* and *PYK1*; Chambers *et al.*, 1990) participate in transcriptional activation rather than repression.

How do long-range interactions enhance silencing?

Silencers appear to be implicated in the establishment rather than in the maintenance of the repressed state of chromatin, since the excision from chromosomes of the *HMR* region without the associated silencers retained the repressed state of its chromatin (Loo and Rine, 1994). Thus, the cooperative event is likely to occur during the initial steps of silencing, for example by facilitating the spreading of the repressed structure into adjacent chromatin and/or by enhancing the nucleation of factors at silencers.

The relatively weak silencing ability of an individual *HML* silencer (see Figure 7a) may be reinforced by the spreading of silent chromatin emanating from a distant silencer. If the two silencers flank a target gene, the propagated structures could meet and stabilize repression between the two silencers (Figure 7b). However, the cooperativity mediated by proto-silencers, which do not act as a silencing 'initiation sites' on their own, argues for another hypothesis in which the more distant site enhances the efficiency of the first silencer (Figure 7c). Alternatively, the role of the auxiliary element might be to modulate the intervening chromatin in order to render it more competent for silencing, for example by imposing a particular nucleosome positioning. This hypothesis is in agreement with the idea that spreading involves the

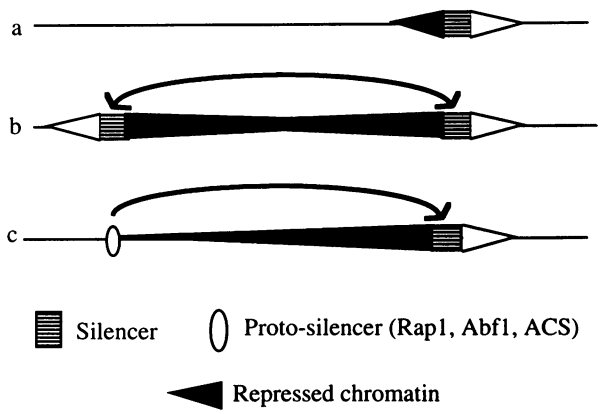

Fig. 7. Long-range cooperative effects observed at the silent *HML* locus. This figure presents a model interpreting results presented in this paper. (**a**) In the presence of a single silencer (e.g. E or I), only a low degree of silencing is observed, acting only on promoters in close proximity. (**b**) In the presence of both silencers, the repression of the intervening chromatin is much more efficient. The two silencers seem to cooperate bidirectionally since both of them are reinforced. (**c**) The presence of any one of the three individual proto-silencer elements can enhance the activity of a distantly located silencer without acting as a silencer on its own. Here, the cooperativity seems to work unidirectionally, reinforcing the strength of the silencer without transforming the individual binding site into a silencer. The direction of the black triangle indicates the extent of the spreading of the repressed chromatin emanating from a silencer. Whether these cooperative effects can act for the spreading directed outside of the two silencers is not known, but is suggested by studies of Mahoney and Broach (1989). We indicate this here with open triangles toward the outside of the domain.

association of silencing factors with nucleosomes (Hecht *et al.*, 1995) and that, in our constructs, the intervening DNA is mainly bacterial, which is not expected to confer a particular nucleosome positioning on its own. However, the nucleosomal positioning along a *URA3* gene is not modified upon its repression near a telomere, suggesting that a particular nucleosome array is not essential for silencing (Wright *et al.*, 1992).

The observed cooperativity between silencers can also be interpreted as an enhancement of the local concentration of binding sites for Sir proteins in a small volume of nuclear space. This can be achieved locally by inserting multiple silencer sequences (Shei and Broach, 1995) or at a distance by clustering silencers through higher order organization of the chromatin. We do not know whether the functional cooperativity observed reflects direct interaction between distant elements. However, since *in vitro* experiments showed that E, I and the α2 promoter can associate in the presence of nuclear scaffold extracts to form DNA loops (Hofmann *et al.*, 1989), we favour the interpretation that direct interaction, which may also be a transient interaction, between proteins bound to both silencers and auxiliary sites accounts for the observed long-range cooperativity. *In vitro* loop formation could be competed by an excess of free Rap1 binding sites, implicating Rap1 directly in these *in vitro* interactions (Hofmann *et al.*, 1989). The requirement for Rap1 is likely to reflect its ability to bind other proteins such as Sir1 or the Sir3–Sir4 complex, to form a tertiary complex, since *in vitro* looping was not observed with highly purified bacterially expressed Rap1 alone (T.Laroche and S.M.Gasser, personal communication), and because attempts to demonstrate Rap1 dimerization *in vitro* have failed (E.Gilson, M.Roberge, D.Rhodes and S.M.Gasser, unpublished observations).

