Requirements for Activity of the Yeast Mitotic Recombination Hotspot HOT1:
RNA Polymerase I and Multiple Cis-Acting Sequences

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ABSTRACT

When inserted at novel locations in the yeast genome, the Saccharomyces cerevisiae recombination hotspot HOT1 stimulates mitotic exchange in adjacent sequences. HOT1 is derived from the rDNA repeat unit, and the sequences required for the recombination-stimulatory activity closely correspond to the rDNA transcription enhancer and initiation site, suggesting there is an association between high levels of RNA polymerase I transcription and increased recombination. To directly test whether RNA polymerase I is essential for HOT1 activity, a subunit of RNA polymerase I was deleted in a strain in which rRNA is transcribed by RNA polymerase II. HOT1 is completely inactive in this strain. Deletion analysis and site-directed mutagenesis were used to further define the sequences within the rDNA enhancer required for HOT1 activity. These studies show that the enhancer contains at least four distinct regions that are required for hotspot activity. In most cases mutations in these regions also decrease transcription from this element, further confirming the association of recombination and transcription.

RECOMBINATION is not uniformly distributed along a chromosome. Sites that produce a local stimulation of recombination have been identified in a number of organisms. One of these hotspots, HOT1, is derived from the rDNA repeat unit of Saccharomyces cerevisiae. When inserted at novel locations in the yeast genome, this hotspot is able to stimulate mitotic recombination up to 200-fold but it does not affect meiotic recombination (Keil and Roeder 1984; Voelkel-Meiman et al. 1987).

Several lines of evidence suggest that the ability of HOT1 to stimulate recombination requires high levels of RNA polymerase I transcription: (1) the sequences required for hotspot activity extensively overlap the enhancer and transcription initiation site for rDNA (Voelkel-Meiman et al. 1987), (2) mutations in these sequences that decrease HOT1 activity also decrease transcription (Stewart and Roeder 1989), (3) HOT1 must be oriented so that transcription initiated from this element will proceed across both of the recombining sequences of an intrachromosomal recombination substrate (Keil and Roeder 1984), (4) the activity of the enhancer is position and orientation dependent for stimulating both recombination and transcription (Voelkel-Meiman et al. 1987), and (5) insertion of the rDNA transcription termination site downstream of HOT1 abolishes the hotspot activity and terminates transcription (Voelkel-Meiman et al. 1987).

An additional test of this proposed linkage between recombination and transcription would be to determine whether RNA polymerase I is necessary for HOT1 activity. It had not been possible to directly address this issue since RNA polymerase I is normally essential for transcribing rDNA. However, RNA polymerase I can be made nonessential in yeast by placing the transcription of rDNA under the control of RNA polymerase II regulatory sequences (NogI et al. 1991). The sequences encoding the 35S rRNA, the precursor rRNA for the 18S, 25S, and 5.8S rRNAs, were fused to the GAL1 promoter and terminator sequences at the 5' and 3' ends, respectively, such that 35S rRNA is a galactose-inducible transcript produced by RNA polymerase II. Yeast strains that contain this plasmid and a disruption of a gene encoding one of the RNA polymerase I subunits are viable when grown on media containing galactose as a carbon source. Using this system, we have found that RNA polymerase I is essential for HOT1 activity.

In addition, sequences within the rDNA enhancer necessary for HOT1 activity were studied. To obtain maximal HOT1 activity, a 320-bp EcoRI-HpaI fragment, E, that contains the rDNA transcription enhancer and a 255-bp Smal-EcoRI fragment, I, that contains the transcription initiation site are required (Voelkel-Meiman et al. 1987). The cis-acting sequences in these elements necessary for the recombination-stimulatory activity of HOT1 have not been well defined. To this point we have limited our investigation to defining the enhancer sequences that are necessary for HOT1 activity. Using linker insertion mutagenesis, Stewart and Roeder (1989) identified two regions, which we call Ea and Eb, that are required for HOT1 activity. Using oligonucleo-
tide-directed mutagenesis and deletion analysis, we more precisely defined sequences of the enhancer that are required for HOT1 activity. We find that at least four separate areas of the enhancer are necessary.

MATERIALS AND METHODS

Strains and media: The yeast strains used were derived by transformation of RLK88-3C [MATa his4-260 leu2-3,112 ura3-52 ade2-1 thr1-112 his3-11 trp1-1 Leu2+] from M. Casadaban), which was used for propagating double-stranded plasmids; JM109 (recA1 supE44 endA1 hsdR17 gyrA96 thi-1  thd-1 lacI(lac-proAB) F’ (traD36 proAB) lacI(lacZAM15) (YANISCH-PERRON et al. 1985), which was used to propagate bacteriophage M13 recombinants and vectors carrying the bacteriophage fl origin to produce single-stranded derivatives; and RZ1032 (HirKL16 P0/45 (hisA1-62) 2td ungl thi1  mel1) (KUNKEL et al. 1991), which was used to produce ura-containing M13 templates for oligonucleotide-directed mutagenesis.

