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

R. Prusty · R. L. Keil

**Abstract** *HOT1* is a mitotic recombination hotspot derived from yeast rDNA. To further study *HOT1* function, trans-acting *HOT1* 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.

**Keywords** Transcription-stimulated recombination · *HOT1* · Recombination hotspot · *SCH9* · Protein kinase A (PKA)

**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 \textit{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 \textit{gal10-URA3-gal10} duplication in a strain lacking either \textit{GAL4}, a positive regulator of galactose-induced gene expression, or \textit{GAL80}, a repressor of Gal4p-induced transcription. They reported a fifteen-fold increase in the rate of recombination in \textit{gal80Δ} 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 \textit{ADE6} promoter in \textit{Schizosaccharomyces pombe} is replaced with the strong \textit{ADH1} promoter, recombination in \textit{ADE6} is increased seven-fold in mitosis and 25-fold during meiosis (Guldener et al. 1996).

To understand the mechanism of action of \textit{HOT1} we isolated trans-acting \textit{HOT1} recombinant mutants (\textit{hrm}) that alter \textit{HOT1}-stimulated exchange (Lin and Keil 1991). The \textit{hrm2-1} mutation decreases \textit{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 \textit{ade2} colonies, compared to the large, red colonies formed by the Hrm+ control strains. The \textit{HRM2} gene was cloned based on its ability to complement both the growth and \textit{HOT1}-recombination defects of \textit{hrm2-1} strains. \textit{HRM2} is identical to \textit{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 \textit{SCH9} increases stress resistance and longevity of non-dividing yeast cells (Fabrizio et al. 2001), but decreases the life span of replicating cells (Defoix et al. 1999). \textit{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 \textit{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 \textit{Escherichia coli} strain MC1066 [ \textit{leuB trpC pyrF:: Tn5} (KanR) 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2301</td>
<td>\textit{MAT} \textit{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</td>
<td>Lin and Keil (1991)</td>
</tr>
<tr>
<td>K2302</td>
<td>\textit{MAT} \textit{his4-260::URA3::his4-Δ} leu2::ADE5,7::leu2-3,112 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1</td>
<td>Lin and Keil (1991)</td>
</tr>
<tr>
<td>K4691</td>
<td>\textit{sch9}-Δ1 a in K2307</td>
<td>This study</td>
</tr>
<tr>
<td>K4693</td>
<td>\textit{sch9}-Δlin K2302</td>
<td>This study</td>
</tr>
<tr>
<td>K5651</td>
<td>\textit{gpa2-Δ1} a in K2302</td>
<td>This study</td>
</tr>
<tr>
<td>K5656</td>
<td>\textit{gpa2-Δlin} K2307</td>
<td>This study</td>
</tr>
<tr>
<td>HRM142</td>
<td>\textit{MAT} \textit{hrm2-1 his6-2::URA3::his6-Δ::HOT1} leu2::ADE5,7::leu2-3,112::HOT1 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1</td>
<td>This study</td>
</tr>
<tr>
<td>HRM201</td>
<td>\textit{MAT} leu2::ADE5,7::leu2-3,112::HOT1 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1</td>
<td>This study</td>
</tr>
<tr>
<td>HRM221</td>
<td>\textit{hrm2-1 lin HRM201}</td>
<td>This study</td>
</tr>
<tr>
<td>HRM759</td>
<td>\textit{MAT} \textit{sch9-Δ1} leu2::ADE5,7::leu2-3,112::HOT1 ade2-1 ade5 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1</td>
<td>This study</td>
</tr>
<tr>
<td>HRM764</td>
<td>\textit{MAT} \textit{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</td>
<td>This study</td>
</tr>
<tr>
<td>RLK98-2A</td>
<td>\textit{MAT} \textit{his4-260} leu2::ADE2::ade2-1 trp1-HIII ura3-52 lys2-ΔBX::CAN1::LYS2 can1 \textit{rDNA:: ADE2} \textit{rDNA:: URA3}</td>
<td>Keil and McWilliams (1993)</td>
</tr>
<tr>
<td>RLK183-2B</td>
<td>\textit{sch9-Δlin RLK98-2A}</td>
<td>This study</td>
</tr>
</tbody>
</table>

