Recombination between DNA repeats in yeast *hpr1Δ* cells is linked to transcription elongation

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The induction of recombination by transcription activation has been documented in prokaryotes and eukaryotes. Unwinding of the DNA duplex, disruption of chromatin structure or changes in local supercoiling associated with transcription can be indirectly responsible for the stimulation of recombination. Here we provide genetic and molecular evidence for a specific mechanism of stimulation of recombination by transcription. We show that the induction of deletions between repeats in *hpr1Δ* cells of *Saccharomyces cerevisiae* is linked to transcription elongation. Molecular analysis of different direct repeat constructs reveals that deletions induced by *hpr1Δ* are specific for repeat constructs in which transcription initiating at an external promoter traverses particular regions of the DNA flanked by the repeats. Transcription becomes *HPRI* dependent when elongating through such regions. Both the induction of deletions and the *HPRI* dependence of transcription were abolished when a strong terminator was used to prevent transcription from proceeding through the DNA region flanked by the repeats. In contrast to previously reported cases of transcription-induced recombination, there was no correlation between high levels of transcripts and high levels of recombination. Our study provides evidence that direct repeat recombination can be induced by transcriptional elongation.

Keywords: deletions/DNA repeat recombination/*HPRI*/transcription elongation/transcription-induced recombination

Introduction

An interesting aspect of the genetic control of recombination is its putative connection to transcription. Recombination has been shown to be stimulated by transcription in both prokaryotes and eukaryotes. The first evidence for this phenomenon was obtained by the isolation of the recombination hotspot *HOT1* (Keil and Roeder, 1984), a sequence from the rDNA region of *Saccharomyces cerevisiae* which is involved in the regulation of transcription by RNA polymerase I (Voelkel-Meiman *et al.*, 1987). The ability of *HOT1* to enhance recombination is related to its capacity to promote transcription (Stewart and Roeder, 1989). Stimulation of recombination by the activation of

RNA polymerase II-mediated transcription of *GAL10* has been also shown (Thomas and Rothstein, 1989). Other examples of transcription-induced recombination are recombination at *ADH1* in *Schizosaccharomyces pombe* (Grimm *et al.*, 1991), mating-type switching (Klar *et al.*, 1981) and Ty recombination in *S.cerevisiae* (Nevo-Caspi and Kupiec, 1994), intra- and interchromosomal recombination (Nickoloff and Reynolds, 1990; Nickoloff, 1992) and gene targeting (Thyagarajan *et al.*, 1995) in mammalian cells, and site-specific recombination in the immunoglobulin genes (Blackwell *et al.*, 1986; Leung and Maizels, 1992; Lauster *et al.*, 1993; Oltz *et al.*, 1993). Stimulation of recombination by transcription has also been reported in virus and bacteria (Bernardi and Bernardi, 1988; Dul and Drexler, 1988a,b; Bourgeaux-Ramoisy *et al.*, 1995; Vilette *et al.*, 1995).

Certainly, transcription-driven recombination may be an indirect consequence of the effect of transcription on DNA. Thus, the unwinding of the DNA duplex, the changes in the local supercoiling of DNA or the disruption of chromatin structure associated with transcription could provide a better accessibility of recombination proteins to the transcribed DNA, could lead to DNA structures hypersensitive to endogenous nucleases or could facilitate a pairing reaction. There are additional reports that indirectly suggest that these types of alterations in the DNA structure can induce recombination. Thus, it has been shown in *S.cerevisiae* that the natural sites for the formation of double-strand breaks initiating meiotic recombination map in promoter regions (Nicolas *et al.*, 1989), and that mutations in the DNA topoisomerase structural genes, *TOP1*, *TOP2* (Christman *et al.*, 1988) and *TOP3* (Wallis *et al.*, 1989), and in the transcriptional regulators involved in chromatin structure, *SIR2* (Gottlieb and Esposito, 1989), *SPT4* and *SPT6* (Malagón and Aguilera, 1996), confer hyper-recombination between different types of repeats. In addition, it has been shown *in vitro* that: (i) transcription-driven site-specific recombination is linked to the negative DNA supercoiling transiently built by an advancing RNA polymerase (Dröge, 1993), and (ii) transcription activates RecA-promoted homologous pairing of nucleosomal DNA (Kotani and Kmiec, 1994).

However, a direct role for the transcription machinery itself in recruiting recombination proteins to transcribed genes cannot be dismissed. Thus, it has been shown that the binding of yeast transcription factors to a promoter increases meiotic recombination (White *et al.*, 1991, 1993), that the meiotic recombination hotspot located in the *Eβ* gene of the mouse major histocompatibility complex is adjacent to the binding motifs of known transcription factors (Shenkar *et al.*, 1991) and that the human recombination signal sequence-binding protein RBP2N functions as a transcriptional repressor (Dou *et al.*, 1994). In this sense, the recent identification of the RecA-like protein

Fig. 1. Frequency of recombination of the different DNA repeat systems used in this study. The data represent the median value of three or four fluctuation tests, each one performed with six independent colonies. The DNA repeat systems are drawn to scale. The shadowed box represents the 0.6 kb internal *LEU2* open reading frame used as the repeat. Thin lines represent pBR322 sequences, and boxes represent yeast DNA sequences. Strains used were AWI-1B (wild-type) and AYW3-4D (*hpr1Δ*). Restriction sites shown are *Cla*I (C), *Nsi*I (N), *Sma*I (M), *Sph*I (Sp) and *Ssp*I (S). Values in kb indicate the distances between repeats.

