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ORIGINAL PAPER

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SCH9, a putative protein kinase from *Saccharomyces cerevisiae*, affects *HOT1*-stimulated recombination

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Abstract *HOT1* is a mitotic recombination hotspot derived from yeast rDNA. To further study *HOT1* function, *trans*-acting H *OT1* recombination mutants (*hrm*) that alter hotspot activity were isolated. *hrm2-1* mutants have decreased *HOT1* activity and grow slowly. The *HRM2* gene was cloned and found to be identical to *SCH9*, a gene that affects a growth-control mechanism that is partially redundant with the cAMP-dependent protein kinase A (PKA) pathway. Deletion of *SCH9* decreases *HOT1* and rDNA recombination but not other mitotic exchange. Although high levels of RNA polymerase I transcription initiated at *HOT1* are required for its recombination-stimulating activity, *sch9* mutations do not affect transcription initiated within *HOT1*. Thus, transcription is necessary but not sufficient for *HOT1* activity. *TPK1*, which encodes a catalytic subunit of PKA, is a multicopy suppressor of the recombination and growth defects of *sch9* mutants, suggesting that increased PKA activity compensates for *SCH9* loss. *RAS2* ^{val19}, which codes for a hyperactive *RAS* protein and increases PKA activity, suppresses both phenotypic defects of *sch9* mutants. In contrast to *TPK1* and *RAS2* ^{val19}, the gene for split zinc finger protein 1 (*SFP1*) on a multicopy vector suppresses only the growth defects of *sch9* mutants, indicating that growth and *HOT1* functions of Sch9p are separable. Sch9p may affect signal transduction pathways which regulate proteins that are specifically required for *HOT1*-stimulated exchange.

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Introduction

Homologous recombination does not occur with equal likelihood in all portions of the genome. Instead, there are localized regions in which the frequency of exchange is relatively high, termed hotspots, or relatively low, termed coldspots. Exchange at hotspots appears to constitute a significant fraction of the recombination that occurs in the genome as a whole. Hotspots (for reviews see Lichten and Goldman 1995; Paques and Haber 1999; Aguilera et al. 2000; Petes 2001) are thought to enhance the rate-limiting step in genetic exchange, which is generally considered to be initiation. Studies of recombination hotspots have provided insights into the complex processes involved in the initiation of genetic exchange.

HOT1 is a mitotic recombination hotspot derived from the non-transcribed spacer (NTS) regions of the rDNA repeat unit (Keil and Roeder 1984). When inserted at novel locations in the yeast genome, in *HOT1* stimulates both inter- and intra-chromosomal recombination at adjacent sequences. It has been proposed that this hotspot may play a role in the maintenance of sequence homogeneity among the tandem rDNA repeat units (Keil and Roeder 1984). However, the mechanism(s) by which *HOT1* stimulate(s) mitotic exchange remain(s) unknown.

HOT1 sequences correspond closely to the enhancer and initiation sites for transcription of rDNA by RNA polymerase I (Voelkel-Meiman et al. 1987; Huang and Keil 1995). Evidence from previous work indicates that high levels of transcription initiated within *HOT1* by RNA polymerase I are necessary for the recombination-stimulating activity of this hotspot (Voelkel-Meiman et al. 1987; Stewart and Roeder

1989; Huang and Keil 1995). In large part, *cis*-acting mutations within the *HOT1* element that decrease the recombination activity also decrease transcription (Stewart and Roeder 1989; Huang and Keil 1995), suggesting a close association between recombination and transcription.

Several other reports have also suggested that high levels of transcription stimulate recombination in yeast. Thomas and Rothstein (1989) tested the recombination frequency of a *gal10-URA3-gal10* duplication in a strain lacking either *GAL4*, a positive regulator of galactose-induced gene expression, or *GAL80*, a repressor of Gal4p-induced transcription. They reported a fifteen-fold increase in the rate of recombination in *gal80*Δ strains in which transcription is constitutive. Using various promoter mutants they showed that sequences within the integrated plasmids were responsible for the altered recombination. When the weak *ADE6* promoter in *Schizosaccharomyces pombe* is replaced with the strong *ADH1* promoter, recombination in *ADE6* is increased seven-fold in mitosis and 25-fold during meiosis (Grimm et al. 1991).

To understand the mechanism of action of *HOT1* we isolated *trans*-acting H*OT1* recombination mutants (*hrm*) that alter *HOT1*-stimulated exchange (Lin and Keil 1991). The *hrm2-1* mutation decreases *HOT1* activity and mitotic recombination in the native rDNA array, but does not affect mitotic exchange in other portions of the genome. This mutation also affects cell growth, as evidenced by the small size and the pale pink color of *ade2* colonies, compared to the large, red colonies formed by the Hrm^+ control strains. The *HRM2* gene was cloned based on its ability to complement both the growth and *HOT1*-recombination defects of *hrm2-1* strains. *HRM2* is identical to *SCH9*, a gene that encodes a putative kinase involved in a nutrient-sensing signaling pathway (Toda et al. 1988; Crauwels et al. 1997). Mutation of *SCH9* increases stress resistance and longevity of non-dividing yeast cells (Fabrizio et al. 2001), but decreases the life span of replicating cells (Defossez et al. 1999). *SCH9* also affects critical cell size at Start in the cell cycle (Jorgensen et al. 2002).

Materials and methods

Strains and media

Yeast strains used in these studies (Table 1) were derived from K2307 or K2302 (Lin and Keil 1991) by transformation or genetic crosses. Null mutant strains were generated by PCR-mediated gene disruption using the *loxP-kanMX-loxP* cassette (Guldener et al. 1996) to precisely replace the entire ORF with a gene that confers resistance to G418 ($G418^R$). Appropriate gene disruptions by this cassette were confirmed by Southern analysis (Sambrook et al. 1989) or PCR. The *Escherichia coli* strain MC1066 [*leuB trpC pyrF::*Tn*5* (Kan^R) *araT lacX74 del strA hsdR hsdM*; obtained from M. Casadaban] was used for propagating plasmids.

