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Connections between Transcriptional Activators, Silencers, and Telomeres as Revealed by Functional Analysis of a Yeast DNA-Binding Protein

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General regulatory factor I (GRFI) is a yeast protein that binds in vitro to specific DNA sequences at diverse genetic elements. A strategy was pursued to test whether GRFI functions in vivo at the sequences bound by the factor in vitro. Matches to a consensus sequence for GRFI binding were found in a variety of locations: upstream activating sequences (UASs), silencers, telomeres, and transcribed regions. All occurrences of the consensus sequence bound both crude and purified GRFI in vitro. All binding sites for GRFI, regardless of origin, provided UAS function in test plasmids. Also, GRFI binding sites specifically stimulated transcription in a yeast in vitro system, indicating that GRFI can function as a positive transcription factor. The stimulatory effect of GRFI binding sites at UASs for the *PYK1* and *ENO1* genes is significantly enhanced by flanking DNA elements. By contrast, regulatory sequences that flank the GRFI binding site at *HMR E* convert this region to a transcriptional silencer.

Eucaryotic chromosomes are complex, dynamic structures involved in the propagation and expression of genetic information. Numerous functional questions have arisen from the analysis of chromosome structure. For example, as most of the DNA in eucaryotes is packaged in nucleosomes, what prevents nucleosomes from blocking the action of other proteins that need access to the DNA? Regulatory elements in eucaryotes, such as transcriptional enhancers and silencers, can exert their effects at great distances. How are the influences of such elements transmitted and controlled, so as not to disrupt inappropriate targets? Are there principles governing the placement of DNA sequences involved in maintaining chromosome structure? To answer some of these questions, we have been studying proteins that regulate the mating type loci of *Saccharomyces cerevisiae* (for a review, see reference 21). The active mating type loci, *MAT***a** and *MAT*α, express pairs of genes controlled by upstream activating sequences (UASs). Silent storage forms of the mating type genes, *HML*α and *HMR***a**, are repressed by transcriptional silencer elements located more than 1,000 base pairs (bp) from the promoters for these genes (2, 10, 18). The silencers, or *E* regions, that flank the silent loci are also *ARS* elements (autonomously replicating sequences), presumed to be origins of DNA replication in yeast. Two DNA-binding factors have been identified that recognize specific sequences within the silencer at *HMR E* (12, 41, 43). One factor, *ARS* binding factor I (ABFI) or silencer binding factor B (SBF-B), binds sequences adjacent to a subset of *ARS* elements. The other factor, general regulatory factor I (GRFI) or repressor activator protein 1 (RAP1), binds sequences within the silencers at *HML* and *HMR*, and at the UAS for the *MAT*α genes. In addition, GRFI binds a conserved sequence, the ribosomal protein gene box, located within UASs of genes involved in translation (28, 39, 46, 50). GRFI appears to be either identical to or closely related to translation upstream factor, a factor that binds ribosomal protein gene box sequences (22, 23). Perhaps the most surprising revelation was that GRFI binds with high affinity to specific sequences within the $C_{1-3}A$ repeat region of yeast telomeres (12).

The diversity of sequences bound by GRFI in vitro raises problems in understanding its function within the cell. Is GRFI a single factor or a family of related factors? Does GRFI act at each of these different elements in vivo and, if so, what roles does it perform? In the present study, we have employed a strategy designed to test whether GRFI functions in vivo at the sequences that are bound by the factor in vitro. Previous comparisons of different binding sites for unfractionated GRFI had suggested the sequence 5′-RMACCCANNCAYY-3′ (R, A or G; M, A or C; Y, T or C) as a consensus for high-affinity recognition by the factor (12). A data base of yeast DNA sequences was searched for all occurrences of the consensus sequence, which were then assayed for their ability to bind both crude and purified GRFI in vitro. Since earlier investigations suggested that GRFI could function as a positive transcription factor, all GRFI binding sequences regardless of their origin were examined for UAS function in vivo, correlating the binding affinity of each sequence with UAS activity. In addition, we tested the ability of GRFI to stimulate transcription in a yeast in vitro system. The results of our analysis have provided evidence that GRFI does act at the sequences recognized by the factor in vitro. However, the functional consequences of GRFI binding are markedly different, depending on the nature of the sequences flanking the GRFI binding site.

MATERIALS AND METHODS

Computer-assisted searches of DNA sequences. Recently published yeast DNA sequences and those in the GenBank data base were searched for sequence matches by using computer programs obtained from the University of Wisconsin Genetics Computer Group (15).

Synthetic oligonucleotides and oligonucleotide-Sepharose resin. Oligonucleotides were produced with an Applied Biosystems DNA synthesizer and were purified and an-

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nealed as described previously (8). The oligonucleotides contain sequences derived from GRFI binding sites in yeast DNA, as well as sequences on each end that allow ligation to other restriction fragments. (The sequences of most of the oligonucleotides are shown in Table 2.) Sequences of the GAL4 (8), EI (12), DED1 (12), SNR47, HIS35, and centromere-binding protein 1 (CP1; 9) oligonucleotides can be found elsewhere. (For further discussion of SNR47 and HIS35, see N. F. Lue, A. R. Buchman, and R. D. Kornberg, Proc. Natl., Acad. Sci. U.S.A., in press). For the DNA affinity chromatography, an oligonucleotide was synthesized that had two tandem copies of the GRFI binding site at the *MAT*α UAS. One strand had the sequence 5′-TAA CAAAACCCAGACATCATTAACAAAACCCAGACAT CAT-3′. The other strand was complementary to the first, such that four-base 5′ protruding ends were formed when the two strands were annealed. The double-stranded oligonucleotide was coupled to Sepharose CL-2B by a modification of a previously described procedure (27). Oligonucleotide (300 μg) was allowed to react with 4 ml of CNBr-activated Sepharose beads. The coupling efficiency, measured spectrophotometrically at 260 nm, was about 70%.

Plasmid and probe DNAs. The pGH derivatives were created by ligating the indicated oligonucleotide with 1.6-kbp *Sal*I-*Eco*RI and 7.3-kbp *Bam*HI-*Sal*I fragments from p10GH (29). Similarly, most pCT plasmids were constructed by joining the indicated oligonucleotide with 1.1-kbp *Eco*RI-*Cla*I and 7.9-kbp *Cla*I-*Bam*HI fragments from pCT136 (30). pCTΔ and pGHΔ contained no inserted oligonucleotide. pCT-LSR1 was created by ligation of the LSR1 oligonucleotide with a 9-kbp *Xho*I-*Eco*RI fragment of pCTΔ. The source of *HMR E* DNA for the construction of pCT-EO/Alpha was the plasmid pJR315 (27a), which contains a 490-bp fragment of *HMR E* inserted into the polylinker region of the plasmid. pCT-EO/Alpha was created by ligation of a 535-bp *Hin*dIII-*Bam*HI fragment of *HMR E* DNA with 1.1-kbp *Bam*HI-*Cla*I and 7.9-kbp *Hin*dIII-*Cla*I fragments of pCT-Alpha. pCT-EN/Alpha was constructed similarly except that the *HMR E* DNA was obtained from a derivative of pJR315 which has the *E*-region DNA inserted in the opposite orientation with respect to the polylinker restriction sites. pCT-EOmtIIA/Alpha was constructed in a manner identical to that of pCT-EO/Alpha, but the source of *HMR E* DNA was a derivative of pJR315 which carried a single base change in the GRFI binding site, pJR315-IIA (27a). A 265-bp *Sau*3AI-*Hin*dIII fragment of *HMR E* DNA was excised from pJR315 and inserted into the polylinker region of the vector pEMBL18 (27a) between the *Bam*HI and *Hin*dIII sites, producing pEMBL-E265. The *HMR E* DNA was liberated from pEMBL-E265 as a *Hin*dIII-*Eco*RI fragment, which was ligated either with 1.6-kbp *Sal*I-*Eco*RI and 7.0-kbp *Hin*dIII-*Sal*I fragments of p10GH or with 1.1-kbp *Eco*RI-*Cla*I and 7.9-kbp *Cla*I-*Hin*dIII fragments of pCT136, producing pGH-E265 and pCT-E265, respectively. pCZ-TEL was created by joining a 7.9-kbp *Eco*RI-*Cla*I fragment of pCT-TEL with a 1.0-kbp *Eco*RI-*Cla*I fragment of pCZΔ (Lue et al., in press). pCX-TEL was generated by recircularization of a 8.9-kbp *Xho*I-*Eco*RI fragment of pCZ-TEL. pCXΔ was created by recircularization of a 8.9-kbp *Xho*I-*Eco*RI fragment of pCZΔ. Probe DNAs were restriction fragments derived from plasmids that were labeled with [α-^{32}P]dATP (Amersham Corp.) and DNA polymerase Klenow fragment (Pharmacia Fine Chemicals) by replacement synthesis (31). Specific radioactivity of probes varied from 5 × 10^3 to 1 × 10^4 cpm/fmol.

Protein-DNA binding assays. Generally, nitrocellulose filter-binding assays and gel electrophoresis mobility shift assays were performed as described previously (12). Binding reactions were done in 20 μl of buffer A (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5], 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, 0.6 μM leupeptin, 5 μg of antipain per ml) containing 50 mM KCl, 1 to 5 fmol of ^{32}P-labeled probe DNA, 2 μg of bovine serum albumin, 1 μg of poly(dI-dC), and various amounts of yeast protein and competitor DNA. After incubation at room temperature, reactions were either filtered through nitrocellulose or electrophoresed in 3% NuSieve agarose (FMC Corp.) gels. In the filter-binding competition assays used to quantitate the relative affinities of oligonucleotides for GRFI, each assay contained 0.2 ng of purified GRFI and 1.25 fmol of ^{32}P-labeled probe, a 0.3-kbp *Bam*HI fragment from pCT-ENO1. Oligonucleotides were tested at five different concentrations that varied by factors of 2 and bracketed the 50% competition point. Measurements were made in triplicate and averaged. The 50% competition point was determined by interpolation.

