Transcriptional regulatory elements of the *RAS2* gene of *Saccharomyces cerevisiae*

J.Liszewicz, J.Brown, D.Breviario¹, T.Sreenath⁺, N.Ahmed, R.Koller[§], and R.Dhar*

Laboratory of Molecular Virology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA and ¹Istituto Biosintesi Vegetali CNR, 20133 Milan, Italy

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ABSTRACT

We have analyzed a series of 5' deletions of the *RAS2* gene to investigate its complex transcriptional regulation in the yeast *Saccharomyces cerevisiae*. Two positive transcriptional regulatory elements were identified. Element A regulates two of the three clusters of *RAS2* transcripts. This element is capable of activating a heterologous promoter and contains two copies of the sequence CCTCGCCCC. Although one copy is sufficient for partial transcriptional activation, both copies are required for maximal *RAS2* induction. Deletion of one copy resulted in a reduced level of *RAS2* mRNA, selective loss of cluster II transcripts and reduced ability to activate the heterologous *CYC1* promoter. Each of the 9 bp C rich repeats of element A is part of a sequence with extensive homology to a transcriptional regulatory element upstream of the human epidermal growth factor receptor (EGFR) gene. Element B contains a tandem duplication of a 21 nucleotide sequence TACATATATATATCTTAG and activates cluster I *RAS2* transcripts in the absence of Element A. The physiological role of these deletions was determined by assaying their ability to support growth on a nonfermentable carbon source. *RAS2* promoter deletions containing either element A or B were able to overcome this growth defect characteristic of *ras2* mutants cells. Deletion of both elements resulted in an insufficient amount of *RAS2* protein for growth on a non-fermentable carbon source.

INTRODUCTION

Saccharomyces cerevisiae contains two *RAS* genes, *RAS1* and *RAS2*, that encode proteins that are highly homologous to the mammalian *RAS* proteins (1). Either one of the two *RAS* genes can support growth in rich medium but disruption of both genes results in spores that fail to germinate (2, 3, 4). The lethality of *ras1 ras2* strains can be overcome by extragenic suppressors that increase intracellular cAMP levels or the activity of cyclic AMP-dependent protein kinase(s) (5, 6, 7, 8). Cells mutated in

the *RAS1* gene (*ras1 RAS2*) have no growth-related phenotype (1), whereas cells carrying a mutation in the *RAS2* gene (*RAS1 ras2*) sporulate in rich media, hyperaccumulate storage carbohydrates such as glycogen and trehalose, and are unable to grow on a nonfermentable carbon source (3, 9). We have shown that the inability of *ras2* cells to grow on nonfermentable carbon sources is due to the low level of *RAS1* gene expression in these culture conditions (10). The combination of *ras2* gene disruption and low *RAS1* expression results in an overall low amount of *RAS* product that is insufficient for cell growth. Low level expression of the *RAS1* gene during growth on nonfermentable carbon sources is due to transcriptional repression carried out by the *SR46* gene product. Mutations in this gene result in the expression of high levels of *RAS1* mRNA and restore the ability of *RAS1 ras2* mutants to grow on nonfermentable carbon sources. Overproduction of *RAS1* does not overcome the *ras2* hypersporulation phenotype (10), indicating that *RAS2* protein has a function in sporulation for which overproduction of *RAS1* protein cannot substitute.

RAS1 and *RAS2* gene products are differentially expressed under a wide variety of culture conditions (11). *RAS1* gene expression appears to be regulated at the transcriptional level, since the amount of *RAS1* protein synthesis parallels the amount of *RAS1* mRNA in cells grown in a variety of culture conditions (11). The mechanism controlling *RAS2* gene expression is more complex, and involves both transcriptional and translational modulation. When cells are grown in dextrose, *RAS2* mRNA levels are high in early and mid-log phase, but *RAS2* protein synthesis is suppressed early in cell growth (11). Under these culture conditions, *RAS2* transcripts have multiple 5' ends (11). These have been grouped into three major clusters with 5' ends mapping around positions 45 bp (I), 180 bp (II) and 235 bp (III) upstream of the translation initiation codon. Nutrient deprivation resulting in G1 arrest and sporulation of diploids leads to an overall reduction of *RAS2* mRNA due to the selective repression of two of the three clusters of *RAS2* transcripts (11). For example, nitrogen starvation results in repression cluster II and III transcripts, while cluster I and II transcripts are reduced during sulfur starvation. This reduction in *RAS2* mRNA has little effect

* To whom correspondence should be addressed

Present addresses: ⁺American Red Cross, Jerome Holland Laboratory, 15601 Crabbs Branch Way, Rockville, MD 20855 and [§]Laboratory of Genetics, National Cancer Institute, Bethesda, MD 20892, USA

on *RAS2* protein synthesis, suggesting an increased rate of *RAS2* mRNA translation in starvation conditions (11). The combined transcriptional and translational regulation of yeast *RAS2* gene expression ensures that *RAS* protein will be produced over a wide range of physiological conditions. To understand the role of transcriptional control in the regulation of *RAS2* gene expression we have analyzed the effect of upstream deletions on growth phenotype and *RAS2* gene expression.

