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Dissection of a Carboxy-Terminal Region of the Yeast Regulatory Protein RAP1 with Effects on Both Transcriptional Activation and Silencing

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RAP1 is an essential sequence-specific DNA-binding protein in *Saccharomyces cerevisiae* whose binding sites are found in a large number of promoters, where they function as upstream activation sites, and at the silencer elements of the *HMR* and *HML* mating-type loci, where they are important for repression. We have examined the involvement of specific regions of the RAP1 protein in both repression and activation of transcription by studying the properties of a series of hybrid proteins containing RAP1 sequences fused to the DNA-binding domain of the yeast protein GAL4 (amino acids 1 to 147). GAL4 DNA-binding domain/RAP1 hybrids containing only the carboxy-terminal third of the RAP1 protein (which lacks the RAP1 DNA-binding domain) function as transcriptional activators of a reporter gene containing upstream GAL4 binding sites. Expression of some hybrids from the strong *ADH1* promoter on multicopy plasmids has a dominant negative effect on silencers, leading to either partial or complete derepression of normally silenced genes. The GAL4/RAP1 hybrids have different effects on wild-type and several mutated but functional silencers. Silencers lacking either an autonomously replicating sequence consensus element or the RAP1 binding site are strongly derepressed, whereas the wild-type silencer or a silencer containing a deletion of the binding site for another silencer-binding protein, ABF1, are only weakly affected by hybrid expression. By examining a series of GAL4 DNA-binding domain/RAP1 hybrids, we have mapped the transcriptional activation and derepression functions to specific parts of the RAP1 carboxy terminus. Activation requires only a small region from amino acids 630 to 695, whereas derepression is observed only when the carboxy-terminal 150 amino acids (678 to 827) are present in the hybrids. Several GAL4 DNA-binding domain/RAP1 hybrids function only as activators or as derepressors, supporting the idea that at least some activation and silencing functions of RAP1 reside in separate parts of the protein.

The yeast protein RAP1 (also known as TUF, GRF1, or TBA) has been independently identified in studies of promoters (19, 20), mating-type gene silencers (6, 8, 46, 47), and telomeres (3). RAP1 binding sites are found upstream of a large number of genes, including many ribosomal protein and glycolytic enzyme genes, where deletion analyses indicate that they are upstream activation sites (UASs) (references 9, 36, 40, 50, 54, and 56 and references therein). In contrast, the RAP1 binding site at the *HMR* silent mating-type locus is required for full repression of transcription (5, 23). At telomeres, high-affinity RAP1 binding sites are found within the terminal poly($C_{1-3}A$) tracts at a frequency of about 1/40 bp (27). These observations raised the question of whether RAP1 is a multifunctional regulatory protein or is instead a member of a family of factors, each with distinct functions.

Purification of the RAP1 protein and the cloning of the *RAP1* gene (46) have made it possible to address this question directly. The RAP1 protein is essential for viability, and recent genetic studies have demonstrated that RAP1 does in fact play a role in both activation and silencing of transcription and in the regulation of telomere structure. Conditional-lethal (temperature-sensitive) *rap1* mutants (*rap1^{ts}*) are defective in transcriptional activation of *MATα* mating-type genes (14, 24), and one *rap1^{ts}* mutant also shows a partial defect in silencing of the *HMR* locus (24). Other *rap1* mutant alleles that are specifically defective in silencing at the *HMR* locus have been isolated (52). The silencing-defective (*rap1^{s}*) mutants grow normally and thus are not defective in any essential transcriptional activation functions. These results indicate that the activation and silencing properties of RAP1 are at least in part genetically separable, suggesting that they might reside in different functional domains of the protein. Both *rap1^{ts}* and *rap1^{s}* mutants have altered telomere structures. *rap1^{ts}* mutants have shorter telomeric poly($C_{1-3}A$) tracts when grown at semipermissive temperatures (10, 28), whereas *rap1^{s}* mutants display an opposite phenotype of telomere elongation (52). Genetic experiments suggest that RAP1 is bound at telomeres in vivo (28), a fact which is borne out by direct biochemical experiments (10).

In the studies described here, parts of the *RAP1* gene have been fused to sequences encoding a heterologous DNA-binding domain from the yeast GAL4 protein (amino acids 1 to 147, hereafter referred to as $GAL4_{BD}$ [29]), and the resulting $GAL4_{BD}$/RAP1 hybrid proteins have been assayed for their effects on transcriptional activation and silencing. We reasoned that if specific domains of the native RAP1 were involved in either silencing or activation, $GAL4_{BD}$/RAP1 hybrids might substitute for the native protein when targeted to either promoters or silencers containing GAL4 DNA-binding sites. Alternatively, the hybrids might have a dominant negative phenotype (18) whereby they interfere with silencing or activation by making inappropriate or nonproductive interactions with native RAP1 or RAP1-interacting factors. By studying hybrids containing different segments of the RAP1 protein fused to the same GAL4

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DNA-binding sequence, one might be able to map specific activation and silencing functions.

We show that $GAL4_{BD}$/RAP1 hybrids containing as few as 49 amino acids from a region near the carboxy terminus of RAP1 (amino acids 630 to 678) can activate transcription of a UAS_{GAL1}-*lacZ* reporter gene, suggesting that this part of the native RAP1 protein may participate in transcriptional activation. Although none of the $GAL4_{BD}$/RAP1 hybrids examined can substitute for RAP1 at an *HMR* silencer containing synthetic GAL4 DNA-binding sites, overexpression of many of these hybrids (which do not contain the RAP1 DNA-binding domain) interferes with silencing at the *HMR* locus, even in the absence of a GAL4 binding site at the silencer. The dominant negative (derepressing) effect on silencing requires an intact RAP1 carboxy-terminal domain (amino acids 667 to 827) but does not require sequences sufficient for transcriptional activation. Derepression of the wild-type *HMR* locus by the $GAL4_{BD}$/RAP1 hybrids is partial. Because the *HMR* silencer is a redundant regulatory element (5, 23), we have examined the effect of hybrids on several different mutated but functional silencers. Removal of the A element (an autonomously replicating sequence [ARS] consensus element) results in a silencer that is very sensitive to hybrid protein expression. In the absence of hybrid protein, the ΔA silencer is fully functional but is completely derepressed by the $GAL4_{BD}$/RAP1 hybrids. Likewise, deletion of the E element (a RAP1 binding site [6, 47]) also results in complete derepression by $GAL4_{BD}$/RAP1 hybrids. In contrast, a silencer deleted for the B element (a binding site for the ABF1 protein [6, 47]) is affected very little by hybrid expression.