Growth media containing G418 for yeast was prepared by adding 200 mg of G418 sulfate (Geneticin, Life Technologies) per liter of YPD. Other growth media for yeast (Lin and Keil 1991) and bacteria (Sambrook et al. 1989) were prepared as described previously.

Table 1 Yeast strains used

Strain	Genotype	Source
K2307	*MAT* **a***his4-260::URA3::his4*-Δ::HOT1 leu2::ADE5,7::leu2-3,112::HOT1 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1	Lin and Keil (1991)
K2302	*MAT* **a***his4-260::URA3::his4*-Δ leu2::ADE5,7::leu2-3,112 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1	Lin and Keil (1991)
K4691	*sch9*-Δ1 [a] in K2307	This study
K4693	*sch9*-Δ1in K2302	This study
K5651	*gpa2*-Δ1 [a] in K2302	This study
K5656	*gpa2*-Δ1in K2307	This study
HRM138	*hrm2-1*in K2307	Lin and Keil (1991)
HRM142	*MAT*α *hrm2-1 his-260::URA3::his4*-Δ::HOT1 leu2::ADE5,7::leu2-3,112::HOT1 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1	This study
HRM201	*MAT***a** leu2::ADE5,7::leu2-3,112::HOT1 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1	This study
HRM221	*hrm2-1*in HRM201	This study
HRM759	*MAT*α *sch9*-Δ1 leu2::ADE5,7::leu2-3,112::HOT1 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1	This study
HRM764	*MAT*α *sch9*-Δ1 his4-260::URA3::his4-Δ::HOT1 leu2::ADE5,7::leu2-3,112::HOT1 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1	This study
RLK98-2A	*MAT*α*his4-260 leu2-3,112 ade2-1 trp1-HIII ura3-52 lys2*-ΔBX::CAN1::LYS2 can1 rDNA*:: ADE2* rDNA*:: URA3*	Keil and McWilliams (1993)
RLK183-2B	*sch9*-Δ1in RLK98-2A	This study

[a] *sch9*-Δ1 and *gpa2*-Δ1 are precise deletions of *SCH9* and *GPA2* respectively, and contain the *loxP-kanMX-loxP* module (Guldener et al. 1996)

DNA and RNA manipulations

Restriction and modification enzymes were purchased from various manufacturers and used according to their recommendations. Standard protocols were used for plasmid (Sambrook et al. 1989) and yeast (Rose et al. 1990) DNA isolation. Primer extension analysis was performed on 50-μg samples of total cellular RNA from yeast as previously described (Huang and Keil 1995). Levels of transcripts were determined using a Molecular Dynamics PhosphorImager 425E in the Macromolecular Core Facility of the M. S. Hershey Medical Center. Normalized percentage transcriptional activity was determined as described previously (Huang and Keil 1995).

HRM221, an *hrm2-1* mutant strain containing the *HOT1::leu2::ADE5,7::leu2* construct required for the colony-color sectoring assay (described below), was used to clone the wild-type *HRM2* gene from a yeast-genomic library constructed in YCp50 (Rose et al. 1987). To localize the *HRM2* gene on the genomic insert in this plasmid (pL3033), $\gamma\delta$ transposon mutagenesis (Guyer 1983) was performed. Transposon insertions in this genomic insert were isolated and tested for their ability to complement the growth and recombination phenotypes of *hrm2-1* mutants.

A multicopy plasmid containing only *TPK1* was constructed by inserting the 1.9-kb *Bst*YI fragment containing *TPK1* into the compatible *Bam*HI site of YEplac112 (Gietz and Sugino 1988). The multicopy *TPK2* plasmid was constructed by inserting the 1.9-kb *Bgl* II fragment of pAPK8-1 (provided by K. Tatchell) that contains *TPK2* into the *Bam* HI site of YEplac112 (Gietz and Sugino 1988). The *RAS2* ^{val19} plasmid was kindly provided by J. Broach.

Fluctuation tests, statistical analysis, and genetic analysis

At least three cultures from three independent transformants were used in fluctuation tests to determine recombination rates (Lin and Keil 1991). Calculation of recombination rates and statistical analyses were performed as described previously (Yuan and Keil 1990). Cultures used to assay *HOT1*-stimulated recombination were grown in SC, while cultures employed for assessment of rDNA exchange were grown in SC-ura since the frequency of loss of the *URA3* marker from the rDNA is relatively high. Tetrad analysis was conducted by standard procedures (Rose et al. 1990).

Multicopy suppressors

A multicopy yeast genomic DNA library (Carlson and Botstein 1982) was transformed into HRM221, which contains the *hrm2-1* mutation as well as the *HOT1*-specific colony-color sectoring recombination substrate. Suppressors of the slow-growth and pale-pink color phenotypes of this strain were identified. Plasmids containing genes that suppressed these phenotypes were recovered into *E. coli* (Hoffman and Winston 1987). Recovered plasmids were retransformed into the original HRM221 strain to assess the plasmid dependence of the suppression. Genomic DNA contained within plasmids of interest was identified by sequencing insert DNA at the vector-insert border. The gene within the insert that is responsible for suppression was identified by insertion of linkers at unique restriction sites in the fragment, or by deletion of portions of the insert using convenient restriction sites. Finally, subcloning of the gene of interest confirmed its identity.