RAD51 as a component of the human RNA polymerase II holoenzyme is particularly intriguing (Maldonado *et al.*, 1996).

The genetic and molecular analysis of mutants affected in both recombination and transcription should contribute greatly to the understanding of the connection between both processes. One such mutant is *hpr1Δ* of *S.cerevisiae*. Null *hpr1Δ* strains show a strong induction (50- to 1000-fold) of recombination, leading to deletions between direct repeats (Aguilera and Klein, 1990). This makes the *HPR1* gene particularly interesting for its special relevance in the genomic stability of DNA repeats. In addition, *hpr1Δ* mutants show high frequencies of chromosome loss (Santos-Rosa and Aguilera, 1994) and are thermosensitive (*ts*) for growth (Fan and Klein, 1994). Recently, it has also been reported that *HPR1* functions as a transcription factor required for the activation of different promoters (Zhu *et al.*, 1995). These data suggest that *HPR1* participates in both transcription and recombination between repeats. This is supported further by the observations that mutations in different transcription factors suppress either the hyper-recombination (Piruat and Aguilera, 1996; Santos-Rosa *et al.*, 1996) or the *ts* phenotypes (Fan *et al.*, 1996; Uemura *et al.*, 1996) of *hpr1Δ* and that *hpr1Δ*-dependent deletions between *GALI10* repeats are stimulated under conditions of activation of transcription (Fan *et al.*, 1996).

In order to understand, on the one hand, how deletions between repeats occur in connection with transcription and, on the other hand, the mechanism by which deletions are induced in *hpr1Δ* mutants, we have performed a

molecular analysis of the effect of *hpr1Δ* on a number of different direct repeat constructs. After showing that the hyper-rec phenotype is not general but specific to particular repeats, we demonstrate that deletions occurring in *hpr1Δ* cells depend on the progression of transcription through particular DNA regions. This is the first time that it has been shown that transcription elongation can induce recombination between repeats.

Results

The hyper-recombination phenotype of *hpr1Δ* is only observed in particular DNA repeats

We have determined the effect of the *hpr1Δ* mutation on recombination of three previously characterized DNA direct repeat constructs (Prado and Aguilera, 1995) which are based on the same 0.6 kb repeat sequence and separated by intervening regions of 5.57 kb (LY), 31 bp (L) and 2.51 kb (LU construct). All these recombination substrates are contained on *CEN*-based plasmids that replicate autonomously. Figure 1 shows that the frequency of recombination of *hpr1Δ* cells was 56.5 times above the wild-type value for the LY construct. However, *hpr1Δ* does not have a significant effect on the levels of recombination (deletions) of the L and LU direct repeat constructs. These results indicate that the hyper-recombination phenotype produced by *hpr1Δ* is not general, but particular to DNA repeat constructs, consistent with our previous hypothesis that the function of *HPR1* should not be equally relevant for all chromosomal regions or sequences (Santos-Rosa and Aguilera, 1994). Indeed, a very weak and basal hyper-

rec phenotype (<5-fold) is observed in all repeat constructs studied in *hpr1Δ* cells. We will refer to those repeat constructs showing an increase of recombination of <5-fold as 'non-hyper-rec', and to those showing an increase of >20-fold as 'hyper-rec'.

The hyper-recombination phenotype of *hpr1Δ* is not dependent on either the DNA sequence per se or the length of the intervening region

We have suggested that the hyper-recombination phenotype of *hpr1Δ* cells should be caused by an increase in the frequency of initiation events (Aguilera and Klein, 1990; Santos-Rosa and Aguilera, 1994) and that spontaneous deletions in our direct repeat constructs initiate primarily at the intervening region (Prado and Aguilera, 1995). Since the LY (hyper-rec in *hpr1Δ* strains) and LU (non-hyper-rec) direct repeat constructs differ only in a 3.03 kb *Clal-SmaI* fragment from pBR322, present in the intervening region of LY and absent from LU (see Figure 1), we considered the possibility that such a 3.03 kb fragment contained a *cis*-element required for the *hpr1Δ*-induced recombination events. In order to identify such a putative *cis*-element, we made DNA repeat constructs carrying deletions of the intervening region of LY. The analysis of recombination of such DNA repeats is shown in Figure 1.

We first confirmed that when the 0.6 kb direct repeats were flanking almost exclusively the LY-specific 3.03 kb fragment (LYΔNS construct), recombination was stimulated in *hpr1Δ* cells to the same levels as construct LY (47- versus 57-fold). Further analysis revealed that the sequences conferring the *hpr1Δ*-dependent hyper-rec phenotype could be located in the right side of the 2.84 kb internal fragment (compare LR and LP constructs).

