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 independent 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).

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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 *GAL7* 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 *Eco*RI-*Hpa*I fragment, E, that contains the rDNA transcription enhancer and a 255-bp *Sma*I-*Eco*RI 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 trp1-HIII lys2-ΔBX can1^R* (LIN and KEIL 1991)], NOY408-1a [*MATa rpa135::LEU2 ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100* containing plasmid pNOY102 (NOGI *et al.* 1991)], and NOY408-1b (*MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100* containing plasmid pNOY102 (NOGI *et al.* 1991). Plasmid pNOY102 is a high copy number plasmid containing *URA3* and the 35S rDNA sequences fused to the *GAL7* promoter at the 5′ end and the *GAL7* terminator at the 3′ end (NOGI *et al.* 1991).

Deletion of the *DAT1* gene was accomplished by transforming *Hin*dIII-cleaved pUC19*dat1Δ::LEU2* (WINTER and VARSHAVSKY 1989) into derivatives of RLK88-3C containing appropriate recombination substrates. Proper deletions were identified by Southern analysis (SAMBROOK *et al.* 1989).

The *Escherichia coli* strains were as follows: MC1066 [*leuB trpC pyrF*::Tn*5* (Kan^R) *araT lacX74 del strA hsdR hsdM* (obtained from M. CASADABAN)], which was used for propagating double-stranded plasmids; JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiD (lac-proAB)* F′ (*traD36 proAB$^+$ lacI^q lacZΔM15*) (YANISCH-PERRON *et al.* 1985)], which was used to propagate bacteriophage M13 recombinants and vectors carrying the bacteriophage f1 origin to produce single-stranded derivatives; and RZ1032 [HfrKL16 PO/45 (*lysA*{61–62}) *dut1 ung1 thi1 relA1* (KUNKEL *et al.* 1991)], which was used to produce uracil-containing M13 templates for oligonucleotide-directed mutagenesis.

Yeast media containing glucose were prepared as described previously (LIN and KEIL 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 pL1038, which contains the *LYS2* gene and the 3.9-kb *Sal*I-*Xho*I fragment of *CAN1* inserted in pBR322 (LIN and KEIL 1991), was cleaved at the unique *Sal*I site, treated with the Klenow fragment of DNA polymerase I to make it blunt ended, and ligated to the 830-bp *Eco*RI-*Stu*I fragment containing *TRP1,* which had also been made blunt ended by treatment with the Klenow fragment. The resulting plasmid, pG306, was cleaved at the unique *Xho*I site in *LYS2,* made blunt ended with the Klenow fragment, and ligated with a blunt ended 575-bp *Eco*RI fragment containing *HOT1.* Plasmid pG312 contains *HOT1* in the active orientation while plasmid pG314 contains the inactive orientation.

These plasmids, pG306, pG312 and pG314, were cleaved at the unique *Stu*I 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 pNOY102 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 assaying the effect of enhancer mutations on *HOT1* activity, plasmid pL524, which contains part of *HIS4* in YIp5 (VOELKEL-MEIMAN *et al.* 1987), was cleaved at the unique *Bam*HI site that had been inserted in the *HIS4* sequences. A *Bgl*II-*Bam*HI-*Xba*I-*Eco*RI-*Bgl*II polylinker was inserted. The resulting vector was cleaved with *Xba*I and *Eco*RI and a 250-bp *Xba*I-*Eco*RI fragment containing the RNA 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 *Xba*I-*Eco*RI fragment was constructed by ligating a *Xba*I linker at the *Sma*I site at the 5′ end of the initiation site. A 540-bp *Eco*RI-*Bam*HI fragment containing the f1 origin from pUC-f1 (Pharmacia) was blunt ended by treatment with the Klenow fragment and inserted at the unique *Sma*I site of this vector to create pG133. This vector was cleaved with *Bam*HI and *Xba*I, and a 320-bp *Bam*HI-*Xba*I fragment containing the enhancer was inserted to produce pG141. The original *Eco*RI and *Hpa*I ends of the enhancer had previously been converted to *Bam*HI and *Xba*I 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 pG1 was used for the production of random mutations. This contains the 320-bp *Eco*RI-*Hpa*I enhancer fragment of *HOT1* with the *Hpa*I site replaced by a *Xba*I linker inserted into *Eco*RI and *Xba*I cut M13mp19. A *Bam*HI linker was then inserted at the *Eco*RI 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 (NER *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 49 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 mutagenizing, 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°, 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 *Bam*HI and *Xba*I, 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 pG133 vector was digested with *Bam*HI and *Xba*I, 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 Amp^R transformants were recovered for each of the two initial pools of oligonucleotides, inoculated into LB + Amp media and grown overnight. DNA

