Two DNA-Binding Factors Recognize Specific Sequences at Silencers, Upstream Activating Sequences, Autonomously Replicating Sequences, and Telomeres in Saccharomyces cerevisiae

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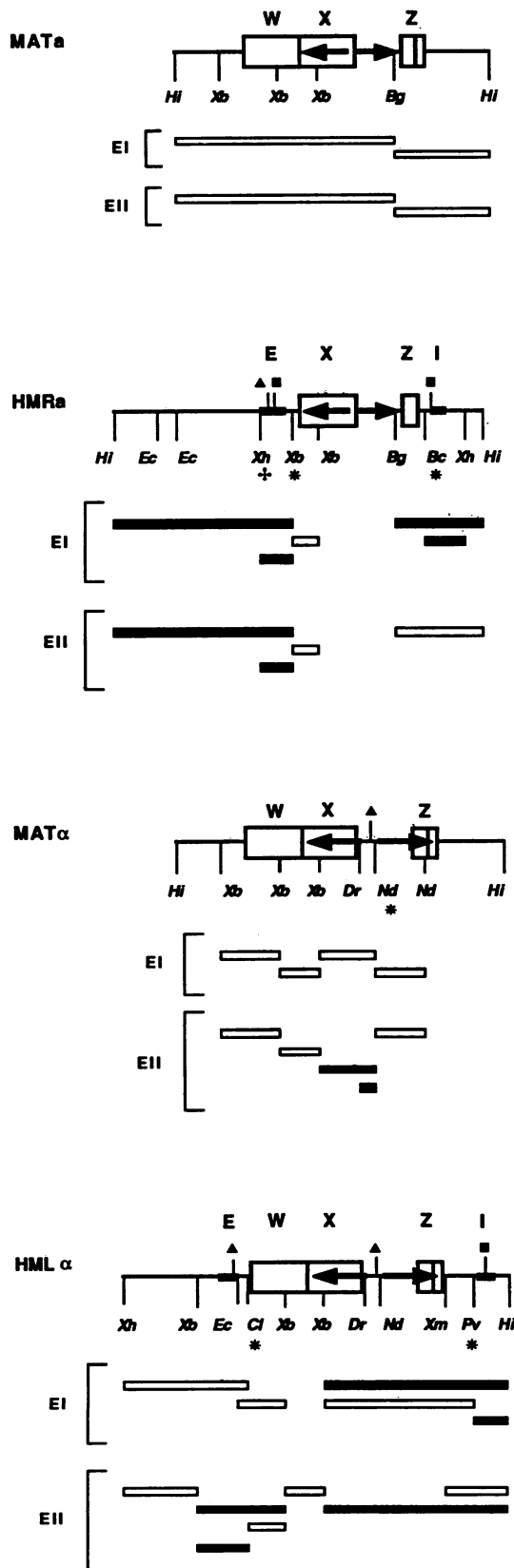
Two DNA-binding factors from Saccharomyces cerevisiae have been characterized, GRFI (general regulatory factor I) and ABFI (ARS-binding factor I), that recognize specific sequences within diverse genetic elements. GRFI bound to sequences at the negative regulatory elements (silencers) of the silent mating type loci HML E and HMR E and to the upstream activating sequence (UAS) required for transcription of the MAT α genes. A putative conserved UAS located at genes involved in translation (RPG box) was also recognized by GRFI. In addition, GRFI bound with high affinity to sequences within the (C$_{1-3}$A)-repeat region at yeast telomeres. Binding sites for GRFI with the highest affinity appeared to be of the form 5'-(A/G)(A/C)ACCCAN NCA(T/C)(T/C)-3', where N is any nucleotide. ABFI-binding sites were located next to autonomously replicating sequences (ARSs) at controlling elements of the silent mating type loci HMR E, HMR I, and HML I and were associated with ARS1, ARS2, and the 2μm plasmid ARS. Two tandem ABFI binding sites were found between the HIS3 and DED1 genes, several kilobase pairs from any ARS, indicating that ABFI-binding sites are not restricted to ARSs. The sequences recognized by ABFI showed partial dyad-symmetry and appeared to be variations of the consensus 5'-TATCATTNNNNACGA-3'. GRFI and ABFI were both abundant DNA-binding factors and did not appear to be encoded by the SIR genes, whose products are required for repression of the silent mating type loci. Together, these results indicate that both GRFI and ABFI play multiple roles within the cell.

Eucaryotic chromosomes appear to be organized in domains governing such processes as gene expression, DNA replication, and chromosome condensation. In an effort to define the biochemical components directing these events, we have been attracted to the features of the mating type loci of Saccharomyces cerevisiae (reviewed in reference 33). Haploid yeast cells of the a or α mating type express different genes at MAT, in the middle of chromosome III (Fig. 1). The alternate forms of this locus, MATa and MATα, encode distinct regulatory factors a1 and a2 or α1 and α2, but have extensive regions of homology, referred to as W, X, and Z (2, 61). There are, in addition, silent copies of genes specifying each mating type stored on the left and right arms of chromosome III at HMLα and HMRa, respectively. In homothallic strains, the mating type of successive generations switches in a tightly regulated manner (29, 35, 80). Switching is accomplished by gene conversion whereby sequences at MAT are replaced by those at HML or HMR (33, 47). This process is initiated with the cleavage of MAT DNA in Z by a specific endonuclease that is the product of the HO gene (49, 50). The silent copies of the mating type loci are controlled by negative regulatory sequences located over 1,000 base pairs (bp) away from the promoters for these genes. Deletion analysis of HML and HMR has revealed cis-acting regulatory elements, E and I, that flank each locus on the left and right sides, respectively (1, 24). Sequences within E are essential for maintaining repression, and those at I are of lesser significance. The products of four nonessential genes, SIR1, SIR2 (MAR1), SIR3 (MAR2, CMT), and SIR4, are also required for repression of HML and HMR (28, 46, 48, 66, 67).

The negative regulatory process at the silent mating type loci is remarkably general. Other genes, such as TRP1 and LEU2, can be repressed by the SIR proteins when placed in the vicinity of the E and I elements (12). Furthermore, a tRNA gene is regulated in a SIR-dependent manner when inserted at HMRa (71). The SIR gene products are also responsible for blocking cleavage by HO endonuclease of Z sequences at HML and HMR, thereby preventing their interconversion (49, 50). In many ways the E element behaves as a negative version of enhancers in higher eucaryotes, or upstream activating sequences (UASs) in yeast, and so has been termed a silencer. The E element exerts it effects in a position- and orientation-independent manner and can repress multiple promoters simultaneously even at distances greater than 2,500 bp (12). These findings have been taken to suggest that the SIR proteins, through the E and I elements, create a domain of altered chromatin structure, impeding the access of a variety of factors to the underlying DNA. In this regard, it is notable that the E and I regions are not only transcriptional regulatory elements, but are also autonomously replicating sequences (ARSs) (1, 13, 24), presumed to be origins of DNA replication in yeast cells (reviewed in reference 15). Studies with a temperature-sensitive sir3 strain have shown that after a shift from the restrictive to the permissive temperature, repression is established only after S phase has been completed, suggesting a link between DNA replication and the mechanism of repression (59).

The elaborate features of the mating type loci indicate that these regions are rich in targets for factors directing DNA replication, recombination, and gene expression. We have attempted to identify some of these factors by looking for activities that specifically bind regulatory sequences of the silent mating type loci. This report presents the characteri-

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zation of two DNA-binding factors, GRFI (general regulatory factor I) and ABFI (*ARS*-binding factor I), that recognize specific sequences at the *E* and *I* elements. The results suggest that these factors are not limited to regulating the mating type genes, but rather play other roles at many additional loci in the yeast genome.

MATERIALS AND METHODS

Enzymes and reagents. Restriction enzymes were purchased from either New England BioLabs or Boehringer Mannheim. Unless indicated otherwise, the reagents used were from Sigma Chemical Co.

Synthetic oligonucleotides. Oligonucleotides were produced with an Applied Biosystems DNA synthesizer and were purified and annealed as described previously (9). The oligonucleotides contain sequences derived from binding sites of the different factors as well as sequences on each end that allow ligation to other restriction fragments. Oligonucleotide sequences are listed in Table 1. Sequences of the mtIIA and mtIIB oligonucleotides can be derived from information in Fig. 4A and those for mtIA and mtIB from Fig. 9A. The sequence of the GAL4 oligonucleotide can be found elsewhere (9).

Plasmid and probe DNAs. Standard recombinant DNA methods were used in these experiments (57). Plasmids pJDB-*SIR1*, pJDB-*SIR2*, and pJDB-*SIR3* are derivatives of the yeast vector pJDB207 (5) carrying inserts with the indicated *SIR* gene. DNA fragments containing the *SIR1* and *SIR2* genes were gifts of J. Hicks (39). The *SIR3* gene was isolated from a YEP24-based library by complementation of the *sir3-8* mutation (J. Rine, unpublished). Plasmid pG2-*SIR4* directs the synthesis of an 88-kilodalton carboxy-terminal fragment of *SIR4* protein from an inducible *GAL1* promoter (42; W. J. Kimmerly and J. Rine, unpublished). This fragment of the *SIR4* gene is sufficient to complement null alleles of *SIR4*. The *GAL1-SIR4* fusion was inserted into the high-copy vector pJDB207 along with a segment carrying the yeast *GAL4* gene (51) to maximize *SIR4* expression (55).

For ease in manipulating *HMR E* sequences, a 490-bp *Xho*I-*Xba*I fragment containing *E* (Fig. 1B) was excised from the plasmid Δ60 (1) and inserted into the *Hinc*II site of the polylinker in the vector pEMBL18 (45). The resulting con-

FIG. 1. Landmarks of the yeast mating type loci and summary of filter-binding studies. Maps of each of the loci are shown with the homologous regions *W* (723 bp), *X* (704 bp), and *Z* indicated by the open boxes and the controlling elements of the silent loci, *E* and *I*, shown with thick black lines. Coding regions are denoted with black arrows—a1 or α1 by rightward arrows and a2 or α2 by leftward arrows—at the appropriate loci. Locations of restriction enzyme cleavage sites are shown below each map; abbreviations: Hi, *Hind*III; Xb, *Xba*I; Bg, *Bgl*II; Ec, *Eco*RI; Xh, *Xho*I; Bc, *Bcl*I; Dr, *Dra*I; Nd, *Nde*I; Cl, *Cla*I; Xm, *Xmn*I; Pv, *Pvu*II. The *Xho*I site at *HMRa* marked with a cross shows the position of an inserted *Xho*I synthetic linker in the plasmid Δ60 (1). Results of nitrocellulose filter-binding assays are summarized by the bars below each map. The position of each bar indicates the restriction fragment tested. Black bars denote fragments showing greater than 30% reduced binding in the presence of the indicated competitor oligonucleotide (10 to 50 ng). White bars denote fragments showing less than 15% effect of added oligonucleotide. Restriction enzyme sites used as ^{32}P-labeled ends for the DNase I protection studies are indicated with asterisks. The actual locations of factor-binding sites determined by DNase I protection mapping are shown with symbols above each map—▲, GRFI sites; ■, ABFI sites.

