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A test of the CoHR motif associated with meiotic double-strand breaks in *Saccharomyces cerevisiae*

Stuart J. Haring[1], Lucas J. Lautner[1], Josep M. Comeron[1,2] & Robert E. Malone[1,2+]

[1]Department of Biological Sciences, and [2]Genetics Program, University of Iowa, Iowa City, Iowa, USA

Meiotic recombination is not random along chromosomes; rather, there are preferred regions for initiation called hotspots. Although the general properties of meiotic hotspots are known, the requirements at the DNA sequence level for the determination of hotspot activity are still unclear. The sequence of six known hotspots in *Saccharomyces cerevisiae* was compared to identify a common homology region (CoHR). They reported that the locations of CoHR sequences correspond to mapped double-strand break (DSB) sites along three chromosomes (*I, III, VI*). We report here that a deletion of CoHR at *HIS2*, a hotspot used to identify the motif, has no significant effect on recombination. In the absence of CoHR, DSB formation occurs at a high frequency and at the same sequences as in wild-type strains. In cases where the deletion of sequences containing the CoHR motif has been shown to reduce recombination, we propose that it may be a reflection of the location of the deletion, rather than the loss of CoHR, *per se*.

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INTRODUCTION

Genetic recombination has long been demonstrated to be an essential feature of meiosis (Baker *et al*, 1976; Lichten, 2001). It is important for the creation of genetic variation and is required for proper segregation of homologous chromosomes during the reductional division in almost all eukaryotes (Baker *et al*, 1976; Lichten, 2001). A feature of meiotic recombination is that it does not occur randomly along chromosomes; rather, it occurs more frequently in preferred regions dubbed hotspots. In *Saccharomyces cerevisiae*, hotspots are associated with high levels of recombination initiation via the formation of double-strand breaks (DSBs). Several meiotic recombination hotspots have been extensively studied in *S. cerevisiae* (e.g., Nicolas *et al*, 1989; White *et al*, 1991; Cherest & Surdin-Kerjan, 1992; Malone *et al*, 1992). These hotspots have many similar properties (e.g., high frequencies of DSBs and gene conversion (GC)); however, one property not shared is an easily identifiable DNA sequence required for DSB formation.

In an attempt to define a DNA motif important for recombination initiation, Blumental-Perry *et al* (2000) examined DNA sequences within 1 kilobase pair (kbp) of six well-characterized recombination hotspots in *S. cerevisiae*. A moderately degenerate motif, called CoHR (common homology region), was identified by sequence alignment (Fig 1A). Comparative sequence analysis was subsequently used to identify CoHR sites on chromosomes *I, III* and *VI*. The authors reported a correlation between the locations of CoHR sequences (Blumental-Perry *et al*, 2000) and mapped sites of DSB formation (Game, 1992; Zenvirth *et al*, 1992; Klein *et al*, 1996; Baudat & Nicolas, 1997). Blumental-Perry *et al* (2000) proposed that the CoHR sequence might provide the basis for understanding meiosis-induced chromatin changes that enable DSBs to occur at defined chromosomal sites. To substantiate the importance of the CoHR sequence, the authors examined published data from the *ARG4* hotspot (de Massy & Nicolas, 1993). Most deletions at *ARG4* that encompass the CoHR sequence also reduce conversion and DSBs.

One of the hotspots used to identify the CoHR motif was *HIS2*, which contains two DSB sites: DSB-C is located in the coding region (at +680 relative to the start of the coding region) and DSB-B is located about 200 bp downstream (at +1200) (Bullard *et al*, 1996; Fig 1B). Blumental-Perry *et al* (2000) reported that the 5′ region of *HIS2* was used for identification of CoHR; however, the CoHR motif is actually located at the 3′ end of *HIS2*, 10 bp downstream from the end of the coding region (+1014 to +1063). Unlike the CoHR motif at some other hotspots, the CoHR motif at *HIS2* is located neither at the 5′ end of the gene nor at the sites of DSB formation.