Yeast media containing glucose were prepared as described previously (LIN and KEEIL 1991). Yeast synthetic media containing galactose (SCGal) were made by the same formulations except 2% galactose was substituted for the glucose. Bacterial media were prepared as described in SAMBROOK et al. (1989).

Recombination substrates: To produce an intrachromosomal recombination substrate in NOY408-1a and NOY408-1b plasmid pl5038, which contains the LYS2 gene and the 3.9 kb Salt-Xhol fragment of CAN1 inserted in pBR322 (LIN and KEEIL 1991), was cleaved at the unique SalI site, treated with the Klenow fragment of DNA polymerase I to make it blunt ended, and ligated to the 850-bp EcoRI-Stu fragment containing TRP1, which had also been made blunt ended by treatment with the Klenow fragment. The resulting plasmid, pc306, was cleaved at the unique Xhol site in LYS2, made blunt ended with the Klenow fragment, and ligated with a blunt ended 575-bp EcoRI fragment containing HOT1. Plasmid pc312 contains HOT1 in the active orientation while plasmid pc314 contains the inactive orientation.

These plasmids, pc306, pc312 and pc314, were cleaved at the unique SalI site in LYS2 and transformed into NOY408-1a and NOY408-1b to produce a LYS2-CAN1-TRP1-LYS2 recombination substrate. These transformants were grown on SCGal–ura–trp to select for the plNOY102 plasmid and the LYS2 duplication. Southern analysis was used to identify transformants that contained a single properly integrated plasmid.

To produce the intrachromosomal recombination substrate for assays of the effect of enhancer mutations on HOT1 activity, plasmid pl524, which contains part of HIS4 in YEp5 (VOELKEL-MEIMAN et al. 1987), was cleaved at the unique BamHI site that had been inserted in the HIS4 sequences. A BglII-BamHI-Xhol-EcoRI-BglII polylinker was inserted. The resulting vector was cleaved with XhoI and EcoRI and a 250-bp XhoI-EcoRI fragment containing the DNA pol I transcription initiation site was inserted such that transcription initiated in this fragment would proceed across the HIS4 coding sequences when integrated into the yeast genome. This XhoI-EcoRI fragment was constructed by ligating a XhoI linker at the 5′ end of the initiation site. A 540-bp EcoRI-BamHI fragment containing the fl origin from pUC-fl (Pharmacia) was blunt ended by treatment with the Klenow fragment and inserted at the unique SalI site of this vector to create pcG133. This vector was cleaved with BamHI and XhoI, and a 320-bp BamHI-XhoI fragment containing the enhancer was inserted to produce pcG141. The original EcoRI and Hpal ends of the enhancer had previously been converted to BamHI and XhoI sites, respectively, by the addition of linkers.

Enhancer mutations of three different types were produced for these studies: random mutations created by site-directed mutagenesis using a pool of mixed oligonucleotides, block mutations also produced by site-directed mutagenesis, and deletion mutations produced using restriction sites inserted in the enhancer element (STEWART and ROEDER 1989).

Random mutations: Plasmid pcG1 was used for the production of random mutations. This contains the 320-bp EcoRI-Hpal enhancer fragment of HOT1 with the Hpal site replaced by a XhoI linker inserted into EcoRI and XhoI cut M13mp19. A BamHI linker was then inserted at the EcoRI site. Uracil-containing templates (KUNKEL 1985; KUNKEL et al. 1991) of this vector produced in RZ1032 were hybridized to a pool of mixed oligonucleotides that had been phosphorylated. Different pools of mixed oligonucleotides (NEK et al. 1988) were synthesized to mutagenize Ea (72 bp) and Eb (39 bp) (see RESULTS). The pools of oligonucleotides used to mutagenize Ea and Eb were 82 and 46 nucleotides long respectively. For the Ea pool, the middle 72 nucleotides of the oligonucleotides were synthesized with 98% of the nucleotide of the wild-type sequence and 0.667% of each of the other three nucleotides at each position. This ratio was used to maximize the yield of single and double mutations in the final pool (MAKris et al. 1988). For the Eb pool, the middle 39 nucleotides were synthesized with 96.37% wild-type nucleotide and 1.21% of each of the other three nucleotides at each position. The five nucleotides at each of the ends of these oligonucleotides, which are outside of the regions we were interested in mutating, were synthesized with 100% of the nucleotide corresponding to the wild-type sequence of the enhancer. Having regions of perfect homology at the ends of these oligonucleotides should permit the recovery of mutations across the entire Ea and Eb regions.

