MOLECULAR AND CELLULAR BIOLOGY, Aug. 1989, p. 3464–3472
0270-7306/89/083464-09$02.00/0

Vol. 9, No. 8

Transcription by RNA Polymerase I Stimulates Mitotic Recombination in *Saccharomyces cerevisiae*

SUE ELLEN STEWART AND G. SHIRLEEN ROEDER*

Department of Biology, Yale University, New Haven, Connecticut 06511

Received 27 March 1989/Accepted 16 May 1989

The recombination-stimulating sequence *HOT1* is derived from the ribosomal DNA array of *Saccharomyces cerevisiae* and corresponds to sequences that promote transcription by RNA polymerase I. When inserted at a chromosomal location outside the ribosomal DNA array, *HOT1* stimulates mitotic recombination in the adjacent sequences. To investigate the relationship between transcription and recombination, transcription promoted by *HOT1* was directly examined. These studies demonstrated that transcription starts at the RNA polymerase I initiation site in *HOT1* and proceeds through the chromosomal sequences in which recombination is enhanced. Linker insertion mutations in *HOT1* were generated and assayed for recombination stimulation and for promoter function; this analysis demonstrated that the same sequences are required for both activities. These results indicate that the ability of *HOT1* to enhance recombination is related to, and most likely dependent on, its ability to promote transcription.

The mechanisms by which cells control the frequency and distribution of recombination events are poorly understood. One approach to examining this regulation is to isolate chromosomal sequences that locally stimulate exchange. To this end, a *cis*-acting sequence that enhances mitotic recombination was isolated from a genomic library of the yeast *Saccharomyces cerevisiae* (15). This hotspot sequence, called *HOT1*, was identified as part of the ribosomal DNA (rDNA) array. Subcloning analysis showed that the sequences necessary for *HOT1* activity are localized on two noncontiguous fragments (Fig. 1). A 255-base-pair (bp) fragment (I) containing the initiation site of the 35S rRNA transcript and a 320-bp fragment (E) containing an enhancer of polymerase I transcription are both required to stimulate exchange (35).

When inserted at the *HIS4* locus on chromosome III, *HOT1* stimulates intrachromosomal recombination between direct repeats of *HIS4* sequences. Stimulation is observed only when *HOT1* is oriented such that a transcript initiating in the I region would proceed toward the recombining segments. This orientation specificity is conferred by the I region; the enhancer fragment functions in either orientation (35). When a putative termination signal for RNA polymerase I transcription (17) is inserted between *HOT1* and the adjacent sequences involved in recombination, the stimulation of exchange is eliminated (35). These observations suggest that transcription by RNA polymerase I, initiating in *HOT1* and proceeding through the adjoining sequences, is responsible for the stimulation of recombination.

To confirm and expand on previous observations, the mechanism of *HOT1* action was investigated by examining the association between *HOT1*-stimulated recombination and *HOT1*-promoted transcription. Linker insertion mutagenesis was used to identify regions within *HOT1* that are necessary for hotspot activity. Patterns of transcription were examined in strains carrying the *his4* duplication used to assay intrachromosomal recombination. These studies demonstrated that transcription from *HOT1* extends through the regions involved in recombination. Furthermore, mutations that alter *HOT1*-stimulated recombination coordinately affect *HOT1*-promoted transcription.

MATERIALS AND METHODS

Strains and media. All yeast strains used were derived by transformation of strain RLK1-3C (α *his4-260 ade2-1 ura3-52* Can^R [15]). Synthetic complete (SC) and SC media lacking histidine and uracil were prepared as described by Sherman et al. (28). Medium containing 5-fluoro-orotic acid was prepared by supplementing SC medium lacking uracil to 0.5 mM uracil and 0.88% 5-fluoro-orotic acid (2). All plasmid constructions and preparations were performed with *Escherichia coli* DB6507 (hsdR hsdM *recA13 thr-1 leuB thi-1 endA proA2 supE44 pyr*F::Tn*5* Kan^r) and R895 (*recA* Δ*lacU169* hsdR *pyrF*::Tn*5* Kan^r [8]). Bacterial media are described by Maniatis et al. (24).

Plasmid constructions. The *HOT1* E region on an *Eco*RI-*Hpa*I fragment and the I region on a *Sma*I-*Eco*RI fragment were inserted in a *Bgl*II-*Bam*HI-*Xba*I-*Eco*RI-*Bgl*II polylinker (35) in the relative orientation found in the intact rDNA array. The DNA sequence of the resulting 613-bp *HOT1 Bgl*II fragment is shown in Fig. 2; the E and I sequences are separated by a 20-bp linker-derived sequence containing the *Xba*I restriction site. For in vitro linker insertion mutagenesis, this *Bgl*II fragment was inserted into a modified pBR322 vector. A derivative of pBR322 lacking the *Hin*dIII site was digested with *Eco*RI, blunt ended with Klenow fragment, and ligated with *Bgl*II linkers. The 613-bp *HOT1 Bgl*II fragment was inserted at this new *Bgl*II site in both orientations to generate plasmids pSES5 and pSES6 (Fig. 3).

In vitro mutagenesis. The *HOT1 Bgl*II fragment was mutagenized by linearization of plasmids pSES5 and pSES6, followed by insertion of a linker sequence. The pSES6 plasmid was linearized with restriction endonucleases. Enzymes with unique sites in *HOT1* sequences (*Hin*dIII and *Sna*BI) were used as recommended by the supplier (New England BioLabs, Inc.). Enzymes that recognize multiple sites (*Rsa*I, *Xmn*I, and *Ava*I) were used for partial restriction digests, using 0.5 to 12 U of enzyme per μg of plasmid DNA for 1 to 15 min.

The pSES5 plasmid was linearized at random positions

* Corresponding author.