Purification of GRFI. Cells of the yeast strain BJ926 (α/**a** *trp1/+prc1-126/prc1-126 pep4-3/pep4-3 prb1-1122/prb1-1122 can1/can1*) were grown in YPD medium (2% Bacto-peptone [Difco Laboratories], 1% yeast extract, 2% dextrose) until the culture reached an optical density at 600 nm of 6.0 and then harvested by centrifugation. The pellet was washed in an equal volume of buffer A and suspended in the same volume of buffer A, which was subsequently adjusted to 0.4 M KCl. Cells were broken at 4°C by glass bead disruption using 10 30-s cycles of a bead beater (Biospec Products). The cell lysate was centrifuged in a Sorvall SS-34 rotor at 12,000 × *g* for 10 min; the supernatant was decanted and centrifuged in a Beckman Ti60 rotor at 252,000 × *g* for 3 h at 4°C. The clarified extract (36 ml) was adjusted to 100 mM KCl by dilution with buffer A and then loaded onto a column of Affi-Gel Blue resin (2.5 by 6.5 cm; Bio-Rad Laboratories) at a rate of 40 ml/h. The column was washed sequentially with 100 ml of buffer A, 100 ml of buffer A with 0.5 M KCl, and 100 ml of buffer A with 1.5 M KCl. Fractions containing GRFI from the 1.5 M KCl step were combined and dialyzed against buffer A with 100 mM KCl. The mixture was centrifuged for 20 min at 17,400 × *g* in a Sorvall SS-34 rotor to remove insoluble material and then mixed with 400 μg each of poly(dI-dC) and poly(dA-dT). After a 15-min incubation at 4°C, the fractionated extract was applied to a 0.5-ml column of oligonucleotide-Sepharose at a rate of 5 ml/h. The column was washed sequentially with 4 ml of buffer A with 0.1 M KCl, 2.5 ml of buffer A with 0.3 M KCl, and 5 ml of buffer A with 1.0 M KCl. Peak fractions of GRFI activity in the 1.0 M KCl step were combined and adjusted to 0.1 M KCl and reapplied to the oligonucleotide-Sepharose column, which was eluted by the same procedure. Fractions containing GRFI were stored by freezing in liquid nitrogen. Protein concentrations were determined by the method of Bradford (7) with bovine serum albumin as the standard.

Recovery of GRFI after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified GRFI from the oligonucleotide-Sepharose column was electrophoresed in a 1.5-mm SDS–7.5%-polyacrylamide gel, which was calibrated with high-molecular-weight protein standards (Bio-Rad). Proteins were eluted from gel slices as described before (20), precipitated, and renatured by suspension in 10 μl of buffer A containing 50 mM KCl and 6 M guanidine hydrochloride, followed by dilution with 0.5 ml of buffer A containing 0.1 mg of bovine serum albumin. After incubation

TABLE 1. Matches to the GRFI consensus sequence

Gene	Product	Function	Position[a]	Orientation[b]	Reference
MATα1	Regulatory protein	Mating type	−144 AUG, −104 TS	−	5
MATα2	Regulatory protein	Mating type	−108 AUG, −79 TS	+	5
TEF2	Elongation factor	Translation	−425 AUG	+	22
RP28-1	Ribosomal protein	Translation	−233 AUG	−	33
RP39A	Ribosomal protein	Translation	−226 AUG, −176 TS	+	39
RP51B	Ribosomal protein	Translation	−286 AUG	+	1
ADH1	Alcohol dehydrogenase	Glycolysis	−652 AUG, −613 TS	+	6
ENO1	Enolase	Glycolysis	−456 AUG, −416 TS	+	48
PYK1	Pyruvate kinase	Glycolysis	−643 AUG, −610 TS	+	13
BCY1	Protein kinase regulatory subunit	Cell growth	−404 AUG	+	47
LSR1	Spliceosomal RNA	mRNA splicing	−196 TS	−	3
SIR3d	Unknown		−233 AUG	+	42
PHO5	Acid phosphatase		+405 AUG	+	4
25S rRNA	rRNA		+114 25S start	−	19
Telomere			Within $(C_{1-3}A)_n$	+	40

[a] Position is given as the number of base pairs between the GRFI consensus sequence, 5′-RMACCCANNCAYY-3′, and the translational start codon (AUG) or the nearest transcriptional start (TS) site for each gene.

[b] Orientation is indicated with respect to the direction of transcription of each gene. In the "+" orientation the GRFI consensus sequence is in the coding strand. For the telomere, orientation is given as if the adjacent chromosomal DNA were transcribed away from the telomere.

of the diluted protein for 1 h at 22°C, 5 μl of each fraction was assayed for GRFI activity. Greater than 50% of the original GRFI activity was recovered after electrophoresis and renaturation.

Assays for UAS function. Plasmid DNAs were introduced into the yeast strain 5C (**a** *his3-Δ200 ura3-52*) by a lithium acetate transformation procedure (24), selecting for uracil prototrophy on minimal medium (0.67% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose) supplemented with histidine. For the pGH plasmids, transformants were streaked onto agar plates containing minimal medium with an appropriate carbon source. Plates were incubated at 30°C for several days and inspected for cell growth. For the pCT plasmids, transformants were grown in minimal medium (2% sucrose and 2% galactose for pCT-GAL4) supplemented with histidine until the culture reached an optical density at 600 nm of 1.0. Cells were harvested by centrifugation, washed, and suspended in an amount of buffer (100 mM Tris hydrochloride, pH 7.5; 5% glycerol; 5 mM β-mercaptoethanol; 1 mM phenylmethylsulfonyl fluoride) equal to the volume of the cell pellet. Cells were broken by glass bead disruption and the extract clarified by centrifugation for 10 min at 14,000 × *g* in a microcentrifuge. β-Galactosidase enzyme assays were performed with *o*-nitrophenyl-β-D-galactoside as described previously (32). Assays were performed twice for each strain, and results were averaged.

In vitro transcription reactions. Yeast nuclear extract was prepared from strain BJ926 as described elsewhere (Lue et al., in press). For each reaction, 8.5 μl of extract (20 mg of protein per ml) was mixed with 12 μl of preincubation buffer (20 mM HEPES [pH 7.6], 160 mM potassium acetate, 8 mM phosphoenolpyruvate, 1 mM dithiothreitol, 0.1 mM EDTA, 20% glycerol, 0.25 mg of bovine serum albumin per ml, and 1.6 U of RNasin [Promega Biotec] per μl) containing 0.3 μg of competitor oligonucleotide and incubated at 22°C for 20 min. Template DNA (0.3 μg of plasmid DNA in 1 μl of 10 mM Tris hydrochloride [pH 7.5]–1 mM EDTA) was added to each reaction along with 3 μl of solution containing 2.5 mM each of the four ribonucleoside triphosphates and 83 mM magnesium acetate. Incubation was continued for 1 h at 22°C, and the reaction was stopped by the addition of 300 μl of 0.1 M sodium acetate–10 mM EDTA. The mixtures were extracted four times with equal volumes of phenol-chloroform (1:1 [vol/vol]), and the products were precipitated by the addition of ammonium acetate (to 2.5 M) and ethanol (2.5 volumes). Procedures for the RNase protection assay using complementary ^{32}P-labeled RNA probes were as described previously (30).

RESULTS

Matches to the GRFI consensus sequence. A computer data base (GenBank) and recently published yeast DNA sequences were searched for occurrences of the GRFI consensus sequence, 5′-RMACCCANNCAYY-3′. The number of matches that were found was greater than anticipated (Table 1). Fourteen separate occurrences of the GRFI consensus sequence were located, whereas an average of four to five would have been the number expected for a random sequence with the same length and composition as the yeast data base. Also, only 2 of the 14 matches were within coding or transcribed regions (*rRNA* and *PHO5*), even though the data base is strongly weighted towards these sequences. For the most part, matches with the consensus sequence were located upstream of yeast genes having diverse but important functions. As expected from our earlier work, matches were found upstream of the *MATα* genes and several genes involved in translation (*RP28-1*, *RP39A*, *RP51B*, and *TEF2*). In addition, matches were revealed upstream of three genes encoding enzymes of glycolysis (*ADH1*, *PYK1*, and *ENO1*). The matches at *ADH1* and *PYK1* were far from the promoters for these genes but were at the same distance (−610 bp) and orientation with respect to the start of transcription. A match was found upstream of a gene encoding the regulatory subunit for the cyclic-AMP-dependent protein kinase, *BCY1*. Also, the *LSR1* gene had an occurrence of the consensus sequence. *LSR1* produces a spliceosomal RNA that recognizes the conserved sequence at branch sites of

introns (36). A GRFI consensus sequence occurs upstream of a gene located next to *SIR3* (we refer to the gene here as *SIR3d*, for *SIR3* downstream gene). This gene is transcribed into an abundant RNA (25) but its function has not been determined. All matches in 5'-flanking regions of genes were located upstream of TATA sequences. In several cases (*MATα*, *ENO1*, and *RP39A*), deletion analysis indicates that the GRFI consensus sequence is within the UAS for these promoters (14, 39, 44). Finally, we had noticed in our earlier study that the $C_{1-3}A$ repeat region of yeast telomeres contains matches to the GRFI consensus sequence.

