A yeast protein that influences the chromatin structure of UAS$_G$ and functions as a powerful auxiliary gene activator

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GRF2, an abundant yeast protein of M_r ~127,000, binds to the GAL upstream activating sequence (UAS$_G$) and creates a nucleosome-free region of ~230 bp. Purified GRF2 binds to sequences found in many other UASs, in the 35S rRNA enhancer, at centromeres, and at telomeres. Although GRF2 stimulates transcription only slightly on its own, it combines with a neighboring weak activator to give as much as a 170-fold enhancement. This effect of GRF2 is strongly distance-dependent, declining by 85% when 22 bp is interposed between the GRF2 and neighboring activator sites.

Studies of eukaryotic chromosomes have revealed an interplay between sequence-specific and nonspecific DNA-binding proteins. Highly sequence-specific proteins, often present in small amounts, serve as activators and repressors of many genes. At the same time, nearly all of the DNA in a chromosome is associated with histones in the form of nucleosomes, independently of the DNA sequence and with no known regulatory function. These two types of protein–DNA interaction may be mutually exclusive. Sites of specific protein binding often lie within histone-free "nuclease-hypersensitive" regions in vivo (for review, see Elgin 1988). Histones have been shown to compete with some sequence-specific proteins for DNA binding and to prevent initiation by RNA polymerase II in vitro (Wasylyk and Chambon 1979; Gottesfeld and Bloomer 1982; Knezetic and Luse 1986; Lorch et al. 1987; Matsui 1987; Workman and Roeder 1987). How are the conflicting requirements of sequence-specific and nonspecific DNA-binding proteins reconciled? It has been suggested that proteins present at the time of DNA synthesis gain access to the DNA, and the resulting associations are essentially irreversible. Alternatively, there may be special proteins that influence the accessibility of the DNA, or there may be mechanisms of remodeling chromatin structure, so that the final state is determined by relative affinities rather than by relative rates of binding. We report here on a yeast protein, referred previously to as factor Y, whose function may relate to these issues.

Factor Y was discovered (Fedor et al. 1988) during studies of specific protein binding to UAS$_G$, a 100-bp stretch of DNA between the GAL1 and GAL10 genes of yeast that is required for transcriptional activation in the presence of galactose (Johnston and Davis 1984; West et al. 1984). UAS$_G$ contains four binding sites for the gene activator protein GAL4 (Bram and Kornberg 1985; Giniger et al. 1985). One of these four sites also binds factor Y, which was distinguished from GAL4 by its abundance in extracts made from a gal4 deletion strain and by challenge with synthetic oligonucleotides that bind only factor Y or only GAL4. From digestion experiments on yeast minichromosomes, it was inferred that factor Y creates a nucleosome-free region of ~230 bp containing UAS$_G$, flanked by an array of nucleosomes in defined locations on either side. This effect of factor Y does not depend on other sequences at UAS$_G$, because a factor Y-binding oligonucleotide interposed between unrelated sequences gave the same structure of chromatin as that observed at UAS$_G$ (Fedor et al. 1988). Factor Y is not required for UAS function, because a single GAL4-binding site that does not bind factor Y will suffice for gene activation. Therefore, the question arises concerning what functional purpose may be served by the nuclease-hypersensitive site created by factor Y, and whether this action occurs more generally in the yeast genome or is limited to UAS$_G$. We now find that factor Y-binding sites are associated with many UASs and other functional DNA elements in yeast and that factor Y greatly augments transcriptional stimulation by a neighboring thymidine-rich sequence in a manner consistent with its effects on chromatin structure. In light of these functional consequences of factor Y-binding, we

have renamed the protein general regulatory factor 2 (GRF2).

Results

Candidate sites for GRF2 binding

The abundance of GRF2 in yeast extracts suggested a role for the protein at other chromosomal locations besides UAS$_G$. Preliminary evidence pointed to GRF2 binding at sites near *CEN4* and upstream of the *PYK* gene (N.F. Lue, unpubl.). By comparing the sequences of UAS$_G$, *CEN4*, and the *PYK* gene, we derived the consensus binding sequence 5'-YNNYYACCCG-3'. A computer-assisted search of a data base of yeast sequences revealed 99 matches to this sequence or its complement. Matches were chosen for further study when there was genetic evidence for functional significance of the site, when the match to the consensus was of particular interest to us, or when the match was associated with a structural element (e.g., the centromere or the telomere). On this basis, candidate sites for GRF2 binding were selected from the regions upstream of the *ACT* (Gallwitz et al. 1981), *TRP1* (Dobson et al. 1984), *TRP3* (Zalkin et al. 1984), *TRP5* (Zalkin and Yanofsky 1982), *PYK* (Nishizawa et al. 1989), *RAP1* (Shore and Nasmyth 1987), and *GAL10* (Johnston and Davis 1984; West et al. 1984) genes, from the 35S rRNA enhancer (Morrow et al. 1989), from telomeres (Shampay et al. 1984), and from *CEN4* (Mann and Davis 1986) (Fig. 1; Table 1). All candidate sites upstream of polymerase II promoters were in the same orientation with respect to the direction of transcription.

Variations of the consensus sequence were also considered. Inspection of the DNA sequence near the candidate site upstream of the *TRP1* gene revealed a neighboring site, ~30 bp away, with a single mismatch to the consensus sequence (a G in the fourth position). Others have reported that a protein that bound to the candidate site in the 35S rRNA enhancer also bound to a site in the promoter with a single mismatch to the consensus sequence [a G instead of a pyrimidine in the most 5' position (Morrow et al. 1989)]. Finally, we noticed that the X region of telomeres contains many near matches to the consensus sequence, differing only in having a T in the most 3' position (Walmsley et al. 1984). When a second search of the data base was performed with the sequence 5'-YNNYYACCCT-3', 186 matches were found. From this search, one 40-bp sequence from the X region of telomeres with tandemly repeated matches to the consensus sequence was chosen for further study (Fig. 1; Table 1).

Purification of GRF2

To establish that candidate sites were bound by a single protein, GRF2 was purified to homogeneity. The purification was monitored by filter-binding and gel mobility-shift assays. A whole-cell extract from the protease-deficient yeast strain BJ926 was fractionated on Matrex Red

and quaternary amine columns. A procedure was then developed to isolate GRF2 from this partially fractionated material on an affinity resin consisting of the binding site for GRF2 from *CEN4* covalently coupled to Sepharose 2B-CL. To avoid contamination by proteins that bind DNA independently of sequence, the partially fractionated material was passed through a column of sheared salmon sperm DNA coupled to Sepharose and was applied to the affinity column under conditions of comparatively high ionic strength. Preliminary filter-binding experiments revealed that GRF2 would bind specifically to its site in 200 mM KCl, so the nonspecific DNA column and the specific affinity column were loaded at this salt concentration. The affinity column was washed with 300 mM KCl and eluted with 1 M KCl. SDS-PAGE of the eluate revealed a prominent species of 127 kD but also many additional proteins (not shown). The material was reapplied to the affinity column in the presence of 0.15 mg/ml poly[d(I-C)] and 200 mM KCl, and eluted as before. After the second pass over the affinity column, only a single polypeptide of 127 kD remained (Fig. 2a). Filter-binding assays demonstrated a 6500-fold enrichment of GRF2 in a yield of 0.34% for the entire procedure (Table 2). These data lead to an estimate of 1000–2000 molecules of GRF2 per diploid cell.