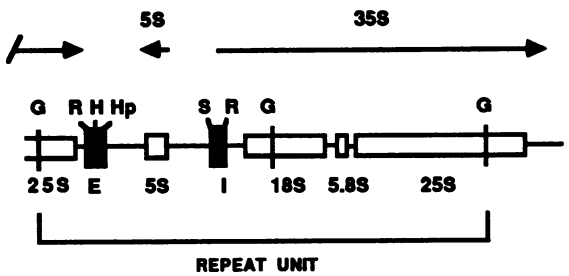

FIG. 1. Organization of the rDNA of *S. cerevisiae*. There are about 140 copies of the rDNA repeat unit, organized as a tandem array of directly repeated elements on chromosome XII. Open boxes represent the mature rRNA species; arrows indicate transcription units. The 35S rRNA precursor is transcribed by RNA polymerase I, and the divergent 5S transcript is transcribed by RNA polymerase III. *HOT1* was originally isolated on the *Bgl*II fragment encompassing the nontranscribed spacer. The enhancer (E) and initiator (I) fragments that comprise the active subclone of *HOT1* are shown (■). Abbreviations for restriction sites: R, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; S, *Sma*I; G, *Bgl*II. Not all sites are indicated.

with DNase I in the presence of Mn^{2+} as described by Heffron et al. (13). In this reaction, 20 μg of CsCl gradient-purified plasmid was incubated with 1.5×10^{-2} U of DNase I (Pharmacia, Inc.) at 24°C for 20 min in 20 mM Tris (pH 7.5)–1.5 mM $MnCl_2$–0.1 mg of bovine serum albumin per ml. The DNA was then extracted with phenol-$CHCl_3$ and ethanol precipitated.

Linear molecules were purified from agarose gels by adsorption to an NA45 DEAE membrane according to the

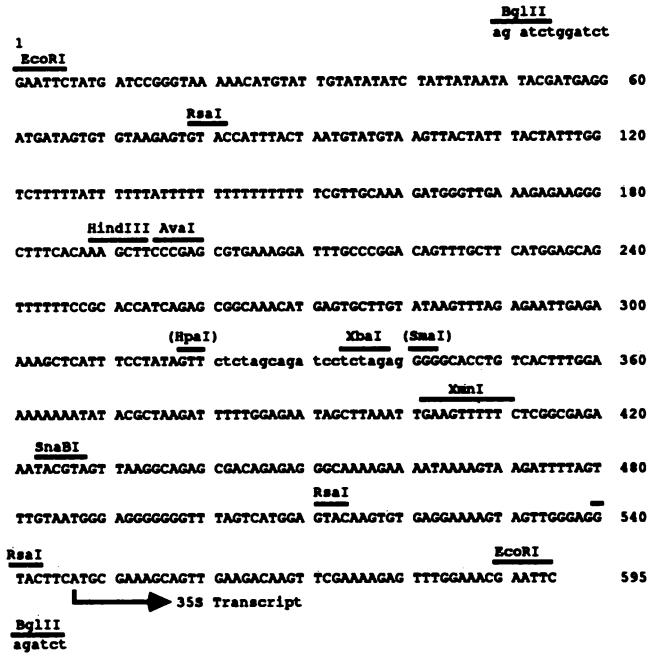

FIG. 2. Nucleotide sequence of the *HOT1 Bgl*II fragment used for in vitro mutagenesis. The 320-bp *Eco*RI-*Hpa*I E fragment and the 255-bp *Sma*I-*Eco*RI I fragment were joined on a *Bgl*II fragment as described in the text. Sequences in uppercase are *HOT1* sequences; those in lowercase are linker sequences. Restriction sites are shown above the sequence; (*Hpa*I) and (*Sma*I) are partial sites. Sequences are numbered beginning at the *Eco*RI site of E, proceeding through the *Xba*I linker region, and ending at the *Eco*RI site of I. The initiation site of the 35S rRNA transcript is indicated by an arrow starting at position 547.

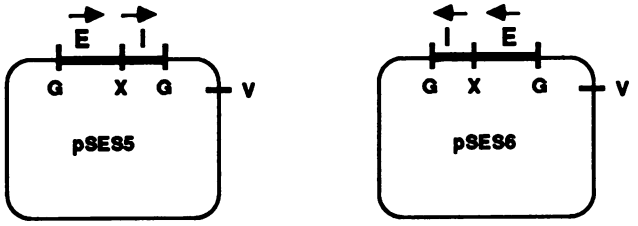

FIG. 3. Plasmids used for in vitro mutagenesis. The *HOT1 Bgl*II fragment was inserted into a pBR322 derivative as described in the text. The arrow above the I fragment indicates the direction of transcription. The E and I fragments are oriented as they are in the intact rDNA array; the arrow above the E fragment represents the orientation of the fragment with respect to I. Abbreviations for restriction sites: G, *Bgl*II; X, *Xba*I; V, *Eco*RV.

directions of the supplier (Schleicher & Schuell, Inc.). In most constructions, the staggered ends of linear molecules were removed or filled in with Klenow polymerase (New England BioLabs). In the case of pSES6 linearized with *Hin*dIII, the 5′ protruding ends were removed by using mung bean nuclease (New England BioLabs). Linear molecules were ligated in the presence of 8-bp phosphorylated *Xho*I linkers (New England BioLabs) under conditions of 50 to 100 linker ends per plasmid end. Ligation products were then transformed into *E. coli*, selecting for resistance to ampicillin and tetracycline. Plasmid DNA was prepared from transformants and screened for *Xho*I sites within *HOT1* sequences by restriction mapping with *Xho*I and *Eco*RV. To remove extra linkers, plasmids containing linker inserts were digested with *Xho*I and recircularized by ligation at low DNA concentrations.

Additional mutant *HOT1* fragments were constructed by deleting sequences between selected linker mutations. In these constructions, pairs of mutant pSES5-based plasmids were digested with *Xho*I (in the linker) and *Sal*I (in pBR322). The appropriate fragments from each plasmid were gel purified and ligated to create deletions of sequences between the linker inserts. The following mutations were created in this manner (Fig. 4): mutation 165 (from mutations 128 and 87); mutation 166 (from mutations 87 and 124); mutation 167 (from mutations 124 and 82); mutation 168 (from mutations 82 and 79); mutation 169 (from mutations 79 and 118); mutation 170 (from mutations 118 and 92); and mutation 171 (from mutations 78 and 121).

DNA sequence analysis. Linker insertions were localized precisely by dideoxy sequencing (27), using double-stranded plasmids pSES5 and pSES6 containing mutant *HOT1* inserts as templates. Denatured templates were prepared by the method of Hattori and Sakaki (12). Synthetic oligonucleotide primers complementary to pBR322 sequences on either side of the *HOT1* inserts in pSES5 and pSES6 were obtained from the Yale University DNA Synthesis Facility. Annealing of primers and subsequent sequencing reactions were performed according to the instructions provided in the Sequenase kit (United States Biochemical Corp.). Sequencing reactions were electrophoretically fractionated on 6% acrylamide–8 M urea gels.