MATERIALS AND METHODS

Yeast strains and methods. Growth media and standard methods for manipulating yeast cells were as described by Sherman et al. (45). Transcriptional activation by $GAL4_{BD}$/RAP1 hybrids was assayed in strain GGY:171 (*ura3-52 leu2-2,112 his3Δ200 Δgal4 Δgal80* UAS_{GAL1}-*lacZ* ([15]) grown in synthetic complete (SC) liquid medium lacking histidine (SC-His) and containing 2% galactose, 2% glycerol, and 2% ethanol as a carbon source. The UAS_{GAL1}-*lacZ* reporter gene is integrated into the chromosome at the *URA3* locus. Expression of the *HMR* silent mating-type locus was examined in strain W303-1B (*HMLα MATα HMRa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and in isogenic derivatives of this strain containing altered *HMR* loci. The *HMRE* silencer mutants consist of deletions and 8-bp *Xho*I linker insertions. The deletion endpoints are as follows (see reference 5): *hmrΔA*, bp 352 to 358; *hmrΔB*, bp 300 to 256; *hmrΔE*, bp 331 to 324; and *hmrΔEB*, bp 331 to 256. Derivatives of the *hmrΔEB* silencer containing one or five copies of a GAL4 binding site were created by ligation of the oligonucleotide 5′-TCGACGGAAGACTCTCCTCCGC-3′ (and its complement, 5′-TCGAGCGGAGGAGAGTCTTCCG-3′) into the *Xho*I site at the silencer deletion. The *hmr*::*TRP1* loci were constructed by replacing an *Xba*I-*Bgl*II fragment of *HMR*, containing part of both the **a***2* and **a***1* genes, with an *Eco*RI-*Bgl*II fragment containing the *TRP1* gene (52). All of the silencer mutants and *hmr*::*TRP1* derivatives were integrated at the chromosomal *HMR* locus, replacing the wild-type sequences (4, 41). DNAs were introduced into these strains by lithium acetate transformation (21). Expression of **a***1* mRNA from *MAT***a** was examined in strain W303-1A, which is isogenic to W303-1B, differing only at the *MAT* locus (4).

Plasmids. Wild-type GAL4 was expressed from plasmid pMA210, and the $GAL4_{BD}$ alone [GAL4(1–147)] was expressed from the related plasmid pMA424 (30). Both of these plasmids contain the *HIS3* gene as a selectable marker and the 2μm origin, which results in high-copy-number maintenance in yeast cells. Constructs in which $GAL4_{BD}$/RAP1 hybrid genes are transcribed from the *ADH1* promoter were derived from plasmid pMA424. To obtain all three possible reading frames from the unique *Sal*I site in the polylinker of pMA424 [which follows the GAL4(1–147) coding sequence], we filled in, successively, the preceding *Eco*RI and *Bam*HI sites in this vector by using the Klenow fragment of DNA polymerase I. Different RAP1 carboxy-terminal fragments (extending beyond the coding sequence to a distal *Xba*I site) were generated by either *Xho*I linker insertion at restriction sites, exonuclease III or BAL31 digestion from unique restriction sites, and addition of *Xho*I linkers, or in two cases digestion at restriction sites followed by Klenow fragment repair of ends [RAP1(679–827) from a *Bst*B1 site and RAP1(691–827) from a *Hin*dIII site]. The precise locations of *Xho*I sites were determined by DNA sequencing. Most amino-terminal deletions encoding portions of the RAP1 carboxy terminus were cloned as *Xho*I-*Xba*I fragments into *Sal*I-*Xba*I-digested pMA424 (or the appropriate derivative) so that *RAP1* coding sequences were fused in frame to the $GAL4_{BD}$. Derivatives of these constructs containing, in addition, carboxy-terminal deletions of *RAP1* sequences were created by a series of multiway ligations, details of which are available upon request. Briefly, internal restriction sites within the 3′ part of *RAP1* (either *Stu*I, *Bst*B1, *Hin*dIII, or *Bgl*II) were used to join together appropriate amino- and carboxy-terminal deletions. Some $GAL4_{BD}$/RAP1 fusions were also placed under the control of the *RAP1* promoter on an integrating vector, as follows. *Hin*dIII-*Xba* fragments containing the $GAL4_{BD}$/RAP1 hybrid genes (from the pMA424-based vectors described above) were joined to a ~1.1-kb *Eco*RI-*Hin*dIII fragment containing the *RAP1* promoter (to −15 bp from the ATG) and ligated into the *Eco*RI-*Xba*I backbone of the *HIS3* integrating vector pRS303 (48). The resulting constructs were targeted for integration at the *HIS3* locus by digestion with *Pst*I. All of the $GAL4_{BD}$/RAP1 constructs described here encode short stretches of amino acids from the pMA424 vector polylinker, novel sequences introduced by the *Xho*I linker, and in the case of carboxy-terminal deletions, novel termini resulting from read-through into vector sequences used in the construction. The predicted protein sequences are known for all of the constructs described here and are given in the appropriate figures.

Measurement of transcriptional activation by GAL4 and GAL4/RAP1 hybrids. Transcriptional activation was measured by using the integrated UAS_{GAL1}-*lacZ* reporter gene in strain GGY:171, which is a *gal4* mutant. β-Galactosidase activities were measured as described previously (16), using cultures grown from colonies picked directly from transformation plates. Because duplicate measurements of individual transformants showed very little variation (typically <10%), whereas differences between transformants from the same plate could be as high as 20 to 30%, four individual transformants of each construct were assayed. The averaged values are reported. The absolute β-galactosidase values for a given construct could vary considerably (by as much as 30 to 40%) when transformations and assays were repeated for both wild-type GAL4 protein and different $GAL4_{BD}$/RAP1 hybrids. However, the relative values obtained for GAL4 protein and several $GAL4_{BD}$/RAP1 hybrids assayed on the

same day were always found to be nearly constant (±10%) in repeated independent assays of fresh transformants done over a period of time. Therefore, GAL4 wild-type and several $GAL4_{BD}$/RAP1 hybrids were always included as controls in all assays in which new hybrids were tested. As a result, all values reported for $GAL4_{BD}$/RAP1 hybrids have been normalized by using a value of 1,900 U for wild-type GAL4.