The binding activity and integrity of the isolated polypeptide were characterized in two ways. First, GRF2 binding activity was shown to reside in the 127-kD polypeptide. An SDS-polyacrylamide gel was divided into 16 slices, and proteins eluted and renatured from each slice were analyzed in filter-binding assays with a probe derived from UAS$_G$. Binding activity was found only in the slice corresponding to the range 121–141 kD, with a 24% recovery of total activity applied to the gel (Fig. 2b). Second, the binding activities in the whole-cell extract, in the purified material, and in the material eluted from the gel were analyzed by a gel electrophoresis mobility-shift assay, with a probe derived from UAS$_G$. All three preparations gave the same gel shift, indicating that no substantial proteolysis occurred during the purification, and again that the GRF2 binding activity was uniquely associated with the 127-kD polypeptide. Further evidence that the GRF2 binding activities in the three preparations were identical came from competitive inhibition of binding to the UAS$_G$ probe in all cases by an oligonucleotide containing the candidate site for GRF2 binding from upstream of the *TRP5* gene (Fig. 2c).

GRF2 binding to candidate sites

Oligonucleotides representing each of the candidate sites for GRF2 binding (Table 1) were inserted between the *Bam*HI and *Eco*RI sites of the polylinker in the shuttle vector pCZ. A 220-bp *Bam*HI fragment of the resulting plasmids, with the oligonucleotide at one end, was used as probe for gel mobility-shift assays. All candidate sites bound purified GRF2, and, in all cases tested except for the PYK site, the mobility shift that resulted from the purified protein was the same as that given by whole-cell extract (Fig. 3). The PYK probe formed two
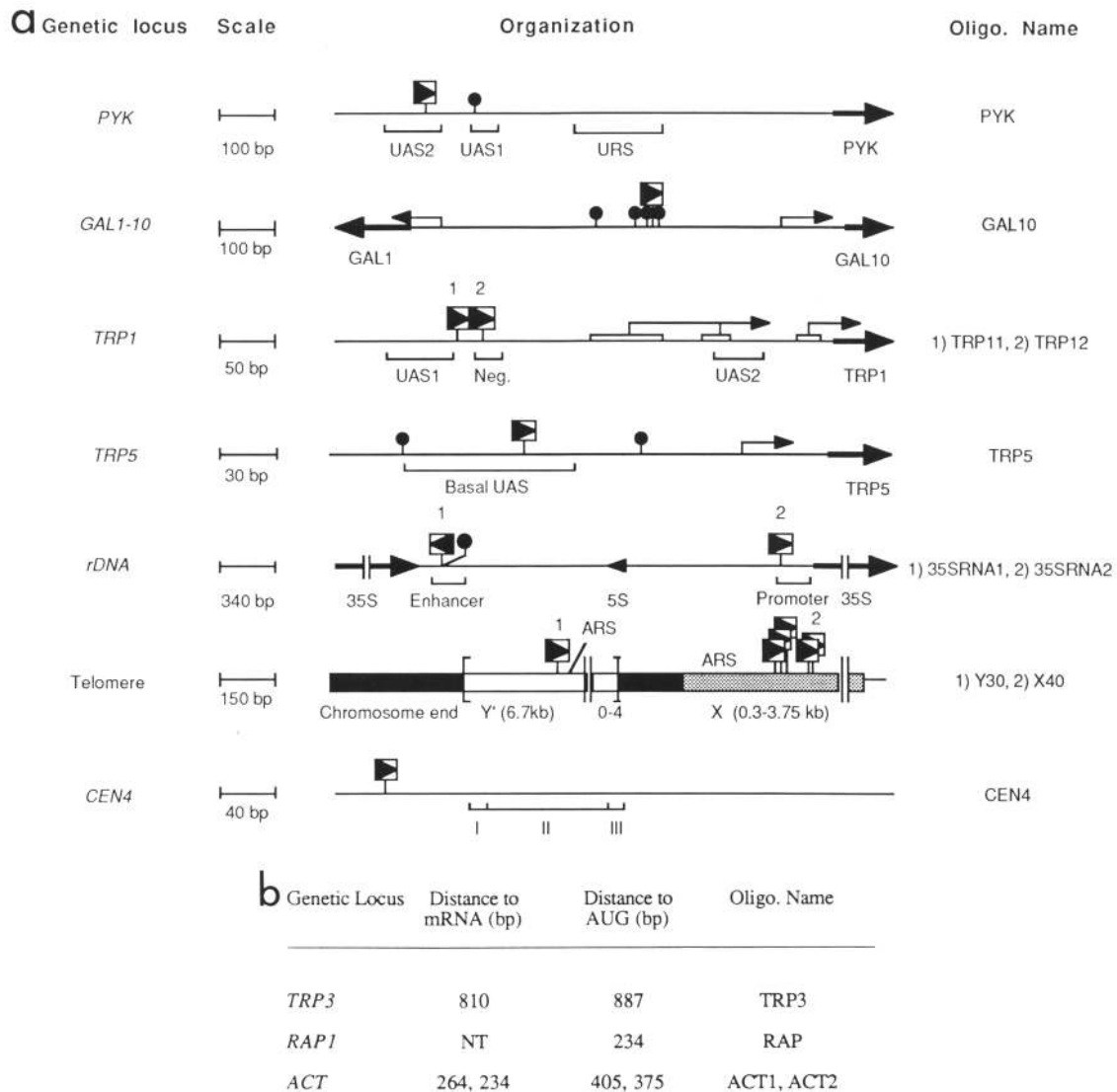
Figure 1. GRF2-binding sites. (*a*) GRF2-binding sites associated with functionally defined elements. Orientations of GRF2-binding sites are indicated with a rightward-pointing triangle corresponding to the consensus sequence in the coding strand (or strand with 3′ end at the right). Functionally defined elements are as follows: [*Pyruvate kinase (PYK)* gene] two UAS elements, an upstream repression element (URS), and a binding site for GRF1 (TUF/RAP1) (Buchman et al. 1988b; Nishizawa et al. 1989); (*GAL1–GAL10* intergenic region) four GAL4 protein-binding sites (Johnston and Davis 1984; West et al. 1984); (*TRP1* gene) UAS1 and a negative element, which regulate expression of the upstream set of transcripts, and UAS2, which regulates expression of the downstream set of transcripts (Dobson et al. 1984; Kim et al. 1986); (*TRP5* gene) two GCN4 protein-binding sites and, between these sites, a UAS responsible for basal level transcription of the *TRP5* gene (Moye and Zalkin 1987); (rDNA genes) the 35S RNA enhancer and promoter, the 5S RNA gene, and a binding site for ABF1 in the enhancer (Buchman and Kornberg 1990; Morrow et al. 1989; see text); (telomere) $C_{1-3}A$ repeat sequences (black bar), the Y′ region (open bar) with its ARS consensus sequence, and the X region (shaded bar) with its ARS consensus (Shampay et al. 1984; Walmsley et al. 1984; Button and Astell 1986). Positions of some matches to the GRF2 consensus sequence that were not tested for binding are also shown (not marked with a numeral). [Centromere 4 (*CEN4*)] conserved centromere DNA element (CDE) I, which binds CP1 (Bram and Kornberg 1987; Cai and Davis 1989), CDE II, which is rich in A and T, and CDE III, most important for centromere function (Hegemann et al. 1988). (*b*) Other GRF2-binding sites. Positions of GRF2 sites are given with respect to the transcription start site (distance to mRNA) and the translation start site (distance to AUG). Positions were determined from the sequences of *TRP3* (Zalkin et al. 1984), *RAP1* (Shore and Nasmyth 1987), and *ACT* (Gallwitz et al. 1981). All sites have the GRF2 consensus sequence in the coding strand. (NT) Not tested; (▶) GRF2 binding sites; (●) Binding sites for other proteins; (➡) Coding sequences; and (┌➡) RNA transcripts.

