The EMBO Journal vol.6 no.2 pp.461–467, 1987

Identification of silencer binding proteins from yeast: possible roles in SIR control and DNA replication

David Shore, David J.Stillman[1], Andrea H.Brand[2] and Kim A.Nasmyth

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

[1]Present address: Department of Cellular, Viral and Molecular Biology, University of Utah School of Medicine, Salt Lake City, UT 84132, USA

[2]Present address: Department of Biochemistry and Molecular Biology, 7 Divinity Avenue, Harvard University, Cambridge, MA 02138,USA

Communicated by K.Nasmyth

The 'silent' yeast mating-type loci (*HML* and *HMR*) are repressed by sequences (*HMLE* and *HMRE*) located over 1 kb from their promoters which have properties opposite those of enhancers, and are called 'silencers'. Both silencers contain autonomously replicating sequences (*ARS*). Silencer activity requires four *trans*-acting genes called *SIR* (silent information regulator). We have identified two DNA binding factors, SBF-B and SBF-E, which bind to known regulatory elements at *HMRE*. SBF-B binds to a region involved in both the silencer and *ARS* functions of *HMRE*, but does not bind to *HMLE*. This factor also binds to the unlinked *ARS1* element. SBF-E recognizes a sequence found at both silencers. These results suggest that the two silencers may be composed of different combinations of regulatory elements at least one of which is common to both. Neither factor appears to be a *SIR* gene product. Hence the *SIR* proteins may not directly interact with the silencer control sites.

Key words: ARS elements/DNA binding proteins/mating type/silencer/transcriptional control

Introduction

The yeast *Saccharomyces cerevisiae* contains three copies of mating-type information (*a* or α), all on chromosome III, at loci called *HML*, *HMR* and *MAT*. Normally only the DNA at the *MAT* locus is expressed, producing two transcripts, the products of which act as master regulators to determine cell type (either *a*, α or *a*/α diploid) [for reviews see Herskowitz and Oshima (1981) or Nasmyth (1982a)]. Although the *HML* and *HMR* loci contain complete copies of the structural genes and promoters for α and *a* information, respectively, they are not transcribed. Instead, these loci are subject to a position effect, whereby *cis*-acting flanking sequences, called E, located over 1 kb from their promoters are required to repress their transcription (Abraham *et al.*, 1984; Feldman *et al.*, 1984). Repression also requires the action of four genes called *SIR1-4* (silent information regulator) (Rine, 1979; Klar *et al.*, 1979; Rine *et al.*, 1979; Haber and George, 1979). The *sir* mutants are all recessive and unlinked to the mating-type loci, and thus code for *trans*-acting negative regulators of the silent loci.

The E region at *HMR* has been shown to act, in an orientation independent manner, from both sides of the *HMR* locus and from a distance of up to 2.5 kb from an affected promoter (Brand *et al.*, 1985). These properties are like those of transcriptional enhancer elements [for a review see Serfling *et al.* (1985)], and because it exerts an opposite effect upon transcription to that of an enhancer, the E region has been called a 'silencer'. *SIR*-mediated repression of other yeast promoters (both pol II and pol III) occurs when they are placed near the *HMRE* silencer, suggesting that the system acts in some general manner to block transcription (Brand *et al.*, 1985; Brand, 1986; Schnell and Rine, 1986). Further evidence for a global effect of the silencer on nearby sequences was presented by Nasmyth (1982b), who showed that a DNase I hypersensitive site at or near the *HO* endonuclease cleavage site is masked in a *SIR*-dependent manner at *HML* and *HMR*, but not at *MAT*. The silencer thus appears to block the access of transcription machinery and *HO* endonuclease to nearby chromatin. It remains to be demonstrated whether or not *HMLE* can act in an orientation- and position-independent manner or whether it can effect heterologous promoters. By analogy with *HMRE* (*SIR*-dependent repression of mating-type promoters) and for the purpose of simplicity in this discussion we shall refer to *HMLE* as a silencer, although the two elements may have some functional differences.

Both *HMRE* and *HMLE* contain *ARS* elements [putative origins of DNA replication; Stinchcomb *et al.* (1979)] and it is tempting to speculate that DNA replication is somehow involved in establishing repression. Strong support for this notion was provided by Miller and Nasmyth (1984), who showed that passage through S phase is required to re-establish repression after a shift to the permissive temperature in a *sir3-ts* strain.

The *HMRE* silencer is composed of three distinct regulatory elements, called A, E and B (Brand, 1986; Brand *et al.*, 1987). No single element is essential for silencer function and only deletion of E results in a measurable loss of *SIR* control. The E element contains an 11-bp sequence, called the E-box, which is also found at the *HMLE* silencer. The E element is flanked by elements A and B, which appear to have *ARS* activity (Brand, 1986; Brand *et al.*, 1987). The A element contains an 11-bp *ARS* consensus sequence (Broach *et al.*, 1982), which is also found near the E-box at the *HMLE* silencer. The A and B elements also play a role in silencer function which becomes apparent when E is mutated or deleted. In this case both A and B become essential for silencer function. The three control elements at the *HMRE* silencer are therefore redundant. Two of the three elements are required for silencer function: either E and A or E and B can provide complete function, whereas elements A and B together provide partial function.