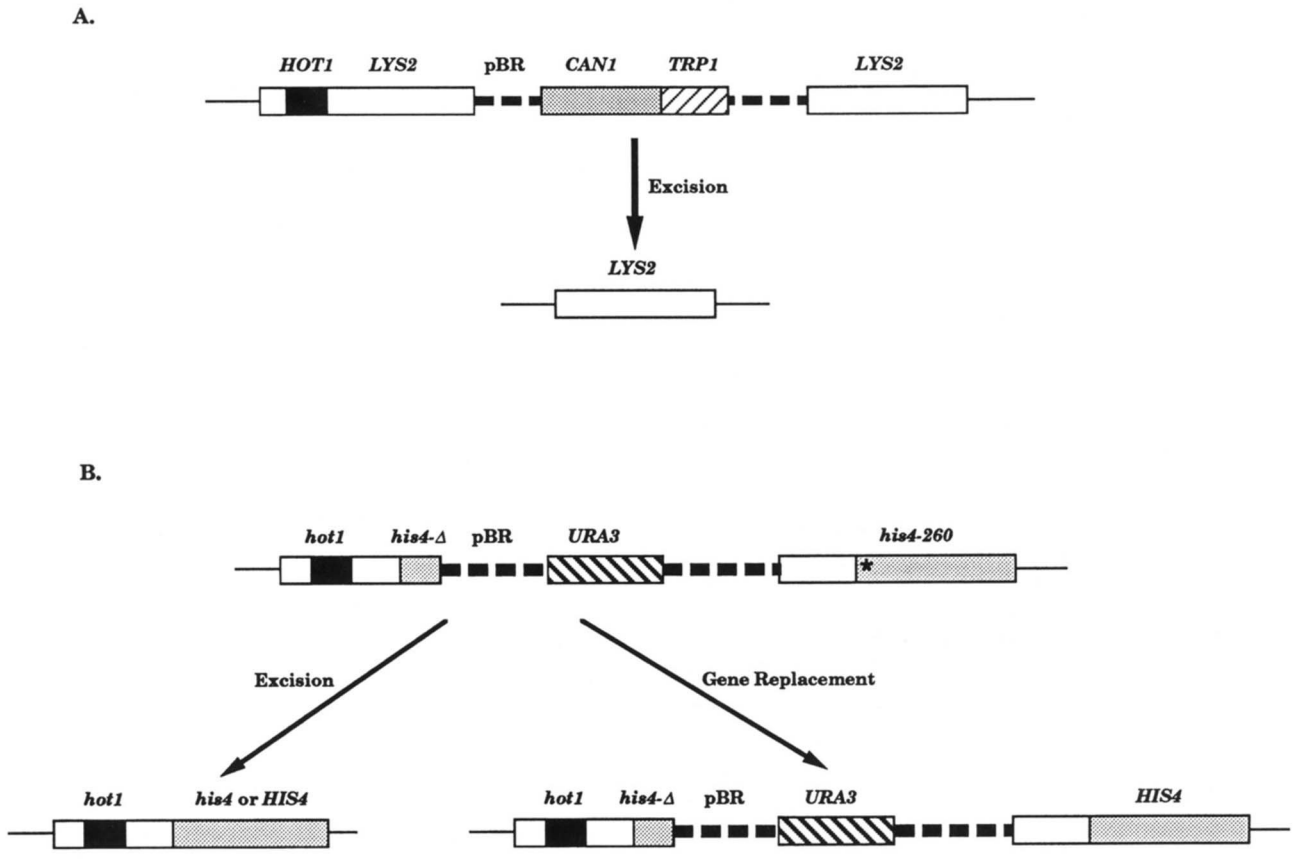


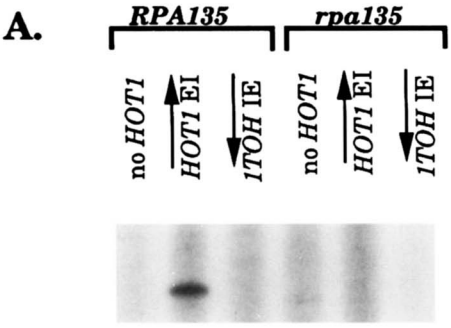
FIGURE 1.—Intrachromosomal recombination substrates. (A) To measure the effect of *rpa135* on *HOT1* activity, a direct duplication of *LYS2* genes separated by *CAN1, TRP1* and pBR sequences was constructed by integrative transformation at the normal *LYS2* chromosomal locus. Excision of this duplication produces Can^R 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-FOA^R) 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 *Cla*I 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 *Cla*I, 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. Dideoxy-sequencing (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 *Bam*HI and *Xba*I, and ligated into the purified *Bam*HI-*Xba*I 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 *Xho*I linker insertion mutations previously constructed by STEWART and ROEDER (1989) were used to construct the deletion mutations. Their mutations that we



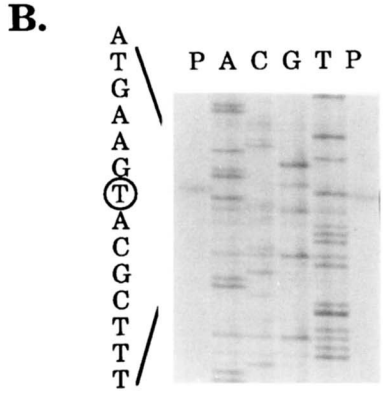


FIGURE 2.—Transcription does not occur from *HOT1* in *rpa135* strains. (A) Transcripts from *HOT1* were identified by primer extension in *RPA135* and *rpa135* strains. Each reaction contained 50 μg of total RNA. *HOT1* EI is active *HOT1* while *1TOH* IE is inactive *HOT1*. (B) Primer extension product 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.

