Insulators are sequences that uncouple adjacent chromosome domains. Here we have shown that *Saccharomyces cerevisiae* Rap1p and Abf1p proteins are endowed with a potent insulating capacity. Insulating domains in Rap1p coincide with previously described transcription activation domains, whereas four adjacent subdomains spanning the whole of the Abf1p C terminus (440–731) were found to display autonomous insulating capacity. That both Rap1p and Abf1p silencing domains either contain or largely overlap with an insulating domain suggests that insulation conveys some undefined chromosome organization capacity that also contributes a function in silencing. Together with Reb1p and Tbf1p, previously involved in the activity of *Saccharomyces cerevisiae* subtelomeric insulators, insulating potential emerges as a supplementary common property of General Regulatory Factors (GRFs). Thus GRFs, which bind to sites scattered throughout the genome within promoters, would not only play a key role in regulating gene expression but also partition the genome in functionally independent domains.

Abf1p, Reb1p, and Rap1p are three yeast transcription factors known as General Regulatory Factors, or GRFs. They share a number of characteristics. First, they are abundant and essential. Second, one or several binding sites for at least one of these factors are found in a very large number of promoters. Third, these proteins behave as obligate synergizers: their binding motifs usually have little intrinsic regulatory activity but instead amplify the effect of neighboring regulatory sites. They can enhance both activation or repression of transcription. These properties were recognized in early studies (1), then reconfirmed by more recent, systematic analysis (2, 3). Fourth, they are highly multifunctional proteins. Of the three known DNA motifs that are part of silencers in *Saccharomyces cerevisiae*, two are binding sites for GRFs (Rap1p and Abf1p), and the third binds the ORC replication complex (4). Reb1p critically assists polII transcription at the rDNA and further plays a central role at silencers of another budding yeast, *Kluyveromyces lactis*. In addition, Rap1 and Abf1 can both regulate DNA replication initiation. Finally, Abf1p was recently shown to play a role in nucleotide excision repair (5).

Besides having comparable effects, the three GRFs seem to share a common mechanism of action. Indeed, the binding site for one GRF within a promoter can be exchanged with another, and even more compellingly, protein domains can be swapped among GRFs without loss of function (6, 7). It has been hypothesized that GRF binding causes a local opening of chromatin, which then permits increased binding of other transcription factors (1, 8, 9). This idea is further substantiated by the fact that the SWI/SNF chromatin remodeling complex appears to compensate for the effect of deleting a Reb1p site in the GAL1 promoter or an Abf1p site in ARS1, on transcription and replication initiation, respectively (10, 11). Although distortions of DNA known to be induced by GRFs at their binding sites may conceivably be involved, the existence of mechanisms independent of their DNA-binding properties per se was inferred from the observation that activities of GRFs can be transferred to some extent to heterologous DNA-binding domains. Rap1p and Abf1p were recently shown to be involved in the targeting of the NuA4 histone acetylase complex and of the TFIID complex itself involved in the recruitment of the RNA polymerase II machinery (12, 13). However, this concerns only a small subset of promoters, which points to an additional determinant of specificity (14, 15). Overall, the mechanism of action of GRFs is poorly understood at present but is probably very indirect.