T4 DNA polymerase and T4 DNA ligase were used to convert the oligonucleotide-template complexes into covalently closed circular DNA (KUNKEL et al. 1991) that was then transformed into JM109. More than 10,000 plaques for each library were scraped from plates and inoculated into liquid media along with 5 ml of saturated JM109 culture. After overnight growth at 37°C, DNA was prepared from these cultures by alkaline lysis followed by cesium chloride-ethidium bromide gradients (SAMBROOK et al. 1989). The mutated enhancers were isolated from these vectors by digesting with BamHI and XhoI, separating the 320-bp enhancer fragment from the plasmid backbone on an agarose gel, and extracting the DNA from the agarose in an Elutrap (Schleicher & Schuell).

The pcG133 vector was digested with BamHI and XhoI, and the large plasmid backbone was purified by agarose gel electrophoresis and recovered in an Elutrap. The mutated enhancers were ligated into this plasmid and transformed into MC1066. More than 10,000 AmpR transformants were recovered for each of the two initial pools of oligonucleotides, inoculated into LB + Amp media and grown overnight. DNA
Figure 1.—Intrachromosomal recombination substrates. (A) To measure the effect of rpa135 on HOT1 activity, a direct duplication of LYS2 genes separated by CAN1, TRP1 and pBR sequences was constructed by integrative transformation at the normal LYS2 chromosomal locus. Excision of this duplication produces CanR recombinants since these strains contain a can1-100 mutation at the normal chromosomal location. (B) The recombination substrate used to identify hot1 mutations consists of a direct duplication of heteroallelic his4 mutations flanking URA3 and pBR sequences constructed by integrative transformation. The effect of potential hot1 mutations on recombination was determined from the frequency of Ura- (5-FOAR) recombinants produced by excision and the frequency of His+ recombinants produced by excision or gene replacement.

From these cultures were purified by alkaline lysis and cesium chloride-ethidium bromide gradients (SAMBROOK et al. 1989). Purified DNA was transformed into JM109 and independent transformants were isolated. Single-stranded phagemid DNA was prepared from 96 and 24 of the transformants derived from the pools of Ea and Eb oligonucleotides, respectively. The enhancer regions of each were sequenced. From the Ea library 59.4% contained mutations while 62.5% of the Eb library contained mutations. Using the Poisson distribution and this percentage, the number of yeast transformants that must be screened to saturate each region (i.e., to examine each nucleotide change at each position) was calculated. For Ea and Eb ~2000 and 930 transformants, respectively, must be examined. We actually screened 2560 Ea transformants and 1200 Eb transformants.

Plasmids pG141, pG133 and the libraries containing Ea and Eb mutations were targeted to integrate at his4 in RLK88-3C by cleavage at the unique CluI site in the his4 sequences (VOELKEL-MEIMAN et al. 1987). Independent yeast transformants were streaked on SC-ura medium and a single colony from each streak was screened for the level of HOT1 activity by determining the frequency of Ura- and His+ recombinants from patch tests (KEIL and ROEDER 1984). Potentially interesting transformants were analyzed by Southern analysis to determine if they contained a single properly integrated copy of the transforming plasmid. The transforming plasmids were recovered from each transformant that passed all of these tests by digesting 1 μg of yeast mini-prep DNA with ClaI, ligating, and transforming into JM109 by electroporation (DOWER et al. 1988) using a Gene Pulser (Bio-Rad). Restriction analysis of the recovered double-stranded plasmids confirmed that their restriction pattern was normal. Dideoxysequencing (SANGER et al. 1977) of the enhancer regions of each of these putative mutants using single-stranded DNA (VIEIRA and MESSING 1987) identified the mutation(s) in each element. To quantify the effect of the mutation(s) on HOT1 activity, each recovered plasmid was retransformed into RLK88-3C and three independent transformants containing a single properly integrated copy of each plasmid were subjected to fluctuation tests as described below.

Block mutations: The oligonucleotides used to construct the block mutations were synthesized such that ten nucleotides to each side of the block that was being mutagenized were identical to the wild-type sequence of the enhancer. Mutant enhancers containing the various block mutations were synthesized from pG1 as described above for the mixed oligonucleotide pools. The mutant enhancers were sequenced, excised with BamHI and XbaI, and ligated into the purified BamHI-XbaI plasmid backbone of pG133. These plasmids were transformed into RLK88-3C as described above. Three independent transformants containing a single properly integrated copy of the various mutations were tested by fluctuation analysis (see below) for their effect on HOT1 activity.