\textit{Schizosaccharomyces pombe} 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 \textit{ADE6} promoter in \textit{Schizosaccharomyces pombe} is replaced with the strong \textit{ADH1} promoter, recombination in \textit{ADE6} is increased seven-fold in mitosis and 25-fold during meiosis (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), γδ 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 BstYI fragment containing TPK1 into the compatible BamHI 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 hyperecombination phenotype and the growth defects of the hrm2-1 mutant, HRM221. To assay HOT1 activity, the
The sequence of the \textit{HOT1}::\textit{leu2}::\textit{ADE5,7}::\textit{leu2} duplication used was (Lin and Keil 1991). Excision of the \textit{ADE5,7} gene produces white \textit{ade2} ade5 sectors in an otherwise red \textit{ade2} colony. While the \textit{Hrm} \textsuperscript{+} strain produces colonies with approximately five to fifteen sectors, \textit{hrm2-1} strains produce colonies that usually have less than three white sectors (Fig. 1; compare wt/normal sized colonies but did not show \textit{Hrm} \textsuperscript{+} levels of sectors, produces colonies with approximately five to fifteen.

Table 2 Effects of \textit{hrm2-1} and \textit{sch9-\Delta1} on mitotic recombination

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Recombination rate</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>\textit{HOT1}</td>
</tr>
<tr>
<td></td>
<td>Ura\textsuperscript{+}</td>
</tr>
<tr>
<td>\textit{SCH9}</td>
<td>33.1</td>
</tr>
<tr>
<td>\textit{hrm2-1}</td>
<td>4.4**</td>
</tr>
<tr>
<td>\textit{sch9-\Delta1}</td>
<td>7.1**</td>
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</tbody>
</table>

* \textit{P < 0.05}, ** \textit{P < 0.01} as compared to \textit{SCH9}; recombination rates are not significantly different between \textit{hrm2-1} and \textit{sch9-\Delta1} strains. nd, not determined.

Table 3 Colony morphology and generation time

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Colony morphology\textsuperscript{a}</th>
<th>Generation time (min) \textsuperscript{b,c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type and mutants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{HRM2} (\textit{SCH9})</td>
<td>Large red colonies; many white sectors</td>
<td>98</td>
</tr>
<tr>
<td>\textit{hrm2-1}</td>
<td>Small pink colonies; few white sectors</td>
<td>217**</td>
</tr>
<tr>
<td>\textit{sch9-\Delta1}</td>
<td>Small pink colonies; few white sectors</td>
<td>239**</td>
</tr>
<tr>
<td>Cloning \textit{SCH9}</td>
<td></td>
<td></td>
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<tr>
<td>\textit{SCH9}/\textit{YCp}</td>
<td>Large red colonies; many white sectors</td>
<td>108</td>
</tr>
<tr>
<td>\textit{SCH9}/\textit{YEp} \textit{SCH9}</td>
<td>Large red colonies; many white sectors</td>
<td>188**</td>
</tr>
<tr>
<td>\textit{hrm2-1}/\textit{YEp}</td>
<td>Large red colonies; many white sectors</td>
<td>98</td>
</tr>
<tr>
<td>\textit{hrm2-1}/\textit{YEp} \textit{SCH9}</td>
<td>Large red colonies; many white sectors</td>
<td>108</td>
</tr>
<tr>
<td>\textit{YEp}/\textit{TPK1}: \textit{YEp}/\textit{SFP1}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{SCH9}/\textit{YEp}</td>
<td>Large red colonies; many white sectors</td>
<td>108</td>
</tr>
<tr>
<td>\textit{SCH9}/\textit{YEp} \textit{TPK1}</td>
<td>Large red colonies; many white sectors</td>
<td>128**</td>
</tr>
<tr>
<td>\textit{SCH9}/\textit{YEp} \textit{SFP1}</td>
<td>Large red colonies; many white sectors</td>
<td>191</td>
</tr>
<tr>
<td>\textit{hrm2-1}/\textit{YEp}</td>
<td>Medium red colonies; many white sectors</td>
<td>150**</td>
</tr>
<tr>
<td>\textit{hrm2-1}/\textit{YEp} \textit{SFP1}</td>
<td>Medium red colonies; many white sectors</td>
<td>172**</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Of strains containing the \textit{leu2}::\textit{ADE5,7}::\textit{leu2} duplication.