Assays for silencing of the *HMR* locus. To examine whether $GAL4_{BD}$/RAP1 hybrids could restore function to a silencer deleted for its normal RAP1 binding site, a *gal4*::*LEU2* derivative of strain W303-1B was constructed in which either one or five copies of a synthetic GAL4 DNA-binding site oligonucleotide (see above) were placed into a deletion of the E and B elements at *HMR* marked by an *Xho*I linker (5). These strains were then transformed with either the *ADH1*-promoted or *RAP1*-promoted $GAL4_{BD}$/RAP1 hybrid constructs and assayed for the ability to mate with an **a** tester strain. In the absence of hybrid protein expression, strains containing an A element and GAL4 binding site(s) at the *HMR* silencer are completely defective in mating as a result of derepression of the *HMR* locus. The presence of wild-type GAL4 protein in these cells does not restore repression (data not shown). Even a partial restoration of repression by GAL4 or $GAL4_{BD}$/RAP1 hybrids should be detectable by an increase in ability of these *MAT*α cells to mate with the *MAT***a** tester strain. Patch mating assays of cells transformed with pMA424 and $GAL4_{BD}$/RAP1 derivatives of this plasmid were performed either by replica plating 1-day-old patches directly from SC-His plates onto SD plates containing a lawn of the appropriate tester strain or by growing patches on YEPD for 12 h before replica plating onto tester lawns. Strains containing stably integrated copies of $GAL4_{BD}$/RAP1 hybrid genes were grown as patches on YEPD plates for 12 h before replica plating onto tester lawns. Quantitative mating assays were performed as described previously (49). Although we have no direct evidence that the A element/GAL4 binding site silencers are potentially functional, we do know that insertion of a number of different UAS-associated RAP1 binding sites into the same ΔEB silencer (in single or multiple copies) results in complete restoration of silencing, as judged by a mating assay (45a).

The ability of $GAL4_{BD}$/RAP1 hybrids to interfere with silencing was measured by two different assays. In strains containing an intact *HMR***a***1* gene, steady-state **a***1* mRNA levels were measured by a quantitative S1 nuclease protection assay (34) using total RNA from exponentially growing cultures in liquid SC-His medium containing 2% glucose. *SIR3* message was assayed as an internal control. In *hmr*::*TRP1* strains, derepression was assayed by plating 5-μl drops of 10-fold serial dilutions of saturated cultures (grown in liquid SC-His medium) onto SC-His plates and SC plates lacking both histidine and tryptophan (SC-His-Trp plates) (52). Silencing of the *TRP1* gene renders these strains phenotypically Trp^-. Derepression of the *hmr*::*TRP1* locus can be measured by an increase in the ability of these strains to form colonies in the absence of tryptophan.

RESULTS

$GAL4_{BD}$/RAP1 hybrid proteins activate transcription. The ability of RAP1 to activate transcription was tested by fusing parts of the gene to sequences encoding the $GAL4_{BD}$. This amino-terminal fragment of GAL4 is capable of sequence-specific DNA binding but is unable to activate transcription (30). The RAP1 protein (827 amino acids) contains two essential domains, one of which encompasses the middle third of the protein (approximately amino acids 360 to 600) and is necessary for sequence-specific DNA binding (2a, 17). The carboxy-terminal third of the protein is not necessary for DNA binding, but several linker insertion and deletion mutations in this part of the gene are lethal. The amino-terminal third of RAP1 has no known function, and strains with large deletions of this region (e.g., amino acids 44 to 315) are viable (51a). We have therefore focused our attention on a series of fusions that join the $GAL4_{BD}$ to sequences within the carboxy-terminal third of RAP1. None of the hybrids described here are capable of binding to sequences recognized by native RAP1.

A series of $GAL4_{BD}$/RAP1 carboxy-terminal hybrid genes has been constructed (see Materials and Methods). For an initial set of hybrid constructs, two different promoters were used to express the hybrid genes. In one vector, the hybrid genes are transcribed from the strong *ADH1* promoter on a multicopy plasmid, conditions which should lead to a high level of hybrid protein expression (2, 30). The same constructs were also placed under the control of the *RAP1* promoter and integrated into the yeast chromosome in single copy, where expression of the hybrids should be similar to that of native RAP1, whose transcript is present at much lower levels than that of *ADH1* (23a). We expected that the hybrid proteins would be targeted to UAS_{GAL} elements in the chromosome and therefore tested them for the ability to activate a UAS_{GAL1}-*lacZ* reporter gene in strains lacking wild-type GAL4 protein. (UAS_{GAL1} contains four binding sites for the GAL4 protein). As shown in Fig. 1 (rows 1 to 6), a number of $GAL4_{BD}$/RAP1 hybrid proteins containing only the carboxy-terminal third of RAP1 (which is by itself incapable of DNA binding) are able to activate the reporter gene to significant levels, whether expressed from the *ADH1* promoter on a multicopy plasmid or from the *RAP1* promoter in single copy (values given in parentheses). One hybrid [$GAL4_{BD}$/RAP1(630–827)] has greater than one-third the activity of native GAL4, a potent activator in yeast cells, yet fusions from amino acids 635 and 653 in RAP1 activate poorly and not at all, respectively. These data suggest that sequences between amino acids 630 and 653 are required for maximal activity of these particular $GAL4_{BD}$/RAP1 hybrids (see below). The failure of the amino acid 653 fusion to activate cannot be due to decreased stability, since Western immunoblots indicate that it is present in amounts comparable to, or slightly greater than, those of the activation-competent fusions (data not shown).