gel mobility-shift bands, indicative of a second protein factor binding. The PYKL1 probe, however, containing the same GRF2-binding site in the opposite orientation and with some flanking sequences, did not appear to bind a second protein factor. From these observations and further evidence (data not shown), we conclude that the second factor bound at the *Eco*RI junction between the oligonucleotide and vector DNA. The X40 oligonucleotide, with multiple matches to the GRF2 consensus sequence, bound only one molecule of GRF2.

Table 1. *Binding properties, intrinsic UAS activity, and sequences of the oligonucleotides*

Oligonucleotide[a]	Relative affinity[b]	β-gal (U/mg)[c]		Orientation in pCZ or pCT[d]	Sequence[e]
		pCZ	pCT		
					YNNYYACCCG
35SRNA2	1	NT[f]	NT	NA[f]	GATCCGGAACT**GGGTTACCCG**GGGCAC
Y30	0.78	2.8	NT	+	GATCCTGTTGT**CTCTTACCCG**GATGTTCAG
X40	0.68	2.3	1.5	+	GATCCTCACTG**CACTTACCCT**GCCATTACCCTACCATCG
35SRNA1	0.64	NT	NT	NA	GATCCTACATG**TTTTTACCCG**GATCATG
ACT1	0.51	2.9	NT	+	GATCCATTTCT**CTGTCACCCG**GCCTCG
TRP5	0.24	1.5	NT	−	AATTCTATACCT**TTATTACCCG**AAAGGG
TRP5N	NT	2.2	NT	+	GATCCTATACCT**TTATTACCCG**AAAGGG
RAP1	0.22	2.3	2.2	+	GATCCCAATGG**CCGCTACCCG**CCTCTTG
RAPmtA	<0.00014	0.62	NT	+	GATCCCAATGG**CCGCTACCtG**CCTCTTG
RAPmtB	<0.00008	0.63	NT	+	GATCCCAATGG**CCGCTACtCG**CCTCTTG
CEN4	0.16	2.4	1.1	−	AATTTATATG**TATTTACCCG**GCGAAT
TRP12	0.14	16	NT	+	GATCCACGCGC**CGCTCACCCG**CACGGG
GAL10	0.10	2.8	NT	−	AATTCCTTCGGAGGG**CTGTCACCCG**CTCGGCGG
PYK	0.05	0.58	NT	−	AATTCAGATTT**CGCTACCCG**AAAGTG
PYKL1	0.02	4.1	NT	+	GATCCTCCGGCAGAAAGATTT**CGCTACCCG**AAAGTG
PYKL2	NT	25	NT	+	GATCCAGATTT**CGCTACCCG**AAAGTTTTTCCGGCAG
ACT2	0.02	4.1	NT	+	GATCCTTTTCT**TCTTTACCCG**CCACGG
TRP3	<0.004	4.6	NT	−	AATTCCTTTATT**TGCCACCCG**CACCTG
TRP11	0.0003	0.87	NT	+	GATCCAAGAAA**CGTgCACCCG**CCCGTG
Δ	NA	0.76	NT	NA	
DED48	NA	4.1	3.7	NA	AATTCTTTCCTTTTTTCTTTTTGCTTTTTCTTTTTTTTTCTCTTGAAC

[a]Oligonucleotides beginning 5'-GATC had complementary strands beginning 5'-AATT and terminating to leave the 5'-GATC of the first strand protruding. Oligonucleotides beginning 5'-AATT had complementary strands beginning 5'-GATC and terminating to leave the 5'-AATT of the first strand protruding. The complement to DED48 began 5'-TCGA and left the 5'-AATT of the first strand unpaired to allow insertion between the *Eco*RI and *Xho*I sites of the polylinker.

[b]Relative affinity refers to the amount of each oligonucleotide needed to compete 50% of the binding in a standard filter-binding reaction expressed as a ratio in comparison to the highest affinity oligonucleotide, 35SRNA2.

[c]β-gal lists the expression of β-galactosidase from pCZ or pCT constructs with the indicated oligonucleotide inserted into the polylinker.

[d]In +(−) orientation, the GRF2 consensus was in the coding (noncoding) strand in pCZ or pCT.

[e]The consensus and sequences related to the consensus within each oligonucleotide are indicated by boldface type. Mismatches to the consensus are indicated by lowercase letters. A second binding site for GRF2 in X40 is underlined.

[f](NT) Not tested; (NA) not applicable.

The results of the gel mobility-shift analysis allowed an estimate to be made of the relative affinities of the various sites for GRF2. The ratio of bound to total probe was determined, leading to the following order of affinities: Y30 > ACT1 > RAP1 > X40 > TRP5 > TRP12 > ACT2 = PYK = PYKL1 > RAPmtA = RAPmtB = TRP11. The affinities of the weak binding sites, TRP11 and TRP3, and the two mutant binding sites, RAPmtA and RAPmtB, were significantly overestimated, because of the presence of a fortuitous weak binding site for GRF2 overlapping the *Sma*I and *Kpn*I sites of the polylinker in pCZ. This binding sequence, 5'-TCGG-TACCCG-3', differed from our consensus in the fourth position but had detectable affinity for GRF2 in filter-binding reactions (N.F. Lue, unpubl.).

A more quantitative comparison of the affinities of

the binding sites was derived from filter-binding experiments in which the oligonucleotides were added in various amounts as competitors of GRF2 binding to a fixed amount of probe from pCZTRP12. Poly[d(I-C)] was also added to keep the total DNA concentration constant. The affinities of the sites, judged from the amounts required for 50% inhibition of binding to probe, varied over a 50-fold range (Table 1). The order of affinities was 35SRNA2 > Y30 > 35SRNA1 > ACT1 > X40 > TRP5 > RAP1 > CEN4 > TRP12 > GAL10 > PYK > ACT2 > PYKL1 > TRP3 > TRP11 > RAPmtA > RAPmtB, in good agreement with the results from gel mobility-shift assays.