TABLE 1. List of oligonucleotides

Oligonucleotide	Length (bp)	Sequence[a]
EI	28	GAT CCA ATA CAT CAT AAA ATA CGAA CGA GT TAT GTA GTA TTT TAT GCT T GCT TCG A
Sym	31	GAT CCA ATA TAT CGT TAT TAA CGAT ATA TTA GT TAT ATA GCA ATA ATT GCTA TAT AAT TCG A
DED1	42	GAT CCA ATG CAT CAT TCT ATA CGTG TCA TTC TGA ACGAG GCG GT TAC GTA GTA AGA TAT GCAC AGT AAG ACT TGCTC CGC TTA A
EII	31	GAT CTT ATA TTG CAA AAA CCC ATC AACC TTG AA TAT AAC GTT TTT GGG TAGT TGG AAC TTA A
Alpha	31	GAT CCA TCC CAA ACA AAA CCC AGAC ATC ATG GT AGG GTT TGT TTT GGG TCTG TAG TAC TTA A
HML29	29	GAT CCA AAT CAA AAA CCC ATT CATA AGA G GT TTA GTT TTT GGG TAA GTAT TCT CTT AA
HML35	35	GAT CCA AAA ATC AAA AAC CCA TTCA TAA GAT ACT G GT TTT TAG TTT TTG GGT AAGT ATT CTA TGAC TTAA
TEF2	31	GAT CCC ATT CAT GTT GCA CCC ACAC ATT TAG GG TAA GTA CAA CGT GGG TGTG TAA ATC TTA A
TEL	38	GAT CCC ACA CCC ACA CAC CCA CACA CCC ACA CAC C CAG GG TGT GGG TGT GTG GGT GTGT GGG TGT GTGG GTCT TAA

[a] Both strands of each oligonucleotide are shown, with the top strand listed, 5' to 3', from left to right.

struct, pJR315 (45), contains the E DNA oriented with the original XbaI site adjacent to a BamHI site in the polylinker. This BamHI site was cleaved and used as the labeled end in DNase I protection studies. Similarly, a BclI-HindIII fragment containing HMR I (Fig. 1B) was inserted into pEMBL18 between BamHI and HindIII sites, placing the original BclI end next to an EcoRI site in the polylinker. The EcoRI site of the resulting plasmid, pEMBL/HMRI, was cleaved and used for ³²P-labeling the probe in DNase I protection studies of HMR I sequences.

Probes containing synthetic oligonucleotides used for gel electrophoresis mobility shift assays were derived from three-piece plasmid constructions. The GRFI-type oligonucleotides were ligated to 1.6-kbp SalI-EcoRI and 7.3-kbp BamHI-SalI fragments from p10GH (54). Digestion of the resulting plasmids with EcoRI liberated a 0.4-kbp fragment containing each oligonucleotide joined to sequences from pBR322. The ABFI-type oligonucleotides were ligated with a 670-bp BamHI-EcoRI fragment of ARS1 DNA from the plasmid Δ173 (76) (this deletion lacks the ABFI binding site at ARS1) and a 7.0-kbp EcoRI-HindIII fragment of YRP14/CEN4 (36). The resulting plasmids were digested with HindIII and BglII to liberate 100-bp fragments containing each oligonucleotide joined to ARS1 sequences.

All probe DNAs were labeled with [α-³²P]dATP (Amersham) and T4 DNA polymerase (New England Biolabs) by replacement synthesis (57). Specific radioactivity of probes varied from 5×10^3 to 1×10^4 cpm/fmol.

Growth of yeast strains. For the production of extracts, cells were grown in YP medium (2% Bactopeptone [Difco], 1% yeast extract) containing 2% glucose or sucrose. Plasmid-containing strains were grown first in minimal medium (0.67% yeast nitrogen base, 0.5% ammonium sulfate, plus uracil and histidine) and then transferred to YP medium for the last 10 generations to increase yield. In the case of cells containing pG2-SIR4, sucrose was used as the carbon source and maximal induction of SIR4 protein was achieved by adding galactose to 2% for the final 2 h of growth. Cells were harvested at an A_{600} value of 1 to 6. Plasmid DNAs were introduced into strain SF657-2d by a standard transforma-

tion procedure (4), selecting for leucine prototrophy on minimal medium supplemented with histidine and uracil.

Whole-cell and nuclear extracts. Yeast whole-cell extracts were prepared as described previously (9) by glass bead disruption of cells in buffer A (25 mM HEPES [N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid, pH 7.5], 5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, 0.6 μM leupeptin, 50 mM KCl) containing 0.3 M ammonium sulfate. Protein concentration was measured by the method of Bradford (8) and varied from 3 to 30 mg/ml, depending on the amount of cells used.

Nuclear extract was prepared from spheroplasts of BJ926 cells as described (9) except that the extract was concentrated by precipitation with 90% saturated ammonium sulfate in buffer A. The precipitate was suspended in one-tenth the original volume of buffer A and dialyzed against the same buffer for use in the binding experiments. The concentrated nuclear extract contained 30 mg of protein per ml.

Nitrocellulose filter-binding assays. Binding reactions were carried out in 20 μl of buffer A containing 4 to 10 fmol of ³²P-labeled probe DNA, 1 μg of either poly[d(I-C)] or salmon sperm DNA, and various amounts of whole-cell extract, nuclear extract, or competitor DNA. Reaction mixes were incubated for 10 to 20 min at 22°C and then filtered and counted as described elsewhere (9). The following DNA concentrations were used for testing competitors: 0.1 to 0.5 μg of plasmid DNAs, 0.05 to 0.2 μg of restriction fragments, and 0.25 to 50 ng of synthetic oligonucleotides (1 ng of a 30-bp oligonucleotide corresponds to 50 fmol). Whole-cell extracts were used in screening restriction fragments for GRFI binding, and poly[d(I-C)] was used as carrier DNA. Salmon sperm DNA greatly inhibits detection of this factor. When screening restriction fragments for ABFI-binding sites, nuclear extract was used, as this was sixfold-enriched for the activity compared with whole-cell extracts. Variation in individual assays was less than 15%. Tests for specific binding were carried out in parallel assays with and without competitor DNA. Binding was judged to be specific when there was more than a 30% reduction of bound probe on

addition of the appropriate amount of competitor. When there was no specific binding, the effect of added competitor was less than 15%, taking into account its contribution to the total DNA in the reaction.

DNase I protection mapping. Binding reactions were carried out for 10 min at 22°C in 20 μl of buffer A containing 20 to 60 kcpm of ^{32}P-labeled probe, 1 μg of poly[d(I-C)], concentrated nuclear extract, and in some cases 100 ng of competitor oligonucleotide. DNase I digestion and analysis were done as described elsewhere (9) except that electrophoresis was in 4 or 8% polyacrylamide–urea gels. Gels were calibrated with labeled DNA markers of known size and with restriction fragments derived from the probe that could be related to the published DNA sequence.

Gel electrophoresis mobility shift assays. DNA binding was performed with whole-cell extracts as in the nitrocellulose filter-binding assays. Reaction mixes were loaded directly onto 3% NuSieve agarose (FMC) gels with electrophoresis for 2 to 4 h in buffer containing 44 mM Tris-borate, pH 8.3, and 1.25 mM EDTA. Gels were dried and analyzed by autoradiography on Kodak XR5 film for 1 to 24 h at −70°C with a Du Pont Cronex Lightning-Plus intensifying screen. Comparisons of probes containing different synthetic oligonucleotides were done with DNAs labeled under identical conditions and having approximately the same specific radioactivity.

Estimates of the minimum abundance of ABFI and GRFI were based on the amount of whole-cell extract required to form complexes with half the labeled probe in the binding reaction, assuming a yield of 5 pg of total protein per haploid cell and 1 molecule of factor per complex. No adjustments were made for the efficiency of extraction, the fraction of active molecules, or the affinity of binding sites, so the figures given are likely to be considerably below the actual amounts.

RESULTS

Two factors bind to specific sequences at *HMR E*. Segments of the silent mating type loci were screened in nitrocellulose filter-binding assays (9, 10) (see Materials and Methods) for specific interactions with components of yeast extracts. Both *E* and *I* elements from *HML* and *HMR* showed specific binding by factors in whole-cell and nuclear extracts. The properties of the *E* region at *HMR* were of special interest. A fragment containing 490 bp of *HMR E* (Fig. 1, *Xho*I-*Xba*I segment) exhibited a high level of specific binding: 80% of the total binding was abolished by competition with the same unlabeled DNA fragment (in the presence of an excess of carrier DNA). This behavior contrasted with that of adjacent sequences from the *X* region (Fig. 1, 330-bp *Xba*I-*Xba*I fragment), which showed no specific binding under the same conditions. Furthermore, some of the specific binding to the *HMR E* fragment was prevented by competition with fragments containing each of the other *E* and *I* regions.

The precise locations of sequences recognized by these factors at *HMR E* were determined by DNase I protection mapping (9) with a yeast nuclear extract and a ^{32}P-labeled probe containing 490 bp of *E* (Fig. 2A). Nuclear extract protected two separate regions of the probe from DNase I digestion, corresponding to nucleotides 258 to 280 and 310 to 334 in the numbering of Abraham et al. (1). Both protected sites lay within the boundaries of *E* as defined by deletion analysis (1). To distinguish between the factors protecting these regions, two double-stranded oligonucleotides were synthesized, EI and EII (Table 1), each containing se-

quences from one of the protected regions. These oligonucleotides were added to the binding reactions in 250-fold molar excess over the probe, but in amounts which constituted less than 10%, by weight, of the total DNA. Addition of EI oligonucleotide, containing sequences from the protected site closest to *X*, completely blocked the protection of this region from DNase I, but had no effect on the protection of sequences at the EII site. The converse was seen when the EII oligonucleotide was added, demonstrating that there are at least two factors with different specificities that bind *HMR E* DNA.

A gel electrophoresis mobility shift assay (25, 26) was used to investigate the simultaneous binding of these factors to probe DNA. The analysis was performed with a 529-bp ^{32}P-labeled probe containing 490 bp of *HMR E* (the additional 39 bp were derived from a polylinker used in manipulating this segment) and a yeast whole-cell extract. Three complexes were detected when extract was mixed with probe, which increased in abundance when more extract was added (Fig. 2B). The two fastest-migrating complexes were not fully resolved, but they could be completely separated when shorter probe DNAs were used (not shown). Formation of the various complexes was prevented by competition with oligonucleotides as follows: the slowest-migrating complex by either EI or EII, the complex with intermediate mobility only by EII, and the fastest-migrating complex only by EI. These data indicate that the two fastest-migrating complexes each contained one of the two factors bound to probe DNA, while the slowest-migrating complex contained both factors. In keeping with the DNase I protection experiments above, this analysis demonstrates the presence in whole-cell extracts of two different factors capable of binding independently to *HMR E* DNA.