Cooperativity at a distance does not necessarily imply formation of a stable looped domain, nor do we propose that silencers or proto-silencer are 'boundaries' for repression, since this infers a limitation or directionality to the repressed chromatin structure. Indeed, it has been argued that looping between silencers does not occur because the repressed chromatin extends into flanking domains (Mahoney and Broach, 1989). Our data suggest that long-range interaction or looping does occur to help establish silencing, but that these interactions do not confer directionality or limitation of the repressed structure to the region between the two silencers. Indeed, the interaction may be transient. It has been amply demonstrated in higher eukaryotic systems that some classes of elements that affect long-range transcriptional regulation (such as LCR elements or SAR sequences, Laemmli *et al.*, 1992; Grosveld *et al.*, 1994) do not alone serve as barriers or insulator elements; on the other hand, elements like the *Drosophila scs* and *scs'* elements do provide boundary or insulator function, but are not necessarily enhancers of transcription (Vazquez *et al.*, 1994). Cooperative, and often transient, long-range interactions like those described here for silencing have also been implicated in the transcriptional regulation of both prokaryotic and eukaryotic promoters (Amouyal *et al.*, 1989; Müller *et al.*, 1989). Interestingly, in *Drosophila*, the PRE elements controlled by the Polycomb group also appear to cooperate at a distance to stabilize the silencing complexes (reviewed in Pirrotta, 1995).

The roles of Sir proteins in long-range interactions between silencers

The Sir3 and Sir4 proteins form both homo- and heterodimers, and are thought to form large multimeric complexes at silencers or at telomeres (Chien *et al.*, 1991; Gilson *et al.*, 1993a; Palladino *et al.*, 1993; Liu *et al.*, 1994; Moretti *et al.*, 1994; Cockell *et al.*, 1995). Since the C-terminal domain of Rap1 also binds Sir3 and Sir4, and may target the complex to chromatin (Moretti *et al.*, 1994; Cockell *et al.*, 1995), the long-range interactions between Rap1 or its targeted C-terminal domain and a silencer could well be achieved through Sir protein interactions. It is not known whether the ORC complex can target Sir3 and Sir4 directly, or whether its effect is mediated through Sir1. Sir1 was shown to enhance the establishment of silencing, but is not essential for maintenance of the repressed state at either the *HM* loci or at telomeres (Pillus and Rine, 1989; Aparicio *et al.*, 1991; Stone *et al.*, 1991; Chien *et al.*, 1993). Thus, it is worth noting that in our *HML* constructs containing both E and I silencers, or a single Rap1 site and I, both are highly dependent on cooperativity and are derepressed almost completely in the absence of Sir1 (Figure 2) unlike the native *HMLα* locus (Pillus and Rine, 1989). Consistently, a single Abf1 site, which does not seem to be capable of targeting Sir1 (Chien *et al.*, 1993), cooperates less well at a distance than a Rap1 or ARS consensus (Figure 6). Taken together, such observations suggest that Sir1 may be involved in the long-range interactions between silencers and auxiliary elements.

As shown for telomeric repression, the silencing of the *HML::LEU2"lacZ* construct is highly sensitive to variation in the dosage of silencing factors. This was interpreted previously in terms of limiting factors that are required for spreading (Locke *et al.*, 1988; Renauld *et al.*, 1993), yet it is also consistent with evidence for long-range interactions between silencers. The higher order reaction required for loop formation involves both occupancy of the distal sites and elements that bridge between the sites. A low concentration of silencer, binding factors can abolish the cooperative effect because of a reduced site occupancy; too high concentrations could prevent the establishment of silencing by competing for long-range interactions. Consistently, overexpression of *SIR1* or *SIR3* results in a slight derepression of an *HML* region containing both silencers (Figure 2A), although the same elevated *SIR3* expression does not affect the residual silencing found at an *HML* region containing a single silencer (our unpublished results).