Purification of GRFI. To compare the binding properties of GRFI with the functional properties of the sequences it recognizes, we first purified GRFI to near-homogeneity. The purification was followed by using a nitrocellulose filter-binding assay with ^{32}P-labeled DNA probes containing sequences from different classes of GRFI sites. Specificity of binding was demonstrated with oligonucleotide competitors (8). The ENO1, TEF2, EII, and TEL probes contained consensus sequences or GRFI binding sites (in the form of synthetic oligonucleotides, listed in Table 2, joined to 375 bp of pBR322 DNA) from the *ENO1*, *TEF2*, *HMR E*, and yeast telomere sequences, respectively. Fractionation was also monitored with probes that detect two other sequence-specific DNA-binding proteins in yeast extracts, CP1 (9) and ABFI (12). ABFI binding sites are located next to a subset of yeast *ARS*s and in some UAS regions. CP1 recognizes sequences located in a conserved domain of yeast centromeres and in some intergenic regions.

A yeast whole-cell extract was loaded onto an Affi-Gel Blue column in buffer containing 100 mM KCl. The column was eluted in two steps with buffers containing 0.5 M and 1.5 M KCl (Fig. 1A). Under these conditions, 70% of the total protein flowed through the column and most of the bound protein was eluted in the 0.5 M KCl step. Almost all of the GRFI was found in the 1.5 M KCl fraction (Fig. 1A shows the data for just the ENO1 probe, but all other GRFI probes, i.e., EII, TEF2, and TEL, gave the same profile on this and subsequent columns). CP1 activity was also contained in the 1.5 M KCl fraction; ABFI, however, was mainly eluted in the 0.5 M KCl step. Pooled fractions of GRFI from the 1.5 M KCl step were dialyzed to 100 mM KCl, mixed with 400 μg each of poly(dA-dT) and poly(dI-dC), and applied to a DNA affinity resin. This column contained Sepharose beads covalently attached to a synthetic oligonucleotide with two high-affinity binding sites for GRFI (see Materials and Methods). The oligonucleotide column was washed extensively with buffer containing 100 mM KCl and eluted in two steps with buffers containing 300 mM KCl and then 1.0 M KCl. All of the GRFI was retained by the DNA affinity column, and most of the activity eluted in the 1.0 M KCl step. No CP1 or ABFI activity was detected in this fraction (data not shown). The 1.0 M KCl fractions from the oligonucleotide-Sepharose column were diluted to 100 mM KCl and reapplied to the same column, which was eluted again with buffers containing 0.3 M KCl and 1.0 M KCl, and the 1.0 M KCl fractions were pooled. Overall, this procedure resulted in a 7,650-fold purification of GRFI from the whole-cell extract, with a yield of 11% (Table 3).

Fractions containing GRFI from the oligonucleotide-Sepharose column were analyzed by SDS-PAGE, and the proteins were detected with a silver stain (Fig. 1B). The 1.0 M KCl fractions from both applications of the DNA affinity column had one major polypeptide (>80%) with a mobility close to that of the β-galactosidase marker (116 kilodaltons [kDa]). GRFI from the second run of the oligonucleotide-Sepharose column was electrophoresed on a similar SDS-polyacrylamide gel, which was divided into 16 slices. Proteins were eluted from each slice, renatured, and assayed for GRFI by a filter-binding assay with the ENO1 probe (Fig. 1C). The vast majority of GRFI activity was in the 110- to 140-kDa slice, a finding consistent with the size of the major polypeptide detected in the stained gel (Fig. 1B).

The integrity of purified GRFI was judged by comparison with crude GRFI from whole-cell extracts by using gel electrophoresis mobility shift assays. The purified GRFI from the oligonucleotide-Sepharose column and the GRFI renatured after gel electrophoresis both produced a single protein-DNA complex when mixed with ^{32}P-labeled ENO1 probe (Fig. 2A). The complex observed with purified GRFI had the same mobility as the predominant complex formed with whole-cell extract. Formation of this complex was blocked by adding excess ENO1 oligonucleotide as competitor (10 ng). The only difference in this assay between the crude and purified GRFIs was that the purified GRFI lacked Y factor (M. J. Fedor, N. F. Lue, and R. D. Kornberg, J. Mol. Biol., in press), another DNA-binding factor that recognizes different sequences in the probe (Y factor binds fortuitously to this probe in a region of the pUC18 polylinker that overlaps the *Sma*I and *Kpn*I restriction sites [A. R. Buchman and N. Lue, unpublished data]). No complexes of the probe with Y factor or with Y factor plus GRFI were detected in the assays containing purified GRFI (Fig. 2A). These experiments indicate that purified GRFI is largely free of other DNA-binding factors and that the purified material is not grossly altered in size compared to the activity in whole-cell extracts. Also, the results indicate that GRFI is largely, if not entirely composed of the single polypeptide observed in SDS-PAGE.

Another characteristic of crude GRFI that we noted in our previous study was that two specific complexes were formed in gel electrophoresis mobility shift assays with a probe containing the TEL oligonucleotide (Table 2). This oligonucleotide has three occurrences of the GRFI consensus sequence match 5'-ACACCCACACACC-3', two of which are nonoverlapping (indicated by underscores below the sequence in Table 2). It was suggested that the two complexes were produced by binding of one or two molecules of GRFI to the probe DNA. Purified GRFI was tested in gel electrophoresis mobility shift assays with a probe containing the TEL oligonucleotide (Fig. 2B). The complexes produced with purified GRFI comigrated with the two complexes generated with whole-cell extract. The faster-migrating complex had the same mobility as the single complex formed with an ENO1 probe, which contains one GRFI consensus sequence match, a result consistent with the notion of two available GRFI binding sites in the TEL oligonucleotide.

Matches to the GRFI consensus sequence bind GRFI in vitro. Matches to the GRFI consensus sequence showed significant variation in structure and might not necessarily bind the same protein. Hence, it was of interest to determine whether all occurrences of the GRFI consensus sequence were recognized by both crude and purified GRFI. Initially, many of these regions were tested by using restriction fragments from each gene as probes in filter-binding and electrophoretic mobility shift assays. This is illustrated below for the *ADH1* and *SIR3d* genes. Later, it became more convenient to use small synthetic oligonucleotides to define more precisely the properties of each binding site. We have focused our attention on the GRFI binding sites that are not located at genes involved in translation, as these genes are being intensively studied by others (22, 39, 49).

TABLE 2. Comparison of GRFI binding and UAS function for different oligonucleotides

Oligo. or Insert[a]	fmole 50% competition[b]	pCT ß-gal. units[d]	pGH *HIS3* phenotype[e]	Sequence[f]
Δ		0.69	–	
GAL4		70	+	
PHO5	70	2.5	NT	GATCCTGTTTTGAACCCATACACTGGTGAAG
HML35	57	6.0	+/–	GATCCAAAATCAAAAACCCATTCATAAGATACTG
HML29	>10⁴	0.43	–	GATCCAAATCAAAAACCCATTCATAAGAG
BCY1	28	8.1	NT	GATCCAACAAAGCACCCAATCACCACCCTTG
RDNA	5.8	13	NT	GATCCGGTTTTACACCCAAACACTCGCATAG
EII	29	19	+	GATCTTATATTGCAAAAACCCATCAACCTTG
mtIIA	>10⁴	0.37	–	GATCTTATATTGCAAAAACaCATCAACCTTG
mtIIB	[9.0][c]	0.37	–	GATCTTATATTGCAcAAACCCATCAACCTTG
Alpha	2.5	24	+	GATCCATCCCAAACAAAACCCAGACATCATG
TEF2	3.1	39	+	GATCCCATTCATGTTGCACCCACACATTTAG
LSR1	3.0	44	NT	TCGAGCCACCCACACCCATACACCCCATTAG
TEL	1.7	105	+	GATCCCACACCCACACACCCACACACCCACACACCCAG
ENO1	3.5	230	++	GATCCGAGCTTCCACTAGGATAGCACCCAAACACCTGG
ENO1mtA	>10⁴	1.2	–	GATCCGAGCTTCCACTAGGATAGCACaCAAACACCTGG
ENO1mtB	4.0	49	NT	GATCCGAGCaaCCACTAGGATAGCACACAAACACCTGG
ENO28	3.2	23	NT	GATCCTAGGATAGCACCCAAACACCTGG
PYK1	2.8	249	++	GATCCTGTACACCCAGACATCGGGCTTCCAG
PYK1mtA	3.0	15	NT	GATCCTGTACACCCAGACATCGGGCaagCAG
E265		0.02	–	
EN/Alpha		<0.02	NT	
EO/Alpha		<0.02	NT	
EOmtIIA/Alpha		2.7	NT	

[a] Oligonucleotides were inserted between the *Bam*HI and *Eco*RI sites of pCT or pGH (Fig. 5A and B) with the exception of LSR1, which was inserted between the *Eco*RI and *Xho*I sites. Consequently, all GRFI binding sequences except LSR1 are in the same orientation in these constructions.

[b] The relative binding affinity of each oligonucleotide is shown as the amount in fmoles required to compete away 50% of GRFI binding in a standard nitrocellulose filter-binding assay with purified GRFI and a probe (1.25 fmol) containing the ENO1 oligonucleotide.

[c] Although the mtIIB oligonucleotide was a good competitor for GRFI binding in the filter-binding assay, this same sequence when inserted into plasmid DNA has less than 5% of the affinity of EII oligonucleotide for GRFI (12). Since the binding affinity of free mtIIB oligonucleotide does not reflect the poor binding ability of this sequence when inserted into the pCT or pGH plasmids, it is shown in brackets.

[d] UAS function of each oligonucleotide was assessed from the expression of *CYC1-lacZ* fusions in pCT derivatives, determined by measuring the specific activity of β-galactosidase (in nanomoles per minute per milligram of protein) in extracts of yeast cells containing each plasmid.