Unexpectedly, the analysis of 12 DNA repeat constructs carrying serial deletions of the 3.03 kb region covering the pBR322 sequences immediately to the left of *URA3* (Δ-repeat constructs) revealed that such a region could be deleted from LY without significantly affecting the *hpr1Δ*-dependent hyper-rec phenotype (data not shown). The two Δ-repeat constructs shown in Figure 1 (Δ20 and Δ21) have frequencies of recombination 20–35 times above the wild-type value, an increase significantly higher than that of LU (3.8-fold). Since the Δ-repeat constructs retained the 2.2 kb right side fragment of the intervening region of LY, it seemed possible that such fragments could contain a second *cis*-element able to induce recombination in *hpr1Δ* cells (see Δ21 in Figure 1). The 0.8 kb internal region of the yeast *URA3* sequence, contained in the aforementioned 2.2 kb fragment, was indeed essential for conferring the hyper-rec phenotype (see LYΔP construct). However, these results raised the paradox of why the LU repeat did not show a strong hyper-rec phenotype in *hpr1Δ* cells, even though it contained the 2.2 kb fragment.

It could be possible that LU contained an additional *cis*-element with a dominant-negative effect on the 2.2 kb fragment. One candidate for such a *cis*-element was the extra 54 bp containing the putative *URA3* terminator of transcription located on the left side of the intervening region of LU, which is absent from the Δ-repeat constructs. The addition of such a 54 bp *URA3* sequence to Δ21 (see LNB construct, Figure 1) abolished the strong *hpr1Δ*-dependent hyper-rec phenotype. However, LNA, identical

to LNB but with the intervening DNA region in the opposite orientation, shows a strong *hpr1Δ*-dependent hyper-rec phenotype. These results indicate that the DNA sequence alone is not sufficient to explain the hyper-rec phenotype conferred by *hpr1Δ*, since the same intervening sequence confers hyper-recombination in one orientation (LNA) but not in the opposite orientation (LNB).

Another factor that is not sufficient to explain the *hpr1Δ*-induced hyper-rec phenotype is the length of the intervening region. Not only do constructs with the same size of intervening region (Δ21, LNA and LNB) show different recombination phenotypes, but the length of the intervening region of *hpr1Δ*-dependent hyper-rec repeats ranges from 2.2 to 5.57 kb, and that of the non-hyper-rec constructs ranges from 0.6 to 2.54 kb. Consistent with this conclusion, a DNA repeat construct (LA) carrying the 0.6 kb *leu2* repeats and a 3.6 kb *ADE2* region as intervening DNA does not show *hpr1Δ*-dependent hyper-rec phenotype (frequencies of 5×10^4 and 8×10^4 in *hpr1Δ* and wild-type cells, respectively).

Therefore, the different recombination behaviour of the DNA direct repeats used in this study in *hpr1Δ* cells has to be caused by other factors besides the presence of specific DNA sequences or the length of the intervening region.

The strong *hpr1Δ* hyper-rec phenotype is linked to transcription progression through particular DNA regions

Since it has been suggested that HPR1 is a global positive regulator of transcription (Zhu *et al.*, 1995), and mutations in the SRB2 general transcription factor completely suppress the hyper-recombination phenotype of *hpr1Δ* (Piruat and Aguilera, 1996), we decided to determine the transcription pattern of the different repeat constructs analysed. We have mapped and quantified by Northern analysis the transcripts produced by nine different DNA repeat constructs and a recombinant *LEU2*⁺ plasmid, used as control. (It is important to note that all recombinant *LEU2*⁺ plasmids are identical, regardless of the DNA repeat from which they are derived.) By using different DNA probes (YIp5, pBR322, *URA3*, *ACT1*, the *LEU2* internal repeat and a *LEU2* 5' end-specific fragment), we were able to map and quantify all the different transcripts produced by each repeat construct. All transcripts covered either the *LEU2* or the *URA3* gene. With the exception of the *URA3* promoter-driven transcripts, all other transcripts detected in each DNA repeat construct were driven from the *LEU2* promoter located outside of the repeats, upstream of the *leu2Δ3'* truncated copy (see Figure 1). This conclusion was suggested by the observation that all such transcripts hybridized with both *LEU2* probes, regardless of whether or not they also hybridized with pBR322 (data not shown), and was confirmed by RNase A protection analysis (data not shown). All episomal *LEU2*-derived RNAs initiate at the *LEU2* transcription initiation region (Andreadis *et al.*, 1984) and are transcribed in the same direction as the *LEU2* gene. In consequence, the different length of the transcripts is caused by different termination sites.

The analysis of the repeats that do not show a strong *hpr1Δ*-dependent hyper-rec phenotype (Figure 2A) reveals that the major transcripts derived from each construct terminated either downstream of the first repeat, directly

Fig. 2. Transcription analysis of five direct repeat systems with a 'non-hyper-rec' phenotype in *hpr1Δ* cells. (A) Northern analysis of strains AWI-1B (wild-type) and AYW3-4D (*hpr1Δ*) transformed with the pRS314 derivative plasmids containing the different direct repeat systems shown in each diagram. The probes used were the 598 bp *ClaI*-*EcoRV* internal *LEU2* fragment, corresponding to the repeated sequence, and the 581 bp *ClaI* internal *ACT1* fragment. The same filter was first hybridized with the *LEU2* probe, and then rehybridized with the *ACT1* probe, with prior removal of the *LEU2* signals. The DNA repeat systems are not drawn to scale (see Figure 1 for a scaled drawing). The size and location of each transcript derived from the *leu2* repeat, whose 3' end has been made to coincide in the diagram with the position of the corresponding band in the gel, is indicated with an arrow. The promoter (P_{rm}) and termination (Ter) sites of transcription of *LEU2* are indicated. Shadowed areas represent the repeated sequences. The *LEU2* transcript corresponding to the *LEU2* endogenous chromosomal band is indicated. (B) Relative amount of the transcripts derived from each repeat system. The data represent the total of all transcripts initiating at the episomic *LEU2* promoter (transcripts represented with arrows in the diagrams). All values were normalized with respect to the endogenous *LEU2* and *ACT1* mRNA values. The value corresponding to the 1.05 kb transcript of LU in the wild-type strain was taken as 1. The amount of *LEU2* transcript corresponding to the *Leu*⁺ plasmid was calculated as the difference between the total amount of *LEU2* transcript observed in the gel and the amount of endogenous *LEU2* transcript inferred from the rest of the transformants. ND: not determined.