In this paper we report the identification of two silencer binding factors, SBF-E and SBF-B, which recognizes the sequences in the E and B elements at *HMRE*, respectively. SBF-E also binds to the E-box sequence at the *HMLE* silencer. At the two SBF-E binding sites the conserved E-box sequence comprises one-half of the region protected from DNase I cleavage by the factor. Non-homologous sequences contribute to the binding energy as the two sites differ in their affinity for the factor. SBF-B binds within the B element at *HMRE* but appears not to recognize sequences at *HMLE*. The involvement of this protein in DNA

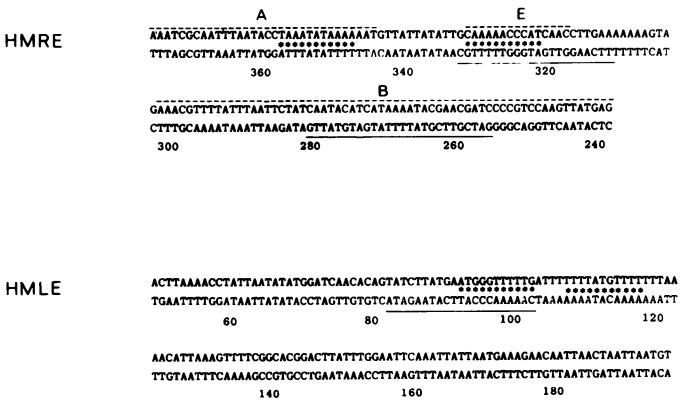

Fig. 1. Sequence of *HMRE* and *HMLE* DNAs used in this study. Numbering is according to Abraham *et al.* (1984) and Feldman *et al.* (1984), respectively. For both DNAs the locations of *ARS* consensus sequences (5′ T/ATTTATPuTTTT/A 3′) and the conserved E-box sequence (see text) are indicated by asterisks. For *HMRE* the location of elements A (345−376), B (239−302) and E (318−332), as determined by deletion analysis (Brand, 1986; Brand *et al.*, 1987) are indicated above the sequence by broken lines. The binding sites of factors described in this paper (see Figures 4 and 6), as defined by DNase I protection, are indicated by bars below the sequence.

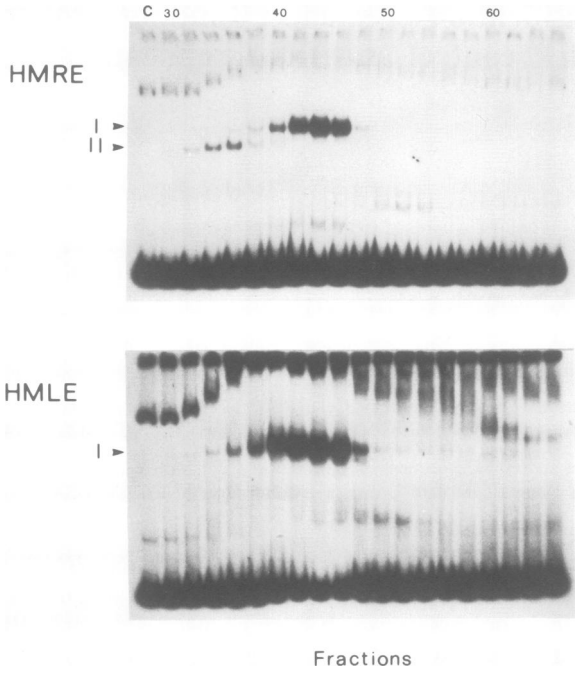

Fig. 2. Heparin−agarose chromatography of silencer binding factors. Elution profile of heparin−agarose column assayed by the bandshift method using *HMRE* (top) or *HMLE* (bottom) probes. Probes were labeled at the *Xba*I site of pUC13 and contained 138 bp of *HMRE* DNA or 147 bp of *HMLE* DNA plus vector DNA (~100 bp) out to the *Pvu*II site (see Materials and methods). Approximately 1% of the protein loaded onto the column was bound and eluted. Each binding reaction contained 2 μl of column fraction (average 0.4 μg protein) in a 20 μl reaction mix with 3 fmol (5 ng) of end-labeled probe (about 2500 c.p.m. Cerenkov radiation). Film was exposed overnight to dried gels. Lanes marked 'c' (minus protein controls) are lanes in which 2 μl of A50 buffer was added to the binding reaction in place of column fraction.

replication is supported by the observation that it also binds to *ARS1*. By examining extracts from *sir* mutants we show that neither factor is likely to be the product of a known *SIR* gene and we discuss the possible implications of this result for the mechanism of silencer function.

Results

Sequence-specific binding activities at the HMLE and HMRE silencers

To identify sequence-specific DNA binding activities, yeast whole cell extracts were first fractionated by heparin−agarose chromatography and then assayed by either of two methods: the 'bandshift' method, whereby protein−DNA complexes are separated from free DNA by electrophoresis in acrylamide gels (Fried and Crothers, 1981; Garner and Revzin, 1981) or by DNase I footprinting (Galas and Schmitz, 1978). The probes used to analyse the column fractions contain sequences 239−376 from *HMRE* (Abraham *et al.*, 1984) and 48−195 from *HMLE* (Feldman *et al.*, 1984; Brand, 1986; Brand *et al.*, 1987). The DNA sequences of these fragments are shown in Figure 1, where homologous sequences are identified, and regions of *HMRE* shown to be involved in *ARS* function and/or *SIR* control are indicated.

Figure 2 shows the analysis of a heparin−agarose column in which bound proteins have been eluted with a linear gradient of increasing ammonium sulphate concentration. Both the *HMRE* and *HMLE* probes are assayed by the bandshift method. In each case the first lane (c, control) shows the migration of probe in the absence of added protein: the upper bands and material not entering the gel probably correspond to incompletely digested probe (see Materials and methods). The bandshift assay (using 0.4 μg protein from each fraction) detects two chromatographically distinct activities, eluting at ~0.1 M and 0.15 M ammonium sulphate, which form a complex with the *HMRE* fragment (upper panel). The two protein−DNA complexes (labelled I and II) show a slight, but reproducable difference in mobility. The *HMLE* fragment (lower panel) appears to be bound by a single factor with the identical elution properties as the complex I *HMRE*-binding factor. The *HMLE* gel has been overexposed to demonstrate the absence of an activity similar to the complex II activity seen with the *HMRE* probe. The origin of other minor bands has not been determined. Typically 1% of the protein loaded on a heparin−agarose column was bound and eluted by ammonium sulphate, and for both activities we estimate that at least a 100-fold purification was achieved by this chromatography step.