Results

HRM2 is identical to *SCH9*

The *hrm2-1* allele was identified in a hunt for mutants that specifically alter *HOT1* activity (Lin and Keil 1991). In addition to decreased levels of *HOT1*-stimulated exchange (Fig. 1; compare wt/YCp to *hrm2-1*/YCp), *hrm2-1* mutants show a lower level of rDNA recombination (Lin and Keil 1991; Table 2) and a growth defect producing small, light pink colonies as compared to the large, red colonies produced by *HRM2 ade2* cells (Table 3). The wild-type *HRM2* gene was cloned by virtue of its ability to complement the *HOT1* hyporecombination phenotype and the growth defects of the *hrm2-1* mutant, HRM221. To assay *HOT1* activity, the

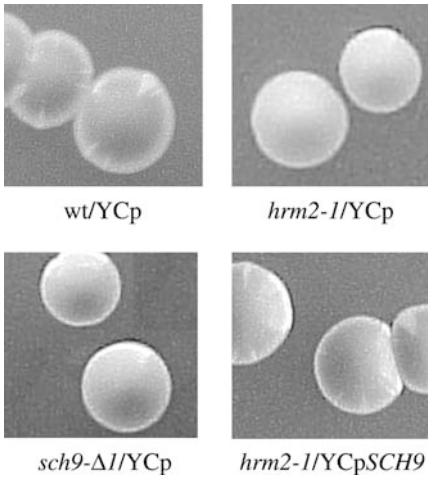

Fig. 1 *hrm2-1* affects *HOT1*-stimulated recombination. When present in an *HRM2* (*SCH9*) *ade2 ade5* strain, the *HOT1::leu2::ADE5,7::leu2* duplication produces red colonies with white sectors that result from excision of the *ADE5,7* gene. The level of *HOT1* activity can be visually assessed by the number of white sectors present in a colony. The *hrm2-1* mutation reduces *HOT1*-stimulated exchange, and few if any white sectors are observed in *hrm2-1* colonies. The *SCH9* gene on a centromeric plasmid (YCp) corrects the recombination defect of *hrm2-1* mutants. Deletion of *SCH9* reduces *HOT1*-stimulated recombination

Table 2 Effects of *hrm2-1* and *sch9*-Δ1on mitotic recombination

Genotype	Recombination rate					
	HOT1		rDNA	Non-*HOT1*		
	Ura$^-$ (×10^5)	His$^+$ Ura$^+$ (×10^6)	Ura$^-$ (×10^5)	Ura$^-$ (×10^6)	His$^+$ Ura$^+$ (×10^6)	Can^R (×10^5)
SCH9	33.1	8.4	8.7	6.8	2.2	4.7
hrm2-1	4.4**	2.4*	2.2*	nd	nd	2.4
sch9-Δ1	7.1**	2.2*	2.5*	5.9	2.7	5.3

* $P < 0.05$, ** $P < 0.01$ as compared to *SCH9*; recombination rates are not significantly different between *hrm2-1* and *sch9*-Δ1 strains. nd, not determined

qualitative colony-color sectoring assay involving recombination of a *HOT1::leu2::ADE5,7::leu2* duplication was used (Lin and Keil 1991). Excision of the *ADE5,7* gene produces white *ade2 ade5* sectors in an otherwise red *ade2* colony. While the Hrm$^+$ strain produces colonies with approximately five to fifteen sectors, *hrm2-1* strains produce colonies that usually have less than three white sectors (Fig. 1; compare wt/YCp to *hrm2-1*/YCp). From approximately 75,000 transformants obtained from a centromeric yeast library, six wild-type sized colonies that had ten or more white sectors were identified (Fig. 1, see *hrm2-1*/YCp *SCH9*). In addition, nineteen transformants generated normal sized colonies but did not show Hrm$^+$ levels of sectoring. The six strains in which both phenotypes reverted were plated on media containing 5FOA to induce loss of the library plasmid. Two of the six strains exhibited decreased levels of *HOT1*-recombination and grew poorly upon plasmid loss, indicating that genomic DNA present on these plasmids complemented the *hrm2-1* mutant phenotypes. Plasmids were rescued from these transformants and retransformed into the original HRM221 (*hrm2-1*) strain. Both plasmids restored normal growth (Table 3) and wild-type levels of *HOT1*-specific recombination. Restriction mapping of these two plasmids indicated that the inserts they contained were identical (data not shown).

Transposon mutagenesis (Guyer 1983) of one of these plasmids, pL3033, was used to localize the complementing gene. Three independent plasmids containing transposon insertions that destroyed the ability of pL3033 to complement the recombination and growth defects of *hrm2-1* mutants were isolated. Sequencing from the ends of these three transposons showed that in each case the transposon had inserted within the ORF of the previously characterized *SCH9* gene (Toda et al. 1988).

Precise deletions of the *SCH9* ORF (*sch9*-Δ1) in strains K2307 and K2302 were constructed by PCR-mediated gene disruption (Guldener et al. 1996). *sch9*-Δ1 (G418^R) strains exhibit a *HOT1* -specific hyporecombination phenotype (Fig. 1, see *sch9*-Δ1/YCp) and the same growth defects as the *hrm2-1* mutant (Table 3). One of the *sch9*-Δ1 transformants, K4691, was mated with the *hrm2-1* strain HRM142. Random spore analysis showed that approximately 50% of the spores (25 spores out of 52) were G418^R and half (27 spores) were G418^S. All spores examined had decreased levels of *HOT1*-stimulated recombination similar to *hrm2-1* control strains. Colonies obtained from all 52 spores were also small and exhibited the pale pink color of the *hrm2-1* mutant. These results show that the G418^R marker is less than 2 cM from the *HRM2* locus and indicate that *SCH9* is identical to *HRM2*. To confirm this, the sequences of *SCH9* from our wild-type and *hrm2-1* strains were determined. The sequence of *SCH9* in our wild-type strain is identical to that reported in the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). The sequence of this gene in the *hrm2-1* strain contains two closely linked mutations: an A inserted in a run of 4As extending from bases 1844–1847 of the wild-type *SCH9* reading frame and an A to T transversion at base 1849 (the sequence of this gene in *hrm2-1* beginning at base 1844 of the *SCH9* reading frame is AAAAATTATC, where the inserted A is