METHODS

Strains

The following yeast *Saccharomyces cerevisiae* strains were used.

JC-482: *MAT α ura3-52 leu2 his4-539*

JC-482/5: *MAT α ura3-52 leu2 his4-539 ras2::HIS4*

RAS1 ras2 strain JC-482/5 contains a *RAS2* null mutation obtained by replacement of the entire *RAS2* coding region with the *HIS4* gene. This strain was the recipient for transfection of *RAS2* promoter deletions shown in Fig. 1. Plasmid YAR contains the *RAS2* 3.2 kb *EcoRI HindIII* fragment cloned into the *HindIII* site of the yeast centromere plasmid YCp50 (12).

Growth conditions

Yeast cells were grown in either SD minimal media containing 0.67% yeast nitrogen base (YNB), 2% dextrose, and the necessary amino acids, or SGE media containing the nonfermentable carbon source glycerol: ethanol at a final carbon concentration of 2 percent (12). JC-482/5 cells containing the various *RAS2* promoter deletions were tested for ability to use a non-fermentable carbon source by first growing them to stationary phase in SD media. Cells were then harvested by centrifugation, washed twice with SGE, and resuspended in SGE at an optical density (600nm) of approximately 0.1 (5×10^5 cells/ml). Growth at 30°C was monitored by measuring the O.D.₆₀₀ and by cell counts in a hemocytometer over a period of 65 hours.

RNA blot-hybridization

Total RNA was extracted according to published procedures (13). 10 μ g of each RNA sample was denatured and fractionated on 1% agarose gels in the presence of formaldehyde. The RNA was transferred to nitrocellulose filters (Schleicher and Schuell) and hybridized with a ³²P-labelled 498 base pair (bp) *RsaI* fragment from the 3' end of the *RAS2* gene. This fragment was labelled by nick-translation and specifically recognized *RAS2* RNA. No cross hybridization with *RAS1* mRNA was detected with this probe. As an internal control for the amount of RNA loaded on the gel, all RNA blots were rehybridized with the nick translated *BglIII* fragment from the coding region of the *S. cerevisiae CDC25* gene (14). This control also showed that *RAS2* promoter deletions do not grossly affect general RNA transcription. Autoradiographs were subjected to densitometry.

S1 endonuclease analysis

A 661 bp *HinPI* fragment corresponding to the 5' region of the *RAS2* gene (15, 16) was cloned into double stranded M13 DNA. A 5' end-labelled oligonucleotide complementary to the 3' end of this insert served as a primer for synthesis of a probe from the single-stranded M13 template. Out of 661 nucleotides, 425 nucleotides map upstream of the translational initiation codon ATG. 5×10^3 cpm of the noncoding strand was mixed with 50 μ g of total RNA in 25 μ l of 0.8 M NaCl, 0.5 M Pipes pH 7.8,

0.01 M EDTA and incubated for 12 hrs at 65°C. The hybridized samples were then treated with S1 endonuclease (Miles Laboratories) at a final concentration of 300 U/ml at 37°C for 30 min in a buffer containing 4 mM ZnSO₄, 30 mM sodium acetate pH 4.6, 250 mM NaCl and 20 μ g/ml denatured salmon sperm DNA. The samples were precipitated with ethanol, dissolved in 5 μ l of loading buffer (80% formaldehyde, 10 mM NaOH, 1 mM EDTA and 100 μ g/ml xylene cyanol and bromophenol blue), denatured for 3 min at 90°C and fractionated on 6% polyacrylamide gels containing 8 M urea (11).

³⁵S labelling of proteins and immunoprecipitation

The following procedure was previously described in detail (11). Yeast cultures in exponential growth phase were labelled with 20 μ Ci/ml of ³⁵S methionine (800 Ci/mmol) for pulse labelling proteins. After 10 minutes of labelling, protease inhibitors TLCK, TPCK and PMSF were added to a final concentration of 100 μ g/ml and cells were rapidly chilled. Cells were washed with PBS containing 1 mM PMSF. The cells were then disrupted and resuspended in modified RIPA buffer (20 mM MOPS, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 1% Apoprotein, pH 7.0) and protein extract was clarified by centrifugation. Extracts containing 10⁷ TCA-precipitable cpm were used per reaction to immunoprecipitate *RAS* proteins with monoclonal anti-*RAS* antibody Y13-259, a generous gift of A. Papageorge (7).