An ARS-related binding activity

Concentration by ammonium sulphate precipitation of the heparin−agarose column fractions which yield the complex II binding activity allowed us to determine the region of protein binding by DNase I footprinting. The protected region (see below) lies within the B element at *HMRE*, and we shall refer to this binding activity as SBF-B.

We were unable to detect a specific binding activity in this concentrated fraction against the *HMLE* region (inclusive of a 370-bp *Hha*I fragment), either by DNase I footprinting or by the bandshift method, despite the fact that *HMLE* has *ARS* activity (Feldman *et al.*, 1984). We have therefore performed DNA 'competition' experiments to ask directly whether other *ARS* elements contain a binding site for this protein. In these experiments a small amount of labeled DNA probe (in this case *HMRE*) is mixed with protein extract in the presence of a 100-fold mass excess of unlabelled 'competitor' DNA, which contains either vector sequences alone, or vector sequences plus either the probe or test *ARS* fragment. The competing DNA is present on the plasmid pUC13. The total DNA added is kept constant (0.5 μg) and the amount of specific competitor is varied by increasing the proportion of insert-containing plasmid DNA. In this way a change in signal can be due only to specific binding to

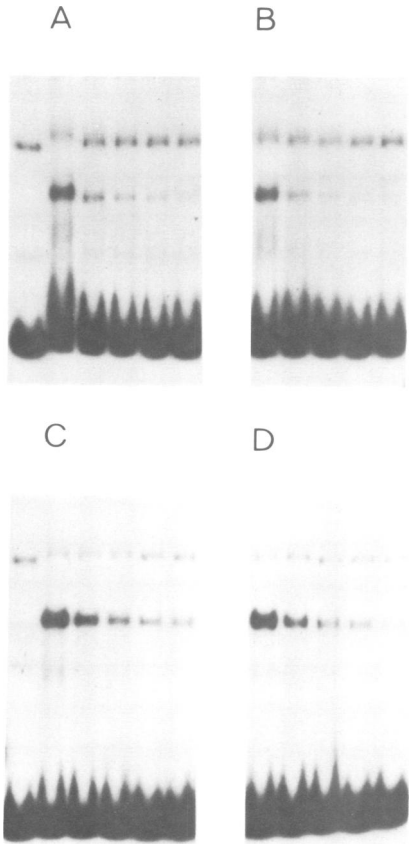

Fig. 3. *HMRE* and *ARS1* DNAs compete for binding to a common factor. Band-shift competition assays performed with 1 μg of protein from concentrated heparin−agarose column fractions (34−36, see Figure 2). The labeled DNA probes are the 138-bp fragment of *HMRE* **(panels A** and **B)** and a *Hin*dIII−*Nae*I fragment from *ARS1* **(panels C** and **D)**. Each reaction (20 μl) contained 3 fmol (5 ng) of labeled DNA. The leftmost lanes in **panels A** and **C** are controls to which no extract has been added: the upper bands represent a small amount of undigested probe. In each panel the amount of insert-containing competitor DNA increases from left to right: the five experimental lanes contain 0, 100, 200, 300 and 500 ng of either *HMLE* DNA **(panels A** and **C)** or *ARS1* DNA **(panels B** and **D)**.

