Alternative Mechanisms of Transcriptional Activation by Rap1p*

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Single Rap1p DNA-binding sites are poor activators of transcription of yeast minimal promoters, even when fully occupied in vivo. This low efficiency is due to two independent repression mechanisms as follows: one that requires the presence of histones, and one that requires Hrs1p, a component of the RNA polymerase II mediator complex. Both repression mechanisms were greatly reduced for constructs with tandemly arranged sites. In these constructs, UASrpg sequences (ACACCCATACATTT) activated better than telomere-like sequences (ACACCCACACACC) in an orientation-dependent manner. Both mutations in the SWI/SNF complex and a deletion of amino acids 597–629 of Rap1p (Tox domain) decreased synergistic effects of contiguous telomeric sites. Conversely, deletion of amino acids 700–798 of Rap1p (Sil domain) made UASrpg and telomeric sites functionally indistinguishable. We propose that the Sil domain masks the main transactivation domain of Rap1p in Rap1p-telomere complexes, where the Tox domain behaves as a secondary activation domain, probably by interacting with chromatin-remodeling complexes. Rap1p DNA-binding sites in ribosomal protein gene promoters are mainly UASrpg-like; their replacement by telomeric sequences in one of these promoters (RPS17B) decreased transcription by two-thirds. The functional differences between UASrpg and telomeric sequences may thus contribute to the differential expression of Rap1p-regulated promoters in vivo.

The yeast transcriptional factor repressor activator protein 1 (Rap1p)1 is a context-dependent regulator. It activates the transcription of a large number of heavily transcribed genes, including those encoding glycolytic enzymes, ribosomal proteins, and several components of the transcriptional machinery. It also behaves as a transcriptional repressor, as it is essential for silencing the HML and HMR loci. Finally, Rap1p binds to yeast telomeres and is required for their structural stability and transcriptional repression (reviewed in Refs. 1–3). In this paper our goal was to understand the genetic determinants of the various and in some cases contradictory roles of Rap1p.

Rap1p binds to a variety of related DNA sequences (4). We have shown it builds up structurally distinct complexes with two versions of its consensus binding sequence, the UASrpg sequence (5′-ACACCCATACATTT-3′) and the telomere consensus sequence (5′-ACACCCACACACC-3′) (5, 6). Crystallographic and chemical footprinting data indicate that, when bound to telomeric sequences, the two nearly identical Myb-like domains of the Rap1p DNA-binding domain make very similar contacts to two identical DNA half-sites (ACACCC), (5, 7, 8). Some of these contacts are not possible in Rap1p-UASrpg complexes, because of the presence of Ts in the second half-site (ACATT). We thus proposed that high affinity binding of Rap1p to a UASrpg requires rearrangement of the C-terminal moiety of the DNA-binding domain of Rap1p (6); such a rearrangement appears not to be necessary for binding to different versions of the telomeric sequence (8, 9). The structural differences between different Rap1p-DNA complexes have functional significance (5, 6); here, we provide a further genetic analysis showing that these complexes are functionally different because they activate transcription through different mechanisms.

The very low transcriptional activity of synthetic reporters encompassing a single UASrpg or telomeric site in Rap1p-based activation is inconsistent with the identical affinity of Rap1p for single and tandemly arranged sites in vitro (5). Here, we show that single sites are completely occupied in vivo, despite their low transcriptional activity, by two approaches. First, we observed the footprint of Rap1p in a single UASrpg in vivo, using the characteristic KMnO4-hypersensitivity site at the base T8 of the UASrpg when occupied (5, 10). Second, we measured the ability of a single UASrpg to block activation through an overlapping UASgal site, an experimental design known as binding interference (11).

When assayed as tandem repeats, UASrpgs showed a strong synergistic effect, which was orientation-dependent, whereas telomeric sequences showed a lower synergism, irrespective of orientation (5, 6). This contrasts with the results from single sites, which were not affected by the orientation or the version of the site (5). One of the main goals of this study was the identification of mutations affecting these functional differences. We thus tested many mutations affecting key steps of transcriptional activation; and those that clarify the mechanisms of transactivation by Rap1p are included in this paper and are described below.

For histone depletion, histone H4 can be selectively depleted in the yeast strain UKY403, in which its sole copy is under the control of the repressible GAL1 promoter (12). This strain loses 50% of its normal complement of nucleosomes when transferred from a galactose-containing medium, where it grows normally, to one containing glucose, where it stops growing after a single cell cycle (12). Nucleosome loss activates many (but not all) yeast reporters in vivo (12, 13). It affects transcription of about 25% of yeast genes either by increasing (15%) or

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1 The abbreviations used are: Rap1p, repressor activator protein 1; bp, base pair; rp, ribosomal protein; UAS, upstream activator sequence.
decreasing (10%) the mRNA levels (14). These results show the pivotal role of histones in both activation and silencing of many yeast genes.