Binding of the two factors to sequences in the EI and EII oligonucleotides was quantitated with nitrocellulose filter-binding assays. The probe for these assays was the same ^{32}P-labeled DNA fragment employed in the previous DNase I protection and electrophoretic mobility shift experiments. An amount of whole-cell extract was used which retained approximately 50% of the probe on filters in the presence of 1 μg of carrier DNA. Various amounts of each of three different oligonucleotides (GAL4, EI, and EII, Table 1) were tested as competitors for their ability to diminish the amount of bound probe (Fig. 2C). As much as 50 ng of a 25-bp oligonucleotide, containing a binding site for the yeast *GAL4* regulatory protein (9), did not significantly reduce the amount of bound probe. In contrast, 50 ng of EI oligonucleotide decreased binding by 29% (0 ng point on EII curve), and the same amount of EII oligonucleotide reduced binding by 65% (0 ng point on EI curve). With 50 ng of both EI and EII oligonucleotides, total binding was diminished by 90% (50 ng point on either EI or EII curve). Titrations with the EI and EII oligonucleotides were performed in the range of 0.5 to 50 ng of one oligonucleotide in the presence of a saturating amount of the other (50 ng). In each case, half-maximal competition was achieved with 0.5 ng and saturation was reached at 5 to 10 ng. Comparison of the molar amounts of probe DNA and oligonucleotide in these assays revealed that the oligonucleotides bound each factor with an affinity of about 1/3 that of the larger probe. The half-lives of complexes formed at the EI and EII sites were 4.5 and 7.5 min, respectively (not shown), indicating that the binding affinities of each factor for *HMR E* were high. The additive relationship observed for binding of the two factors in both electrophoretic mobility shift and filter-binding experiments showed that there were no substantial cooperative effects.
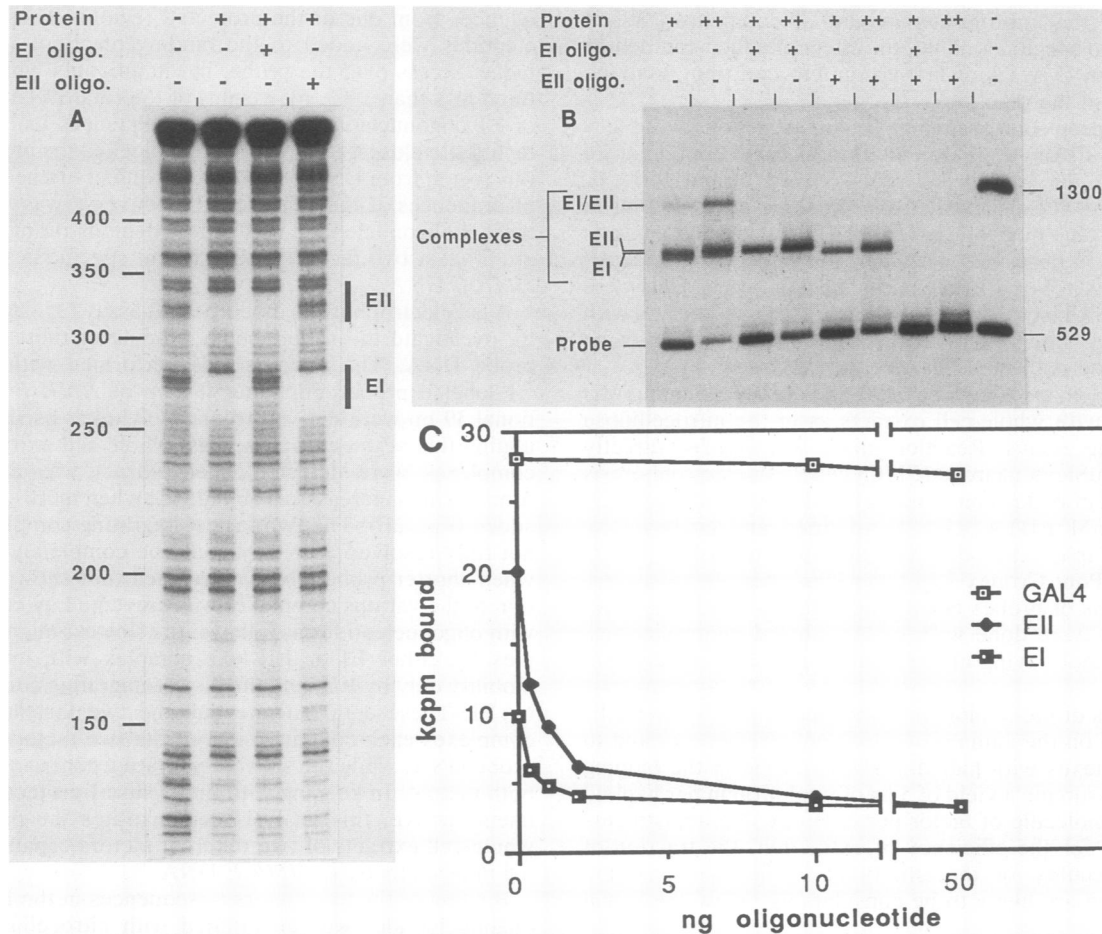
FIG. 2. Two factors bind to *HMR E* DNA. (A) DNase I protection mapping of *HMR E*-binding factors was performed with 15 μg of nuclear extract and a probe containing 490 bp of *HMR E*. The probe was an *Hind*III-*Bam*HI fragment of pJR315, ^{32}P-labeled at a *Bam*HI site next to the original *Xba*I site of *HMR E* DNA (see Fig. 1) in this plasmid. The products were analyzed by electrophoresis in a 4% polyacrylamide–urea gel. Coordinates on the left indicate nucleotide numbers according to the sequence of Abraham et al. (1). Regions of the probe protected by nuclear extract and blocked with the addition of oligonucleotide are shown with solid bars on the right. (B) Gel electrophoresis mobility shift assays were performed with 20 kcpm of a 529-bp *HMR E* probe (*Hind*III-*Bam*HI fragment of pJR315) and either 1.25 (+) or 2.5 μg (++) of an SF657-2d whole-cell extract. Products were analyzed by electrophoresis in a 3% NuSieve agarose gel. The far right lane contains the probe DNA and a 1,300-bp marker fragment, showing the range of sizes separated on this gel. Labels on the left point out complexes blocked by the indicated oligonucleotides. (C) Nitrocellulose filter-binding assays were carried out with 60 kcpm of the same *HMR E* probe as in panel B, 1 μg of poly[d(I-C)], and 2.5 μg of SF657-2d whole-cell extract. Assays contained the indicated amounts of each oligonucleotide, except that titrations of EI oligonucleotide were done in the presence of 50 ng of EII oligonucleotide and titrations of EII oligonucleotide were done in the presence of 50 ng of EI oligonucleotide. Measurements were performed in triplicate, and the averages are plotted without subtracting background.

Based on further findings of binding sites for these activities, described below, the two factors were named ABFI (*ARS*-binding factor I) and GRFI (general regulatory factor I) and operationally defined by specific competition for binding with the EI and EII oligonucleotides, respectively.

ABFI and GRFI are abundant DNA-binding factors not encoded by the *SIR* genes. The possibility that ABFI or GRFI might be encoded by one of the *SIR* genes was investigated by examining extracts from yeast strains carrying wild-type or mutant alleles of the four *SIR* genes (Table 2). Whole-cell extracts were prepared from each strain and assayed with the gel electrophoresis mobility shift and nitrocellulose filter-binding methods described above (data not shown). All mutant strains produced the same amount of ABFI (EI-competed binding) and GRFI (EII-competed binding) as wild-type strains (e.g., BJ926, SF657-2d, XR160-12b, and DBY703). Significant alterations in the charge or size of a

factor might have been detected as a shift in electrophoretic mobility of complexes, but no mobility differences were observed with extracts from *sir* strains. The relationship between ABFI, GRFI, and the *SIR* genes was also examined by analyzing extracts from strains constructed to overproduce each of the *SIR* gene products (Table 2). These strains contained high-copy yeast plasmids (50 to 100 per cell) carrying one of the *SIR* genes (39, 73). No change in the amount of ABFI or GRFI was found, nor were any new complexes detected in electrophoretic mobility shift assays. These results indicate that ABFI and GRFI are not encoded by the *SIR* genes. The very abundance of the factors suggests broader roles for ABFI and GRFI. Measurements of the amount of complex formed in filter-binding and electrophoretic mobility shift experiments, discussed above, gave minimum estimates (see Materials and Methods) of 500 molecules of ABFI and 1,000 molecules of GRFI per haploid

TABLE 2. Yeast strains used in this study

Strain[a]	Relevant genotype	Source
SF657-2d	*SIR*	C. Fields
BJ926	*SIR/SIR*	E. Jones
XR160-12b	*SIR*	Rine and Herskowitz (66)
JRY1030	*sir1-21*	Rine and Herskowitz (66)
JRY1059	*sir1-47*	Rine and Herskowitz (66)
JRY1013	*sir2-540*	Rine and Herskowitz (66)
JRY1022	*sir2-45*	Rine and Herskowitz (66)
JRY1027	*sir2-30*	Rine and Herskowitz (66)
JRY1044	*sir2-37*	Rine and Herskowitz (66)
JRY1020	*sir3-49*	Rine and Herskowitz (66)
JRY1028	*sir3-32*	Rine and Herskowitz (66)
JRY1056	*sir3-52*	Rine and Herskowitz (66)
JRY1060	*sir3-31*	Rine and Herskowitz (66)
JRY1032	*sir4-37*	Rine and Herskowitz (66)
JRY1045	*sir4-33*	Rine and Herskowitz (66)
JRY50	*sir4-9*	Rine and Herskowitz (66)
JRY51	*sir4-351*	Rine and Herskowitz (66)
DBY703	*SIR cir*[0]	D. Botstein
YWK55	*sir3::LYS2 cir*[0]	W. Kimmerly
YRS477	*sir4::HIS3 cir*[0]	R. Schnell
SF657-2d(pJDB-*SIR1*)	*SIR*	This work
SF657-2d(pJDB-*SIR2*)	*SIR*	This work
SF657-2d(pJDB-*SIR3*)	*SIR*	This work
SF657-2d(pG2-*SIR4*)	*SIR*	This work

[a] JRY1013 through JRY1060 are derivatives of XR160-12b (66). YWK55 and YRS477 are derivatives of DBY703 (45).

cell, more than might be expected to act solely at the silent mating type loci.

GRFI binds sequences at the core of both silencers at *HML* and *HMR* and at the UAS for the *MATα* genes. Additional functions for ABFI and GRFI were investigated by searching for other binding sites for each factor, both within and outside the mating type genes, with the following strategy. First, restriction fragments from loci of interest were screened in filter-binding assays for specific competition of binding with EI and EII oligonucleotides. Sites recognized by the factors were precisely located by DNase I protection mapping, and the sequences protected by each factor were compared for homology. Finally, hypotheses about the recognition sequences for these factors were tested by using synthetic oligonucleotides in filter-binding and gel electrophoresis mobility shift assays.

To search for binding sites for GRFI, sequences of the mating type loci were subdivided into restriction fragments (Fig. 1), which were tested as probes in nitrocellulose filter-binding assays for specific competition with EII oligonucleotide. In some cases unlabeled restriction fragments were also tested as competitors for GRFI binding to an *HMR E* DNA probe. Besides *HMR E*, two other segments contained high-affinity GRFI-binding sites (greater than 30% competition with EII oligonucleotide)—the *E* region at *HML* and the promoter region for the mating type genes at *HMLα* and *MATα*. No significant GRFI binding was detected at *MAT*a or the *I* regions. Binding sites at *HML E* were localized by DNase I protection mapping (Fig. 3A) by using an *Xho*I-*Cla*I probe (Fig. 1) ^{32}P-labeled at the *Cla*I site. On addition of nuclear extract, a region of the probe was protected from nuclease digestion corresponding to nucleotides 80 to 100 in the numbering of Feldman et al. (24). Addition of excess EII oligonucleotide blocked the protection of this region, whereas an excess of EI oligonucleotide

had no effect, demonstrating specificity for GRFI. Previous sequence comparisons of *HML E* and *HMR E* revealed several small regions of homology, the most extensive being the sequence 5'-CAAAAACCCAT-3' (24), which overlaps the regions protected by GRFI at *HMR E* and *HML E*.