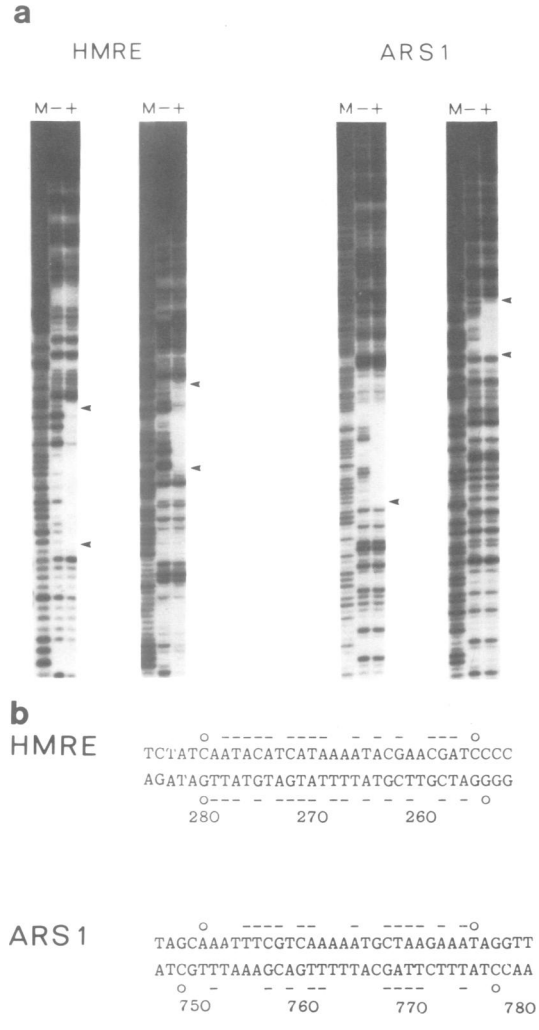

Fig. 4. SBF-B binding sites at *HMRE* and *ARS1*. **(a)** DNase I footprints. The *HMRE* 138-bp fragment, cloned into the *Sma*I site of pUC13, was labeled either at the *Eco*RI site (top strand) or *Xba*I site (bottom strand) and the probes contained vector sequences out to the *Pvu*II sites. Each reaction contained 12 fmol (20 ng) of labeled DNA. The *ARS1* probe was labeled at the *Bgl*II site (top strand, position 853) or at the *Xba*I site in pUC13 (bottom strand). Protein fractions used were obtained by concentration of heparin−agarose fractions yielding the complex II activity (see Figure 2); 5 μg of protein was used in each 20 μl reaction. In each set, the lanes marked M contain the products of the A + G reactions (Maxam and Gilbert, 1980). which serve as markers, and + or − indicate whether the DNase I reaction has been carried out in the presence or absence of extract. Protection of the top strand is shown on the left, the bottom strand on the right. In places where the borders of the protected regions can be determined precisely, we have indicated this with an arrow. **(b)** Summary of the footprint data. A bar above a base indicates a DNase I cleavage site which is protected by added extract. Open circles indicate the definable borders of the footprints, i.e. clear DNase I cleavage sites which have not been protected by added extract.

the insert DNA. The ability of the labeled probe to compete with the cold DNA and bind factor is then analysed by the bandshift method. Of several *ARS* elements tested [*ARS1*, *ARS2* (Tschumper and Carbon, 1982), *HO ARS* (Kearsey, 1984), *HMRI* (Abraham *et al.*, 1984), and *HMLE*; see Materials and methods] *ARS1* showed clear competition with the *HMRE* probe. The results of binding competition experiments with *HMRE* and *ARS1* are shown in Figure 3.

We have mapped the binding sites at *HMRE* and *ARS1* by DNase I footprinting, and the results are shown in Figure 4. For both *HMRE* and *ARS1* the binding sites do not coincide with the so-called *ARS* consensus sequence, but rather are 80 or 100 bp away from this site. The region containing the binding site at *ARS1* has been implicated in the *ARS* activity of *ARS1* by deletion analysis (Celniker *et al.*, 1984). The two binding sites do not show particularly extensive homology with each other (see Figure 4b).

SBF-E binds to both silencers

The elution profile of the heparin−agarose column suggests that a common protein (or protein complex) binds to both *HMRE* and *HMLE* sequences. To determine whether in fact the two silencer DNAs are recognized by a common factor we have again performed binding competition experiments. Figure 5 shows the results of a series of such experiments in which labelled DNA from either *HMRE* or *HMLE* is competed with an excess of cold DNA from either locus. It is clear from these experiments that the two DNA fragments, *HMRE* and *HMLE*, compete with each other for a common binding factor. In addition, it appears that the factor has a higher affinity for the *HMRE* site, as lower concentrations of this fragment are required to compete away binding to either labelled fragment. We shall refer to this binding activity as SBF-E.

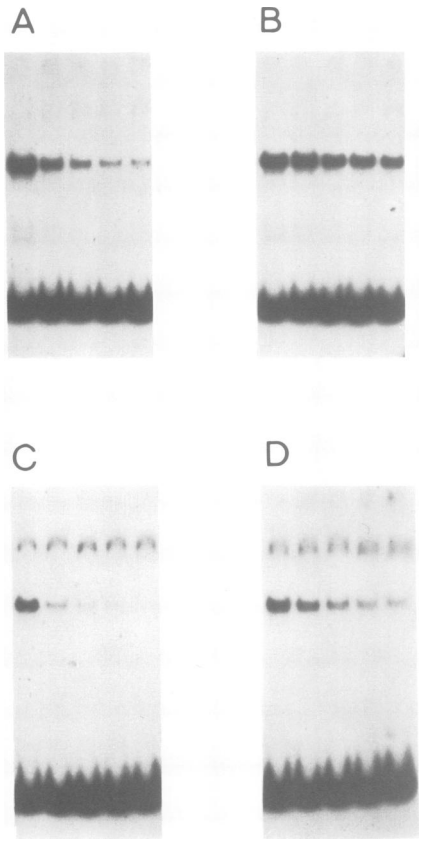

Fig. 5. *HMRE* and *HMLE* compete for binding to a common factor. Bandshift assays were performed with 3 fmol (5 ng) of *HMRE* labeled probe **(panels A** and **B)** or *HMLE* labeled probe **(panels C** and **D)**. Each reaction (20 μl) contained 0.5 μg of protein. The competitor DNAs *HMRE* **(panels A** and **C)** and *HMLE* **(panels B** and **D)** were present at 0, 100, 200, 300, 500 ng (left to right). The uppermost bands in **panels C** and **D** are uncut probe.

The SBF-E binding sites are related but different

We have mapped precisely the sequences protected from DNase I cleavage at *HMRE* and *HMLE* by SBF-E. The results are shown in Figure 6. Protection of both strands has been mapped using G and G+A sequencing ladders (Maxam and Gilbert, 1980) as size standards. Where it is possible to resolve the boundaries of the footprints to within one to two phosphodiester bonds we have indicated this by an arrow.

The size and precise location of a DNase I footprint should provide an upper limit to the extent of a protein binding site and give some information about the sequences which are important for binding. The conserved 11-bp E-box sequence (5′ CA_5C_3AT 3′) is present at both protected regions, but rather than being centered about the borders, in both cases it begins at the edge of the footprint. The remaining halves of the protected regions are largely non-homologous (2 out of 11 matches), as shown in Figure 6b.

