Properties of the transcriptional enhancer in *Saccharomyces cerevisiae* telomeres

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ABSTRACT

Saccharomyces cerevisiae chromosomes end with the sequence $C_{2-3}A(CA)_{1-4}$, commonly abbreviated as $C_{1-3}A$. These sequences can function as upstream activators of transcription (UAS's) when placed in front of a *CYC1-lacZ* fusion gene. When $C_{1-3}A$ sequences are placed between the *GAL1,10* UAS and the *CYC1-lacZ* fusion, the $C_{1-3}A$ UAS still functions and the amount of β -galactosidase produced in cells grown on glucose is as much or more than that for cells grown on either glycerol medium, or cells grown on glucose medium containing a plasmid with just the $C_{1-3}A$ UAS. These data indicate that the UAS is immune from glucose repression from the upstream *GAL1,10* UAS. Because $C_{1-3}A$ sequences are bound *in vitro* by the transcription factor *RAP1*, the UAS activity of yeast telomere sequences was compared with that of a similar UAS from the tightly regulated ribosomal protein gene *RP39A*, which also contains a *RAP1* binding site. While transcription from the ribosomal protein gene UAS was responsive to cell density, the amount of transcription from the $C_{1-3}A$ UAS was nearly the same at all cell densities tested. These data show that the transcriptional activation by $C_{1-3}A$ sequences is not regulated by cell density.

INTRODUCTION

Telomeres are the physical ends of eukaryotic chromosomes. They protect internal sequences from exonucleolytic degradation and end-to-end fusion, and provide a solution to the dilemma of how to replicate completely a double-stranded DNA end (reviewed in [1]). These properties suggest that specific telomere binding proteins play a role in the replication and maintenance of telomeres. In the study reported here, it was our goal to devise a genetic screen for mutants in telomere-binding proteins. In the course of this work, we found that yeast telomeric DNA sequences function as an upstream transcriptional activator (UAS) with some unusual properties.

It was shown several years ago that by placing a recognition site for the DNA binding protein *lexA* between the *GAL1,10* UAS and its TATA box and then supplying *lexA* protein in *trans*, one could attenuate the transcriptional activation of the *GAL1,10* UAS. It was also shown that placing the site at a number of positions between the UAS and TATA box would inhibit

transcription [2]. Although the mechanism by which the *lexA* protein interferes with transcription activation is still unclear (reviewed in [3]), this result suggested a general approach for assaying yeast DNA-binding proteins *in vivo*. In place of the *lexA* binding site, one can insert the sequence of interest and then ask for interference with *GAL4*-mediated transcription activation. One can then look for mutants that partially or completely remove this inhibition, some of which may be in the gene encoding the DNA-binding protein which caused the initial repression of transcription. We have conducted such an experiment with yeast telomeric DNA sequences ($C_{1-3}A$ repeats) to search for proteins that interact with yeast telomeres. During the course of these experiments, we found that the yeast telomere sequence $C_{1-3}A$ functions as a UAS (others have also reported that telomere sequences can act as a UAS [4]) and is unaffected by glucose repression from the upstream *GAL1,10* UAS. This result is in marked contrast to a similar fusion made to the *HIS3* UAS in which the *HIS3* transcription was reduced about 80% on glucose by the upstream *GAL1,10* UAS [5].

Because $C_{1-3}A$ sequences are bound *in vitro* by the transcription factor *RAP1*, the UAS activity of yeast telomere sequences was compared with that of a similar UAS from the tightly regulated ribosomal protein gene *RP39A*, which also contains a *RAP1* binding site. The control of ribosomal protein gene transcription is linked to cell growth rate (reviewed in [6]). We tested the activity of the $C_{1-3}A$ UAS and the tripartite *RP39A* UAS at different cell densities. While transcription from the ribosomal protein gene UAS was responsive to cell density, the amount of transcription from the $C_{1-3}A$ UAS was nearly the same at all cell densities tested.

