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Changes in chromatin structure at recombination initiation sites during yeast meiosis

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Transient double-strand breaks (DSBs) occur during *Saccharomyces cerevisiae* meiosis at recombination hot spots and are thought to initiate most, if not all, homologous recombination between chromosomes. To uncover the regulatory mechanisms active in DSB formation, we have monitored the change in local chromatin structure at the *ARG4* and *CYS3* recombination hot spots over the course of meiosis. Micrococcal nuclease (MNase) digestion of isolated meiotic chromatin followed by indirect end-labeling revealed that the DSB sites in both loci are hypersensitive to MNase and that their sensitivity increases 2- to 4-fold prior to the appearance of meiotic DSBs and recombination products. Other sensitive sites are not significantly altered. The study of hyper- and hypo-recombinogenic constructs at the *ARG4* locus, also revealed that the MNase sensitivity at the DSB site correlates with both the extent of DSBs and the rate of gene conversion. These results suggest that the local chromatin structure and its modification in early meiosis play an important role in the positioning and frequency of meiotic DSBs, leading to meiotic recombination.

Key words: chromatin/hot spots/meiosis/recombination/yeast

Introduction

Genetic recombination in meiotic cells occurs at levels that are several orders of magnitude higher than in somatic cells, and contributes not only to genetic diversity but also ensures the segregation of homologous chromosomes in the first of the two meiotic divisions when the cells halve their chromosome content (reviewed in Baker *et al.*, 1976; von Wettstein *et al.*, 1984). This reductional division, unique to meiosis, is the milestone of gametogenesis in all sexually reproducing organisms. It is preceded by the period of meiotic prophase in which replicated homologous chromosomes (homologs) search for one another, pair, synapse and recombine with each other in temporal and functional relationships that are not fully understood (reviewed and discussed in Esposito and Klapholz, 1981; Carpenter, 1987; Resnick, 1987; Hawley, 1988; Roeder, 1990; Kleckner *et al.*, 1991; Atcheson and Easton-Esposito, 1993; Hawley and Arbel, 1993). The traditional view holds that chromosomes must synapse before they recombine but recent data from yeast demonstrate that the earliest steps in recombination precede synapsis, and therefore may play a role in the recognition of homologs (Kleckner *et al.*, 1991; Goyon and Lichten, 1993). In addition to this early role in chromosome dynamics, reciprocal recombination events which result in the exchange of chromosome arms are also thought to be essential for the proper segregation of the homologs at the reductional division because they create a physical connection between homologs until they are properly oriented on the spindle at metaphase I, and then segregate to opposite poles (reviewed in references cited above). To understand meiosis, we must decipher the mechanisms of meiotic recombination and their controlling factors.

One crucial and poorly understood issue is how meiotic cells acquire their high recombinational ability. The differentiation of the meiotic cell type may include changes in the recombination machinery and/or modification in the structure of the chromosomal substrate, for example, through an increase in the accessibility of the recombination initiation sites. The state of chromatin is known to play a role in the control of the target site accessibility in yeast mating-type switching (Nasmyth, 1982) and is invoked in the initiation of recombination at the mouse Eβ meiotic recombination hot spot (Shenkar *et al.*, 1991) as well as in switch-site activation in immunoglobulin gene rearrangement (Berton and Vitetta, 1990). An accessibility model has also been proposed to account for the sex-specific difference in genetic recombination observed in many eukaryotes, including man (Thomas and Rothstein, 1991). Furthermore, the recent study of Wu and Lichten (1994) and the present report indicate that the features of chromatin structure that are established before meiosis play a role in determining where meiotic recombination events initiate in *Saccharomyces cerevisiae.*

To uncover the regulatory mechanisms for the enhancement of genetic recombination in meiotic cells, we have investigated the developmental behavior of the recombination substrate, i.e. the change in structure of the chromosomes during meiosis of the yeast *S.cerevisiae*. For this purpose, the advantages of *S.cerevisiae* are the relatively good synchrony of meiotic cells which allows time course studies of meiotic events, including recombination landmarks (Padmore *et al.*, 1991; Goyon and Lichten, 1993) and the exploitation of the recent identification of meiotic recombination initiation sites (Nicolas *et al.*, 1989; Cao *et al.*, 1990; Nag and Petes, 1993) which allows the physical study of chromatin structures in these probable key sites.