We have also constructed and tested $GAL4_{BD}$/RAP1 hybrids containing more N-terminal RAP1 sequences and hybrids in which the internal RAP1 DNA-binding domain is replaced with the $GAL4_{BD}$. None of these hybrids are more active than those shown in Fig. 1, although several hybrids containing nearly the complete RAP1 protein have activities comparable to those of the most active carboxy-terminal $GAL4_{BD}$/RAP1 hybrids (data not shown). These experiments suggest that the carboxy terminus of RAP1 may contain the predominant (or sole) activation domain on the protein and that this domain is not masked by more N-terminal sequences in the context of the hybrid proteins that we have examined.

Mapping of a small transcriptional activation domain in $GAL4_{BD}$/RAP1 hybrids. An additional series of $GAL4_{BD}$/RAP1 hybrids was constructed in order to map the amino acids required for transcriptional activation. These hybrids were expressed from the *ADH1* promoter on a high-copy-number vector. As shown in Fig. 1 (rows 7 to 10), further

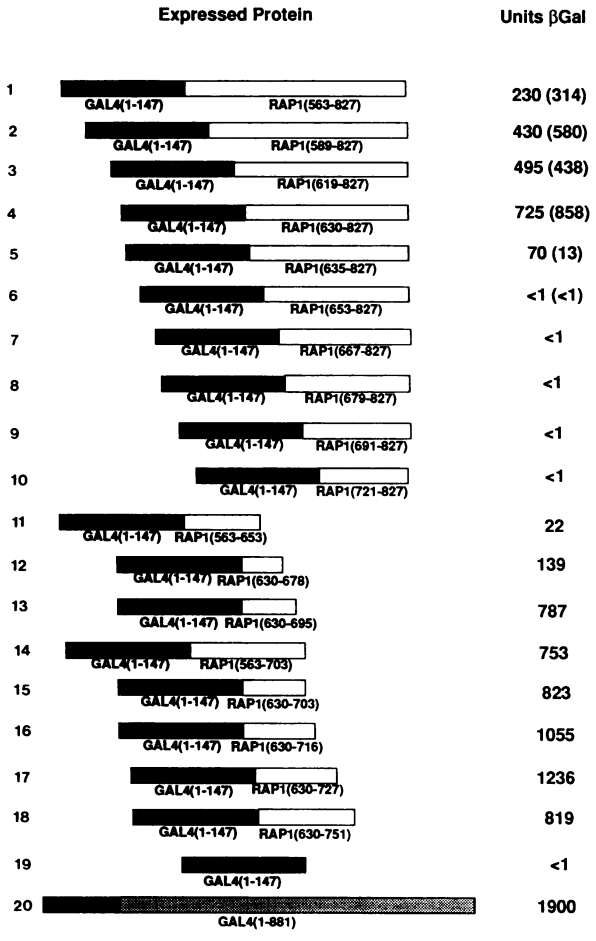

FIG. 1. Transcriptional activation of a UAS_{GAL1}-*lacZ* reporter gene by $GAL4_{BD}$/RAP1 hybrid proteins: deletion analysis of a RAP1 transcriptional activation domain. β-Galactosidase (βGal) levels were measured for strains containing plasmids expressing various $GAL4_{BD}$/RAP1 hybrid genes and for strains containing plasmids expressing full-length GAL4 or the $GAL4_{BD}$ alone as controls. The values given in parentheses for the first six hybrids are for strains containing integrated $GAL4_{BD}$/RAP1 hybrid genes expressed from the *RAP1* promoter. All other values are for strains containing high-copy-number plasmids (2μm based), expressing the hybrids from the strong *ADH1* promoter (see Materials and Methods). Filled boxes represent GAL4 amino acid sequences, and open boxes represent the fused RAP1 sequences. The amino acid sequences (one-letter code) at amino-terminal fusion junctions are as follows, where the first slash indicates the end of the $GAL4_{BD}$ (amino acid 147) and the second slash indicates the end of junction sequences and the beginning of RAP1 carboxy-terminal sequences: RAP1(563), S/PEFGIRRA/AE; RAP1(589), S/PELIPGDRSVEA/PG; RAP1 (619), S/PELIPGDRSVER/AA; RAP1(630), S/PELIPGDPSSG/SY; RAP1(635), S/PEFGIRRA/EN; RAP1(653), S/PELIPGDPSE/DL; RAP1(655), S/PEFPGIRRAV/SN; RAP1(667), S/PELIPGDRSVER/HE; RAP1(679), S/PELIPGDP/NE; RAP1(691), S/PEFG/IS; RAP1 (702), S/PELIPGDRSVER/LF; and RAP1(721), S/PELIPGDPSSG/HE. The carboxy-terminal endpoints of the remaining hybrids are as follows, where the slash indicates the end of RAP1 sequences and the beginning of vector-encoded sequences (Z is a stop codon): RAP1(562–653), ND/PSTCSQANSGRISYDLZ; RAP1(630–678), FS/LESIGTSERFZ; RAP1(630–695), PP/ARPAAKLIPGELMIYD FYYZ; RAP1(653–703); RAP1(630–703), FL/RSTCSQANSGRISY DLZ; RAP1(630–716), QF/IRSTCSQANSGRISYDLZ; RAP1(630–727), GD/PLDLQPSZ; and RAP1(630–751), FS/PLDLQPSZ.

deletions beyond amino acid 653, with endpoints at amino acids 667, 679, 691, and 721, are also incapable of transcriptional activation when fused to the $GAL4_{BD}$. These results support the idea that sequences between amino acids 630 and 653 define an amino-terminal endpoint of a RAP1 transcriptional activation domain, as measured by this assay. To determine the carboxy-terminal boundary of this domain, we tested $GAL4_{BD}$/RAP1 hybrids which contained amino-terminal RAP1 endpoints at either amino acid 562 or 630 and carboxy-terminal endpoints ranging from amino acids 653 to 751. All of these hybrids are transcriptional activators (Fig. 1), although the two deletions extending upstream of amino acid 695, with carboxy-terminal endpoints at amino acids 653 and 678, give values only 10 and 19%, respectively, of those of the hybrids with the same amino termini that extend to the end of RAP1 (compare row 1 with row 11 and row 4 with row 12). In contrast, all of the carboxy-terminal deletions up to and including the amino acid 695 deletion yield consistently high activation values (>700 U). Taken together, these data suggest that a short region of RAP1, extending from amino acids 630 to 695, functions as a transcriptional activation domain when fused to the $GAL4_{BD}$.