[e] UAS function of each oligonucleotide was assessed from the expression of *GAL1-HIS3* fusions in pGH derivatives. The histidine growth requirement of a *his3* yeast strain containing each pGH plasmid is indicated as follows: –, auxotrophy when grown on sucrose or glucose; +/–, a small proportion of cells show prototrophy when grown on sucrose; +, prototrophy when grown on sucrose (sucrose plus galactose for pGH-GAL4) and auxotrophy when grown on glucose; ++, prototrophy when grown on sucrose or glucose. Some oligonucleotides were not tested (NT) in the pGH constructs.

[f] The sequence of one strand of each oligonucleotide is listed 5′ to 3′ from left to right. The complementary strand of each oligonucleotide anneals with the 5th base from the left to the last base shown and has a four-base 5′-protruding end which is 5′-AATT-3′ for all oligonucleotides. The GRFI consensus sequence region of each oligonucleotide is shaded. Base changes in mutant oligonucleotides (mt) are in lower-case type.

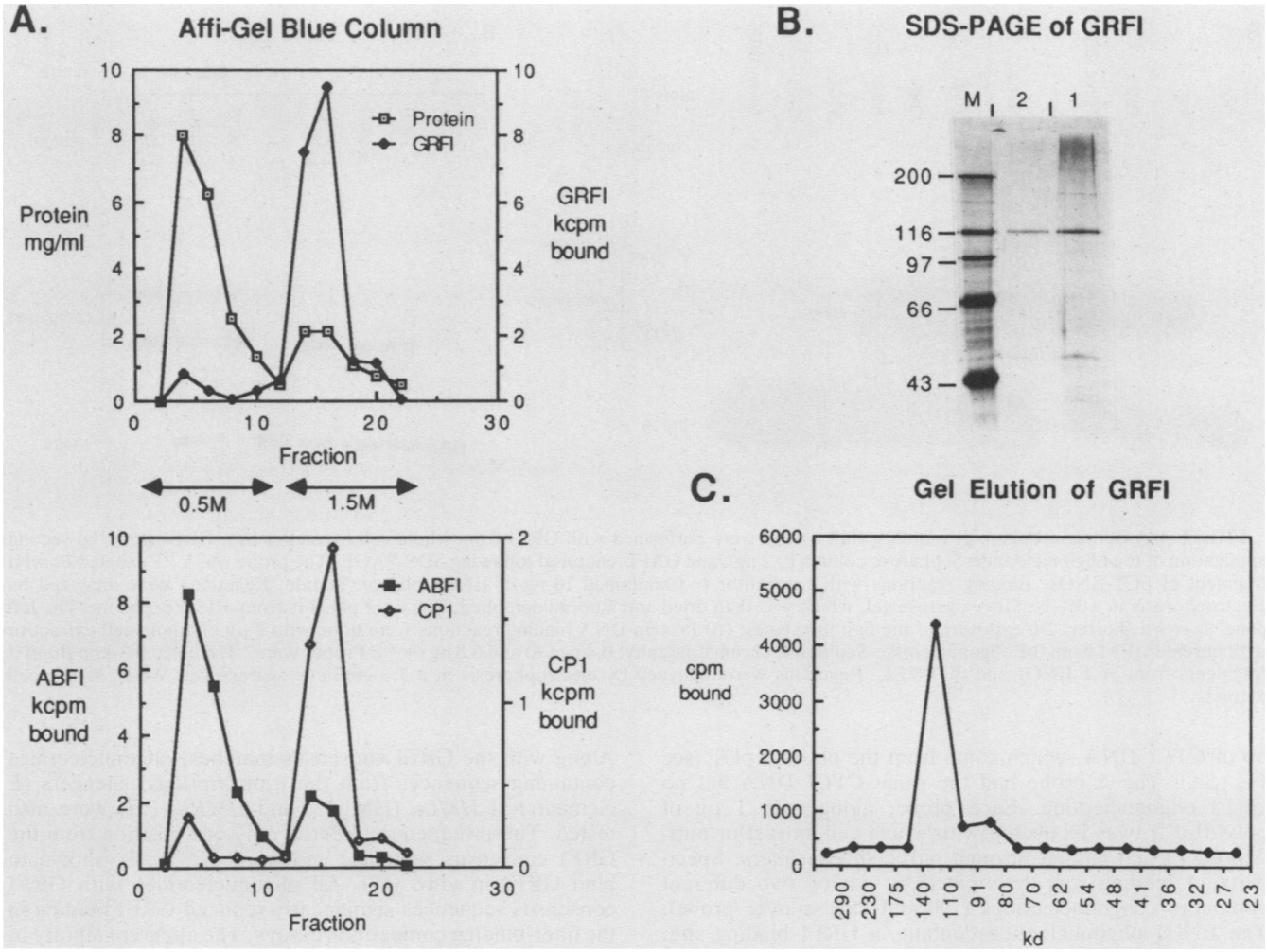


FIG. 1. (A) Yeast whole-cell extract was applied to an Affi-Gel Blue column, which was sequentially eluted with steps of buffer containing 0.5 M KCl and 1.5 M KCl. Fractions (10 ml) were taken, and every other fraction was assayed for protein and specific DNA-binding activities. Nitrocellulose filter-binding assays were performed with ^{32}P-labeled probes: GRFI, 0.4-kbp *Eco*RI fragments from pGH-ENO1, pGH-EII, pGH-TEF2, and pGH-TEL; ABFI, 0.4-kbp *Eco*RI fragment from pGH-DED1 (12); CP1, a 1.3-kbp *Hin*dIII-*Bam*HI fragment from the GAL2 gene (9). Pairs of assays were carried out for aliquots of each fraction, with and without 10 ng of the appropriate competitor oligonucleotide, and the difference was plotted. The column profile for all the GRFI probes was the same, so just the values for the ENO1 probe are shown. (B) Pooled fractions from the 1.0 M KCl steps of the oligonucleotide-Sepharose column were analyzed by SDS–7.5% PAGE, and proteins were visualized with a silver stain (51). Lane 1, 75 ng of protein from the first application of the column. Lane 2, 35 ng of protein from the second application of the column. Lane M, High-molecular-weight protein standards: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (43 kDa). (C) Purified GRFI from the second application of the oligonucleotide-Sepharose column was analyzed by SDS-7.5% PAGE; the gel was then divided into 16 slices. Proteins were eluted from each slice, renatured, and assayed for GRFI activity in nitrocellulose filter-binding assays. The probe was a ^{32}P-labeled 0.4-kbp *Eco*RI fragment of pGH-ENO1. Counts retained on the filters are plotted for each gel slice.

The results of filter-binding assays with crude and purified GRFIs with five different ^{32}P-labeled probes are presented in Fig. 3A. The SIR3d and ADH1 probes were restriction fragments containing the GRFI consensus sequence at the *SIR3d* and *ADH1* genes, respectively. The ENO1 and LSR1 probes were derived from synthetic oligonucleotides (Table 2) which contained the GRFI consensus matches from these genes. Oligonucleotides in these probes were joined to 252

TABLE 3. Purification of GRFI

Fraction	Protein (mg)	Vol (ml)	Activity (U)	Sp act (U/mg)[a]	Purification	
					Yield (%)	Fold
Whole-cell extract	1,400	36	476,000	340	100	1
Affi-Gel Blue	42	60	428,400	10,200	87	30
Oligonucleotide-Sepharose	0.02	2	52,000	2,600,000	11	7,650

[a] 1 U of DNA binding activity is the amount required to retain 1 fmol of labeled probe on a nitrocellulose filter.

