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Maximal Stimulation of Meiotic Recombination by a Yeast Transcription Factor Requires the Transcription Activation Domain and a DNA-Binding Domain

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ABSTRACT

The DNA sequences located upstream of the yeast *HIS4* represent a very strong meiotic recombination hotspot. Although the activity of this hotspot requires the transcription activator Rap1p, the level of *HIS4* transcription is not directly related to the level of recombination. We find that the recombination-stimulating activity of Rap1p requires the transcription activation domain of the protein. We show that a hybrid protein with the Gal4p DNA-binding domain and the Rap1p activation domain can stimulate recombination in a strain in which Gal4p-binding sites are inserted upstream of *HIS4.* In addition, we find recombination hotspot activity associated with the Gal4p DNA-binding sites that is independent of known transcription factors. We suggest that yeast cells have two types of recombination hotspots, α (transcription factor dependent) and β (transcription factor independent).

RECOMBINATION events are not distributed evenly along eukaryotic chromosomes. Regions with high (hotspots) and low (coldspots) levels of recombination exist (reviewed by Lichten and Goldman 1995). In the yeast *Saccharomyces cerevisiae*, meiotic recombination hotspots are usually located between, rather than within, genes and are associated with high levels of local meiosis-specific double-strand DNA breaks (DSBs). These breaks occur within regions of "open" chromatin (Wu and Lichten 1994), although the patterns of DSBs do not reflect in detail the patterns of nuclease sensitivity in the chromatin (Fan *et al.* 1995; Wu and Lichten 1995).

The *HIS4* hotspot region contains binding sites for the transcription activators Rap1p, Gcn4p, Bas1p, and Bas2p (Arndt *et al.* 1987; Tice-Baldwin *et al.* 1989; Devlin *et al.* 1991). Mutations that eliminate the binding sites for Rap1p or Bas2p cause loss of hotspot activity (White *et al.* 1991, 1993). In addition, loss of Bas1p or Bas2p causes loss of hotspot activity (White *et al.* 1993), whereas loss of Gcn4p has little effect (White *et al.* 1992). Although these results suggest a possible linkage between transcription and recombination, deletion of the TATAA sequence, required for efficient expression of *HIS4*, has no effect on hotspot activity (White *et al.* 1992). Thus, transcription factors are required for hotspot activity in a role that is not directly linked to transcription (White *et al.* 1993). Similar conclusions have been reached in recent studies of the *M26* hotspot of *S. pombe* (Kon *et al.* 1997).

A number of observations indicate that the transcriptional activator Rap1p is important for hotspot activity at the *HIS4* locus. First, as described above, alteration of the Rap1p-binding site upstream of *HIS4* eliminates hotspot activity (White *et al.* 1991). Second, overproduction of Rap1p stimulates hotspot activity and this effect is eliminated in strains with a mutation of the Rap1p-binding site (White *et al.* 1991). Third, when the normal transcription-factor-binding sites upstream of *HIS4* are replaced with a 51-bp insertion of telomeric DNA, strong hotspot activity is detected (White *et al.* 1993; Fan *et al.* 1995); telomeric sequences bind Rap1p (Longtine *et al.* 1989; Liu and Tye 1991) as well as a number of other proteins. The DSBs associated with the telomeric insertion upstream of *HIS4* occur immediately adjacent to, but not within, the telomeric repeats (Xu and Petes 1996). In addition, duplication of the Rap1p site that is present in a single copy in the sequences upstream of *HIS4* in wild-type strains strongly stimulates recombination, and mutation of one of these sites results in loss of this stimulation (White *et al.* 1993; Fan *et al.* 1995). Thus, Rap1p, in the absence of the other transcriptional activators of *HIS4* expression, can stimulate hotspot activity.

Rap1p has diverse cellular roles (reviewed by Shore 1994). Rap1p is an essential DNA-binding protein, and this binding induces a bend in DNA *in vitro* (Gilson *et al.* 1993). Rap1p is required for the activation of transcription at a number of loci (Shore 1994) including *HIS4* (Devlin *et al.* 1991). At other loci (the silent *MAT* loci and the telomeres), Rap1p represses transcription

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(Kurtz and Shore 1991; Hardy *et al.* 1992; Moretti *et al.* 1994). As described above, Rap1p binds to telomeres and different mutant Rap1 proteins result in either telomere lengthening or shortening (reviewed by Sussel and Shore 1991; Kyrion *et al.* 1992); on the basis of these and other results, it has been suggested that telomere length is regulated in yeast by a mechanism that "counts" the number of Rap1p molecules bound to the telomere (Marcand *et al.* 1997). Rap1p localizes in clusters at the nuclear periphery, presumably reflecting these telomeric associations (Klein *et al.* 1992). Finally, Rap1p can stimulate the formation of quadruplexes of telomeric DNA (Gilson *et al.* 1994; Giraldo and Rhodes 1994).

Some of the functions described above require Rap1p to interact with other proteins, including Sir3p, Sir4p, Rif1p, and Rif2p (Hardy *et al.* 1992; Klein *et al.* 1992; Moretti *et al.* 1994; Wotton and Shore 1997). The interactions with Sir3p and Sir4p are required for silencing of gene expression at the telomeres (Moretti *et al.* 1994) and localization of Rap1p clusters to the nuclear periphery (Palladino *et al.* 1993), whereas the interactions with Rif1p and Rif2p are required for normal telomere length regulation as well as telomere silencing (Hardy *et al.* 1992; Wotton and Shore 1997).

The domains of the 827-amino-acid Rap1p required for the activities described above are diagrammed in Figure 1. The region of the protein between amino acids 43 and 279 is required for the bend induced in DNA by Rap1p binding (Gilson *et al.* 1993). The DNA-binding region extends from amino acids 361 to 596 (Henry *et al.* 1990). The acidic transcriptional activation domain (amino acids 630 to 695) is adjacent to the DNA-binding domain (Hardy *et al.* 1992). The carboxy terminal quarter of the protein contains the domains required for telomeric silencing (amino acids 667–827; Buck and Shore 1995) and the regulation of telomere length (amino acids 726–827; Sussel and Shore 1991; Lustig and Petes 1993). This region also has binding sites for Sir3p, Sir4p, Rif1p, and Rif2p (Hardy *et al.* 1992; Moretti *et al.* 1994; Wotton and Shore 1997).

Given the diverse cellular functions of Rap1p, a number of explanations of the recombination-stimulating effect of the protein are possible. First, the binding of Rap1p may open the chromatin, allowing access of the enzyme complex that forms the initiating double-strand DNA breaks; this complex presumably contains Spo11p (Keeney *et al.* 1997), which has been shown to possess DNA double-strand endonucleolytic activity during meiosis. Second, Rap1p may interact directly with proteins of the recombination-initiating complex, tethering the complex to the hotspot region. Third, the DNA bend induced by Rap1p binding may act as a recombinogenic signal. Fourth, binding of Rap1p to the hotspot may result in localization of this region to a cellular compartment that has high levels of recombination. As described below, by analyzing strains with various deletions of Rap1p and by examining Gal4p/Rap1p fusions, we show that the maximal recombination-stimulating activity of Rap1p requires both DNA binding and activation domains. The domains of the protein required to bend DNA, to control telomere silencing and telomere length, and to localize Rap1p to the nuclear periphery are not required to stimulate recombination, eliminating explanations 3 and 4 above. Further, as these dispensable domains contain the regions of the protein required for interactions with Sir3p, Sir4p, Rif1p, and Rif2p, none of the known Rap1p-interacting proteins are involved in meiotic recombination initiation at *HIS4*.

MATERIALS AND METHODS

Media: Standard media were utilized (Guthrie and Fink 1991). Sporulation plates contained 1% potassium acetate, 0.1% yeast extract, 0.05% glucose or galactose, 6 μg/ml adenine, and 2% agar.

Plasmids: The plasmids pAJL1000 and pDTK109 contain altered forms of *RAP1* inserted into the CEN-containing vector pRS316 (Sikorski and Hieter 1989). The plasmid pAJL100 (provided by A. Lustig) has the *rap1-17* allele, encoding Rap-1p with a C-terminal deletion, whereas the plasmid pDTK109 contains the *rap1-ΔN* allele, encoding a Rap1p lacking amino acids near the N terminus (Figure 1). The pDTK109 plasmid was constructed by joining an *Eco*RI-*Xba*I fragment containing the *rap1-ΔN* allele [derived from pM672 (provided by D. Shore)] with *Eco*RI-*Xba*I-treated pRS316.