It is now well established that the genomes of higher eukaryotes are organized into chromosomal domains that are independently regulated (16). At the boundaries between domains have been found insulators, DNA sequences that prevent the influence of enhancers and silencers from spreading into adjacent regions, and therefore uncouple adjacent domains (17). The genome of *S. cerevisiae* was originally thought to be free of long-range regulations and of proper chromosomal domains. However, studies culminating in the computational analysis of whole genome expression data have revealed stretches of co-regulated genes (18). In parallel, we and others have identified chromatin insulators in this organism and in two instances GRFs were implicated. We showed that Reb1p is involved in the insulator activity of *S. cerevisiae* subtelomeric sequences called STARs, for Subtelomeric Antisilencing Regions (19, 20). Reb1p shares this role with Tbf1p, a DNA-binding protein the few known properties of which are compatible with a GRF-like function (21, 22). The Upstream Activating Sequence of the ribosome protein genes TEF (UAS-rpg) is another recently discovered yeast insulator. Its active core consists of three Rap1p binding sites (23). We therefore decided to address whether the capacity to organize a chromatin insulator is a general property of GRFs. Rap1p and Abf1p were indeed found to contain insulator protein domains, suggesting that GRFs are involved in boundary effects throughout the yeast genome.
**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—Yeast strains described in this study are derivatives of W303-1A, and were obtained following standard genetic manipulations as described in Ref. 20. The variegated expression of URA3 and TRP1 was monitored as described in detail in Ref. 19 by assessing the ratio of colonies growing on a medium selecting for the repression of URA3 and/or the expression of TRP1 versus the total number of colonies growing on a non-selective medium. To correct for the variability inherent to silencing assays, 6 to 12 cultures seeded with independent colonies were assayed in parallel for any given strain.

**Plasmids**—The Gal4-Rap1 chimera harbors Gal4p region 1–94 tagged at its N terminus with a hemagglutinin (HA) epitope and are expressed from the pCUP promoter (20) and are tagged at its N terminus with a hemagglutinin (HA) epitope and are expressed from the pYCAL vector, parallel experiments with some chimeras obtained in both systems as well as in the pCUP system showed that these can be used indiscriminately. Indeed, protein expression levels (24) and results obtained with reporter activation domains found in Rap1 and Abf1 also behave as insulators.

**RESULTS**

**A Silencing Discontinuity Assay for Insulating Factors**—We previously devised a Gal4-based tethering assay to identify proteins that recapitulate insulator properties in S. cerevisiae (20). Strain GF101 carries four tandem binding sites for the transcription factor Gal4p (4×UASg) placed between two reporter genes, URA3 and TRP1, at a fragmented VII-L telomere (Fig. 1A). A STAR derived from a subtelomeric Y element (Y-STAR) was interposed between TRP1 and the telomere. URA3 OFF colonies are detected by virtue of their growth on medium containing 5-fluoro-orotic acid (FOA). We sensitized the assay by deleting Rif1, which increases telomeric silencing. TRP1 is predominantly repressed because only 0.7% of the colonies grew on medium lacking tryptophan (Fig. 1A, line 1, SC-W). The fraction of URA3 OFF colonies was as high as 70%, even though the reporter is 1.5 kb away from the telomere (Fig. 1A, line 2). Therefore it appears that TRP1 and URA3 are only marginally protected from the high level of silencing by the intervening STAR. Not a single colony from GF100Δr1f cultures grew on SC-W+FOA of 5 × 10⁶ cells, showing that in the few colonies where TRP1 is ON, URA3 is also expressed. The 4×UASg sequence, and the Gal4p DNA-binding domain peptide (GBD), have no apparent effect on silencing by themselves (20) (Fig. 1, lines 1–3).

A GBD chimera endowed with insulating capacities is expected to recapitulate insulator properties in (i) improving TRP1 expression, by creating an insulated, silencing-free domain together with the Y-STAR, and (ii) allowing the growth of colonies on SC-W+FOA through the uncoupling of URA3 and TRP1, leaving URA3 silencing essentially unaffected. This is illustrated in Fig. 1B. We have previously shown (20) that GBD chimeras containing Thf1p or Reb1p moieties, as well as some transcription activation domains from higher eukaryotes, fulfill these criteria. Therefore, we first tested whether the transcription activation domains found in Rap1 and Abf1 also behave as insulators.