Deletion mutations: The XhoI linker insertion mutations previously constructed by STEWART and ROEDER (1989) were used to construct the deletion mutations. Their mutations that we
TABLE 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Construct</th>
<th>Median frequency of Can(^{\text{r}}) recombinants ((\times 10^3))</th>
</tr>
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<tr>
<td>RPA135</td>
<td>No HOT1</td>
<td>2.3</td>
</tr>
<tr>
<td>RPA135</td>
<td>HOT1 EI</td>
<td>560</td>
</tr>
<tr>
<td>RPA135</td>
<td>IT0H IE</td>
<td>2.8</td>
</tr>
<tr>
<td>rpa135::LEU2</td>
<td>No HOT1</td>
<td>2.9</td>
</tr>
<tr>
<td>rpa135::LEU2</td>
<td>HOT1 EI</td>
<td>3.3</td>
</tr>
<tr>
<td>rpa135::LEU2</td>
<td>IT0H IE</td>
<td>4.8</td>
</tr>
</tbody>
</table>

HOT1 EI indicates the active orientation of the recombination hotspot while IT0H IE indicates the inactive orientation.

**Effect of RNA polymerase I mutation on HOT1 activity**

A 80-bp BamHI-XhoI fragment from this plasmid was ligated to the 200-bp XhoI-XhoI fragment of p92 containing the 3' end of the enhancer. The resulting enhancer fragment was ligated into BamHI-XhoI-cleaved pG133. Three independent transformants for each of these deletion mutations were examined for HOT1 activity as described for the block mutations.

**Analysis of HOT1 activity**

HOT1 activity was assayed essentially as described previously (Keil and ROEDER 1984). However, after growth in liquid SC, 5 \(\mu\)l of appropriate dilutions of each culture were spotted on SC, SC+5-fluoroorotic acid (5-FOA), and SC−his plates. The number of colonies in each spot was counted under a dissecting microscope at 10× magnification. The median frequency of Ura− and His+ recombinants for each mutation was determined (LEA and COULSON 1949). The frequencies were then converted to the percentage activity where wild-type HOT1 activity (pG141) is defined as 100% and the activity of the enhancer-deleted mutation (pG133) is 0%. The formula for converting to percentage activity is [(recombination frequency of mutation − recombination frequency of pG133) / (recombination frequency of pG141 − recombination frequency of pG133)] \(\times 100\%\).

**Transcription analysis**

Primer extension analysis was used to analyze the effect of various enhancer mutations on RNA polymerase I transcription from the transcription initiation site. Total RNA was prepared by the hot phenol method (ELION and WARNER 1984) from YEPD-grown cultures that were harvested at an OD\(_{600}\) of 1.0. Primer extension analysis was conducted essentially as described by STEWART and ROEDER (1989) except that the primer was complementary to the sense strand of HIS4 from nucleotides 40–70 downstream of the HOT1 insertion site. Correct initiation of transcription from HOT1 produces a major product 129 nucleotides long. To normalize the amount of RNA used from the different mutations, a second primer that is complementary to sequences of the CYH2 gene from nucleotides 20–49 downstream of the translation start site (KÄUFER et al. 1983) was used. The major CYH2 transcript produces an 80-nucleotide-long product in primer extension.

These primer extension products were separated on a 7 M urea 8% polyacrylamide gel and the gel was dried. After autoradiography the regions of the gel containing primer extension products were excised and the levels of transcripts were quantitated using a Betascope 603 blot analyzer (Betalen). The percentage transcriptional activity was determined by the formula [(Mt − Nt) / (Wt − Nt)] \(\times 100\%\), where Mt, Nt, and Wt are the normalized transcriptional activities for

**Figure 2**.—Transcription does not occur from HOT1 in rpa135 strains. (A) Transcripts from HOT1 were identified by primer extension in RPA135 and rpa135 strains. Each reaction contained 50 \(\mu\)g of total RNA. HOT1 EI is active HOT1 while IT0H IE is inactive HOT1. (B) Primer extension product from the RPA135 strain containing active HOT1 was run adjacent to the dideoxy-sequence of this same region. Transcription initiated at the circled T in the HOT1 I fragment, which is the same as the normal initiation site in the rDNA array.
Requirements for HOT1 Activity

the enhancer mutation, the enhancer deletion (pG133), and the wild-type enhancer (pG141), respectively. The normalized transcriptional activities were calculated from the formula (H

as expected in the rpu135 disruption

RESULTS

RNA polymerase I activity is essential for the recombination-stimulatory activity of HOT1: Yeast strain NOY408-1a contains pNOY102, an autonomously replicating, high copy number yeast vector containing the 35S rDNA sequences fused to the RNA polymerase II transcription regulatory sequences of GAL7 (Nogi et al. 1991), and a disruption of RPA135, the gene encoding the second largest subunit of RNA polymerase I. NOY408-1b is an isogenic strain containing pNOY102 but a wild-type RPA135 gene. To assay the effect of HOT1 on recombination in these strains, direct duplications of LYS2 sequences separated by CAN1, TRP1, and pBR (Figure 1A) were constructed by transformation. A 575-bp EcoRI fragment containing HOT1 was present in one of the LYS2 genes. Both the orientation of HOT1 that is expected to stimulate exchange (the active orientation), where transcription initiated in HOT1 can proceed across both copies of LYS2, and the opposite orientation (inactive) were tested. Strains containing these duplications did not grow on medium supplemented with canavanine due to the presence of the CAN1 gene. However, these strains contain the can1-100 mutation at the normal chromosomal locus so Can" recombinants can arise by an exchange event that excises the CAN1, TRP1, and pBR sequences along with one copy of LYS2 in the duplication (Figure 1A).