The plasmids pDTK116-BC, pDTK118-BAC, pDTK121-BA contain fusion genes encoding proteins with the DNA-binding domain from Gal4p (amino acids 1–147 of Gal4p) attached to various Rap1p-derived domains inserted in the vector pRS316; the synthesis of the fusion gene in these plasmids is controlled by the *RAP1* promoter. The portion of the Rap1p represented in each plasmid is shown in Figure 1. The plasmid pDTK116-BC was constructed by transferring the *Eco*RI-*Xba*I fragment from p136 (Buck and Shore 1995) into *Eco*RI/*Xba*I-treated pRS316. The plasmid pDTK118-BAC was constructed by transferring a *Kpn*I-*Not*I fragment encoding the fusion protein (derived from p292; Buck and Shore 1995) into *Kpn*I/*Not*I-treated pRS316.

The plasmid pDTK121-BA was derived from pDTK118-BAC. pDTK118-BAC was treated with *Bam*HI and *Not*I, deleting the DNA sequences encoding the Rap1p portion of the fusion protein, and a *Bam*HI-*Not*I fragment encoding amino acids 630–695 of Rap1p was inserted. This DNA fragment was produced by using the oligonucleotides 739 (5′ CAGTAGGATCC TATCTTACGCTATACCAGAAAAC 3′) and 706 (5′ GTATT GCGGCCGCTTATGGTGGAAAGCTTATGGTATC 3′) in a PCR reaction with undeleted pDTK118-BAC DNA; this fragment was then treated with *Bam*HI and *Not*I. The plasmid pDTK122-B (encoding a protein with the Gal4p DNA-binding domain but no Rap1p protein sequences) was constructed by related procedures. The double-stranded oligonucleotide formed by annealing 13030 (5′ GATCTAATGATAA 3′) and 13031 (5′ GGCCTTATCATTA 3′) was ligated to *Bam*HI/*Not*I-digested pDTK118-BAC. The altered regions of pDTK121-BA and pDTK122-B were confirmed by sequencing.

The plasmid pQF1 was used to replace the transcription-factor-binding sites located upstream of the wild-type *HIS4* gene with duplicated Gal4p-binding sites. Oligonucleotides with the sequences 5′ TCGA**CGGAAGACTCTCCTCCG**CAC TGTATTACCATAGTACCG**CGGAAGACTCTCCTCCG**C 3′

and 5′ TCGAG**CGGAGGAGAGTCTTCCG**CGGTACTATGG TAATACAGTG**CGGAGGAGAGTCTTCCG** 3′ were annealed together and inserted into the *Xho*I site of pPD5 (a YIp5 derivative; White *et al.* 1993). The DNA sequences shown in boldface represent the Gal4p-binding sites (Feldmann *et al.* 1994) and the DNA sequences located between the two binding sites are the same as those used previously to separate two Rap1p-binding sites (White *et al.* 1993); these intervening sequences have been previously demonstrated to lack hotspot activity in the absence of Rap1p-binding sites.

The plasmids used to generate mutations of various yeast genes were the following: (1) pLSD64 (provided by D. Shore; *rap1::LEU2* allele), (2) pKD18 (provided by E. T. Young; *adr1-Δ2::URA3* allele), (3) pBM2387 (provided by M. Johnston; *gal4::hisG-URA3-hisG* allele), (4) pNKY85 (provided by S. Keeney; *leu2Δ::hisG-URA3-hisG* allele), and (5) pNKY349 (provided by N. Kleckner; *rad50S*).

Strains: All strains used in this study were derived from the haploid strains AS13 (*MAT***a** *leu2 ura3 ade6 rme1*) and AS4 (*MAT*α *trp1 arg4 tyr7 ade6 ura3 spt22*; Stapleton and Petes 1991). The relevant genotypes of the haploid and diploid strains used in our study are summarized in Tables 1 and 2.

The diploid strains DTK438 and DTK453 were used to examine the effects of *rap1-ΔN* and *rap1-17* on *HIS4* hotspot activity. To construct an AS13-derived haploid strain with the *rap1-ΔN* mutation, we transformed the haploid strain HF4U (Fan *et al.* 1995) with pDTK109 (encoding *rap1-ΔN*) to yield DTK342. HF4U is isogenic with AS13 except for the following alterations: (1) replacement of *HIS4* with *his4-lopc*, (2) replacement for wild-type *RAD50* gene with the *rad50S* allele (Alani *et al.* 1990), and (3) a spontaneous mutation of the *URA3* gene used to insert *rad50S* sequences (Fan *et al.* 1995). The wild-type *RAP1* gene in DTK342 was then inactivated by transformation with *Bgl*II/*Xba*I-treated pLSD64, a plasmid containing a *rap1::LEU2* allele. The net result of these manipulations was DTK354, in which *RAP1* was replaced with a plasmid-borne *rap1-ΔN* allele.

To create an AS13 derivative bearing *rap1-17*, we crossed DTK354 (described above) to DTK355. DTK355 was derived from DNY25 (which contains a *his4-lopc* mutation; Nag *et al.* 1989) by using pGAL-HO to perform a mating-type switch (Herskowitz and Jensen 1991). The diploid DTK356 is a derivative of the DTK354 × DKT355 diploid that had lost the pDTK109 plasmid. The plasmid pAJL1000 (encoding *rap1-17*) was introduced into this strain, creating DTK359. This diploid was sporulated, and a plasmid-bearing spore with the genotype *a rap1*::*LEU2 rad50S* was identified (DTK366).

A similar process was used to create AS4-derived *rap1-17* and *rap1-ΔN* strains. A *rad50S* derivative of AS4 (DNY107; Nag and Petes 1993) was transformed with a *Bgl*II fragment derived from pNKY85 (strain DTK387) and a Ura⁻ derivative of the resulting transformant was selected on medium contain 5-fluoroorotate. This strain (DTK388) had the *leu2Δ::hisG* allele. DTK388 was mated to LS1-*leu2Δ*. LS1-*leu2Δ* was derived from LS1 (Symington and Petes 1988) by a two-step transplacement with the *Kpn*I-treated plasmid p*leu2-RI* (provided by G. S. Roeder); this plasmid contains a mutant *leu2* gene caused by "fill-in" of an *Eco*RI site in the coding sequence cloned in YIp5. LS1 is isogenic with AS4 except LS1 has the opposite mating type and lacks a number of restriction site alterations in the region between *LEU2* and *CEN3* (Symington and Petes 1988; Stapleton and Petes 1991). The diploid formed by crossing DTK388 and LS1-*leu2Δ* (DTK389) was transformed with a *Bgl*II-*Xba*I fragment derived from pLSD64 to generate DTK400 (heterozygous for *rap1::LEU2*). DTK410 and DTK411 were created by transformation of DKT400 with pDTK109 (encoding *rap1-ΔN*) and pAJL1000 (encoding *rap1-17*), respectively. DTK410 was sporulated and dissected, and a spore containing pDTK109 with the genotype α *rap1::LEU2* was isolated (DTK431). Similarly, a spore derived from DTK-411 containing pAJL1000 with the genotype α *rap1::LEU2* was isolated (DTK451). The diploid DTK438 was constructed by mating DTK354 and DTK431, and the diploid DTK453 was generated by mating DTK366 and DTK451.

In the diploid strain QFY120, the transcription-factor-binding sites located upstream of *HIS4* were replaced by duplicated Gal4p-binding sites. In addition, the strain was heterozygous for the *his4-lopc* allele. This strain was constructed by mating QFY32 and QFY33. The haploids QFY32 and QFY33 were derived from PD80 and PD63 (Detloff *et al.* 1992), respectively, by two-step transplacement with *Pvu*II-treated pQF1d; the resulting substitution is *his4-301.* The genotypes and progenitor strains for PD80 and PD63 are shown in Table 1. Derivatives of QFY32 (QFY38) and QFY33 (QFY39) with mutant *gal4* genes were constructed by transformation of the parental strains with *Sac*I/*Kpn*I-treated pBNM2387 DNA, followed by selection of Ura⁻ derivatives of the resulting Ura⁺ transformants. The diploid QFY122 was obtained by mating QFY38 and QFY39. Derivatives of QFY38 (QFY51) and QFY39 (QFY52) with a deletion of *ADR1* were generated by transformation of the parental strains with *Sac*I/*Sph*I-treated pKD18. The diploid QFY132 was obtained by crossing QFY51 and QFY52.

To examine the recombination-stimulating effects of various Gal4p/Rap1p fusion proteins, we transformed QFY122 with the following plasmids (name of transformed strain in parentheses): pRS316 (DTK191), pDTK116-BC (DTK192), pDTK118-BAC (DTK193), pDTK121-BA (DTK197), and pDTK122-B (DTK206). A related set of strains was constructed by transforming the *rad50S* diploid QFY135 with the same plasmids: PRS316 (DTK216), pDTK116-BC (DTK217), pDTK-118-BAC (DTK218), pDTK121-BA (DTK219), and pDTK-122-B (DTK220). QFY135 was constructed by mating the haploids QFY44 and PG31, *gal4::hisG* derivatives of QFY36 and QFY37, respectively. QFY36 and QFY37 were Ura⁺ *rad50S* transformants obtained by transformation of HF5U (QFY36) and HF6U (QFY37) with *Eco*RI/*Bam*HI-treated pNKY349.