The binding site for GRFI in the α1/α2 promoter was mapped in a similar manner, using an *Xba*I-*Nde*I probe ^{32}P-labeled at the *Nde*I site. Nuclear extract protected a region from digestion covering nucleotides 1606 to 1628 in
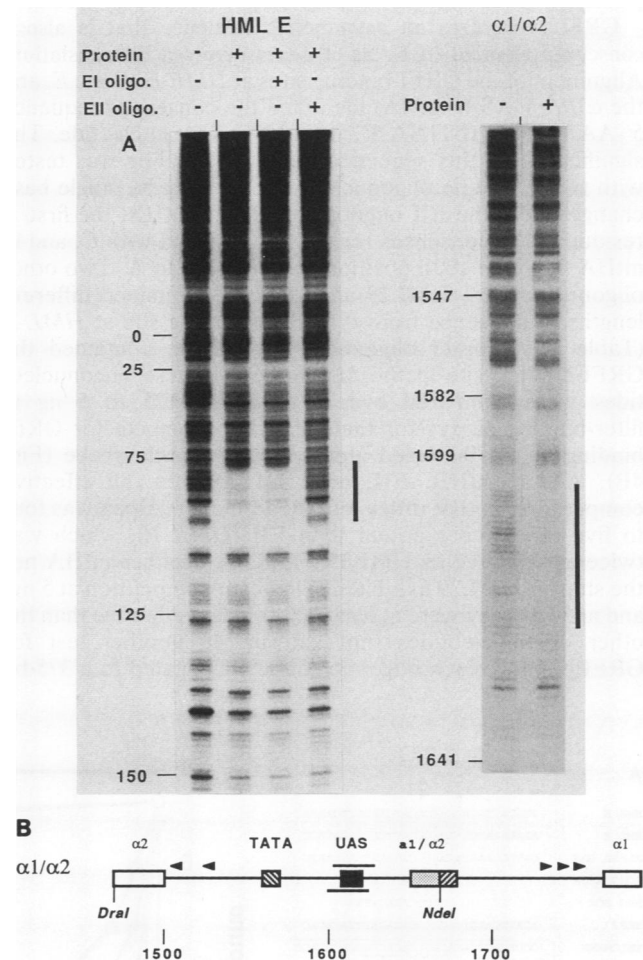

FIG. 3. DNase I protection mapping of GRFI-binding sites at *HML E* and the α1/α2 promoter region. (A) Analysis of the *HML E* region was performed with 60 μg of nuclear extract and a 1.3-kbp *Xho*I-*Cla*I probe, ^{32}P-labeled at the *Cla*I site (Fig. 1). Coordinates on the left indicate nucleotide numbers according to the sequence of Feldman et al. (24). Analysis of the α1/α2 promoter region was carried out with 15 μg of nuclear extract and a 630-bp *Xba*I-*Nde*I probe, ^{32}P-labeled at the *Nde*I site (Fig. 1). Coordinates on the left indicate nucleotide numbers as in the sequence of Siliciano and Tatchell (75). Electrophoresis was in an 8% polyacrylamide–urea gel. (B) Landmarks of the α1/α2 promoter region are depicted. The coding regions for the genes are represented by open boxes, and sites of transcription initiation are indicated with arrows. The bidirectional UAS element is shown as a black box. The hatched rectangles represent TATA boxes, and the stippled region denotes sequences responsible for a1/α2-mediated repression in diploids. The coordinates below refer to nucleotide numbers of Siliciano and Tatchell (75). The lines above and below the UAS show the sequences on each strand protected from DNase I digestion by GRFI. Data for the top strand are presented in panel A; data for the bottom strand are not shown.

the published numbering scheme (75). Specific competition of binding to this sequence by EII oligonucleotide is demonstrated below in filter-binding assays. Siliciano and Tatchell (75) defined three elements governing αl and α2 transcription from an extensive deletion analysis of the promoter region (Fig. 3B), TATA box sequences that position the site of transcription initiation, a regulatory region responsible for al/α2-dependent repression in diploids, and a centrally located upstream activating sequence (UAS) required for both αl and α2 transcription. The UAS was completely contained within the sequences protected by GRFI.

GRFI recognizes an asymmetric sequence that is also a conserved element in UASs of genes involved in translation. Alignment of the GRFI-binding sites at *HMR E*, *HML E*, and the αl/α2 UAS (Fig. 4A) identified the consensus sequence 5'-AAAACCCANNNA-3', where N is any nucleotide. The significance of this sequence for GRFI binding was tested with two synthetic oligonucleotides differing by single base changes from the EII oligonucleotide. In mtIIB, the first A residue of the consensus region was replaced with C, and in mtIIA, C in the sixth position was changed to A. Two other oligonucleotides, HML29 and HML35, contained different lengths of sequence from the GRFI-binding site at *HML E* (Table 1). Another oligonucleotide, alpha, contained the GRFI-binding site at the *MATα* UAS. These oligonucleotides were compared over a range of 0.25 to 5 ng in filter-binding assays for their ability to compete for GRFI binding to a ^{32}P-labeled alpha oligonucleotide probe (Fig. 4B). Alpha, mtIIB, EII, and HML35 were all effective competitors, in that order, of GRFI binding. Alpha was four to five times more potent than EII (*HMR E*), which was twice as effective as HML35 (*HML E*). Neither mtIIA nor the shorter HML29 exhibited significant competition at 5 ng, and at 50 ng they were at least 200-fold less effective than the other oligonucleotides (not shown). In another test for GRFI binding, each oligonucleotide was ligated to a 375-bp

*Hind*III-*Bam*HI fragment of pBR322 and used as a probe in gel electrophoresis mobility shift assays (Fig. 5A and B). The results paralleled those of the filter-binding study, with an exception in the case of mtIIB (see below). On addition of extract, a single complex of identical mobility was formed with these probes. Complex formation increased with more protein and could be abolished with excess oligonucleotide. Based on the amount of complex produced, the relative affinities of these sites are alpha > EII > HML35 > mtIIB. No complex was formed with the mtIIA and HML29 probes, in keeping with the filter-binding results. Surprisingly, the mtIIB probe formed 20-fold less complex than the EII probe (right panel, Fig. 5A), even though it was a better competitor in the filter-binding assay. It seems that some structural difference in these two situations affects the ability of GRFI to bind the mtIIB site. Perhaps the oligonucleotide can assume a conformation that promotes high-affinity binding, which the larger DNA probe cannot form due to the adjacent pBR322 sequences.

The occurrence of a binding site for GRFI at the αl/α2 UAS suggested that GRFI might generally be involved in stimulating transcription. While considering this possibility, we noticed a strong similarity between the *MATα*-binding site and a putative UAS element conserved at genes involved in translation. Computer-assisted comparisons of sequences upstream of ribosomal protein genes had previously uncovered the conserved sequence 5'-ACCCATA CAT(C/T)(A/T)-3' (52), which deletion studies have implicated as a UAS element (69, 89). Others have found an abundant DNA-binding factor that recognizes this sequence at the *RP51A*, *TEF1*, and *TEF2* genes (37). It was thought that this element might specifically regulate genes involved in translation. Accordingly, the conserved sequence was named the RPG box (ribosomal protein gene box) and the binding factor was called TUF (translation upstream factor). We noticed, however, that the consensus sequence for GRFI
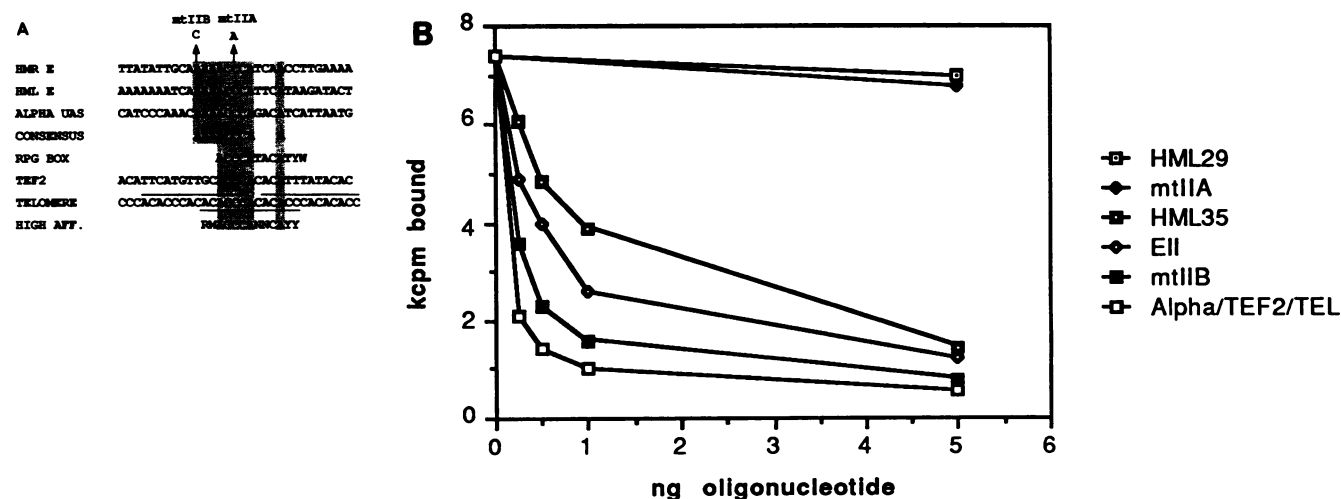