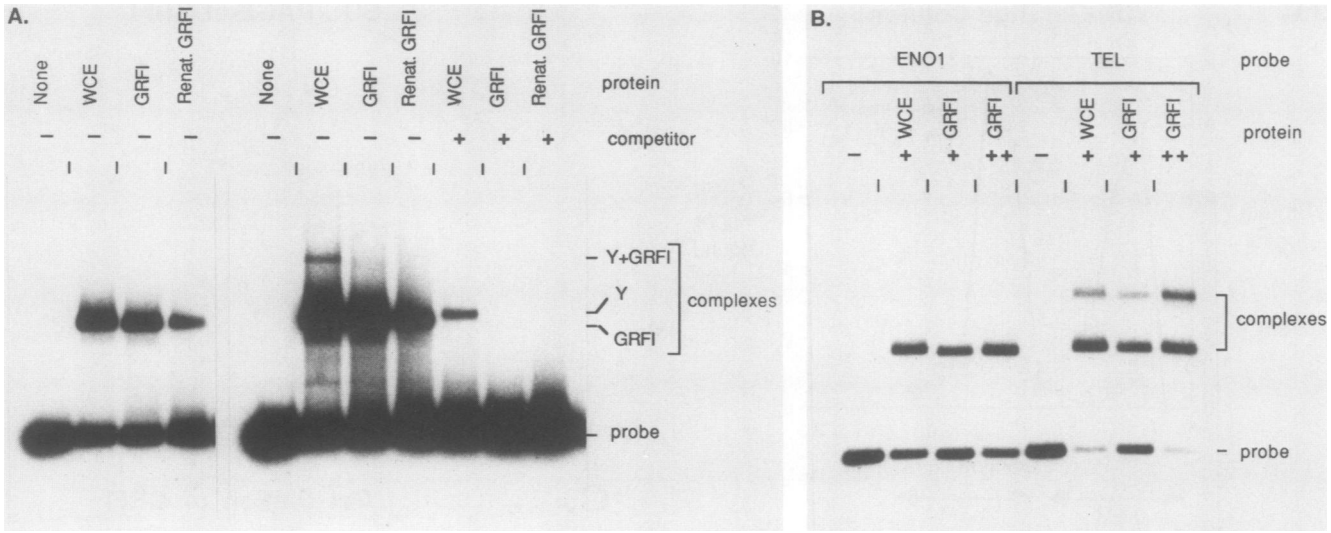


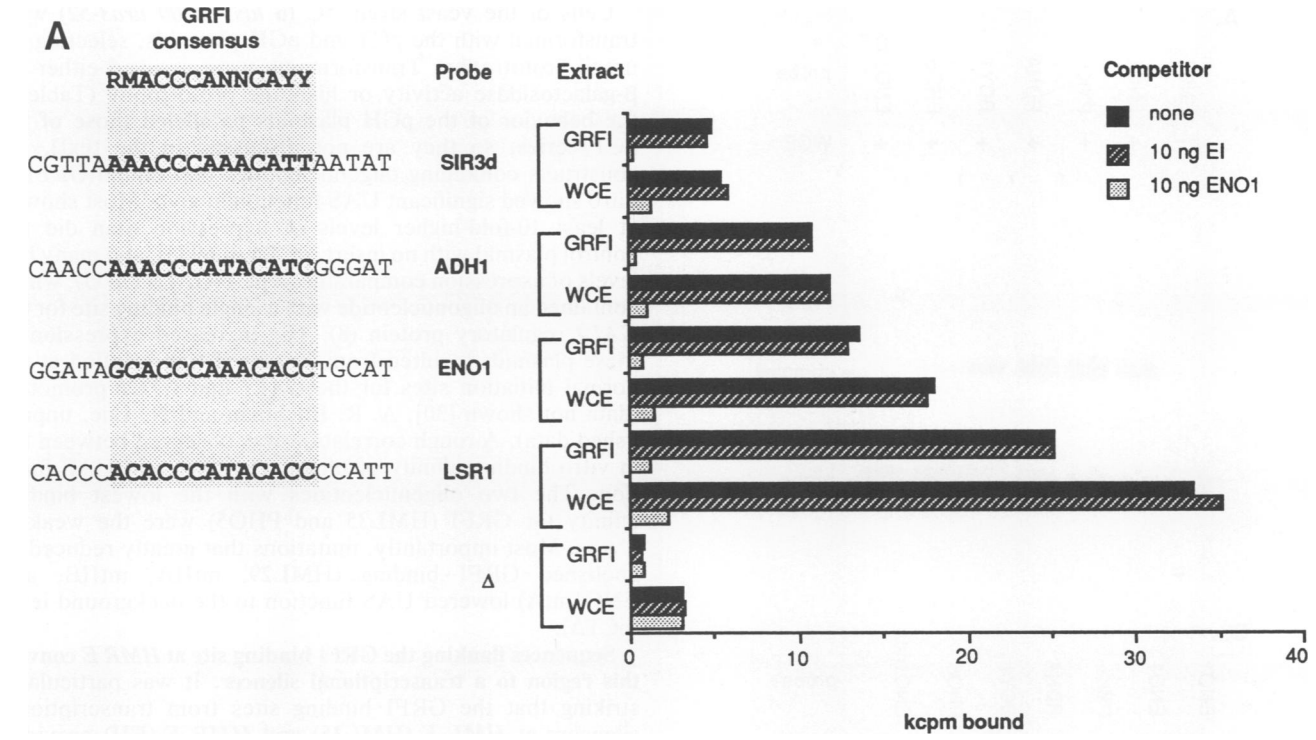
FIG. 2. (A) Gel electrophoresis mobility shift assays were performed with GRFI from whole-cell extract (1 μg), GRFI from the second application of the oligonucleotide-Sepharose column (0.2 ng), and GRFI renatured following SDS-PAGE. The probe was a ^{32}P-labeled *Bam*HI fragment of pCT-ENO1. Binding reactions with competitor (+) contained 10 ng of ENO1 oligonucleotide. Reactions were analyzed by electrophoresis in a 3% NuSieve agarose gel, which was then dried and autoradiographed. The right panel is from a 15-h exposure. The left panel shows a shorter, 2-h exposure of the first four lanes. (B) Protein-DNA binding reactions were done with 2 μg of whole-cell extract or with purified GRFI from the oligonucleotide-Sepharose chromatography: 0.4 ng (+) and 0.8 ng (++). Probes were ^{32}P-labeled 0.3-kbp *Bam*HI fragments from pCT-ENO1 and pCT-TEL. Reactions were analyzed by electrophoresis in a 3% NuSieve agarose gel. WCE, Whole-cell extract.

bp of *CYC1* DNA, which came from the plasmid pCT (see Fig. 5A). The Δ probe had the same *CYC1* DNA but no GRFI oligonucleotide. Each probe, along with 1 μg of poly(dI-dC), was incubated with whole-cell extract or purified GRFI and passed through nitrocellulose filters. Specificity of binding was determined by adding two different competitor oligonucleotides (100-fold excess over probe). The ENO1 oligonucleotide contains a GRFI binding site. The EI oligonucleotide binds the factor ABFI but does not bind GRFI (12). Probes containing matches to the GRFI consensus sequence all showed significantly greater levels of binding than the control probe Δ, with either whole-cell extract or purified GRFI. In all cases, binding was blocked by excess ENO1 but not EI oligonucleotide, a result which indicates specificity. The same relative affinities of GRFI binding were found with whole-cell extract and purified GRFI: LSR1 > ENO1 > ADH1 > SIR3d. These probes were also tested in gel electrophoresis mobility shift assays with purified GRFI (Fig. 3B). GRFI generated a single complex with each probe, and complex formation could be blocked with excess ENO1 oligonucleotide (10 ng). Although the size of each complex varied, binding of GRFI reduced the mobility of each probe by a constant amount that was equivalent to the addition of 300 bp. Thus, each of these regions appeared to contain a single binding site for GRFI.

To compare the binding of GRFI in vitro with the functional properties of these sequences in vivo, it was more useful to study the properties of small synthetic oligonucleotides (28 to 38 bp) containing the GRFI consensus sequences (Table 2). Each oligonucleotide was tested for GRFI binding in two ways. In one assay, oligonucleotides were examined for their ability to block the binding of purified GRFI to labeled ENO1 probe in a standardized filter-binding assay (see Materials and Methods). The amount of free oligonucleotide needed to cause a 50% reduction in the amount of bound probe (1.25 fmol total) is given in Table 2. Along with the GRFI consensus matches, oligonucleotides containing sequences from the transcriptional silencers (*E* elements) at *HMLα* (HML35) and *HMRa* (EII) were also tested. These sequences differ at only one position from the GRFI consensus sequence and were previously shown to bind GRFI in vitro (12). All oligonucleotides with GRFI consensus sequences significantly reduced GRFI binding in the filter-binding competition assays. The apparent affinity of these oligonucleotides for GRFI varied over a 40-fold range, with the following order of binding: TEL > Alpha (*MATα*) > PYK1 > LSR1 > TEF2 > ENO1 > RDNA > BCY1 > EII (*HMR E*) > HML35 (*HML E*) > PHO5.

In the second assay, the synthetic oligonucleotides were tested as probes in gel electrophoresis mobility shift experiments with whole-cell extract and purified GRFI. The probes contained each oligonucleotide fused to 286 bp of DNA that had been derived from the pCT plasmids (see Fig. 5A). Assays with many of these probes are shown in Fig. 4. A single protein-DNA complex of the same mobility was formed with each probe on addition of either whole-cell extract or purified GRFI. The relative affinity of these probes was the same with either crude or purified GRFI: PYK1 > ENO1, RDNA > BCY1 > PHO5. This order of binding affinity was in agreement with that found in the filter-binding competition assay (Table 2).

In our initial study we showed that mutations either within or flanking the GRFI consensus region of EII and HML35 could greatly reduce binding. This is illustrated in Table 2 by the properties of HML29 and mtIIA, which are altered forms of HML35 and EII, respectively. Each of these oligonucleotides is reduced over 100-fold in its ability to compete for GRFI binding in the filter binding assay. Another mutant form of EII oligonucleotide mtIIB, exhibited mixed binding properties. The free oligonucleotide was an effective competitor of GRFI binding in the filter-binding assay (Table 2). However, when joined with other DNA sequences, mtIIB-containing probes were greatly reduced (20-fold) in GRFI



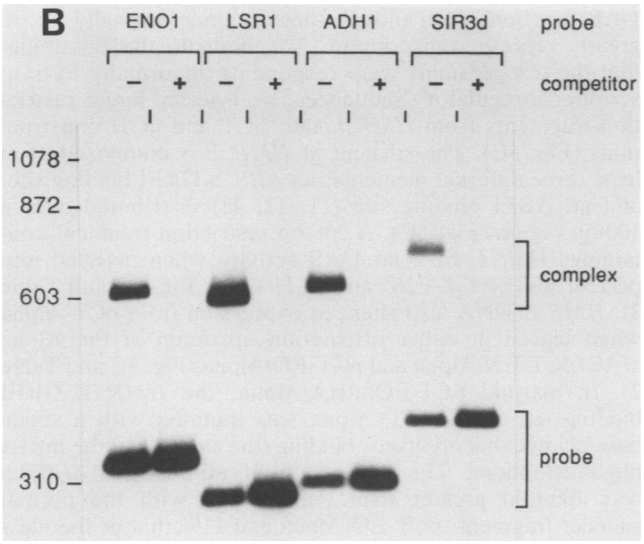


FIG. 3. (A) Purified GRFI from the oligonucleotide-Sepharose chromatography (GRFI) was compared to GRFI in whole-cell extracts (WCE) by nitrocellulose filter-binding assays with five different ^{32}P-labeled probes: SIR3d, a 365-bp *Eco*RI-*Xmn*I fragment from the *SIR3d* gene; ADH1, a 310-bp *Eco*RV-*Sph*I fragment from the *ADH1* gene; ENO1, a 324-bp *Bam*HI fragment from pCT-ENO1; LSR1, a 282-bp *Eco*RI-*Bam*HI fragment from pCT-LSR1; Δ, a 251-bp *Xba*I-*Bam*HI fragment from pCTΔ. Binding reaction mixtures contained the indicated amount of competitor oligonucleotide and the following amounts of protein: SIR3d, 8 μg of whole-cell extract or 1.6 ng of purified GRFI; ADH1, 4 μg of whole-cell extract or 0.8 ng purified GRFI; ENO1, 1 μg of whole-cell extract or 0.2 ng of purified GRFI; LSR1, 1 μg of whole-cell extract or 0.2 ng of purified GRFI; Δ, 8 μg of whole-cell extract or 1.6 ng of purified GRFI. (B) Binding reactions with purified GRFI and the ENO1, LSR1, ADH1, and SIR3d probes were performed as in panel A and analyzed in gel electrophoresis mobility shift assays. Some binding reactions contained 10 ng of ENO1 oligonucleotide as competitor. Reactions were electrophoresed in a 3% NuSieve agarose gel, dried, and autoradiographed. Numbers in the left margin indicate the size and position of φX174 *Hae*III DNA markers.