**Insulating Domains in Rap1p Coincide with Transcription Activation Domains**—Two transactivation domains were previously described in Rap1p. The first is an 87-amino acid region with a BRCT motif found in the first one-third of the protein. The second is 65 amino acids long and resides in the last third of the protein (see Fig. 1A). Chimeras carrying either the N-terminal third of the protein or only the BRCT motif were expressed in strain GF100Δr1f. This slightly increased the fraction of TRP1 ON colonies (Fig. 1A, lines 4 and 5) but did not affect URA3 silencing (Fig. 1A, lines 5 and 8). Importantly, about 10% of the TRP1 ON cells were now able to grow in the presence of FOA (Fig. 1A, lines 6 and 9). The BRCT region of Rap1p therefore composes with the definition of insulating domains. Mutation of residues 206–208 of Rap1p into alanines, which abolishes the transactivating potential of the BRCT domain (25), also abolished its insulating capacity (data not shown).

Expression of chimeras containing the C-terminal activation domains (amino acids 630–695), or a longer version extending into the DNA-binding domain (amino acids 563–695) also allowed the growth of colonies on SC-W+5-FOA medium (Fig. 1A, lines 12 and 15). However, this not only caused full TRP1 derepression but also a large decrease of URA3 silencing (Fig. 1A, lines 11 and 14). We could not ascertain whether this is because of strong protection against silencing, as previously shown for potent insulators (20), or because of direct transactivation of the TRP1 and/or URA3 promoters by the chimera.

Close reexamination of the initial studies that delimited the C-terminal transcription activation domain indicated that while region 630–695 corresponds to the minimal domain endowed with transcription activation potential, extending it up to position 727 significantly augments its activity (26). Moreover, a domain located C-terminal to position 653 also possesses autonomous transcription activation potential, as revealed upon truncation of the Rap1p C terminus (27). Strikingly, regions 630–727 and 653–727 clearly behaved as insulators (Fig. 1A, lines 16–21). They increased the proportion of TRP1 ON colonies (going up to almost 100% for 630–727; line 16), did not modify URA3 silencing (lines 17 and 20), and accordingly allowed a large fraction of the colonies to grow on SC-W+FOA medium (lines 18 and 21).

The chimera containing Rap1p C-terminal to position 727 had no distinguishable effect (Fig. 1A, compare lines 1–3 and 22–24). The same was true for a shorter chimera containing only the C-terminal region with high similarity to human Rap1 (hRap1) (28) (Fig. 1A, lines 25–27) or the corresponding region from the human peptide (data not shown).

We then addressed whether the insulating activity of the Rap1p C-terminal activation domain could still be observed in the context of a larger chimera containing an intact silencing domain. For this we used a strain with a wild-type Rif1 gene. In this situation both URA3 and TRP1 genes are predominately expressed (Fig. 1C, lines 1–2), and silencing effects can be detected. When GBD alone was expressed a few colonies grew on SC-W+5-FOA medium (Fig. 1C, line 3). Expression of GBD chimeras carrying either the 563–827 or 653–827 Rap1p regions resulted in strong and coupled silencing of both URA3 and TRP1 (Fig. 1C, lines 4–9). This presumably arose as a consequence of the potent, bi-directional silencing relay activity of the domain targeted at the 4×UASg (29). The potent repressive effect of these chimeras therefore surpasses their insulating potential.