RESULTS

Construction of promoter deletions

A 3.2 kb *EcoRI HindIII* DNA fragment containing the *RAS2* gene was cloned into the *HindIII* site of the yeast centromere vector YCp50 (12). The resulting plasmid, YAR, contains the complete protein coding region of the *RAS2* gene plus 1723 bp upstream of the coding region (Fig. 1). We previously published the DNA sequence encoding the *RAS2* protein and 250 nucleotides upstream from the translational initiation codon ATG (15). In this report we extend the sequence further to 1723 bp upstream of the ATG codon (Fig. 2).

Two types of *RAS2* promoter deletions were constructed and are shown in Fig. 1. Series I was generated by digestion of YAR with *XbaI* endonuclease followed by treatment with *Bal31* exonuclease (IBI). *XbaI* linkers were ligated to the deletion endpoints, and the plasmids were recircularized and transformed into *E. coli*. Each of these plasmids has a deletion of the *XbaI* fragment (−1492 to −642) as well as a *Bal31* generated deletion extending downstream of −642 and upstream of −1492. The sequences upstream of −642 from the translation initiation codon were then reinserted into the *XbaI* site at −642, and the 3' deletion endpoint was determined by dideoxy sequencing. All of the series I deletions have a 5' endpoint at −642 and are named according to their 3' end points. Series II deletions were generated by digesting each series I mutant with *XbaI* to delete the 852 bp *XbaI* fragment from nt −1492 to −642. These are designated with an $\times$ and the 3' deletion endpoint. Thus, for each 3' deletion endpoint we have constructed two 5' endpoints: one at −641 (series I), and the other at −1491 (series II)(Fig. 1). These *RAS2* promoter deletions were transformed into *S. cerevisiae* strain JC482/5 (*RAS1 ras2*).

Identification of *RAS2* transcriptional regulatory elements

Cells growing exponentially in a medium containing dextrose as

a carbon source (SD medium) show three major RNA species corresponding to the three major clusters of RNA that differ in the length of their leader sequences (11). Transcripts were grouped in these clusters according to their pattern of differential enrichment in various nutritional conditions (11). The 5' ends of the major RNA species from clusters I, II and III map at -45, 180, and -235, respectively.

It was found that series I deletions from -642 through -479 had little or no effect on the steady state levels of *RAS2* mRNA in cells growing exponentially in SD media (Fig. 3A). Deletions extending downstream of -479 showed a progressive decrease in the amount of *RAS2* mRNA compared to wild type levels. Densitometry indicated a 2-fold decrease in the amount of total *RAS2* RNA in deletions extending downstream of -444, while

cluster I RNA levels remained fairly constant in deletions extending as far downstream as -168. Thus the decrease in total *RAS2* RNA resulted from a decrease of clusters II and III transcripts. These results are shown in greater detail by 5' end analysis of *RAS2* RNA from the series I deletions discussed below (Fig. 4). The deletion mutant carrying just 36 nucleotides upstream of the *RAS2* translation initiation codon (-36) resulted in a 20-fold reduction in *RAS2* RNA determined by densitometry. These results suggest that a positive regulatory element is located downstream of -479, and that it affects primarily cluster II and III mRNA levels.

All yeast strains transformed with plasmids deleted of the *XbaI* fragment (series II deletions) showed the presence of a cryptic *RAS2* RNA (Fig. 3B). The 5' end of this RNA is located between the *EcoRI* site at -1723 and the *XbaI* site at -1493 (data not shown). This cryptic RNA probably does not serve as a template for *RAS2* protein synthesis since its leader contains multiple termination codons in all three reading frames, and cryptic RNA levels did not correlate with *RAS2* protein levels (compare Figs. 3B and 3C).