binding ability in either gel electrophoresis mobility shift or filter-binding assays (12). mtIIB is the only oligonucleotide we have found that exhibits different binding properties in the two assays. A mutation was also introduced into the consensus sequence region of the ENO1 oligonucleotide. The C residue in the sixth position of the consensus sequence was changed to A in ENO1mtA (Table 2), which was the same alteration as in mtIIA. Like mtIIA, ENO1mtA did not compete for GRFI binding in the filter-binding assay (Table 2). Also, only very low levels of GRFI binding were detected with an ENO1mtA probe in gel electrophoresis mobility shift assays (Fig. 4).

GRFI binding sites provide UAS function in vivo. The sequences that GRFI binds in vitro are located in regions with very different functions in vivo—silencers, UASs, telomeres, and transcribed regions—making it difficult to define the role GRFI plays in gene expression or chromosome maintenance. Many of the binding sites that have been identified are located in UAS elements. Most strikingly, at the *MAT*α genes the GRFI binding site coincides precisely with a 12-bp region that is a bidirectional UAS required for the expression of the α1 and α2 regulatory proteins (12, 44). This result strongly suggests that GRFI can function intrinsically as a positive transcription factor when bound upstream of a promoter. We pursued this idea further by testing the ability of GRFI-binding oligonucleotides to provide UAS function, regardless of whether these sequences are normally located at a UAS, silencer, telomere, or transcribed region. Oligonucleotides were placed in two plasmids containing different reporter genes. The pCT plasmids (Fig. 5A) contained a *CYC1-lacZ* (promoter-coding region) fusion, and the pGH plasmids (Fig. 5B) contained a *GAL1-HIS3* fusion.

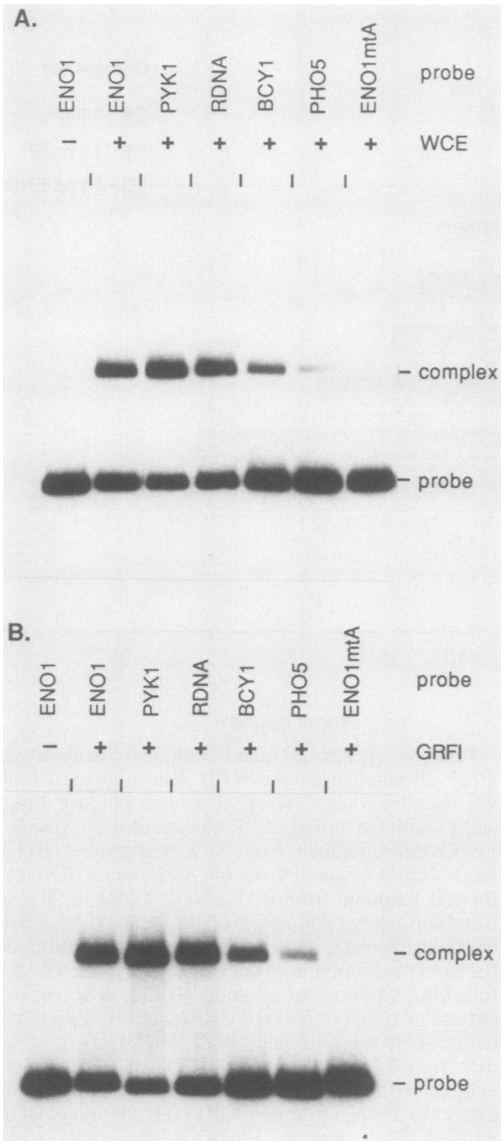


FIG. 4. Purified GRFI from the oligonucleotide-Sepharose chromatography was compared to GRFI in whole-cell extracts (WCE) by gel electrophoresis mobility shift assays with five different ^{32}P-labeled probes: 0.3-kbp *Bam*HI fragments from pCT-ENO1, -PYK1, -RDNA, -BCY1, -PHO5, and -ENO1mtA. Binding reactions contained 10 ng of an oligonucleotide that binds Y factor (Fedor et al., in press) in order to block the formation of these complexes with the whole-cell extract. (A) Reaction mixtures contained 2 μg of whole-cell extract. (B) Reactions contained 0.4 ng of purified GRFI. Products were analyzed by electrophoresis in 3% NuSieve agarose gels, which were then dried and autoradiographed.

In both plasmids, the oligonucleotides were inserted 210 bp upstream of the site of transcription initiation, next to a promoter lacking a UAS. Each of these plasmids contains a yeast centromere sequence (CEN4), causing them to be maintained at about 1 copy per cell. If the inserted oligonucleotide is bound by GRFI in vivo and if GRFI can intrinsically provide UAS function, then all of the oligonucleotides that bind GRFI in vitro would be expected to increase transcription of the reporter gene. This would be revealed by an increase in β-galactosidase enzyme activity from the pCT plasmids or complementation of histidine auxotrophy by the pGH plasmids.

Cells of the yeast strain 5C (**a** *his3 Δ200 ura3-52*) were transformed with the pCT and pGH plasmids, selecting for uracil prototrophy. Transformants were assayed either for β-galactosidase activity or histidine prototrophy (Table 2; the behavior of the pGH plasmids paralleled those of the pCT series, so they are not discussed in the text). All constructs containing oligonucleotides that bound GRFI in vitro showed significant UAS function in vivo. Most showed at least 10-fold-higher levels of expression than did the control plasmid with no insert, pCTΔ (0.69 U), and many had levels of expression comparable to pCT-GAL4 (70 U), which contained an oligonucleotide with a single binding site for the *GAL4* regulatory protein (8). The increased expression of these plasmids resulted from increased transcription at the normal initiation sites for the *GAL1* and *CYC1* promoters (data not shown [30]; A. R. Buchman and N. Lue, unpublished data). A rough correlation was observed between the in vitro binding affinity of oligonucleotides and UAS function. The two oligonucleotides with the lowest binding affinity for GRFI (HML35 and PHO5) were the weakest UASs. Most importantly, mutations that greatly reduced or abolished GRFI binding (HML29, mtIIA, mtIIB, and ENO1mtA) lowered UAS function to the background level (pCTΔ).

Sequences flanking the GRFI binding site at *HMR E* convert this region to a transcriptional silencer. It was particularly striking that the GRFI binding sites from transcriptional silencers at *HML E* (HML35) and *HMR E* (EII) provided UAS function, even though these regions normally act to greatly repress transcription. To eliminate the possibility that the test plasmids were responding abnormally to transcriptional regulatory sequences, we inserted larger restriction fragments from *HMR E* into pCT and pGH constructions (Fig. 5C). The silencer at *HMR E* is composed of at least three different elements: an *ARS*, a GRFI binding site, and an ABFI binding site (11, 12, 43) distributed over a 100-bp region (Fig. 5C). A 265-bp restriction fragment containing *HMR E* gave no UAS activity when inserted into pCT or pGH (pCT-E265 and pGH-E265, Fig. 5C and Table 2). *HMR E* DNA also silenced expression from pCT-Alpha when placed in either orientation upstream of the Alpha UAS (pCT-EN/Alpha and pCT-EO/Alpha; Fig. 5C and Table 2). In plasmid pCT-EOmtIIA/Alpha, the *HMR E* GRFI binding site in pCT-EO/Alpha was mutated with a single base change that abolishes binding (the same as in the mtIIA oligonucleotide). The expression of pCT-EOmtIIA/Alpha was 100-fold greater than the plasmid with the normal silencer fragment, pCT-EO/Alpha and 11% that of the plasmid with no silencer DNA, pCT-Alpha. These results agree with previous studies of the role of the GRFI binding site at *HMR***a** (11, 27a) and demonstrate that the pCT plasmids respond normally to transcriptional control sequences. It is interesting that the same base change in a GRFI binding site had opposite effects on gene expression in two different contexts (pCT-EII and pCT-mtIIA versus pCT-EO/Alpha and pCT-EOmtIIA/Alpha). This difference can be understood in terms of GRFI activating the adjacent promoter in pCT-EII or the surrounding silencer in pCT-EO/Alpha through interactions with different regulatory factors at these elements.