$GAL4_{BD}$/RAP1 hybrid proteins interfere with silencing when expressed from the *ADH1* promoter on multicopy plasmids. To identify a RAP1 domain involved in transcriptional silencing, the effect of $GAL4_{BD}$/RAP1 hybrids on the *HMR* silent locus was examined. Either of two consequences of hybrid protein expression might be expected: restoration of function to a silencer containing a GAL4 binding site in place of the RAP1 binding site, or disruption of silencing. Disruption of silencing might result either from nonproductive interactions at the silencer or titration of an essential silencer factor present in limiting amounts. We find that expression of $GAL4_{BD}$/RAP1 hybrids from the *ADH1* promoter on high-copy-number plasmids interferes with silencer function. None of the hybrids that we have tested can restore function to a silencer containing GAL4 binding sites in place of the RAP1 binding site, whether expressed from the *ADH1* promoter on multicopy plasmids or from the *RAP1* promoter in single copy in the chromosome.

The inhibitory effect of hybrid protein expression on silencing was assayed by two different methods. In the first method, transcription of the *HMRa1* gene was measured directly by a quantitative S1 nuclease protection assay (34). We also examined the effect of hybrids on silencing of an *hmr::TRP1* locus (4, 35), by measuring the growth of appropriate strains in the presence and absence of tryptophan. The *hmr::TRP1* locus has been shown to be an accurate and sensitive measure of silencing at *HMR*: growth of *hmr::TRP1* strains in media lacking tryptophan is strongly correlated with levels of *HMRa1* mRNA in strains containing either mutated silencers or *rap1* alleles defective in silencing (52). A particular advantage of the *hmr::TRP1* assay is that it is very sensitive to low levels of depression, perhaps in part because it allows one to examine the properties of individual cells.

Because the silencer at *HMR* is a redundant regulatory element, the effects of hybrid proteins were also examined in the context of mutated silencers, deleted either for the A element (an ARS consensus sequence), the B element (an ABF1 binding site), or the central E element (a RAP1 binding site). Single deletions of either the A or B element have no effect on silencing, whereas a deletion of the RAP1 binding site results in partial (~10%) derepression (5, 23). Mutation of any two sites abolishes repression. In these

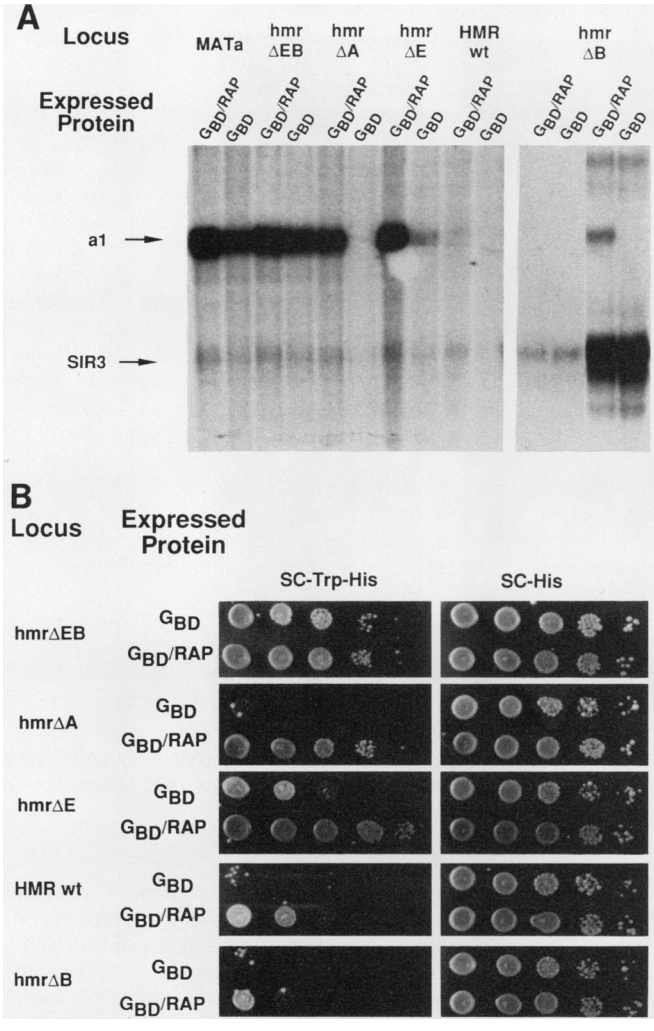

FIG. 2. Effect of $GAL4_{BD}$/RAP1(653–827) hybrid protein overexpression on silencing at wild-type and mutant *HMR* silent mating-type loci. (A) Quantitative S1 nuclease protection assays of steady-state **a***1* mRNA in a *MAT***a** strain (W303-1A) and in MATα strains (W303-1B and derivatives) with a ΔEB, ΔA, ΔE, wild-type (AEB), or ΔB *HMR* silencer, expressing the $GAL4_{BD}$/RAP1(653–827) hybrid or the $GAL4_{BD}$ alone, as indicated. Only the primary spliced **a***1* mRNA is shown, together with *SIR3* mRNA which serves as an internal control (34). An overexposure of the ΔB silencer lanes (rightmost two lanes) is shown alongside the original exposure to indicate the low but detectable level of **a***1* expression in the hybrid-containing strain. (B) The effect of $GAL4_{BD}$/RAP1 hybrid protein overexpression on the growth of strains containing *hmr*::*TRP1* loci with wild-type and mutated silencer elements, as indicated. Tenfold serial dilutions of overnight liquid cultures grown selectively for the expression plasmid (SC-His) were spotted onto SC-His-Trp (left) or SC-His (right) plates and allowed to grow for 2.5 to 3 days before being photographed.

experiments, no GAL4 DNA-binding site has been placed at the silencer element, and all of the strains tested contain wild-type copies of *RAP1* and *ABF1*, both of which are essential genes (12, 46).