FIG. 4. Comparison of GRFI-binding sites. (A) Nucleotide sequences at GRFI-binding sites were aligned for optimal homology (18, 62) and are listed, 5' to 3', from left to right. Identical nucleotides are shaded. The alterations in the mtIIA and mtIIB oligonucleotides are shown above the *HMR E* sequence. A candidate consensus sequence for high-affinity (high aff.) recognition is shown at the bottom. Overlapping occurrences of the high-affinity consensus in the telomere sequence are indicated with lines. The following nucleotide codes are used: R, A or G; Y, C or T; M, A or C; W, A or T. (B) Nitrocellulose filter-binding assays were performed with 2.5 μg of SF657-2d whole-cell extract, 20 kcpm of ^{32}P-labeled alpha oligonucleotide, 1 μg of poly[d(I-C)], and various amounts of added competitor oligonucleotides. Data are presented as in Fig. 2C. Symbols used for the different oligonucleotides are shown on the right, with the oligonucleotides being listed, top to bottom, in order of increasing ability to compete binding. The competition data for the alpha, TEF2, and TEL oligonucleotides were not significantly different, so only the values for the alpha oligonucleotide were plotted.
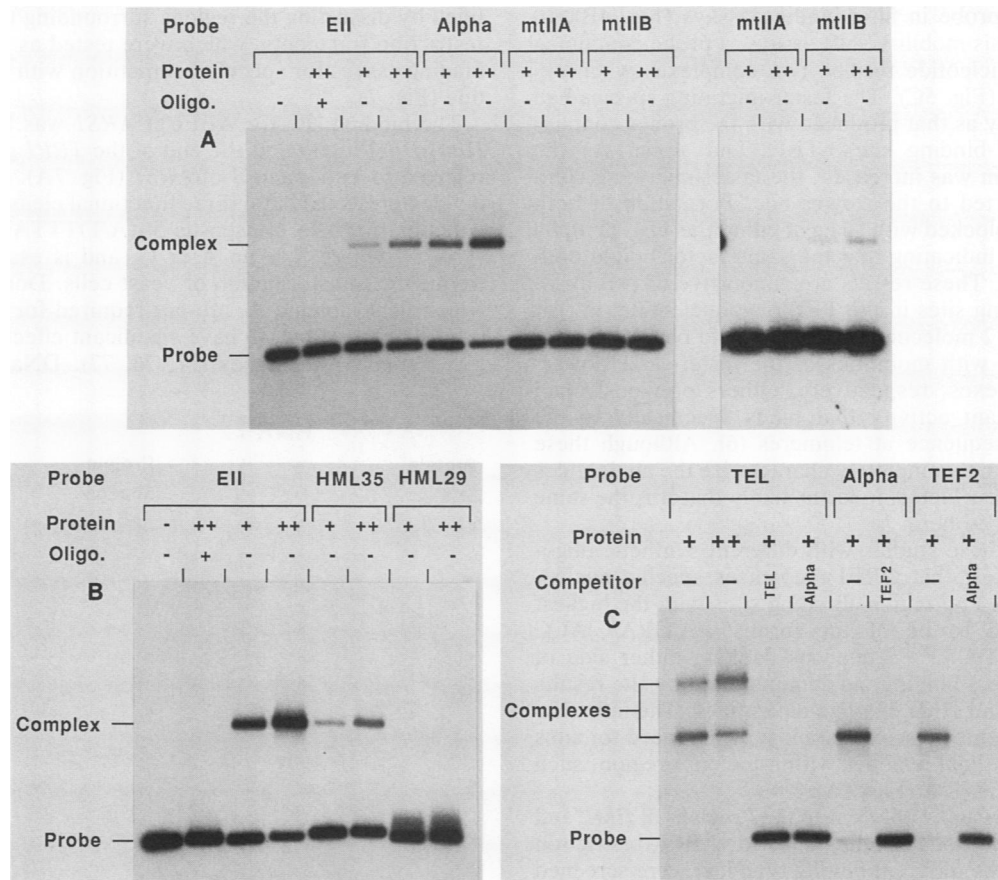
FIG. 5. Complexes of GRFI with different binding sites. (A) Each binding reaction mix contained either 0.625 (+) or 1.25 µg (++) of whole-cell extract (SF657-2d) and 20 kcpm of 0.4-kbp probes derived from the oligonucleotides indicated at the top. In one experiment, 50 ng of competitor EII oligonucleotide was added. Reactions were analyzed by electrophoresis in a 3% NuSieve agarose gel. The right panel is a longer exposure of the lanes with the mtIIA and mtIIB probes. (B) Gel electrophoresis mobility shift assays were carried out as in panel A, except that either 1.25 (+) or 2.5 µg (++) of whole-cell extract was used with the EII, HML35, and HML29 probes. (C) Gel electrophoresis mobility shift assays were performed as in panel A with the TEF2, alpha, and TEL probes and 2.5 µg (+) or 5 µg (++) of whole-cell extract. Some reaction mixes contained 10 ng of the indicated competitor oligonucleotide.

binding had overlapping homology with the RPG box (Fig. 4A). Indeed, the binding site for GRFI at the *MAT*α UAS differed at only one position from the RPG box.

To determine whether TUF and GRFI might be the same activity, an oligonucleotide was synthesized (Table 1 and Fig. 4A) containing sequences from the highest-affinity TUF-binding site, located upstream of the *TEF2* gene (37). This oligonucleotide was tested both as competitor and as probe in the same manner as the GRFI-binding oligonucleotides described above. In filter-binding assays, the TEF2 oligonucleotide was as effective as the alpha oligonucleotide at competing for GRFI binding (Fig. 4B). When examined in gel electrophoresis mobility shift assays, a complex of identical mobility was observed with the alpha and TEF2 probes (Fig. 5C). Addition of 10 ng of alpha oligonucleotide blocked complex formation with the TEF2 probe. Conversely, 10 ng of TEF2 oligonucleotide abolished the complex of GRFI with the alpha probe, indicating that the same DNA-binding factor recognized both sequences.

GRFI-binding sites are present within repeated sequences at yeast telomeres. Comparison of the binding sites for GRFI at the mating type loci with those at RPG boxes (37, 52) suggested the sequence 5'-(A/G)(A/C)ACCCANNCA(T/C)(T/C)-3' as a candidate for high-affinity recognition. One

form of this sequence, 5'-ACACCCACACACC-3', was particularly interesting because its unusual composition was reminiscent of sequences repeated at the ends of yeast chromosomes. Yeast telomeres are composed of an irregular repeat of poly(C_{1-3}A), extending for several hundred base pairs at each chromosome end (14, 72, 88). Shorter stretches of poly(C_{1-3}A) (25 to 150 bp) are also present between other telomere-associated sequences, Y' and X (88). Examination of published yeast telomere sequences (14, 21, 72, 88) revealed that each of them contained two to four matches to the high-affinity GRFI consensus sequence, 5'-ACACCCA CACACC-3'. We investigated the possibility that GRFI might bind the (C_{1-3}A) repeat at telomeres by studying the properties of a synthetic oligonucleotide, TEL (Table 1 and Fig. 4A), whose sequence was derived from a terminal repeat (72). This sequence was of special interest because it contained three overlapping versions of the high-affinity consensus sequence (indicated with lines in Fig. 4A). If the notion of GRFI sequence recognition was correct, the TEL oligonucleotide should bind 1 or 2 molecules of GRFI but not 3, since the third site would be occluded by occupancy of the other two.

The TEL oligonucleotide was as effective as the alpha oligonucleotide at competing for GRFI binding to an alpha

oligonucleotide probe in filter-binding assays (Fig. 4B). In gel electrophoresis mobility shift assays, a probe containing the TEL oligonucleotide formed two complexes when extract was added (Fig. 5C). The faster-migrating species had the same mobility as that produced with the probes containing other GRFI-binding sites, TEF2 and alpha. As the amount of protein was increased, the faster-migrating complex was converted to the slower one. Formation of both complexes was blocked with 10 ng of either the TEL or alpha oligonucleotide, indicating that the same factor bound both oligonucleotides. These results are supportive of two available GRFI-binding sites in the TEL oligonucleotide, in that binding of 1 and 2 molecules of GRFI would be expected to produce species with mobilities of the faster- and slower-migrating complexes, respectively. Others previously had found an abundant activity that binds specifically to the $(C_{1-3}A)$ repeat sequence at telomeres (6). Although these investigators did not completely characterize the nucleotides recognized by their factor, it seems likely that it is the same as GRFI, described here.

In summary, these studies with different synthetic oligonucleotides suggest that GRFI recognizes specific nucleotides in a 10- to 13-bp region. Binding sites with the highest affinity appeared to be of the form 5'-(A/G)(A/C)ACC CANNCA(T/C)(T/C)-3'. Sequences flanking either side of this core can affect binding, as demonstrated by the results with the mtIIB and HML29 oligonucleotides. The influence of the flanking sequences seemed most pronounced for sites containing suboptimal residues within the core region, such as HMR E and HML E (Fig. 4A).

ABFI binds sequences adjacent to the *I* regions at *HML* and *HMR*. To find additional binding sites for ABFI, restriction fragments from the different mating type loci were screened in filter-binding assays as above, but in this case specific binding was revealed by competition with the EI oligonucleotide (Fig. 1). In contrast to GRFI, no significant binding of ABFI was detected at HML E or MATα, nor was binding detected at MATa DNA. However, besides HMR E, the *I* regions of both HML and HMR contained ABFI-binding sites. The nucleotides recognized by the factor at the *I* elements were mapped more precisely by DNase I protection. The probe for the HMR *I* region contained the BclI-HindIII segment, ^{32}P-labeled at the BclI end (Fig. 1). Nuclear extract protected a region of the probe (Fig. 6) covering nucleotides 182 to 202 (1). Protection was blocked by EI but not by EII oligonucleotide, demonstrating ABFI specificity. For HML *I* DNA, the probe was a PvuII-HindIII fragment, ^{32}P-labeled at the PvuII site (Fig. 1). Two separate regions of the probe were spared from digestion when nuclear extract was added (Fig. 6). Protection of nucleotides 58 to 80 (24), the region closest to the labeled end, was abolished by EI but not by EII oligonucleotide, indicative of ABFI binding. Neither oligonucleotide affected the more distal protected region, suggesting that a factor other than ABFI or GRFI recognizes this sequence.

ABFI-binding sites occur next to a subset of yeast *ARS*s. The binding sites for ABFI are located close to *ARS*s at HMR E, HMR I, and HML I (1, 13, 24). The possibility that ABFI-binding sites might be more generally associated with *ARS*s was investigated by screening plasmid DNAs containing these elements for the ability to compete for ABFI binding to an HMR E DNA probe in filter-binding assays (see Materials and Methods). DNAs with ARS1, ARS2, and the 2μm plasmid ARS all diminished binding to HMR E DNA and also competed for binding to *I* region probes (not shown). Segments containing ABFI-binding sites were further iden-

tified by dissecting the regions surrounding these *ARS*s into restriction fragments, which were tested as probes in filter-binding assays for specific competition with EI oligonucleotide (Fig. 7).

The binding site for ABFI at *ARS1* was localized to the HinfI-DdeI interval at the end of the *TRP1* gene in a region referred to as domain B of *ARS1* (Fig. 7A). *ARS1* had been divided previously into three functional elements. Domain A contains the *ARS* consensus 5'-(A/T)TTTATRTTT(A/T)-3' (17, 78), where R is an A or G, and is essential for high-frequency transformation of yeast cells. Domains B and C, which flank domain A, are not required for high-frequency transformation, but do have significant effects on the stability of minichromosomes (17, 76, 77). DNase I protection
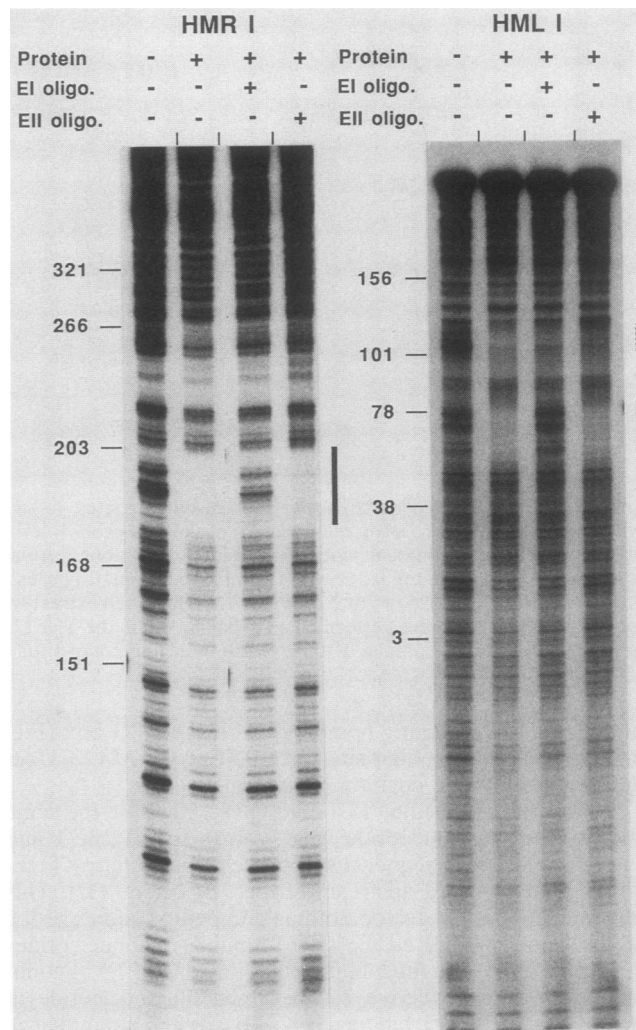