Primer extension analysis showed that HOT1-specific transcripts proceeding across the duplication were produced in RPA135 strains when HOT1 was in the active orientation but not in RPA135 strains containing the inactive orientation of HOT1 in the duplication (Figure 2A). The transcripts produced from the active orientation of HOT1 in RPA135 strains begin at the same initiation site as is used in the normal array of RNA repeats (Figure 2B). As expected, in the rpa135 disruption strains no HOT1-specific transcripts were detected with either the active or inactive orientation of HOT1.

In RPA135 strains grown on media with galactose as the carbon source, HOT1 behaved as expected based on previous studies (Keil and Roeder 1984; Voelkel-Meiman et al. 1987). The active orientation of HOT1 in the LYS2 duplication stimulated excision 240-fold while the inactive orientation did not affect the level of this recombination (Table 1). In contrast, the active orientation of HOT1 did not alter the level of excisive exchange in strains containing a disruption of rpa135 indicating that RNA polymerase I is essential for the recombination hotspot activity of this element. In the inactive orientation HOT1 did not affect the level of excision in the rpa135-disruption strain.

Identification of enhancer sequences required for HOT1 activity: Within the 320-bp EcoRI to HpaI fragment that contains the rDNA enhancer, Ea (Figure 3) is a 72-bp region that has been shown to be important for HOT1 activity (Stewart and Roeder 1989). To more precisely identify the sequences within Ea that are essential for the recombination hotspot activity, random mutations were initially generated in this region. The logic for the approach was that since Ea is fairly large it was not feasible to produce mutations at each nucleotide and determine their effects on HOT1 activity and transcription. Instead, after mutagenesis using mixed oligonucleotides (Ner et al. 1988), a library of potential hot1 mutations was introduced into RLK88-3C by targeted transformation to produce the recombination substrate diagrammed in Figure 1B. The effect of the hot1 mutation on the level of both Ura' recombinants, from excision events, and His' recombinants, from excision or gene replacement events, can be determined from this substrate. Patch tests were used to rapidly screen the library to identify transformants with lower levels of recombination. Southern analysis was performed to ensure that the transformants with low levels of exchange had a single properly integrated plasmid containing a hot1 element. Plasmids containing putative hot1 mutations were isolated by excision (Roeder and Fink 1980), recovered into E. coli, and the enhancer was sequenced. Selected mutations were retransformed into yeast and at least three independent transformants

![Figure 3](image-url)

**Figure 3.** Landmarks within the enhancer fragment of HOT1. REB1 and ABF1 are the binding sites for Reb1p and Abf1p, respectively. DR1 and DR2 are short direct repeats identified by inspection of the sequence. Ea and Eb are regions previously shown to be required for HOT1 activity. PolyT identifies an AT-rich region in Ea, while IR1 and IR2 identify short inverted repeats in Ea. The restriction sites for EcoRI, HindIII and HpaI are abbreviated RI, H and Hp, respectively.

**Table 1.** Summary of transformation experiments with HOT1 enhancer mutants. The level of excisive exchange in strains containing the RPA135 disruption was normalized against the wild-type rDNA enhancer (pG141), respectively. The normalized level of exchange for each enhancer mutation, the enhancer deletion (pG133), and the wild-type enhancer (pG141), respectively. The normalized level of exchange for each enhancer mutation, the enhancer deletion (pG133), and the wild-type enhancer (pG141), respectively. The normalized level of exchange for each enhancer mutation, the enhancer deletion (pG133), and the wild-type enhancer (pG141), respectively. The normalized level of exchange for each enhancer mutation, the enhancer deletion (pG133), and the wild-type enhancer (pG141), respectively.
were analyzed to quantify the effect of the mutation. For wild-type HOT1 the median frequencies of Ura− and His+ recombinants from this substrate are $5.5 \times 10^{-2}$ and $4.0 \times 10^{-3}$, respectively. These are defined as 100% activities (Figure 4A). Without the enhancer the frequencies of Ura− and His+ recombinants were 5.5 and 4.0%, respectively. These are defined as 0% activities.