Figure 2 shows the dominant negative (derepressing) effect of the $GAL4_{BD}$/RAP1(653–827) hybrid on wild-type and mutated silencers, measured by both steady-state *HMR***a***1* mRNA levels and the ability of *hmr*::*TRP1* reporter strains to grow in the absence of tryptophan. The $GAL4_{BD}$/RAP1(653–827) hybrid fully derepresses two of the mutated silencers, the ΔA and ΔE silencers, whereas $GAL4_{BD}$ alone has no effect on these silencers. (Note that the ΔE silencer is partially derepressed [~10%] in the absence of hybrid protein, as discussed above.) This derepression is reflected by full expression of **a***1* mRNA (equal to that in a strain containing a completely defective silencer or in a *MAT***a** strain; Fig. 2A) and by full growth of an *hmrΔA*::*TRP1* strain on media lacking tryptophan (Fig. 2B). The $GAL4_{BD}$/RAP1(653–827) hybrid also leads to weak derepression of both the wild-type (AEB) and ΔB silencers. The two assays reflect this fact either by lower steady-state mRNA levels or by a decreased ability of *hmr*::*TRP1* strains to grow in the absence of tryptophan compared with the ΔA and ΔE silencer strains. For the ΔB silencer strain, **a***1* mRNA is not detected in the original exposure; however, a longer exposure of this gel indicates a low level of expression in the strain containing the $GAL4_{BD}$/RAP1(653–827) hybrid but none at all in the $GAL4_{BD}$ alone control (Fig. 2A, rightmost lanes). The behavior of the *hmr*::*TRP1* and *hmrΔB*::*TRP1* strains is somewhat curious and worth noting. One might expect that lower expression of *TRP1* in these strains would result in slower growth among all of the cells in the population. Instead, it appears that a lower fraction of cells are able to grow and form colonies in the SC-Trp plates. This might reflect a cell-to-cell variation in both hybrid protein levels and resultant *hmr*::*TRP1* expression and a threshold required to allow growth in the absence of tryptophan. Alternatively, repression of *HMR* might be metastable in cells expressing the hybrids, as has been observed in cells containing silencing-defective *rap1* alleles (51b, 52). Metastable repression at *HML* has been observed in strains containing *sir1* mutations or *cis* silencer mutations (31, 38).

As mentioned above, derepression by the $GAL4_{BD}$/RAP1(653–827) hybrid, and all other hybrids tested (Fig. 1, rows 1 to 5), occurs only when they are expressed from the *ADH1* promoter on multicopy plasmids. None of these hybrids had any detectable effect on silencing when expressed from the *RAP1* promoter (data not shown), although many were strong transcriptional activators in this context. Of these initial six hybrids, the $GAL4_{BD}$/RAP1(653–827) hybrid, which fails to activate transcription, results in the strongest disruption of silencing. The other hybrids, with amino-terminal endpoints ranging from amino acid 562 to amino acid 635, show a weaker but qualitatively similar derepression effect on the different silencers (data not shown). All of these more amino-terminal hybrids derepress the ΔE silencer and have partial effects on the ΔA silencer, as judged by the S1 protection assay. However, none has any measurable effect on a wild-type or ΔB silencer, both of which are partially derepressed by the $GAL4_{BD}$/RAP1(653–827) hybrid (Fig. 2). The reduced ability of these larger hybrids to derepress silencers may be directly or indirectly due to their toxic effect on cells. Strains containing these plasmids grow more slowly than do cells containing control (GAL4 or $GAL4_{BD}$) or more carboxy-terminal $GAL4_{BD}$/RAP1 plasmids [e.g., $GAL4_{BD}$/RAP1(653–827)] and show a higher rate of plasmid loss (data not shown).

Derepression by $GAL4_{BD}$/RAP1 hybrids requires an intact RAP1 carboxy-terminal domain. The amino- and carboxy-terminal boundaries of the RAP1 domain responsible for silencer derepression were mapped by examining a series of additional $GAL4_{BD}$/RAP1 hybrids. Derepression was assayed in wild-type and mutant silencer backgrounds, and the data for the *hmrΔA*::*TRP1* strain (which is particularly

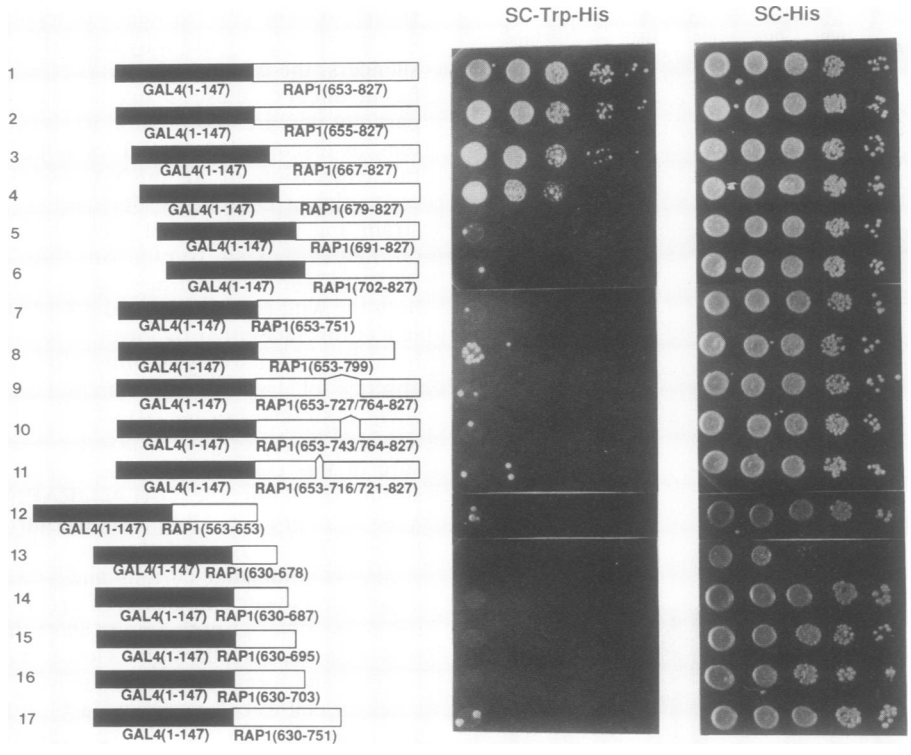

FIG. 3. Deletion analysis of a carboxy-terminal domain of RAP1 whose overexpression results in derepression of the silent *hmrΔA::TRP1* locus. A series of $GAL4_{BD}$/RAP1 hybrids, expressed from the *ADH1* promoter on 2μm high-copy-number vectors (see Materials and Methods), was assayed as described for Fig. 2.