MATERIALS AND METHODS

Yeast and bacterial strains and methods. All yeast and bacterial media, yeast lithium acetate and bacterial transformation procedures have been previously described [7]. All experiments reported here were conducted in yeast strain KR14-86 (*MAT α ade2-1 ade8-18 ura3-52 trp1 Δ leu2-3,112 his4*) and KR9-14 (*MAT α ade2-1 ade8-18 ura3-52 trp1 Δ met2*). All plasmid constructions were performed in *E. coli* strain MC1000 (*r⁻ m⁻ pyrF::Mu trp lac*). Assays for β -galactosidase activity in yeast were performed as described by Guarente [8]. All values reported for β -galactosidase activity are the results of at least three independent cultures of the same transformant where each culture

was assayed in triplicate. Results similar to those reported here (Table I) were obtained with 3 or more separate transformants, and using yeast centromere vectors bearing similar constructs to those described below and in Table IA (data not shown). Yeast grown in galactose medium were always started from other cultures grown for ~10 generations on a non-fermentable carbon source prior to being tested for β -galactosidase activity in order to avoid any residual effects of glucose repression. All β -galactosidase assays were performed on cells grown to densities of 1×10^7 to 15×10^7 cells/ml, with the exception of those assays presented in Figure 2. We found no significant difference in the amount of β -galactosidase produced per cell over this range of cell densities for cells bearing YEpGCAZ, YEpGZ, YIpGZ, YIpGCAZ, YIpCAZ, YIpZ, YIpcaZ, YIpgtZ, YEpGcaZ and YEpGgtZ (described below).

Plasmid constructions. Plasmids were constructed using the methods described previously [7]. The plasmids YEpGZ, YEpGCAZ, YIpGZ, YIpGCAZ, YIpZ, YIpCAZ, YEpGcaZ, YEpGgtZ, YIpcaZ, and YIpgtZ were constructed from pLGSD5 ([9]). YEpGZ is the same plasmid as pLGSD5 and contains the 2μ origin of replication, the *URA3* gene and a *GAL1,10* UAS-*CYC1* TATA region-*lacZ* fusion (fig. 1). This plasmid contains a single *XhoI* site between the *GAL1,10* UAS and the *CYC1* TATA region. YEpGZ was linearized with *XhoI* and a 125 bp *Sall*-*XhoI* fragment containing 81 bp of $C_{1-3}A$ from YTCA-1X [7] was ligated into this site to generate YEpGCAZ. This plasmid contains the $C_{1-3}A$ fragment in the orientation (reading the top strand 5' to 3') *GAL1,10* UAS-*XhoI*- $G_{1-3}T$ -*Sall*/*XhoI*-*CYC1* TATA (see fig. 1). Both YEpGZ and YEpGCAZ were converted to yeast integrating plasmids (YIp versions) by cutting with *HindIII*, isolating the largest fragments and recircularizing by ligating at low DNA concentration. This procedure removes the 2μ origin of replication so that these plasmids cannot replicate autonomously in the cell.

YIpGZ and YIpGCAZ were used to construct, respectively, YIpZ and YIpCAZ. YIpGZ and YIpGCAZ were individually cut with *StuI* (which cuts only once in these plasmids in the *URA3* gene) and *XhoI*, treated with T4 DNA polymerase to render the ends flush, and the largest fragments were isolated from an agarose gel. These fragments were individually ligated to the 0.4 kb *StuI*-*SmaI* restriction fragment from the *URA3* gene (isolated from the YIp5 plasmid [10]) and the ligation mix was used to transform the bacterial strain MC1000. In order to isolate those plasmids with an intact *URA3* gene, transformants were tested for their ability to grow on minimal media lacking uracil (the yeast *URA3* gene will complement the *E. coli pyrF* mutation [11]). These plasmids, called YIpZ and YIpCAZ, contain a precise deletion of the *GAL1,10* UAS and a *XhoI* site at the 3' end of the *URA3* gene. The plasmids YIpZ and YEpGZ were cut with *XhoI* and ligated to the $C_{1-3}A$ oligonucleotides (sequence shown in fig. 1) to form YIpcaZ, YIpgtZ, YEpGcaZ and YEpGgtZ. YIpcaZ and YEpGcaZ are in the same orientation as YEpGCAZ, while the gt plasmids have the inserts in the reverse orientation. All plasmids were transformed into yeast using the lithium acetate procedure. Yeast integrating plasmids (YIp plasmids) were first cut with *StuI* to target the plasmids to the *URA3* gene. Yeast transformed with the YIp plasmids were screened by Southern hybridization to isolate those strains that contained only one plasmid per genome.