From 2560 initial transformants 17 showed decreased levels of recombination and contained a single point mutation in the Ea region. Fourteen of these 17 mutations cluster in the region from position 175 to 186. Close examination of the sequence in this area shows that there is a Gbp palindrome (nucleotides 168-173 and 181-186) separated by 7 bp. Three of these 14 mutations (N1, N34, and N14) lie in the inverted repeat on the right (IR2), whereas the other 11 mutations in this region lie between the palindromic sequences. Two,
N15 and N13, contain a mutation in IR1, in which no single mutations were isolated. Again, most of these mutations have relatively small effects on HOT1 activity. These elements containing multiple mutations reinforce the importance of this palindromic region for HOT1.

An obvious concern in identifying mutations by the procedure used is whether the Ea fragment was randomly mutagenized. Two lines of evidence suggest that mutagenesis was fairly random. First, the enhancers from 42 randomly picked transformants that had wild-type HOT1 activity were cloned and sequenced (data not shown). Twenty of these had mutations in Ea and there is no obvious clustering of mutations in any region. None of these mutants contained mutations in the first 12 or the last five nucleotides of Ea, indicating that these regions may not have been as heavily mutagenized as other areas. Second, mutations in the multiply mutant enhancers that occur outside of the palindromic region (Figure 4B) appear to be randomly distributed except for the ends of the Ea element. Based on deletion mutations (see below) the first 12 nucleotides of Ea play, at most, a minor role in HOT1 activity.

To confirm and extend the observations concerning the importance of the palindromic region, we did block mutagenesis of this region (Figure 4C). Mutations G188 and G190 cover the left and the right palindromes, respectively, as well as some of the nucleotides separating these palindromes. Both of these mutations dramatically decrease HOT1 activity, 50- to 100-fold. Block mutations just to the left of this palindromic sequence, G186 and G184, only modestly decrease HOT1 activity.

An obvious structural feature at the left end of Ea is the tract of 27 T's interrupted by two A's (Figures 3 and 4). Point mutations in this region from the random mutagenesis strategy described above do not affect the activity of HOT1 (data not shown). However, more drastic modification of this region could affect HOT1 activity. Block mutagenesis was used to alter this region. Destroying the left half of this poly(dt) tract, G216; the right half, G220; or modifying the middle portion to produce two shorter stretches of poly(dt), G218; produces at most a twofold decrease in HOT1 activity (Figure 4D). However, replacement of the entire poly(dt) tract with a sequence that is not T-rich, G182, produces a 25-fold decrease in HOT1 activity.

DATIN, a poly(dt)-binding protein, does not affect HOT1 activity: WINTER and VARSHAVSKY (1989) characterized the DATIN protein, encoded by the DAT1 gene, that binds to uninterrupted tracts of 9 T's in vivo. Since the poly(dt) tract in Ea is necessary for full HOT1 activity, it was of interest to determine whether DATIN plays a role in stimulating recombination. Deletion of DAT1 has little effect on the recombination-stimulatory activity of HOT1 (Table 2).

**TABLE 2**

<table>
<thead>
<tr>
<th>Insert</th>
<th>Genotype</th>
<th>Percent HOT1 activity (Ura-/His+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>DAT1</td>
<td>0/0</td>
</tr>
<tr>
<td>None</td>
<td>dat1::LEU2</td>
<td>0/1</td>
</tr>
<tr>
<td>HOT1</td>
<td>DAT1</td>
<td>100/100</td>
</tr>
<tr>
<td>HOT1</td>
<td>dat1::LEU2</td>
<td>86/78</td>
</tr>
</tbody>
</table>

**Eb mutations that affect HOT1 activity are clustered:** Random mutagenesis was also used to determine the sequences of the 39-bp Eb region that affect HOT1 activity. From 1200 transformants ten mutants with a single base-pair mutation that dramatically affected HOT1 activity were isolated (data not shown). Twenty of these mutations in Ea and Eb were necessary, but not sufficient for HOT1 activity. From 40 transformants that had wild-type activity were sequenced, twenty of these mutations isolation (Figure 5). These mutations decrease HOT1 activity 5- to 50-fold. All of these mutations cluster between nucleotides 272 and 279 of Eb. One double mutant, C26, contains mutations at both nucleotides 272 and 279 and decreases HOT1 activity almost 100-fold. Recovery of an Eb mutation with such a low level of HOT1 activity, especially for the level of excision (Ura- recombinants), is surprising. In strains where the entire 130-bp HindIII-HpaI fragment that contains Eb is deleted, there is only a 10-fold decrease in the level of excision [(Table 3) VOELKEL-MEIMAN et al. 1987; STEWART and ROEDER 1989].

To insure that the mutagenesis of Eb was random, the Eb region from 40 transformants that had wild-type activity was sequenced. Of the 18 transformants that contained mutations, there was no apparent clustering (data not shown).