At the *ARG4* hot spot of meiotic recombination, the frequency of homologous gene conversion increases almost 10^3-fold during meiosis. It reaches 17% of total

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Table I. Yeast strain relevant genotypes

Diploid	Haploid parents	*ARG4*	RAD50	Reference
ORD149	ORT118.2	RV	+	de Massy and Nicolas (1993); Rocco *et al.* (1992)
	ORT126	Bg	+	
ORT195	ORT191	Inv *Eco*47–*Sna*BI, RV	+	Rocco *et al.* (1992)
	ORT196	Inv *Eco*47–*Sna*BI, Bg	+	
ORD307	ORT305	RV	rad50S	de Massy and Nicolas (1993)
	ORT311	Bg	rad50S	Rocco *et al.* (1992)
ORD392	ORT380	ΔEA464, RV	+	de Massy and Nicolas (1993)
	ORT366	ΔEA464, 464, Bg	+	
ORD 805	MGD357-7A	Δ5	+	de Massy and Nicolas (1993)
	MGD357-7D	Δ5	+	
ORD887	ORD1449-20C	poly1, RV	+	de Massy and Nicolas (1993)
	ORD1751-70D	poly1, Bg	+	
ORD1132	ORT389	Inv *Hpa*I–*Sna*BI, RV	+	Rocco *et al.* (1992)
	ORT138	Inv *Hpa*I–*Sna*BI, Bg	+	
	YKN1419	+	+	this study

meioses for a marker located in the promoter region at position −118 relative to the first base pair (+1) of the *ARG4* coding sequence (Schultes and Szostak, 1990). The *cis*-acting region required for this high conversion frequency has been mapped by deletion and inversion studies between positions −465 and −37 (Nicolas *et al.*, 1989; Schultes and Szostak,1991; Rocco *et al.*, 1992; de Massy and Nicolas, 1993). It includes a site for a meiosis-specific double-strand break (DSB) mapped around position −185 to −200 (Sun *et al.*, 1989; 1991). The results that suggest the role of meiotic DSBs in initiation of recombination in *S.cerevisiae* are: (i) DSBs are located in close proximity to the peak of gene conversion (Schultes and Szostak, 1990; Sun *et al.*, 1991); (ii) DSBs appear prior to the appearance of heteroduplex DNA and crossover products (Padmore *et al.*, 1991; Goyon and Lichten, 1993); (iii) the amount of DSBs in various deletions, and in a replacement of the *ARG4* promotor region, is correlated with the frequency of gene conversion (Sun *et al.*, 1989; de Massy and Nicolas, 1993) and (iv) the meiotic DSBs are not unique to the *ARG4* locus and its vicinity (Sun *et al.*, 1989). DSBs have been identified in several highly recombinogenic artificial constructs (Cao *et al.*,1990; Wu and Lichten, 1993), at the *CYS3* locus (M.Vedel and A.Nicolas, personal communication) and along chromosome III (Zenvirth *et al.*, 1992; Nag and Petes, 1993; Wu and Lichten, 1994), suggesting that a substantial fraction of meiotic recombination events may be initiated by localized DSBs. Nothing is known about the molecular mechanism(s) involved in the formation of DSBs. Two alternative but not mutually exclusive hypotheses are that (i) the specificities and activities of a yet unknown site-specific endonuclease active in meiosis determine the position and the frequency of DSBs, like the multi-site-specific endonuclease that initiates homologous recombination in the yeast mitochondria genome (Nakagawa *et al.*, 1992) and (ii) transitions of local chromatin structure at the DSB sites regulate the accessibility to a nuclease that is not necessarily meiosis specific. An accessibility-based regulation would be consistent with the observation that the formation of the DSB at *ARG4* is position but not sequence specific (de Massy and Nicolas, 1993) and can explain why DSBs occur in numerous intergenic regions including transcription promoters that may share structural rather than sequence similarities (Sun *et al.*, 1989; Zenvirth *et al.*, 1992; Nag and Petes, 1993; Wu and Lichten, 1994).

In this report, we focus on the role of local chromatin structure in the initiation of meiotic recombination. Micrococcal nuclease (MNase) digestion of isolated meiotic chromatin followed by indirect end-labeling revealed that (i) the *ARG4* and *CYS3* DSB sites colocalize with the regions hypersensitive to MNase digestion, (ii) the sensitivity of these MNase sites increases significantly during meiosis and (iii) the MNase sensitivity correlates with DSBs varying in amount and position in several hyper- and hypo-recombinogenic constructs. We conclude that the features of local chromatin structure and its potentiation in early meiosis determine the position and efficiency of meiotic DSBs. The data are discussed along with those of a parallel and complementary study performed with DNase I by Wu and Lichten (1994).