Recombination assay. The stimulation of recombination by *HOT1* sequences was measured in the intrachromosomal recombination assay described by Voelkel-Meiman et al. (35) and is diagrammed in Fig. 5. *HOT1* activity was initially characterized by patch tests (35). The frequencies of His^+ and Ura^- recombinants were quantitated by fluctuation tests. For most mutants, nine cultures (three from each of

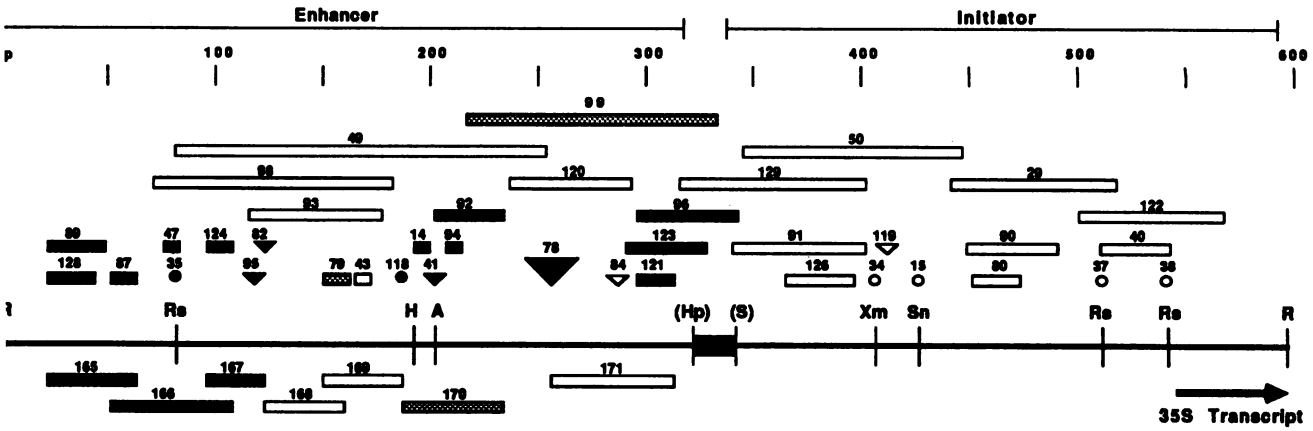

FIG. 4. Map of *Xho*I linker mutations. The position of each linker insertion is indicated along the map of *HOT1*; precise locations of mutations are given in Table 1. Simple insertion of the CCTCGAGG linker is indicated by a circle; deletion of *HOT1* sequences accompanied by linker insertion is indicated by a rectangle (the length of the rectangle represents the approximate extent of the deletion); duplication of *HOT1* sequences accompanying linker insertion is represented by a triangle (the size of the triangle is proportional to the extent of the duplication). Solid figures represent mutations with no significant loss of activity (at least 35% of wild-type *HOT1*); shaded figures represent mutations with intermediate activity (10 to 35%); open figures represent mutations that exhibited severe loss of *HOT1* activity (less than 10%). Numbers above the figures are the mutation numbers referred to in the text and tables. Abbreviations for restriction sites: R, *Eco*RI; Rs, *Rsa*I; H, *Hin*dIII; A, *Ava*I; (Hp), partial *Hpa*I site; (S), partial *Sma*I site; Xm, *Xmn*I; Sn, *Sna*BI.

three independent transformants) were grown and plated as described by Voelkel-Meiman et al. (35). For mutants 98, 49, 99, 91, 129, 50, 90, 29, and 122, six cultures (three from each of two independent transformants) were assayed. The

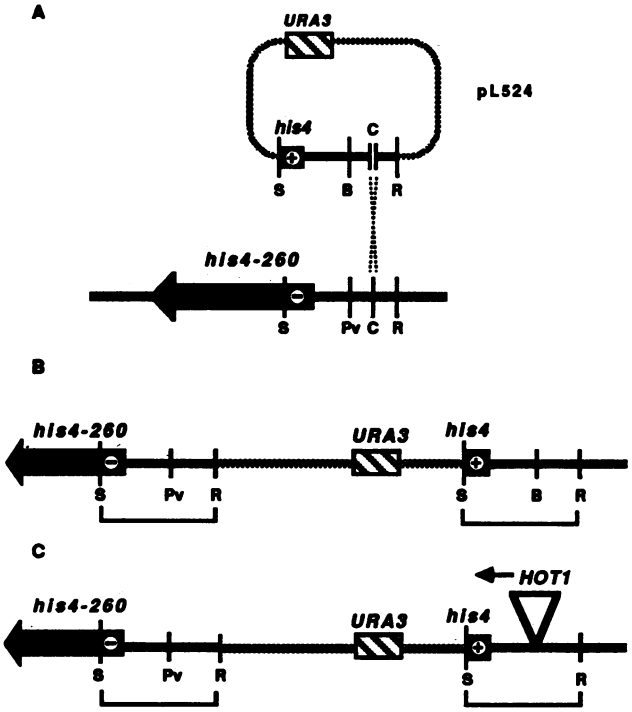

FIG. 5. Intrachromosomal recombination assay. (A) Integration of plasmid pL524 (35) at the *HIS4* locus on chromosome III. The *Pvu*II site located upstream of *HIS4* was converted to a *Bam*HI site on pL524. Plasmid pL524 was cut at the *Cla*I site to facilitate integration at homologous sequences on the chromosome. Integrants were selected as Ura$^+$ transformants. (B) The *his4* duplication. Brackets indicate a 2.8-kilobase block of homologous sequences able to undergo recombination. (C) Insertion of a *HOT1* *Bgl*II fragment at the *Bam*HI site in the *his4* duplication. *HOT1* is shown in the active orientation such that a transcript initiating in the I region could traverse the duplication. Symbols: ◀, *HIS4* locus on the chromosome; – site of the mutant *his4-260* allele; ■, truncated wild-type *HIS4* coding region; +, wild-type information at the site of the *his4-260* mutation; ▬, sequence adjacent to the *HIS4* locus; ▧, *URA3* gene on pL524; ▩, pBR322 vector sequence. Abbreviations for restriction sites: S, *Sal*I; B, *Bam*HI; C, *Cla*I; R, *Eco*RI; Pv, *Pvu*II. Not all sites are indicated.

method of the median (21) was used to calculate the median mitotic frequencies of His$^+$ and Ura$^-$ recombinants. Relative recombination activities of mutant *HOT1* strains were normalized to the activity of the wild-type *HOT1* sequence.