Studies of the *HIS2* hotspot (Haring *et al*, 2003) and the *ARG4* hotspot (Nicolas *et al*, 1989; de Massy & Nicolas, 1993) have demonstrated that sequences at the DSB sites can profoundly affect the frequency of DSB formation. Since the CoHR site at *ARG4* is within the region of DSB formation, it is not surprising that a deletion encompassing this CoHR motif has an effect on hotspot activity; the DSB site is altered as well. It is difficult to determine the contribution of CoHR to hotspot activity by studying hotspots like *ARG4* (Liu *et al*, 1995), *ARE1* (YCR048W) (Liu *et al*, 1995) or *CYS3* (de Massy *et al*, 1995), because CoHR sites lie

[1]Department of Biological Sciences, and [2]Genetics Program, University of Iowa, 204 Biology Building East, Iowa City, Iowa 52242, USA
[+]Corresponding author. Tel: +1 319 335 1112; Fax: +1 319 335 1069;
E-mail: robert-malone@uiowa.edu

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A

Locus	Sequence	Matches	Quality score
CoHR consensus	**TAGRTKGAAC ARRATRAWCR AAAAAAAAAA CTGTKKCKHS RCMABHDAWK**		
ARG4	**AAGA**AC**GAAC AAA**·**TAATCG AAAAAAAAAA** ACTG**TTCTAG GCCAGATATG**	42/50	13.54
CYS3	**TTTT**CCTCGA G**GATTTTTAT** GG**AAAAAAAA GTGTGGCGCC ACAACTGGCT**	30/50	11.84
HIS4	**TTGGTTGAAC AATTGAATGT ACCAAAGGAG CGTGTTGTTG** TGG**AAGAGAA**	28/50	12.11
ARE1	G**AGATGGTGT AGAGTGAAAA AAAAAAAAAA ATCTGGCTTG GCCATCAAAT**	42/50	13.30
HIS2	**TA**TC**TTGAAC** T**GGATGTACA** T**AAAAAAAAA CTGT**CATA**TA AACTCATG**TA	37/50	12.01
HIS2-ΔCoHR	CTTT**TGG**GCC **AATATATA**AT CTGA<u>TC</u>**A**GCT **GTGTAG**AAGT **GAATGATCTT**	23/50	

B

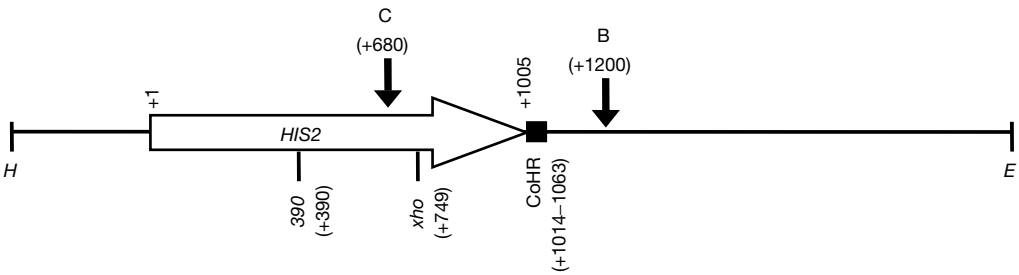

Fig 1 | CoHR sequences and features at *HIS2*. (**A**) Comparison of the CoHR sequences of five known meiotic recombination hotspots (modified from Fig 1 of Blumental-Perry *et al*, 2000) and the sequence created by deleting CoHR at *HIS2*. Sequences and base designations are as in Fig 1 from Blumental-Perry *et al* (2000), except that the poly(A) region has been corrected from 12 to 10 bp. Matches (in bold) represent base pairs matching the CoHR consensus. The quality score has been taken from Table 1 of Blumental-Perry *et al* (2000). A comparison of the fusion created by Δ*CoHR* with the CoHR consensus is shown. Underlined bases represent the fusion junction created by Δ*CoHR*. (**B**) Important features at *HIS2*. The 2.6 kbp *Hind*III–*Eco*RI fragment containing *HIS2* is shown. The direction of transcription of *HIS2* is designated by the horizontal arrow. DSB sites are shown by vertical arrows, with C and B representing DSB-C and DSB-B, respectively. The location of CoHR is designated by the solid box at the end of the *HIS2* coding region. Alleles used to measure gene conversion are designated by *390* and *xho* (Malone *et al*, 1992, 1994). *E* = *Eco*RI; *H* = *Hind*III.

within sequences at DSB sites. How can one determine the contribution of CoHR to hotspot activity, when removing CoHR alters the DSB site? Since CoHR is separable from the locations of DSB formation at *HIS2*, this hotspot allows us to test the importance of the CoHR motif; CoHR can be altered without altering sequences at the DSB sites.

