The Influence of a Palindromic Insertion on Plasmid Integration in Yeast

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Dedicated to the memory of Professor Vera Johanides

Summary

Palindromic sequences act as potential initiators of mitotic and meiotic recombination in yeast. We investigated the influence of a 102 base pair (bp) palindromic insertion present on a non-replicative plasmid on recombination with the yeast chromosome. First we show that the frequency of transformation was not significantly affected by the presence of the perfect palindrome in the *CYC1* gene. However, as revealed by molecular analysis of transformants, both the proportion and the distribution of recombination events involving chromosomal homology were changed. Our results indicate that the palindrome present on the plasmid molecule was not efficiently processed *in vivo* to a double-strand break (DSB). We conclude that, in the absence of DNA replication, palindromic sequences are not potent initiators of recombination, but may influence subsequent step(s) and are preferentially lost during recombination process.

Key words: palindrome, recombination, yeast, plasmid integration

Introduction

Meiotic recombination in yeast is initiated by double-strand breaks (DSBs) induced by the endonuclease Spo11 after chromatin remodelling early in the prophase I. Some regions become particularly accessible for the endonucleolytic attack and are known as »hot spots« for genetic recombination that ensures proper segregation of homologues in the first meiotic division (*1,2*). In mitotic cells, homologous genetic recombination is induced by different agents that damage DNA and presents a powerful mechanism for DNA repair. Recombination detected in the absence of exogenous source of damage is thought to reflect the repair of spontaneous lesions occurring in DNA. Mutations in more then 60 yeast genes increase the spontaneous recombination rate. Interestingly, a number of these genes is not involved in DNA repair, but is implicated in replication, transcription or remodelling of the chromatin suggesting that these processes may contribute to the generation of recombinogenic lesions (*3*). However, it is not clear whether each lesion has to be processed first in a DSB in order to initiate recombination or there are some other structures that may act as initiators like, for example, single-stranded breaks or gaps.

The best characterised hot spots for mitotic recombination in yeast involve DNA sequences in chromosomal, mitochondrial or plasmid DNA recognised by specific

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endonucleases that introduce DSBs (*4*). Less specific recombinogenic sequences involve repeats organised in different motifs like microsatellites, minisatellites, triplet repeats, direct or inverted repeats (*5*). Palindromes are of special interest as possible initiators of recombination due to their ability to form cruciform structures. Such structures are cut *in vitro* by the enzymes involved in the resolution of recombinational intermediates (*6,7*) and similar structures could be responsible for the replication of block-induced recombination (*8*). In yeast meiosis long palindromes are processed to DSBs (*9,10*) and strong stimulation of recombination in the presence of a long, perfect palindrome has also been described in vegetative yeast cells. It was found that the inverted dimmer of the *URA3* gene (1.0 kb) can stimulate recombination in the adjacent region up to 17.000-fold (*11*).

Yeast transformation with exogenous non-replicative DNA is based on genetic recombination with homologous sequence present in the genome. This process is essential for efficient manipulation of the yeast genetic material but may also be used as a model for the study of genetic recombination. Here we investigated whether the presence of a palindromic sequence on a plasmid molecule will stimulate recombination with the yeast chromosome. We first described construction of the plasmids containing palindromic and non-palindromic insertions in the yeast *CYC1* gene. The efficiency of transformation with these plasmids was compared and the Ura$^+$ transformants obtained were further characterised by molecular techniques. We showed that the palindromic sequence present on the non-replicative plasmid did not increase the efficiency of transformation, but had strong influence on the spectrum of recombination events observed.

Material and Methods

Plasmid construction

The plasmids used in this work are integrative vectors containing the yeast *URA3* gene and the yeast *CYC1* region. The plasmid pAB218-2 (6,5 kb) was constructed by deleting the *Sal* I – *Sal* I and the *Aat* II – *Hin*d III fragments in the plasmid pAB218 (*12*). The plasmid pAB218-4 was constructed by inactivation of recognition sites for the enzymes *Sph* I and *Kpn* I in the plasmid pAB218-2 using exonucleolytic and polymerase activity of the Klenow fragment. We assumed that the recognition site for *Sph* I was inactivated by deletion and for *Kpn* I by insertion of four bp. The plasmids pAB218-5 and pAB218-6 were constructed by insertion of the 102 bp synthetic fragments into the *Eco*R I site of the plasmid pAB218-4. The palindromic insertion in the plasmid pAB218-5 is a dimer of the multiple cloning site (51 bp *Eco*R I – *Hin*d III fragment) found in the plasmid pUC19 (*13*) obtained by ligation of the two *Hin*d III sites. It should be noted that the overall size of the perfect palindrome created in this way was 110 bp since the *Eco*R I site in the pAB218-4 is flanked by T:A and A:T base pairs. In the plasmid pAB218-6, one copy of the multiple cloning site was replaced by the synthetic polylinker created by annealing of the two 51-mers: 5'-
-AAT-TCG-ACG-TCA-CTA-GTA-AAG-ATC-TAT-CCC-