after entering the intervening region, or at the *LEU2* terminator located downstream of the second *leu2* repeat. The latter case was found mainly for DNA repeats containing short intervening regions (L and LP). However, in the repeat constructs with a strong *hpr1Δ*-dependent hyper-rec phenotype (Figure 3A), all major transcripts covered the intervening DNA, traversing DNA regions

Fig. 3. Transcription analysis of four direct repeats with a strong hyper-rec phenotype in *hpr1Δ* cell. One *Leu*⁺ recombinant is also analysed as control. (A) and (B) For details see legend of Figure 2.

that were not present in the non hyper-rec constructs. Among these regions, we find the sense strands of either the bacterial *Amp* (LR, LY) or *Tet* genes (LNA), or the nonsense strand of the yeast *URA3* gene ($\Delta 20$).

There was no difference between wild-type and *hpr1Δ* strains in the amount of transcripts produced by the non-hyper-rec constructs (Figure 2B). However, in the strongly hyper-rec constructs, the levels of all transcripts driven from the external *LEU2* promoter in *hpr1Δ* were 2–3 times lower than in wild-type (Figure 3B). Therefore, our results indicate that transcription becomes *HPR1* dependent when it progresses through particular regions located between the repeats, and this property is unique to DNA repeat constructs that show a strong hyper-rec phenotype in *hpr1Δ* strains.

A comparative analysis of transcription of all repeat constructs (Figures 2 and 3) reveals that the size of all transcripts driven from the *LEU2* external promoter are identical in both wild-type and *hpr1Δ* cells, indicating that the sites of transcription initiation and termination do not depend on *HPR1*. Also, the overall levels of transcripts terminating at known yeast transcription termination sequences were much higher (>10-fold in wild-type cells) than those of the transcripts terminating at bacterial or yeast sequences which do not naturally function as transcription terminators in yeast (compare LU and LNB from Figure 2 with LY, $\Delta 20$ and LNA from Figure 3).

Fig. 4. Effect of the *CYC1* transcription terminator (*CYC1ter*) on transcription and recombination of the hyper-rec LNA direct repeat system. Northern analysis, relative amounts of transcripts and frequencies of recombination are shown for the direct repeat systems LNA (without *CYC1ter*), LNAT and LNATD (with *CYC1ter*). The frequency of recombination shown for each transformant strain is the median frequency of three fluctuation tests made with six independent colonies each. For details see legend to Figure 2.

This is independent of the *hpr1Δ* mutation. In addition, it is interesting to note that the *LEU2*⁺ transcript of the *LEU2*⁺ recombined plasmid is significantly less abundant than that of LU or LNB, even though they all terminate at natural yeast terminators. We do not have a clear explanation for this. A particular topological configuration of the *LEU2*⁺ recombined plasmid or some other structural features as well as the primary sequence of the *LEU2*⁺ mRNA might be among the factors that could be affecting either transcription efficiency or transcript stability of the episomal *LEU2*⁺ mRNA.

The relevant differences between the hyper-rec and non-hyper-rec repeat constructs observed in this study are illustrated by the results of the non-hyper-rec LNB (Figure 2) and the hyper-rec LNA (Figure 3) repeat constructs, which differ only in the orientation of the intervening region. Whereas in LNB the strong *URA3* terminator of the intervening sequence is located downstream of the first *leu2* repeated sequence, in LNA the *URA3* terminator is at the other end of the intervening region, upstream of the second *leu2* repeated sequence. As a consequence, the transcript of LNB, initiated at the external *LEU2* promoter, only covers the first *leu2* repeated sequence (1.05 kb transcript). However, all transcripts of LNA initiating at the same *LEU2* promoter proceed through the first *leu2* repeated sequence, traverse the intervening region and terminate at a region at the 5' end of *URA3* (2.25 kb transcript). (Only a minor proportion of the transcripts do not terminate at such a region.) In addition, the LNB transcripts were equally abundant in *hpr1Δ* and wild-type strains, whereas the LNA transcripts were ~3 times less abundant in *hpr1Δ* than in wild-type strains.

Our results also suggest that the lower levels of transcripts observed for the hyper-rec constructs in *hpr1Δ* strains, as well as the hyper-rec phenotype, are not a consequence of differences in transcription termination between *hpr1Δ* and wild-type cells. Figures 2 and 3 show that transcription terminates at the same 5' region of

URA3 in both the non-hyper-rec LYΔP and the hyper-rec LNA construct. (This region contains transcription termination sequences of the upstream open reading frame.) However, although the *hpr1Δ* mutation does not affect either recombination or the level of transcripts in LYΔP, it conferred a hyper-rec phenotype as well as a significant reduction (2- to 3-fold) in the overall levels of transcripts in LNA.