In series II deletions with 3' ends at -444 or downstream, RNA comigrating with *RAS2* mRNA was barely detectable. Cells containing deletion -X444 had significantly less *RAS2* RNA than deletion -444, and in deletion -X36, *RAS2* RNA was undetectable (Fig. 3B). These results suggest that a second

Figure 1. Diagrammatic representation of the 5' upstream region of the *RAS2* gene showing internal deletions. I, II and III represent the major clusters of initiation sites for *RAS2* mRNA synthesis. The positions of regulatory elements A and B are indicated. Series I deletions were made by *Bal* 31 exonuclease digestion starting from the *XbaI* site at nucleotide -642. The numbers correspond to the 3' deletion end point. The first nucleotide upstream of the translational initiation codon ATG is -1. Series II deletions were made by deleting the 852 bp *XbaI* fragment from each of the series I deletions, and are designated with an X in front of the 3' deletion end point. JC482/5 cells were transformed with each of the deletions. Their ability to grow on SGE medium is indicated with a + or -.

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-1723  EcoRI  GAATTCCTCCACCTGGCCACCTCCAGATATTTAACTACTACACGAAATAGATGCAAAATGTGTGCA
-1658  TTCTATGCCGAATTGTAACGAGAGGATAGATAAGTTCTTGAAGAAAGACTTCAATAAAGATCACC
-1593  AAACACAAGTAAGACTGCTCAATAATATCAACAAGATTATGAAAGACTGATGCCATCGCTGGAG
-1528  GAGAAATGCATGTCTCATCCATTATGCTGGATAATCTAGACAGATTGACGTCGCCGTTAGAATT
-1463  GGCCTATGAAGTCGCAATCAAGAACACAGAAATCTCTAGAGGTTTAAAGACGGGTGTGGACAACC
-1398  ATCCAGCAATGCACCTCCATCATGAACTAATGGAAGAAATAGAGAGCAAACTAAACAGCAAACTCG
-1333  TCGCAGGCACTGAAGAGCGAATCAAGAAGAGAGCCATGGCTGCCAAGCAGGAGCGGCGCAACA
-1268  TTACTCCGCGACGACACACCAACAGACGACTCAAGAACGACGCAAACTACGGAGGAGCAGGCAC
-1203  GAGAGCCGAAGACCACTGTTAACAACAACTCAAGAAAAAAGAGCCAAAGCTGCCAATACC
-1138  AACAACGCCGATCCAGAGACCAAAAAACGCAAGAGGAGAGTTGCCACACAGCCGTTTCCCAAG
-1073  CACTATCAGCACGGCACTGCCGTCATAATGGCAGGATAGGTACATCTACAGCGTCCAGGGGAG
-1008  TTAGCAGCGTCGGAACAGCAACAACAGCAGGATATCAAGACCAAAAAACCAAGACTACGCGAAC
-948  CGCACTACTGCTACTGTAACCAAGTGGTCATACGGGGAATGGTGGGTGTGATGGCGCAGACTG
-878  TGAGCTAGAAATGGTTCCATTGCGCATGTATTGACTCGAACTCTACCTAAGGCAAGTGGTATTGC
-813  GACGACTGCAAAAAAACTGTGAATAGCTCTCGCGGATATCGCCTTAAGAAATATCTACTAAATA
-748  CCGAATATATACATATATATATCTTAGCTACATATATATATATCTTAGGTTTAAACGGGCGT
-683  GGCCTATCAATGGATCTGCAAGATCTGGGCTACCGGTCTAGATAAAATATTCAAATCGATG
-618  GTGATGACGGCAGCATAAAACGTAATAATGATGAATGACTTGTACGGCGTAGCAGCGCCAGC
-553  AGCTCAGTCTAATCGCCGTACAGTGGGTGGTGGCTAATCGCTTAGGCTTTTATTCAGAAAGGA
-488  CCTTTTCATTCACCTCGCCCTTGGAGGTTCTGCTGCTGACCCCTTGGCCCTAGCCTCGCCCTC
-423  TGCCTCATGTCTGCTGGAGCGTGACATTAGGACGGAAGCTAGGACGCGCTTCAGAAATT
-358  AAACGGAACATGACAGCAAGACGAAACAAAGCTGGAATAAGGGATCTGGGAAACGGGAAACA
-293  AGGTTACATCAGCAAACTTTCTACCGAAAATCTGGGTAAACATTATAGTACCCCAACATCTT
-228  AAGTTTTCAGCGTGTCTCTCTTATCGCTAGAAATCGCTCATTTCAAGCGTAACGCAATCCGGC
-163  TTTGTTTTTTTTTCCATTTATTCATCCACTCTTATCTGACTCTTCTGCACTATATTAATCAAC
-98  TAGGAGAAATTAATCTGAGCAGAAAGATACGAGAAATACGGATAAAAAACCAAGTTAACCGT
-33  TTTGCAATTGAAGGAGATATACAGAAAAAAATGCTTCTTGAACAAGTCGAACATAAGAGAGTA

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Figure 2. Nucleotide sequence of the 5' noncoding region of the *RAS2* gene. The first nucleotide of the initiation codon is designated +1. I, II, and III refer to the major 5' ends for *RAS2* mRNA synthesis. Horizontal arrows show the 9 nucleotide repeat sequence within element A. The boxed sequence indicates the 21 nucleotide tandem duplication of element B.