-ATC-CTA-GGT-GAG-AGC-CGC-GGA -3' and 5'-AGC-
-TTC-CGC-GGC-TCT-CAC-CTA-GGA-TGG-GAT-AGA-
-TCT-TTA-CTA-GTG-ACG-TCG -3'. The *CYC1* region of the plasmids used in this study is presented in Fig. 1. All DNA manipulations were done by standard procedures (*14*).
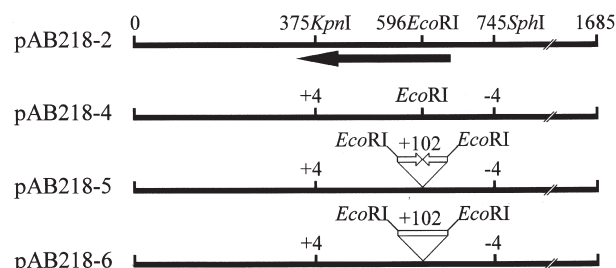
Fig. 1. The plasmids used in this study; only the part homologous to the yeast *CYC1* region is presented. In the plasmids pAB218-4, pAB218-5 and pAB218-6 the recognition sites for *Kpn* I were inactivated by insertion, and for *Sph* I by deletion of four base pairs. The plasmid pAB218-5 contains a 102 bp palindromic insertion in the *Eco*R I site (two white arrows) and the plasmid pAB218-6, a non-palindromic insertion (white box). Black arrow indicates the position of the *CYC1* open reading frame

Yeast transformation and analysis of transformants

Yeast transformation by a modified spheroplast procedure and Southern-blot analysis of transformants was described previously (*15,16*). Genomic DNA of transformants obtained with plasmids pAB218-2 and pAB218-4 was digested with *Pvu* II and DNA of transformants ob-
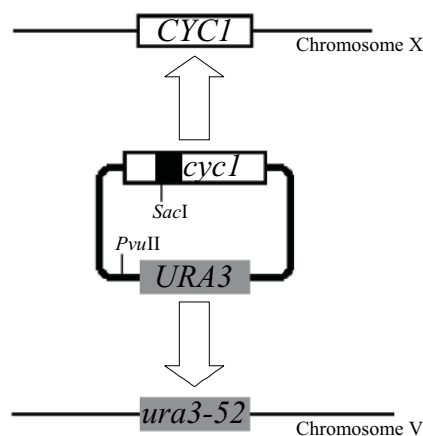
Fig. 2. Experimental system; the plasmids used in this study contained two yeast genes *CYC1* and *URA3*, so that Ura$^+$ transformants could arise either by plasmid integration in the yeast *CYC1* locus or by recombination with the chromosomal *ura3-52* allele. The exact nature of the recombination event leading to uracil prototrophy was determined by Southern blot analysis. Genomic DNA of transformants obtained with plasmids pAB218-2 and pAB218-4, that did not contain heterologous insert, was digested with *Pvu* II. Genomic DNA of transformants obtained with plasmids pAB218-5 and pAB218-6 was digested with *Sac* I which cuts the plasmid molecule within the heterologous insert (Black box)

tained with plasmids pAB218-5 and pAB218-6 with *Sac* I (Fig. 2). Labelled plasmid pBR322 was used as a probe to detect the chromosomal target for recombination. This analysis also revealed the structure of the two *CYC1* regions generated by integration of the plasmids pAB218-5 and pAB218-6 into the yeast *CYC1* locus (Fig. 3). The yeast strain used for transformation was FF18-52 (*MAT*a, *leu2-3,112, ura3-52, ade5, trp1-289, can1*). Statistical analysis was performed according to Glantz (*17*).