The plasmid YIpRPG was derived from pMR672A (see [12]; kindly provided by J. Woolford). The same method that

Rotenberg and Woolford used to convert pMR10 to pMR11 [12] was used to convert pMR672A to YIpRPG.

RESULTS

The YIp plasmids contain no *ARS* and were integrated in single copy at the *URA3* locus. The YEp plasmids are extra chromosomal replicating vectors by virtue of the 2μ origin of replication. All plasmids contain the gene for β -galactosidase (*lacZ*) fused to the *CYC1* TATA region (designated Z in the

Fig. 1. The plasmids used in this study. The structure of the family of plasmids used in this study is diagrammed. The large arrow is the 125 bp CA fragment and the small arrows are the 35 bp oligonucleotide. The letters (CA, ca or gt) indicate the insert's orientation (see Methods). Examples of integrating plasmid names are shown. The sequences of the B) 125 bp insert with 81 bp of $C_{1-3}A$ and C) the 35 bp $C_{1-3}A$ oligonucleotide are shown. The complementary strand to both inserts results in a duplex with a 5' TCGA overhang on each end. The sequences which conform to *RAP1* binding sites as determined by Buchman et al. [4] are underlined. The 35 bp oligonucleotide contains no exact matches to this consensus and only one 12/13 match and is underlined with a dashed line. The nonconforming base is in italics. Both the 125 bp fragment containing 81 bp of $C_{1-3}A$ and the 35 bp $C_{1-3}A$ oligonucleotide are bound by a factor in crude yeast cell extracts prepared by the method of Berman et al. [13] (data not shown). Subsequent work by a number of groups indicates that the factor that binds to these sequences in a gel retardation assay is the *RAP1* protein [4, 14, 15]. D) The *RAP1* consensus sequence is shown.

plasmid name). These constructs alone do not support detectable levels of β -galactosidase production in yeast, but require the addition of an upstream activator sequence, or UAS, to activate transcription. The addition of the 365 bp *GALI,10* UAS upstream of the *CYC1* TATA region places the *lacZ* gene under *GAL4* control [9] (the *GALI,10* UAS is designated by G in the plasmid name). We have inserted short stretches of the yeast telomeric DNA sequence $C_{1-3}A$ into the *XhoI* I site (fig. 1) (designated by CA in the plasmid name) and placed these constructs into cells and assayed them for β -galactosidase activity (Table I). Cells were grown on glucose, glycerol or galactose in order to gauge the effect of the $C_{1-3}A$ sequences upon the *GALI,10* UAS under conditions of transcriptional repression (glucose medium), lack of repression and lack of transcriptional activation (glycerol medium) and transcriptional activation (galactose medium).

As had been previously reported, cells bearing plasmids containing only the *GALI,10* UAS and no $C_{1-3}A$ sequences produced no β -galactosidase on glucose and glycerol media and large amounts of enzyme when grown on galactose media (Table I A) [9]. Those cells bearing plasmids that contained the *GALI,10* UAS and a 125 bp insert with 81 bp of $C_{1-3}A$ sequences produced β -galactosidase on glucose and glycerol media, although the amount of enzyme produced was lower than that for cells grown on galactose medium. This observation is in contrast to a similar experiment with *HIS3* where *HIS3* gene expression was

reduced ~80% on glucose media by an upstream *GALI,10* UAS. The amount of enzyme produced in cells bearing YIpGCAZ and YEpGCAZ was proportional to plasmid copy number. These data suggested that 1) the $C_{1-3}A$ sequences could function as a UAS because they could activate transcription under conditions where the *GALI,10* UAS is quiescent, and 2) the $C_{1-3}A$ UAS was immune from the glucose repression effect of the upstream *GALI,10* UAS. Cells bearing the GCAZ plasmids produced less β -galactosidase on galactose media than cells with the analogous GZ plasmids. These data suggest that the protein(s) which activate transcription from the $C_{1-3}A$ UAS may also interfere with the transcription activation from the *GALI,10* UAS.