Meiotic analysis: For genetic analysis, diploids were sporulated on plates at 18° for 4–6 days and dissected by standard methods onto plates containing rich growth medium; the colonies were then replica plated to various omission media. Spores derived from strains heterozygous for the *his4-lop* and *his4-lopc* alleles have high levels of postmeiotic segregation, which are detectable as sectored His⁺/His⁻ colonies on medium lacking histidine (Nag *et al.* 1989). To ensure that all sectored colonies were detected, we used a microscope to examine all colonies on histidine omission medium. For those strains containing an autonomously replicating plasmid, all data were derived from tetrads in which at least one spore colony retained the plasmid. For physical studies of meiotic cells (determination of the level of meiosis-specific DSBs), cells were sporulated in liquid medium at 24°, as described previously (Nag and Petes 1993).

Isolation and analysis of DNA and RNA: DNA was isolated from meiotic or premeiotic cells by methods described previously (Nag and Petes 1993). To monitor DSBs at the *HIS4* hotspot, we treated DNA samples with *Bgl*II and examined the resulting fragments by Southern analysis. The hybridization probe was an *Xho*I-*Xba*I fragment of plasmid pDN42 containing sequences derived from the 5′ end of *HIS4* (Nag and Petes 1993). Previous studies (Fan *et al.* 1995) show that the DNA fragment resulting from the DSB at the hotspot is ∼2 kb and the unbroken *Bgl*II fragment is ∼3 kb. The amount of hybridization to these two fragments was determined using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The DSB percentage was calculated by dividing the amount

TABLE 1

Haploid strains

Name[a]	*HIS4* upstream alteration[b]	*HIS4* coding sequence alteration[c]	Other relevant alterations[d]	Progenitor strain
AS13	WT	WT	WT	
DNY9	WT	*his4-lop*	WT	AS13
DNY25	WT	*his4-lopc*	WT	AS13
PD57	*his4-Δ52*	WT	WT	AS13
PD80	*his4-Δ52*	*his4-lopc*	WT	PD57
MW33	*his4-51*	*his4-lop*	WT	DNY9
HF3	*his4-51*	*his4-lop*	*rad50S*	MW33
HF4U	WT	*his4-lopc*	*rad50S*	DNY25
MW68	*his4-202*	WT	WT	PD57
MW72	*his4-202*	*his4-lopc*	WT	MW68
HF5U	*his4-202*	*his4-lopc*	*rad50S*	MW72
DTK355	WT	*his4-lopc*	α	DNY25
DTK342	WT	*his4-lopc*	*rad50S*; pDTK109 (*rap1-ΔN*)	HF4U
DTK354	WT	*his4-lopc*	*rad50S; rap1Δ::LEU2*; pDTK109 (*rap1-ΔN*)	DTK342
DTK366	WT	*his4-lopc*	*rad50S; rap1Δ::LEU2*; pAJL1000 (*rap1-17*)	Spore of DTK359
QFY32	*his4-301*	*his4-lopc*	WT	PD80
QFY36	*his4-301*	*his4-lopc*	*rad50S*	HF5U
QFY38	*his4-301*	*his4-lopc*	*gal4::hisG*	QFY32
QFY44	*his4-301*	*his4-lopc*	*rad50S gal4::hisG*	QFY36
QFY51	*his4-301*	*his4-lopc*	*gal4::hisG adr1::URA3*	QFY38
AS4	WT	WT	WT	
LS1[e]	WT	WT	*a*	XJ24-24a
LS1-*leu2Δ*	WT	WT	*a leu2*	LS1[e]
DNY107	WT	WT	*rad50S*	AS4
MW30	*his4-51*	WT	WT	AS4
PD63	*his4-Δ52*	WT	WT	AS4
MW73	*his4-202*	WT	WT	PD63
HF6U	*his4-202*	WT	*rad50S*	MW73
DTK387	WT	WT	*leu2Δ::hisG-URA3-hisG rad50S*	DNY107
DTK388	WT	WT	*leu2Δ::hisG rad50S*	DTK387
DTK431	WT	WT	*leuΔ::hisG rap1Δ::LEU2*; pDTK109 (*rap1-ΔN*)	Spore of DTK410
DTK451	WT	WT	*leu2Δ::hisG rap1Δ::LEU2*; pAJL1000 (*rap1-17*)	Spore of DTK411
QFY33	*his4-301*	WT	WT	PD63
QFY37	*his4-301*	WT	*rad50S*	HF6U
PG31	*his4-301*	WT	*rad50S gal4::hisG*	QFY37
QFY39	*his4-301*	WT	*gal4::hisG*	WFY33
QFY52	*his4-301*	WT	*gal4::hisG adr1::URA3*	QFY39

[a] AS13-related and AS4-related strains are grouped separately. Strains described previously are the following: DNY9 and DNY25 (Nag *et al.* 1989); AS13 and AS4 (Stapleton and Petes 1991); DNY107, HF3, HF4U, HF5U, and HF6U (Fan *et al.* 1995); PD57, PD63, and PD80 (Detloff *et al.* 1992); MW68, MW72, and MW73 (White *et al.* 1993); LS1 (Symington and Petes 1988); MW30 and MW33 (White *et al.* 1991).

[b] The wild-type (WT) strains have binding sites for the transcriptional activators Rap1p, Bas1p, Bas2p, and Gcn4p, and strains with the *his4-Δ52* allele are deleted for all of these binding sites. The *his4-51* allele disrupts the Rap1p-binding site. In strains with the *his4-202* and *his4-301* insertions, the normal upstream sequences are replaced by insertions that contain three Rap1p- or two Gal4p-binding sites, respectively.

[c] The *his4-lop* and *his4-lopc* alleles are 26-bp palindromic insertions in the coding sequence of *HIS4*; heteroduplexes heterozygous for these insertions have a poorly repaired DNA mismatch, leading to high levels of postmeiotic segregation (Nag *et al.* 1989).

[d] Unless indicated, all strains derived from AS13 have the genotype *a leu2 ura3 ade6 rme1* and all strains derived from AS13 have the genotype α *trp1 arg4 tyr7 ade6 ura3*. In addition, both AS4 and AS13 have restriction site alterations located between *LEU2* and *CEN3* that were used to map the position of crossovers in a previous study (Symington and Petes 1988; Stapleton and Petes 1991).

[e] LS1 is isogenic with AS4 except that LS1 is of the opposite mating type and lacks restriction site alterations located between *LEU2* and *CEN3* (Symington and Petes 1988).

TABLE 2

Diploid strains

Name[a]	Parental strains[b]	Plasmid	Relevant genotype[c]
DNY26	DNY25 × AS4	None	13 × 4; *his4-lopc/HIS4*
PD81	PD80 × PD63	None	13 × 4; *his4-Δ52/his4-Δ52 his4-lopc/HIS4*
MW118	MW33 × MW30	None	13 × 4; *his4-51/his4-51 his4-lop/HIS4*
MW154	MW72 × MW73	None	13 × 4; *his4-202/his4-202 his4-lopc/HIS4*
DTK389	DTK388 × LS1-*leu2*Δ	None	4 × 4; *leu2Δ::hisG/leu2 rad50S/RAD50*
DTK400	DTK389	None	4 × 4; *leu2Δ::hisG/leu2 rad50S/RAD50 rap1Δ::LEU2/RAP1*
DTK410	DTK400	pDTK109	4 × 4; *leu2Δ::hisG/leu2 rad50S/RAD50 rap1Δ::LEU2/RAP1*
DTK411	DTK400	pAJL1000	4 × 4; *leu2Δ::hisG/leu2 rad50S/RAD50 rap1Δ::LEU2/RAP1*
DTK356	DTK354 × DTK355	None	13 × 13; *his4-lopc/his4-lopc rap1Δ::LEU2/RAP1 rad50S/RAD50*
DTK359	DTK356	pAJL1000	13 × 13; *his4-lopc/his4-lopc rap1Δ::LEU2/RAP1 rad50S/RAD50*
DTK438	DTK354 × DTK431	pDTK109	13 × 4; *his4-lopc/HIS4 leu2/leu2Δ::hisG rap1::LEU2/rap1::LEU2 rad50S/RAD50*
DTK453	DTK366 × DTK451	pAJL1000	13 × 4; *his4-lopc/HIS4 leu2/leu2Δ::hisG rap1::LEU2/rap1::LEU2 rad50S/RAD50*
QFY120	QFY32 × QFY33	None	13 × 4; *his4-301/his4-301 his4-lopc/HIS4*
QFY122	QFY38 × QFY39	None	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG*
QFY132	QFY51 × QFY52	None	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG adr1::URA3/adr1::URA3*
DTK191	QFY122	pRS316	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG*
DTK192	QFY122	pDTK166-BC	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG*
DTK193	QFY122	pDTK118-BAC	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG*
DTK197	QFY122	pDTK121-BA	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG*
DTK206	QFY122	pDTK122-B	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG*
QFY135	QFY44 × PG31	None	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG rad50S/rad50S*
DTK216	QFY135	pRS316	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG rad50S/rad50S*
DTK217	QFY135	pDTK116-BC	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG rad50S/rad50S*
DTK218	QFY135	pDTK118-BAC	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG rad50S/rad50S*
DTK219	QFY135	pDTK121-BA	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG rad50S/rad50S*
DTK220	QFY135	pDTK122-B	13 × 4; *his4-301/his4-301 his4-lopc/HIS4 gal4::hisG/gal4::hisG rad50S/rad50S*

[a] The references to strains previously described are the following: DNY26 (Nag *et al.* 1989), PD81 (Detloff *et al.* 1992), MW118 (White *et al.* 1991), and MW154 (White *et al.* 1993).