Figure 3. Effect of 5' upstream deletions on *RAS2* gene expression. Cells were grown on SD media to mid log phase. Panel A: Northern blot analysis of *RAS2* RNA from the series I deletions (see Fig. 1 legend). RNA isolated from transformants was fractionated by electrophoresis, transferred to nitrocellulose, and hybridized with labelled *RAS2* fragment. Deletion numbers refer to the nucleotides present upstream from the translation initiation codon. A labelled fragment from the *CDC25* gene was used as a control. Strain JC482 contains the wild-type *RAS2* gene, and YAR indicates the *RAS2* disruption strain JC482/5 transformed with plasmid YAR containing the wild-type *RAS2* gene and 5' upstream region. Panel B: Northern blot analysis of *RAS2* RNA from the series II deletions. A cryptic *RAS2* RNA band was present in all of these mutants. Panel C: *RAS2* protein immunoprecipitation. JC482/5 cells transformed with the various *RAS2* deletion constructs were pulse-labelled for 10 minutes with ³⁵S-methionine, extracts were prepared and *RAS2* protein was immunoprecipitated as described in materials and methods. *ras2*⁻ indicates untransformed JC482/5 cells. The lower band is a nonspecific protein that precipitated with normal serum.

positive regulatory element resides on the *Xba*I fragment between -642 and -1493 from the *RAS2* translation initiation codon. Given the results seen with series I deletions, it would appear that in the absence of a downstream regulatory element, an upstream element enhances the production of cluster I but not cluster II and III transcripts.

To determine if the rate of *RAS2* protein synthesis levels

parallels the steady state *RAS2* RNA levels, *RAS2* protein was immunoprecipitated from ³⁵S methionine pulse-labelled extracts of cells carrying each of the various promoter deletions. It has been established that the pulse labelling period of 10 minutes reflects the rate of *RAS2* protein synthesis (11). Some of these are shown in Fig. 3C. Series II deletions with 3' endpoints at -444 (not shown) or downstream had a lower rate of *RAS2*

Figure 4. 5' termini of *RAS2* mRNA mapped by S1 endonuclease digestion. JC482/5 cells carrying the series I deletion mutants shown in Fig. 3A were grown on SD media, RNA was isolated, and 5' ends were mapped as described in materials and methods. The band at the top of the figure is the full-length probe fragment, probably hybridized with plasmid DNA. I, II, and III represent the three *RAS2* transcript clusters. Their major 5' ends map 45, 180 and 235 nucleotides, respectively, upstream of the translation initiation codon (shown in Figs. 1 and 2) and are indicated by arrows. JC482 is the wild-type *RAS1 RAS2* strain. YAR refers to the plasmid containing the entire *RAS2* gene and upstream noncoding region transformed into the *ras2* disruption strain.

protein synthesis than their series I counterparts (Fig. 3C and data not shown). As deletions extended further downstream from -444, a progressive decrease in the amount of *RAS2* protein was observed. Therefore, the level of *RAS2* protein paralleled the RNA levels.

S1 Analysis

To further characterize the effect of *RAS2* 5' deletions on the pattern of the three *RAS2* mRNA clusters we mapped the 5' ends of *RAS2* RNA isolated from JC482/5 cells transformed with each of the series I *RAS2* 5' deletion mutants. The cells were grown on SD, RNA was extracted at the exponential phase of growth, and the 5' ends of *RAS2* RNAs were mapped by S1 analysis (Fig. 4). Plasmid YAR produced the three major clusters of *RAS2* transcripts and their overall pattern was similar to that of the wild-type *RAS2* strain JC482. However, there is a slight enrichment of the major cluster I transcript in cells carrying the plasmid. The plasmid deleted of the *Xba*I fragment (-1492 to -642) also showed a wild-type *RAS2* RNA pattern (data not shown). Deletion mutants -444 and -434 showed a selective loss of cluster II transcripts, but no change in the amount of cluster I and III *RAS2* RNAs. When the deletion was extended to -423, cluster III RNA was also reduced. These results correlate with the Northern analysis of these mutants shown in Fig. 3A. Additional variations in cluster III transcript levels were observed; for example, deletions -568 through -509 produce greater levels of cluster III RNA than the wild-type gene or the undeleted plasmid copy of *RAS2*. Disappearance of all three transcription initiation sites occurred only when all three transcription initiation sites were deleted (-36). This deletion lead to the appearance of a new species