Sequences flanking the GRFI binding site at *ENO1* and *PYK1* enhance UAS function. Although there was a good correspondence between UAS function and the binding affinity of oligonucleotides for GRFI, the correlation was not perfect. For example, the RDNA oligonucleotide had a higher affinity for GRFI in vitro than did the EII oligonucle-

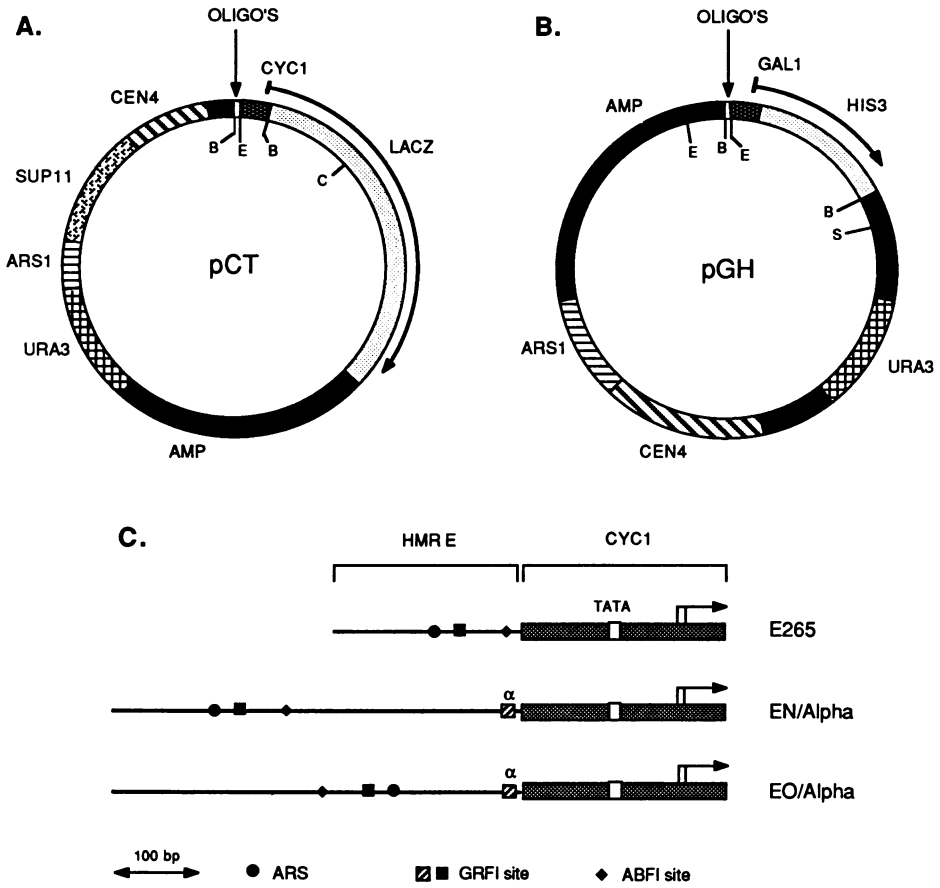


FIG. 5. (A) Structures of the pCT plasmids (9 kbp) are depicted with different segments represented as follows: ■, pUC18; ■, *CYC1* promoter; ▨, *lacZ* coding region; ▩, *URA3*; ⊡, *SUP11*; ▧, *CEN4*; ▤, *ARS1*; □, polylinker region and oligonucleotides. The arc with arrow shows the transcribed region of the *CYC1-lacZ* gene fusion. Restriction enzyme cleavage sites used for constructing these plasmids and for producing DNA probes are abbreviated: B, *Bam*HI; C, *Cla*I; E, *Eco*RI. The polylinker region has the following configuration of restriction sites: *Hin*dIII-*Sph*I-*Pst*I-*Sal*I-*Xba*I-*Bam*HI-*Eco*RI-*Sac*I-*Kpn*I-*Sma*I-*Xho*I. (B) Structures of the pGH plasmids (8.9 kbp) are depicted with different segments represented as follows: ■, pBR322; ■, *GAL1* promoter; ⊡, *HIS3* coding region; ▩, *URA3*; ▧, *CEN4*; ▤, *ARS1*; □, oligonucleotides and polylinker region. The arc with arrow shows the transcribed region of the *GAL1-HIS3* gene fusion. Restriction enzyme sites used to construct these plasmids and to produce DNA probes are abbreviated: B, *Bam*HI; E, *Eco*RI; S, *Sal*I. (C) An enlarged view of the *CYC1* promoter and upstream region is shown for the plasmids pCT-E265, -EN/Alpha, and -EO/Alpha. The arrow indicates the transcription initiation sites. □, Major TATA sequence; ■, different elements of the silencer at *HMR E*; ▨, Alpha (*MAT*α) GRFI binding site.

otide, yet it was a weaker UAS (Table 2). Small discrepancies such as this were expected, since factors other than just the binding affinity might significantly affect UAS activity, such as the precise geometry of the binding site with respect to the promoter or the fortuitous creation of other protein binding sites in the construction. Even so, the level of UAS activity of the ENO1 and PYK1 constructions appeared anomalously large compared to the other high-affinity oligonucleotides. Expression of plasmids carrying the PYK1 and ENO1 oligonucleotides was 5- to 10-fold greater than plasmids with Alpha, TEF2, or LSR1 and was over twice that of TEL, which has two GRFI binding sites. UAS regions in yeast, like enhancers in higher eucaryotes, are often compound elements composed of multiple binding sites for the same or several different transcription factors. Deletion analysis of the *ENO1* promoter suggests that this gene has a compound UAS (14, 48). We noticed that the ENO1 and PYK1 sequences shared a common heptanucleotide sequence, 5′-GCTTCCA-3′, on opposite sides of the GRFI consensus region (indicated by the lines above the sequences in Table 2). Others have suggested that the related sequence motif 5′-CTTCC-3′ is a UAS element common to genes involved in glycolysis (34). Accordingly, we investigated the importance of sequences flanking the GRFI consensus region of PYK1 and ENO1 by synthesizing altered forms of these oligonucleotides, ENO1mtB, ENO28, and PYK1mtA (Table 2), which were assayed for GRFI binding in vitro and UAS function in vivo. PYK1mtA, ENO1mtB, and ENO28 all showed reduced UAS function compared with the wild type sequences (Table 2). The amount of expression observed with the flanking sequence alterations was still significantly above the background level of pCTΔ and was within the range of the other high-affinity GRFI sequences. By contrast, alterations in the regions flanking the GRFI consensus sequence had no measurable effect on GRFI binding in vitro. The mutant oligonucleotides did not differ significantly from the wild-type sequences in the standard filter-binding competition assay (Table 2). Also, when tested as labeled probes for GRFI binding in gel electrophoresis mobility shift assays, no decrease in binding affinity was detected for the probes with flanking sequence alterations (Fig. 6). Thus, sequences adjacent to the GRFI binding sites at ENO1 and PYK1 significantly enhance UAS function without affecting GRFI binding affinity. Comparison of the

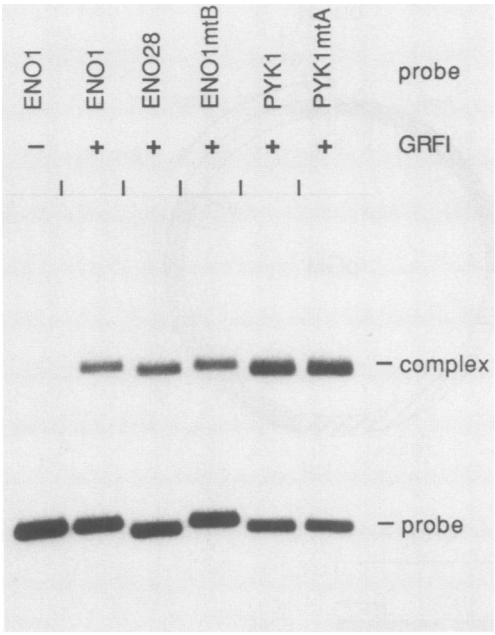


FIG. 6. The effects of alterations in regions flanking the GRFI binding sites in PYK1 and ENO1 were measured in gel electrophoresis mobility shift assays. Each assay contained 0.4 ng of purified GRFI. Probes were ^{32}P-labeled *Bam*HI fragments from pCT-ENO1, -ENO28, -ENO1mtB, -PYK1, and -PYK1mtA. Products were analyzed by electrophoresis in a 3% NuSieve agarose gel, which was then dried and autoradiographed.

UAS activity of ENO1, ENO1mtA, ENO1mtB, and ENO28 suggests a synergistic interaction between the GRFI consensus region and the flanking DNA sequences (Table 2).

GRFI binding sites specifically stimulate transcription in vitro. Previously, we characterized the properties of a yeast nuclear extract system that accurately initiated transcription at the *CYC1* and *PYK1* promoters (30). Specific transcription in vitro required TATA sequences in the template, but was not affected by the presence or absence of a GRFI binding site upstream, in contrast to the behavior of the same templates (pCT-PYK1 and pCTΔ) in vivo. There were several possible explanations for this discrepancy. First, we found that GRFI in nuclear extracts was substantially degraded due to contamination with protease from the enzyme used to lyse yeast cell walls. Also, studies of another UAS element, a thymidine-rich sequence at the *DED1* promoter, revealed that the stimulatory effect of this sequence in vitro was significantly increased by moving the element very close to the TATA box (Lue et al., in press). Accordingly, changes were made in the nuclear extract preparation to reduce the activity of proteases, and different templates, pCZ-TEL and pCX-TEL, were constructed that have multiple GRFI binding sites placed just 60 and 24 bp, respectively, upstream of the *CYC1* TATA sequence. A control template, pCXΔ, was also made that lacks the GRFI binding sites of pCX-TEL. Nuclear extract was pretreated with different competitor oligonucleotides (0.3 μg) and then incubated with template DNAs (0.3 μg) under conditions permitting transcription in vitro. Transcription from the *CYC1* promoter was measured by an RNase protection assay. The products of each transcription reaction were annealed to complementary ^{32}P-labeled RNA probes, digested with RNase, and analyzed by gel electrophoresis (Fig. 7). The presence of GRFI binding sites in the template (pCZ-TEL and pCX-TEL) caused a sixfold increase in transcription relative to the control template (pCXΔ) when the competitor oligonucleotide did not bind GRFI: ENO1mtA, SNR47, and HIS35 (Lue et al., in press). Preincubation of the extract with competitors capable of binding GRFI (TEL and ENO1) reduced the level of transcription of templates with GRFI binding sites (pCZ-TEL and pCX-TEL), but not of the control template (pCXΔ). Hence, transcription of these templates in vitro was specifically stimulated in a GRFI-dependent manner.