Of note, all chimeric proteins were expressed at roughly
Fig. 1. Delimitation of insulating domains in Rap1p. The construct introduced by telomere fragmentation at telomere VII-L in the GF100 reporter strain is shown at the bottom of panels A and C. GF100 as used in panel A was further modified by deleting the RIF1 open reading frame, which boosts telomeric silencing. The parental GF100 strain was used in panel C to assay chimeras displaying silencing capacity. Arrows indicate the 5' to 3' direction of transcription of reporter genes. The Y-STAR from telomere XII-L in reverse orientation is represented as a stippled box and four high affinity binding sites for the transcription factor Gal4p as ovals (4xUASg). Tandem arrowheads represent telomere repeats. The chimeras are represented on the left, with the Gal4p DNA-binding domain as a black box. Rap1p portions fused to the Gal4p DBD are shown aligned with the full-length Rap1p protein. Rap1p has 827 amino acids and can be subdivided into three regions: a central DNA binding domain (DBD) with two Myb repeats and flanking domains of approximately equal size that have been shown to display some functional redundancy (50, 51). The C terminus of Rap1p contains regions involved in transcriptional activation and stimulation of meiotic recombination (black box) (26, 51), mating-type and telomeric silencing (stippled box) (26, 52), and telomere length control (amino acids 726–827). The N terminus of Rap1p contains a BRCT motif characterized by conserved patches of hydrophobic residues, which can activate transcription and remodel chromatin (25). Cultures were serially diluted and grown on distinct medium: synthetic complete (SC); SC/H110015-FOA, which only allows growth of cells that do not express URA3; SC-W cells that express TRP1; and SC-W/H110015-FOA cells that display both URA3 OFF and TRP1 ON, i.e. cells in which TRP1 is insulated from the silencing environment. Each diamond indicates the ratio of colonies growing on a given medium versus SC for single culture. The histogram bar represents the average of the values obtained for expression of a given chimera. When not a single colony grew out on selective medium from 10^5 ml of undiluted culture, the ratio was estimated to 2.10^-5. B, interpretative scheme. Silent chromatin is represented as a gradient to indicate that silencing decreases with increasing distance from the telomere. Insulators delimit a domain largely protected from silencing while allowing efficient propagation of silent chromatin beyond this domain. D, HA-tagged Gal4p fusion proteins were detected by immunoblotting using an anti-HA monoclonal antibody. Equal amounts of yeast extract were loaded, as verified by Ponceau staining. Two independent yeast transformants were analyzed for the expression of each construct, as indicated above two consecutive lanes. GBD, Gal4p DNA-binding domain; ScR, S. cerevisiae Rap1, with the residue coordinates indicated afterward; HuR, human Rap1; Abf1, residue 604–731 moiety from Abf1p. Yeasts harbored either a wild-type (+) or a deleted copy (Δ) of the HDF1 gene as indicated below the panel. Shown is a composite of different gels processed in parallel. The extrapolated position of molecular weight markers is indicated on the left (in kilodaltons). Aberrant migration was noted for three of the chimeras: the GBD-ScR1:361 and GBD-Abf1 chimeras migrate at a higher position than expected, and the GBD-ScR121:208 at a lower position. For some of the constructs, the two analyzed strains expressed slightly different levels of the GBD chimera, less than 3-fold as assessed independently. The GBD-ScR563:695 and 630:695 chimeras were expressed at a lower level than the average (2–3-fold), presumably because of the presence of a toxic domain. The GBD-ScR1:361 chimera was generally found expressed at higher levels than the average (3-fold), as shown here. However, clones that expressed more average levels gave an identical silencing/insulation pattern as that shown in panel A, lines 4–6.
similar levels (Fig. 1D, see also “Experimental Procedures”). Furthermore, there appeared to be no correlation between slight differences in relative expression levels and the amplitude of associated silencing/insulation effects. For instance, the GBD-Rap1p 563-695 and 630-695 chimeras were expressed at lower levels than the average but displayed strong anti-silencing and insulation effects. Conversely, the GBD-Rap1p 727-827 and 784-827 chimeras, as well as the human equivalent of the latter had no effect in either silencing or insulation although expressed at average levels.

In summary, Rap1p protein harbors two insulating domains, one that coincides with the BRCT motif (amino acids 121–208) and the other in the C terminus corresponding to the previously characterized transactivation domain (amino acids 630–727). Subdomains of the latter region (630–695 and 653–727) were also active. Interestingly, the Rap1p silencing domain largely overlaps with the 653–727 insulating region, raising the intriguing possibility that the latter function contributes to silencing capacity.

Cooperating Insulating Domains in the C Terminus of Abf1p—Because the transcription activation domain of Abf1p appears to coincide with the minimal silencing domain, we decided to perform dissection analysis of Abf1p in search of insulating domains in the GF100 strain harboring a wild-type RIF1 gene. Chimeras not endowed with silencing capacity were assayed in parallel in GF100/H9004 rif1 (data not shown).