**HRS1** was identified as a suppressor of a hyper-recombinant phenotype produced by hpr1 mutations (15). Deletion of the **HRS1** gene impairs transcription of many chromosomal genes, but it also enhances transcription of many promoters when assayed as episomal plasmids, by an unknown mechanism (15).

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Chromatin remodeling is mediated by multisubunit complexes that alter nucleosome structure in an ATP-dependent manner (19). Here we assay mutations in two of these complexes, the SWI/SNF complex and the RSC complex. These complexes have distinct roles in the cell. The SWI/SNF complex is relatively rare, dispensable, and associated with transcriptional activation, and the RSC complex is abundant, essential, and related to cell cycle progression (19). The relevance of these complexes in transcription regulation has been much debated. Deletion of key components of the SWI/SNF complex affects the levels of about only 1% of the yeast transcripts (20, 21). However, the SWI/SNF complex is required for mitotic transcription, probably because of the condensed state of mitotic chromatin (22). Human and yeast SWI/SNF complexes directly reorganize the chromatin structure in vitro to facilitate the binding of transcription factors (23, 24).

Rap1p is a large protein (827 amino acids) with multiple functions, some of which are associated with various domains in the primary protein structure (see Ref. 3 for a review). Analysis in vivo of the functional regions within Rap1p revealed that the DNA-binding domain (amino acids 342–596) is the only portion of Rap1p essential for growth (25). However, removal of the so-called activation (Act) domain (amino acids 627–690, (26)) strongly decreases growth rates and compromises the transcription of several, but not all, Rap1p-regulated genes (25). Deletion of the so-called silencing (Sil) domain (amino acids 700–798) results in a milder growth defect, an increase in the transcription of several Rap1p-regulated genes, and the loss of transcriptional silencing through Rap1p both at telomeres and at the HML and HMR loci (25–27). This domain is the target for interactions with telomeric proteins Rif1p, Rif2p, Sir2p, Sir3p, and Sir4p (26, 28–31). Finally, deletion of amino acids 597–629 (the Tox domain) has minor physiological effects but increases the amount of mutated Rap1p present in the cell (25, 26). It also eliminates the cellular toxicity produced by the overexpression of Rap1p both in *Saccharomyces cerevisiae* and in *Schizosaccharomyces pombe* (32, 33). Rap1p alleles truncated at the C-terminal domain, as well as *sir* mutants, show a general de-repression in telomere-proximal genes (14).

Our results indicate that the architecture of Rap1p-based promoters determines their ability to alleviate various overlapping mechanisms of transcriptional repression. Single sites partially counteracted histone repression, but their transcriptional activation was limited by a putative Hrs1p-mediated transcriptional control. Tandemly arranged telomeric sites eliminated both types of repression, but the resulting complex was not fully active due to internal inhibition in the Rap1p molecule by the Sil domain. The characteristic high activation through tandemly arranged UASrpsgs, which we refer to as the RPE effect (6), appeared in this context as the result of the suppression of this internal inhibition. We propose that this is probably mediated by the remodeling of the C-terminal portion of the Rap1p molecule required for its binding to UASrpsgs (6).

The physiological significance of the RPE effect is unknown. No mutation has been reported so far to abolish it, and so the question cannot be directly addressed. We present two complementary approaches as follows: a whole yeast genome search for UASrpsgs and telomeric sequences in gene promoters, and the substitution of a UASrpg by a telomeric sequence in a natural, Rap1p-controlled promoter. Both analyses suggest that the functional differences between both types of sequences apply to natural promoters and that they control the levels of expression of Rap1p-regulated genes in vivo.

**MATERIALS AND METHODS**

**Plasmids and Strains**

The yeast strains used in this paper are listed in Table I. Strain YPH499 was from the Yeast Genetic Stock Center (YGSC), Berkeley, CA. Strain SCR101ΔU was constructed by transformation of the parent SCR101 (35) with a deleted version of the *URA3* gene, lacking the sequences between the endogenous *StuI* and *EcoRV* sites (36). Transformants were plated directly onto SD plates (6.7 g/liter yeast nitrogen base without amino acids (Difco), 20 g/liter glucose, and 2% agar (Difco)) supplemented with 0.1% casamino acids (Difco) plus 0.1% tryptophan, 0.02% uracil, and 900 mg/liter 5-fluoro-orotic acid (Fluorochem, UK) (37). Plasmid pSLFA-178K is a derivative of pLGA-312 (38), constructed by S. L. Forsburg. It contains the sequences from -178 (XhoI) to the *BamHI* site of the CYC1 promoter. Plasmids pPRLG and pTEL were constructed by inserting copies of the appropriate oligonucleotides into the XhoI site of pSLFA-178K (5). These oligonucleotide sequences (upper sequences only) are as follows: RPEG1, 5'-TCGACACCTTAT-3';TEL1, 5'-TCGACACCTTATCAG-3'; RPEG2, 5'-TCGACACCC- ATATATATATATATATAC-3';TEL2, 5'-TCGACACCC- CATATATATATATGAG-3'; and RPEG12, 5'-TCGACACTCATAATC-3' and TEL12, 5'-TCGACACTCATAT-3'. For convenience, the orientation of these oligonucleotides in the upper strand will be referred to as "direct." In vivo binding interference experiments were performed by