RNA isolation. Cells were grown to an A_{600} of 0.5 in SC liquid medium. Total RNA was prepared by the method of Elder et al. (5), followed by treatment with 10 U of DNase I (Worthington Diagnostics) in 10 mM NaCl–40 mM Tris (pH 7.5)–6 mM $MgCl_2$ for 15 min at 37°C. RNA was then extracted with phenol-$CHCl_3$ and ethanol precipitated. Pellets were washed four times with 0.5 ml of 70% ethanol, dried under vacuum, and suspended in water for quantitation. RNA was divided into equal portions and stored in ethanol at −70°C.

Northern (RNA) analysis. For analysis by Northern blot hybridization, 10-μg samples of total RNA were denatured in 50% formamide–6% formaldehyde–MOPS buffer (24). Samples were separated on 1% agarose gels containing 6% formaldehyde. RNA was transferred without prior treatment to a Nytran membrane (Schleicher & Schuell) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridizations were performed as described by Thomas (32), with addition of 0.1% sodium dodecyl sulfate at 42°C (for DNA probes) or 65°C (for RNA probes). After hybridization, filters were washed once at room temperature in 2× SSC–0.1% sodium dodecyl sulfate for 30 min and three times in 0.2× SSC–0.1% sodium dodecyl sulfate at 65°C for 30 min and then exposed to Kodak XAR5 film (Eastman Kodak Co.), using two intensifying screens.

DNA probes were prepared by randomly primed synthesis on isolated DNA fragments, using Klenow polymerase (9).

RNA probes were prepared by in vitro transcription of fragments inserted into the Bluescript transcription vector (Stratagene), using the method described by Krieg and Melton (20).

Primer extension analysis. A 19-base oligonucleotide primer (designated HIS) complementary to chromosomal sequences located 54 to 72 bases downstream from the site of *HOT1* insertion was obtained from Operon Research. The HIS primer was 5′ end labeled by using T4 polynucleotide kinase (New England BioLabs) as described by Maniatis et al. (24). Labeled primer was ethanol precipitated in the presence of 2 μg of carrier tRNA and stored at −20°C.

For primer extension analysis, 50 μg of total cellular RNA was annealed with 1.5 ng of end-labeled HIS primer. Extension reactions were performed as described by Fouser and Friesen (10). Extension products were fractionated by electrophoresis on 8 M urea–6% acrylamide gels. Promoter activity was determined by densitometric scanning of autoradiograms of primer extension products. To size extension products, a sequence ladder was generated by using the HIS oligonucleotide to prime Sequenase reactions on the denatured template pL623 (35), a derivative of pL524 (Fig. 5) with the wild-type 613-bp *HOT1 Bgl*II fragment inserted at the *Bam*HI site.

RESULTS

In vitro mutagenesis of *HOT1*. The *HOT1* enhancer and initiator sequences were inserted into a synthetic polylinker to generate a 613-bp *Bgl*II fragment (see Materials and Methods). The sequence of this *Bgl*II fragment is shown in Fig. 2. The *Bgl*II fragment was inserted into a pBR322 vector in both orientations, producing plasmids pSES5 and pSES6 (Fig. 3). *HOT1* in these plasmids was mutagenized in vitro by insertion of an 8-bp *Xho*I linker at a number of restriction sites and at random locations generated by limited DNase I digestion. Thirty-nine independent mutations distributed throughout the E and I regions were recovered (Fig. 4). Each mutation was localized by restriction mapping of the *Xho*I linker site and then characterized by DNA sequencing. The exact position of each insertion is indicated in Table 1. Linker insertions at restriction sites exhibited the sequence alterations predicted. Mutations that were generated by DNase I digestion often showed deletion or duplication of *HOT1* sequences accompanying the insertion of the linker, probably a consequence of the ability of DNase I to make closely spaced, staggered double-strand cuts in the presence of Mn^{2+} (24). These alterations are diagrammed in Fig. 4 and described in Table 1. To more completely analyze the effects of mutations in the enhancer region, seven additional deletion mutations were constructed by pairwise combinations of original *Xho*I linker mutations, as described in Materials and Methods. These deletions are indicated as mutations 165 to 171 in Fig. 4 and Table 1.

Effect of *HOT1* mutations on recombination. *HOT1* was assayed for its effect on the frequency of intrachromosomal recombination. In this assay, plasmid pL524 containing a truncated *HIS4* gene was transformed into a haploid yeast strain carrying a mutation at the *HIS4* locus (*his4-260*; Fig. 5A). Integration of the plasmid generated a duplication of *HIS4* sequences separated by vector sequences and the *URA3* gene (Fig. 5B). Mitotic recombination between the repeated *his4* sequences can generate an intact His^+ locus. These events were selected by plating cells on medium lacking histidine. Recombination events that result in the excision of the vector sequences (e.g., reciprocal crossing