Structure of the *CYC1* locus	Plasmids	
	pAB218-5	pAB218-6
	7	4
	8	8
	3	14
	0	5
total analysed:	18	31

☐ *CYC1*

▨ *URA3*　　　— genomic DNA

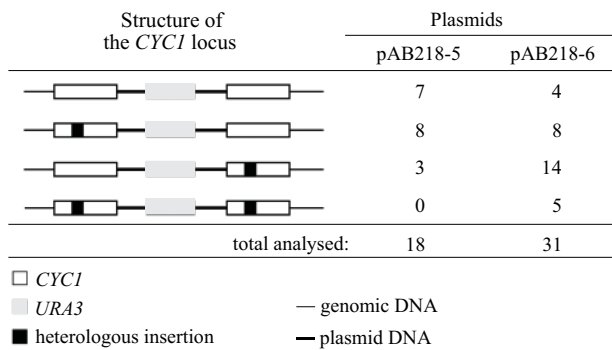■ heterologous insertion　　▬ plasmid DNA

Fig. 3. Integration of the plasmids pAB218-5 and pAB218-6 in the yeast *CYC1* region; heterologous insertion could be present on either side of the *URA3* gene, lost or duplicated. The number of transformants belonging to each class is presented

Results and Discussion

Yeast transformation with circular non-replicative plasmids occurs with very low frequency and was used for detection of lesions in DNA that strongly stimulate plasmid integration, like DSBs (*18,19*), psoralene adducts (*20*) or UV-induced lesions (*21*). However, if transformation experiments are followed by molecular analysis of transformants, very fine, but biologically relevant effects can also be detected. For example, it was shown that a single base pair substitution may influence the spectrum of recombination events detected by Southern blot hybridisation by decreasing plasmid integration to the homologous region present in the yeast genome

(*16*). This approach was applied here to study the influence of heterologous insertions on integration by homologous recombination. The results obtained with the plasmid pAB218-5 were of special interest since it contained a 102 bp palindromic sequence inserted in the *CYC1* gene. Such structures are widely used as cruciform substrates for biochemical analysis of the enzymes involved in the resolution of recombinational intermediates and we wondered whether they are also processed to DSBs *in vivo* in the absence of DNA replication.

First we asked whether the changes introduced in the *CYC1* region present on the plasmid molecule will influence the frequency of transformation (Table 1). Each plasmid was cut in the *CYC1* region by the restriction enzyme *Eco*R I and the ratio between the numbers of transformants obtained with circular and linearized DNA is presented. Different plasmids used in this study, including the plasmid pAB218-5, transformed yeast cells with comparable efficiencies with fluctuations characteristic for this type of experiment. This result suggested that the presence of the palindromic sequence on the plasmid molecule was not sufficient to stimulate recombination. However, it should be noted that the processing of the cruciform structure on the plasmid pAB218-5 to a DSB would create DNA ends without homology to the yeast *CYC1* gene. In this case, plasmid integration might depend on exonucleolytic removal of terminal heterologies that prevent strand invasion. In other words, although plasmid integration was not stimulated by the presence of the palindromic sequence we could not conclude that such structures were not processed to the DSBs in the yeast cells.

In our previous study we transformed yeast cells with the plasmid pAB218-5 cut within the palindromic sequence by the endonuclease *Hin*d III. We found that even in the presence of short terminal heterologies transformation occurred almost exclusively by integration to the *CYC1* region (*22*). Therefore, we could expect that the processing of the palindromic sequence to a DSB *in vivo* would also result in targeted integrations to the *CYC1* region. In order to determine the proportion of transformation events due to the integration of circular plasmids in the *CYC1* region we performed Southern

Table 1. The results of transformation experiments

Plasmid	Transformants[a]			Relative efficiency[b]		
	1	2	3	1	2	3
pAB218-2	18	27	12	0.25	0.27	0.41
pAB218-2 / *Eco*R I	73	100	29			
pAB218-4	26	7	17	0.21	0.15	0.18
pAB218-4 / *Eco*R I	127	47	96			
pAB218-5	19	57	22	0.15	0.49	0.29
PAB218-5/ *Eco*R I	125	116	76			
pAB218-6	23	43	8	0.16	0.17	0.16
pAB218-6 / *Eco*R I	146	252	50			

[a] the numbers of transformants obtained in three independent experiments are presented; protoplasts were transformed with 2 g of circular plasmid or with 0.4 g of plasmid DNA linearized with *Eco*R I

[b] relative efficiency of transformation denotes the ratio between the numbers of transformants obtained with circular and linearized plasmid

Table 2. Distribution of recombination events between plasmid molecule and two homologous loci present in the yeast genome