* nmoles of ONPG cleaved / min / mg protein

Element A (-478 to -371)

CACCTCGCCCCCTTGGAGGTTCTGCCTGACCCCTTTGCCCT
 AGCCTCGCCCCCTGCGCCATGTCTCTGCCTGGAGCGTGACATTTA
 GGACGGAGAAGCTAGGCAGG

Figure 5. Activation of a heterologous promoter. The diagram shows the location of *RAS2* fragments 1 through 8 that were inserted upstream of the *CYC1* promoter in plasmid pUAS *CYC1* which contains the *CYC1* promoter fused to the *lacZ* gene. The fragment end points and their β -galactosidase activities in SGE and in SD media are shown in the table. β -galactosidase activity was measured as described (26). Fragments 4 and 7 were also cloned in the opposite orientation and are designated 4 rev and 7 rev. *CYC1* is plasmid pUAS *CYC1* with no insertion. Below the table is shown the DNA sequence of element A. Arrows indicate the 9 bp repeats.

with a 5' end mapping at nt. -32, suggesting the presence of additional regulatory elements either downstream of nt. -32 or upstream of nt. -641.

Activation of a heterologous promoter

The results presented above suggest the presence of at least two positive transcriptional regulatory elements, one lying between nt. -479 and -423 and the other within the 852 bp *Xba*I fragment (from nt. 1493 through nt. -642). To further characterize these two elements, we cloned various fragments of the *RAS2* 5' sequence upstream of the minimal heterologous *CYC1* promoter in plasmid p UAS (18). Transcriptional activation of the reporter gene *lacZ* was measured in cells grown in SD and in SGE media (Fig. 5).

Fragments originating upstream of -638 (fragments 1, 2, 3 and 8) were unable to drive transcription from this heterologous promoter. However, sequences between nucleotides -371 and -642 (fragment 4) were able to promote β -galactosidase expression. Fragments 4 (-371 to -642) and 7 (-371 to -478)

provided comparable β -galactosidase activities, indicating that the sequences between -371 and -478 were sufficient for transcriptional activation comparable to that achieved with larger fragments. This fragment, designated element A, contains two copies of the sequence CCTCGCCCC separated by 43 bp. Fragments 5 and 6 each contained one of these 9 bp direct repeats and provided only partial activation of the *lacZ* gene. Thus while one copy was sufficient to activate transcription from the heterologous *CYC1* promoter (fragments 5 and 6) both copies were required for maximal activation (fragment 7).

These findings are consistent with the results of the Northern and S1 analyses where a decrease in cluster II mRNA was associated with removal of one copy of the 9 bp repeat (-444), and further decreases in cluster II and III RNAs resulted from deletion of the second 9 bp repeat (-423). Fragments 4 and 7 were active when inserted in reverse orientation, suggesting that element A is a classical yeast upstream activation site (UAS). When reversed, fragment 4 showed a decrease in activity of approximately 10-fold, probably as a result of moving element A sequences further upstream. This is consistent with the observation that insertion of sequences between the *CYC1* UAS and its TATA element resulted in reduced promoter activity (18).

Element B

The *Xba*I fragment (-1492 to -642) was unable to activate transcription from the *CYC1* promoter (Fig. 5, fragment 2) suggesting that sequences contained within this fragment do not contain a typical UAS element. However, Fig. 3A and B show that this fragment was necessary for activation of cluster I transcription in the absence of element A. To further characterize

Figure 6. Northern analysis of element B constructs. Amount of *RAS2* RNA from cells containing deletions -168 and X168 are compared to *RAS2* RNA from constructs with the *Xba*I fragment inserted in reverse orientation upstream of -X168, and with the *Xba*I fragment inserted 600 bp further upstream in the *Bam*HI site of plasmid -X168. Oligo 53 (ATATATACATATATATATCTTAGCT-ACATATATATATATCTTAGCG) contains the sequence from -744 to -693 (Fig. 2) and was cloned into the *Xba*I site of -X168. The cryptic *RAS2* RNA is seen in cells that do not contain a functional element B. *CDC25* RNA served as a control.

transcription elements present in this region, the 852 bp *Xba*I fragment was reinserted in both orientations either at its original location on plasmid -X168 in its original orientation (-168), in reverse orientation (XRev-168), or 600 bp further upstream at the *Bam*HI site in the pBR322 sequence of plasmid -X168. Fig. 6 shows that this fragment could activate transcription of cluster I *RAS2* RNA in both orientations but was inactive when it was moved to the *Bam*HI site upstream of its natural location.