Table 3 Colony morphology and generation time

Genotype	Colony morphology[a]	Generation time (min) [b,c]
Wild type and mutants		
HRM2 (*SCH9*)	Large red colonies; many white sectors	98
hrm2-1	Small pink colonies; few white sectors	217**
sch9-Δ1	Small pink colonies; few white sectors	239**
Cloning *SCH9*		
SCH9/YCp	Large red colonies; many white sectors	108
SCH9/YCp *SCH9*	Large red colonies; many white sectors	101
hrm2-1/YCp	Small pink colonies; few white sectors	188**
hrm2-1/YCp *SCH9*	Large red colonies; many white sectors	98
YEp/*TPK1*; YEp/*SFP1*		
SCH9/YEp	Large red colonies; many white sectors	103
SCH9/YEp *TPK1*	Large red colonies; many white sectors	108
SCH9/YEp *SFP1*	Large red colonies; many white sectors	128**
hrm2-1/YEp	Small pink colonies; few white sectors	191
hrm2-1/YEp *TPK1*	Medium red colonies; many white sectors	150**[d]
hrm2-1/YEp *SFP1*	Medium red colonies; many white sectors	172**[d]

[a]Of strains containing the *leu2::ADE5,7::leu2-3,112::HOT1* duplication
[b]In synthetic medium lacking appropriate nutrients to maintain plasmid selection when necessary
[c]All standard deviations were less than 10% of the generation time
** $P < 0.01$ as compared to the appropriate control (*SCH9*,-*SCH9* /YCp or *SCH9* /YEp)
[d] $P < 0.01$ as compared to *hrm2-1*/YEp

underlined and the T resulting from the transversion is indicated in bold). Following the inserted A, 12 novel amino acids are encoded prior to the occurrence of a premature termination codon leading to the loss of approximately 200 amino acids normally present at the C-terminus of the wild-type protein.

sch9-Δ1 decreases *HOT1*-stimulated recombination

Effects of *sch9*-Δ1 on *HOT1*-stimulated recombination were quantitated using a *HOT1::his4*-Δ::URA3::his4-260 duplication (Fig. 2A, and Lin and Keil 1991). This contains a *URA3* gene and pBR322 sequences flanked by mutant *his4* genes. The frequency of Ura^- excision or Ura^+His^+ gene replacement recombinants (Fig. 2A) provides a measure of *HOT1* activity. The frequency of both excision and gene replacement decreased approximately four- to five-fold in *sch9*-Δ1 strains as compared to the wild-type strain (Table 2), indicating that loss of Sch9p function decreases both types of *HOT1*-stimulated recombination events. Recombination rates for *sch9*-Δ1 mutants are not statistically different from those observed for *hrm2-1* strains (Table 2). Homologous mitotic recombination stimulated by *HOT1* has been proposed to be involved in maintaining homogeneity among rDNA repeats (Keil and Roeder 1984). The effect of *sch9*-Δ1 on mitotic intrachromosomal rDNA exchange was assayed by determining the rate of loss of a *URA3* gene inserted in the rDNA array of haploid strains (Fig. 2C, and Lin and Keil 1991). In *sch9*-Δ1 strains the rate of rDNA recombination decreased approximately four-fold, similar to the effect observed in *hrm2-1* mutants (Table 2, and Lin and Keil 1991). These results suggest that *SCH9* affects rDNA exchange, possibly due to altered *HOT1* activity in the native rDNA locus. This provides reassurance that *HOT1* activity measured at the ectopic *HIS4* locus is indicative of its normal function in rDNA.

To determine whether loss of *SCH9* is recessive with respect to its effect on *HOT1* recombination, diploid strains heterozygous (*sch9*-Δ1/*SCH9*) and homozygous (*sch9*-Δ1/*sch9*-Δ1) for the *sch9*-Δ1 mutation were generated. Both strains were heterozygous for the *HOT1::his4*-Δ::URA3::his4-260 duplication. Rates of Ura^- excision and His^+Ura^+ gene replacement in *SCH9*/*sch9*-Δ1 diploids were similar to those in *SCH9*/*SCH9* diploids, while those of the *sch9*-Δ1 / *sch9*-Δ1 diploids were five-fold lower (data not shown). Rates of non-*HOT1* recombination remained unaltered in all diploids. This indicates that, like the *hrm2-1* mutation (Lin and Keil 1991), deletion of *SCH9* is recessive with respect to its effect on *HOT1* activity.

We used two different recombination substrates to determine whether *sch9*-Δ1 also alters non- *HOT1* recombination levels. First, a *his4*-Δ::URA3::his4-260 duplication lacking *HOT1* was used. In this instance the effect of *sch9*-Δ1 was monitored with the same recombination substrate used to measure *HOT1* activity. Second, a *lys2::CAN1::LYS2* recombination substrate lacking *HOT1* (Fig. 2B) was assayed. The *sch9*-Δ1 mutation did not affect mitotic recombination in either of these substrates (Table 2), indicating that *sch9* mutations affect *HOT1*-stimulated exchange but not non-*HOT1* recombination. Taken together, these findings show that loss of *SCH9* specifically affects rDNA and *HOT1*-stimulated recombination. Furthermore, since the effects are similar to those observed in *hrm2-1* strains, it appears the mutations in *hrm2-1* lead to loss of function.

To determine if overexpression of *SCH9* affects *HOT1* activity, *hrm2-1* or *sch9*-Δ1 strains were transformed with single-copy (YCp) or multicopy (YEp) vectors containing *SCH9*. The levels of *HOT1*-stimulated recombination in these transformants were not statistically different from each other (data not shown).