To begin to define the sequences required for SBF-E binding we have made oligonucleotides corresponding to the conserved 11-bp E-box sequence and the full DNase I-protected regions at both *HMLE* and *HMRE* (each 22 bp). The oligonucleotides were cloned into the *Sal*I site of pUC13 and used as competitors to measure their affinities for SBF-E relative to the native *HMLE* and *HMRE* sites. The results of these competition experiments are shown in Figure 7. The conserved 11-bp sequence, when in the context of the pUC vector, is a poor binding site com-

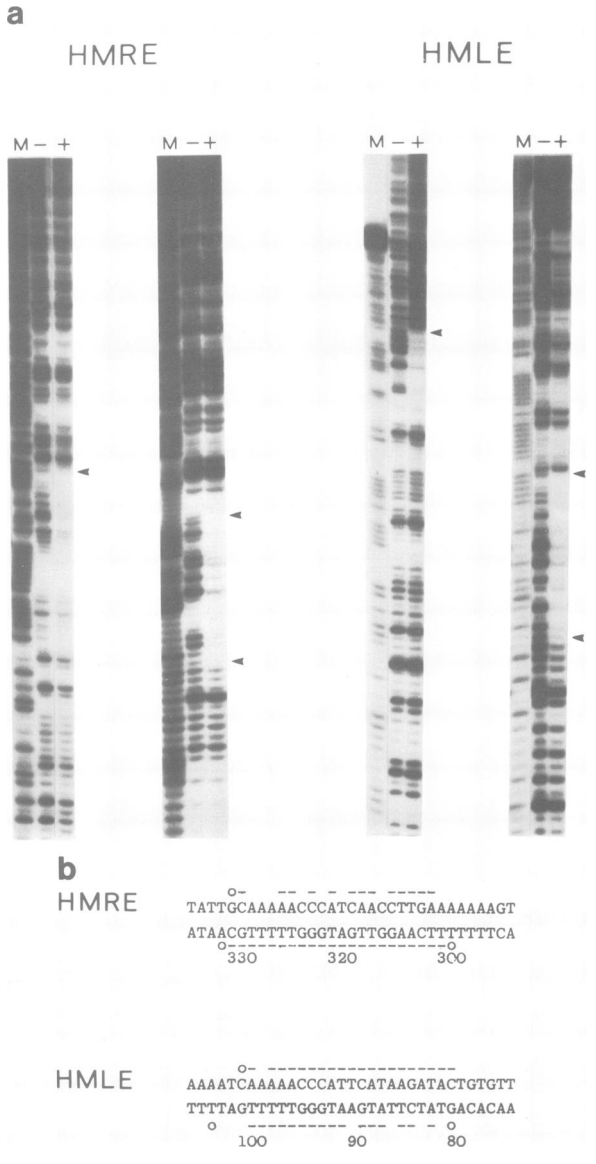

Fig. 6. SBF-E binding sites at *HMRE* and *HMLE*. **(a)** Footprint analysis of *HMRE* and *HMLE* E-box binding activity. The *HMRE* probes were labeled at the *Eco*RI site of pUC13 (top strand) or the *Xba*I site (bottom strand) and contain about 100 bp of vector sequence out to the *Pvu*II site. The *HMLE* probes were labeled at the *Xba*I site in pUC13 from clones of both orientations and also contained vector sequences to the *Pvu*II site. Each lane contains 12 fmol (20 ng) of labeled DNA. One microgram of protein (pooled and concentrated fractions from the heparin−agarose column, Figure 2) was used in each 20-μl reaction. The lanes marked M contain the products of the A + G reactions (Maxam and Gilbert, 1980) and those marked + or − indicate whether the DNase I reaction was carried out in the presence or absence of extract. Protection of the top strand is shown on the left, bottom on the right, in both cases. In places where borders of the protected regions can be determined to within one to two phosphodiester bonds, we have indicated this with an arrow. **(b)** Analysis of the footprint data is depicted as in (a).

pared to either *HMLE* or *HMRE*. This would indicate a difference in affinity of at least 10-fold under the conditions of the competition experiments. In contrast, when the complete DNase I-protected region is cloned into the same vector site, binding is about the same as that obtained with the corresponding *HM* loci (each with about 50 bp of flanking sequences). The sequences within the protected regions alone are therefore sufficient for binding, and the difference in affinity between the *HMLE* and

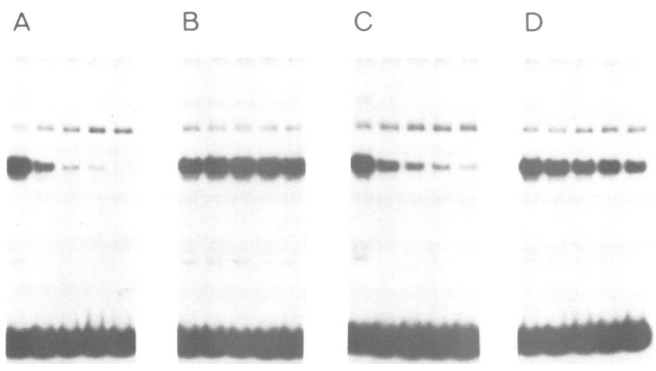

Fig. 7. E-box binding at *HMRE* was analysed by competition with cloned synthetic oligonucleotides. The probe in all panels is the 138-bp *HMRE* fragment (3 fmol, or 5 ng labeled DNA). Each 20 μl binding reaction contained 0.5 μg of protein. Competitor DNAs are *HMRE* **(A)**, the 11-bp E-box oligonucleotide cloned into pUC13 **(B)**, the 22-bp *HMRE* oligonucleotide in pUC13 **(C)**, and the 22-bp *HMLE* oligonucleotide in pUC13 **(D)**. Total DNA is kept constant at 0.5 μg and the amounts of insert-containing competitor are 0, 100, 200, 300 and 500 ng from left to right.