Inspection of the sequence within the *Xba*I fragment showed the presence of a 21 bp tandem duplication that may play an important regulatory role (Fig. 2). To determine if this duplication was responsible for the activation of *RAS2* transcription, we replaced the *Xba*I fragment of plasmid -168 with a 53 bp synthetic oligonucleotide containing the 21 bp repeat (Fig. 6). Northern blot analysis of the mRNA isolated from yeast cells carrying this construct showed that oligo 53 could substitute for the *Xba*I fragment in the production of cluster I *RAS2* mRNA (Fig. 6). We conclude that the 21 bp repeats present in the *Xba*I fragment represent a positive transcription regulatory element. It is possible that this 21 bp repeat is functional only when it is brought closer to downstream promoter elements, as was the case when the *Xba*I fragment was inserted into deletion -168. However, insertion of the *Xba*I fragment in reverse orientation upstream of deletion -168 resulted in cluster I transcription. In this case the 21 bp repeat was 877 bp upstream of the 5' end of cluster I transcripts, 225 bp further upstream than its native position of -652 from cluster I 5' ends. This suggests that function of the 21 bp repeat is not dependent upon proximity to downstream promoter elements.

Growth on Ethanol

RAS1 ras2 cells are unable to grow on nonfermentable carbon sources due to a low level of *RAS1* mRNA synthesis (10). An extragenic suppressor mutation (*sra6*) that results in increased *RAS1* RNA overcomes this phenotype (10). Therefore, *RAS1* protein is able to substitute for *RAS2* for growth on nonfermentable carbon sources as long as a sufficient amount of *RAS* protein is produced.

To determine if *RAS2* promoter deletions that result in decreased *RAS2* expression result in this classic *ras2* phenotype, we transferred *RAS1 ras2* cells carrying the various promoter deletions from SD medium to SGE medium, and monitored their growth. Fig. 7A shows the growth curve for several of the series I deletions. Cells with the wild-type *RAS2* gene entered exponential growth at approximately 30 hours after transfer, while cells deleted for *RAS2* failed to enter exponential growth phase by 65 hours. Deletions -479 and -168 entered exponential phase at about the same time as wild type cells, indicating that the *Xba*I fragment was able to support growth on SGE in the absence of Element A. Only deletion -36 was unable to grow on SGE, consistent with the absence of transcripts of wild type length (Fig. 4) and the severely reduced amount of *RAS2* protein (Fig. 3C) in this mutant.

In the absence of the *Xba*I fragment (series II deletions), cells with deletions to -423 and greater had not entered exponential phase by 65 hours in SGE (Fig. 7B). These results are consistent with the marked reduction of *RAS2* transcripts observed in these deletions (Fig. 3B). Thus in the absence of element B, the decrease in *RAS2* mRNA and the inability to use ethanol as a carbon source correlate with the deletion of both 9 bp repeats in Element A.

Transcriptional activation from the *Xba*I fragment supported

growth in SGE in either orientation, but not when it was inserted at the BamHI site 600 bp further upstream (Fig.7C), consistent with *RAS2* mRNA levels produced by these constructs (Fig.6).

Figure 7. Growth curves in nonfermentable carbon source. Cells containing the various deletion plasmids were grown to stationary phase in SD and then transferred to SGE medium. Optical density was measured for a period of 65 hours. Optical density at 600 nm was plotted on the Y axis and the X axis represents hours after transfer to SGE. wt is strain JC482 containing the wild-type *RAS2* gene. A. Series I deletions. wt, strain JC482; *ras2*, JC482/5 *ras2* disruption strain. The numbers of the others indicate the *RAS2* 3' deletion end point. B. Series II deletions. C. Element B constructs. 53 oligo, the 53 nt oligo containing element B cloned upstream of deletion -X168; X in BamHI-X168, the 852 bp *Xba*I fragment cloned into the BamHI site of the plasmid carrying deletion -X168; Xrev-168, the *Xba*I fragment cloned into its normal position but in reverse orientation in the plasmid carrying -X168. The deletion constructs not shown here were also tested for growth in SGE medium and the results are shown in Fig. 1.