| Name | Relevant genotype | Source |
|------|-------------------|--------|
| MHY308 | MATa ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-52 trp1-Δ419 Hdh1::HIS3-hdh2::LEU2/ pMH310/TRP1 CEN3 ARS1 HHD2 | Han & Grunstein, 1988 |
| UKY403 | MATa ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-52 trp1-Δ419 Hdh1::HIS3-hdh2::LEU2/ pUK421/TRP1 CEN3 ARS1 UASgal-Hhd2 | Han & Grunstein, 1988 |
| AYW3-1B | MATa ade2 his3 ura3 trp1 leu2-6::URA3-ADE2 leu2-k | Santos-Rosa et al., 1996 |
| SSAB-2C | AYW3-1B Hrs1Δ LEU2 | Santos-Rosa et al., 1996 |
| CY26 | MATa ade2 his3 leu2 lys1 ura3 trp1 | J. Pérez-Martin, CNB |
| CY258 | CY26 sir1::LEU2 | J. Pérez-Martin, CNB |
| BY4741 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | EUROSCARF |
| Y01586 | BY4741 snf2::kanMX4 | EUROSCARF |
| Y04686 | BY4741 rec1::kanMX4 | EUROSCARF |
| Y03586 | BY4741 sir1::kanMX4 | EUROSCARF |
| SCR101 | MATa ade2 his3 leu2 ura3 trp1 rap1-1 UASgal-rlp25-Rap1 | Goncalves et al., 1996 |
| SCR101ΔU | SCR101 ura3 | This study |

For additional information see the following web page: web.wi.mit.edu/young/chromatin/.
Mechanisms of Transcriptional Activation by Rap1p

**Extraction of Genomic DNA**—Cells resuspended in 500 μl of Solution A were spheroplasted by adding 100 mM KMKO₄ for 1 min on ice. The reaction was stopped by the addition of an equal volume of 140 mM Tris-HCl, pH 7.4, 20 mM EDTA. After addition of 50 mM potassium acetate was added, mixed thoroughly, and left on ice for 30 min. Proteins were then spun down in a microcentrifuge for 15 min at 4 °C. DNA on the supernatant was then extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 1 volume of isopropyl alcohol.

**Rap1p-binding sites** were identified by reiterated extension of appropriate oligonucleotides. Fig. 2 shows results from YHP499 strain clones either non-transformed (panel A) or transformed with pRPG1d and pRPG2d, respectively. Panel D, strain transformed with the mutant construct pRPG2-12. Positions of various UASs are indicated by arrows. × indicates the mutation in Rap1g. Tracks labeled DNA correspond to genomic DNA modified in vivo, and tracks labeled in vitro correspond to cells treated with KMKO₄. In this case, the two tracks correspond to two times of treatment.

**RESULTS**

**In vivo Footprinting of Rap1p by KMKO₄**—We treated yeast cells bearing plasmids including UASρg sites with KMKO₄ in vivo. This treatment damaged specific DNA bases, which can be observed by reiterated extension of appropriate oligonucleotides. Fig. 1 shows results from YHP499 strain clones either non-transformed (panel A) or transformed with pRPG1d (panel B) or pRPG2d (panel C) plasmids. We observed two bands for each UASρg, corresponding to bases T8 and A7 (lower and upper band of each pair in Fig. 1, respectively). This pattern differed from the single strong KMKO₄ -hypersensitivity site at position T8 observed elsewhere (25). The intensity of the KMKO₄ hypersensitivity sites in **Fig. 1** suggests that both constructs were similarly occupied in vivo, despite their very distinct transcriptional
activity (about 20-fold (5), see below). We verified that the bands shown in Fig. 1 were actually originated by the binding of Rap1p to the UASrpg sequences by repeating the experiment with strains transformed with the plasmid pRPG2-12. This construct bears two mutated sites (RPG-12 (47)); it displays a severely reduced affinity for Rap1p binding (47) and a negligible transcriptional activation (5). This construct did not show the characteristic KMnO4 hypersensitivity sites due to Rap1p binding (Fig. 1, panel D).