In order to show that the 125 bp fragment could function as a UAS on its own, the *GALI,10* UAS was deleted from YIpGCAZ to form YIpCAZ. Cells bearing a single copy of this plasmid contained the same amount of β -galactosidase when they were grown on glucose, glycerol or galactose. The amount of β -galactosidase produced in glucose-grown cells bearing YIpGCAZ and YIpCAZ was similar, further demonstrating that the *GALI,10* UAS did not repress the downstream $C_{1-3}A$ UAS. These data indicate that this fragment can function alone as a UAS and that this UAS is not under carbon source control.

Besides the 81 bp of $C_{1-3}A$ sequences, the 125 bp fragment contains small amounts of pBR322 (4 bp) and a telomere associated sequence called Y' (40 bp). In order to establish that $C_{1-3}A$ sequences could function as a UAS in the absence of other sequences, plasmids containing synthetic oligonucleotides consisting of only 35 bp of $C_{1-3}A$ in both orientations (denoted ca and gt) were constructed and transformed into yeast (Fig. 1). This fragment also functioned as a UAS in promoting β -galactosidase production in plasmids containing only the $C_{1-3}A$ UAS, and in constructs containing the *GALI,10* UAS upstream of the $C_{1-3}A$ UAS (Table 1 B). In all cases, the $C_{1-3}A$ UAS allowed a level of β -galactosidase production that was about 10-fold above the control plasmid with no UAS (YIpZ). The value for untransformed cells was the same as that for YIpZ (data not shown). As with the 125 bp fragment, the 35 bp $C_{1-3}A$ UAS, was also immune from glucose repression of the upstream *GALI,10* UAS. When the 35 bp $C_{1-3}A$ insert was 3' to the *GALI,10* UAS, approximately the same amount of β -galactosidase was made on glucose (repressing) media as on glycerol (nonrepressing) media. These data show that $C_{1-3}A$ sequences alone could act as a UAS and that they were immune from the glucose repression activity of the *GALI,10* UAS. The 35 bp $C_{1-3}A$ UAS functions in both orientations as those cells bearing YIpgtZ or YIpcaZ produced similar amounts β -galactosidase (Table I). Interestingly, we have found that 140 bp of the telomere sequence from *Oxytricha nova* (C_4A_4) also has weak UAS activity in yeast (data not shown), but it is not clear if transcription activation by $C_{1-3}A$ and C_4A_4 occur by the same mechanism.

While this work was in progress, several groups reported the isolation, cloning and/or characterization of a protein, called, alternatively, *RAP1*, GRFI, TUF or TBA, that can bind to sequences in yeast telomeres [4, 13, 14, 15, 16]. All of these activities are believed to be the same protein, and will be referred to here as *RAP1*. The *RAP1* protein was originally isolated as a protein that can bind to the E-box of the yeast silent mating type cassettes [14, 15], and the consensus binding site has been defined as A/G C/A ACCCANNCA C/T C/T [4] which occurs 5' to many genes as well as at yeast telomeres. The consensus sequence from the yeast telomere is a high-affinity *RAP1* binding

Table I: β -galactosidase produced in yeast KR14-86 bearing the indicated plasmids

β -galactosidase units (nmol/min $\times 10^7$ cells)			
A. 81 bp $C_{1-3}A$ Insert			
Carbon source: Plasmid	glucose	glycerol	galactose
YIpGZ	0.027	0.037	6.4
±	0.005	0.015	0.30
YIpGCAZ	1.4	1.0	2.7
±	0.15	0.26	0.05
YEpGZ	0.047	0.035	73.
(pLGSD5) ±	0.013	0.009	3.5
YEpGCAZ	22.	17.	48.
±	8.2	2.8	7.3
YIpCAZ	1.1	0.63	0.82
±	0.33	0.32	0.19
YIpZ	0.034	0.024	0.031
±	0.027	0.015	0.014
B. 35 bp $C_{1-3}A$ Insert			
Carbon source: Plasmid	glucose	glycerol	galactose
YIpcaZ	0.50	0.17	0.28
±	0.19	0.049	0.019
YIpgtZ	0.89	0.38	0.51
±	0.36	0.25	0.23
YEpGcaZ	7.4	8.9	59.
±	0.59	4.8	6.3
YEpGgtZ	12.	6.1	51.
±	1.3	0.92	3.2