used are p35, with a *Xho*I linker inserted after nucleotide 80 of the enhancer; p82, with a *Xho*I linker after nucleotide 120 and nucleotides 115 to 120 duplicated; and p124, with a *Xho*I linker replacing nucleotides 97 to 104. Each of these vectors was cleaved with *Xho*I and treated with the Klenow fragment, and a *Bam*HI linker was inserted. The *Bam*HI-*Xba*I enhancer fragments from each of the resulting vectors were purified and ligated into the purified *Bam*HI-*Xba*I plasmid backbone of pG133. This produces enhancers containing deletions of nucleotides 1–80, 1–114, and 1–104 from the p35, p82 and p124 vectors, respectively. To construct an enhancer with nucleotides 81–114 deleted, a *Bam*HI linker was inserted at the *Eco*RI site at the 5′ end of the enhancer in p35. The 80-bp *Bam*HI-*Xho*I fragment of this plasmid containing the 5′ end of the enhancer was ligated to the 200-bp *Xho*I-*Xba*I fragment of p82 containing the 3′ end of the enhancer, and the resulting 280-bp *Bam*HI-*Xba*I enhancer fragment was ligated into the *Bam*HI-*Xba*I plasmid backbone of pG133. An enhancer with nucleotides 81–104 deleted was constructed in a similar fashion, except the 80-bp *Bam*HI-*Xho*I fragment with the 5′ end of the enhancer was ligated to the 216-bp *Xho*I-*Xba*I fragment of p124. The enhancer with nucleotides 97–114 deleted was constructed by inserting a *Bam*HI linker at the *Eco*RI site at the 5′ end of the enhancer in p124. The 96-bp *Bam*HI-*Xho*I fragment from this plasmid was ligated to the 200-bp *Xho*I-*Xba*I fragment of p82 containing the 3′ end of the enhancer. The resulting enhancer fragment was ligated into *Bam*HI-*Xho*I-cleaved pG133. Three independent transformants for each of these deletion mutations were examined for *HOT1* activity as described for the block mutations.