Other evidence for long-range interactions in yeast chromosomes is provided by the observation that the silencing mediated by telomeric repeats inserted internally within yeast chromosomes is enhanced by their proximity to a telomere (Stavenhagen and Zakian, 1994), and that the level of *HMR* silencing is dependent upon its chromosomal location again in relation to telomeres (Thompson *et al.*, 1994a). These findings, together with the fact that *HML* and *HMR* are located at 13 and 25 kb respectively from the two telomeres of chromosome III, suggest that telomeres and silent mating type loci could also interact to form large chromatin domains at both ends of this chromosome (Gilson *et al.*, 1993a). Indeed, in the case of yeast silencers, a long-range cooperative effect could be achieved either by a close association of the two elements or by targeting them to a common subnuclear compartment, such as that defined by the clustering of Rap1, presumably reflecting telomeres in foci near the nuclear periphery (Palladino *et al.*, 1993; M.Gotta and S.M.Gasser, in preparation). A molecular analysis of these synergistic effects may shed light on how the higher order organization of chromosomes aids in the formation and maintenance of silent chromatin domains.

Materials and methods

Molecular biology methods

Plasmid growth and extraction, Southern blot analysis and restriction enzyme digests were carried out as described in Sambrook *et al.* (1989). Yeast media and methods were as described in Rose *et al.* (1990). Yeast transformation was done according to a modified lithium acetate protocol described by Hill *et al.* (1991), using 10 μg of DNA fragment for integration or 0.1 μg of intact plasmid DNA for extrachromosomal replication, and 60 μg of denaturated salmon sperm DNA.

Plasmid constructions

Plasmids with both *HML*-E and *HML*-I regions were derived from pUC18 where an *Xba*I–*Spe*I fragment carrying E is fused to an *Xmn*I fragment carrying I (Figure 1). In these constructs, single *Kpn*I and *Bcl*I sites are present between the two *HML* fragments. The wild-type E and I regions (1067 and 1212 nt, respectively) were excised from pVO3-Bam26 (a gift from J.Broach). The e_1 allele was excised from pD15-89 which contains a *Bgl*II linker substituted for 34 nucleotides within the E silencer, inactivating both the Rap1 site and the ARS sequence (Mahoney *et al.*, 1991). The e_2 allele is derived from e_1 by deleting the D region from the *Bgl*II linker inserted at E to the *Spe*I site (Figure 1). The i allele was excised from pDM25 which contains a 280 nt deletion of I [$I^{\Delta 242}$ in Mahoney and Broach (1989)] which removes both the Abf1 site and the ARS sequence. The ΔB allele corresponds to the deletion of the *Xmn*I–*Eco*RV 592 bp fragment carrying the Abf1 site of I (Figure 1). The dimer of Gal4 DNA binding sites [$(USA_G)_2$] was cloned by inserting a *Bam*HI–*Bgl*II DNA fragment containing these sites (Chien *et al.*, 1993) into the *Bcl*I site. Four 44 nt long oligonucleotides (α, β, ε, ε^m) were inserted at the *Spe*I site of I. Their sequences are shown in Figure 5A. The integrity of their sequence after cloning and their orientation were checked both by PCR and by sequencing.

A 3.4 kb *LEU2"lacZ* reporter gene DNA, corresponding to the *LEU2'* from −220 to +39 bp fused to the *'lacZ Bam*HI–*Dra*I fragment from pMC1403 (Casadaban *et al.*, 1983), was inserted into the *Kpn*I site of the above plasmids carrying various combinations of *HML* silencers, $(USA_G)_2$ and oligonucleotides. The hybrid protein with β-galactosidase activity is expected to be under the control of a minimal *LEU2* promoter (Tu and Casadaban, 1990).