FIG. 6. DNase I protection mapping of ABFI-binding sites at HMR I and HML I. Analysis of the HMR I region was carried out with 30 μg of nuclear extract and a 740-bp EcoRI-HindIII fragment from the plasmid pEMBL/HMRI, ^{32}P-labeled at the EcoRI site. This EcoRI site lies next to the original BclI site in HMR I DNA (see Fig. 1 and Materials and Methods). Coordinates on the left are nucleotide numbers according to Abraham et al. (1). Analysis of the HML I region was performed with 30 μg of nuclear extract and a 0.4-kbp PvuII-HindIII fragment (see Fig. 1), ^{32}P-labeled at the PvuII site. Coordinates on the left indicate nucleotide numbers according to Feldman et al. (24). The striped bar on the right marks a region of protection that was not affected by either oligonucleotide. Products were analyzed by electrophoresis in 8% polyacrylamide–urea gels.

analysis of *ARS1* was performed with an *Hin*dIII-*Bgl*II probe, ^{32}P-labeled at the *Bgl*II site (Fig. 7A). Addition of nuclear extract protected nucleotides 752 to 775 (86) from digestion (Fig. 8), and protection was prevented with EI but not EII oligonucleotide, demonstrating specificity for ABFI. Partial protection of sequences around nucleotide 700 was also observed to be blocked by EI oligonucleotide. This may be caused by weak binding by ABFI, by other factors that interact with ABFI, or by the intrinsic bending of DNA in this region (76). The sequences flanking *ARS2* contain two kinds of repeated yeast elements, a tRNA gene and solo δ sequences (87). Analysis of the *ARS2* region in filter-binding assays indicated that neither the tRNA gene nor δ sequences contained high-affinity ABFI-binding sites (Fig. 7B). Rather, the binding site(s) for the factor lay in the *Xho*I-*Eco*RI interval beyond the divergent δ element and over 150 bp away from the *ARS* consensus sequence. As the nucleotide sequence of this region has not been determined, the *ARS2*-associated ABFI site was not pursued further.

The region of the 2μm plasmid that specifically competed for ABFI binding has many interesting features. The 2μm circle *ARS*, required for plasmid propagation (13), is located in the *Xmn*I-*Xba*I region (Fig. 7C) adjacent to a large inverted repeat sequence involved in plasmid rearrangement. On the other side of the *ARS* is a region important for efficient segregation of the plasmid during mitosis (40, 41, 44). At the core of this stability region, called *REP3*, is a 63-bp sequence repeated 5.5 times (*Hpa*I-*Ava*I interval). For optimal plasmid segregation, *REP3* must be present in *cis*, and two plasmid-encoded proteins, *REP1* and *REP2*, must also be supplied (40, 44). Beyond the *REP3* region lies a segment of DNA (*Pvu*I-*Hpa*I) that is transcribed bidirectionally. A 1,950-base RNA emanates from the edge of *REP3*, while the *D* reading frame mRNA terminates in this region. As yet, no specific functions have been assigned to either transcript (41, 84). The *Pvu*I-*Xba*I segment of 2μm plasmid DNA was divided into smaller restriction fragments which were tested as probes for ABFI binding in nitrocellulose filter-binding assays (Fig. 7C). No binding was detected at the *ARS* and inverted repeat sequences. Instead, the ABFI binding site was located near the end of the *REP3* repeats, overlapping an *Hpa*I site. This location was confirmed by DNase I protection mapping with an *Ava*I-*Pvu*I probe, ^{32}P-labeled at the *Ava*I site (Fig. 8). Nuclear extract altered the pattern of protection of a large region of the probe, nucleotides 2872 to 2975 (30), which extends from the edge of the *REP3* repeats to the end of the *D* mRNA. However, addition of EI oligonucleotide only abolished the protection of nucleotides 2953 to 2975, indicating ABFI binding at this sequence. Presumably, factors other than GRFI or ABFI bind to the rest of the protected region, as addition of EII oligonucleotide had no effect. Some *cir*^{0} derivatives are among the strains listed in Table 2 that were assayed for ABFI and GRFI. All these strains produced normal levels of each factor, demonstrating that neither is encoded by the 2μm plasmid.

Binding sites for ABFI are not limited to *ARS*s. From the results presented so far, it is tempting to speculate that the function of ABFI is related to DNA replication. Indeed, the ABFI-binding sites at *HMR E*, *HMR I*, *HML I*, and *ARS1* were less than 100 bp away from core *ARS* elements, thought to be origins of cellular DNA replication, and the binding site at *ARS1* coincided with domain B, which augments *ARS* function in vivo (76). Other observations, however, argue against an obligatory relationship between *ARS*s and ABFI-binding sites. Some fully functional yeast

*ARS*s appear to lack ABFI-binding sites, including the histone H2B- (*TRT3* [32, 36]) and H4- (7, 36) associated *ARS*s (not shown), as well as the *ARS* at *HML E* (Fig. 1). Furthermore, the ABFI-binding sites at *ARS2* and in the 2μm plasmid appear to be linked loosely, if at all, to the sequences involved in *ARS* function (e.g., the ABFI-binding site in the 2μm plasmid was more than 700 bp away from the ARS consensus sequence; Fig. 7C). It seemed possible that ABFI might provide a function not limited to DNA replication. Accordingly, other segments of DNA containing a variety of yeast genes were screened for ABFI-binding sites by using them as probes and competitors in filter-binding assays. DNAs with the following yeast genes exhibited no significant ABFI binding: *GAL4* (51), *GAL80* (85), *GAL1*, *GAL10*, *GAL7* (79), *URA3* (68), *LEU2* (64), and *LYS2* (3) (not shown). However, a segment from the vicinity of the *HIS3* and *DED1* genes (82) showed exceptionally high levels of ABFI binding. This region was examined further (Fig. 7D), and the sequences recognized by ABFI were localized to the *Pst*I-*Hpa*II interval between the *HIS3* and *DED1* genes. DNase I protection mapping was performed with a *Bgl*II-*Xho*I probe, ^{32}P-labeled at the *Xho*I site. Nucleotides 799 to 835 (82) were protected from nuclease digestion after addition of nuclear extract (Fig. 8), and all of the observed protection was blocked by EI but not EII oligonucleotide, indicative of ABFI binding. This region of DNase I protection was larger than other ABFI-binding sites (usually 20 to 25 bases). Inspection of the DNA sequence within the protected region suggested a plausible explanation, a tandem duplication of the sequence 5'-TCATTCTNNACGNG-3'. Indeed, gel electrophoresis mobility shift assays gave results consistent with 2 molecules of ABFI binding to this region (not shown). These binding sites for ABFI lay just upstream of a poly(dT) · poly(dA)-rich region, implicated as the UAS for the *DED1* gene (81). No *ARS*s are located within several kilobases of the *HIS3* and *DED1* genes (83).