In Vivo Binding Interference between Rap1p and Gal4p—Gal4p-binding sites (UASgal) consists of two palindromic CGG sequences separated by 11 nucleotides (48). We constructed a hybrid UAS in which an essentially canonical UASgal overlaps a single RPG site (pGR5, Fig. 2). In this construct, Gal4p and Rap1p should interact simultaneously with the same GG doublet. As a control, plasmid pMD1 contains a very similar sequence but lacks the key C/G base pair at position 10 of the UASrpg, which is essential for Rap1p binding (7, 47) (Fig. 2). Note that this residue lies outside the Gal4p-binding sequence in a position that does not affect Gal4p binding (48). Fig. 2 shows that, when transformed into BY4741 cells, this construct behaved as a typical UASgal reporter, silent in glucose (about 3 Miller units) and strongly activated in galactose (over 250 units (48)). Conversely, plasmid pGR5 gave a weak activation in glucose, as a single RPG site (Fig. 2). In this construct, Gal4p and Rap1p binding sites to activate transcription over the basal levels of expression (7, 47). This construct did not show the characteristic KMnO4 hypersensitivity sites due to Rap1p binding (Fig. 1, panel D).

Transcriptional Activation by Rap1p in Histone-depleted Chromatin—We analyzed the behavior of synthetic promoters bearing different Rap1p-binding DNA sites in the histone-depletable strain UKY403, as well as in the isogenic wild type strain MHY308 (Table II). When grown in galactose, the two strains gave very similar results for all constructs assayed, although the values from the UKY403 strain were about 80% of the values from strain MHY308 (Table II, column Gal-H4/wt). Apart from this difference, the behavior of both UASrpg-based (RPG) and telomeric sequence-based (TEL) plasmids was essentially as reported for glucose-grown transformants of unrelated genetic backgrounds (5).

Addition of glucose to strain MHY308 (the “wild type” strain) increased transcription of all constructs 3–4-fold without changing their relative strengths (upper and lower sections of Table II). This was probably associated with the change of carbon source. In contrast, in strain UKY403 (the histone-depletable strain), addition of glucose and the subsequent depletion of chromatin from nucleosomes changed the relative strength of each construct. For example, addition of glucose increased transcription of pSLFΔ-178K only 2-fold in strain MHY308 and more than 20-fold in strain UKY403 (Table II). A similar but reduced effect was observed with constructs bearing single Rap1p-binding DNA sites, which increased their transcription 8.3-fold, on average, in the histone-depleted conditions (Table II). As a result, these constructs showed higher transcription levels in the histone-depleted strains than in the wild type strain (Table II, column Gal-H4/wt). In contrast, constructs with two sites showed a moderate increase (about 3-fold) in their transcription in the histone-depleted strain upon addition of glucose. This brought their transcription rates to 60% (on average) of the corresponding values for the wild type strain. Therefore, histone depletion had opposite effects in constructs encompassing single or tandemly arrayed sites, activating the former and reducing transcription from the latter. Single Rap1p molecules may not completely counteract histone repression, which would be irrelevant in constructs with tandemly arranged sites.

Histone depletion decreased the efficiency of single Rap1p-binding sites to activate transcription over the basal levels of the UAS-less pSLFΔ-178K construct. The ratio between constructs with single sites and the empty vector was, on average, 8.8 in the wild type strain and only 1.9 in the histone-depleted strain (Table II). In addition, the synergistic effect of adjacent sites was also reduced in histone-depleted conditions, where plasmids pTEL2d, pTEL2r, and pRPG2r gave, on average, only 2.1 times more β-galactosidase units than their single-site counterparts. This ratio was 6.2 in the wild type strain (Table II). However, nucleosome loss did not affect the RPG effect; the ratio between pRPG2d and the average of the rest of constructs with two sites was 1.7 in the wild type strain and 2.1 in the histone-depleted strain, a difference well within the expected error (Table II).

Effects of a Δhrs1 Deletion on Transcriptional Activation by Rap1p—Deletion of the HRS1 gene did not affect transcription of the UAS-less pSLFΔ-178K plasmid but increased transcription of the rest of plasmids (Fig. 3). On average, the increase

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3 F. Z. Idrissi and B. Piña, unpublished results.
was 5-fold for plasmids with single sites and 2.4-fold for constructs with two sites. As a result, the relative strength of these plasmids was significantly lower in the Δhrs1 strain relative to the wild type strain. For example, single sites, which in this particular yeast strain gave only 4.3 more units than the UAS-less vector, increased this figure to 17-fold in the Δhrs1 strain. This suggests that Hrs1p acts as a repressor of the transcription of these constructs. As for histone-depleted strains, the apparent synergistic activation of plasmids pTEL1d, pTEL2r, and pRPG2r was severely reduced in the Δhrs1 strain. These plasmids gave, on average, only 2.6 more β-galactosidase units than their single-site counterparts in the mutant background, a ratio that was 5.2 in the wild type strain (Fig. 3). Therefore, the synergistic activation shown by these constructs may reflect their ability both to counteract histone repression and to suppress a putative inhibitory effect of Hrs1p. However, as in histone-depleted conditions, the RPG effect persisted in the Δhrs1 background; the ratio between the activity of pRPG2d and the rest of constructs with two sites was 2.2 in the wild type strain and 1.8 in the Δhrs1 strain (Fig. 3). The same conclusions were reached when integrated instead of episomal constructs were assayed in Δhrs1 strains (data not shown).