UAS function of GRF2-binding sites

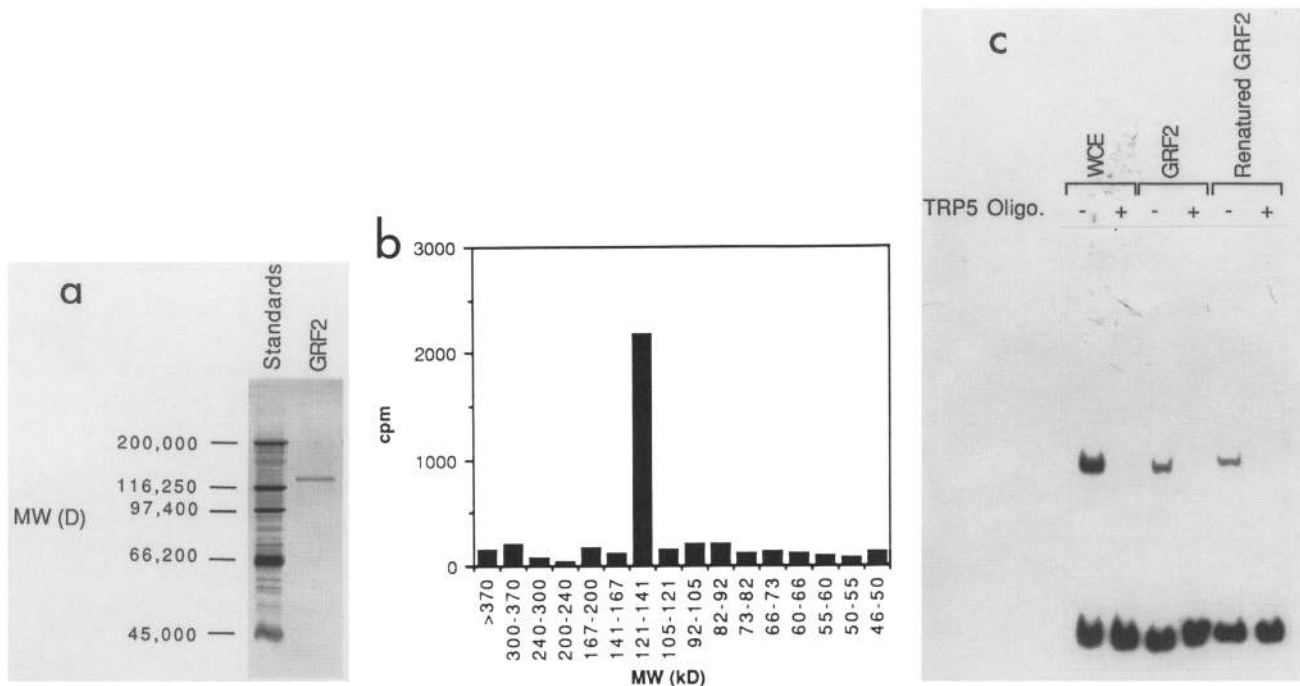As mentioned above, all GRF2-binding sites chosen for

Figure 2. Characterization of purified GRF2. (a) SDS-PAGE of purified GRF2. The peak fraction from the second oligonucleotide column (20 μl) was analyzed, with the following standards for molecular weight (MW) calibration: myosin (200,000), β-galactosidase (116,250), phosphorylase B (97,400), bovine serum albumin (66,200), and ovalbumin (45,000). (b) Recovery of GRF2-binding activity from SDS-PAGE. The peak fraction from the second oligonucleotide column (50 μl) was subjected to SDS-PAGE, the gel was cut in 2.5-mm-thick slices, proteins eluted from the slices were renatured, and GRF2 activity was determined in nitrocellulose filter-binding assays, with the EcoRI–EcoRI fragment from p10GH as probe. Molecular weight range (MW) of each gel slice and radioactivity retained on the filter by protein eluted from the slice (cpm) are shown. (c) Gel electrophoresis mobility-shift assay of GRF2. Whole-cell extract (WCE, 0.5 μg protein), purified GRF2 (GRF2, 0.5 μl of peak fraction from second oligonucleotide column), and GRF2 eluted from an SDS gel and renatured (renatured GRF2, 5 μl of the peak fraction from b) were assayed with the EcoRI–EcoRI fragment of p10GH as probe. The TRP5 oligonucleotide (20 ng) was added as competitor where indicated.

study here from upstream of RNA polymerase II promoters fell within regions previously associated with UAS function by deletion analysis. Thus, the GRF2 site from UAS_G overlaps the binding site for the transcriptional activator GAL4 (Fedor et al. 1988). The GRF2-binding site upstream of the PYK gene lies within a 97-bp region, identified as UAS2, which confers about a twofold effect on transcription in its usual context and about a 45-fold effect when juxtaposed with the TATA box (Nishizawa et al. 1989). The GRF2-binding site upstream of the TRP1 gene (TRP12 oligonucleotide) lies within UAS1, which is responsible for expression of one

subset of transcripts (Kim et al. 1986). Regulated transcription of the TRP5 gene is controlled by sequences that bind the activator GCN4, but basal expression is controlled by sequences between those that bind GCN4, including the GRF2 site (Moye and Zalkin 1987). Within the basal UAS, the GRF2 site neighbors a thymidine-rich sequence similar to those required for basal expression of the HIS3 and DED1 genes, which confer UAS activity in vitro (Fig. 1; Struhl 1985). Finally, the GRF2-binding sites upstream of the 35S rRNA gene, transcribed by RNA polymerase I, occur in functionally important locations. Site 35SRNA1 lies within the poly-

Table 2. Purification of GRF2

Fraction	Protein (mg)	Activity (units)[a]	Yield	Fold purification
Whole-cell extract	480	110,000	(100%)	—
Matrix Red	27	43,900	40	7.1
MPLC-QAM	7.8	12,300	11	7.1
DNA–Sepharose	7.8	7,000	6.4	4.0
Oligonucleotide 1[b]	—	1,680	1.5	—
Oligonucleotide 2[b]	(250 ng)	370	0.34	(6500)