HMRE sites must be due only to sequences within the non-homologous part of the protected regions.

*Neither factor is dependent upon SIR*1-4 gene activity

The four *SIR* genes are *trans*-acting negative regulators of both the *HMR* and *HML* loci and are thus candidates for silencer binding proteins. We have asked, therefore, whether the presence of the two binding activities we have identified is dependent upon the activity of the four *SIR* genes by examining extracts from a series of strains carrying mutations in one of the four genes.

For the *SIR2*, *SIR3* and *SIR4* genes we have made protein extracts from strains containing gene disruption mutations (Shore *et al.*, 1984; M.Marshall, personal communication). A similar disruption of *SIR1* was not available and we have thus tested two independent *sir1* mutants. [These mutants were selected as spontaneous Trp^+ prototrophs in a strain which contains the *TRP1* gene under *SIR* control at the *HMR* locus (Brand *et al.*, 1985; Miller *et al.*, 1984)]. Extracts were prepared, fractionated, and assayed exactly as for the parental Sir^+ strains. For each mutant examined the pattern of footprints on the *HMRE* probe was indistinguishable from wild-type extracts and there was no apparent variation in the quantity of the two activities obtained. In addition, the mobility of protein–DNA complexes in a bandshift assay is unchanged in the *sir* mutants (data not shown).

Discussion

We have described here the identification of two silencer binding factors (SBF-E and SBF-B) and presented evidence relating these factors to the *SIR* control and *ARS* functions of the *HMRE* and *HMLE* silencers.

Properties of SBF-B and SBF-E

The SBF-B binding site at *HMRE*, element B, is involved in both silencer and *ARS* activity (Brand, 1986; Brand *et al.*, 1987). The binding competition experiments presented here show that this factor also recognizes sequences within *ARS1*, in a region called domain B, which is required for optimal *ARS1* function (Celniker *et al.*, 1984). Both of these binding sites occur 80–100 bp from a so-called *ARS* consensus sequence (element A at *HMRE* and domain A at *ARS1*). It is worth noting that nuclease digestion studies of the *TRP1ARS1* minichromosome suggest that SBF-B may be bound stably *in vivo* (Thoma and Simpson, 1985). Two strong micrococcal nuclease cutting sites are exposed in chromatin at positions 737 and 797, which correlate well with the observed boundaries of the *in vitro* footprint in this region (nucleotides 750–773).

We have examined a 370-bp fragment of *HMLE*, containing almost 200 bp to either side of the *ARS* consensus sequence, and failed to find a SBF-B binding site. Other *ARS* fragments tested (*HMRI*, *ARS2* and the *HO ARS* element) also appear not to contain a SBF-B binding site, suggesting that the factor may be specific to a subset of *ARS* elements. Such a possibility is consistent with the work of Maine *et al.* (1984), who have isolated mutations which effect the activity of only particular groups of *ARS* elements. It remains a possibility, though, that we have missed binding sites (particularly at *HMRI* and the *HO ARS*) by examining somewhat smaller fragments containing, on one side, less than 100 bp of flanking sequence about the *ARS* consensus element. It is also possible that SBF-B binds to all *ARS* elements *in vivo* and that we have only been able to detect the strong binding sites in our experiments. It is curious that the *HMRE* and *ARS1* sites, though very similar in affinity, do not show striking sequence homology. The identification of more binding sites would greatly help to resolve these questions.

The observation that *HMLE* does not appear to have an SBF-B binding site, together with the known involvement of this site in silencer function at *HMRE* (Brand, 1986; Brand *et al.*, 1987), suggests that silencers can be constructed from different combinations of regulatory elements. It remains to be seen whether the *HMLE* silencer contains an element analogous to B at *HMRE*, or whether it simply dispenses with this function. In any event, it appears that the *SIR* control machinery can utilize different combinations of replication related factors to produce the silencer effect (see below).

Binding competition experiments with synthetic sites show that the two SBF-E binding sites are composed of adjoining homologous (E-box) and non-homologous sequences, both of which contribute to the energy of binding. The difference in affinity between the two sites is less than a factor of 10 and thus the difference in the free energy of binding is small compared to the overall energy of binding. The apparent bipartite structure of the two binding sites, with no 2-fold rotational symmetry, raises the possibility that each site might be recognized by a unique heterodimer containing a common subunit which recognizes the conserved 11-bp E-box sequence. The common subunit would have to be exchangeable and present in limiting amounts to explain the binding competition results. A simpler interpretation of these results is that a single factor recognizes the two sites and that this factor has a higher affinity for the *HMRE* site. The apparent bipartite structure of the sites may therefore be coincidental.

All of the *sir* mutants that we have examined contain wild-type levels of both SBF-E and SBF-B which show indistinguishable chromatographic properties on heparin–agarose columns. It is unlikely, therefore, that either factor is the product of a known *SIR* gene, although we cannot rule out the possibility that some of the mutants we have examined retain DNA binding activity but are defective in an effector function. This seems unlikely because all of the mutants examined (most of which would produce significantly truncated proteins) give identical bandshift patterns, suggesting that they contain unaltered binding factors.