A transcription terminator impeding transcription from elongating through the intervening region abolishes hyper-recombination

We decided next to confirm directly the hypothesis that the progression of transcription through the intervening region is required for a strong stimulation of recombination and a significant reduction in the level of transcripts in *hpr1Δ* versus wild-type cells (Figure 3). To do so, we put a strong transcription terminator (*CYC1ter*) downstream of the *leu2Δ3'* repeat in the hyper-rec construct LNA. Figure 4 shows that the presence of *CYC1ter* (LNAT construct) efficiently terminates transcription initiated at the *LEU2* promoter, impeding, therefore, the progression of transcription through the intervening region. Consistent with our predictions, such a strong terminator abolished both the hyper-recombination phenotype and the expression pattern of LNA (the new 1.15 kb transcript observed in LNAT was now equally abundant in wild-type and *hpr1Δ* strains). Also, the overall expression levels of the 1.15 kb transcripts of LNAT were eight (wild-type strains) and 25 times (*hpr1Δ*) above the levels of the 2.25 kb transcripts of LNA, consistent with our observation that termination of transcription at strong and natural yeast terminators led to high levels of transcripts in all other repeat constructs.

To confirm that transcription termination was not responsible for the hyper-rec and the transcription phenotypes of *hpr1Δ* cells and that the *CYC1ter* sequence itself was not responsible for the lack of *hpr1Δ*-dependent

DNA repeats		Repressed		Induced	
		wt	<i>hpr1Δ</i>	wt	<i>hpr1Δ</i>
L		6.7	18.2 (x2.7)	8.1	22.3 (x2.8)
LGAL1-1		4.6	14.3 (x3.1)	22.9	72.9 (x3.2)
LGAL1-5		5.1	10.5 (x2.0)	10.6	77.2 (x7.3)
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L		7.8	17.7 (x2.3)	4.7	12.9 (x2.7)
LPHO5		5.0	18.0 (x3.6)	4.3	39.2 (x9.1)

Fig. 5. Frequency of Leu^+ recombinants ($\times 10^4$) in DNA repeats containing the *GAL1,10* or the *PHO5* promoters as the intervening sequence, under repressed and activated conditions. The data shown correspond to the isogenic strains W303-1A (wt) and U678-4C (*hpr1Δ*). Similar results were obtained with wild-type strains FWY-3B and W303-1B, and *hpr1Δ* strains FWY-4B and AYW3-4D (data not shown). The median frequency of three or four fluctuation tests is shown for each transformant strain. Numbers in parentheses indicate the fold increase above the wild-type value. All systems used are based on the same 0.6 kb *leu2* repeat. LGAL1-1 and LGAL1-5 contain the *GAL1,10* promoter as the intervening sequence in the opposite orientation, and LPHO5 contains the *PHO5* promoter. For transcription repression, 2% glucose and 7.5 mM phosphate were used for LGAL and LPHO5, respectively. For induction, 2% galactose and 0.05 mM phosphate were used. Identical conditions were used for the L control system in each case.

hyper-rec phenotype of LNAT, we decided to place the *CYCter* sequence into the intervening region of LNA, 0.6 kb further downstream of the 3' end of the *leu2Δ3'* repeat (LNATD construct). As expected, in this new construct, the *LEU2*-driven transcript is 0.6 kb longer than in LNAT and also terminates at *CYCter* (Figure 4). As can be seen in Figure 4, the LNATD construct shows 11 times more recombinants in *hpr1Δ* than in wild-type cells and led to a 1.75 kb transcript which is 1.7 times less abundant in *hpr1Δ* than in wild-type cells. Therefore, the new repeat construct becomes sensitive to *hpr1Δ*, even though to a lesser extent than LNA, suggesting that the extra 0.6 kb sequence that is transcribed covers part of the region responsible for the phenotypes observed in LNA. In addition, it is clearly observed that transcript levels in both wild-type and *hpr1Δ* cells are significantly less abundant in LNATD as compared with LNAT. These results indicate that the elongation of transcription through the 0.6 kb fragment of the intervening region causes both the general decrease in transcript levels and the *hpr1Δ*-dependent hyper-recombination and transcription patterns of LNATD. Therefore, these experiments exclude any role for transcription termination in the transcription and recombination phenotypes of *hpr1Δ*.

Recombination induced by *hpr1Δ* does not initiate at regulated promoters

If HPR1 were a general activator of transcription (Zhu *et al.*, 1995), we would expect it to perform its function at promoter regions. If recombination events in *hpr1Δ* were a direct consequence of transcriptional activation defects, they should be induced by regulated promoters. To assess this possibility, we constructed 0.6 kb *LEU2* direct repeats carrying as intervening regions the *GAL1*- or the *PHO5*-regulated promoters. The new DNA repeat constructs (LGAL1-1, LGAL1-5 and LPHO5) should show strong hyper-rec phenotypes under activated conditions of transcription and wild-type levels of recombination under repression conditions. Figure 5 shows that this is not the case. In the three DNA repeat constructs tested, there is

a 2.0- to 3.6-fold stimulation of recombination under repressed conditions that increases up to 7.3- (repeats with *GAL1p* as intervening region) or 9.1-fold (*PHO5p*) under induced conditions in *hpr1Δ* strains, implying that the increase in recombination caused by *hpr1Δ* in connection with transcription activation of promoters is at most 3-fold. Therefore, the major recombination events observed in *hpr1Δ* cells do not initiate at regulated promoter sequences nor are they related to any role HPR1 might have in transcription activation.