[a]Units of filter binding activity. One unit is the amount of protein needed to bind 1 fmole of probe in the filter binding assay.
[b]Protein concentrations were too low to be measured for the fractions from the specific affinity oligonucleotide columns and are reported, in parentheses, for the second oligonucleotide column only as an estimate from SDS-PAGE of the material.
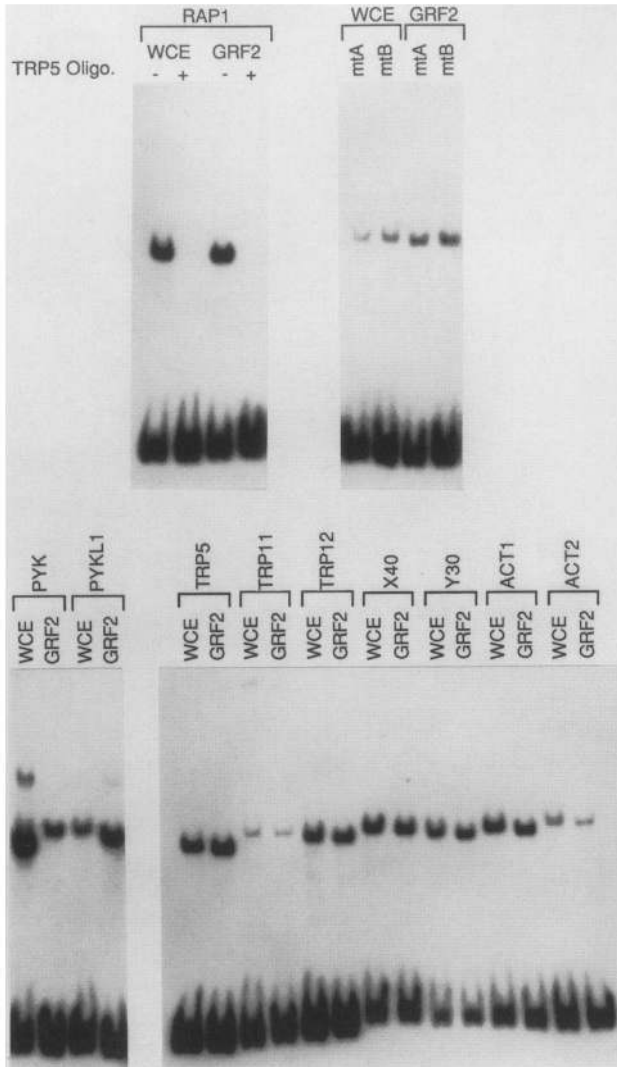
Figure 3. Gel electrophoresis mobility-shift assays of GRF2-binding to candidate sites. Binding reactions contained whole-cell extract (WCE, 2.0 μg protein) or purified GRF2 (GRF2, 0.5 μl of peak fraction from second oligonucleotide column). Probes were *Bam*HI–*Bam*HI fragments from constructs with the oligonucleotide indicated in the polylinker of pCZ. The TRP5 oligonucleotide (20 ng) was added as competitor where indicated. The faint additional gel shift band seen with the PYKL1 probe and purified GRF2 was due to a second molecule of GRF2 binding to the polylinker (see text). Such a band would have been apparent in all lanes on longer exposure of the auto-radiograph.

merase I enhancer, 20 bp from the binding site for a protein referred to as REB2 [which is likely to be the same as a protein we have previously designated ABF1 (Buchman and Kornberg 1990) because an oligonucleotide with the sequence of the REB2 site competes for specific protein binding to other ABF1 sites] and 60 bp from a thymidine-rich sequence (Swanson et al. 1985; Morrow et al. 1989). Site 35SRNA2 is located in the 35S RNA promoter (Morrow et al. 1989).

We pursued the possibility that GRF2 is important for

UAS function with the use of the pCZ constructs from which probes for gel mobility-shift assays were derived. An oligonucleotide inserted in the polylinker of pCZ is located upstream of a yeast *CYC1–Escherichia coli lacZ* fusion gene. UAS function of the inserted oligonucleotide is quantified by measurement of β-galactosidase levels in extracts of yeast cells harboring the construct. Most of the oligonucleotides conferred low but significant UAS activity (Table 1), giving between 1.7 and 4.1 units of β-galactosidase per milligram of protein, as compared with 0.76 U/mg for a construct with no oligonucleotide, pCZΔ. UAS activity was not strictly correlated with affinity of the oligonucleotide for GRF2 (cf. pCZY30 with pCZACT2), or its orientation (cf. pCZTRP5 with pCZTRP5N). The low levels of UAS activity conferred by GRF2 sites were similar to the activity of the basal UAS from the *DED1* gene (pCZDED48, 4.1 U/mg). Any UAS function of the fortuitous weak binding site in the polylinker, present in pCZΔ, was easily distinguished from the effects of the oligonucleotides inserted in the polylinker. UAS activity of the oligonucleotides was dependent on the binding of GRF2 because pCZRAPmtA and pCZRAPmtB, containing mutations in the RAP1 GRF2-binding site, and pCZTRP11, with a very low affinity for GRF2, supported only as much *lacZ* expression as pCZΔ. There was no detectable UAS activity of the PYK oligonucleotide, even though PYK was capable of binding purified GRF2 in vitro, possibly because of interference from the additional protein binding to this oligonucleotide detected in the gel mobility-shift assay. Notably high expression was obtained from the high-affinity TRP12 (15 U/mg) and low-affinity TRP3 (5.15 U/mg) oligonucleotides, and from the PYKL2 (25 U/mg) oligonucleotide. We have no explanation for these high levels but observe that the PYKL2 effect may be due to an additional function of the *PYK* UAS2, mediated by sequences flanking the GRF2 site, present in PYKL2 but not in PYKL1. The UAS activity of GRF2-binding sites showed a modest distance dependence (cf. RAP1 and X40 sites in pCZ with the same sites in pCT, which contains an additional 120 bp between the binding site and the TATA sequence).

Function of GRF2 as an auxiliary activator

The proximity of a GRF2 site to the GAL4-binding sites in UAS$_G$ and the juxtaposition of GRF2 sites to thymidine-rich sequences in the *TRP5* basal UAS and the 35S rRNA enhancer raised the possibility that GRF2 might play an auxiliary role in a compound UAS. That is, GRF2 may not function alone as an important transcriptional activator but may augment the effects of neighboring activators. There are precedents for such effects with the thymidine-rich sequence from upstream of the *DED1* gene (DED48 oligonucleotide). The multifunctional protein ABF1 augments the UAS effect of the DED48 element upstream of the *DED1* gene and in pCZ constructs (Buchman and Kornberg 1990). The UAS effect of two DED48 elements in pCZ is roughly the square of that with one element (Buchman and Kornberg 1990). The repetition of GRF2 sites upstream of the *ACT*

gene suggested the possibility that two GRF2 sites could function in a compound UAS.

We therefore tested combinations of GRF2 sites (RAP1, X40, Y30) with the DED48 element in pCZ. The synergistic effects observed were unexpectedly large (Table 3). RAP1, together with DED48, gave a β-galactosidase level of 36 U/mg, compared with 1.7 and 4.1 U/mg for the two elements tested separately. The synergism was even greater with Y30 (46 U/mg) and X40 (130 U/mg). The large effect of X40 may reflect a preferred alignment of GRF2 and the DED48 element through GRF2 binding to one of the two sites in X40. Doubling the RAP1 site in pCZ(RAP)[2] gave an increase in UAS activity but to a lesser extent than in the combinations of GRF2 sites with DED48.