\textsuperscript{b} In synthetic medium lacking appropriate nutrients to maintain plasmid selection when necessary.

\textsuperscript{c} All standard deviations were less than 10% of the generation time.

\textsuperscript{**} \textit{P < 0.01} as compared to the appropriate control (\textit{SCH9}://\textit{SCH9}/\textit{YCp} or \textit{SCH9}/\textit{YEp}).

\textsuperscript{d} \textit{P < 0.05} as compared to \textit{hrm2-1}/\textit{YEp}.

Qualitative colony-color sectoring assay involving recombination of \textit{A}\textsubscript{HOT1}::\textit{leu2}::\textit{ADE5,7}::\textit{leu2} duplication was used (Lin and Keil 1991). Excision of the \textit{ADE5,7} gene produces white \textit{ade2} ade5 sectors in an otherwise red \textit{ade2} colony. While the \textit{Hrm} \textsuperscript{+} strain produces colonies with approximately five to fifteen sectors, \textit{hrm2-1} strains produce colonies that usually have less than three white sectors (Fig. 1; compare wt/normal sized colonies but did not show \textit{Hrm} \textsuperscript{+} levels of sectors. The six strains in which both phenotypes reappeared were plated on media containing 5FOA to induce loss of the library plasmid. Two of the six strains exhibited decreased levels of \textit{HOT1}-recombination and grew poorly upon plasmid loss, indicating that genomic DNA present on these plasmids complemented the \textit{hrm2-1} mutant phenotypes. Plasmids were rescued from these transformants and retransformed into the original \textit{HRM221} (\textit{hrm2-1}) strain. Both plasmids restored normal growth (Table 3) and wild-type levels of \textit{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, \textit{pL3033}, was used to localize the complementing gene. Three independent plasmids containing transposon insertions that destroyed the ability of \textit{pL3033} to complement the recombination and growth defects of \textit{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 \textit{SCH9} gene (Toda et al. 1988).

Precise deletions of the \textit{SCH9} ORF (\textit{sch9-\Delta1}) in strains K2307 and K2302 were constructed by PCR-mediated gene disruption (Guldener et al. 1996). \textit{sch9-\Delta1} (G418 \textsuperscript{R}) strains exhibit a \textit{HOT1}-specific hyporecombination phenotype (Fig. 1, see \textit{sch9-\Delta1}/\textit{YCp}) and the same growth defects as the \textit{hrm2-1} mutant (Table 3). One of the \textit{sch9-\Delta1} transformants, K4691, was mated with the \textit{hrm2-1} strain HRM142. Random spore analysis showed that approximately 50% of the spores (25 spores out of 52) were G418 \textsuperscript{R} and half (27 spores) were G418 \textsuperscript{S}. All spores examined had decreased levels of \textit{HOT1}-stimulated recombination similar to \textit{hrm2-1} control strains. Colonies obtained from all 52 spores were also small and exhibited the pale pink color of the \textit{hrm2-1} mutant. These results show that the G418 \textsuperscript{R} marker is less than 2 cM from the \textit{HRM2} locus and indicate that \textit{SCH9} is identical to \textit{HRM2}. To confirm this, the sequences of \textit{SCH9} from our wild-type and \textit{hrm2-1} strains were determined. The sequence of \textit{SCH9} in our wild-type strain is identical to that reported in the \textit{Saccharomyces} Genome Database (http://www.yeastgenome.org/). The sequence of this gene in the \textit{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 \textit{SCH9} reading frame and an A to T transversion at base 1849 (the sequence of this gene in \textit{hrm2-1} beginning at base 1844 of the \textit{SCH9} reading frame is AAAA\textsubscript{ATTATC}, where the inserted A is
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** duplication 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).
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.