Effects of Mutations in Chromatin Remodeling Complexes on Transcriptional Activation by Rap1p—Disruption of the SWI/SNF complex notably reduced the transcriptional activation of all constructs with Rap1p-binding DNA sites (Table III). In the BY4741 strain background, disruption of the ATPase subunit Snf2p reduced transcription from pRPG1d to 40%, whereas transcription from pRPG2r, pTEL2d, and pTEL2r was reduced to 10% and transcription from pRPG2d to 50%. A snf2Δ mutation in the CY26 strain background had similar effects. In contrast, Δrsc1 and Δrsc2 deletions had little effect on all assayed constructs (not shown), although they seriously affected growth rates, which drop to about a division every 4 h in selective media. These ratios are similar to those of the Δsnf2 strain (not shown).

The ratios between plasmids in SWI/SNF complex mutants showed a pattern similar to that observed in histone-depleted conditions. The synergistic effect produced by contiguous sites in plasmids pTEL2d, pTEL2r, and pRPG2r was lost in SWI/SNF mutant strains; these plasmids produced between 1.5 and 1.6 more β-galactosidase units than pRPG1d, less than an additive effect (Table III). However, the RPG effect persisted in the strains lacking the SWI/SNF complex, in which pRPG2d gave, on average, 4.6 times more β-galactosidase units than the rest of constructs with two sites. This figure is slightly higher than the one corresponding to wild type strains (Table III). Although this increment may be due to some pleiotropic effect of these mutations, our data indicate that the RPG effect occurred in the absence of the SWI/SNF complex, and it did not depend on RSC1 or RSC2 genes.

Dissection of the C-terminal Domain of Rap1p—Strain SCR101 contains a chromosomal copy of the RAP1 gene under control of the GAL1-10 promoter. This strain was supplemented with a single copy plasmid encompassing variants of the Rap1p protein expressed from its natural promoter. In this background, the episomal Rap1p variant is the only Rap1p source when the cells are grown in glucose (25). The results for three of these variants, as well as for an intact copy of the RAP1 gene, are shown in Fig. 4.

Basal expression of the pSLFΔ-178K vector was extremely low in this genetic background, about 1 Miller unit, and identical to the wild type strain (Fig. 4). These effects were opposite those found in the Rap1pΔSil background, both showing a very strong synergistic, orientation-dependent activation (Fig. 4). The Rap1pΔSil allele was thus insensitive to the differences between RPG and TEQ sequences. The effect of the ΔSil mutation did not depend on the presence of the SIR complex, for deletion of the SIR4 gene did not significantly alter the expression of any of the assayed plasmids (data not shown).

Rap1pΔTox deletion generally decreased activation through Rap1p DNA-binding sites (Fig. 4). Transcription from plasmids pRPG1, pTEL1, and pRPG2d was approximately halved, whereas transcription from plasmids pRPG2r, pTEL2d, and pTEL2r was reduced to 10–20% of the corresponding values in the wild type strain (Fig. 4). These effects were opposite those found in the Rap1pΔSil background. In addition, the Rap1pΔTox background, plasmids pTEL2d, pTEL2r, and pRPG2r gave β-galactosidase activities that were only 1.5–3.5 times higher than for pRPG1d and pTEL1d, thus showing low synergism, if any, above a simple additive effect. In contrast, pRPG2d gave 35 times more activity than pRPG1d or pTEL1d, indicating that its synergistic capacity was not affected by ΔTox deletion (Fig. 4).

RPG Effect in a Natural Context—Fig. 5, panel A, shows a sequence analysis of 234 putative Rap1p-binding sites in the yeast genome. Panel B shows the results of a similar analysis when the sites corresponding to ribosomal protein gene promoters were excluded (non-rp promoters, 177 sites). The results from the 64 sites corresponding to ribosomal gene (rp) promoters are shown in panel C. Of the 13 positions analyzed, 