Discussion

The central conclusion of this study is that recombination leading to deletions between DNA repeats can be induced by transcription elongation. We provide evidence that the recombination events occurring in yeast *hpr1Δ* cells are associated with the elongation of transcription through particular DNA regions flanked by repeated sequences.

Recombination induced by *hpr1Δ* is associated with transcriptional elongation

We have shown that the recombination phenotype of *hpr1Δ* is not general but is specific to particular DNA repeat constructs. This is consistent with our previous hypothesis that the hyper-rec phenotype of *hpr1Δ* cells should not be general for all chromosomal regions (Santos-Rosa and Aguilera, 1994). Whether or not a direct repeat construct has a strong hyper-rec phenotype (50-fold stimulation of recombination) or not (<5-fold) does not depend on either the DNA sequence *per se* or the length of the intervening region (Figure 1), but on transcription elongation. All tested repeat constructs are transcribed from the same upstream *LEU2* external promoter. However, a characteristic unique to the strongly hyper-rec constructs is that the transcripts driven from such a *LEU2* promoter elongate through specific regions contained in the intervening DNA flanked by the repeats (Figure 3). The constructs in which transcription do not proceed through such DNA regions, either because they are not

contained in the intervening DNA or because a terminator impedes transcription from entering the intervening region, are not strongly hyper-rec (Figure 2). Indeed, a strongly hyper-rec construct could be converted into non-hyper-rec by inserting a strong transcription terminator between the upstream repeat unit and the intervening region (Figure 4).

Further evidence that hyper-recombination is linked to transcription elongation is provided by the observation that the levels of transcripts driven from the *LEU2* promoter are significantly lower (3-fold) in *hpr1Δ* than in wild-type cells in the strongly hyper-rec constructs, but identical in non-hyper-rec constructs. This rule has no exception for any of the repeat constructs studied. Thus, even the few transcripts that elongate through the intervening region in the non-hyper-rec constructs are equally abundant in wild-type and *hpr1Δ* cells (Figure 2). Therefore, it is the passing of transcription through specific regions in the intervening DNA which is linked to both the lower levels of transcripts and the higher incidence of recombination in *hpr1Δ* cells.

The reduction in the levels of transcription of the hyper-rec constructs in *hpr1Δ* cells could be consistent with the recent observation implying that HPR1 participates in transcriptional activation of promoters (Fan and Klein, 1994; Zhu *et al.*, 1995). However, expression from the *LEU2* promoter is not dependent on HPR1 (see endogenous *LEU2* in Figures 2–4), nor is it obvious how different transcribed DNA sequences can affect the strength of its promoter directly. Our results suggest that the lower levels of transcription observed in *hpr1Δ* cells in the hyper-rec constructs are caused by defective elongation. Such defective elongation can either slow down the rate of transcription (Krassimir *et al.*, 1994) or lead to abortive transcripts, presumably undetectable by Northern analysis, given the instability of mRNA not terminating at the appropriate termination signals (Brawerman, 1987; Ross, 1995). Indeed, we have observed that, regardless of the strain background used, the transcripts driven from the same *LEU2* promoter accumulate at higher levels (>10-fold) when terminating at natural yeast terminators than when terminating at weak non-natural terminators. The exception is construct LNATD which shows lower levels of transcripts terminating at the *CYCter* sequence (Figure 4). This suggests that the elongation through particular DNA regions can also lead to a reduction in transcript levels.

Transcription termination is not responsible for the different recombination and transcription phenotypes reported here. Transcription terminates at the same region in two direct repeats (LYΔP and LNA) with opposite patterns of recombination. In addition, the *CYCter* terminator of transcription abolishes the recombination and transcription phenotypes of *hpr1Δ* when located immediately downstream of the transcribed repeat (LNAT), but not when located 0.6 kb further downstream (LNATD) in the intervening region (Figure 4). We have also shown that the suggested promoter–activator function of HPR1 (Zhu *et al.*, 1995) is not related to the hyper-recombination phenotype of *hpr1Δ* cells either, since two different regulated promoters are not able to strongly induce deletions between repeats when used as intervening regions.

Therefore, our result suggests that a defective elongation

of transcription is a cause of the hyper-recombination phenotype and its associated reduction in transcript levels in *hpr1Δ* cells. This defective elongation is not equally relevant in all DNA regions. Only when the elongation through specific DNA regions is clearly altered (either retarded, stalled or arrested) does it produce a detectable reduction in the levels of transcripts (3-fold) and a strong hyper-rec phenotype (>50-fold). One strand of the *Amp*, *Tet* or *URA3* regions are candidate regions where the RNA polymerase could pause in the absence of HPR1. In this sense, the LNATD construct is particularly revealing, because it shows that transcription through the 0.6 kb region including the *Tet* gene reduces the transcript levels in wild-type and, to a major extent, in *hpr1Δ* cells. It is likely that the proper progression of transcription throughout this region is HPR1 dependent.

How does transcription elongation induce deletions between repeats?