RESULTS

Comparison of CoHR sites and DSB sites on chromosome *III*

It was reported that the location of CoHR sites correlates with DSB sites on chromosomes *I*, *III* and *VI* (Blumental-Perry *et al*, 2000). The precise correlation of CoHR and DSB sites on chromosomes *I* and *VI* is difficult to determine, because the breaks on these chromosomes were mapped by pulse-field gel electrophoresis (Zenvirth *et al*, 1992; Klein *et al*, 1996) and therefore have lower resolution. However, Baudat & Nicolas (1997) mapped DSB formation on chromosome *III* to an average resolution of ±167 bp. We therefore re-examined the relationship between CoHR and DSB sites on this chromosome. Analysis of all 75 DSB sites (⩾0.1% breaks) on chromosome *III* revealed that 6.7% (5/75) are located at positions indistinguishable from CoHR sites (including the *ARE1* and *HIS4* sites used to define CoHR). We then examined the distance between DSB sites and CoHR sites by asking how far from each CoHR the nearest DSB site is located. If CoHR plays a role in the determination of hotspots, one might expect a DSB site relatively close to each CoHR site. We find that 76% (16/21) of chromosome *III* CoHR sites have a measurable DSB site within 2 kbp. The distribution of distances between CoHR sites and the nearest DSB site is shown in Fig 2A (black bars); the mean distance between a CoHR site and its closest DSB is 4.36 ± 6.79 kbp. However, given that there are 75 DSB sites along the 317 kbp of chromosome *III*, it is not clear whether these data are different from what would be expected if CoHR sites were distributed randomly along chromosome *III*. The calculated distribution of distances from CoHR sites to the nearest DSB site for a random distribution (see Methods) along chromosome *III* is also shown in Fig 2A (grey bars); the mean distance is 4.66 kbp with a 95% confidence interval of 2.03–7.59 kbp. We note that the observed distance distribution does not significantly differ from the random distribution ($\chi^2 = 9.31$, df = 14, $P = 0.81$). Since the observed mean distance falls easily within the 95% confidence interval of the mean for a random distribution, the mean values are also not significantly different. It appears that there is no significant association of CoHR sequences with DSB sites on chromosome *III*.

In the simplest hypothesis, the closer a DSB site is to a CoHR motif, the stronger the DSB site should be. The frequency of breaks for each DSB site within 4.36 kbp (the mean distance observed in Fig 2A) of a CoHR was plotted against the distance from the CoHR. Fig 2B demonstrates that there is no correlation ($r = -0.08$; $P = 0.52$) between the strength of a DSB site and its distance from a CoHR site. Owing to the high level of degeneracy of the putative CoHR sequence, CoHR sites were identified based on a