ABFI recognizes a dyad-symmetric sequence. Alignment of the nucleotides at seven ABFI-binding sites revealed a clear consensus sequence, which was also consistent with the notion of two tandem ABFI sites upstream of the *DED1* gene (Fig. 9A). The consensus sequence was composed of two segments of similarity separated by a variable region, 5'-TATCATTNNNN<u>ACGA</u>-3', each half of which contained nucleotides that were identical at all the sites (underlined). Furthermore, when reduced to the form 5'-TCRT<u>NNNN</u>NA<u>Y</u>GA-3', the two halves of the consensus sequence were related by an axis of dyad symmetry (underlined) which fit the helical geometry of DNA (i.e., the half-sites were separated by about one turn of the DNA helix). These suppositions regarding the features recognized by ABFI were tested with synthetic oligonucleotides, two of which differed by only a single base change from the EI oligonucleotide. In the mtIA oligonucleotide, a stretch of four A residues within the central variable region of the consensus sequence was increased to five A residues. Thus, each half-site has been retained but the geometry relating them has been altered. In the oligonucleotide mtIB, the invariant C residue in the left half of the consensus sequence was changed to A. Also, a dyad-symmetric oligonucleotide, Sym, was produced whose sequence was based on the predominant residues of a hypothetical half-site (Fig. 9A and Table 1). These oligonucleotides, along with an oligonucleotide containing the *DED1*-binding sites (Table 1), were tested at 0.5 to 10 ng in filter-binding assays for their ability to compete for ABFI binding to an *HMR E* DNA probe (Fig. 9B). The DED1 and Sym oligonucleotides appeared to be equally effective com-
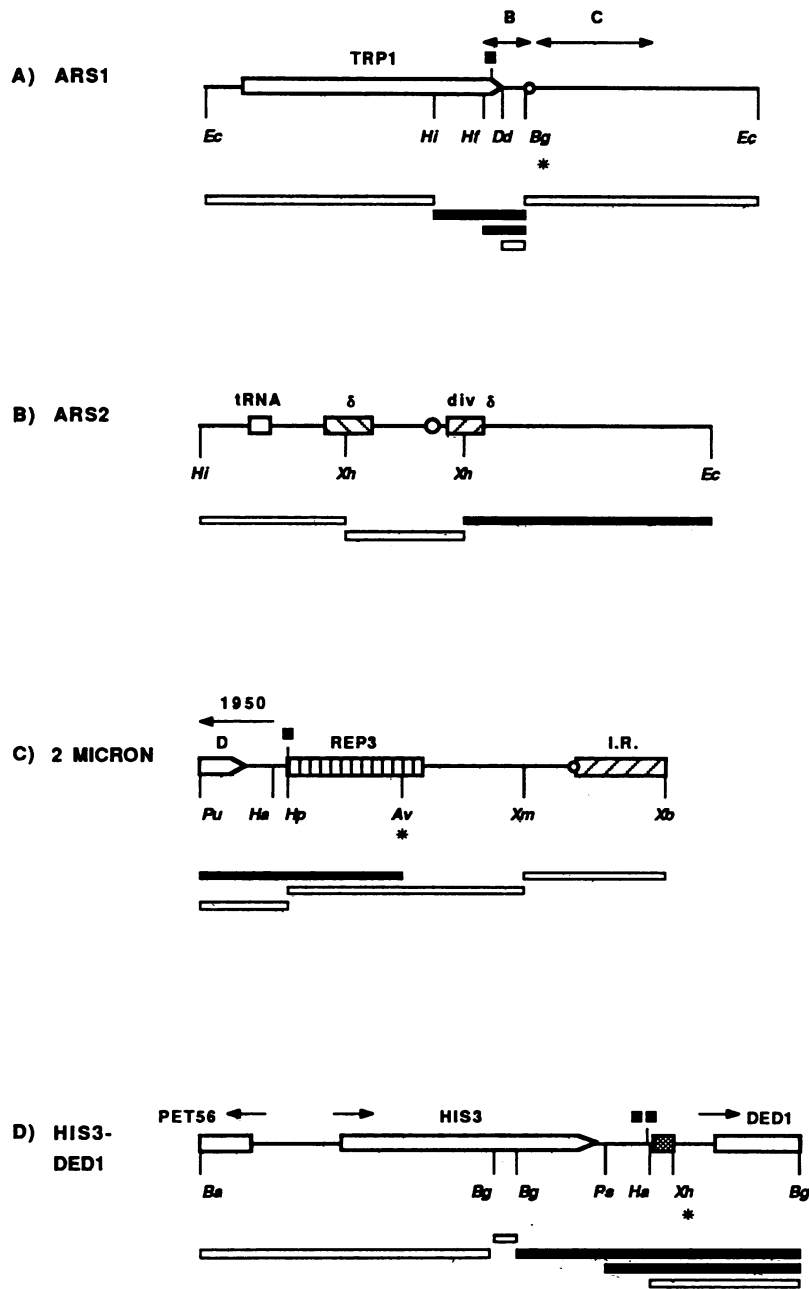
FIG. 7. Landmarks at *ARS1*, *ARS2*, 2μm plasmid *ARS*, and *HIS3-DED1* regions, with summary of filter-binding results. Restriction enzyme cleavage sites for the separate regions are abbreviated as in Fig. 1 with the addition of the following: Hf, *Hin*fI; Dd, *Dde*I; Pu, *Pvu*I; Ha, *Hpa*II; Av, *Ava*I; Ba, *Bam*HI; Ps, *Pst*I. Bars below the maps symbolize the filter-binding results with the corresponding restriction fragments. Black bars indicate fragments which exhibited greater than 50% competition of binding with added EI oligonucleotide (10 to 50 ng). White bars denote fragments showing no significant competition. Restriction sites used for DNase I mapping are marked with asterisks, and the actual positions of ABFI-binding sites are indicated by solid squares above the maps. (A) A 1.45-kbp *Eco*RI-*Eco*RI segment surrounding *ARS1* (17, 78, 86). The *TRP1* coding region is shown as the unshaded polygon. Domains B and C of *ARS1* are indicated above the map, and the *ARS* consensus sequence position (domain A) is denoted by the open circle. (B) A 2.3-kbp *Hin*dIII-*Eco*RI segment of *ARS2* (87). The tRNA^{Gln} gene is shown as the unshaded box. The δ and divergent δ sequences are indicated with hatched rectangles, and the position of the *ARS* consensus sequence is shown by the open circle. (C) A 1.2-kbp *Pvu*I-*Xba*I segment of 2μm plasmid DNA (30, 41, 84). The large inverted repeat area is shown by the hatched box, the *REP3* repeat region with a vertically striped rectangle, and the *D* reading frame with an unshaded polygon. The 1,950-base RNA is indicated with the arrow above the map, and the *ARS* consensus sequence is marked by an open circle. (D) A 1.67-kbp *Bam*HI-*Bgl*II segment of the *HIS3-DED1* region (82). The coding regions of the *PET56*, *HIS3*, and *DED1* genes are shown as unshaded boxes. Arrows above indicate transcription initiation sites. The stippled box represents the poly(dT) · poly(dA)-rich region implicated as the *DED1* UAS (81).
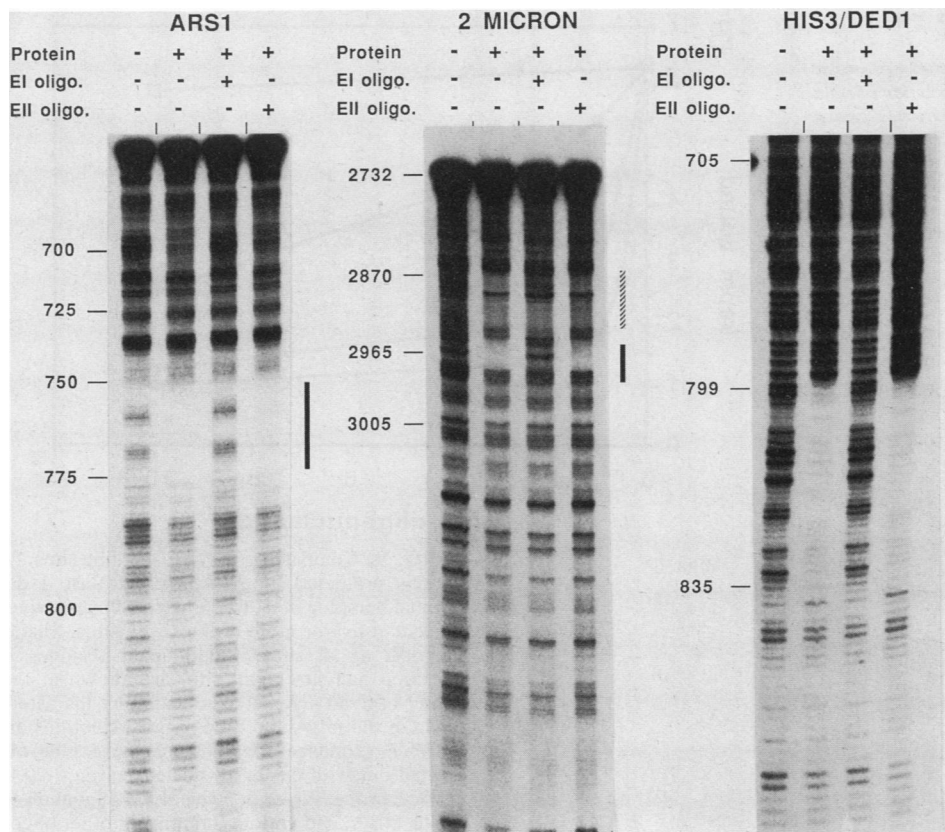
FIG. 8. DNase I protection mapping of ABFI-binding sites at *ARS1*, 2μm plasmid, and *HIS3-DED1* DNAs. Analysis of *ARS1* sequences was carried out with 30 μg of nuclear extract and a 237-bp *Bg*II-*Hin*dIII probe, ^{32}P-labeled at the *Bg*II site (Fig. 7A). Products were separated by electrophoresis on an 8% polyacrylamide–urea gel. Coordinates on the left refer to the nucleotide numbers according to Tschumper and Carbon (86). Analysis of the 2μm plasmid sequences was performed with 30 μg of nuclear extract and a 1.2-kbp *Pvu*I-*Ava*I probe, ^{32}P-labeled at the *Ava*I site (Fig. 7C). Electrophoresis was in a 4% polyacrylamide–urea gel. Coordinates on the left refer to the nucleotide numbers of the published sequence (30). Analysis of the *HIS3-DED1* region was carried out with 15 μg of nuclear extract and a 600-bp *Bg*II-*Xho*I probe, ^{32}P-labeled at the *Xho*I site (Fig. 7D). Products were separated by electrophoresis in an 8% polyacrylamide–urea gel. Coordinates on the left indicate nucleotide numbers according to the sequence of Struhl (82).

petitors and were about twice as potent as the EI oligonucleotide, which was four times as effective as the mtIB oligonucleotide. No significant competition was found with 10 ng of the mtIA oligonucleotide. Results with higher amounts of this variant (not shown) demonstrated that it was over 100-fold less effective than the EI oligonucleotide in competing for ABFI binding. Some of these oligonucleotides were also tested as probes in gel electrophoresis mobility shift assays. The probes were constructed by ligating the EI, Sym, mtIA, and mtIB oligonucleotides to a fragment of *ARS1* DNA which lacks its own ABFI site (see Materials and Methods). When extract was added to these probes, a single predominant complex was formed which increased with more protein and was abolished by competition with excess EI oligonucleotide (Fig. 9C). Based on the amount of complex formed, the relative affinities of these oligonucleotides were the same as determined in the filter-binding assays, Sym > EI > mtIB. No complex was detected with the mtIA oligonucleotide. Together, these results support the proposed sequence requirements for binding of ABFI.

DISCUSSION

We have characterized two yeast DNA-binding factors, ABFI and GRFI, detected originally by their specific binding to *HMR E* DNA. The *SIR* gene products are also thought to