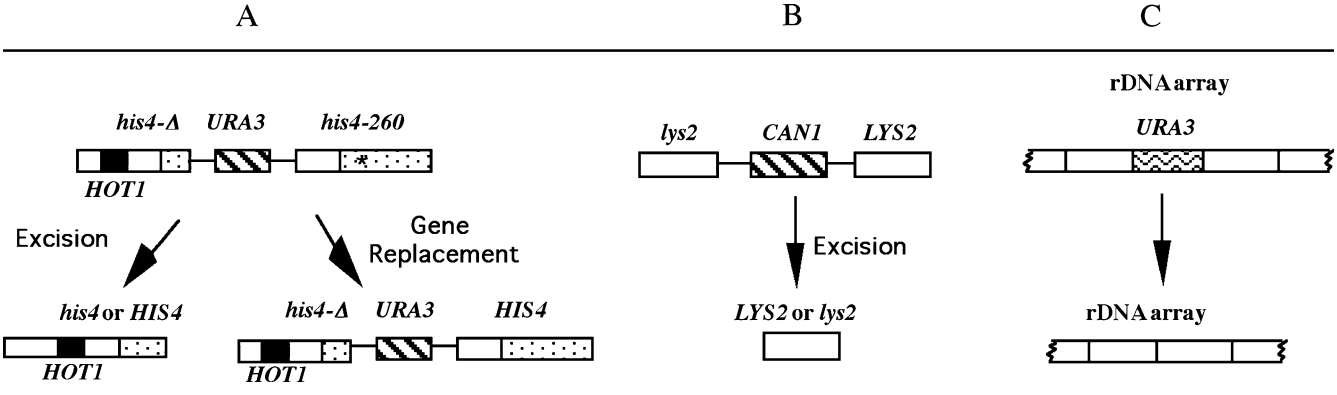

Fig. 2A–C Recombination substrates. **A** The *HOT1::his4*-Δ::URA3::his4-260 duplication contains two mutant *HIS4* genes, *his4*-Δ and *his4-260* (indicated by *), flanking a *URA3* marker and pBR322 sequences. *HOT1* is present 5′ of the *his4*-Δ gene. Excision events result in the loss of *URA3,* producing Ura^- ($5FOA^R$) cells. Gene replacement events involve repair of the *his4-260* point mutation using information from the *his4*-Δ gene. This occurs without loss of *URA3*, producing His^+ Ura^+ cells. **B** The *lys2::CAN1::LYS2* duplication contains the *CAN1* gene flanked by the *lys2*-ΔBX and *LYS2* genes. The rate of Can^R recombinants assays non-*HOT1* exchange. **C** In this recombination substrate the *URA3* gene is inserted in the rDNA array. Loss of this marker assays rDNA exchange and produces Ura^- ($5FOA^R$) cells

In addition, the growth and colony color of the YEp *SCH9* transformants were indistinguishable from those of the YCp *SCH9* transformants.

SCH9 does not affect transcription initiated at *HOT1*

Several lines of evidence from previous work indicate that the recombination-stimulating activity of *HOT1* requires high levels of transcription initiated within this element: (1) deletion of the largest subunit of RNA polymerase I abolishes *HOT1* activity (Huang and Keil 1995); (2) premature termination of transcription initiated at *HOT1* abolishes recombination activity (Voelkel-Meiman et al. 1987); (3) the transcription initiation site must be oriented such that transcription proceeds through the recombining genes (Voelkel-Meiman et al. 1987), and (4) most *cis*-acting mutations that decrease *HOT1* activity also decrease transcription (Stewart and Roeder 1989; Huang and Keil 1995). We assayed for effects of the *sch9*-Δ1 mutation on transcription initiated within *HOT1* sequences at *his4*, and on transcription of rDNA. Levels of transcription initiated within *HOT1* sequences at *his4* were quantitated using primer extension analysis (Stewart and Roeder 1989; Huang and Keil 1995). The steady-state level of transcripts from *HOT1* in an *sch9*-Δ1 strain is not decreased compared to the level observed in the wild-type strain [Fig. 3A (lanes marked *his4*) and B (dark bars)]. The finding that *HOT1* transcription is not reduced in *sch9*-Δ1 strains indicates that altered transcription is not the cause of the reduced *HOT1* activity in these mutants. The simplest interpretation is that the *HOT1* hyporecombination phenotype of *sch9*-Δ1 strains is independent of transcription initiated within this element. These results do not rule out the possibility that *sch9* mutants have subtle effects, not detected by this assay, on *HOT1*-dependent transcription at specific stages of the cell cycle. Such changes occurring during a stage of the cell cycle when *HOT1*-stimulated exchange is highly active might alter this recombination.