**Eb and Ea are necessary, but not sufficient for HOT1 enhancer activity:** Two sites in the enhancer, REB1 and ABF1 (REB2), that bind proteins have been identified based on DNase I footprint analysis (MORROW et al. 1989). Block mutations that alter the REB1-binding site show only minor effects on HOT1 activity (Figure 6A). DNase I footprint analysis showed that the mutations M1 and M2 abolished the REB1-binding site although the M3 mutant still bound Reb1p (data not shown).
Deletion of the first 80 bp of the enhancer (G198), which removes both the REBl- and the ABFl-binding sites, only reduces HOTl activity threefold (Figure 6B). Examination of the sequence of the enhancer identified 12 bp direct repeats, DR1 and DR2, in which 11 of the 12 bp are identical (nucleotides 80–91 and 104–115). These repeats are separated by 12 bp. Deletion G284, which removes DR1 and the nucleotides separating DR1 and DR2 (nucleotides 81–104), reduces HOTl activity threefold (Figure 6B). Deleting DR2 (G286, nucleotides 97–114) or DR1 and DR2 (G251, nucleotides 81–114) decreases the activity fourfold. Unexpectedly, deletions G282 and G200 that simultaneously remove REBl, ABFl and at least DRl (as well as the intervening sequences) completely abolish HOTl activity (Figure 6B). Thus, in addition to Ea and Eb, some sequences within the first 104 nucleotides of the EcoRI-HpaI enhancer fragment are necessary for HOTl activity.

**Effect of hotl mutations on transcription:** Since stimulation of recombination is associated with high levels of transcription for HOTl, the hotl mutations, which were isolated based on their effect on recombination, may also alter transcription. Any deviation from this relationship would suggest that certain sequences in the enhancer are preferentially involved in one activity or the other. Using primer extension the relative level of transcription from hotl mutations was determined (Figures 4 and 5). The vast majority of the mutations examined have similar effects on both HOTl activity and on transcription. Two exceptions to this generalization are observed. First, when only the 190-bp EcoRI-HindIII fragment is present, recombination is reduced 10-fold but there is only a minor (20%) reduction in transcription (Table 3). Second, two of the point mutations in Ea, N42 and N1 that slightly decrease HOTl activity actually increase transcription (Figure 4A). These point mutations are in the inverted repeat region of Ea. Other point mutations in this region decrease both recombination and transcription. It is not clear why these two mutations behave differently but their effects on both recombination and transcription are rather small.

**DISCUSSION**

**RNA polymerase I is essential for HOTl activity:** Deleting the gene encoding the second largest subunit of
RNA polymerase I destroys the recombination-stimulatory activity of HOT1 and abolishes transcription initiation from the RNA polymerase I transcription initiation site in HOT1. This further supports the contention that there is an association between high levels of transcription and localized increases of mitotic recombination. Our results do not directly show that active transcription is required for the recombination hotspot activity. It could be argued that efficient binding of RNA polymerase I to HOT1 is all that is required to stimulate recombination. However, our results in conjunction with other features of HOT1 argue that transcription from HOT1 is required. First, HOT1 shows directionality (Keil and Roeder 1984). It only stimulates recombination in sequences adjacent to one side. Those are the sequences across which transcription initiated within the I fragment would proceed. Second, insertion of the RNA polymerase I transcription termination site between HOT1 and the recombining sequences abolishes hotspot activity (Voelkel-Meiman et al. 1987). If only binding of RNA polymerase I were required to stimulate recombination, it is difficult to explain these two features of HOT1.

The HOT1 enhancer has a complex structure: Our results suggest that at least four separate regions within the enhancer are required for HOT1 activity. These include two separate regions in Ea (the poly(dT) tract and sequences in the vicinity of IR1 and IR2), a region within Eb, and a region in the first 104 nucleotides of the enhancer fragment that is less defined and may be complex. The existence of this last region is implied from experiments defining the sequences that are sufficient for HOT1. Deletions that remove most of the sequences to the left of Ea completely destroy HOT1 activity. We tested shorter deletions in this area that together span the entire region. None of them affected HOT1 activity nearly as dramatically as the complete deletions. Several explanations are possible for this finding. First, there may be sequences in the region to the left of Ea that have overlapping functions. Thus HOT1 could still be active if some of the sequences are present but not when all of them are deleted. Second, these sequences may provide a buffer that normally protects HOT1 from antagonistic activities in adjacent sequences. Third, the particular junction formed by the large deletions G282 and G200 (Figure 6B) may produce a sequence that poisons HOT1 activity. We do not favor the third possibility since two different deletions of this region abolish HOT1 activity. It seems unlikely that both produce a poison sequence when a number of other mutations tested do not have this effect.