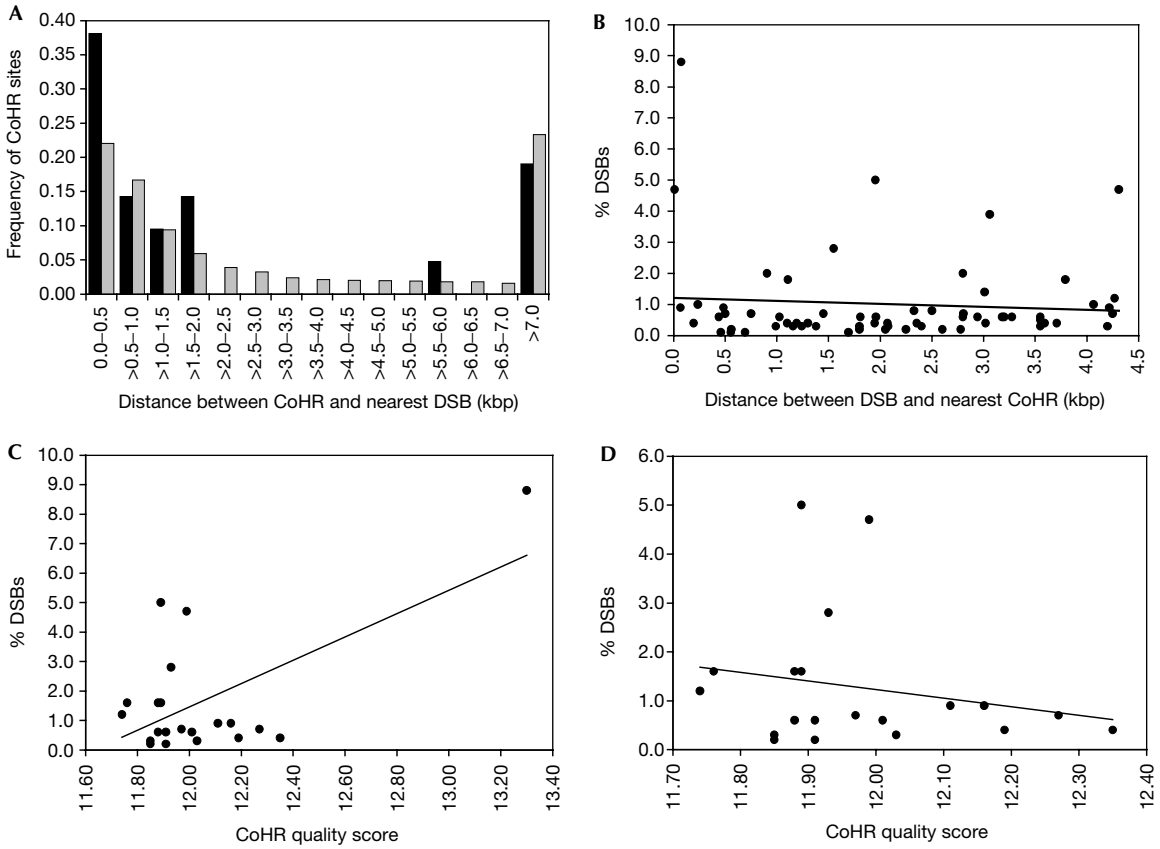

Fig 2 | Relationships between CoHR sites and DSB sites on chromosome *III*. (**A**) Distribution of the distance between CoHR sites and the nearest DSB sites. The actual distribution, determined by combining data from Baudat & Nicolas (1997) and Blumental-Perry *et al* (2000), is represented by black bars. The random distribution determined by 1,000 independent trials (see Methods) is represented by grey bars. (**B**) Plot of DSB strength versus distance to nearest CoHR. All detectable DSBs within 4.36 kbp of a CoHR motif were examined. (**C**) Plot of DSB strength nearest to each CoHR versus CoHR quality score. (**D**) Plot of DSB strength nearest to each CoHR versus CoHR quality score, as in (**C**), excluding the *ARE1* data point.

quality score system; only CoHR sites scoring above 11.7 were considered significant (for an explanation, see Blumental-Perry *et al*, 2000). Blumental-Perry *et al* (2000) calculated the quality scores of CoHR sites present in various constructs at *ARG4* (de Massy & Nicolas, 1993) and argued that they might be responsible for the alterations in the frequency of DSBs observed. There is a significant correlation ($r = 0.61$; $P = 0.0031$) between the CoHR quality score and the strength (i.e., frequency) of the DSB nearest each CoHR (Fig 2C) when all 21 CoHR sites on chromosome III are examined. However, examination of the data (Fig 2C) suggested to us that most of the correlation came from the *ARE1* data point (8.8% DSBs; quality score = 13.30). *ARE1* was, of course, one of the loci used to define CoHR. If we remove the *ARE1* data point (Fig 2D), there is no correlation between the strength of DSB and quality score ($r = -0.21$; $P = 0.38$). If we exclude both the sites on chromosome *III* used to define CoHR (*HIS4* and *ARE1*), there is also no significant correlation ($r = -0.20$; $P = 0.41$).

The CoHR motif at *HIS2* has no effect on recombination

The data from chromosome *III* suggest that CoHR might not be important for the creation of DSBs. To test directly the role of CoHR, we precisely deleted (Δ*CoHR*) the entire 50 bp sequence described in Blumental-Perry *et al* (2000) (Fig 1A). GC was measured in diploids homozygous for Δ*CoHR*. In the absence of the CoHR motif, conversion of the *his2-390* allele is 10.0% (LJL1-1; Table 1). This is not significantly different (G-test; $P = 0.11$) from its isogenic counterpart containing an intact CoHR sequence (RM193; 12.9%). Conversion was also analysed in an isogenic Δ*CoHR* diploid (LJL1-2) containing the *his2-xho* allele. The conversion of this marker was 14.9% compared to 14.1% conversion in its CoHR-containing counterpart (RM169; Table 1); there is no statistical difference (G-test; $P = 0.67$). GC remains very high at *HIS2* in the absence of CoHR.