Abf1p region 604–662 somewhat repressed TRP1 and URA3 expression (Fig. 2A, compare lines 1–2 and 4–5). It also exhibited significant uncoupling activity, because about 1% of the URA3 OFF colonies were now TRP1 ON (Fig. 2A, line 6). Abf1p region 604–662 also displayed silencing and insulating activities, both to a lesser extent than the longer 604–731 chimera. This is consistent with the observation that region 663–731 has insulating capacity (Fig. 2A, line 12 and confirmed in GF100Δrif1). Thus, Abf1p regions 604–662 and 663–731 cooperate both for silencing (as previously reported in Ref. 24) and for insulation. The Abf1p C-terminal region up to position 607, for which no activity had been described so far, is also endowed with insulating potential (Fig. 2A, line 13–15 and confirmed in GF100Δrif1). Furthermore, the insulating activity of the full 440–731 C-terminal region essentially masked its silencing capacity (Fig. 2A, line 16–18). This stands in sharp contrast with the behavior of the Rap1 C terminus for which, on the contrary, silencing activity predominated over insulation (see above).

A detailed analysis recently showed that two short regions within the 604–662 multifunctional domain, 624–628 and 644–648, are essential for cell viability (24). We found that the 644–648 mutation rendered both 604–662 and 604–731 chimeras null for silencing (Fig. 2B, compare lines 4–5 and 10–11 and lines 13–14 and 19–20). The mutation also completely inactivated region 604–662 for insulation. In contrast, it only had a moderate impact on insulation by the 604–731 chimera (Fig. 2B, lines 21 and 12). This is probably due to the presence in this region of a second insulation domain, within residues 662–731 (see above). In contrast, mutation of amino acids 624–
628 had little effect and actually slightly improved the insulating ability of the 604–662 chimera (Fig. 2B, line 18).

A very short domain, between amino acids 633–662, has been shown to be almost as active as full-length Abf1p in stimulating transcription and replication (24). Strikingly this domain also displayed robust silencing and insulator capacity (Fig. 2B, lines 25–27). The complementary 604–633 subdomain is devoid of transactivating potential (440–604, 604–633, 662–731) and can also insulate in a cooperative albeit partially redundant manner. The minimal silencing domain of Abf1p (604–662) is therefore also endowed with insulating capacity, and an adjacent region (662–731) plays an accessory role in silencing and cooperates in insulation.

In conclusion, the transcription activation domain of Abf1p (amino acids 633–662) is also an insulation domain, which parallels our findings in Rap1p. However, adjacent subdomains of Abf1p devoid of transactivating potential (440–604, 604–633, 662–731) can also insulate in a cooperative albeit partially redundant manner. The minimal silencing domain of Abf1p (604–662) is therefore also endowed with insulating capacity, and an adjacent region (662–731) plays an accessory role in silencing and cooperates in insulation.

**Coupled Insulating and Silencing Capacities of the Rap1p C Terminus**—The silencing domain of Abf1p (604–731) is also an insulator. It was plausible that we had failed to detect a similar
activity in the silencing domain of Rap1p (655–827) simply because Rap1p silencing activity is much stronger. In that case we reasoned that we might be able to uncover insulating capacity by modulating the transcriptional activity of one of the reporter genes.

To address this possibility, we used strain GF101, in which the order of URA3 and TRP1 is reversed relative to GF100 (Fig. 3A). This strain also has 4xLexA sites upstream of the TRP1 gene promoter so that expression of a LexA-Gal11 chimera, which recruits the RNA polymerase II holoenzyme (30), permits transactivation of TRP1. The control chimera consisting of the GBD alone was expressed in a /H9004 rif1 background to provide a control setting of high silencing that would compare with that observed upon targeting of silencing domains to the 4xUASg, which is indeed the case as far as Abf1p is concerned (Fig. 3A, compare lines 1–2 and 7–8). Of note, expression of the LexA DNA-binding domain alone had no influence on the silencing pattern of this strain (data not shown).