Plasmid	Transformants analysed	Homologous target	
		CYC1	*ura3-52*
pAB218-2	51	35 (69 %)	16 (31 %)
pAB218-4	48	29 (60 %)	19 (40 %)
pAB218-5	50	18 (36 %)	32 (64 %)
pAB218-6	50	31 (62 %)	19 (38 %)

blot analysis of transformants (Table 2). Similar values were observed for the plasmids pAB218-2 (69 %) and pAB218-4 (60 %) that had two 4 bp insertions/deletions within the 1682 bp *CYC1* region. Even with the plasmid pAB218-6 that had an additional insertion of 102 bp, 62 % of transformation events were still due to the integration in the *CYC1* region. In other words, these mutations did not affect recombination with homology present in the yeast genome, while in our previous work we found that a single base pair substitution in the 856 bp *CYC1* region reduced plasmid integration (*16*). This difference can be due to different types of mutations, size of uninterrupted homology and total length of homology used in these studies. However, when the 102 bp insertion in the plasmid pAB218-6 was replaced by the palindromic insertion of the same size (plasmid pAB218-5) only 36 % of transformation events were produced by plasmid integration to the *CYC1* region. This difference is statistically significant (P=0.016) indicating that only the palindromic insertion influenced recombination with homology. Interestingly, the proportion of integrations to the *CYC1* region was decreased, rather then increased. This result strongly suggests that the integration was not initiated by the DSB created by *in vivo* processing of the palindromic sequence present on the plasmid molecule.

Southern blot analysis revealed another difference between the spectra of transformation events obtained with the plasmids pAB218-5 and pAB218-6. Due to the presence of recognition site for the restriction enzyme *Sac* I in the insert we could distinguish four types of recombination events in the *CYC1* region: the insertion could be present in the left or in the right copy of the *CYC1* region, duplicated, or lost during recombination process (Fig. 3). First we noticed that 19/31 transformants obtained with the plasmid pAB218-6 contained the insertion in the right copy of the *CYC1* region and only 3/18 with the plasmid pAB218-5 and this difference is highly significant (P=0.006). Palindromic sequence was lost in 7/18 transformants analysed and no duplication was observed indicating disparity in conversion process (P<0.045). Such disparity was not observed with non-palindromic insertion found in the plasmid pAB218-6, in agreement with the results of other studies investigating the influence of insertions on homologous recombination in yeast (*23*). We also observed that the integration events with heterology present only in the right copy of the *CYC1* region were less frequent for the plasmid pAB218-5 then for the plasmid pAB218-6 (P=0.087). It may be that the palindromic insertion can affect some other step(s) of recombination process like, for example, the resolution of the recombination intermediate formed between the plasmid molecule and the yeast chromosome.

Conclusions

The results presented can be summarised as follows: in our experimental system, only the palindromic insertion present on the plasmid molecule influenced recombination with the yeast chromosome. Both the proportion and the distribution of recombination events involving chromosomal *CYC1* region were different in comparison with the plasmid bearing non-palindromic insertion of the same size. However, no significant increase in the frequency of transformation was observed indicating that the presence of palindromic sequence is not sufficient to stimulate recombination. Our results are consistent with the model proposed by Gordenin (*11,24*) where replication of the DNA containing inverted repeats creates a hot spot for recombination due to the formation of secondary structures in single-stranded regions. Analogous structures could also occur during different steps of homologous recombination like, for example, strand exchange and influence the final outcome of the process.

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Utjecaj palindromske insercije na integraciju plazmida u kvascu

Sažetak

Palindromske sekvencije djeluju kao mogući poticatelji mitotičke i mejotičke rekombinacije u kvascu. U ovom je radu istraživan utjecaj palindromske insercije od 102 para baza, prisutne na nereplikativnom plazmidu, na rekombinaciju s kvaščevim kromosomom. Najprije je pokazano da prisutnost palindroma u genu *CYC1* ne utječe bitno na učestalost transformacije. Međutim, molekularna analiza transformanata pokazala je promjenu kako u udjelu, tako i raspodjeli rekombinacijskih događaja s homologijom prisutnom u genomu kvasca. Naši rezultati pokazuju da se palindrom na nereplikativnom plazmidu ne prevodi uspješno u dvolančani lom *in vivo*. Na temelju prikazanih rezultata može se zaključiti da u odsutnosti replikacije DNA palindromi ne djeluju kao snažan poticatelj, ali mogu utjecati na druge etape procesa homologne rekombinacije koja dovodi pretežno do gubitka takvih sekvencija.