HIS2 retains a high frequency of DSBs in absence of CoHR

The persistence of high recombination levels at *HIS2* in the absence of CoHR implies a high frequency of DSB formation. The measurement of DSBs at *HIS2* in Δ*CoHR* diploids demonstrated that breaks still occur at high frequency (Table 2). The sites of DSB formation in Δ*CoHR* diploids were indistinguishable from the normal DSB-B and DSB-C sites (Fig 3). Quantitation of the breaks at DSB-B and DSB-C revealed that DSB formation is slightly reduced at both sites (Table 2). DSB-B is reduced from

Table 1 | GC analysis of strains containing and lacking the CoHR motif at *HIS2*

Diploid	Relevant genotype	Total GC	Total tetrads	GC (%)
RM193[a]	*his2-390/HIS2*	80	622	12.9
LJL1-1	*his2-390-ΔCoHR/HIS2-ΔCoHR*	33	329	10.0
RM169[a]	*his2-xho/HIS2*	55	391	14.1
LJL1-2	*his2-xho-ΔCoHR/HIS2-ΔCoHR*	47	315	14.9

[a]Data taken from Malone *et al* (1994) and Haring *et al* (2003).

Table 2 | DSB analysis of strains containing and lacking the CoHR motif at *HIS2*

Diploids[a]	Relevant genotype[b]		Isogenic *RAD50* derivatives	No. of exp.	DSB-B (%)	DSB-C (%)	Total (%)
SJH5-0	*his2/HIS2*	*rad50S/rad50S*	RM193	4	2.8±1.1	1.9±0.9	4.7±2.0
LJL1-5			RM169				
LJL1-3	*his2-ΔCoHR/HIS2-ΔCoHR*	*rad50S/rad50S*	LJL1-1	8	1.7±0.2	0.9±0.3	2.6±0.5
LJL1-4			LJL1-2				

[a]The data from diploids SJH5-0 and LJL1-5 were combined, and the data from LJL1-3 and LJL1-4 were combined. All these strains are isogenic.
[b]The *rad50S* mutation was used, because DSBs form and accumulate in the presence of this allele.

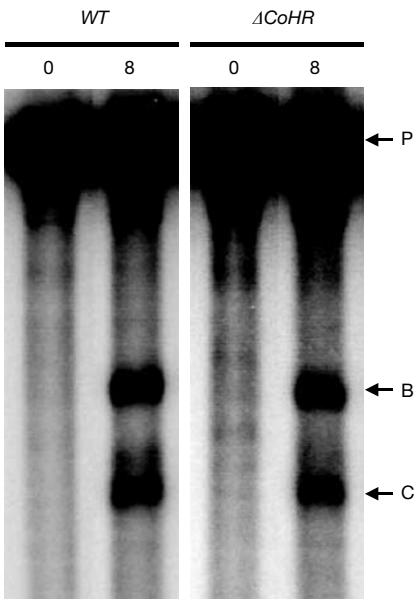

Fig 3 | DSB formation at *HIS2* in the presence and absence of the CoHR site. *WT* refers to a diploid in which CoHR is intact at *HIS2*; Δ*CoHR* refers to a diploid in which CoHR is deleted at *HIS2*. The numbers above each lane represent hours in sporulation. DSB-B, DSB-C and the parental (unbroken) fragments are designated by horizontal arrows labelled B, C and P, respectively.

2.8±1.1 to 1.7±0.2%; DSB-C is reduced from 1.9±0.9 to 0.9±0.3%. However, neither of these differences is significant at the 95% confidence level (Mann–Whitney test; $P=0.21$ for DSB-B; $P=0.07$ for DSB-C).

Medium resolution mapping of HIS2 DSB sites

The CoHR deletion has no significant effect on GC or DSB formation at *HIS2*. To examine whether CoHR affects the precise locations of DSBs, the technique of medium-resolution mapping was employed (Liu *et al*, 1995; Diaz *et al*, 2002; Haring *et al*, 2003). Examination of the break pattern revealed that most (if not all) of the breaks present in diploids with an intact CoHR motif also occur when CoHR is absent. The pattern of breaks at DSB-C demonstrates that the positions of at least 13 break sites are indistinguishable (Fig 4A). At first glance, the pattern of breaks at DSB-B appears to be altered (Fig 4B), because Δ*CoHR* reduces all band sizes by 50 bp. To account for this, the wild-type lane was resized to mimic the electrophoresis of fragments 50 bp smaller. This lane could then be aligned with the lane containing breaks from a Δ*CoHR* diploid; at least 11 breaks occur at the same sequence positions (Fig 4C).