Our data also suggest there may be a fifth region that affects HOT1 activity. This sequence, which is within the HindIII-HpaI fragment, appears to act as a suppressor of HOT1 that antagonizes the activity of the Eb sequences. The presence of such a sequence is implied by the A44 and C26 mutations in Eb (Figure 5) that decrease the frequency of Ura- recombinants more than when the HindIII-HpaI fragment is completely deleted (Table 3). Stewart and Roeder (1989) also suggested a suppressor in this region based on a deletion that increased HOT1 activity ~30%. We attempted to identify hyperrecombination mutations in these screens but none were found. The patch tests used in our studies to initially screen potential mutations may not be sensitive enough to identify such a small increase or the suppressor sequences may lie outside the regions subjected to mutagenesis.

Association of mitotic recombination and transcription: In general, mutations that dramatically decrease HOT1 activity also substantially decrease the transcriptional activity of the enhancer (Figures 4 and 5), further supporting the contention that there is an association between mitotic recombination and high levels of transcription. The most dramatic departure from this relationship is when the 130-bp HindIII-HpaI fragment is deleted. This produces a 10- to 30-fold decrease in HOT1 activity but only a 20% decrease in transcription (Table 3). These results agree with the localization of the transcription enhancer within the EcoRI-HindIII fragment (Elion and Warner 1984). However, a perplexing finding is that single-base pair mutations in the Eb region contained in the HindIII-HpaI fragment can dramatically reduce transcription as much as 12-fold (Figure 5). This suggests that the transcriptional effect of the HindIII-HpaI fragment is complex. A possible explanation is that this fragment contains separate stimulatory and repressor sequences. The sequences defined by the mutations in Eb are important for the stimulatory activity. In wild type the stimulatory and repressor effects appear to cancel out each other. However, when the stimulatory sequences are inactivated by mutation, the repressor sequences substantially decrease transcription. A similar explanation was given above for the unexpectedly low levels of recombination observed with some of the Eb mutations.

Several features about this model are important to note. The sequences required to stimulate transcription and recombination overlap and may be the same. However, their effects are not identical since HOT1 activity is substantially increased by the addition of the HindIII-HpaI fragment while transcription is not (Table 3). This indicates that the interaction between the stimulatory and repressor activities is different for transcription and recombination.

Our data do not provide any evidence regarding the location of the proposed repressor sequences in the HindIII-HpaI fragment. They could be the same or different sequences for recombination and transcription. However, Stewart and Roeder (1989) found a mutation in the HindIII-HpaI fragment that increased both recombination and transcription. This suggests that the repressor sequences may be the same for these two activities.

It is interesting to note that in an in vitro assay the HindIII-HpaI fragment by itself provided maximum en-
hancer activity (SCHULZ et al. 1993). If the suppressor that is implied by the above argument is inactivated during preparation of the in vitro extract, our model would predict that this fragment by itself would stimulate transcription.

Mitotic vs. meiotic recombination hotspots: In addition to the studies on HOT1, other studies indicate an association between high levels of mitotic recombination and high levels of transcription in organisms ranging from bacteria to mammals (for a recent review see GANGLOFF et al. 1994). In Schizosaccharomyces pombe it has also been reported that high levels of transcription stimulate meiotic exchange (GRIMM et al. 1991), but in S. cerevisiae it has been demonstrated that the meiotic recombination hotspots at ARG4 and HIS4 do not depend on high levels of transcription (SCHULTZ and SZOSTAK 1991; WHITE et al. 1992). Furthermore, HOT1 does not act as a meiotic hotspot (KEIL and ROEDER 1984). However, the ARG4 meiotic hotspot requires a poly(dT) tract of 14 bases for much of its activity (SCHULTZ and SZOSTAK 1991) and HOT1 requires a long poly(dT) tract for its mitotic hotspot function. The function of the poly(dT) tracts for these hotspots is not clear. It is possible that they serve different roles for the two hotspots. Poly(dT) tracts have been shown to act as upstream promoter elements in a number of systems (for examples see STRUHL 1985; FASHENA et al. 1992). In fact, in addition to our demonstration that the poly(dT) sequence in HOT1 plays a role in RNA polymerase I transcription, it has been shown that this same tract activates RNA polymerase II transcription in vivo (LORCH et al. 1990). It has been suggested that poly(dT) tracts may provide an entry site for RNA polymerases (RUSSELL et al. 1983) and this may be the role played by this tract in the rDNA/HOT1 enhancer. Poly(dT) tracts have also been reported to be located in nuclease sensitive sites (BOETTCHER 1990; LOSA et al. 1990). This may be the case for such tracts at meiotic recombination hotspots like the one at ARG4 (SCHULTZ and SZOSTAK 1991). Both of these functions may result from the ability of poly(dT) tracts to disrupt nucleosome formation (KUNKEL and MARTINSON 1981; NELSON et al. 1987; PRUNELL 1982). Since HOT1 does not stimulate meiotic exchange (KEIL and ROEDER 1984), it is clear that a poly(dT) tract is not sufficient to produce a meiotic hotspot.

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