To determine if the 21 bp direct repeats of Element B were responsible for supporting growth in SGE in the absence of Element A, we tested the construct containing oligo 53 cloned upstream of deletion -X168 for growth in SGE (Fig.7C). Insertion of oligo 53 enabled cells carrying deletion -X168 to use ethanol as a carbon source, providing further evidence that Element B functions as an activator of *RAS2* gene expression. Together these results show that deletions upstream of the *RAS2* gene that result in reduced *RAS2* RNA and protein levels (Figs. 3, 4, and 6) correlate with the inability to use ethanol as a carbon source.

DISCUSSION

Deletion analysis of the 5' coding and noncoding regions of the *RAS2* gene allowed us to identify two major transcriptional regulatory elements, A and B, involved in *RAS2* gene expression. Additional minor upstream regulatory elements are most likely present, as suggested by subtle changes in expression of different transcript clusters. Fine mapping of the *RAS2* upstream region should allow characterization of these additional elements. Deletion of element A, located between -371 and -478 bp from the *RAS2* translation initiation codon, resulted in the reduction of cluster II and III transcripts. This element contains two copies of the sequence CCTCGCCCC that appear to be critical for activation. Only cluster II transcripts were reduced when a single copy of this sequence was deleted. A fragment from the *RAS2* upstream region containing one copy of the 9 bp repeat was capable of activating the heterologous promoter, UAS *CYC1*, although maximum activation required both copies.

The sequences including the downstream 9 bp repeat from element A have 14/17 bp homology with an upstream element of the human epidermal growth factor receptor (*EGFR*) gene (*EGFR*= GCCTCGgCCC GCGcTA, *RAS2*= GCCTCGc-CCCtGCGCcA) (19). The sequences including the upstream 9 bp repeat from element A have 9/11 bp homology with the *EGFR* element. This *EGFR* upstream sequence is protected from DNase I digestion by GCF-1 protein, which represses transcription of the *EGFR* gene and a number of other eukaryotic genes. It has been suggested that the *EGFR* element may act as both a negative and a positive regulatory element. Other elements have been shown to have both positive and negative roles in transcription. For example, the sequences recognized by RAP1 (GRF1) can act as either activators or repressors of transcription depending on their context (20, 21). The octamer sequence (ATGCAAT), is involved in either positive or negative regulation of immunoglobulin gene transcription, depending on which octamer binding factor is present in the cell (22-25). We have previously shown that *RAS2* RNA clusters II and III are specifically repressed during nitrogen deprivation (11). It will be of interest to determine if factors binding to element A, which is required for transcription of *RAS2* RNA clusters II and III, mediate repression of these clusters during nitrogen starvation. Also of interest is the ability of human GCF-1 to recognize and bind to the 9 bp repeat sequences in element A, and conversely, the presence in yeast extracts of a factor that binds to the human *EGFR* sequence.

Element B is a 21 bp tandem duplication of the sequence TACATATATATATCTTAG that maps 215 bp upstream of element A. In the absence of element A, element B can support transcription of cluster I mRNA but not clusters II and III. Element B remained functional when the *Xba*I fragment was inverted, moving this element 600 bp further upstream.

Therefore, this element functions in either orientation, and as far upstream as 877 bp from the 5' end of the cluster I transcript. However, element B did not activate transcription of the *CYC1* promoter and is therefore not a classical UAS.

Perhaps element B is a TATA-like element. Element B contains sequences demonstrated to function as TATA elements for other yeast promoters (27) and that are potential TFIID recognition sequences (28). TATA elements can function as far upstream as 200 bp from the 5' ends of transcripts (27). In contrast, element B functions as far as 600 bp upstream. TATA elements may influence the level of transcription (29, 30), the site of mRNA initiation (27), or both. Element B does not affect the site of mRNA initiation, but does specifically increase the level of cluster I transcription in the absence of element A.

We have previously shown that the relative abundance of different *RAS2* RNA clusters changes during nitrogen starvation and sulfur starvation (11). However, *RAS2* protein levels remain constant due to an increase in the efficiency of *RAS2* mRNA translation (11). These observations suggest that the choice of 5' ends may determine translation efficiency. Since we have defined upstream deletions that affect the levels of particular 5' ends, we can now address this question. For example, deletion of element A results in selective reductions of cluster II and III transcripts. During sulfur starvation only cluster III transcripts are detectable (11). It will be interesting to determine if during sulfur starvation *RAS2* mRNA is detectable in cells with element A deletions. Further, if the expected decrease in the rate of *RAS2* RNA synthesis occurs, is there a corresponding decrease in the rate of *RAS2* protein synthesis, or is there a compensatory increase in translational efficiency of *RAS2* mRNA? This approach should allow us to elucidate the role of 5' end selection in determining the translation efficiency of *RAS2* mRNA.

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