sensitive to derepression; Fig. 2) are presented in Fig. 3. The amino-terminal boundary of the derepression domain clearly falls between amino acids 667 and 691: the $GAL4_{BD}$/RAP1(667–827) hybrid fully derepresses, the $GAL4_{BD}$/RAP1(679–827) hybrid derepresses partially (colony formation on SC-His-Trp plates is approximately 10-fold lower than on SC-His plates), and the $GAL4_{BD}$/RAP1(691–827) hybrid fails to derepress ($<10^{-4}$ efficiency of colony formation in the absence of tryptophan, which is indistinguishable from the value for the $GAL4_{BD}$ control; Fig. 2B). Consistent with this conclusion, hybrids with more distal amino-terminal endpoints at amino acids 702 (Fig. 3, row 6) and 721 (data not shown) are also inactive. Similar results were obtained when this series of amino-terminal deletions was assayed on strains carrying the wild-type (AEB) and ΔE silencers (data not shown). Neither of the carboxy-terminal deletions, with endpoints at amino acids 751 and 799, displayed any ability to derepress the *hmrΔA::TRP1* locus (Fig. 3, rows 7 and 8), nor did they affect any of the other silencers (data not shown). These data indicate that sequences between amino acids 800 and the carboxy terminus at 827 are required for derepression. We do not know whether this result reflects a direct involvement of these sequences in derepression or a requirement for folding and/or stability of the hybrids. However, these derepression results stand in marked contrast to the transcriptional activation studies, in which a large number of carboxy-terminal deletions show full activation potential (Fig. 1, rows 13 to 18).

We have also tested a series of internal deletions within the carboxy terminus that either cover or are adjacent to amino acids that are altered in silencing-defective *rap1*^{s} mutants (amino acids 726, 727, and 747 [52]). None of these internal deletion hybrids affect the *hmrΔA* silencer (Fig. 3, rows 9 to 11), nor do they affect any other silencer (data not shown), pointing to a role for this region in derepression. These data also suggest that the regions from amino acids 667 to 691 and 800 to 827 are together not sufficient for derepression, further supporting the idea that an intact carboxy terminus, beginning at the 667-to-691 region, is required. Finally, we examined a series of hybrids with RAP1 amino-terminal endpoints at either 562 or 630 and carboxy termini from 653 to 751 (Fig. 3, rows 12 to 17). Because these hybrids are strong activators of transcription, we presume that they must be at least in part stable and correctly folded. However, none of these hybrids affect silencing (Fig. 3). We conclude from this finding that RAP1 sequences sufficient for activation in the $GAL4_{BD}$/RAP1 hybrids are incapable of derepressing the *HMR* silent locus.

DISCUSSION

We have shown that carboxy-terminal sequences of the RAP1 protein, when fused to the $GAL4_{BD}$, can activate transcription from a UAS_{GAL1}-*lacZ* test gene. Furthermore, expression of these $GAL4_{BD}$/RAP1 hybrids from a strong promoter leads to derepression of the normally silent *HMR* mating-type locus. We have mapped these activation and derepression properties by examining a larger number of hybrids containing differing amounts of RAP1 carboxy-terminal sequence. Transcriptional activation requires only a small region between amino acids 630 and 695. Deletions extending into this region from the amino terminus (amino acid 635) and the carboxy terminus (amino acid 678) have partial function, suggesting that both ends of this small domain make independent contributions to activation. Derepression of the silent *HMR* locus by $GAL4_{BD}$/RAP1 hybrids

requires sequences from amino acid 667 to the carboxy terminus at amino acid 827. Although the activation and derepression domains defined in these studies partially overlap (in the region between amino acids 667 and 695), many hybrids have one activity (either activation or derepression) yet lack the other. These observations provide further support for the idea that the activation and derepression functions of RAP1 can be, at least in part, genetically separated (52). In addition, these data identify for the first time a potential activation domain in native RAP1.

Direct evidence that RAP1 is a transcriptional activator comes from recent studies of *rap1*^{ts} mutants and point mutations in the RAP1 binding site at the *MAT*α UAS (14, 24). Results presented here suggest that amino acids 630 to 695 of RAP1 might mediate activation by the native protein. Such an interpretation must be viewed with some caution until this part of the native protein is subjected to detailed mutagenesis studies. We cannot rule out the possibility, for example, that the $GAL4_{BD}$/RAP1 hybrids are transcriptional activators by virtue of the fortuitous exposure of an activating surface not normally present on native RAP1. Ma and Ptashne (29) have shown that sequences from the *Escherichia coli* genome, all of which encoded peptides with a large net negative charge, are capable of activating transcription in yeast cells when fused to the $GAL4_{BD}$. We note that the 66-amino-acid region from 630 to 695 in RAP1 has a net negative charge of −12. Although the mechanism of activation by either RAP1 or the $GAL4_{BD}$/RAP1 hybrids is unknown, one possibility is that direct protein-protein interactions with other factors (coactivators) are important at many promoters. For example, *GCR1* and *GAL11* are required for full activity of a number of genes with RAP1 UAS elements and for synthetic UAS_{RAP1} promoters (13, 37, 42). These proteins may not bind to DNA sequences by themselves and might work in part by interacting directly with RAP1 at promoters. Our results lead to the (testable) hypothesis that the region of RAP1 from amino acids 630 to 695 might be sufficient for such an interaction. Alternatively, RAP1 may work by providing access in the chromatin template for other DNA-binding proteins which are themselves direct activators, as may be the case at the *HIS4* gene (11).