[b] In crosses in which one haploid was derived from AS13 and the other from AS4, the AS13-derived strain is listed first.

[c] The expression 13 × 4 indicates that one haploid was AS13 derived and the other AS4 derived. The expressions 4 × 4 and 13 × 13 signify that both haploids in the cross were AS4 or AS13 derived, respectively.

of hybridization in the 2-kb fragment by the sum of the amounts of hybridization to the 2- and 3-kb fragments. Because the percentage of DSBs is affected by the efficiency of sporulation, we normalized the percentage of DSBs at the *HIS4* hotspot to the percentage of DSBs at the *ARG4* hotspot; DSBs at the *ARG4* locus were detected using an *Eco*RV-*Bgl*II fragment derived from plasmid pAK1 as a hybridization probe for *Bgl*II-treated yeast DNA samples (Fan *et al.* 1995). The normalization was done by multiplying the percentage of DSBs at the *HIS4* locus in each individual experiment by the correction ratio (maximum percentage of DSBs at the *ARG4* locus divided by the percentage of DSBs at the *ARG4* locus in each individual experiment).

Total RNA was prepared (Solomon *et al.* 1992) from cells grown in liquid cultures. Standard Northern analysis was done (Sambrook *et al.* 1989). The nylon filter with the transferred RNA was hybridized to a mixture of two probes, the same *HIS4* probe used in the Southern analysis (described above) and an actin gene probe; the *ACT1* probe was a 1-kb gel-purified *Hin*dIII-*Xho*I fragment of plasmid pGAL1-*ACT1* (provided by R. Sia, Duke University). Levels of hybridization were quantified using a PhosphorImager (Molecular Dynamics).

RESULTS

As described in the Introduction, Rap1p is required for the meiotic recombination hotspot observed upstream of *HIS4.* Rap1p has diverse cellular roles and the protein domains required for these roles have been characterized (Figure 1). To determine which protein domains of Rap1p were required for hotspot activity, we examined the recombination-stimulating properties

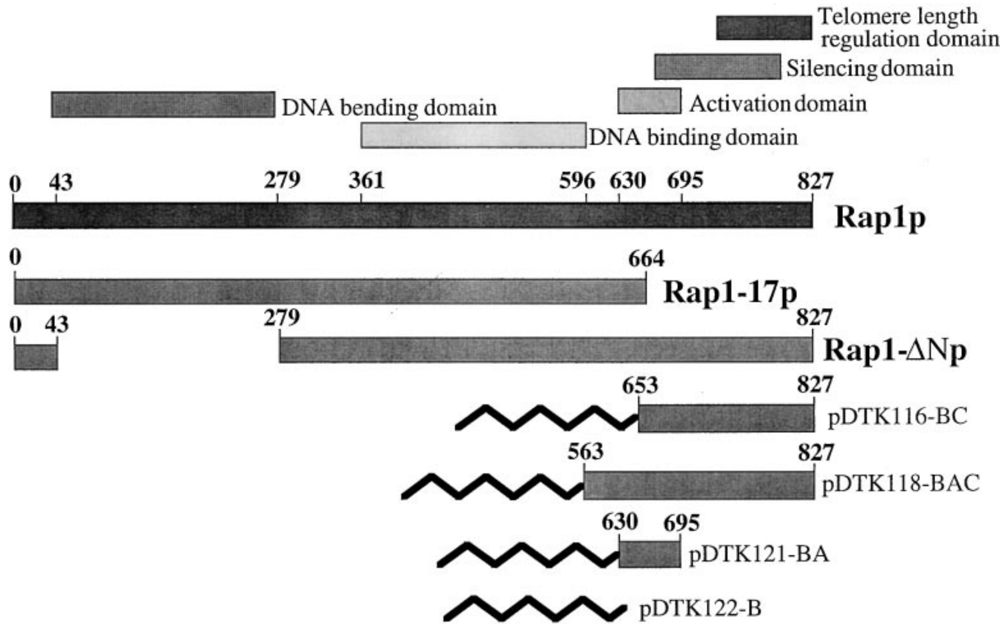

Figure 1.—Rap1p functional domains in Rap1p deletion and Gal4p/Rap1p fusion proteins. The full-length Rap1p is 827 amino acids. The various functional domains of the proteins [summarized in Shore (1994) and references therein] are shown at the top of the figure. The extent of the deletions encoded by the *rap1-17* and *rap1-ΔN* mutant alleles and the portion of Rap1p in various Gal4p/Rap1 fusion proteins (plasmids encoding these fusions indicated on the right) are shown at the bottom of the figure. All fusion proteins contain the DNA-binding domain of Gal4p (amino acids 1–147 of Gal4p, indicated by zigzag lines).

of deletion derivatives of Rap1p. In addition, we studied the effects of fusion proteins with the Gal4p-binding domain and various segments of Rap1p on recombination in strains in which the normal *HIS4* upstream sequences were replaced with Gal4p-binding sites. These two studies show that maximal hotspot activity requires proteins that have both a DNA-binding domain and a transcription activation domain.

Analysis of the effects of aminoterminal and carboxyterminal deletions of Rap1p on *HIS4* hotspot activity: One convenient method of monitoring *HIS4* hotspot activity is by measuring the frequency of aberrant segregation (non-2:2 segregation) of a heterozygous mutation within the *HIS4* coding sequence by tetrad analysis. In many of our previous studies, we have used mutant alleles resulting from 26-bp palindromic insertions (*his4-lopc* or *his4-lop*) near the beginning of the *HIS4* gene. In one such strain, DNY26 (Nag *et al.* 1989; Fan *et al.* 1995), the frequency of aberrant segregation of *his4-lopc* was 51% (Table 3). As described in the Introduction, this very high level of meiotic recombination is dependent on the binding of a number of transcription factors in the region upstream of *HIS4*, including Rap1p, Bas1p, and Bas2p. As shown in Table 3, a deletion that removes all of these transcription-factor-binding sites (*his4-Δ52*, strain PD81) or the Rap1p-binding site (*his4-51*, strain MW118) substantially reduces the frequency of aberrant segregation (White *et al.* 1991; Detloff *et al.* 1992); these alterations also eliminate meiosis-specific DSBs associated with hotspot activity (Fan *et al.* 1995). In addition, overproduction of Rap1p further stimulates *HIS4* recombination (White *et al.* 1991). Replacement of the wild-type *HIS4* promoter with two copies of the Rap1p-binding site is sufficient to initiate recombination at wild-type levels, while three copies of the binding site create the strongest meiotic recombination initiation site yet observed (*his4-202*, MW154; Table 3). In general, the frequency of aberrant segregation of markers within the *HIS4* coding sequence is correlated with the frequency of crossovers between *HIS4* and the linked *LEU2* gene (Table 3).

To determine the role of the nonessential functional domains of Rap1p in stimulating *HIS4* hotspot activity, two diploid strains were constructed, each with a different mutant allele of *RAP1.* The strain DTK453 contained the *rap1-17* mutant allele (Kyrion *et al.* 1992), encoding a Rap1p with a C-terminal deletion (Figure 1). The diploid DTK438 contained *rap1-ΔN* (Gilson *et al.* 1993), encoding a Rap1p with a deletion near the N terminus. Both strains were heterozygous for the *his4-lopc* allele. These strains were sporulated, tetrads were dissected, and we monitored the frequency of aberrant segregation of *his4-lopc* (Table 3).

The aberrant segregation of *his4-lopc* in DTK453 was 38%, significantly ($P < 0.001$ by Fisher exact test for both comparisons) above the level of aberrant segregation seen in a strain lacking the *HIS4* upstream activation region (PD81) or in a strain that lacks only the Rap1p DNA-binding site (MW118). The aberrant segregation of *his4-lopc* in DTK438 (37%) was nearly identical to DTK453 and significantly greater than observed for PD81 or MW118. For both DTK453 and DTK438, the frequencies of aberrant segregation for *his4-lopc* were significantly reduced compared to the frequency observed in the wild-type strain DNY26.