TABLE 1. Effect of linker mutations in *HOT1* on recombination

Insert	Position of mutation[a]	Mitotic frequency ($\times 10^{-4}$)		% of *HOT1* activity[b]	
		His^+	Ura^-	His^+	Ura^-
Controls					
No insert		3.5	2.1	0	0
HOT1		68.2	192.8	100	100
Enhancer					
89[c]	del 20–40	51.6	127.8	74.3	65.9
128	del 20–38	40.8	98.7	57.7	50.6
165	del 20–57	36.1	87.1	50.4	45.6
87	del 51–57	43.1	147.3	61.2	76.1
47	del 76–80	31.0	120.0	42.5	61.8
35	80	35.1	94.8	48.8	48.6
166	del 51–104	31.1	95.1	42.7	48.8
124	del 97–104	41.0	86.9	58.0	44.5
95	dup 111–115	30.4	92.2	41.6	47.2
167	del 97–115	26.7	90.7	35.9	46.5
82	dup 115–120	38.7	110.1	54.4	56.6
79	del 152–161	19.1	58.3	24.1	29.5
168	del 120–161	7.1	6.2	5.6	2.1
43	del 166	6.8	17.6	5.1	8.1
93	del 119–170	4.4	7.5	1.4	2.8
169	del 152–186	5.2	8.9	2.6	3.6
98	del 72–176	3.8	ND[d]	1.1	ND
49	del 80–257	2.3	ND	0	ND
118	186	40.6	125.7	57.3	64.8
14	del 190–193	59.8	179.0	87.0	92.8
E: H-Hp[e]	del 1–193	2.5	2.8	0	0.4
41	dup 196–199	35.0	112.0	48.7	57.6
92	del 199–226	42.9	84.4	60.9	43.2
94	del 204	56.3	199.1	81.6	103.3
170	del 187–226	11.0	40.9	11.6	20.3
78	dup 223–258	31.1	76.5	42.7	39.0
84	dup 281	3.8	5.5	0.5	1.8
120	del 239–291	4.2	4.8	1.1	1.4
171	del 258–313	5.9	8.9	3.7	3.6
123	del 291–327	91.8	238.3	136.5	123.9
121	del 298–313	43.1	86.8	61.2	44.4
96	del 298–338	65.8	164.6	96.3	85.2
99	del 218–330	7.2	25.3	5.7	12.2
E: R-H[f]	del 194–320	8.8	26.7	8.2	12.9
Initiator					
126[c]	del 368–396	2.9	3.3	0	0.6
91	del 342–401	3.8	ND	0.5	ND
129	del 317–400	4.6	ND	1.3	ND
50	del 345–445	4.2	ND	1.2	ND
34	406	2.8	2.0	0	0
119	dup 401–408	5.4	11.2	2.9	4.8
15	425	3.2	3.6	0	0.8
80	del 451–471	2.6	1.4	0	0
90	del 450–488	2.8	ND	0	ND
29	del 442–515	2.1	ND	0	ND
37	512	3.8	2.7	1.1	0.3
38	541	3.2	3.1	0	0.5
40	del 513–541	3.1	2.2	0	0.1
122	del 502–565	2.3	ND	0	ND

[a] Position of each mutation is given in reference to the *HOT1* sequence shown in Fig. 2; mutations are ordered according to position. A single number indicates insertion of the *Xho*I linker CCTCGAGG following the position given. del, Deletion of *HOT1* sequence accompanied by insertion of linker; dup, duplication of *HOT1* sequences flanking the linker insert.

[b] Calculated as 100 × [(frequency with mutation)−(frequency with no insert)]/[(frequency with *HOT1*)−(frequency with no insert)].

[c] Mutation number.

[d] ND, Not determined.

[e] Deletion of the *Eco*RI-*Hin*dIII enhancer subfragment (see Fig. 4). This deletion was constructed by Voelkel-Meiman et al. (35) and contains no linker insert.

[f] Deletion of the *Hin*dIII-*Hpa*I subfragment (see Fig. 4). This deletion was constructed by Voelkel-Meiman et al. (35) and contains no linker insert.

over) cause the cell to become Ura$^-$; these events were selected by plating on medium containing 5-fluoro-orotic acid, a drug toxic to Ura$^+$ cells. The introduction of the *HOT1* E and I sequences into the *his4* duplication (Fig. 5C) caused a 19-fold increase in the frequency of His$^+$ prototrophs and a 92-fold increase in Ura$^-$ recombinants (Table 1). These results were consistent with those obtained by Voelkel-Meiman et al. using identical constructions (35).

The intrachromosomal recombination assay was used to assess the effect of linker insertion mutations on *HOT1* activity. The original set of 39 linker insertion mutations, seven additional deletions in the enhancer region (mutations 165 to 171), and deletions of the *Eco*RI-*Hin*dIII and the *Hin*dIII-*Hpa*I subfragments of the enhancer (Fig. 4) were included in these tests. Mutated *HOT1* *Bgl*II fragments were introduced into the *his4* duplication (Fig. 5C) in the orientation in which wild-type *HOT1* stimulates recombination. Recombination frequencies in the resulting strains were determined in fluctuation tests and compared with values for a strain with no insert and a strain with the wild-type *HOT1* *Bgl*II fragment. Table 1 lists the mitotic His$^+$ and Ura$^-$ recombination frequencies for each mutant and indicates the percentage of recombination activity relative to wild-type *HOT1* (100%) or no *HOT1* (0%). The relative activities effected by the mutations are also represented diagrammatically in Fig. 4, where they have been assigned to three classes: (i) no significant loss of activity (defined as at least 35% of the wild-type hotspot activity); (ii) intermediate activity (10 to 35% of the wild-type activity), and (iii) severe loss of activity (less than 10% of the wild-type activity).

Most mutations in the enhancer region did not significantly disrupt *HOT1* activity. However, mutations localized to two separate regions within the enhancer, marked by mutations 43 at position 166 and 84 at position 281, reduced the frequency of recombinants to less than 10% of that observed with the intact *HOT1* insert. In the region marked by mutation 43, the large deletions 93 and 169 also showed severe reductions in hotspot activity. Mutation 79, a small deletion near but not covering mutation 43, resulted in a reduction of activity to an intermediate level, whereas a larger deletion (deletion 168) covering mutation 79 but not mutation 43, was severely reduced. The active linker insertions 82 (position 120) and 118 (position 186) define the boundaries of this essential region. In the region of the enhancer defined by mutation 84, deletions 120 and 171, covering the 84 site, also showed severe reductions in hotspot activity. The required sequences are delimited by the active insertions at positions 258 (mutation 78) and 290 (mutation 123). The presence of an essential site in this region was unexpected, because deletion of the entire *Hin*dIII-*Hpa*I subfragment of E (the construct designated E: R-H in Table 1) resulted in only a sevenfold reduction in *HOT1* activity, a result consistent with the observations of Voelkel-Meiman et al. (35). A large deletion (mutation 99) covering most of the *Hin*dIII-*Hpa*I fragment also resulted in only a moderate reduction in recombination activity.

In the I region, nearly all linker mutations reduced *HOT1* activity to background level; only mutation 119, an insertion at position 408, resulted in a low level of activity. These results suggest that most of the sequences in the *Sma*I-*Eco*RI fragment containing the 35S rRNA initiation site are essential for *HOT1* activity.