The pe1URA3ΔB plasmid contains a *Hin*dIII *URA3* fragment excised from pAF101 (Thierry *et al.*, 1990) inserted at the *Kpn*I site of a plasmid DNA carrying both the e_1 and ΔB alleles, with the *URA3* promoter located near ΔB. For disrupting the *HMR* locus, we constructed phmr(eURA3i), a derivative of pUC18, which carries the *URA3* fragment flanked by the *Eco*RI–*Xho*I fragment distal to *HMR*-E, excised from pJA82.6 with the *Xho*I linker insertion number 268 (Abraham *et al.*, 1984) and by the *Xho*I–*Hin*dIII fragment distal to *HMR*-I, excised from pJA82.6 Δ59–296 (Abraham *et al.*, 1984).

Yeast strain constructions

All the strains used in this work derived from S150-2B (*MATa leu2-3,112 ura3-52 trp1-289 his3D gal2*) (a gift from J.Broach). The replacement of the wild-type *HMLα* region by the *LEU2"lacZ* reporter construct was done in two steps. First, the *HMLα* region was disrupted with *URA3* by transforming pe1URA3ΔB cut with *Xba*I (to the left of the E region) and *Not*I (to the right of the I region). The transformants were checked by a Southern blot analysis and the resulting strain named GA210. Second, an *Xba*I–*Not*I fragment from the plasmids carrying the *LEU2" lacZ* reporter gene was used to replace a *URA3* gene inserted at the *HML* locus in GA210 by co-transformation (Rudolph *et al.*, 1985) with pRS315 (Sikorski and Hieter, 1989). The LEU^+ transformants were replica-plated to SD–URA, SD+URA and SD+URA+5-fluoroorotic acid (5FOA). The URA^-, $5FOA^R$ colonies were analysed both by Southern blot analysis and by PCR using one primer located outside the transformed DNA fragment and the other within the *LEU2"lacZ* reporter gene. The E (respectively I) silencer alleles were checked by the presence, in the case of e_1 (respectively I), or by the absence, in the case of E (respectively ΔB and i), of a *Bgl*II site (respectively *Eco*RV). The strains carrying the $(UAS_G)_2$ sequence were checked by the presence of the *Xba*I site. In addition, the strains carrying either the α, ε or β sequence were checked by PCR using one primer corresponding to the inserted oligonucleotide and a primer located outside the transformed DNA fragment.

The *sir1* and *sir3* derivatives of these strains were obtained by a one-step gene replacement procedure using, respectively, pKL12 (Stone *et al.*, 1991) or pJI23.2 (Ivy *et al.*, 1986). The *HMR* region was disrupted with a *URA3* gene in a one-step gene replacement procedure by transforming phmr(eURA3i) containing a *URA3* gene flanked by null alleles of the *HMR*-E and *HMR*-I silencers (see plasmid construction). A functional *HMR* region was introduced by transformation with pJA82.6 (here termed pHMR), a centromere-containing plasmid carrying the 4.92 kb *Hin*dIII fragment which includes the entire *HMR* region (Abraham *et al.*, 1984). An *HMR* region containing a deletion of the *HMR*-E silencer was introduced by transformation with pJA82.6Δ77–268 (here termed phmr; Abraham *et al.*, 1984). A *gal4* derivative of GA210 was obtained by a one-step gene replacement procedure using pSJ4LEU2 (a gift of S.Gangloff), in which *LEU2* is inserted at the *Xho*I site of the *GAL4* gene. This *gal4* strain was used to integrate the reporter constructs carrying the $(UAS_G)_2$ sequence.

For overexpressing *SIR1* or *SIR3*, we transformed with the multicopy plasmid YEpSIR1 (here termed pSir1; Stone *et al.*, 1991), or pKAN63 (here termed pSir3; Ivy *et al.*, 1986). In both cases, the genes are under control of their natural promoters. For overexpressing *SIR4* or its C-terminal part from an *ADC1* promoter, we transformed with a multicopy plasmid carrying a 2 μ origin called pADH-SIR4 (here termed pSir4) or pADH-SIR4C (here termed pSir4C; Cockell *et al.*, 1995). DNA encoding the UAS_G binding domain of Gal4 (GBD) and the hybrid between GBD and aa 653–827 of Rap1 (GBD–Rap1) are carried by the *HIS3/CEN* plasmid pRS313 (Buck and Shore, 1995).