TABLE II

| Plasmid | Transcriptional activity (Miller units) of different plasmids in a histone-depletable yeast strain |
|---------|---------------------------------------------------------------------------------------------|
|         | Galactose                                                                 | Glucose                                                        |
|         | MHY308 (wt)                                                                               | MHY308 (wt)                                                    |
| pTEL1d  | 17 ± 0.4                                                                                  | 10 ± 1.5                                                      |
| pTEL2d  | 34 ± 0.3                                                                                  | 75 ± 6.2                                                      |
| pRPG1d  | 31 ± 1.2                                                                                  | 140 ± 12                                                      |
| pRPG1r  | 27 ± 1.7                                                                                  | 180 ± 11                                                      |
| pRPG2d  | 26 ± 1.3                                                                                  | 170 ± 14                                                      |
| pRPG2r  | 29 ± 1.5                                                                                  | 180 ± 14                                                      |
| pTEL1r  | 19 ± 5.1                                                                                  | 520 ± 10                                                     |
| pTEL2r  | 21 ± 5.5                                                                                  | 520 ± 10                                                     |
| pRPG2d  | 30 ± 4.3                                                                                  | 520 ± 10                                                     |
| pRPG2r  | 29 ± 4.3                                                                                  | 520 ± 10                                                     |
| pTEL1d  | 19 ± 5.1                                                                                  | 520 ± 10                                                     |
| pTEL2d  | 19 ± 5.1                                                                                  | 520 ± 10                                                     |
| pRPG1d  | 23 ± 6.0                                                                                  | 100 ± 6.3                                                    |
| pRPG1r  | 21 ± 6.0                                                                                  | 230 ± 16                                                    |
| pRPG2d  | 33 ± 3.4                                                                                  | 100 ± 6.3                                                    |
| pRPG2r  | 31 ± 3.4                                                                                  | 100 ± 6.3                                                    |
| pTEL1r  | 24 ± 3.4                                                                                  | 520 ± 10                                                     |
| pTEL2r  | 22 ± 3.4                                                                                  | 520 ± 10                                                     |
| pRPG2d  | 120 ± 16                                                                                   | 520 ± 10                                                     |
| pRPG2r  | 120 ± 16                                                                                   | 520 ± 10                                                     |
| pTEL1d  | 160 ± 17                                                                                   | 630 ± 7.5                                                     |
| pTEL2d  | 160 ± 17                                                                                   | 630 ± 7.5                                                     |
| pRPG1d  | 19 ± 1.8                                                                                  | 630 ± 7.5                                                     |
| pRPG1r  | 29 ± 1.8                                                                                  | 630 ± 7.5                                                     |
| pRPG2d  | 120 ± 7.8                                                                                  | 550 ± 97                                                    |
| pRPG2r  | 120 ± 7.8                                                                                  | 550 ± 97                                                    |

a wt, wild type.
b Histone-depleted conditions were used.
positions 8, 12, and 13 showed different base distribution between rp promoters and the rest. Whereas position 8 hardly revealed sequence specificity in non-rp promoters, Ts prevailed (67%) in rp promoters. Position 12 showed an even distribution of Cs and Ts (47 and 42%, respectively) in non-rp promoters, whereas there is a strong preference for Ts in rp promoters (75%). For position 13, there is a preference for Cs in non-rp promoters (47%) and for Ts in rp promoters (50%). A similar analysis of all UASrpg from rp promoters (50) (186 sites, Fig. 5, panel D) also revealed a clear preference for Ts at positions 8, 12, and 13 (67, 73, and 47%, respectively). All these data demonstrate a strong preference for RPG-like sequences in the UASrpgs of rp promoters, which is not observed for Rap1p-binding sites in non-rp promoters.

These results point to the relevance of RPG-like sequences for the physiology of rp promoters. Fig. 6 shows an experiment aimed at testing this hypothesis. Plasmid pSN36 essentially contains all promoter sequences from the RSP17B gene, fused to the β-galactosidase gene. When introduced into BY4741 cells, it gave a relatively strong β-galactosidase activity, around 200 Miller units. Replacement of the natural UASs in this promoter (two UASrpgs and a poly(T) track) by a sequence similar to the UAS of plasmid pRPG2d (pSR320) did not alter the expression of the construct, except for a slight increase up to 250 units. However,
when the same sequences were replaced by a sequence similar to the UAS from pTEL2d (pST47), the expression of the construct dropped by two-thirds, down to 70 units (Fig. 6). These results indicate that the RPG effect is at least partially responsible for the characteristic high expression of rp genes.

**DISCUSSION**

Synergism between adjacent activator proteins is attributable to several mechanisms. Binding of many activators to their cognate DNA sequences requires the formation of homo- or heteroduplexes. This is the case for most members of the nuclear receptor family (51) and for factors binding to DNA through leucine-zipper motifs, like Jun and Fos (52). However, functional synergism may also occur between factors that do not cooperate for binding to DNA, as is the case of adjacent Rap1p molecules (5, 6). In these UASs, synergism may reflect a cooperative action either for binding to nucleosomes (53) or for interaction with transcriptional cofactors (54–56). Our footprinting data in vivo showed a similar sensitivity to KMnO4 for single and for tandemly repeated UASrpg sites in constructs whose transcription differed by 10–20-fold, suggesting a similar occupancy for single and double sites. The finding that a single Rap1p site prevents binding of Gal4p to its cognate sites further demonstrates that Rap1p binds to isolated sites, although it does not activate through them but marginally. In this regard, Rap1p molecules binding to single sites behave as positive control mutants that bind DNA but do not activate transcription. This phenotype has been related to lack of interaction with key coactivators (Ref. 57 and references therein).