TABLE 1

Effect of RNA polymerase I mutation on *HOT1* activity

Genotype	Construct	Median frequency of Can^R recombinants ($\times 10^4$)
RPA135	No *HOT1*	2.3
RPA135	$\overrightarrow{HOT1}$ EI	560
RPA135	$\overleftarrow{1TOH}$ IE	2.8
rpa135::LEU2	No *HOT1*	2.9
rpa135::LEU2	$\overrightarrow{HOT1}$ EI	3.3
rpa135::LEU2	$\overleftarrow{1TOH}$ IE	4.8

$\overrightarrow{HOT1}$ EI indicates the active orientation of the recombination hotspot while $\overleftarrow{1TOH}$IE indicates the inactive orientation.

Analysis of *HOT1* activity: *HOT1* activity was assayed essentially as described previously (KEIL and ROEDER 1984). However, after growth in liquid SC, 5 μl 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)] × 100%.

Transcription analysis: Primer extension was used to analyze the effect of various enhancer mutations on RNA polI 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 (Betagen). The percentage transcriptional activity was determined by the formula [(Mt − Nt)/(Wt − Nt)] × 100%, where Mt, Nt, and Wt are the normalized transcriptional activities for

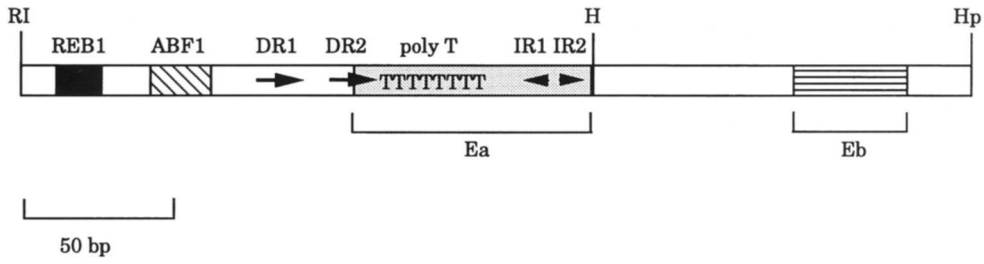


FIGURE 3.—Landmarks within the enhancer fragment of *HOT1*. REB1 and ABF1 are the binding sites for Reb1p and Abf1p, respectively. DR1 and DR2 are short direct repeats identified by inspection of the sequence. Ea and Eb are regions previously shown to be required for *HOT1* activity. PolyT identifies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

the enhancer mutation, the enhancer deletion (pG133), and the wild-type enhancer (pG141), respectively. The normalized transcriptional activities were calculated from the formula (H − Hb)/(C − Cb), where H and C are the number counts in the *HOT1* and *CYH2* primer extension bands, respectively. Hb and Cb are the number of background counts for H and C, respectively, obtained by counting an area the same size as H or C immediately above the H or C band.

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 *Eco*RI 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 do 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^R 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 rDNA 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 *Eco*RI to *Hpa*I 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