The combined effects of GRF2 and the activator protein GAL4 were also examined, but no synergism was found. Yeast harboring pCTG01P (with a single GAL4-binding site 120 bp upstream of the CYC1 TATA box) or pCTRAP1G01P (with GRF2 and GAL4-binding sites) grown on a galactose-containing medium expressed 160 and 100 U/mg of β-galactosidase per milligram of protein, respectively. When grown on 2% galactose with 0.5% glucose to diminish the stimulatory effects of GAL4 through catabolite repression (Johnston 1987), yeast harboring pCTG01P or pCTRAP1G01P gave expression levels of 20 and 22 U/mg, respectively. (The abundance of GRF2, determined by filter binding, was about the same in extracts from cells grown on galactose and glucose.) Reversing the orientation of the GRF2-binding site in pCTCENG01P gave no greater effect on expression (19 U/mg).
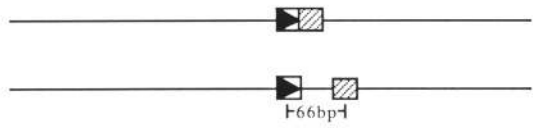
The synergistic actions of GRF2 and DED48 elements were strongly dependent on the distance between the elements. When 22 bp was interposed between the elements, the level of transcription was reduced 6.5-fold, and with a 121-bp spacing, the level was essentially the same as that in the absence of a GRF2 element.

Stimulation of transcription by GRF2 sites in vitro

Templates for transcription in vitro were constructed with the high-affinity RAP or mutant RAPmtB GRF2-binding sites upstream of the CYC1 promoter and G-minus transcription unit (Sawadogo and Roeder 1985) used for transcription assays with yeast nuclear extracts (Lue et al. 1989a). Transcription was performed over a range of template concentrations and revealed a small stimulation by a functional GRF2-binding site (Fig. 4), comparable to that observed in vivo (Table 1). Filter-binding assays demonstrated that the nuclear extract used in these experiments contained enough GRF2 to fully occupy its binding site in the templates (data not shown). Despite the stimulatory effect of GRF2 on transcription in vitro and our previous observation of an effect of the DED48 element (Lue et al. 1989b), we were unable to detect any synergism between the two elements in vitro (data not shown).

Table 3. *Combinatorial effects of a GRF2-binding site and the DED48 element*

	RAP1	X40	Y30	
pCZ(GRF2)DED48	36	130	46	u/mg
pCZ(GRF2)CZDED48	20	20	-	
pCT(GRF2)CZDED48	5.9	5.0	-	
pCZGRF2	2.2	2.3	2.8	
pCZDED48	4.1 (DED48 only)			
pCZ(RAP1)[2]	4.7 (two RAP1 sites)			

Expression of β-galactosidase from constructs containing a GRF2-binding site, the DED48 element, or both is indicated along with a schematic representation of the center-to-center distance (bp) between the elements where appropriate. The two circles represent nucleosomes positioned with respect to the GRF2-binding site at UAS$_G$ (Fedor et al. 1988). (GRF2) refers to RAP1, X40, and Y30 oligonucleotides in expression constructs, as indicated. (▶) GRF2 site; (▨) DED48 element.
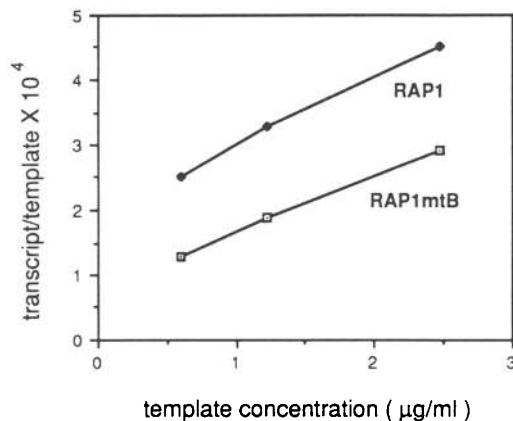
Figure 4. Effect of GRF2 on transcription in vitro. Templates for transcription were pRAPCG−, containing the high affinity GRF2-binding oligonucleotide RAP1, and pRAPmtBCG−, containing the mutant form of this oligonucleotide, RAP1mtB, with no detectable affinity for GRF2.

Discussion

The most striking aspects of GRF2 to emerge from the present study are the widespread occurrence of its binding sites in the yeast genome, frequently in association with UAS elements, and the synergistic effect on transcription with a thymidine-rich element. A GRF2-binding site and a thymidine-rich element, which separately stimulate transcription 3- and 5.4-fold, respectively, together give a 170-fold stimulation. This synergism may be due to the effect of GRF2 on chromatin structure. We showed previously that GRF2 created a nucleosome-free region surrounding its binding site in two constructions with very different sequences flanking the site (Fedor et al. 1988). Although we have not demonstrated the occurrence of nucleosome-free regions in the plasmids studied here, such regions would be expected to extend ~95 bp from the GRF2-binding site in the direction of the thymidine-rich elements in the plasmids. The occurrence of nucleosome-free regions would give rise to synergistic effects on transcription if a thymidine-rich element were effective as a UAS when located in such a region but less effective when associated with a nucleosome. The packaging of thymidine-rich elements in nucleosomes remains to be demonstrated, but incorporation of even a part of an element in a nucleosome may affect its function, because deletion of only 18 of 48 residues inactivates the DED48 element (Lue et al. 1989b); the small stimulatory effects of DED48 alone (4.7 U/mg) may reflect such a partial association. This line of interpretation, though conjectural, is consistent with the dependence of the synergism on the distance between the GRF2 site and thymidine-rich element (Table 3). As the distance increases, the synergism declines; and at a distance of 165 bp, where the thymidine-rich element would be expected to lie within a nucleosome, the synergism is undetectable.

The distance dependence of the GRF2 effect and its possible mechanism of action are altogether different from those of GAL4 protein, the thymidine-rich binding factor, and other transcriptional activators. The effects of GAL4 do not diminish when its binding site is moved from 50 to 150 bp upstream of the CYC1 promoter (data not shown), whereas the effects of the thymidine-rich element diminish only twofold over 100 bp (Lue et al. 1989b). Such activators are thought to interact with components of the transcription apparatus, in contrast with an effect of GRF2 possibly mediated through chromatin structure. It is possible that GRF2 can stimulate transcription by direct interaction with the transcription apparatus as well, and the small effect on transcription in vitro may occur by this mechanism.

The proximity of many GRF2-binding sites in the yeast genome to other functional elements (Fig. 1) is in keeping with GRF2 playing an auxiliary role, exerting synergistic effects, and with these effects involving the structure of chromatin. The actual role of GRF2 at most chromosomal locations, however, remains to be established. A deletion analysis of a 35S rRNA transcription unit on a plasmid suggested a stimulatory effect of the GRF2 site in the enhancer (Mestel et al. 1989), but a role of this GRF2 site in transcription termination would also be consistent with the data. Although we found no effect of GRF2 on GAL4 action, others have reported the stimulation by GRF2 (referred to as factor Q) of transcription from a promoter with a GCN4 site replacing the TATA sequence (Chen and Struhl 1989). The role of the GRF2 sites at telomeres is especially intriguing, because there are matches to the consensus sequence throughout the X region, suggesting extensive GRF2 binding.