Nucleosome loss activates transcription of many repressed yeast promoters in vivo, whereas activated promoters either do not change or even reduce their transcription rates in histone-depleted conditions (12, 13, 58). Our results indicate that transcription of the parental pSLF-D-178K reporter and that of constructs with a single Rap1p-binding DNA site were impaired by nucleosomes, since their removal increased the transcription rates. In contrast, transcription of constructs with two adjacent sites was reduced, rather than increased, by histone depletion. The synergistic activation by adjacent Rap1p-binding DNA may thus be the relief of the transcriptional repression by histones, as suggested elsewhere (59, 60). The 40% decrease of transcription observed for plasmids with two Rap1p DNA-binding sites in histone-depleted conditions may indicate a specific enhancement of transcriptional response mediated by the presence of histones (61). However, it may rather result

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**FIG. 5.** Relative frequency of the different bases of Rap1p DNA-binding sites from yeast promoters. Panel A, base composition of 234 putative Rap1p-binding sites found in yeast promoters throughout the whole yeast genome, using the strict consensus sequence RCAC-CCANACAYN. Panel B, subset of 177 sites from the previous set, corresponding to non-rp genes. Panel C, subset of 64 sites from rp gene promoters. Panel D, analysis of 186 UASrpg sites from rp promoters, as defined in Ref. 50, selected with the broad consensus AYCCRNNCM. Note the similarity of distributions in panels C and D. Bar sizes indicate the absolute frequency (number of genes); bases are coded as follows: A, light stripes; T, solid bars; C, dark stripes; G, empty bars.

**FIG. 6.** Base mutation analysis of the RPS17B promoter. Panel A, structure of the fusion of RPS17B promoter to the lacZ gene. Base positions are given relative to the first ATG; the lacZ gene starts just after the G of this codon. Panel B, transcription of the different variants of this promoter; the bars represent Miller units from three independent transformants for each construct; lines indicate standard deviations. The structure of the different promoters is indicated in the left; R stands for RPG-like sequences, and T stands for TEL-like ones. Tn indicates a poly(T) track found in the natural RPS17B promoter (plasmid pSN36, top bar). Note that RPG sites of pSR320 (middle bar) are somewhat different from the natural ones. These sites were substituted by TEL sites in pST47 (bottom bar).
from the profound pleiotropic changes occurring upon histone depletion (12). One way or another, the results showed that the tandemly arranged sites of the plasmids were affected by histone depletion oppositely to plasmids with single or no sites.

The effect of the Δhrs1 mutation suggests that transcription of plasmids with Rap1p DNA-binding sites is repressed by a second mechanism involving the RNA polymerase II mediator complex. This inhibition was stronger for constructs with single sites than for constructs with two sites. Therefore, we propose that the apparent synergistic activation through contiguous Rap1p DNA-binding sites (RPG effect excluded) may also reflect that single Rap1p molecules do not overcome a putative Hrs1p-mediated repressive effect. This effect seems more specific than that of histone H4 depletion, since it did not affect UAS-less CYC1 promoter constructs (15). In any case, the construct pRPG2d still showed a strong synergistic effect over constructs with single sites, as well as its characteristic orientation dependence in the Δhrs1 strain, indicating that the Hrs1p was not a determinant to the RPG effect.

Transcription from all our constructs was compromised in genetic backgrounds lacking the SWI/SNF complex. In agreement with the data obtained under histone-depleted conditions, synergism between adjacent TEL or RPG sequences was particularly sensitive to the loss of the SWI/SNF complex, whereas activation through single sites was less affected. A simple hypothesis is that adjacent sites recruit the SWI/SNF complex and that this recruitment would be inefficient for single sites. In chromatin-depleted conditions, the need for chromatin remodeling complexes would be much reduced; consistently, the efficiency of Rap1p for activation was also reduced. However, the RPG effect was present in all the chromatin-altered backgrounds we assayed. We thus propose that it is caused by the cooperative interaction between adjacent Rap1p/ UASrpg complexes and some component(s) of the transcriptional machinery, irrespective of the structure of chromatin.