These results suggest that the portions of Rap1p that are deleted in Rap1-17p and Rap1p-ΔNp are not essential, but may contribute, to hotspot activity. Alternatively, because the mutated forms of Rap1p are expressed from a plasmid in DTK453 and DTK438, it is possible that

TABLE 3

Analysis of aberrant segregations and crossovers in strains with different alterations of the upstream regulatory region of *HIS4* or with mutations affecting the Rap1p transcription factor

Strain[c]	*HIS4* upstream sequences[d]	Other relevant mutations[e]	No. tetrads with various segregation patterns[a]: Spor. media[f]	Total tetrads	4:4	6:2	2:6	5:3	3:5	Other ab. seg.	% ab. seg.	Crossovers between *HIS4* and *LEU2*[b]: PD	NPD	T	*HIS4-LEU2* (cM)
DNY26	Wild type	*his4-lopc*	Glu	493	244	29	17	88	67	48	51	198	8	202	31
PD81	*his4-Δ52*	*his4-lopc*	Glu	398	313	14	6	26	32	7	21	184	4	79	19
MW118	*his4-51*	*his4-lop*	Glu	325	266	7	2	20	24	6	18	188	1	112	20
MW154	*his4-202*	*his4-lopc*	Glu	321	90	17	9	56	72	77	72	75	23	133	59
DTK453	Wild type	*his4-lopc rap1-17*	Glu	116	72	11	5	12	8	8	38	—	—	—	—
DTK438	Wild type	*his4-lopc rap1-ΔN*	Glu	219	137	11	7	35	18	11	37	—	—	—	—
QFY120	*his4-301*	*his4-lopc*	Glu	459	218	15	7	89	83	47	53	202	6	179	28
QFY120	*his4-301*	*his4-lopc*	Gal	224	121	5	4	33	37	24	46	110	6	81	30
QFY122	*his4-301*	*his4-lopc gal4*	Glu	194	105	10	2	29	26	22	46	80	6	82	35
QFY132	*his4-301*	*his4-lopc gal4 adr1*	Glu	137	68	6	4	25	23	11	50	60	4	53	33

[a] These data represent the segregation patterns of the heterozygous *his4-lopc* or *his4-lop* alleles. For all patterns, the first number represents the wild-type allele and the second, the mutant allele. We use the nomenclature devised for eight-spored fungi: 4:4 (normal Mendelian segregation), 6:2 or 2:6 (single gene conversion events), 5:3 or 3:5 [single postmeiotic segregation (PMS) events], and other ab. seg. (more than one gene conversion and/or PMS event).

[b] PD, NPD, and T indicate parental ditype, nonparental ditype, and tetratype tetrads, respectively. Map distances were calculated using the equation of Perkins (1949). Crossovers could not be calculated for DTK438 and DTk453, because these strains are homozygous for the *LEU2* marker.

[c] The references for data described previously are the following: DNY26 (Fan *et al.* 1995), MW154 (White *et al.* 1993), PD81 (Detloff *et al.* 1992), and MW118 (White *et al.* 1991).

[d] The wild-type *HIS4* upstream region contains binding sites for Rap1p, Bas1p, Bas2p, and Gcn4p, the *his-Δ52* allele represents a deletion for all these sites, and the *his4-51* allele contains point mutations that disrupt the Rap1p-binding site. The *his4-202* allele represents an insertion of telomeric DNA with three Rap1p-binding sites, and *his4-301* signifies insertion of two Gal4p-binding sites; for both alleles, the normal upstream transcription-factor-binding sites have been deleted.

[e] All strains are heterozygous for *his4-lopc* or *his4-lop*, 26-bp palindromic insertions in the *HIS4* coding region. *his4-lop* and *his4-lopc* have equivalent levels of aberrant segregation in wild-type strains (Nag *et al.* 1989). DTK438 and DTK453 contain homozygous deletions of *RAP1* (*rap1Δ::LEU2*); DTK438 contains pDTK109, a plasmid bearing the *rap1-ΔN* allele, while DTK453 contains pAJL1000, a plasmid bearing the *rap1-17* allele.

[f] Strains were sporulated on solid medium containing either 0.05% glucose (Glu, the standard medium) or galactose (Gal).

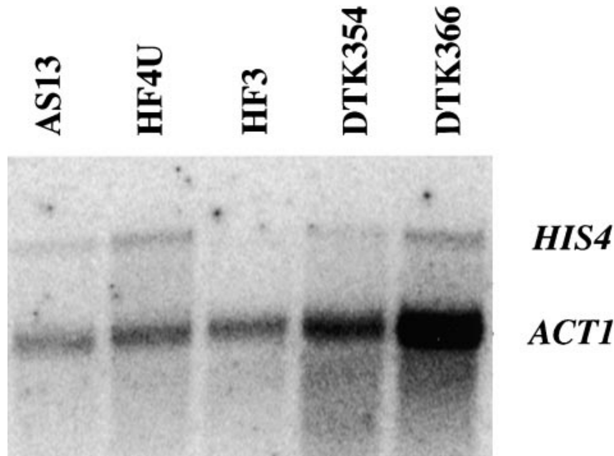

Figure 2.—Rap1-ΔNp and Rap1-17p stimulate *HIS4* transcription. Haploid strains were grown in rich medium and total RNA was isolated from each strain. RNA was examined by Northern analysis using probes to *HIS4* and *ACT1.* AS13 and HF4U have wild-type promoter regions at *HIS4*, whereas HF3 bears the *his4-51* mutation (a disruption of the Rap1p-binding site in the *HIS4* upstream activation region). DTK354 and DTK366 have wild-type *HIS4* promoter sequences but contain the *rap1-ΔN* and *rap1-17* mutations, respectively.

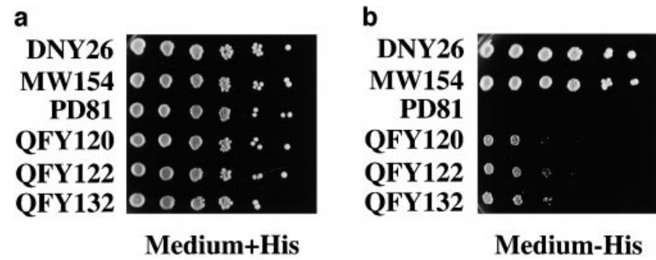

Figure 3.—*HIS4* gene expression in strains with various transcription-factor-binding sites upstream of *HIS4.* Diploid strains were grown in rich growth medium to stationary phase. Undiluted samples were placed in one column of wells in a microtiter dish. Each sample was serially diluted (1:10) in water in adjacent wells within each row. A multipronged device was then used to transfer a small amount of the samples from the microtiter dish to a plate containing solid medium with or without histidine; the strains were then grown at 30° for (a) 3 and (b) 5 days. The relevant features of the strains are the following: DNY26 (wild-type *HIS4* upstream sequences), MW154 (telomeric insertion with three Rap1p-binding sites replacing wild-type upstream sequences), PD81 (deletion of wild-type upstream sequences), QFY120 (replacement of wild-type upstream sequences with duplicated Gal4p-binding sites; *GAL3 ADR1*), QFY122 (replacement of wild-type upstream sequences with duplicated Gal4p-binding sites; *gal4 ADR1*), and QFY132 (replacement of wild-type upstream sequences with duplicated Gal4p-binding sites; *gal4 adr1*).

the level of Rap1p is quantitatively different from the wild-type level of Rap1p. The strains with the mutated forms of Rap1p do not have a global reduction in meiotic recombination relative to the wild-type DNY26 strain. The gene conversion frequencies at the *ARG4* locus were 18% (DTK438), 12% (DTK453), and 9% (DNY26). The recombination distances between *ARG4* and the centromere in the three strains were 12 cM (DTK438), 16 cM (DTK453), and 12 cM (DNY26).

Strains with a null mutation of *RAP1* are not viable. If this lack of viability is a consequence of the role of Rap1p as a transcription factor, Rap1-17p and Rap1-ΔNp should be capable of stimulating *HIS4* transcription. We confirmed this prediction by measuring the level of *HIS4* mRNA (relative to an actin control mRNA) by Northern analysis (Figure 2). Control strains AS13 and HF4U (wild-type promoter sequences and wild-type Rap1p) had similar levels of *HIS4* mRNA. A strain with a deletion of the Rap1p-binding site (HF3) had a level of *HIS4* transcript at least 10-fold reduced from the control strains, whereas strains with the *rap1-17* or *rap1-ΔN* mutations had *HIS4* mRNA levels that were reduced about 4-fold. Thus, the proteins encoded by these mutant genes retain some, but not all, of their transcription-stimulating activity.