DISCUSSION

Properties of GRFI. Our studies with purified GRFI suggest that a single protein binds sequences at very different genetic elements. Direct evidence for this point comes from the work of Shore and Nasmyth (41). These investigators demonstrated that a single polypeptide encoded by the yeast *RAP1* gene binds the silencers at *HML* and *HMR*, the UAS at the *MATα* genes, and ribosomal protein gene box sequences. The size predicted for RAP1 protein from the DNA sequence was 93 kDa; however, Shore and Nasmyth found that the protein migrated anomalously on SDS-PAGE with an apparent size of 120 kDa, a finding in good agreement with our estimate of 116 kDa for GRFI. Combining the present results with those of Shore and Nasmyth, we conclude that a single protein, GRFI or RAP1, binds with high affinity to specific sequences located at silencers, telomeres, transcribed regions, and the UASs of a wide variety of genes. We have found no evidence for multiple factors with related or overlapping specificities. Estimates indicate that there is sufficient GRFI in vivo to occupy the array of sites detected in vitro. Previous measurements with an *HMR E* DNA probe showed that there were at least 1,000 molecules of GRFI per haploid cell (12). By using probes with higher-affinity binding sites (e.g., ENO1 [K_d, 2.5×10^{-11} M; measurements not shown]), this figure can be adjusted to at least 4,000 molecules per cell. Surveys conducted by our group show that GRFI is one of the most abundant specific DNA-binding factors that can be detected in yeast extracts. ABFI (12) and CP1 (9) are other members of this highly abundant class of factors.

Remarkably, our guesses about the specific sequences recognized by GRFI have been completely substantiated. All occurrences of the sequence 5′-RMACCCANNCAYY-3′ were binding sites for the protein in vitro. Some sequences that differ slightly from this consensus sequence, such as those at *HMR E* (EII) and *HML E* (HML35) can also bind GRFI with reasonably high affinity (Table 2). Thus, it is likely that other divergent forms of the consensus sequence are targets for GRFI action in vivo, such as the HOMOL1 box element found upstream of ribosomal protein genes (22, 46). The binding properties of sequences at *HMR E* (EII and mtIIB) and *HML E* (HML35 and HML29) indicate that sequences flanking the consensus region can affect GRFI binding (Table 2). On the other hand, mutations adjacent to the consensus region of PYK1 and ENO1 did not reduce GRFI binding. We interpret this as indicating that GRFI interacts primarily with nucleotides of the consensus region; however, when contacts with the consensus region are suboptimal, interactions with flanking sequences become more important for binding.

Functions of GRFI. Considering the array of different GRFI binding sites, it does not seem that GRFI has a unique or specialized function. Nonetheless, GRFI appears to possess the intrinsic capacity to function as a positive transcrip-

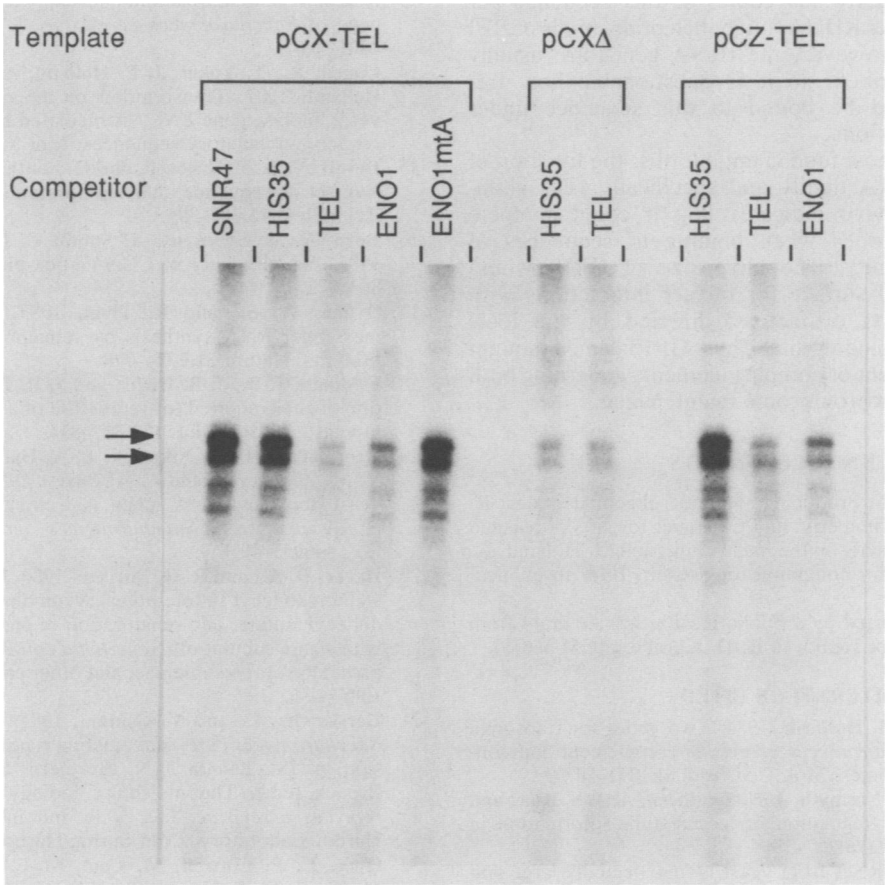


FIG. 7. In vitro transcription reactions were done by incubating nuclear extract, first with competitor oligonucleotides (0.3 μg) and then with template DNA (0.3 μg) and ribonucleoside triphosphates. Products were analyzed by hybridization to ^{32}P-labeled RNA probes, digestion with RNase, and electrophoresis in a 7% polyacrylamide-urea gel, which was then dried and autoradiographed. Arrows indicate the protected fragments generated by transcripts initiated at the *CYC1* promoter.

tion factor when bound upstream of a yeast promoter. All GRFI binding sites showed at least modest levels of UAS activity, and all mutations that abolished binding decreased UAS function to background levels. Furthermore, GRFI binding sites specifically stimulated transcription in an in vitro reaction with yeast nuclear extract. Promoters that contain GRFI binding sites are regulated in different ways in vivo. Hence, GRFI can be compared to the more general class of transcription factors in higher eucaryotes that bind regulatory sequences, such as SP1 (17), CCAAT box binding factors (16, 26, 35), and octamer sequence binding factors (38, 45). Analysis of the promoters of *ENO1*, *RP39A*, and *LSR1* genes has demonstrated a composite UAS structure (14, 39; A. R. Buchman, unpublished data). We observed a strong synergistic relationship between the GRFI binding region and the flanking DNA sequence of *ENO1*, suggesting an interaction between GRFI and another DNA-binding factor. So far, we have been unable to detect an additional factor that binds the ENO1 oligonucleotide. If it exists, this other factor may be much less abundant than GRFI or may not be revealed by the assays we have used.

Like many UAS regions, silencers are multicomponent elements. The silencer at *HMR E* contains an *ARS*, an ABFI binding site, and a GRFI binding site. In addition, proteins encoded by the four *SIR* genes are required for silencer function (37). Analysis of deletions and point mutations has shown that silencer activity is reduced in the absence of a GRFI binding site (11, 27a). Silencers that lack both a GRFI binding site and an ABFI binding site are completely nonfunctional. So, it is presumed that the *SIR* proteins interact with GRFI or ABFI in order to establish the repressed state. However, it remains unclear how the *SIR* proteins act specifically at *HML* and *HMR* and avoid interfering with other regions with ABFI or GRFI binding sites. There may be other elements within the silencer that contribute to *SIR* specificity. Reconstruction experiments indicate that a fully functional silencer requires more than just an *ARS*, an ABFI site, and a GRFI site (27a).

The role of GRFI at telomeres has yet to be defined. As with the TEL oligonucleotide, larger fragments of the $C_{1-3}A$ repeat (40 to 81 bp) provide UAS function in test plasmids (K. Runge and V. Zakian, personal communication). There are no reports, however, of transcripts emanating from the conserved telomere elements that flank the $C_{1-3}A$ region. In lieu of a regulatory function for GRFI, a role in maintaining telomere structure seems plausible. In binding to the $C_{1-3}A$ sequence, GRFI may retard its destruction by nucleases or prevent its packaging in nucleosomes, giving other proteins that maintain the $C_{1-3}A$ sequence access to this region.

It is, perhaps, most difficult to understand the significance of the GRFI binding sites within transcribed regions. The *PHO5* site might easily be dismissed as fortuitous, due to its low binding affinity and low level of UAS function. But the binding site in the rRNA gene is not as readily discounted.

There are approximately 120 copies of this sequence per haploid cell, and the RDNA oligonucleotide binds GRFI with high affinity. However, the rRNA genes are usually very heavily transcribed, so it is questionable how frequently GRFI would be bound to this sequence under normal growth conditions.

Of course, in the most fundamental terms, the function of GRFI is to bind DNA tightly and specifically. The abundance of GRFI in vivo indicates that it could compete effectively with histones when binding to sequences of reasonably high affinity. The large size of GRFI would provide a substantial surface for further interactions with many additional sorts of factors, directed by the local context of the GRFI binding site. Thus, GRFI can be thought of as a basic constituent of complex elements governing both gene expression and chromosome maintenance.

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