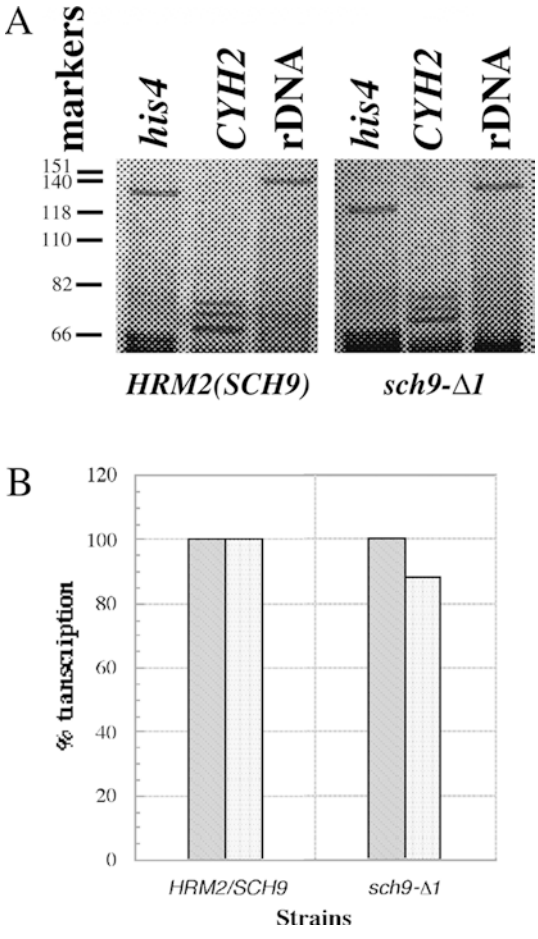

Fig. 3A, B *sch9*Δ mutations do not affect transcription initiated at *HOT1*. **A** Steady-state levels of transcripts initiated within *HOT1* sequences at *his4* or rDNA were measured by primer extension analysis in wild-type and *sch9*-Δ1 strains. A representative experiment is shown. The expected sizes of extension products are 129 nt for the transcript from *HOT1* at *his4* (Huang and Keil 1995) and 139 nt for the 35S rDNA precursor (Bayev et al. 1980; Klemenz and Geiduschek 1980). Three products ranging in size from approximately 68 to 75 nt are produced from *CYH2*, which are consistent with sizes previously reported for the multiple transcription initiation sites of this gene (Schwindinger and Warner 1987). The size standards were supplied with the primer extension kit (Promega) and their positions and lengths (in nt) are indicated next to the gels. The 5′ ends of the primers were labeled using T4 polynucleotide kinase. **B** Primer extension products were quantified using a phosphorimager and normalized against *CYH2* transcripts as an internal control as described in Huang and Keil (1995). The *dark bars* indicate transcription initiated in *HOT1* at *his4* and the *light bars* indicate rDNA transcription. The quantitative data are for the experiment shown in **A**

Since the *sch9*-Δ1 mutation affects rDNA recombination and growth, we also tested whether *sch9*-Δ1 strains are defective in rRNA synthesis. Deletion of *SCH9* does not decrease transcription of rDNA [Fig. 3A (lanes marked rDNA) and B (light bars)]. Thus, the reduced rate of rDNA recombination observed in *sch9*-Δ1 mutants does not result from lower rates of rDNA transcription.

Overexpression of *TPK1* suppresses the *HOT1* recombination defect of *sch9* mutants

Since *hrm2-1* colonies are small and pale pink, transformants containing multicopy plasmids that suppress the growth defects of this mutation are readily identified. Among 85,000 transformants, three large, red colonies were recovered. To confirm that the suppression of the growth defects exhibited by these transformants was plasmid dependent, colonies that had lost these plasmids were examined for growth and color. In all three cases the resulting colonies were small and pale pink, like the parental *hrm2-1* strain (HRM221). To further confirm the plasmid dependence of this suppression, plasmid DNA was recovered into *E. coli* from these transformants (Hoffman and Winston 1987) and retransformed into the original HRM221 strain. All three plasmids

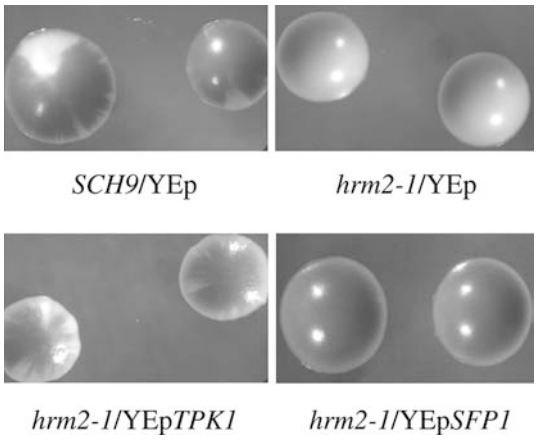

Fig. 4 Overexpression of *TPK1* suppresses the *HOT1*-recombination defect of *sch9* mutants. A multicopy plasmid bearing the *TPK1* gene, but not *SFP1,* alleviates the *HOT1*-recombination defects of *hrm2-1* as visualized by the number of white sectors present in the colony

increased the size of *hrm2-1* colonies, decreased the generation time of these mutants (Table 3; compare the generation time of *hrm2-1*/YEp to that of *hrm2-1*/YEp *TPK1* or *hrm2-1*/YEp *SFP1*), and changed their color from pink to red.

The three plasmids were tested for their ability to complement the *HOT1* recombination defect of *hrm2-1* cells based on the colony-color sectoring assay. Two of the three plasmids suppressed the recombination defect of *hrm2-1* strains (Fig. 4; see *hrm2-1*/YEp *TPK1*). Restriction analysis of these two plasmids showed they were identical. Partial sequence analysis, linker insertion and subcloning showed that *TPK1*, which encodes a catalytic subunit of protein kinase A and has previously been shown to suppress the growth defect of *sch9* strains (Hartley et al. 1994), was responsible for this suppression. To confirm that only *TPK1* is required to suppress the *hrm2-1* defects, a multicopy YEplac112 (Gietz and Sugino 1988) vector carrying a 1.9-kb *Bst*YI fragment that contains only *TPK1* was constructed. This plasmid suppresses both the excisive and gene replacement recombination defects of an *hrm2-1* strain (Table 4). In addition, this plasmid suppresses the *HOT1* and rDNA recombination defects, as well as the growth defects, of an *sch9*-Δ1 strain (Table 4, and data not shown), indicating that *TPK1* is a bypass suppressor of these *sch9* phenotypes.

Overexpression of *TPK1* presumably activates the cAMP-dependent PKA pathway by increasing the pool of catalytically active PKA subunits. To further assess if the PKA pathway is responsible for the observed suppression, $RAS2^{val19}$, a dominant mutation that constitutively activates adenylate cyclase and thus the PKA pathway, was tested for its ability to suppress *sch9* phenotypes. $RAS2^{val19}$ suppresses both the growth and recombination defects of *hrm2-1* and *sch9*-Δ1 mutants (Table 4), supporting the conclusion that activation of the cAMP-dependent PKA pathway suppresses the

Table 4 Suppressors of *hrm2-1* and *sch9*-Δ1

Strain/plasmid	Recombination rate (%)		
	Ura^-	His^+ Ura^+	Can^R
HRM2(*SCH9*/vector	100	100	100
hrm2-1/vector	14**	12**	70
sch9-Δ1/vector	11**	23**	62
hrm2-1/YEp *TPK1*	95	105	94
sch9-Δ1/YEp *TPK1*	48	114	68
hrm2-1/YCp $RAS2^{val19}$	47	64	136
sch9-Δ1/YCp $RAS2^{val19}$	57	117	77
hrm2-1/YEp *TPK2*	10**	nd	36
sch9-Δ1/YEp *TPK2*	20**	nd	59

** $P < 0.01$ as compared to *HRM2*(*SCH9*)/vector; vector = appropriate YCp or YEp control plasmid; nd, not determined

HOT1-specific hyporecombination phenotype of *sch9* mutants.