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
increased the size of hrn2-1 colonies, decreased the generation time of these mutants (Table 3; compare the generation time of hrn2-1/YEp to that of hrn2-1/YEp TPK1 or hrn2-1/YEp SPF1), and changed their color from pink to red.

The three plasmids were tested for their ability to complement the HOT1 recombination defect of hrn2-1 cells based on the colony-color sectoring assay. Two of the three plasmids suppressed the recombination defect of hrn2-1 strains (Fig. 4; see hrn2-1/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 hrn2-1 defects, a multicopy YEpLac112 (Gietz and Sugino 1988) vector carrying a 1.9-kb BstYI fragment that contains only TPK1 was constructed. This plasmid suppresses both the excise and gene replacement recombination defects of an hrn2-1 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, RAS2val19, a dominant mutation that constitutively activates adenylate cyclase and thus the PKA pathway, was tested for its ability to suppress sch9 phenotypes. RAS2val19 suppresses both the growth and recombination defects of hrn2-1 and sch9-Δ1 mutants (Table 4), supporting the conclusion that activation of the cAMP-dependent PKA pathway suppresses the 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 hrn2-1 and sch9-Δ1 strains. When TPK2 is present on a multicopy vector, it suppresses the growth and color defects of hrn2-1 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 Gz 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 hrn2-1 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

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**Table 4 Suppressors of hrn2-1 and sch9-Δ1**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Recombination rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRM2/SCH9/vector</td>
<td>100/100/100</td>
</tr>
<tr>
<td>hrn2-1/vector</td>
<td>14*/12*/70</td>
</tr>
<tr>
<td>sch9-Δ1/vector</td>
<td>11*/23*/62</td>
</tr>
<tr>
<td>hrn2-1/YEp TPK1</td>
<td>95/105/94</td>
</tr>
<tr>
<td>sch9-Δ1/YEp TPK1</td>
<td>48/114/68</td>
</tr>
<tr>
<td>hrm2-1/YCp RAS2val19</td>
<td>47/64/136</td>
</tr>
<tr>
<td>sch9-Δ1/YCp RAS2val19</td>
<td>57/117/77</td>
</tr>
<tr>
<td>hrm2-1/YEp TPK2</td>
<td>10*/nd/36</td>
</tr>
<tr>
<td>sch9-Δ1/YEp TPK2</td>
<td>20*/nd/59</td>
</tr>
</tbody>
</table>

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

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**Fig. 4** Overexpression of TPK1 suppresses the HOT1-recombination defect of sch9 mutants. A multicopy plasmid bearing the TPK1 gene, but not SPF1, alleviates the HOT1-recombination defects of hrn2-1 as visualized by the number of white sectors present in the colony.
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 (ρ0) 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 ρ0 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 hrn2-1 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 G2/M (mitosis) transition and the DNA-damage checkpoint pathway (Xu and Norris 1998). When present in an hrn2-1 strain, YEps 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 hrn2-1/YEp SFP1). YEps SFP1 transfectants of K4691, which contains the sch9-A1 mutation, behave exactly like hrn2-1/YEp SFP1 transfectants, 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-A1 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 HinIII-HpaI 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 HindIII-HpaI 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-Per1 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-Per1 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 z-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 z-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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