As expected, expression of LexA-Gal11 led to a predominant expression of TRP1, irrespective of the Gal4 chimera targeted to the 4xUASg (Fig. 3A, lines 5, 11, 17, 23). This also strongly impaired URA3 silencing in the strain GF101 with the GBD alone but not with the Abf1p 608–731 and Rap1p 563–827 or 653–827 silencing chimeras (Fig. 3A, compare lines 1 and 4, 7 and 10, 13 and 14, 19 and 22). Furthermore, expression of the latter chimeras, but not of GBD alone, induced a large fraction of cells to grow on SC-W+/H11001 FOA (Fig. 3A, lines 12, 18, 24). Thus, these chimeras uncoupled the regulation of adjacent chromosomal domains, allowing at the same time predominant silencing of URA3 and expression of TRP1 (Fig. 3B).

**DISCUSSION**

**GRFs as Genome Partitioners**—Chromosomal insulators were first described in a variety of higher eukaryotes, and more recently in yeast, as specialized sequences that allow adjacent chromosomal domains to be independently regulated (17). In a growing number of cases, promoters themselves were shown to
act as insulators in a transcription-independent manner (23, 31–33). One simple explanation for this would be that some transcriptional regulators are endowed with insulating capacity. The work reported here, together with our previous observations (20), shows that it is indeed the case for Abf1p, Rap1p, and Reb1p, three yeast transcription factors that share a number of distinctive features and are known as General Regulatory Factors. Abf1p and Rap1p contain potent insulating domains, and although not investigated here, DNA binding per se and the DNA-binding domains of Rap1p and Abf1p may further contribute to their insulating activity in the context of full-length proteins.

One prediction would thus be that promoters containing combinations of binding sites for Rap1p, Abf1p, and Reb1p (as well as Thf1p; Ref. 20) should behave as insulators. This is in good accordance with the available data; furthermore, a screen designed to recover yeast genomic fragments that contain insulators has indeed retrieved many such sequences. The documented role of Abf1p and Reb1p in reducing transcriptional interference may be reinterpreted in the light of these new findings (34). An important consequence is that GRFs, which are abundant and bind to many promoters across the genome, may not only play a key role in regulating gene expression but may also partition chromosomes in functionally independent domains. Classic transcription activators endowed with insulating capacity (20) may also contribute to this partitioning although acting at a more restricted number of UASs. This idea reconciles the fact that, although essentially no transcription regulatory sequences other than promoters were found in yeast, most genes actually appear regulated independently despite short intergenic regions (18).

Mechanism of Insulation by GRFs—Chromatin modifications that are known to occur at promoters and that are incompatible with silencing may be envisaged to account for a barrier effect to heterochromatin propagation. However, simply creating a nucleosomal gap appears not sufficient for barrier activity (33). Furthermore, it is more difficult to envision how such local modifications may allow for discontinuous propagation of silencing (Refs. 19, 20, 35 and this study) or induce an enhancer-blocking effect (31), suggesting a more specific mechanism. The ability of GRFs to activate transcription, control replication, and cause insulation seems to be interdependent, as indicated by the overlapping of the protein domains involved. Transcription and replication are compartmentalized processes within the nucleus (36). This probably accounts for the fact that many transcription and replication factors co-purify with a nuclear scaffold fraction (37), as is notably the case for Rap1p in yeast. Another GRF-like factor that emerges as a good candidate for an insulating protein is YY-1 (48). Like CTCF, it is highly conserved among human, mouse, and Xenopus and has nuclear scaffold-associated partners. YY-1 was recently implicated in the reiterated targeting of a repressive complex at D4Z4 repeats in human, which results in the extinction of 4q35 genes through a repression mechanism that displays many common features with heterochromatin-mediated silencing (49). Whether YY-1 has insulating capacity has not been reported as yet.

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