Isoforms of the catalytic subunit of PKA are encoded by three unlinked genes: *TPK1*, *TPK2* and *TPK3*. Although products encoded by the *TPK* genes are closely related, they are only partially redundant in function (Toda et al. 1987; Robertson and Fink 1998; Pan and Heitman 1999; Robertson et al. 2000). Since only *TPK1* was identified in the suppressor hunt, we tested whether another *TPK* gene, *TPK2*, can suppress the phenotypic defects of the *hrm2-1* and *sch9*-Δ1 strains. When *TPK2* is present on a multicopy vector, it suppresses the growth and color defects of *hrm2-1* and *sch9*-Δ1 mutants, but does not suppress the *HOT1* recombination defects (Table 4). This provides further evidence that PKA subunits encoded by the various *TPK* genes share some common physiological roles but also have features that distinguish them from each other.

It has been proposed that upstream regulators of Sch9p include the Gα protein Gpa2p and its coupled receptor Gpr1p, although other models have also been advanced (contrast Xue et al. 1998 with Kraakman et al. 1999 and Lorenz et al. 2000). If the Sch9p activities relevant to *HOT1* are regulated by Gpa2p, deletion of *GPA2* should have the same effect on *HOT1* activity as deletion of *SCH9*. Yeast strains derived from K2307 and K2302 that contain a precise deletion of *GPA2* (K5656 and K5651, respectively) were constructed. Deletion of *GPA2* has no effect on *HOT1* activity (data not shown). With regard to the *HOT1*-related functions of Sch9p, this suggests either that Gpa2p does not regulate Sch9p or conversely that additional upstream components are involved in this regulation.

The growth and recombination defects of *sch9* strains are separable

As described above, *ade2* yeast strains carrying either the *hrm2-1* mutation or the *sch9*-Δ1 allele form small, pale pink colonies unlike the large, red colonies formed by *SCH9* strains (Table 3). The doubling time of the mutant strains is approximately twice that of the wild-type

control (see Table 3, and Toda et al. 1988). It is possible that the decreased rates of *HOT1* and rDNA recombination in *sch9* mutants are simply due to the growth defect of these strains. To test this possibility, petite (ρ^0) derivatives of the wild-type K2307 strains were isolated (Slonimski et al. 1968). The generation time of these petites is 210 min, which is similar to the generation time of *sch9* mutants (Table 3). However, *HOT1* and rDNA recombination rates in the petites are not significantly different from the rates in wild-type ρ^+ strains (data not shown). These results indicate that slow growth does not necessarily produce the reduced recombination phenotypes of these mutants.

Further evidence that extends this conclusion comes from identification of two multicopy suppressors that partially suppress the growth defects of *sch9* but do not correct the *HOT1* hyporecombination phenotypes. As described above, multicopy *TPK2* affects only the growth defects of *sch9* mutants. The other growth-defect-specific multicopy suppressor is *SFP1*, split zinc finger protein, which was identified in our hunt for multicopy suppressors of *hrm2-1* mutant phenotypes (see above). *SFP1*, like *SCH9*, affects critical cell size at Start in the cell cycle (Jorgensen et al. 2002). The effect of the transcription factor encoded by *SFP1* on cell size appears to be linked to its regulation of a number of genes involved in ribosome biogenesis. *SFP1* has also been proposed to affect the G_2/M (mitosis) transition and the DNA-damage checkpoint pathway (Xu and Norris 1998). When present in an *hrm2-1* strain, YEp *SFP1* partially suppresses the growth defects, as evidenced by the decreased generation time (Table 3), but does not affect not the *HOT1* recombination defect (Fig. 4, strain *hrm2-1*/YEp *SFP1*). YEp *SFP1* transformants of K4691, which contains the *sch9*-Δ1 mutation, behave exactly like *hrm2-1*/YEp *SFP1* transformants, indicating *SFP1* is a bypass suppressor of the growth defects of strains that are devoid of Sch9p. The results with *TPK2* and *SFP1* indicate that the growth defects observed in *sch9* mutants are separable from the effects on recombination.

Discussion

To further characterize the recombination-stimulating activity of *HOT1*, *trans*-acting mutations that affect the activity of this recombination hotspot are being studied. Four genes, *HRM1–HRM4*, were identified by mutations that specifically decrease *HOT1*-stimulated exchange (Lin and Keil 1991). Here we report the cloning and characterization of *HRM2*, which is identical to *SCH9*. Examination of the amino acid sequence predicted for the product of *SCH9* revealed motifs that are hallmarks of protein kinases, and Sch9p has been implicated in a growth control mechanism that is partially redundant with the PKA pathway (Toda et al. 1988). Deletion of *SCH9* decreases both *HOT1* and rDNA recombination. As discussed in more detail below, the effect of *SCH9* on *HOT1* activity does not appear to be due to decreased transcription at the hotspot. Several lines of evidence indicate that the effect on recombination involves a signal transduction pathway. First, *TPK1*, which encodes the catalytic subunit of PKA, is a multicopy suppressor of *sch9* mutations, suggesting that lack of Sch9p can be compensated for by overexpression of another signal transduction component. Second, *RAS2* ^{val19}, a constitutive mutation in an upstream component of the PKA pathway, also suppresses the *HOT1* defect of *sch9* mutants. These data indicate that activation of the PKA signaling pathway can compensate for the lack of Sch9p, possibly by permitting illegitimate phosphorylation of substrates.