DISCUSSION

The contribution that DNA sequence, *per se*, makes to the initiation of meiotic recombination has been difficult to resolve. Unlike replication origins (Deshpande & Newlon, 1992) or transcriptional promoters (Kollmar & Farnham, 1993), there is no obvious recombination initiation sequence. At least two types of sequences contribute to hotspot activity: sequences at DSB sites and 'surrounding sequences' (e.g., Wu & Lichten, 1995; Haring *et al*, 2003). Sequences at DSB sites affect breaks at that particular site, whereas surrounding sequences are important for hotspot activity within a region (Haring *et al*, 2003).

The extent of surrounding sequences sufficient to maintain hotspot activity has yet to be determined. Wu & Lichten (1995) and Borde *et al* (1999) showed that an 8.5 kbp *URA3*::*ARG4*::pBR322 construct has different levels of recombination and DSB formation, depending on its position on chromosome *III*. Examination of these constructs by Petes &

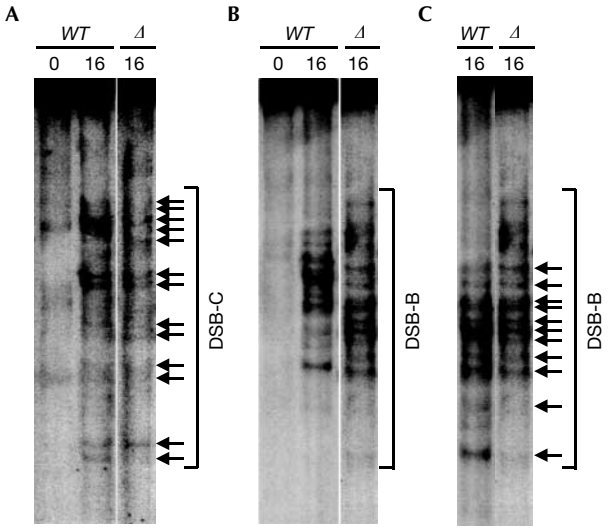

Fig 4 | Medium-resolution mapping of DSBs at *HIS2* in the presence and absence of the CoHR site. DSBs at indistinguishable sequences in wild-type or Δ*CoHR* strains are designated by horizontal arrows. *WT* refers to a wild-type diploid with CoHR intact at *HIS2*; Δ refers to a diploid with CoHR deleted at *HIS2*. Numbers above lanes represent hours in sporulation. (**A**) Mapping of the break pattern at DSB-C. (**B**, **C**) Mapping of the break pattern at DSB-B. The pattern of DSB-B in (**B**) appears altered in a Δ*CoHR* strain, because the DSB fragments also contain the Δ*CoHR* (50 bp) deletion. To account for the 50 bp size difference due to Δ*CoHR*, the *WT* lane from (**B**) was stretched in (**C**) to mimic the running of 50 bp shorter fragments on a gel.

Merker (2002) showed that the recombination activity of inserted sequences can be correlated with the GC content of large surrounding chromosomal regions (30–100 kbp). More than 10 kbp are required to maintain 50% of the recombination activity of a hotspot (Ross *et al*, 1992; Haring *et al*, 2003) when moved to a different location. In all these constructs, DSB formation occurs at the same sites regardless of the location of the construct; only the frequency is affected. Although long-range surrounding sequences are important for hotspot activity in a region, it is less clear as to what local sequences determine the location and frequency of DSBs.

Our analysis of chromosome *III* demonstrates no correlation between the amount of DSB formation and the distance to, or quality score of, the nearest CoHR. Furthermore, the distribution of CoHR sites along chromosome *III* is not significantly different from that expected if CoHR sites were randomly distributed. Taken together, the data for chromosome *III* suggest that CoHR is not important for DSB formation.