The characteristics of the induction of recombination by transcription in *hpr1Δ* cells are different from those of previously reported cases in which high recombination rates are associated with high transcription levels (Voelkel-Meiman *et al.*, 1987; Stewart and Roeder, 1989; Thomas and Rothstein, 1989; Nickoloff, 1992; Vilette *et al.*, 1992). In *hpr1Δ*-induced recombination, high transcription levels (LU, LNB, LNAT) are associated with low recombination levels (Figure 2). Therefore, the strong *hpr1Δ* hyper-rec phenotype cannot be explained easily by an indirect effect of transcription on DNA structure, such as the unwinding of the DNA duplex, the accumulation of local negative supercoiling behind the advancing RNA polymerase or the disruption of the chromatin structure.

We believe that HPR1 is required to allow the RNA polymerase II to traverse specific DNA structures that would otherwise pause or block transcription in the absence of HPR1. A stalled transcription complex might confer nuclease-hypersensitive sites, generate recombinogenic lesions (DNA breaks) or interfere with the replication machinery, causing deletions if flanked by direct repeats (Piruat and Aguilera, 1996). In this sense, illegitimate deletions in *Escherichia coli* have been suggested to be stimulated by collisions between converging replication and transcription machineries (Vilette *et al.*, 1995). Whether or not the transcription machinery could facilitate the recruitment of recombination proteins, as in nucleotide excision repair (Basthia *et al.*, 1996; Hoeijmakers *et al.*, 1996) is certainly unknown. Regardless of the mechanism used, our results suggest that transcription elongation may be a source of mitotic recombination in *S.cerevisiae*.

Materials and methods

Strains and plasmids

The yeast strains used in this study are congenic to W303-1A and are listed in Table I. Plasmids are listed in Table II.

Media and growth conditions

Standard media such as rich medium YEPD, synthetic complete medium (SC) with bases and amino acids omitted as specified, and sporulation medium were prepared as described previously (Sherman *et al.*, 1986). Yeast strains were transformed using the lithium acetate method (Ito *et al.*, 1983) modified according to Schiestl and Gietz (1989).

Table I. Strains

Strain	Genotype	Reference
W303-1A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	R.Rothstein
W303-1B	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	R.Rothstein
U678-4C	<i>MATa ade2-1 can1-100 his3-11,15 hpr1Δ3::HIS3 leu2-3,112 trp1-1 ura3-1</i>	R.Rothstein
AWI-1B	<i>MATα ade2 can1-100 leu2 his3 trp1 ura3</i>	Santos-Rosa and Aguilera (1996)
AYW3-4D	<i>MATa ade2-1 can1-100 his3 hpr1Δ3::HIS3 leu2-3,112 trp1 ura3</i>	this study
FWY-3B	<i>MATα ade2 his3 leu2-3,112 trp1 ura3</i>	this study
FWY-4B	<i>MATα ade2 his3 hpr1Δ3::HIS3 leu2-3,112 trp1 ura3</i>	this study