As shown in Figure 1, Rap1-17p lacks the silencing domain, the domain required for telomere length maintenance, and the binding sites for Sir3p, Sir4p, and Rif1p. The results shown in Table 3 and Figure 2 indicate that none of these functions are essential for the hotspot-activation or transcription-activation functions of Rap1p. As expected from this conclusion, a deletion of *SIR3* had no effect on DSB formation at the *HIS4* hotspot (data not shown). Similarly, the region of Rap1p that generates a DNA bend upon binding (missing in Rap1-ΔNp) is not required for hotspot activity or transcription activation. These results suggest that the essential recombination-stimulating activity of Rap1p might be the DNA-binding and transcription activation domains. Because Rap1p is essential, we could not examine the effects of deletions removing these domains by the same approach. Consequently, we replaced the Rap1p-binding sites upstream of *HIS4* with Gal4p-binding sites and examined the transcription-stimulating effects of various activators that had the Gal4p DNA-binding domain and portions of Rap1p.

Stimulation of *HIS4* gene expression and meiotic recombination activity by insertion of Gal4-binding sites: As in the studies described above, we used diploid strains that were heterozygous for the allele *his4-lopc* and homozygous for various sequences upstream of *HIS4.* We first examined the effects of these alterations on *HIS4* gene expression. As shown in Figure 3, strains with the wild-type upstream sequences (DNY26) or with a telomeric insertion (MW154, containing three Rap1p-binding sites replacing the wild-type upstream sequences) have strong His^+ phenotypes (White *et al.* 1993). A strain (PD81) that lacks transcription-factor-binding sites is His^- (Nagawa and Fink 1985). We constructed the strain QFY120 in which the wild-type transcription-factor-binding sites were replaced by two Gal4p-binding sites separated by a short spacer (see materials and

methods). This substitution (*his4-301*) activated *HIS4* gene expression weakly (Figure 3).

The activation of *HIS4* gene expression in QFY120 in glucose-containing medium is somewhat surprising for two reasons. First, expression of Gal4p is repressed four- to seven-fold in cells grown on glucose-containing medium (Griggs and Johnston 1991). Second, although Gal4p binds to DNA when cells are grown in the absence of galactose, the activation domain is blocked from functioning because of its interactions with Gal80p (Johnston *et al.* 1987; Ma and Ptashne 1987), an interaction that is lost when the cells are grown in galactose (Bhat and Hopper 1992).

To investigate in more detail the possibility of Gal4p-independent activation of *HIS4* expression by the duplicated Gal4p-binding sites, we constructed a derivative of QFY120 (QFY122) that was homozygous for a deletion of *GAL4.* In addition, because the Gal4p-binding site shares sequence homology with the binding site recognized by the transcription factor Adr1p (Cheng *et al.* 1994), we constructed a derivative of QFY120 (QFY132) that lacked both *GAL4* and *ADR1* genes. Both QFY122 and QFY132 were weakly His$^+$ (Figure 3), confirming that the Gal4p-binding sites confer some activation of *HIS4* transcription that is independent of the binding of either Gal4p or Adr1p.

In these strains, we monitored the level of aberrant segregation for the *his4-lopc* allele (as a genetic measure of hotspot activity), as well as the frequency of crossing-over between *HIS4* and the linked *LEU2* gene (Table 3). QFY120, homozygous for the insertion of duplicated Gal4p-binding sites (*his4-301*), had a level of aberrant segregation (53%) for the *his4-lopc* allele that was close to that observed in the wild-type strain DNY26. Sporulation of QFY120 in medium containing galactose, instead of glucose, did not significantly elevate the frequency of aberrant segregation or crossovers above the frequency observed in cells sporulated in glucose-containing medium. In addition, derivatives of QFY120 in which *GAL4* (QFY122) or *GAL4* and *ADR1* (QFY132) were mutated had about the same level of aberrant segregation and crossovers as QFY120 (Table 3). In summary, when duplicated Gal4p-binding sites were inserted upstream of *HIS4*, we observed recombination hotspot activity. Both the frequency of aberrant segregation of *his4-lopc* and the level of *HIS4-LEU2* crossovers were elevated relative to the values observed in PD81, a strain that lacks hotspot activity. The hotspot activity of the Gal4p-binding sites, however, did not require the binding of Gal4p or Adr1p.

At least two possibilities exist for the hotspot activity observed in QFY120 and its derivatives. First, the duplicated Gal4p-binding sites might bind an unknown transcription factor that stimulates meiotic recombination at *HIS4.* Alternatively, some property of the DNA sequence of the insertion may be responsible for hotspot activity. These possibilities will be addressed in more detail in the discussion.

Analysis of the recombination-stimulating properties of fusion proteins containing the DNA-binding domain of Gal4p and activation domains of Rap1p in strains with Gal4p-binding sites upstream of *HIS4*: Although Gal4p does not appear to contribute to hotspot activity (as described above), we previously concluded that Rap1p was capable of contributing to *HIS4* hotspot activity (White *et al.* 1991, 1993). To determine whether the differences in the behavior of these two transcription factors resided in the activation domain of the proteins, we examined the recombination-stimulating properties of proteins with the Gal4p DNA-binding domain and the Rap1p activation domain in strains with two Gal4p-binding sites upstream of *HIS4.* We transformed QFY122 (homozygous for duplicated Gal4p-binding sites and the *gal4* mutation) with five different plasmids: (1) pRS316 (vector alone), (2) pDTK116-BC (*b*inding domain of Gal4p and *C* terminus of Rap1p), (3) pDTK118-BAC (*b*inding domain of Gal4p, complete *a*ctivation domain of Rap1p and *C* terminus of Rap1p), (4) pDTK121-BA (*b*inding domain of Gal4p and complete *a*ctivation domain of Rap1p), and (5) pDTK122-B (*b*inding domain of Gal4p without any Rap1p). The regions of Rap1p in each plasmid are shown in Figure 1. Although the fusion protein encoded by pDTK116-BC has part of the activation domain of Rap1p, this protein is incapable of activating transcription (Hardy *et al.* 1992).

These strains were sporulated and tetrads were dissected. As above, we monitored the aberrant segregation frequency of *his4-lopc* and crossovers between *HIS4* and *LEU2*; only tetrads in which at least one spore retained the plasmid (signalling retention of the plasmid in the diploid cell giving rise to the spores) were included in our analysis (Table 4). As expected, QFY122 with the vector alone had the same frequency of recombination as QFY122 without any plasmid (compare Table 3 and Table 4). Three types of effects were seen in the derivatives of QFY122 with the fusion plasmids. In the strain with pDTK116-BC, both the frequency of aberrant segregation and the level of *HIS4-LEU2* crossovers were significantly ($P < 0.01$) reduced compared to QFY122 with the vector alone. In the strains with pDTK118-BAC and pDTK121-BA, both the frequency of aberrant segregation and the level of crossovers between *HIS4* and *LEU2* were significantly ($P < 0.01$) elevated compared to QFY122 with only the vector. In the strain with the plasmid pDTK122-B, the frequency of aberrant segregation was significantly ($P < 0.02$) elevated, but the frequency of crossovers was not ($P = 0.8$), compared with QFY122 with the vector alone.

These results indicate that fusion proteins with the Gal4p-binding domain and a complete Rap1p activation domain (encoded by plasmids pDTK118-BAC and pDTK-121-BA) stimulate meiotic recombination more effectively than other fusion proteins. A protein with the

TABLE 4

Analysis of the effects of Gal4p/Rap1p fusion proteins on meiotic recombination (aberrant segregation of *his4-lopc* and *HIS4-LEU2* crossovers) in diploid strains (derived from QFY122) with insertions of duplicated Gal4p-binding sites upstream of *HIS4*

		No. tetrads with various segregation patterns[a]								Crossovers between *HIS4* and *LEU2*[a]			
Strain[b]	Plasmid[c]	Total tetrads	4:4	6:2	2:6	5:3	3:5	Other ab.seg.	% ab. seg.	*aa*PD	NPD	T	*HIS3-LEU2* (cM)
DTK191	pRS316 (vector)	458	247	14	13	81	66	37	46	205	18	169	35
DTK192	pDTK116-BC	416	265	13	9	50	49	30	36	235	5	128	21
DTK193	pDTK118-BAC	657	225	27	24	136	120	125	66	213	30	267	44
DTK197	pDTK121-BA	916	366	27	21	185	167	150	60	328	35	383	40
DTK206	pDTK122-B	625	291	26	25	116	104	63	53	271	19	223	33

[a] See Table 3 footnotes.

[b] All strains were derived from QFY122 by transformation with the indicated plasmids.

[c] The plasmid pRS316 is the vector used for all of the pDTK plasmids represented in this table. In these plasmids, synthesis of the Gal4p/Rap1p is controlled by the *RAP1* promoter. All of the pDTK constructs have the DNA-binding domain of Gal4p (amino acids 1–147). pDTK122-B does not have any Rap1p sequence. The portions of Rap1p found in the other plasmids are the following: pDTK116-BC (653–827), pDTK118-BAC (563–827), and pDTK121-BA (630–695).