Transcription in *HOT1*-containing strains. To examine directly whether *HOT1* sequences act to promote transcription at *HIS4*, transcripts were visualized by Northern blot hybridization. Total RNA was isolated from strains carrying the *his4* duplication (Fig. 6A) with or without the *HOT1* insertion. By using a probe located just downstream of the site of *HOT1* insertion, a set of unique transcripts, appearing as discrete bands against a considerable background smear, were detected in the *HOT1*-containing strain (Fig. 6B). The sizes of the discrete transcripts ranged from 900 bases to 13 kilobases, the latter being of sufficient length to extend through the duplicated regions involved in recombination. To monitor the loading of RNA on these gels, filters were also probed with sequences homologous to the *TCM1* gene,

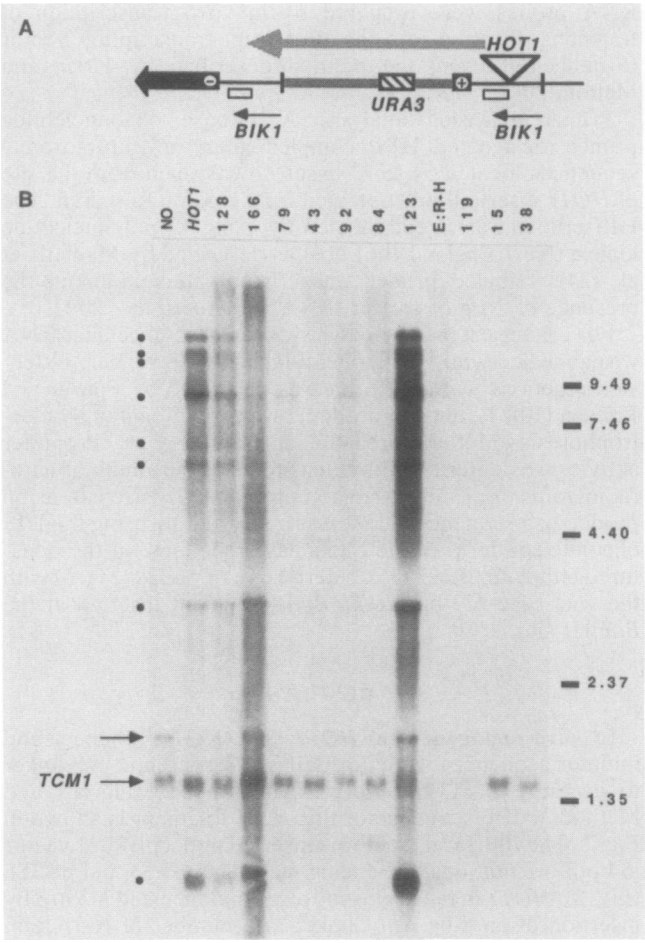

FIG. 6. Northern blot hybridization analysis of *HOT1* transcription. (A) The *his4* duplication. Symbols: ◁▷ putative *HOT1* transcript; ←, *BIK1* transcript (33); ▭, *Acc*I-*Pvu*II DNA fragment located just downstream of the *HOT1* insertion site that was used as a probe for *HOT1* transcripts. Other symbols are as in the legend to Fig. 5. (B) Northern blot probed with the fragment shown in panel A and with a fragment containing the *TCM1* gene (11). RNA was prepared from strains containing the *his4* duplication, fractionated on formaldehyde gels, and blotted to a Nytran membrane. Lane NO, Control strain with no *HOT1* insert; lane *HOT1*, strain carrying the wild-type *HOT1* insert. Strains with mutant *HOT1* inserts are identified by mutation numbers. Symbols: ●, discrete transcripts appearing in *HOT1*-containing strains; →, transcripts observed in all samples. RNA size standards (Bethesda Research Laboratories, Inc.) were visualized by ethidium bromide staining; positions and sizes of these markers are indicated to the right. Single-stranded RNA probes derived from the *Acc*I-*Pvu*II DNA fragment were also used to probe Northern blots. An RNA probe complementary to the *HOT1* transcripts gave essentially the same pattern of transcripts as did the DNA probe; a probe for transcripts from the opposite strand gave no signal (data not shown).

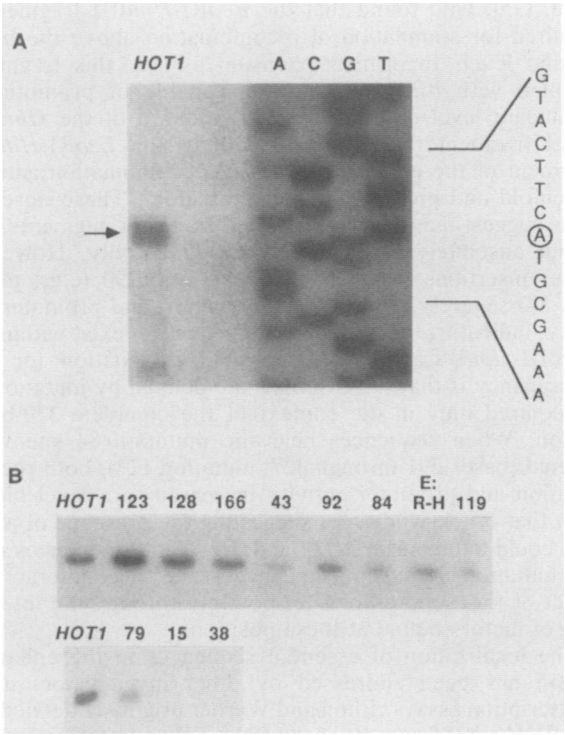

FIG. 7. Primer extension analysis of *HOT1* transcription. (A) Primer extension reactions performed by using RNA from a strain carrying the *his4* duplication with a wild-type *HOT1* insertion and a complementary oligonucleotide primer located 54 to 72 bases downstream of the *HOT1* insertion site. The same oligonucleotide was used to prime dideoxy-chain termination sequencing reactions, using a denatured double-stranded pL524 derivative with the wild-type *HOT1* sequence inserted at the *Bam*HI site (Fig. 5) as a template. →, Major primer extension product ending at the circled nucleotide in the expanded *HOT1* sequence. (B) Major product of primer extension reactions, using RNA from strains carrying mutant *HOT1* inserts. Samples are identified by mutation number.

which encodes the ribosomal protein L3 (11). Similar amounts of *TCM1* transcript were present in all samples (Fig. 6B). The probe for *HOT1* transcripts was also expected to hybridize with a transcript from the *BIK1* gene, which is located within the duplicated sequences (33). A band at about 1.8 kilobases was present in all samples (Fig. 6B); this band could represent the *BIK1* transcript, or it could be a consequence of nonspecific hybridization to the highly abundant 1.79-kilobase rRNA species.