The data clearly indicate that the CoHR sequence at *HIS2* contributes little to hotspot activity. Deleting CoHR does not decrease recombination. To argue that CoHR is important, one would have to propose that there is another CoHR site nearby, or that the fusion resulting from Δ*CoHR* creates a new CoHR site allowing hotspot activity at *HIS2* to remain. However, the next closest CoHR site to *HIS2* is 6.5 kbp away (Blumental-Perry *et al*, 2000). In addition, the fusion sequence created by the deletion does not resemble the CoHR motif; only 23/50 bases match, and there is no poly(A) tract (Fig 1A). Not only do DSB breaks form at high frequency in a strain with the CoHR sequence deleted, but the locations of the individual DSBs appear unchanged as well. Since both DSB formation and GC remain high when CoHR is absent, we conclude that CoHR is not required for *HIS2* hotspot activity.

Speculation

Since CoHR appears to be unnecessary for hotspot activity at *HIS2*, we suspect that CoHR may not be required for other hotspots. In those situations (e.g., *ARG4*) where the deletion of CoHR has been shown to affect recombination, we posit that it was due to loss of the DSB sites themselves or important sequences in the promoter region rather than CoHR. We note that CoHR located at *HIS4* (another hotspot used to define the consensus sequence) is not at the site of DSB formation; it is located in the coding region a few hundred bases away. We predict that a deletion of CoHR at *HIS4*, or of other CoHR sites not located at DSB sites, would have a minimal effect on meiotic recombination. Although the data at *HIS2* indicate that CoHR is not important for hotspot activity, studies of CoHR at other hotspots are necessary to confirm the generality of our results.

METHODS

Strains. Isogenic derivatives of haploids RM96-15A and RM182-55C were crossed to create all diploids (Malone *et al*, 1994). Haploids containing the Δ*CoHR* mutation at *HIS2* were created by two-step gene replacement (Rothstein, 1991) using pLJL2. Relevant genotypes of diploids are as follows: RM193 (*his2-390*/*HIS2*), RM169 (*his2-xho*/*HIS2*), LJL1-1 (*his2-390*-Δ*CoHR*/*HIS2*-Δ*CoHR*) and LJL1-2 (*his2-xho*-Δ*CoHR*/*HIS2*-Δ*CoHR*). Diploids used for DSB analysis are isogenic, except homozygous for *rad50KI81*::*URA3* (*rad50S*; Alani *et al*, 1990).

Plasmids. To create the Δ*CoHR* mutation at *HIS2*, plasmid pSJH1 (Haring *et al*, 2003) was digested with *Sma*I and *Not*I. The digested ends were treated with Klenow and ligated to create pLJL1, eliminating the *Bam*HI site in the multiple cloning site of pSJH1. Primers 362 (5′-ATCCCTTTTGGGCCAATATATAATCT GATCAGCTGTGTAGAAGTGAATGATCTTTCC-3′) and 375 (5′-GGAAAGATCATTCACTTCTACACAGCTGATCAGATTATATATT GGCCCAAAAGGGAT-3′) and pLJL1 were used with the Quick-Change Site-Directed Mutagenesis Kit (Stratagene) to create pLJL2, containing a precise 50 bp deletion of the CoHR motif downstream of *HIS2* (confirmed by DNA sequencing). pLJL2 was targeted for integration by *Bam*HI digestion.

Chromosome *III* comparisons. All comparisons of DSB sites and CoHR sites on chromosome *III* were made by combining data from Baudat & Nicolas (1997) (http://www.yeastgenome.org/DSB_table.shtml) and Blumental-Perry *et al* (2000). Data were graphed using Microsoft Excel, and correlation coefficients were determined using Microsoft Excel and GraphPad Instat.

Generating a random distribution between CoHR and DSB sites. Computer simulations were used to investigate the distance between CoHR sites to the nearest DSB site when CoHR sites are randomly distributed along a chromosome. This process mimics a chromosome 316,613 bp long (i.e., chromosome *III*) with 75 DSB sites assigned to their known physical positions (Baudat & Nicolas, 1997). A total of 21 CoHR sites are then randomly distributed along the chromosome, and the average distance between each

CoHR site and its nearest DSB site is recorded. After 1,000 independent replicates of this process, a distribution of the average distance between CoHR and DSB sites is generated and can be used to obtain confidence intervals and the mean distance. The program used for this analysis is available from J.M.C. upon request.

DSB analysis and medium-resolution mapping. DSB analysis and medium-resolution mapping were carried out as in Haring *et al* (2003). We estimate the precision of medium-resolution mapping to be ± 8 bp.

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