Gal4p-binding domain with no Rap1p (encoded by pDTK122-B) had a smaller stimulatory effect on recombination, whereas a fusion protein with the Gal4p-binding domain, a truncated activation domain, and the C-terminal sequences derived from Rap1p (encoded by pDTK116-BC) suppressed recombination. These effects are unlikely to reflect differing amounts of different Gal4p/Rap1p proteins, because Western analysis indicates similar levels of expression for the different fusions (Hardy *et al.* 1992).

As first shown at the *ARG4* locus (Sun *et al.* 1989), meiotic recombination hotspots in *S. cerevisiae* are sites for DSB formation. At *HIS4*, the level of DSB formation is roughly proportional to the level of hotspot activity, although a basal level of recombination is observed in the absence of a detectable DSB (Fan *et al.* 1995). To examine the level of DSBs at *HIS4* associated with various fusion proteins, we introduced the same five plasmids described above into a derivative of QFY122 that was homozygous for *rad50S* (QFY135), a mutation that prevents the processing of DNA ends resulting from DSB formation (Alani *et al.* 1990). We monitored DSBs at the *HIS4* locus in each strain as described in materials and methods. The frequencies of *HIS4* DSBs (as a percentage of the total *HIS4* DNA) are shown in Figure 4.

As expected, the highest levels of DSB formation (~25%) were observed in strains with the plasmids pDTK118-BAC and pDTK121-BA; these same plasmids were associated with the highest levels of recombination. The levels of DSB in the other three strains were considerably lower, between 3 and 8%. Although this result is consistent with the lower level of recombination observed in these strains, the level of DSBs does not appear to be directly proportional to the level of recombination. For example, although the frequency of aberrant segregation differs by less than a factor of two between the strain with the plasmid pDKT118-BA and the strain with the vector alone, the level of DSBs differs by about a factor of three. In summary, the analysis of DSBs confirms that the highest level of recombination in the QFY122 background requires both the Gal4p-binding domain and a complete Rap1p activation domain. Some hotspot activity (measured by genetic and physical techniques), however, results from the duplicated Gal4p-binding sites in the absence of the binding of known transcription factors.

Analysis of the effects of fusion proteins containing the DNA-binding domain of Gal4p and activation domains of Rap1p on gene expression in strains with Gal4p-binding sites upstream of *HIS4*: We examined the QFY122 derivatives with the same plasmids used to study recombination for growth on medium with and without histidine. Although all strains grew at approximately the same rate on medium with histidine, growth on medium without histidine varied in the different strains (Figure 5). Strains with the plasmids pDTK118-BAC or pDTK121-BA formed colonies much more readily on medium lacking histidine than the strains with pRS316, pDTK116-BC, or pDTK122-B. All of these strains, unlike PD81, however, eventually formed colonies on the histidine-deficient medium. Thus, the same fusion proteins that maximally stimulate *HIS4* meiotic recombination also maximally stimulate *HIS4* gene expression.

DISCUSSION

The main conclusions of this study are the following: (1) maximal rates of meiotic recombination hotspot activity stimulated by the binding of transcription factors require both the DNA binding and activation domains

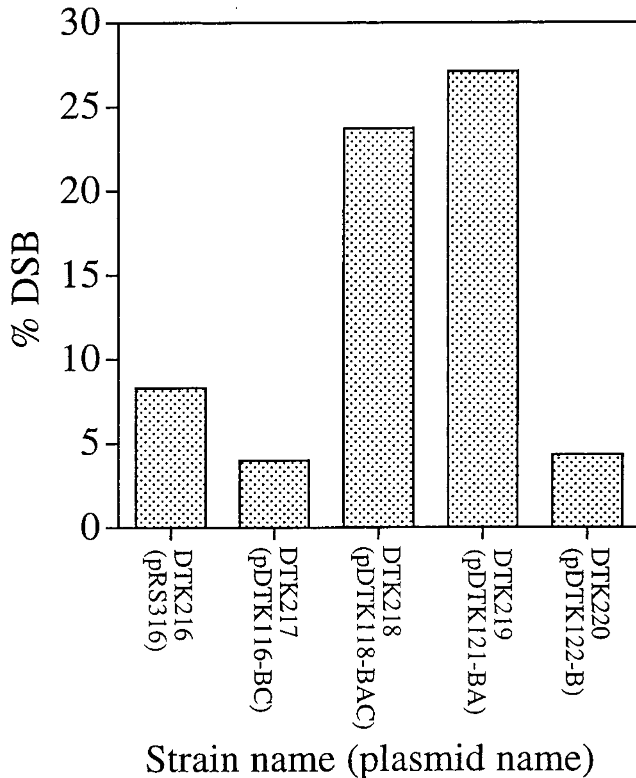

Figure 4.—Meiosis-specific DSBs at the *HIS4* locus in strains in which the wild-type *HIS4* upstream region was replaced with duplicated Gal4p-binding sites. All diploid strains depicted in this figure were homozygous for insertions of duplicated Gal4p-binding sites upstream of *HIS4*, *gal4*, and *rad50S.* In addition, each strain contained a plasmid with vector sequences alone (pRS316) or insertions of genes encoding various Gal4p/Rap1 fusion proteins (Figure 1). All plasmids of the latter class have the Gal4p DNA-binding domain. pDTK122-B encodes a protein with only the Gal4p DNA-binding domain. Other plasmids contain the following domains of Rap1p: pDTK116-BC (silencing and telomere length regulation domains; aa 653–827), pDTK118-BAC (activation, silencing, and telomere length regulation domains; aa 563–827); pDTK121-BA (activation domain; aa 630–695). Maximal levels of DSB formation require the Rap1p activation domain.

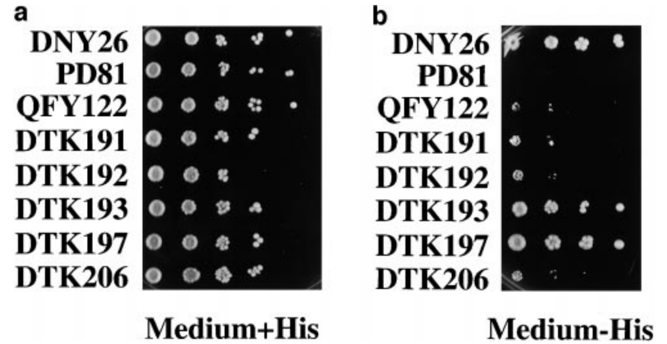

Figure 5.—Analysis of the effects of various Gal4p/Rap1p fusion proteins on *HIS4* gene expression in strains with duplicated Gal4p-binding sites upstream of *HIS4.* Strains were grown to stationary phases in medium lacking uracil (to force retention of plasmids) or rich medium (other strains). The resulting cultures were diluted and plated (as described in Figure 3 legend) on solid medium (a) containing or (b) lacking histidine. The relevant features of each strain are the following: DNY26 (wild-type *HIS4* upstream sequences), PD81 (deletion of wild-type upstream sequences), QFY122 (replacement of wild-type upstream sequences with duplicated Gal4p-binding sites; *gal4 ADR1*), DTK191 (QFY122 + pRS-316 vector), DTK192 (QFY122 + pDTK116-BC), DTK193 (QFY122 + pDTK118-BAC), DTK197 (QFY122 + pDTK121-BA), and DTK206 (QFY122 + pDTK122-B).

of these proteins, and (2) certain DNA sequences appear to stimulate recombination and gene expression without requiring any known bound transcription factors. We suggest that yeast cells have two types of recombination hotspots: α hotspots, in which hotspot activity involves an interaction of the recombination machinery with the activation domain of bound transcription factors, and β hotspots, in which the recombination machinery (without contacting transcription factors) acts on "naked" DNA. Each of these conclusion will be discussed further below.

Transcription-factor-dependent stimulation of recombination activity: We previously concluded that the activity of the wild-type *HIS4* recombination hotspot required the binding of three transcription factors, Rap1p, Bas1p, and Bas2p (White *et al.* 1991, 1993). Replacement of the wild-type upstream binding sites with duplicated or triplicated Rap1p-binding sites also resulted in hotspot activity (White *et al.* 1993), suggesting that Rap1p, in the absence of the other transcription factors, could stimulate meiotic recombination. Because (as described in the Introduction) Rap1p has a wide variety of cellular roles unrelated to its function as a transcription factor, we performed deletion analysis and examined fusions of Gal4p and Rap1p to clarify which portions of the protein were required for hotspot activity. This analysis indicates that maximal recombination activity requires both the DNA binding and the transcription activation domains.