Relationship to SIR control

Why have the genes coding for SBF-B and SBF-E not been identified as *SIR* genes? There may be two reasons. First, it is likely that a silencer can be constructed from combinations of protein–DNA interactions (some related to *ARS* activity), none of which are by themselves essential for silencer function. Clearly the regulatory elements at *HMRE* appear redundant (Brand, 1986; Brand *et al.*, 1987). Hence a mutation in a silencer binding protein might not be expected to give a Sir^- phenotype. SBF-B appears not to bind at the *HMLE* silencer and the binding site at *HMRE* is not necessary for silencer function when the A and E elements are intact. Although SBF-E does bind to both silencers, deletion of the binding site at *HMRE* results in only a partial loss of silencer function. It is not clear whether the absence of this protein–DNA interaction would lead to a phenotype strong enough to have been observed in Rine's extensive screen for *sir* mutants (Rine, 1979). Secondly, it seems likely that both factors have other functions in the cell which might be essential for viability, and thus would not have been detected as *sir* mutants. It is easy to see how this might be the case for SBF-B, as it may have a general role in DNA replication. SBF-E appears to be a relatively abundant DNA binding factor [on the order of 10^3 copies per cell (D.Shore, unpublished results)] and would hence be likely to play a role in other regulatory processes in the cell, perhaps seemingly unrelated to silencer activity. The identification of other binding sites for this factor might therefore be informative.

Our results suggest that the *SIR* proteins themselves are not silencer binding proteins. The *HMRE* silencer, for which we have the most information, is composed of three regulatory sites, and we have described here factors which bind to two of these sites. Neither SBF-E nor SBF-B would appear to be the product of a *SIR* gene yet recent experiments show that the binding sites for the two factors at E and B correspond precisely to the sequences required for regulation (Brand *et al.*, 1987). The third element, A, consists of an 11-bp *ARS* consensus sequence and is thus unlikely to be a *SIR* protein binding site because none of the *SIR* genes is essential for growth (Shore *et al.*, 1984; Ivy *et al.*, 1986). Perhaps SBF-E and SBF-B binding at *HMRE* is a prerequisite for binding of one or several *SIR* proteins, in analogy to the recruitment of the SL1 factor to the human rRNA promoter (Learned *et al.*, 1986). Alternatively, the *SIR* proteins may recognize a protein complex composed of SBF-E and SBF-B at *HMRE*, without specifically recognizing silencer DNA sequences. If SBF-E and SBF-B have other regulatory roles in the cell, the function of the *SIR* gene products may be to interact with these two factors (at *HMRE*) and change their function in such a way as to create a silencer.

Materials and methods

Strains and DNAs

Most yeast extracts were prepared from a protease-deficient strain, BJ2168 (*a leu2 trp1 ura3-52 prb1-1122 pep4-3 prc1-407 gal2*), kindly provided by Melanie Lee. *sir2* and *sir3* gene disruptions of this strain were prepared by the gene replacement technique (Rothstein, 1983). Extracts were also prepared from *sir1* and *sir4* derivatives of strain RS1 (Brand *et al.*, 1985). The origin of DNA fragments used in this study is as follows. A 138-bp fragment of *HMRE* DNA [Abraham *et al.* (1984); *Aha*III site at position 380 to *Alu*I site at position 240] was cloned into the *Sma*I site of pUC13. In early experiments not described here a 524-bp *HMRE* fragment [*Xho*I mutant 268, at position 547 to *Xba*I site at position 24; Abraham *et al.* (1984)] was subcloned into pUC13 and assayed by footprinting for SBF-E and SBF-B binding sites. Two subclones of *HMLE* (Feldman *et al.*, 1984) were constructed: a 370-bp *Hha*I fragment and a 147-bp *Rsa*I fragment (position 47–194) were cloned into the *Sma*I site of pUC13 (Feldman *et al.*, 1984). The following *ARS* fragments were cloned into pUC13: a 454-bp *Hin*dIII to *Nae*I fragment of *ARS1* [Tschumper and Carbon (1980); position 616–1069]; a 633-bp *Xho*I fragment containing *ARS2* (Tschumper and Carbon, 1982); a 75-bp *HO ARS* fragment [h12; Kearsey (1984)]; a 220-bp *HMRI* fragment from the *Bcl*I site at position 100 to the *Xho*I linker mutant $d^2 39$ (Abraham *et al.*, 1984).

DNA manipulations

All DNAs used in this study were twice banded in CsCl–ethidium bromide gradients. Labeled DNA probes used in both footprinting and bandshift experiments were prepared by end-labeling restriction fragments with either [α-^{32}P]dATP or [α-^{32}P]dCTP using reverse transcriptase (Anglia Biotechnology) as directed by the manufacturer. Probes were prepared from fragments cloned into pUC13. After labeling at a restriction site within the pUC13 polylinker a double digest was done to release full-length probe and a small fragment containing polylinker sequences (10–20 bp) which will run off the bottom of a gel. Such a procedure obviates the need to purify the probe after labeling. Often probes were cut at the *Pvu*II sites of pUC13 and thus contain slightly over 100 bp of vector sequences.

Preparation and fractionation of yeast extracts

Yeast cells were grown with vigorous aeration in 10-l bottles of YEPD to a density of $2.5–5 \times 10^7$ cells/ml. Cells were harvested in a Westphalia continuous flow centrifuge and washed with cold distilled water. Extracts were prepared essentially according to Klekamp and Weil (1982). Cells were broken with 0.5-mm glass beads in a 'Bead-beater' with a dry ice cooling chamber. The breakage buffer contained 0.2 M Tris, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.5 mM PMSF, 20 μg/ml pepstatin A, and 20 mM TPCK, TLCK. An S-100 was prepared and ammonium sulphate precipitated (0.35 g ammonium sulphate/ml S-100). The precipitate was resuspended and dialysed against A50 buffer (20 mM Tris, pH 8.0, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 50 mM ammonium sulphate). The dialysed extract was loaded onto a heparin–agarose column (Davison *et al.*, 1979) and washed with at least four column volumes of A50 buffer. Bound proteins were eluted with a gradient of ammonium sulphate (typically A50–A800). Peaks of binding activity were concentrated (typically 30-fold) by ammonium sulphate precipitation (0.35 g/ml) and resuspended in A50 buffer with 25% glycerol. Frozen aliquots were stored at −70°C.