The mechanisms of transactivation by Rap1p were further elucidated by the analysis of C-terminal Rap1p deletions. The main activation domain of Rap1p corresponds to amino acids 629–690 (the Act domain (25, 26)). Our data suggest that the Tox domain (amino acids 597–629) may also encompass a secondary activation domain. Its deletion decreased activation from all constructs, especially of pTEL2d, pTEL2r, and pRPG2r plasmids, which lost all their synergistic potential. In this regard, the Rap1ΔTox strain resembles Δswil or Δsnf2 strains, which also presented a reduced synergism for the same constructs. A suitable explanation would be that the Tox domain functions as a recognition motif for chromatin remodeling factors, like the SWI/SNF complex, and that this is the main trans-activation function of Rap1p-TEL complexes. As mentioned above, we suggest that this function is only fulfilled when two Rap1p molecules are bound to DNA. The link of Rap1p to chromatin remodeling machines via the Tox domain may be relevant for both transcriptional activation and repression (62, 63). Yeast cells in which Rap1TTox is the only source of Rap1p show high RAP1 expression, which is probably repressed by its genomic product Rap1p (4, 25). Although the effect of this deletion on the transcription of other genes has not been reported, the Tox domain probably plays some key role in the cell, which becomes lethal when the amount of Rap1p in the cell exceeds a given value (32, 33).

The Rap1ΔSil allele was unique for many reasons. After an extensive search for mutations suppressing the RPG effect, we failed to find a mutant where activation through adjacent UASrpgs became orientation-independent or where their activity was similar to that of telomeric sequences. On the contrary, all chromatin-related mutations (and the ΔTox deletion) had the opposite effect, i.e. they exacerbated the functional difference between pRPG2d and the rest of the constructs. Yet, in the Rap1ΔSil background, the RPG effect was extended to adjacent telomeric sequences, the activation from which became orientation-dependent. A simple explanation of these results is provided by the following model: the RPG effect was due to a particular activation domain of Rap1p, which interacted with certain component(s) of the transcriptional machinery. This interaction requires at least two Rap1p molecules bound to the promoter and is orientation-dependent. The Sil domain, or one of the proteins it binds, would preclude or mask this activation in Rap1p-TEL complexes. We have previously proposed that the Rap1p DNA-binding domain requires a structural transition in order to accommodate to UASrpg sequences (6). This structural change may affect the C-terminal domain of the protein and decisively alter the Sil domain, preventing it from masking the activation domain of Rap1p. This should be independent of the SIR complex, because deletion of the SIR4 gene did not affect transcription of these reporters. These results are consistent with the reported data which show that deletion of the Rap1p C-terminal domain (similar to our Rap1ΔSil allele) has broader effects on gene transcription than mutations of any of the SIR genes. Recent reports show that binding of Rap1p to different telomeric-like DNA sequences, although divergent from the canonical site, does not require gross structural changes in the Rap1p DNA-binding domain (8, 9). These results are consistent with the idea that these changes are specific to Rap1p-UASrpg complexes.

The characteristics of the transcriptional induction by Rap1p, including the RPG effect, may be central to the functions of Rap1p in vivo. Our analysis of yeast promoters encompassing Rap1p DNA-binding sites showed a strong bias for UASrpg-type sites at ribosomal gene promoters, where Rap1p probably behaves as a true activator. In about 50% of the cases, these genes show an arrangement of UASrpg sites similar to that of our pRPG2d plasmid (50). Note the good conservation of position T8, although the functional significance of this finding is unknown (6). It may reflect additional control levels that have not been detected. Our results suggest that RPG sequences are evolutionarily favored in rp promoters, because the RPG effect may determine their characteristic high expression. Replacement of RPG sequences by telomeric ones significantly decreases expression. In the rest of the promoters, the RPG effect seems not to be relevant, since no special bias for any combination of “C” or “T” was found at the two positions examined. This may be related to the postulated functions of Rap1p as chromatin opening factor (3, 59, 60) or as a facilitator of Gcr1p binding to DNA (65). In telomeres, the presence of Rap1p molecules in an active configuration may be incompatible with the silencing role of Rap1p in subtelomeric genes. The above-mentioned profound effects of the Rap1ΔC allele on the expression of subtelomeric genes may merely reflect the physiological relevance of the non-active (TEL) configuration of Rap1p.

Our studies show that Rap1p can be added to the list of transcriptional factors whose transcriptional potential is allosterically affected by the DNA sequence to which they bind (34, 57, 66–68). Most of these factors are vertebrate nuclear receptors, but there is at least another possible example from yeast, the heme-activated Hap1p factor (64). Our study differs from these in that we have defined genetic correlations between structurally distinct Rap1p-DNA complexes and genes involved in various aspects of transcription regulation. The modulation of Rap1p transcription activity by its DNA cognate binding sequence implies a shift between chromatin-dependent and chromatin-independent mechanisms of transcriptional activation, and it is linked to specific domains of the Rap1p protein.
and to several epistatic gene groups. This analysis may elucidate the behavior of transcription factors like Rap1p, which can play various and even contradictory roles within the same cell in the same metabolic conditions.

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