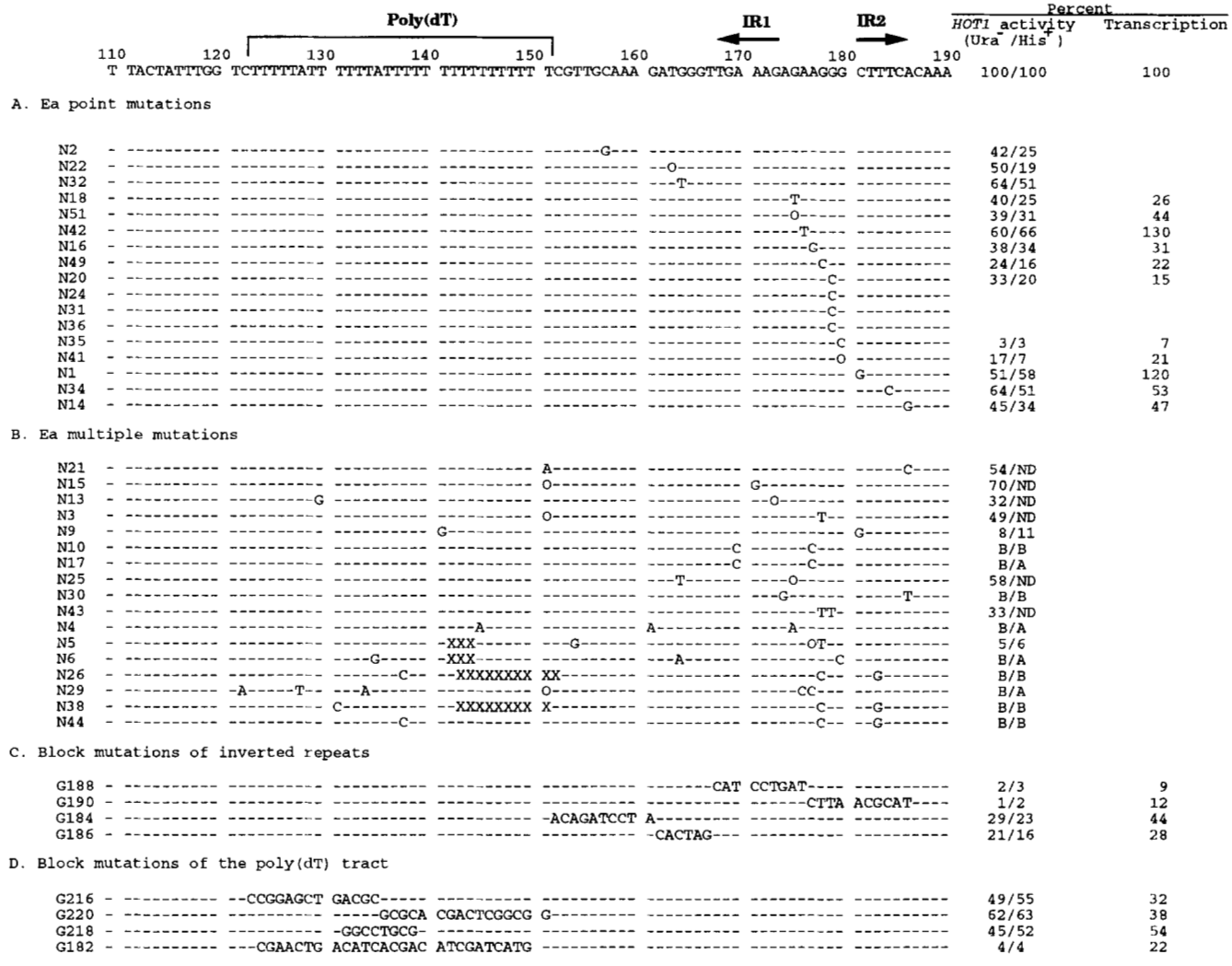


FIGURE 4.—Effect on recombination and transcription of *hot1* mutations in the Ea region of the enhancer. Point mutations (A) and multiple mutations (B) were produced by doping mutagenesis. Block mutations of the inverted repeats (C) and of the poly(dT) tract (D) were produced by site-directed mutagenesis. The altered base pairs are shown for each mutation. O, a base pair deletion; X, the total number of T's in the poly(dT) tract was not unambiguously determined. The effect of the mutations on recombination (*HOT1* activity) is assessed by the frequency of Ura^- and His^+ recombinants. These are given as percentages where the frequency for wild-type *HOT1* equals 100%, while the frequency when the enhancer is deleted from *HOT1* equals 0%. For some of the multiple mutations only qualitative replica-plating assays were performed. A, 0–30% *HOT1* activity; B, 30–50% activity. For some of the multiple mutations the frequency of His^+ recombinants was not determined (ND). The effect of selected mutations on transcription from the I site was quantitated by primer extension. The relative values are given as percentages with wild-type *HOT1* and the enhancer deletion mutation defined as 100% and 0%, respectively.

were analyzed to quantitate the effect of the mutation. For wild-type *HOT1* the median frequencies of Ura^- and His^+ recombinants from this substrate are 5.5×10^{-2} and 4.0×10^{-3}, respectively. These are defined as 100% activities (Figure 4A). Without the enhancer the frequencies of Ura^- and His^+ recombinants are 1.5×10^{-4} and 2.0×10^{-4}. 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 there is a 6-bp 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. Most of the single point mutations recovered show a modest, but reproducible, two- to fivefold decrease in *HOT1* activity (Figure 4A). The only two exceptions are mutations at position 180, which is located just to the left of IR2. Mutation N35, a change of the G at position 180 to a C, gives a 30- to 40-fold reduction in *HOT1* activity, while mutation N41, a deletion of basepair 180, produces a 5- to 15-fold reduction.