Previous studies demonstrate that meiotic hotspot activity at *HIS4* is not directly related to the rate of transcription (White *et al.* 1992). Two alternative models to explain the recombination-stimulating effect of transcription factors are the following: (1) chromatin is remodeled by the transcription factor to allow accessibility of DNA sequences to the recombination machinery [which presumably includes Spo11p (Keeney *et al.* 1997) as well as other proteins], and (2) the activation domain of the bound transcription factor directly contacts the recombination machinery to recruit it to the DNA (as shown in Figure 6a). The second model is similar to the mechanism by which transcription factors are thought to stimulate gene expression (Ptashne and Gann 1997). Although neither of these models can be ruled out by existing data, we favor the second, because the degree of nuclease sensitivity of chromatin (presumably reflecting DNA accessibility) at hotspots does not

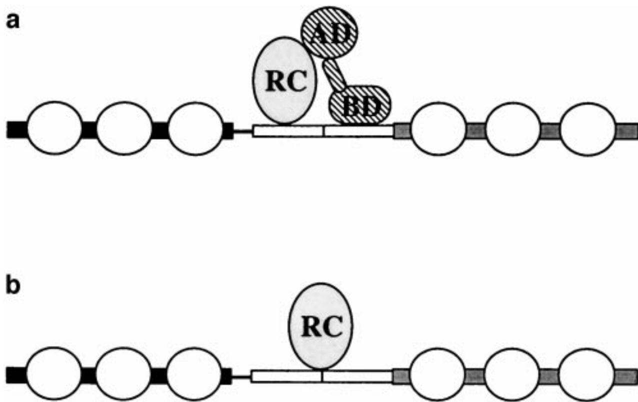

Figure 6.—α- and β-meiotic-recombination hotspots. (a) α-hotspot. A transcription factor (indicated by cross-hatching) with an activation domain (AD) and DNA-binding domain (BD) binds to DNA and interacts with the recombination complex. This interaction targets (and/or tethers) the complex to the DNA, allowing DSB formation. In this diagram, the large white circles indicate nucleosomes bound to coding sequences, and the white rectangles indicate binding sites for the transcription factor. (b) β-hotspot. A region that is devoid of nucleosomes and is not bound by transcription factors is directly accessible to the recombination complex, allowing local DSB formation.

correlate well with the degree of hotspot activity (Wu and Lichten 1995; Fan and Petes 1996). If this model is correct, one interesting issue is whether the recombination and transcription machinery compete for interactions with the transcription factors.

A number of variants of the second model are possible. The activation domain could contact the recombination machinery directly or indirectly through connector proteins. Once the connection is made, the transcription factor could tether the recombination complex to the DNA until the initiating DSB is made or the connection could simply facilitate the entry of the recombination machinery onto the chromosome by a transient association. Because DSBs occur in a local region of ~50–100 bp near the hotspot, rather than at a single position (De Massy *et al.* 1995; Liu *et al.* 1995; Xu and Kleckner 1995; Xu and Petes 1996), a transient association of the transcription factor and the recombination machinery may be more likely. The DSB sites associated with insertion of the triple Rap1p-binding sites upstream of *HIS4* occur in a 50-bp region immediately adjacent to, but not within, the Rap1p-binding sites (Xu and Petes 1996).

Different transcription factors appear to have different abilities to stimulate recombination. *HIS4* hotspot activity in strains with duplicated Gal4p-binding sites upstream of *HIS4* was approximately the same with or without Gal4p, even when cells were sporulated in galactose-containing medium (Table 3). Although we did not monitor synthesis of Gal4p in meiosis, Kolodkin *et al.* (1986) showed meiotic expression of Gal4p-dependent genes when strains were sporulated in galactose-containing medium, suggesting that Gal4p is present under these conditions. The results shown in Table 3, therefore, indicate that Gal4p, in contrast to Rap1p, does not effectively stimulate hotspot activity. We previously found that deletion of Gcn4p, which binds upstream of *HIS4*, has no effect on *HIS4* hotspot activity (White *et al.* 1992). In addition, many intergenic regions in yeast do not represent strong meiotic recombination hotspots (Baudat and Nicolas 1997), although most of these regions contain transcription-factor-binding sites.

The results obtained with the Gal4p/Rap1p fusions (Table 4) add several complicating factors. First, in the strain with the plasmid pDTK116-BC (fusion protein with the Gal4p DNA-binding domain and the C terminus of Rap1p lacking the activation domain), we found a significant reduction of recombination compared to the level of exchange found in the absence of Gal4p. Second, the strain with the plasmid pDTK122-B (Gal4p-binding domain alone) had slightly elevated levels of aberrant segregation for *his4-lopc* without an elevated level of crossovers or DSBs. Although we have no simple explanation for these results, some of these complications may represent interactions between transcription-factor-dependent and transcription-factor-independent hotspot activity (as described below).

The finding that transcription factors stimulate meiotic recombination at the *HIS4* locus (as described above and in White *et al.* 1991, 1993) is consistent with the finding that most meiotic recombination hotspots in *S. cerevisae* map between, rather than within, genes (reviewed by Lichten and Goldman 1995). In *S. pombe*, activity of the artificial *ade6-M26* hotspot also requires binding of the transcription factor Aft1/Pcr1 (Kon *et al.* 1997). Although detailed information about meiotic recombination hotspots is limited for higher organisms, a hotspot located within the major histocompatibility complex (MHC) of the mouse contains transcription-factor-binding motifs and has DNase I-hypersensitive sites (Shenkar *et al.* 1991).

In summary, meiotic recombination hotspots induced by the action of transcription factors may be common in eukaryotes. We term these α-recombination hotspots.

Possible transcription-factor-independent stimulation of recombination activity and gene expression at *HIS4*: In addition to naturally occurring meiotic recombination hotspots, foreign DNA sequences introduced into yeast cells will occasionally stimulate meiotic recombination. For example, high levels of meiotic recombination are associated with integrated pBR322 sequences (Stapleton and Petes 1991; Wu and Lichten 1995) and with an insertion of tandem *Bam*HI sites (Xu and Kleckner 1995). We describe above evidence that duplicated Gal4p-binding sites also have hotspot activity even in the absence of Gal4p. One explanation of this result is that the duplicated Gal4p-binding sites represent an α-recombination hotspot for which the tran-

scription factor has not yet been identified. An alternative possibility is that the duplicated binding sites represent a different type of hotspot (β-hotspot) in which hotspot activity reflects DNA that is accessible to the recombination machinery independent of the binding of a transcription factor (Figure 6b); this DNA may be "naked," that is, unbound by any cellular protein. We favor the second explanation, because it seems more consistent with the observation that insertions of foreign DNA of diverse sequence often have hotspot activity.

Two other issues related to the hypothesis of two types of hotspots should be discussed. First, most recombination hotspots in yeast are located in regions of open chromatin (Wu and Lichten 1994). This result is consistent with the expected location of both α- and β-hotspots. Second, we suggest that naturally occurring hotspots in yeast are likely to be α rather than β. Because the chromosome that initiates recombination is the recipient of information during meiotic exchange (reviewed by Petes *et al.* 1991), if a diploid strain is heterozygous for a hotspot, meiotic gene conversion events will preferentially result in loss of the hotspot. Because α-hotspots are transcription-factor-binding sites, it is likely that other selective forces will balance the loss caused by meiotic recombination. Any β-hotspot that is not associated with a cellular function, however, would be expected to be eventually lost as a consequence of recombination.

There are two modes of stimulating transcription that may be related to our proposal of two types of recombination hotspots. High levels of gene expression in yeast, as in other eukaryotes, usually require the binding of transcription factors to the upstream region (reviewed by Struhl 1995; Ptashne and Gann 1997). Iyer and Struhl (1995), however, showed that poly(A) and poly(G) sequences inserted upstream of the coding sequences stimulate transcription in yeast in the absence of the binding of known transcription factors. They suggested that an intrinsic structure of these simple DNA sequences increased the accessibility of transcription factors to nearby chromosomal regions, stimulating transcription. This explanation is very similar to our explanation of β-hotspots. Our finding that the duplicated Gal4p-binding sites stimulate gene expression of *HIS4* in strains lacking Gal4p (Figure 3) suggests that DNA sequences allowing transcription-factor-independent gene expression may also function as β-hotspots. In addition, we have recently found that tandem arrays of the repeat CCGNN, a nucleosome-excluding sequence, stimulate both meiotic recombination and transcription in yeast (D. T. Kirkpatrick, Y.-H. Wang, M. Dominska, J. D. Griffith, and T. D. Petes, unpublished data).

Minisatellite instability: Mammalian genomes contain regions of DNA (minisatellites) in which short (20 to several hundred bp) repeats are tandemly arranged (Armour and Jeffreys 1992). These regions are often meiotically unstable and the types of alterations that are observed indicate that the instability reflects meiotic recombination (Jeffreys *et al.* 1994). One explanation for the high levels of meiotic instability observed for some minisatellites is that they represent either α- or β-hotspots. For example, the minisatellite located near Ha-Ras that binds the NF-κB transcription factor (Trepicchio and Krontiris 1993) could be an α hotspot. Previously, Wahls (1998) suggested that hyper-variable minisatellites may represent preferred sites for the resolution of Holliday junctions, a late step in recombination. We prefer the hypothesis that the minisatellites are hotspots for the initiation of recombination.

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