site, and three copies of this sequence (in the gt orientation) can function as a UAS in plasmid constructions similar to ours [4]. The 125 bp insert with 81 bp of $C_{1-3}A$ contains 4 *RAP1* binding sites, 3 of which overlap (Fig. 1). Yeast ribosomal protein genes contain a tripartite UAS consisting of three consensus sequences denoted HOMOL1, RPG, and the T-rich region [17], all of which are important for transcriptional control [12]. The consensus for the RPG box matches the *RAP1* consensus sequence. Transcription of these genes is highly regulated (for review see [6]), one level being in response to cell density. Cells approaching stationary phase transcribe *RPG-lacZ* fusions less frequently than cells in log phase, resulting in less β -galactosidase per cell in stationary phase cells than in log phase cells (see below, and KR unpublished observations). Because the *RAP1* protein binds at both telomeres and the RPG box *in vitro* [4], we compared the effect of cell density upon transcription from the $C_{1-3}A$ UAS and the UAS from the ribosomal protein gene *RP39A*.

KR9-14 cells containing a single copy of either YIpCAZ or YIpRPG at the *URA3* locus were constructed. The YIpRPG plasmid is very similar to YIpCAZ except that it contains a 126 bp fragment that contains the HOMOL1 box, the RPG box, and the T-rich region of the *RP39A* gene in place of the $C_{1-3}A$

sequences (see Methods and [12]). This fragment promotes *lacZ* transcription in yeast in an orientation specific manner and contains the RPG box in an orientation equivalent to the GT orientation in YIpgtZ. KR9-14 cells bearing either YIpCAZ or YIpRPG were grown to log phase in glucose medium, and then diluted to various extents into fresh glucose medium and grown for 14 hrs at 30°C. Aliquots of 10^7 cells from cultures at different cell densities were assayed, and the amount of β -galactosidase versus cell density was determined (Fig. 2). The amount of β -galactosidase per 10^7 cells in KR9-14 cells bearing YIpRPG decreased dramatically with increasing cell density, while the amount of enzyme per 10^7 cells in cells bearing YIpCAZ was scattered about the value of ~ 2.6 nmol/min/ 10^7 cells regardless of cell density. Thus, the $C_{1-3}A$ UAS did not respond to changes in cell density as did the tripartite UAS from the ribosomal protein gene *RP39A*. Very similar results were obtained when cells were first grown in glycerol media and then diluted into glucose media for 14 hr (data not shown). In addition, we never observed any effect of cell density upon the amount of β -galactosidase produced in cells bearing YIpgtZ. Cells bearing YIpgtZ contained the same amount of β -galactosidase per cell at cell densities from 1×10^7 /ml to 15×10^7 /ml. These data suggest that the transcriptional control of the *RP39A* gene cannot be explained by a simple model in which the amount of *RAP1* protein binding activity per cell decreases as cell density increases.

A. YIpCAZ

B. YIpRPG

Fig. 2. The *RP39A* UAS, but not the $C_{1-3}A$ UAS, is regulated by cell density. Units of β -galactosidase activity (nmol ONPG hydrolyzed per min per 10^7 cells) from different cultures are plotted against the cell density of the respective culture. A) Cells bearing YIpCAZ. B) Cells bearing YIpRPG. Each point represents the value for a single culture where each culture was assayed in triplicate. The standard deviation of each culture was 10% or less.

DISCUSSION

We have examined several properties of a UAS present in sequences from a cloned yeast telomere. While this work was in progress, several groups reported the isolation of an activity, referred to here as *RAP1*, that can bind to some of the combinations of $C_{2-3}A(CA)_{1-4}$ sequences present at yeast telomeres. These workers have shown that the *RAP1* binding sites from telomeres are high-affinity sites [13, 14], can function as a UAS in yeast [4] and are important for transcription *in vitro* [4]. We have extended these observations with our studies using oligonucleotides and cloned yeast telomere sequences to show that this UAS is immune from glucose-mediated repression from an upstream *GALI,10* UAS (Table I) and that the $C_{1-3}A$ UAS is regulated differently from the tripartite *RP39A* UAS with respect to cell density. One similarity between the *RP39A* UAS and the $C_{1-3}A$ UAS is that ribosomal protein genes show a 2.5 to 4 fold increase in mRNA when shifted from a nonfermentable carbon source to glucose (see [6]). We found that plasmids containing the 35 bp $C_{1-3}A$ oligonucleotide made up to 3 times as much β -galactosidase on glucose media as on glycerol (Table I B), suggesting an increased rate of transcription from the $C_{1-3}A$ UAS on glucose medium compared to a nonfermentable carbon source. The lack of a similar effect in plasmids bearing the 125 bp insert may be due to the multiple *RAP1* binding sites in this fragment or the 44 bp of non- $C_{1-3}A$ sequences interfering with these subtle differences in UAS activity. However, the *RP39A* UAS and $C_{1-3}A$ UAS did not respond in the same way to changes in cell density. These observations indicate that regulation of ribosomal protein gene expression by cell density does not involve major changes in *RAP1* activity, and that the regulation of YIpRPG with respect to cell density may arise from interactions of other proteins with the HOMOL1 box or the T-rich region.