Primer extension assays were performed to determine whether the set of unique transcripts observed in *HOT1*-containing strains initiated at the expected 35S rRNA transcription initiation site in the I region of *HOT1*. A primer located downstream of the site of *HOT1* insertion and complementary to the expected RNA species was annealed with total RNA from a strain carrying the *his4* duplication with a *HOT1* insert. The primary extension product from this reaction comigrated with the band on the sequence ladder corresponding to the known 35S rRNA initiation site (1, 19) (Fig. 7). Thus, *HOT1*-promoted transcription through the *his4* duplication initiated at the RNA polymerase I initiation site.

Effect of *HOT1* mutations on transcription. To examine the effect of linker insertion mutations on *HOT1*-promoted transcription, RNA was prepared from a set of strains carrying the *his4* duplication with different mutant *HOT1* inserts.

TABLE 2. Effect of linker mutations in *HOT1* on transcriptional activity

Insert	% Recombination activity[a]	% Promoter activity[b]
Controls		
No insert	0	0
HOT1	100	100
Enhancer		
128[c]	50.6	128 (138, 118)
166	48.8	60 (54, 79, 48)
79	29.5	32 (22, 35, 38)
43	8.1	9 (7, 18, 4)
92	43.2	44 (45, 43)
84	1.2	11 (14, 16, 4)
123	123.9	294 (262, 326)
E: R-H[d]	12.9	29 (29, 29)
Initiator		
119[c]	4.8	9 (7, 10)
15	0.8	0 (0, 0, 0)
38	0.5	0 (0, 0)

[a] Frequency of Ura⁻ recombinants compared to wild-type *HOT1* (from Table 1).

[b] Calculated as the amount of primer extension product relative to the wild-type control. Values are averages determined from two or three independent experiments; values in parentheses are the percent activities observed in individual experiments.

[c] Mutation number.

[d] See Table 1, footnote *f*.

Transcripts were analyzed on Northern blots as described above (Fig. 6B). Strains carrying mutations that completely eliminated recombination activity (mutants 15 and 38) did not exhibit the pattern of transcripts observed in the wild-type *HOT1* control. Instead, these strains appeared identical to the control strain lacking *HOT1*. In mutant strains with low hotspot activity (mutants 43, 84, and 119), the pattern of discrete transcripts superimposed on a background smear was visible; however, the intensity of this pattern was much fainter than that observed in the wild-type strain. In strains carrying mutations that conferred intermediate to high levels of recombination, the pattern of bands was identical to that of the wild type and the intensity of the pattern was similar to or lower than that of wild type. Finally, mutant 123, which stimulated recombination to a higher level than did the wild type, displayed a more intense pattern of bands than did the wild-type strain. Thus, the nature of *HOT1*-promoted transcripts was not altered in the mutant strains, but the amount of transcription varied in proportion to the level of recombination stimulation.

To more accurately quantitate the level of transcription from mutated *HOT1* fragments, primer extension assays were performed on the set of mutants shown on the Northern blot in Fig. 6B. In this assay, 50 μg of total RNA from each mutant and from the wild-type *HOT1* strain was annealed with excess end-labeled primer. Thus, the amount of extension product recovered was directly proportional to the amount of *HOT1*-promoted transcripts in each sample. The primary extension product from each mutant *HOT1* promoter comigrated with the product from the wild-type promoter (Fig. 7B), confirming that the mutations did not alter the site of transcription initiation. In each independent experiment, the amount of primary product from each mutant was compared to the amount of wild-type product by densitometric scanning of autoradiograms. The relative amounts of transcripts are given in Table 2; relative recombination activities are shown for comparison. As in the Northern blot analysis, the transcriptional activity of each

mutant correlated with its activity in the recombination assay. In strains that exhibited no stimulation of recombination (mutants 15 and 38) and in the control strain with no insert (not shown), no extension product was detected. Mutants with low recombination activity (mutants 43, 84, and 119) produced only about 10% of the amount of extension product observed in the wild-type *HOT1* strain. Mutants with intermediate hotspot activity (mutant 79 and E: R-H) displayed about 30% promoter activity, and those that did not significantly affect recombination (mutants 128, 166, and 92) showed a range of promoter activity from 40% to slightly greater than the wild-type level. Finally, mutant 123, which exhibited more activity than did the wild type in the recombination assay, generated threefold more extension product than did the wild-type *HOT1* promoter.

DISCUSSION

***HOT1*-stimulated recombination is correlated with *HOT1*-promoted transcription.** Previous work suggested that the ability of *HOT1* to stimulate recombination is dependent on its function as an RNA polymerase I promoter (35). The results presented here directly demonstrate that *HOT1* promotes transcription at the *his4* duplication used to assay *HOT1*-stimulated recombination. A unique set of transcripts that traverse the *his4* sequences involved in recombination was observed only in strains containing the *HOT1* insert. These transcripts are presumed to be the products of transcription promoted by RNA polymerase I because they originated at the 35S rRNA initiation site within the I region of *HOT1*. On Northern blots, these products appeared as a set of discrete transcripts superimposed on a heavy background. The appearance of discrete transcripts suggests the existence of several preferred termination or processing sites for *HOT1*-promoted transcripts in the *HIS4* region. The strong background signal, however, indicates that transcription termination occurred throughout the region.

Linker insertion mutations in the E and I regions of *HOT1* coordinately affect *HOT1*-mediated stimulation of recombination and *HOT1*-promoted transcription. In mutants that exhibited decreased or increased recombination activity with respect to the wild type, the distinctive array of *HOT1*-promoted transcripts was retained, but the amount of transcription varied in proportion to recombination stimulation. In mutants with no hotspot activity, no transcription was observed on Northern blots or in the more quantitative primer extension assay; strains carrying these mutations were indistinguishable from the control strain with no *HOT1* insert.