Seventeen mutations that have multiple base changes and showed decreased levels of *HOT1* activity were also identified (Figure 4B). Interestingly, in all of the *hot1* enhancers that contain multiple mutations, at least one of the mutations occurs in the palindromic region. Two,

TABLE 2

Effect of *DAT1* mutation on *HOT1* activity

Insert	Genotype	Percent *HOT1* activity (Ura⁻/His⁺)
None	*DAT1*	0/0
None	*dat1::LEU2*	0/1
HOT1	*DAT1*	100/100
HOT1	*dat1::LEU2*	86/78

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 vitro*. 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).

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 (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 *Hin*dIII-*Hpa*I 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).

Ea and Eb 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 DNaseI 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).

		Percent	
		HOT1 activity (Ura⁻/His⁺)	Transcription
	260 270 280 290		
	AG CGGCAAACAT GAGTGCTTGT ATAAGTTTAG AGAATTG	100/100	100
C20	-- ---------- -C-------- ---------- -------	7/5	8
A22	-- ---------- -C-------- ---------- -------	13/8	16
A4	-- ---------- -C-------- ---------- -------		
A44	-- ---------- -G-------- ---------- -------	2/8	16
A11	-- ---------- -----G---- ---------- -------	5/5	20
A12	-- ---------- -----A---- ---------- -------	12/7	20
C4	-- ---------- -----O---- ---------- -------	8/4	11
C19	-- ---------- -----O---- ---------- -------		
C6	-- ---------- -------C-- ---------- -------	17/4	20
A46	-- ---------- --------C- ---------- -------	21/21	29
C26	-- ---------- -C------A- ---------- -------	2/1	7

FIGURE 5.—Effect on recombination and transcription of *hot1* mutations in the Eb region of the enhancer. Nomenclature is the same as in Figure 4.

TABLE 3

Effects of enhancer deletions on *HOT1* activity and transcription

Enhancer fragment present	Percent *HOT1* activity (Ura^-/His^+)	Percent transcription
*Eco*RI-*Hin*dIII-*Hpa*I	100/100	100
*Eco*RI-*Hin*dIII	10/3	80
*Hin*dIII-*Hpa*I	0/0	2
None	0/0	0

Deletion of the first 80 bp of the enhancer (G198), which removes both the REB1- and the ABF1-binding sites, only reduces *HOT1* 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 *HOT1* 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 REB1, ABF1 and at least DR1 (as well as the intervening sequences) completely abolish *HOT1* activity (Figure 6B). Thus, in addition to Ea and Eb, some sequences within the first 104 nucleotides of the *Eco*RI-*Hpa*I enhancer fragment are necessary for *HOT1* activity.

Effect of *hot1* mutations on transcription: Since stimulation of recombination is associated with high levels of transcription for *HOT1*, the *hot1* 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 *hot1* mutations was determined (Figures 4 and 5). The vast majority of the mutations examined have similar effects on both *HOT1* activity and on transcription. Two exceptions to this generalization are observed. First, when only the 190-bp *Eco*RI-*Hin*dIII 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 *HOT1* 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 *HOT1* activity: Deleting the gene encoding the second largest subunit of