Diffley and Stillman (12) have noted a weak similarity between a carboxy-terminal part of RAP1 (amino acids 633 to 678), the silencer-binding factor ABF1, and the SAN1 protein. Mutations in *SAN1* were isolated as suppressors of certain *sir4* alleles (44), leading to the suggestion that the SIR4 protein, known to be required for silencing, might function by binding directly to the homology regions of both RAP1 and ABF1. Our results indicate that the region of RAP1 from amino acids 630 to 695 constitutes an activation domain and that sequences beyond amino acid 678 are sufficient for derepression. The homologous region of ABF1 might also play a role in activation: ABF1 binding sites appear to be weak activation elements and are found upstream of a number of genes (7). The results presented here are more consistent with the recent isolation of RAP1 silencing-defective (*rap1*^{s}) mutants, all of which map further downstream from the RAP1/ABF1/SAN1 homology region, within the carboxy-terminal domain required for derepression in the $GAL4_{BD}$/RAP1 hybrids (52). Nonetheless, it is important to point out that our experiments do not exclude a role for amino acids 633 to 678 in silencing. Rather, they indicate that overexpression of this region is neither necessary nor sufficient to interfere with silencing in a dominant negative fashion. Targeted mutagenesis of this region might provide further insights into the mechanism of RAP1 function in both activation and silencing.

The magnitude of derepression by $GAL4_{BD}$/RAP1 hybrids depends strongly on the *cis* regulatory elements present at the affected silencer. A wild-type silencer is affected only slightly by hybrid overexpression, yet the ΔA silencer is strongly derepressed. Surprisingly, the ΔB silencer is affected hardly at all by the hybrids. Finally, the ΔE silencer is strongly derepressed by the $GAL4_{BD}$/RAP1 hybrids, even though it lacks a RAP1 binding site. The behavior of the hybrids in different silencer backgrounds is strikingly similar to that of four silencing-defective *rap1*^{s} mutants, all of which result from missense mutations in the RAP1 carboxy terminus at amino acids 726, 727, and 747. *rap1*^{s} mutants display a phenotype in a ΔA silencer background but not in conjunction with either a wild-type or ΔB silencer (52), in which the effect of the hybrids is minimal. Similarly, two strong *rap1*^{s} mutants (one a double-point mutant) also result in the complete derepression of a ΔE silencer (51b), as do the $GAL4_{BD}$/RAP1 hybrids. We do not know whether these data indicate a role for other (telomeric?) RAP1 binding sites at *hmr*Δ*E* silencers or an involvement of RAP1 at the silencer even in the absence of a strong DNA-binding site. Although it is unclear to what extent all of these results reflect in some general way the strengths of the different silencers, it is tempting to try to relate them to a possible mechanism for silencer derepression. We think that it is likely that silencer interference by the $GAL4_{BD}$/RAP1 hybrids results from a protein-protein interaction that either sequesters or inactivates an essential silencing factor. First, derepression occurs when the hybrids are expressed from the *ADH1* promoter on multicopy plasmids, but not from the *RAP1* promoter in single copy, consistent with a titration model. Second, the hybrids interfere with silencing despite lacking a DNA-binding domain that could target them specifically to the silencer element. Although we do not know whether the $GAL4_{BD}$ is necessary for derepression, it seems unlikely that its nonspecific binding to the silencer would be sufficient by itself to disrupt silencing in the absence of an additional protein-protein interaction. One possible explanation for the similar phenotypes of *rap1*^{s} mutants and hybrid overexpression is that the *rap1*^{s} mutants are defective in an interaction with the factor(s) being titrated by the $GAL4_{BD}$/RAP1 hybrids.

What factor might the $GAL4_{BD}$/RAP1 hybrids be interacting with so as to disrupt silencing? Two obvious candidates are the known silencer binding factors, RAP1 and ABF1. The fact that silencers lacking binding sites for one or the other factor are still derepressed by the hybrids would seem to argue against this model. However, we cannot exclude the possibility that either factor is still involved in silencing and subject to interference when its binding site is deleted. Furthermore, our data are consistent with the possibility that the $GAL4_{BD}$/RAP1 hybrids bind to and interfere with both RAP1 and ABF1. Other possible candidates are the four *SIR* gene products (39), all of which are required for efficient silencing but may not bind directly to silencer DNA sequences (32, 46, 47, 51). We note that overexpression of a carboxy-terminal region of SIR4 also causes derepression (22, 32). Finally, the titratable protein in question may be an as yet unidentified factor involved in silencing. Because overexpression of the RAP1 carboxy terminus also results in elongation of poly($C_{1-3}A$) tracts at telomeres (10, 16a), as do *rap1*^{s} mutations (52), the titrated factor may be involved in both silencing and regulation of telomere structure.

We do not know why $GAL4_{BD}$/RAP1 hybrids, when

expressed from the *RAP1* promoter and targeted to silencers containing GAL4 binding site, fail to restore silencing. Perhaps these hybrid proteins are missing another part of RAP1 essential for silencing (possibly the DNA-binding domain) or bind to the silencer in a nonproductive conformation. The ability of these hybrids to interfere with silencing when expressed from the strong *ADH1* promoter may not be subject to the same constraints. One might expect a priori that dominant negative effects would most likely result from expression of partially functional or nonfunctional protein fragments (18).

While we do not know the precise mechanisms by which the various $GAL4_{BD}$/RAP1 fusions function, our results imply that the carboxy terminus of RAP1 is involved in at least two different regulatory interactions. The adenovirus E1A protein, which can also either activate or repress transcription, appears to act through separate functional domains (25, 26, 43, 55), and we have provided evidence that the activation and silencing functions of RAP1 can be similarly dissected. Several other groups have noted that overexpression of regulatory proteins (e.g., full-length GAL4 and steroid hormone receptors) can result in dominant negative transcriptional effects that have been ascribed to titration of limiting transcription factors (1, 15, 33, 53). We have shown here that the approach of creating dominant negative phenotypes may be of particular value in studies of essential regulatory factors in yeast cells whose functions may be diverse and complex. One potentially useful outcome of these experiments is that they identify specific regions of the RAP1 protein that may be involved in distinct protein-protein interactions important for different regulatory processes.

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