Most of the GRF2 sites described here were identified by a search of a data base with a consensus sequence derived from just three sites. Subsequent determination of the relative affinities of all the sites confirmed that some of the bases of the search consensus were critical. Changing the C in positions eight and nine to a T in the RAPmtA and RAPmtB oligonucleotides essentially eliminated binding. Changing the consensus pyrimidine in position four to a G in the TRP11 oligonucleotide was also unfavorable. Less critical, however, was the most 5' pyrimidine, because the 35SRNA2 oligonucleotide, with a G in that position, bound GRF2 with the highest affinity of all sequences tested. There was considerable variation in affinity among sequences consistent at all positions with the consensus (>200-fold difference in affinity between Y30 and TRP3 oligonucleotides), and even among sequences identical at all positions (a 5-fold difference between the ACT1 and GAL10 oligonucleotides). Features of sequences flanking the consensus may be important for high-affinity binding, although no strict correlations are apparent.

All matches to the consensus sequence bound purified GRF2, and there was no evidence of any other abundant binding activity in a crude extract. We presume, therefore, that GRF2 is identical to a protein referred to as REB1, shown by others to bind the 35SRNA1 and 35SRNA2 sites (Morrow et al. 1989). The gene for REB1 has been isolated and shown to be essential in yeast; the sequence encodes a polypeptide of 809 amino acids, less

than expected from the mobility of GRF2 in SDS gels (Q. Ju, B. Morrow, J. Warner, pers. comm.), as has sometimes been found for yeast DNA-binding proteins (e.g., Shore and Nasmyth 1987). A protein likely to be GRF2 has also been identified by virtue of its affinity for the 35SRNA1 and 35SRNA2 sites, as well as additional sites upstream of genes not studied here (D. Stillman, pers. comm.).

GRF2 is the fourth member of a family of DNA-binding proteins we have found that are remarkable for their abundance in yeast extracts, their many binding sites in the yeast genome, and their involvement in diverse chromosomal functions. The first such protein to be identified, CP1, binds to centromere DNA element I and to a site upstream of the *GAL2* gene (Bram and Kornberg 1987). Although CP1 sites possess no intrinsic UAS activity and give no synergistic effect with the DED48 element, CP1 does play a role in centromere function (Bram and Kornberg 1987; Hegemann et al. 1988; Buchman and Kornberg 1990). A second abundant protein, GRF1 (also known as TUF and RAP1), binds to UASs, telomeres, and the mating type silencer (Huet and Sentenac 1987; Shore and Nasmyth 1987; Buchman et al. 1988b). GRF1 is a potent gene activator protein, and its binding site is required for silencer function (Brand et al. 1987; Shore and Nasmyth 1987; Buchman et al. 1988b). Finally, ABF1 binds to UASs, autonomously replicating sequence (ARS) elements, and the mating type silencer and functions as a gene activator protein (Shore et al. 1987; Buchman et al. 1988a; Francesconi and Eisenberg 1989; Buchman and Kornberg 1990). It remains to be seen what relates the functions of these proteins at diverse chromosomal elements, but we may anticipate important structural roles, exemplified by the positioning of nucleosomes by GRF2.

Methods

Plasmids

The pCZ and pCT families of plasmids have been described (Lue et al. 1989b). Both are centromeric yeast shuttle vectors, which carry the *URA3* gene as a selectable marker, and a yeast promoter *CYC1–E. coli LacI/Z* fusion gene with a polylinker upstream of the promoter. In pCZ, the polylinker replaces an *SphI* site 12 bp upstream of the major TATA element of the *CYC1* promoter. In pCT, the polylinker replaces a *XhoI* site 114 bp upstream of the TATA element. In both types of plasmid, the structure of the polylinker is 5'-*HindIII/SphI/PstI/SalI/XbaI/BamHI/EcoRI/SacI/KpnI/SmaI/SalI/XhoI*-3'. pCZΔ contains no insert in the polylinker (Buchman and Kornberg 1990). pCZ(RAP)[2] was constructed by ligation of the large *EcoRI–ClaI* fragment of pCZRAP with the *EcoRI* end-filled into the small *XbaI–ClaI* fragment of pCZRAP with the *XbaI* end filled in. pCZRAPDED48, pCZY30DED48, and pCZX40DED48, containing a GRF2-binding site immediately upstream of the DED48 element, were constructed by inserting the RAP, Y30, and X40 oligonucleotides, respectively, between the *BamHI* and *EcoRI* sites of pCZDED48 (Lue et al. 1989b). pCZRAPCZDED48 and pCZX40CZDED48, containing a GRF2-binding site 66 bp upstream of the DED48 element, were constructed by ligation of the large *ClaI–EcoRI* fragment of

pCZRAP or pCZX40, with the *EcoRI* site filled in, to the small *HindIII–ClaI* fragment from pCZDED48, with the *HindIII* site filled in (Maniatis et al. 1982). pCTRAPCZDED48 and pCTX40CZDED48, containing a GRF2 site 165 bp upstream of the DED48 element, were constructed by ligation of the 211-bp *XbaI–SphI* fragment from pCTRAP or pCTX40, the small *SphI–ClaI* fragment from pCZDED48, and the large *ClaI–XbaI* fragment from pCZDED48. pCTG01P contained the oligonucleotide

GATCATTCGCCGGCCGGCACTGCTCCG
TAAGCGG<u>CCGGCCGAGACGAGGC</u>TTAA

(Gal4-binding site is underlined) inserted in the polylinker of pCT. pCTRAPG01P was constructed by ligation of the large *EcoRI–ClaI* fragment from pCTRAP, with the *EcoRI* site filled in, to the small *XbaI–ClaI* fragment from pCTG01P, with the *XbaI* end filled in. pCTCEN4G01P was constructed by inserting the following oligonucleotide between the *SphI* and the *XhoI* sites of pCT:

```
CATTCGCCGGGTAAATACATATAGGATCCGAATTCGTCGACGGAGCAGTGCCGGCCGCGC
GTACGTAAGCGGCCCATTTATGTATATCCTAGGCTTAAGCAGCTGCCTCGTCACGGCCGGCCGCGAGCT
```

This oligonucleotide binds both GRF2 (binding site in boldface type) and GAL4 (binding site underlined). p10GH, containing a GAL4-binding site upstream of a *GAL1–HIS3* fusion gene has been described (Lorch and Kornberg 1985; Buchman et al. 1988a).

Templates for transcription in vitro, pRAPCG– and pRAPmtBCG–, contained the RAP and RAPmtB oligonucleotides, respectively, upstream of the *CYC1* promoter and a se- and Roeder 1985). These plasmids were constructed by inserting the *BamHI–BamHI* fragment from pGAL4CG– (Lue et al. 1989b) between the *and BglII* sites of pSP73 and then replacing the GAL4-binding oligonucleotide in this plasmid with the appropriate GRF2-binding oligonucleotide.