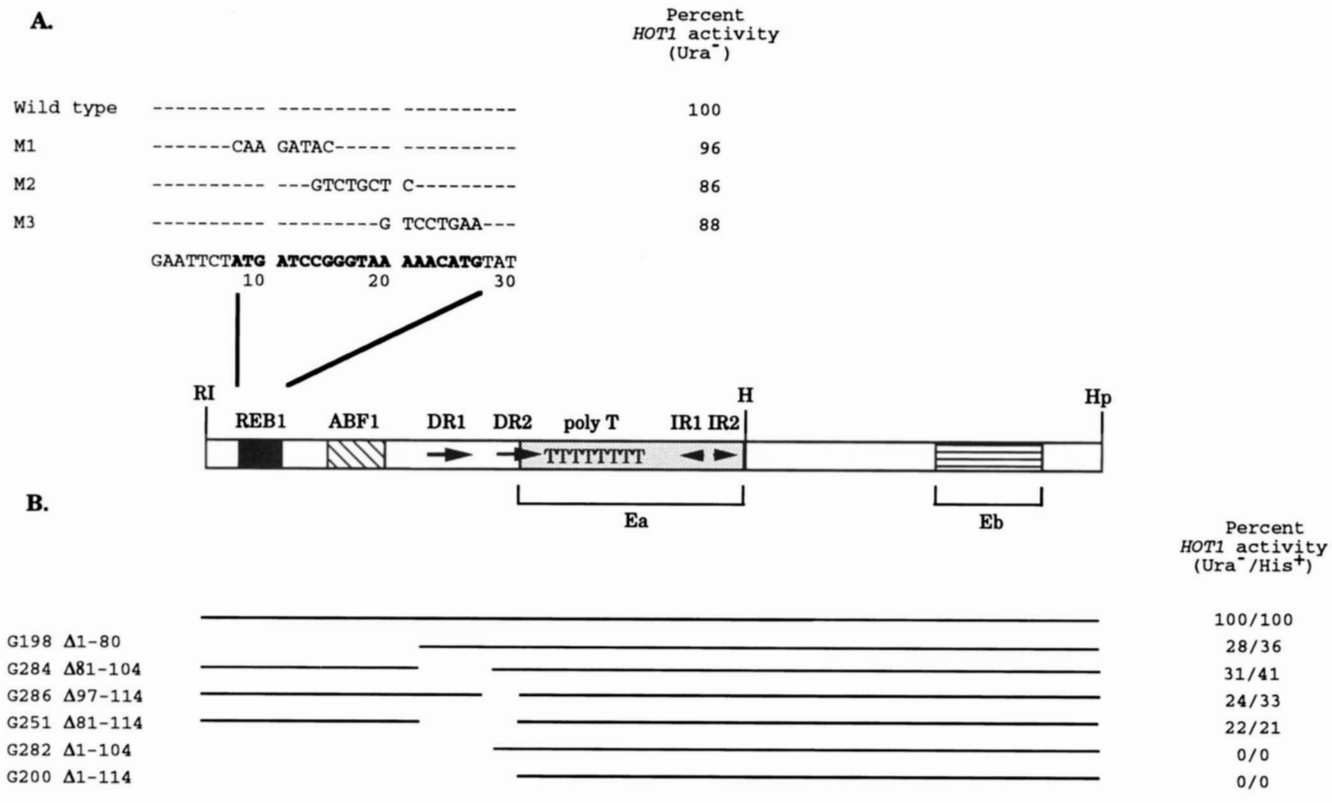


FIGURE 6.—Mutations of regions outside of Ea and Eb. (A) Block mutations of various portions of the REB1-binding site were tested for effects on *HOT1* activity by measuring the frequency of Ura^- recombinants. (B) Various deletions of the enhancer were tested for their effect on *HOT1* activity. The sequences present in each of the enhancer constructs are indicated by the lines. Other nomenclature is as in Figure 4.

RNA polymerase I destroys the recombination-stimulatory activity of *HOT1* and abolishes transcription initiated 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 *Hin*dIII-*Hpa*I 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 *Hin*dIII-*Hpa*I 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 *Hin*dIII-*Hpa*I 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 *Eco*RI-*Hin*dIII fragment (ELION and WARNER 1984). However, a perplexing finding is that single-base pair mutations in the Eb region contained in the *Hin*dIII-*Hpa*I fragment can dramatically reduce transcription as much as 12-fold (Figure 5). This suggests that the transcriptional effect of the *Hin*dIII-*Hpa*I 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 *Hin*dIII-*Hpa*I 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 *Hin*dIII-*Hpa*I fragment. They could be the same or different sequences for recombination and transcription. However, STEWART and ROEDER (1989) found a mutation in the *Hin*dIII-*Hpa*I 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 *Hin*dIII-*Hpa*I fragment by itself provided maximum en-

hancer activity (SCHULTZ *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 (SCHULTES 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 (SCHULTES 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; WANG *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* (SCHULTES 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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