Table II. Plasmids

Plasmid	Description	Source or reference
YIp5	pBR322 containing the <i>URA3</i> gene	Struhl <i>et al.</i> (1979)
pRS314	YCp vector based on the <i>LEU2</i> gene	Sikorski and Hieter (1989)
p426-GAL1	YEpl vector based on the <i>LEU2</i> gene and containing the <i>GAL1</i> promoter and the <i>CYC1</i> terminator	Mumberg <i>et al.</i> (1994)
pYA301	pBR322 with the <i>ACT1</i> gene	Galtwitz and Sures (1980)
pRS314-L	pRS314 containing two 598 bp <i>Clal</i> - <i>EcoRV</i> <i>LEU2</i> fragments repeated in direct orientation, separated by 31 bp of polylinker	Prado and Aguilera (1995)
pRS314-LB	pRS314-L with a <i>Bgl</i> II linker inserted at the unique <i>Nru</i> I site of the polylinker fragment located in between the repeats	Prado and Aguilera (1995)
pRS314-LU	pRS314-L with the 2.5 kb <i>Clal</i> - <i>Sma</i> I fragment of YIp5 located in between the <i>LEU2</i> repeats	Prado and Aguilera (1995)
pRS314-LY	pRS314-LB containing the complete YIp5 sequence inserted at the <i>Bgl</i> II site located in between the <i>LEU2</i> repeats	Prado and Aguilera (1995)
pRS314-LA	pRS314-LB with the 3.6 kb <i>Bam</i> HI <i>ADE2</i> fragment inserted at the <i>Bgl</i> II site located in between the <i>LEU2</i> repeats, leaving the <i>ADE2</i> and <i>LEU2</i> genes in the same orientation	this study
pRS314-LYΔP	pRS314-LY with the 4.15 kb <i>Pst</i> I internal fragment of the YIp5 sequence deleted	this study
pRS314-LP	pRS314-L with the 568 bp <i>Bam</i> HI ^a - <i>Ssp</i> I fragment of pBR322 inserted at <i>Nru</i> I, leaving the <i>Ssp</i> I site distal to the <i>EcoRV</i> - <i>Bgl</i> II <i>LEU2</i> fragment ^b	this study
pRS314-LYΔNS	pRS314-LY with the 1.92 kb <i>Sph</i> I- <i>Nsi</i> I fragment of YIp5 deleted	this study
pRS314-LR	pRS314-L with the 2.73 kb <i>Sma</i> I- <i>Ssp</i> I fragment of YIp5 inserted at <i>Nru</i> I, leaving the <i>Sma</i> I site distal to the <i>EcoRV</i> - <i>Bgl</i> II <i>LEU2</i> fragment ^b	this study
pRS314-LNA	pRS314-L with the 2.16 kb <i>Bam</i> HI ^a - <i>Sma</i> I fragment of YIp5 inserted at <i>Nru</i> I, leaving the <i>Sma</i> I site distal to the <i>EcoRV</i> - <i>Bgl</i> II <i>LEU2</i> fragment ^b	this study
pRS314-LNB	pRS314-LNA with the 2.16 kb insert in the opposite orientation	this study
pBSRS	pBlueScriptII SK ⁻ with the 1.46 kb <i>Ssp</i> I- <i>Eco</i> RI <i>LEU2</i> fragment inserted at <i>Eco</i> RI- <i>Eco</i> RV	this study
pBS-CYCT	pBlueScriptII SK ⁻ with the 0.3 kb <i>Kpn</i> I ^a - <i>Pst</i> I <i>CYC1</i> transcription terminator from p426-GAL1 inserted at the <i>Eco</i> RV- <i>Pst</i> I site of pBlueScriptII-SK ⁻	this study
pRS314-LNAT	pRS314-LNA with the 0.3 kb <i>Hind</i> III <i>CYC1</i> terminator from pBS-CYCT inserted at the <i>Hind</i> III site located between the <i>LEU2</i> repeats	this study
pRS314-LNATD	pRS314-LNA with the with the 0.3 kb <i>Hind</i> III <i>CYC1</i> terminator from pBS-CYCT inserted at the <i>Nru</i> I site located between the <i>LEU2</i> repeats	this study
pRS314-LGAL1-1	pRS314-L with the 479 bp <i>Sac</i> I ^a - <i>Sma</i> I <i>GAL1</i> promoter from p426GAL1 inserted at <i>Nru</i> I, leaving the <i>GAL1</i> and <i>LEU2</i> promoters in the same orientation	this study
pRS314-LGAL1-5	pRS314-LGAL1-1 with the <i>GAL1</i> promoter inserted in the opposite orientation	this study
pRS314-LPHO5	pRS314-LB with a 550 bp <i>Bam</i> HI <i>PHO5</i> promoter inserted at <i>Bgl</i> II	this study
pRS314-Δ2-Δ21	Series of 12 plasmids derived from pRS314-LY by progressive nested deletions starting at the unique <i>Nsi</i> I site	this study

^aRestriction sites blunt-ended prior to ligation.

^bThe *EcoRV*-*Bgl*II *LEU2* fragment corresponds to the *leu2Δ3'* repeat (see Figure 1).

Genetic analysis and determination of recombination frequencies

Genetic analysis was performed as previously published (Sherman *et al.*, 1986).

Recombination frequencies were calculated as the median value of 3–4 fluctuation tests, each one performed with six independent colonies for each transformant studied. Yeast strains were grown on SC-trp. After 3 days, independent colonies were plated on SC-leu to determine the median frequency of *Leu*⁺ recombinants. The viable cell number was determined on SC-trp.

DNA manipulation

Plasmid DNA from *E.coli* was isolated as previously published (Biggin *et al.*, 1980). Yeast DNA was prepared from 5 ml of YEPD cultures as described (Sherman *et al.*, 1986).

Nested deletions were obtained with *E.coli* *Exo*III (Pharmacia) according to published procedures (Henikoff, 1984). Plasmid pRS314-LY was digested with *Sma*I and *Nsi*I prior to *Exo*III treatment. Clones carrying

deletions differing in length by 200–400 bp were selected for further studies.

[³²P]dCTP-labelled DNA probes were prepared as previously described (Feinberg and Vogelstein, 1984).

RNA analysis

Yeast RNA was prepared from 10 ml of SC-trp exponential cultures (Köhler and Dombey, 1991), electrophoresed in formaldehyde-agarose gels and blotted to Hybond-N nylon as described (Thomas, 1980).

RNA-DNA hybridization was performed in 50% formamide 5× SSPE, 1× Denhardt's solution, 1% SDS at 42°C for 16 h. DNA probes used were labelled with [³²P]dCTP.

RNase A protection assays were performed as described (Forrester *et al.*, 1987) with the modifications of Almoguera *et al.* (1995). RNA probes were prepared by *in vitro* transcription from the T3 and T7 promoters of the pBSRS plasmid cut with either *Hind*III or *Eco*RI, respectively. RNA-RNA hybridization was carried out in 80% formamide, 0.4 M NaCl, 1 mM pH 8 EDTA, 40 mM pH 6.7 PIPES at 48°C

for 16 h. The RNA-RNA hybrids were treated with RNase A at 30°C for 30–60 min. The resulting RNA fragments were analysed in 4% PAGE gels. The 768 bp RNAs derived from the *SspI-EcoRI LEU2* region transcribed from either a T3 or T7 promoter (see text) were used as RNA probes.

RNA was quantified from Northern experiments using an InstantImager (Packard, USA). All data from different samples were normalized in relation to the values obtained for the *ACT1* and *LEU2* mRNAs obtained from the endogenous chromosomal genes.

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