interact with this region, but in contrast to the specialized nature of the *SIR* proteins, our results point to less restricted roles for ABFI and GRFI. Even the names used here, *ARS*-binding factor and general regulatory factor, are probably oversimplifications of their functions. Both factors are abundant, and sequences recognized by them appear to be common in the yeast genome. In an independent study of proteins that bind *E* region sequences, Shore et al. (74) found two DNA-binding factors, SBF-B and SBF-E, that appear to be the same as those reported here. Although these investigators searched for binding sites outside the *E* elements, only an additional site for SBF-B at *ARS1* was found. Other high-affinity binding sites were missed in their study, presumably due to limitations of the methods they employed. A key feature of our approach was developing the means to survey numerous loci for factor-binding sites, accomplished by performing nitrocellulose filter-binding assays with synthetic oligonucleotide competitors (9, 10). The analysis of a large number of binding sites detected in this way has had several important consequences. First, the nucleotides recognized by each factor have been characterized in some detail. We have the information needed, for example, to test precisely the functional significance of sequences recognized by these factors with point mutations that abolish binding. Second, the locations of the various binding sites have given
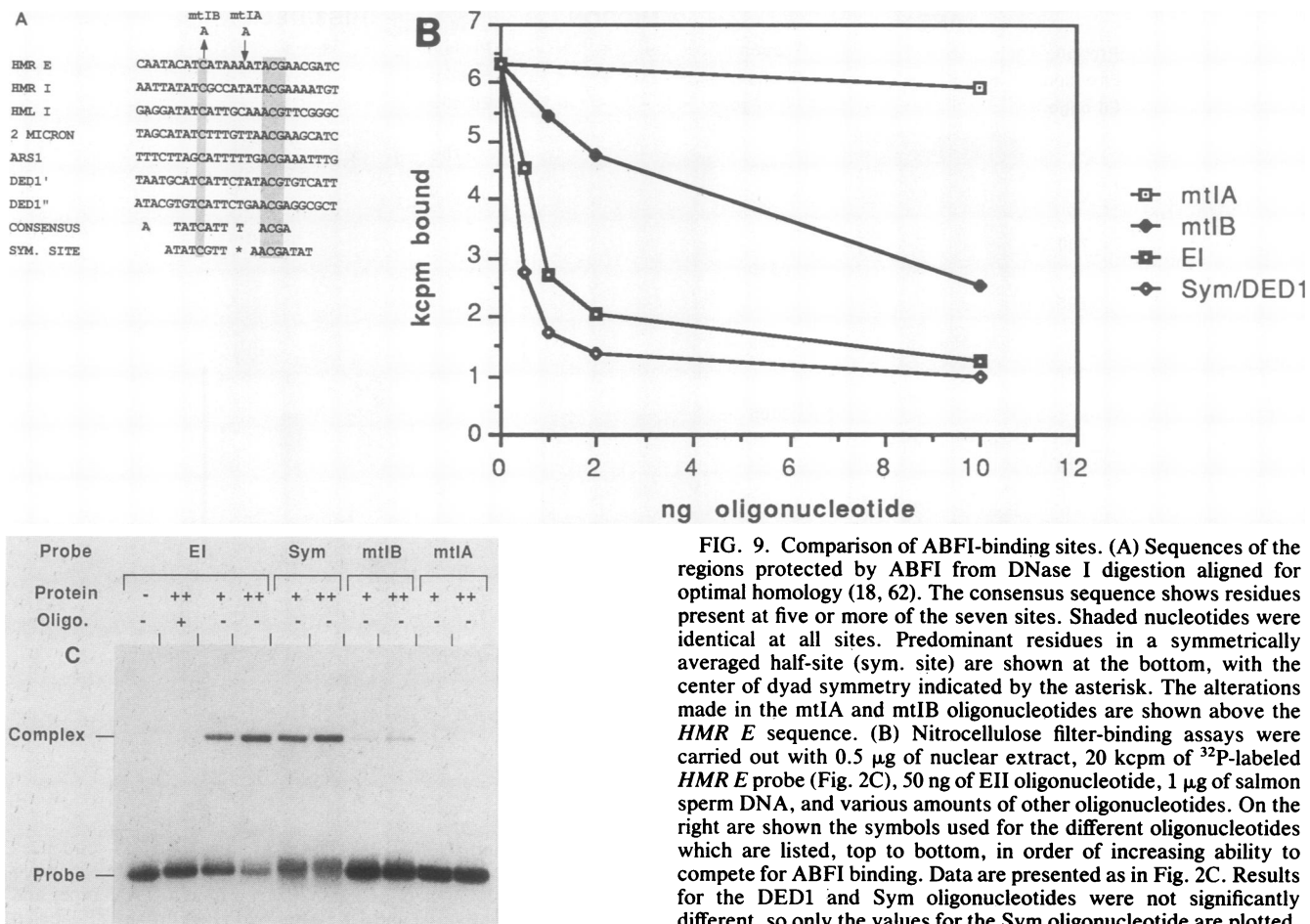
FIG. 9. Comparison of ABFI-binding sites. (A) Sequences of the regions protected by ABFI from DNase I digestion aligned for optimal homology (18, 62). The consensus sequence shows residues present at five or more of the seven sites. Shaded nucleotides were identical at all sites. Predominant residues in a symmetrically averaged half-site (sym. site) are shown at the bottom, with the center of dyad symmetry indicated by the asterisk. The alterations made in the mtIA and mtIB oligonucleotides are shown above the *HMR E* sequence. (B) Nitrocellulose filter-binding assays were carried out with 0.5 μg of nuclear extract, 20 kcpm of ^{32}P-labeled *HMR E* probe (Fig. 2C), 50 ng of EII oligonucleotide, 1 μg of salmon sperm DNA, and various amounts of other oligonucleotides. On the right are shown the symbols used for the different oligonucleotides which are listed, top to bottom, in order of increasing ability to compete for ABFI binding. Data are presented as in Fig. 2C. Results for the DED1 and Sym oligonucleotides were not significantly different, so only the values for the Sym oligonucleotide are plotted. (C) Each binding reaction mix contained either 1.25 (+) or 2.5 μg (+ +) of whole-cell extract (SF657-2d) and 20 kcpm of 100-bp probe DNAs derived from the oligonucleotides indicated at the top. In one reaction mix, 50 ng of competitor EI oligonucleotide was added. Reaction mixes were analyzed by electrophoresis in a 3% NuSieve agarose gel for 2 h.

insights into the roles these factors perform and revealed unexpected connections between diverse genetic elements. Finally, the relative affinities of the different binding sites have provided a physical basis for evaluating their importance. For instance, it is conceivable that some binding sites might be detected which are of such low affinity that they are of no functional significance. As all the binding sites that have been found had affinities within the same order of magnitude, each of them must be considered potential targets for binding in vivo. In the course of this survey, binding sites with affinities fivefold below those at *HMR E* could have been missed. The prime concern, however, was in finding binding sites with affinities equal to or greater than those at *HMR E*, and these were easily detected. Crude extracts were used in this study, so that additional factors might be found which recognize the sequences being tested. Factors other than ABFI and GRFI were detected that bound sequences of 2μm plasmid and *HML I* DNA. Also, in examining the *TEF2* and telomere sequences, it was particularly important to show that GRFI was the predominant cellular factor recognizing these regions. The data obtained with unfractionated extracts suggest that the ABFI and GRFI activities each reside in single proteins. Unique, well-defined complexes were observed in electrophoretic

mobility shift assays, and in filter-binding assays, all oligonucleotides containing binding sites for the same activity competed to the same extent at saturating levels. The possibility remains that each factor is a mixture of multiple proteins with similar physical properties and sequence specificities. This matter will be settled when the factors have been purified to homogeneity and the genes encoding them have been characterized.

ABFI-binding sites. There appear to be two general classes of ABFI-binding sites—those close to *ARS*s and those not at *ARS*s. The binding sites in 2μm plasmid and in the *HIS3-DED1* region could conceivably be involved in directing transcription of the 1,950-base RNA and the *DED1* mRNA, respectively (Fig. 7C and D). This function could also explain an accessory role for ABFI at *ARS*s and in DNA replication. Transcriptional activation has long been suggested from studies of DNA replication in procaryotes (20, 38). More recently, promoter elements of papovaviruses have been shown to assist viral DNA replication in vivo (19, 34, 53, 56). Another precedent comes from studies of a cellular DNA-binding factor in higher eucaryotes, nuclear factor I, which stimulates both adenovirus DNA replication (31, 60, 65) and transcription of the herpes simplex virus thymidine kinase gene (43). If ABFI is a transcription factor,

it may not be a particularly potent one. Deletion studies by Struhl (81) indicate that the most important elements for activating transcription of the *DED1* gene are in the poly(dT) · poly(dA)-rich sequence, not the ABFI-binding sites (Fig. 7D). Also, we have tested the ABFI oligonucleotides for UAS function in specially constructed plasmids (A. R. Buchman and R. D. Kornberg, unpublished). Although these oligonucleotides did stimulate transcription of test genes, the effect was much weaker than that of bona fide UAS elements, such as *GAL4*-binding sites (11, 54) and binding sites for GRFI, discussed below. Accordingly, other roles for ABFI should also be considered. ABFI might, in some general way, assist the binding of additional factors to adjacent regulatory sequences. Alternatively, ABFI-binding sites might serve as a boundary or insulator between adjacent regions of the yeast genome. It is noteworthy that binding sites for ABFI were situated at the interface of different regions—between the *HIS3* and *DED1* genes, between the *D* gene and *REP3* in 2μm plasmid, and between the *E* and *I* elements and the mating type genes.

GRFI-binding sites. In contrast to ABFI, the binding sites for GRFI were found at the center of various elements, suggesting that this factor contributes in significant ways to their function. What is remarkable is the diverse nature of these elements, which act as silencers, UASs, and telomeres. The nucleotides recognized by GRFI at the *MATα* promoter coincide with a sequence that is necessary and sufficient for UAS function (75), implying that GRFI has the inherent ability to act as a positive transcription factor. Support for this conclusion comes from the demonstration that GRFI also recognized a putative UAS element located at genes involved in translation. Indeed, we have found that oligonucleotides containing GRFI-binding sites exhibit potent UAS activity when examined in test plasmids (A. R. Buchman and R. D. Kornberg, unpublished). UAS function was dependent on the ability of the oligonucleotide to bind GRFI in vitro, but did not depend on whether the oligonucleotide sequence was derived from a UAS, a silencer, or a telomere. It would appear, then, that the silencer is a compound element composed, in part, of a sequence that can act in exactly the opposite manner. There is further evidence indicating that GRFI plays a role in silencer function. Point mutations in the binding site at *HMR E* decrease silencer activity and disrupt other *SIR*-mediated processes (W. J. Kimmerly and J. Rine, unpublished). It will be revealing to see what combination of elements—GRFI sites, *ARS*s, and others—are needed to confer negative regulatory properties to the silencer.

We are consequently led to the notion that GRFI participates in both activating and repressing the α1 and α2 genes, depending on whether these genes are at *MAT* or *HML*. In some ways, this idea is analogous to the regulation of the *Escherichia coli araBAD* operon, whose expression is modulated both positively and negatively by the AraC protein (58). Induction of the arabinose genes requires the binding of AraC protein to a site, *araI*, within the promoter region. In the absence of arabinose, repression is accomplished when this same factor binds at both *araI* and a site 210 bp upstream, *araO2*. Thus, the *araI* and *araO2* sites could be compared to the binding sites for GRFI at the UAS and silencer of *HMLα*, except that at *HML* the separation between binding sites is much greater, 1,870 bp, and the *SIR* proteins are also required for repression. A model has been proposed for repression of the *araBAD* genes involving the formation of a DNA loop between AraC proteins bound at *araI* and *araO2* (58). The DNA loop model is also a popular

explanation for the orientation and distance independence of enhancers and silencers in eucaryotes (22, 63). Along these lines, it might be imagined that GRFI is capable of forming such loops between distant regions of DNA. In the case of *HMLα*, a loop between the UAS and the silencer might be made by GRFI that brings a *SIR* complex formed at *E* in proximity with the promoter, thereby repressing transcription. However, it remains to be seen how this mechanism would accommodate the general features of the silencer— i.e., repression of other polymerase II and III promoters that presumably lack GRFI-binding sites, and the occlusion of *HO* endonuclease cleavage sites. Perhaps GRFI and/or the *SIR* proteins can form loops with many additional factors. At this stage, though, alternate roles for GRFI appear equally likely. GRFI might simply aid in recruiting other specific factors to adjacent regulatory sequences (e.g., transcription factors at the UAS and *SIR* proteins at the silencer), either through direct contacts with other factors or by altering nucleosomes in these regions. A key question regarding any model is whether GRFI is actually bound to both the silencer and UAS when *HMLα* is repressed. A definitive answer is not yet available, but the problem seems amenable to study by in vivo cross-linking (27) and DNA footprinting techniques (23).

With regard to the GRFI-binding sites at telomeres, a role in affecting transcription at these regions seems less likely. If GRFI plays a part in telomere function, it is probably just related to a physical property of the protein. Any of the fundamental mechanisms discussed above might apply at telomeres—DNA looping, recruiting other specific factors, or altering nucleosomes. In one of these ways GRFI might help direct the attachment of telomeres to other nuclear structures or assist in the maintenance of the repeated sequence at chromosome ends. It is possible that intrinsic properties of the unusual $(C_{1-3}A)$ repeat DNA are all that is necessary for telomere function and that GRFI serves just to bind part of this region and prevent its removal by exonucleases. GRFI may be used at telomeres due to its overall abundance and because it has the right specificity to recognize a CA-rich sequence. A similar multipurpose role has been put forward for another abundant yeast DNA-binding factor, CP1 (10). This protein binds to a site upstream of the *GAL2* UAS (11) and shows similarity in both sequence specificity and physical properties to a transcription factor in higher eucaryotes called USF (70) or MLTF (16). Yet CP1 also binds to one of the three conserved elements at yeast centromeres, CDEI (10). The properties of CP1, GRFI, and ABFI suggest the existence of a class of multifunctional DNA-binding factors used as general chromosomal building blocks. In different combinations with other factors, these proteins may be used to construct complex structural elements required for both the expression and maintenance of eucaryotic chromosomes.

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