Another potential role for *SCH9* in DNA transactions is revealed by its effect on transposition of Ty1 elements (Scholes et al. 2001). In contrast to the decrease in *HOT1* and rDNA recombination reported here, mutation of *SCH9* leads to increased mobility of Ty1. Given this difference, it is not clear if Sch9p has similar or different roles in these events. In agreement with our results, altered transcription does not appear to play a role in the effect of *SCH9* on transposon movement.

It is interesting that *TPK2*, which encodes an isozyme of *TPK1*, does not suppress the *HOT1* recombination defect of *sch9* mutants. It is possible that excess Tpk1p is able to phosphorylate targets of Sch9p that are essential for *HOT1* activity while Tpk2p can not. However, overexpression of either *TPK1* or *TPK2* partially suppresses the growth defects of *sch9* mutants. This indicates that there may be specific targets regulated by Sch9p that alter *HOT1* recombination while other targets affect growth.

HOT1—more than transcription

As described in the Results section, numerous lines of evidence indicate that the recombination-stimulating activity of *HOT1* requires high levels of transcription. The simplest models for *HOT1* activity envision that increased transcription is sufficient for the recombination-stimulating effect of this element. Increased transcription could facilitate access of the normal mitotic recombination machinery to DNA, or it could introduce recombinogenic lesions. Such simple models are ruled out by our finding that *HOT1* transcription is unaltered in *sch9*-Δ1 mutants although *HOT1*-stimulated recombination is specifically reduced. Thus, transcription is necessary (Stewart and Roeder 1989; Huang and Keil 1995) but not sufficient for *HOT1* activity. Previous findings are consistent with this argument. For instance, Huang and Keil (1995) found a correlation between the recombination and transcription effects of most, but not all, *cis*-acting *HOT1* mutations. The most dramatic exception to the correlation involved deletion of a 130-bp *Hin*dIII-*Hpa*I fragment adjacent to the transcriptional enhancer required for efficient ectopic expression

of rDNA (Elion and Warner 1986; Wai et al. 2001). Deletion of this fragment produces only a 20% decrease in transcription but a 90% to 97% decrease in *HOT1* activity (Huang and Keil 1995). At one time it was proposed that high levels of transcription and pausing of replication at the replication fork barrier (RFB) present in the *Hin*dIII-*Hpa*I fragment were necessary for *HOT1* activity (Kobayashi and Horiuchi 1996). Under this model Sch9p could regulate this pausing. However, it was recently shown that RFB is not required for *HOT1* activity (Ward et al. 2000), arguing this is not the role of Sch9p.

The involvement of a putative protein kinase in the activity of *HOT1* is reminiscent of the requirement for protein kinases for the recombination-stimulating action of *M26* and related meiotic hotspots in *S. pombe* (reviewed in Fox and Smith 1998; Davis and Smith 2001). A cascade involving the Wis1 and Spc1 protein kinases is required to phosphorylate the Atf1-Pcr1 transcription factor. This phosphorylated factor binds to *M26* and related sequences, leading to a localized enhancement of meiotic recombination (Kon et al. 1998; Fox et al. 2000). Binding of Atf1-Pcr1 to *M26* does not increase *ade6* transcription above the basal level (Kon et al. 1997) but instead may stimulate exchange by remodeling chromatin (Mizuno et al. 1997; Fox et al. 2000) to make it more susceptible to double-strand breaks and/or by recruiting recombination machinery (Kirkpatrick et al. 1999; Steiner et al. 2002). It is not clear whether a basal level of *ade6* transcription is required for *M26* hotspot activity (Davis and Smith 2001). As with *M26*, the well-characterized meiotic recombination hotspot at *HIS4* in *S. cerevisiae* also requires a bound transcription activator (White et al. 1991, 1993; Kirkpatrick et al. 1999). The effect of the bound factor on recombination does not result from activation of transcription, as it has been shown that the activity of this hotspot is independent of transcription (White et al. 1992). Meiotic recombination hotspots that require binding of transcription factors have been termed α-hotspots (Kirkpatrick et al. 1999).

One possible model consistent with our characterization of the role of *SCH9* in *HOT1* activity is that mitotic hotspots differ from meiotic α-hotspots by requiring both high levels of transcription and bound factors that recruit recombination proteins or remodel chromatin to make it more recombinogenic. Under this model, Sch9p could be involved in regulating binding of such factors presumably by phosphorylation. Alternatively, Sch9p could regulate a recombination protein that is specifically involved in rDNA and *HOT1*-stimulated exchange, or a protein that affects DNA:RNA hybrids which play a role in at least some forms of transcription-stimulated recombination (Huertas and Aguilera 2003).

Several other protein kinases affect mitotic recombination in yeast, although their effect on transcription-stimulated exchange has not been assessed. Of particular interest to this work is the finding that overexpression of Snf1p, which may affect rDNA chromatin structure due to its histone H3 kinase activity, increases rDNA recombination (Lin et al. 2003). Other kinases affecting mitotic recombination include those encoded by *CDC5* (a serine/threonine protein kinase), *PKC1* (protein kinase C), *DUN1* (a kinase required for induction of DNA repair genes), and *TEL1* and *MEC1* (related kinases affecting DNA damage response and telomere length). In contrast to our findings with *SCH9*, mutation of *CDC5* (Hartwell and Smith 1985; Aguilera and Klein 1988), *PKC1* (Huang and Symington 1994), *DUN1* (Fasullo et al. 1999) or *TEL1* and *MEC1* (Craven et al. 2002) generally enhances mitotic recombination, although some mitotic exchange events are negatively affected in *dun1* strains. The mechanisms by which these kinases affect recombination have not been elucidated.

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