β-Galactosidase assay

β-Galactosidase assays were performed in permeabilized cells (Rose *et al.*, 1990) grown in synthetic media, by collecting the cells when they reached 10^7/ml, by assaying 5×10^8 cells in 1 ml and by stopping the reaction at 30 min. Miller units are thus calculated as $10^3\times OD_{420}/OD_{600}\times30$. At least four independent cultures of the same strain were measured. Under these conditions, the standard deviation of the mean did not exceed 10%, even for low level of expression (<1 Miller Unit). Strains with the reporter gene in either orientation according to the E and I silencers (strains EG5 and EG31, Figure 3) produced the same amount of β-galactosidase activity (0.07 Miller units), which was standardized to 1. In order to avoid any interference with the proper *LEU2* regulation, all the assayed cells were LEU^+ due to either pRS315 or another *LEU2* vector, and were grown in the absence of leucine. For X-gal filter assay, cells were grown directly on filters and were assayed for β-galactosidase activity as described in Breeden and Nasmyth (1985).

Gel retardation experiments

The gel retardation assay and the affinity purification of Rap1 over-expressed in *Escherichia coli* was described in Gilson *et al.* (1993b). The probes are 600 nt *Kpn*I–*Eco*RI fragments excised from plasmid DNA carrying the Rap1 site oligonucleotide in either orientation (probes ε and ε′) or with a mutation (probe ε'^{m}) (see Figure 5A for the sequence of the RAP1 site oligonucleotides), labelled at both ends with [γ-^{32}P]ATP and the T4 polynucleotide kinase. The *HML*-I fragments used for gel retardation and competition studies are the 158 bp *Pvu*II–*Eco*RV and the 240 bp *Eco*RV–*Hin*dIII fragments as shown in Figure 4. The *HMR*-E fragment is the 134 bp *Dra*I–*Alu*I minimal silencer as defined by Brand *et al.* (1987). Specific competitor is a double-stranded oligonucleotide (named 003–004), containing a telomeric Rap1 binding site. All reactions contain either 1 μg of poly d(I–C) (Figure 5), or 0.2 μg of poly d(I–C) and 0.2 μg of *E.coli* single-stranded DNA (Figure 4) as non-specific competitor. Quantitation of the Rap1–DNA complex was performed using a PhosphorImager from Molecular Dynamics.

In vitro DNase I footprinting

Binding reactions (100 μl) contained 10 ng of I-silencer fragment, 0.5 μg of double-stranded poly(dI)·poly(dC), 0–3 molar excess of specific competitor oligo(003–004), and 0–5 μl of affinity-purified Rap1 protein expressed in *E.coli*, as used for the gel retardation experiments above. Binding buffer was 20 mM KCl, 20 mM Tris–HCl (pH 7.5), 0.125 mM spermidine, 0.05 mM spermine, 5 mM $MgCl_2$, 1 mM $CaCl_2$. The binding reaction was performed at room temperature for 20 min and then transferred to ice. Nuclease digestions were done by adding 5 μl of 2 μg/ml DNase I directly to the reaction mixtures in ice, and digesting at room temperature for 5 min. Reactions were terminated by adding 100 μl of stop solution [0.6 M sodium acetate (pH 5.2), 20 mM EDTA (pH 8.0), 50 μg/ml tRNA]. After extraction with phenol/chloroform/isoamyl alcohol (50:49:1), the specific competitor oligo(003–004) was removed over a Centricon 30 micro-concentrator. DNA was then precipitated with ethanol. The 70% ethanol-washed and dried DNA pellets were resuspended in H_2O and used directly for the primer extension analysis. DNase I cleavage sites were located by primer extension assay mainly as described (Shimizu *et al.*, 1991). The primer oligonucleotide, SG105, corresponds to nucleotides 294–324 on the I-silencer sequence (Figure 4A). The DNA–primer mix was denatured at 93°C for 90 s, annealed at 62°C for 4 min, and then extended at 72°C for 3 min. This cycle was repeated 35 times. The DNA was extended after 35 cycles for 10 min at 72°C and the reactions terminated by chloroform extraction and ethanol precipitation. Dideoxy sequencing reactions using *Taq* polymerase were performed on the 398 nt I-silencer fragment as described (Shimizu *et al.*, 1991), using the same primer extension reactions as above. The DNA products were analysed on an 8% polyacrylamide–7 M urea sequencing gel with wedge spacers (Sambrook *et al.*, 1989).

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