DNA binding assays

Bandshift assays were performed according to Arcangioli and Lescure (1985). The binding buffer for both bandshift and footprint assays was 20 mM Tris, pH 7.5, 7 mM $MgCl_2$, 1 mM DTT, 6% glycerol, 100 mM NaCl. Binding reactions (20 μl total volume) always contained an unlabeled carrier DNA (100–500 ng) of salmon sperm DNA, calf thymus DNA or poly d(IC). Some bandshift assays were done in poly d(IC) and 5 mM spermidine, 1 mM $MgCl_2$, conditions which increased the yield of the SBF-E complex. Footprint assays were performed according to Klemenz *et al.* (1982), except that the binding buffer usually contained 100 mM NaCl rather than ammonium sulphate. DMSO could be omitted with no observable effect.

Acknowledgements

We would like to thank J.Kilmartin, J.R.MacIntosh, and J.Fogg for help and advice on the growth and breakage of large volumes of yeast cells, M.Marshal for the generous gift of a *sir4* deletion strain, and H.Pelham and L.Breeden for comments on the manuscript. D.S. and D.J.S. received support from the Jane Coffin Childs Foundation. D.S. would also like to acknowledge support from EMBO.

References

Abraham,J., Nasmyth,K.A., Strathern,J.N., Klar,A.J.S. and Hicks,J.B. (1984) *J. Mol. Biol.*, **176**, 307–331.
Arcangioli,B. and Lescure,B. (1985) *EMBO J.*, **4**, 2627–2633.
Brand,A.H. (1986) Ph.D. thesis, Cambridge University, UK.
Brand,A.H., Breeden,L., Abraham,J., Sternglanz,R. and Nasmyth,K. (1985) *Cell*, **41**, 41–48.
Brand,A.H., Micklem,G. and Nasmyth,K.A. (1987) *Cell*, in press.
Broach,J.R., Li,Y.-Y., Feldman,J., Jayaram,M., Abraham,J., Nasmyth,K.A. and Hicks,J.B. (1982) *Cold Spring Harbor Symp. Quant. Biol.*, **47**, 1165–1173.
Celniker,S.E., Sneder,K., Srienc,F., Bailey,J.E. and Campbell,J.L. (1984) *Mol. Cell. Biol.*, **4**, 2455–2466.
Davison,B.L., Leighton,T. and Rabinowitz,J.C. (1979) *J. Biol. Chem.*, **254**, 9220–9226.
Feldman,J.B., Hicks,J.B. and Broach,J.R. (1984) *J. Mol. Biol.*, **178**, 815–834.
Fried,M. and Crothers,D.M. (1981) *Nucleic Acids Res.*, **9**, 6505–6525.
Galas,D. and Schmitz,A. (1978) *Nucleic Acids Res.*, **5**, 3157–3170.
Garner,M.M. and Revzin,A. (1981) *Nucleic Acids Res.*, **9**, 3047–3060.
Haber,J.E. and George,J.P. (1979) *Genetics*, **93**, 13–35.
Herskowitz,I. and Oshima,Y. (1981) In Strathern,J.N., Jones,E. and Broach,J.R.

(eds), *The Molecular Biology of the Yeast Saccharomyces*. Cold Spring Harbor Laboratory Press, pp. 181–209.
Ivy,J.M., Klar,A.J.S. and Hicks,J.B. (1986) *Mol. Cell. Biol.*, **6**, 688–702.
Kearsey,S. (1984) *Cell*, **37**, 299–307.
Klar,A.J.S., Fogel,S. and Macleod,K. (1979) *Genetics*, **93**, 37–50.
Klekamp,M.S. and Weil,P.A. (1982) *J. Biol. Chem.*, **257**, 8432–8441.
Klemenz,R., Stillman,D.J. and Geiduschek,E.P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 6191–6195.
Learned,R.M., Learned,T.K., Haltiner,M.M. and Tjian,R.T. (1986) *Cell*, **45**, 847–857.
Maine,G.T., Sinha,P. and Tye,B.-K. (1984) *Genetics*, **106**, 365–385.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
Miller,A.M. and Nasmyth,K.A. (1984) *Nature*, **312**, 247–251.
Miller,A.M., Sternglanz,R. and Nasmyth,K.A. (1984) *Cold Spring Harbor Symp. Quant. Biol.*, **49**, 105–113.
Nasmyth,K.A. (1982a) *Annu. Rev. Genet.*, **16**, 439–500.
Nasmyth,K.A. (1982b) *Cell*, **30**, 567–578.
Rine,J. (1979) Ph.D. thesis, University of Oregon, USA.
Rine,J., Strathern,J.N., Hicks,J.B. and Herskowitz,I. (1979) *Genetics*, **93**, 877–901.
Rothstein,R. (1983) *Methods Enzymol.*, **101**, 202–211.
Schnell,R. and Rine,J. (1986) *Mol. Cell. Biol.*, **6**, 494–501.
Serfling,E., Jasin,M. and Schaffner,W. (1985) *Trends Genet.*, **1**, 224–230.
Shore,D., Squire,M. and Nasmyth,K.A. (1984) *EMBO J.*, **3**, 2817–2823.
Stinchcomb,D.C., Struhl,K. and Davis,R.W. (1979) *Nature*, **282**, 39–43.
Thoma,F. and Simpson,R.T. (1985) *Nature*, **315**, 250–252.
Tschumper,G. and Carbon,J. (1980) *Gene*, **10**, 157–166.
Tschumper,G. and Carbon,J. (1982) *J. Mol. Biol.*, **156**, 293–307.

Received on 27 October 1986; revised on 15 December 1986