The observation that the $C_{1-3}A$ UAS sequences used in this study are immune from glucose-mediated repression from the

upstream *GALI,10* UAS is surprising when compared to similar studies performed with the *HIS3* gene. When the 365 bp *GALI,10* UAS was placed upstream of both *HIS3* UAS sequences (the constitutive *A₁₁* UAS and the inducible *GCN4* UAS) and inserted at the chromosomal *HIS3* locus, *HIS3* gene expression was always partially repressed to about 20% of its control value when glucose was the carbon source [5]. These data suggest that *RAP1* protein mediated transcription may be specifically immune from glucose repression. The binding of *RAP1* protein nearer the TATA box may specifically abrogate the effects of glucose repression-mediating proteins that bind at the upstream *GALI,10* UAS.

Buchman *et al.* have suggested that *RAP1* represents a general transcription factor in yeast similar to SP1, CCAAT box binding factor, and octamer binding factor in mammalian cells [4]. However, this proposal does not suggest how *RAP1* protein acts at telomeres. Gasser and co-workers have suggested that *RAP1* may attach yeast telomeres to the nuclear scaffold [18]. Thus, *RAP1* may possess a telomere-specific function distinct from transcription activation. Any hypothesis for *RAP1* action at telomeres must address the fact that yeast cells can support 200–800 new telomeres per cell and a 10–25 fold increase in telomeric *C₁₋₃A* sequence with no effect upon cell growth rate or chromosome stability ([7] and Runge, Wellinger and Zakian, in preparation). The only known phenotype is an increase in the length of terminal *C₁₋₃A* tracts ([7]). Assuming three *RAP1* binding sites per 80 bp (as in the cloned yeast telomere sequences in YIpCAZ), this increase in *C₁₋₃A* sequence exceeds the estimated total number of *RAP1* protein molecules per cell (~4000 molecules per cell [4], vs. 6000–16000 sites [7]). Since *RAP1* appears to bind to telomeres *in vivo* (M. Conrad, J. Wright, and V. Zakian, in preparation), its presence at telomeres may be dispensable for telomere function with regard to replication and end stabilization.

Does the transcriptional activation properties of the *C₁₋₃A* UAS serve a function in telomere replication? In the ciliates *Tetrahymena*, *Oxytricha*, and *Euplotes*, an enzymatic activity has been isolated that adds telomeric DNA repeats to DNA termini in the absence of a complementary DNA strand [19, 20, 21]. This activity, known as telomerase, contains an RNA that is required for function. It has been proposed that this RNA provides the template for the addition of telomeric DNA sequences [19]. In the case of the *Tetrahymena* enzyme, the RNA has been cloned and sequenced. It is most likely an RNA polymerase III transcript and contains 159 nucleotides, 9 of which can serve as a template for *Tetrahymena* telomere synthesis [19]. It is conceivable that *RAP1* protein binding in telomeric DNA sequences serves to promote the transcription of *C₁₋₃A* RNA's that are used in a similar enzyme in *S. cerevisiae*. However, transcription promoted within the telomere would yield a long RNA that contains mostly *C₁₋₃A* sequences, quite different from the RNA in *Tetrahymena* (e.g. a small RNA with only a 9 bp stretch of telomere sequence). Thus, if yeast contains a telomerase with a short RNA template similar to that in *Tetrahymena*, it is unlikely to be transcribed from the terminal *C₁₋₃A* repeats.

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