Computer-assisted search of yeast sequence data base

The Find computer program from the University of Wisconsin Computer Group was used to search the Genbank (release 60.0) sequence data base for matches to GRF2-binding sequences (Devereux et al. 1984).

GRF2-binding assays

GRF2 binding was detected by nitrocellulose filter binding (Buchman et al. 1988b) and gel electrophoresis mobility-shift assays (Chasman et al. 1989) essentially as described. DNA probes were either *BamHI–BamHI* fragments from pCZ or the *EcoRI–EcoRI* fragment from p10GH, labeled at both ends with [α-^{32}P]dATP (3000 Ci/mmole, ICN) with the large fragment of DNA polymerase I (Pharmacia) (Maniatis et al. 1982). Binding reactions contained 10,000–20,000 cpm of probe, 1 μg poly[d(I-C)] (Pharmacia), and 5 μg bovine serum albumin (ICN) in 20 μl of 10% glycerol, 25 mM HEPES (pH 7.5), 5 mM MgCl$_2$, 0.1 mM EDTA, and 50 mM KCl, with protein fraction and competitor oligonucleotide as indicated. Reactions were incubated for 15 min at room temperature before analysis. Reactions for gel mobility-shift assays and the gels contained 0.1% and 0.01% (vol/vol) NP-40, respectively. Gels were quantified with an Ambis Radioanalytic Imaging System (AMBIS Systems, San Diego).

Purification and analysis of GRF2

Protease-deficient yeast strain BJ926 was grown to saturation in 1% yeast extract, 2% Bacto-peptone, 2% glucose, harvested by

centrifugation, washed once with water, and resuspended to 240 ml with buffer A [100 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 20% glycerol, 3 mM dithiothreitol (DTT), 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM benzamidine, 2 μM pepstatin, 0.7 μM leupeptin, and 2 μg/ml chymostatin]. Cells were lysed with 220 ml of glass beads (0.5 mm), using twenty 30-sec cycles of a bead beater (Biospec products), with 90-sec intervals for cooling between cycles. The supernatant was decanted, adjusted to pH 7.5 with 1 M KOH, and centrifuged in a Beckman JA10 rotor at 5000 rpm for 5 min at 4°C. Ammonium sulfate (3 M) was added to the supernatant to a final concentration of 0.9 M, and the mixture was stirred for 30 min and centrifuged in Beckman Ti45 rotor at 45,000 rpm for 90 min at 4°C. One-quarter of the supernatant (whole-cell extract) was dialyzed against buffer B [25 mM HEPES (pH 7.5), 0.1 mM EDTA, 50 mM KCl, 10% glycerol, 1 mM DTT, and protease inhibitors as in buffer A], diluted to 8 mg of protein per milliliter (Bradford 1976) with buffer B, and loaded at 50 ml/hr onto a 50-ml column of Matrex Red (Amicon), 2.5 cm in diameter. The remaining three-quarters was used for other purposes. The column was washed with 3 volumes of buffer B and 2 volumes of buffer B containing 500 mM KCl and was eluted with buffer B containing 1.2 M KCl. Peak protein-containing fractions were pooled, dialyzed against buffer B containing 100 mM KCl, and loaded at 1 ml/min onto a 10-ml quarternary amino methane (ACCEL, Waters) medium pressure liquid chromatography column (Millipore). The column was washed with 14 ml of buffer B containing 100 mM KCl and eluted with a 135-ml gradient of KCl (100–400 mM) in buffer B. Peak fractions were pooled, adjusted to 200 mM KCl and 5 mM MgCl$_2$, and passed at 20 ml/hr through a 10-ml column of salmon sperm DNA–Sepharose (prepared by coupling sheared salmon sperm DNA (Sigma) at 3 mg/ml to CNBr-activated Sepharose CL-2B as described (Kadonaga and Tjian 1986; Buchman et al. 1988b). Protein-containing fractions were pooled and loaded onto a 1-ml column containing 0.2 mg/ml of the double-stranded oligonucleotide

```
GATCCTCGCCGGGTAAATACATATACGGGTAAATACATATAG
     GAGCGGCCCATTTATGTATATGCCCATTTATGTATATCTTAA
```

coupled to CNBr-activated Sepharose CL-2B, without catenation, as described (Kadonaga and Tjian 1986; Buchman et al. 1988b). The column was washed with 6 volumes of buffer B containing 300 mM KCl and 5 mM MgCl$_2$ and was eluted with buffer B containing 1 M KCl and 5 mM MgCl$_2$. Peak protein-containing fractions were pooled, diluted with buffer B containing 5 mM MgCl$_2$ to 200 mM KCl, made 0.15 mg/ml in poly[d(I-C)] and loaded onto the oligonucleotide column, which was washed and eluted as before. The peak fraction containing about half of the purified GRF2 was 250 μl.

Proteins were analyzed by precipitation with 10% (vol/vol) trichloroacetic acid and electrophoresis in SDS–7.5% polyacrylamide gels as described (Laemmli 1970) and visualized with a silver stain (Wray et al. 1981). For recovery of GRF2-binding activity, an SDS gel calibrated with prestained markers (Bio-Rad) was cut into 2.5-mm-thick slices, and protein was eluted and renatured as described (Hager and Burgess 1980).

Assays for UAS function

Plasmids were introduced into yeast strain 15c (α *leu2-31, 12, ura3-52, Δtrp1, his4-580, pep4-3*) by the lithium acetate transformation procedure (Ito et al. 1983; Buchman et al. 1988a), with selection for uracil on minimal medium (0.67% yeast nitrogen base, 0.5% ammonium sulfate, 2% glucose) supplemented with tryptophan, histidine, and leucine. Single colonies

were isolated, purified, and grown in 100 ml minimal medium under uracil selection until the culture reached an OD$_{600}$ of 1. Cells were harvested, washed once in 10 ml of H$_2$0, resuspended in 200 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10 mM magnesium acetate, 5% glycerol, 10 mM β-mercaptoethanol, and 1 mM PMSF and were frozen. Cells were thawed, lysed by glass bead disruption, and centrifuged at 14,000g for 10 min at 4°C in a microcentrifuge. Protein concentrations were determined as mentioned above, and β-galactosidase assays were performed with o-nitrophenyl-β-D-galactoside as described (Miller 1972).

Transcription in vitro

Transcription was performed with nuclear extracts as described (Buchman et al. 1988b; Lue et al. 1989a,b). Following the reaction, transcripts were treated with 10 units T1 RNase (Calbiochem) in 200 μl of 10 mM Tris (pH 7.5), 5 mM EDTA, and 300 mM NaCl for 10 min at room temperature and then treated with SDS (0.5%) and proteinase K (Boehringer–Mannheim, 200 μg/ml) for 20 min at 30°C. Nucleic acids were precipitated with ethanol and analyzed by gel electrophoresis (Lue et al. 1989b). Transcripts were visualized by autoradiography and quantified with an Ambis Radioanalytic Imaging System.

Acknowledgment

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A yeast protein that influences the chromatin structure of UASG and functions as a powerful auxiliary gene activator.

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