Essential sequences in the enhancer and initiator regions. Linker insertion mutagenesis has localized regions within *HOT1* that are required both for the stimulation of recombination and for promoter activity. In the I region, mutations covering the initiation site of the 35S rRNA transcript (1, 19) or adjacent upstream sequences abolish or severely disrupt *HOT1* activity. These results are in agreement with the deletion analysis of Kempers-Veenstra et al. (16), who identified sequences in the region from −192 to +45 of the yeast RNA polymerase I promoter as essential for initiation of transcription. Similar sequence requirements for RNA polymerase I transcription have been described in higher eucaryotes (14, 29).

In the enhancer region, linker insertions between positions 120 and 186 severely reduced *HOT1* activity. The existence of an essential site in the *Eco*RI-*Hin*dIII fragment (bases 1 to 193) is consistent with the observations of Voelkel-Meiman et al. (35), who found that the *Eco*RI-*Hin*dIII fragment is required for stimulation of recombination above the background level. In primer extension assays, this fragment, coupled with the I region, was capable of promoting a significant level of transcription. Addition of the *Hin*dIII-*Hpa*I fragment (bases 194 to 320) to the *Eco*RI-*Hin*dIII fragment of the enhancer increased recombination activity sevenfold and promoter activity threefold. These observations suggest that the *Hin*dIII-*Hpa*I fragment augments, but is not absolutely essential for, *HOT1* activity. However, linker insertions between bases 258 and 290 (e.g., mutation 84) severely reduced recombination and promoter activity, indicating that essential sequences do exist within the *Hin*dIII-*Hpa*I fragment. A possible explanation for this discrepancy is that the essential site defined by mutation 84 is required only in the context of the complete 320-bp E region. When sequences near the mutation 84 site were deleted (bases 291 through 327; mutation 123), both recombination and promoter activity increased to a level higher than that of the wild type, suggesting the presence of a site that could antagonize *HOT1* activity. Thus, sequences near the mutation 84 site may be necessary to counteract the effect of this suppressor site, possibly through the interaction of factors bound at these positions.

The localization of essential sequences in the enhancer region has been addressed by other investigators using transcription assays. Elion and Warner originally defined the *Eco*RI-*Hin*dIII fragment as the RNA polymerase I transcriptional enhancer (6, 7). Since the *Hin*dIII-*Hpa*I subfragment of *HOT1* was included in all of their constructions, the role of this sequence could not be assessed. In an independent study, Mestel et al. constructed and assayed a series of deletions in the enhancer (25). Here, sequences between positions 140 and 194 were found to be essential for enhancer activity; this region includes the essential sequences near mutation 43.

How does transcription stimulate recombination? The results presented by Voelkel-Meiman et al. (35) and this report suggest that transcription by RNA polymerase I effects changes in DNA structure that render the transcribed sequences more recombinogenic. Liu and Wang have proposed that movement of RNA polymerase through a DNA template generates a positively supercoiled domain ahead of the transcription complex and a negatively supercoiled domain behind the polymerase (22). Supercoiled domains in the vicinity of transcription complexes have been observed on plasmid templates in vivo (36) and in vitro (34). *HOT1*-promoted transcription could mediate a localized structural transition of DNA to an open form that is accessible to endonucleases that initiate recombination or to enzymes involved in the processing of recombination intermediates. Alternatively, strand separation of DNA associated with polymerase I-mediated transcription could increase the likelihood of homologous pairing and strand exchange.

A relationship between transcription-induced supercoiling and recombination is suggested by studies of yeast mutants defective in topoisomerase function. Brill and Sternglanz have shown that plasmids carrying a transcriptionally active gene are highly negatively supercoiled when isolated from *top1* mutant strains (3). Increased supercoiling was observed on plasmids carrying the 35S rRNA promoter or strongly expressed genes transcribed by RNA polymerase II. The extent of supercoiling was correlated with transcript length, suggesting that movement of the transcription complex through DNA was responsible for accumulation of supercoils. In addition, Christman et al. have reported that

recombination in the rDNA array is increased 50- to 100-fold in cells lacking topoisomerase function (4). These observations are consistent with the hypothesis that transcription (in this case, by RNA polymerase I) promotes supercoiling and that supercoiling, in turn, stimulates recombination. Perhaps topoisomerase is needed to relax the torsional stress induced by *HOT1*-promoted transcription and thus to prevent a deleteriously high level of recombination in the rDNA array. Experiments are in progress to examine the effect of *HOT1* on recombination in the rDNA in both Top^+ and Top^- strains.

The association of transcription with recombination has been documented by other investigators. In the yeast mating-type switch, the expressed *MAT* locus receives information from the transcriptionally silent donor loci *HML* and *HMR*. In mutant strains in which the *HML* and *HMR* loci are transcribed, these loci are able to act as recipients of information (18). These data suggest that transcription renders expressed loci accessible to the *HO* endonuclease that initiates switching (30). In cultured spleen cells, treatment with specific mitogens selectively induces transcription of immunoglobulin genes encoding heavy-chain constant regions. Directed class switching follows the induction of transcription (23), suggesting that transcription renders these sequences accessible to a site-specific recombinase. In yeast cells, an initiation site for high levels of meiotic gene conversion has been localized to the promoter of the *ARG4* locus (26). Deletion mutations that reduce gene conversion in this region also reduce transcription of *ARG4*. Finally, Thomas and Rothstein (31) have shown that transcription by RNA polymerase II stimulates mitotic recombination in yeast. Induction of transcription through direct repeats of the *GAL10* gene causes a 15-fold stimulation in the frequency of reciprocal exchange between the transcribed repeats (31). These results indicate that transcription stimulates both site-specific and general homologous recombination and that transcription by either RNA polymerase I or RNA polymerase II can stimulate exchange. Thus, transcription may play an important role in controlling the distribution and frequency of recombination events in eucaryotic cells.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM28904 from the National Institutes of Health. S.E.S. is the recipient of Public Health Service Individual National Research Service Award GM11773 from the National Institutes of Health.

The *TCM1* clone was generously provided by J. R. Warner. We thank Jaya Bhargava, Ralph Keil, Bradley Ozenberger, and Karen Voelkel-Meiman for critical reading of the manuscript.

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