Results

MNase-hypersensitive sites in chromatin correlate with the meiotic DSB site at the ARG4 locus

To investigate the role of chromatin structure in the formation of meiotic DSBs, we prepared chromatin fractions from meiotic cells and analyzed the accessibility of the *ARG4* region in the wild type ORD149 strain (Table I) by digestion of the DNA with MNase. In the first experiment, cells were taken in prophase I of meiosis (at t = 8 h after transfer to the sporulation medium), and the chromatin fraction gently prepared and treated with 50 U/ml of MNase. The DNA was extracted and redigested by restriction endonucleases, separated by gel electrophoresis and analyzed by Southern hybridization using a short probe for the sequence adjacent to one of the restriction sites (indirect end-labeling). MNase sensitivity of chromatin and of naked DNA was studied in parallel (Figure 1A). Within the pattern of MNase sites observed in the chromatin extract, we detected a prominent MNase-hypersensitive site in the promotor region of *ARG4* (around position −200), not detected in naked DNA (Figure 1A). Thus, this MNase-hypersensitive site (hereafter named −200-hypersensitive site) is created by local chromatin structure, but not determined by preferential DNA

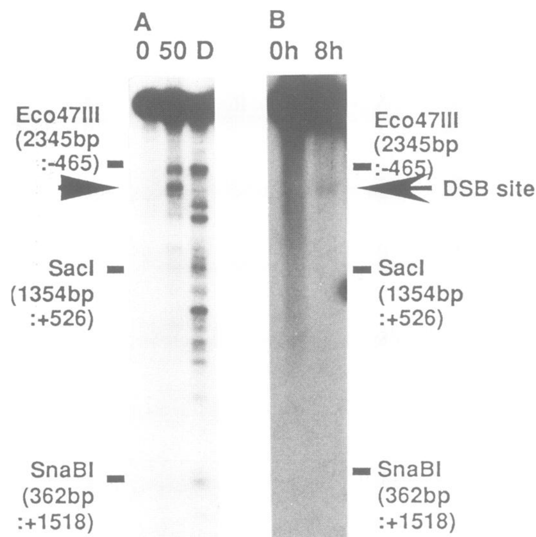


Fig. 1. MNase-hypersensitive site at the meiotic DSB site in *ARG4*. (**A**) MNase-sensitive sites in chromatin and naked DNA. Chromatin and naked DNA were prepared from wild type ORD149 cells at the 8 h time point in meiosis. MNase digestion and detection of MNase-sensitive sites were performed by indirect end-labeling method using the *Sna*BI−*Pst*I probe (see Figure 2C) as described in Materials and methods and mapped by reference to *ARG4* restriction fragments (not shown) of known molecular sizes (bp) and positions (see Figure 2C). Chromatin was treated with 0 and 50 U/ml of MNase (lanes 0 and 50, respectively), deproteinized and redigested by *Pst*I. DNA fragments were separated on 1.2% agarose gels. Naked DNA (D) was digested with 5 U/ml of MNase followed by digestion with *Pst*I. The large arrowhead indicates the position of the MNase-hypersensitive site (mapped at −200) in chromatin. (**B**) Meiotic DSB in *ARG4*. DNA was prepared from the *rad50S* strain (ORD307) at the 0 and 8 h time points in meiosis, and analyzed as described above. The arrow indicates the position of the meiotic DSB.

sequences for MNase or pre-existing single-stranded nicks or gaps. Its position corresponds to the region of the *ARG4* meiotic DSB detected at t = 8 h in the isogenic rad50S strain ORD307 (Figure 1B; see also de Massy and Nicolas, 1993) which accumulates unprocessed DSB molecules (Cao *et al.*, 1990). These results obtained with the MNase corroborate the parallel observation by Wu and Lichten (1994) of the presence of DNase I hypersensitivity in the *ARG4* promoter region in a different genetic background (strain SK1).

Since MNase preferentially cuts in the linker DNA between nucleosomes, the comparison of the pattern of bands in chromatin extracts and naked DNA by high resolution electrophoresis reveals the positions of nucleosomes. In the 5′ region of the *ARG4* locus, nucleosomes seem to be randomly distributed or poorly positioned, since the chromatin digestion and the naked DNA digestion were generally similar in a longer exposure of Figure 1A (data not shown).

MNase sensitivity at the ARG4 DSB site increases during meiosis

To test whether the −200-hypersensitive site is a constitutive feature of the *ARG4* region or is induced in meiosis, we monitored the MNase sensitivity of chromatin in the *ARG4* region during the course of meiosis (Figure 2A). We observed that the general patterns of MNase sensitivity in vegetative (t = 0 h) and meiotic chromatin extracts (t = 4–10 h) do not change except for the −200-hypersensitive site, which exhibits a specific increase in sensitivity during meiosis. Quantitatively, the meiotic increase in sensitivity is ~2.6 ± 0.9-fold (standard deviation observed from four independent sporulation experiments), while the sensitivity at other MNase-sensitive sites (in particular the two MNase-sensitive sites located around positions −450, −60 and +220) is not significantly altered (Figure 2B and C). The increase in MNase sensitivity in meiosis suggests that the formation of the DSB may be potentiated by a change of chromatin conformation.

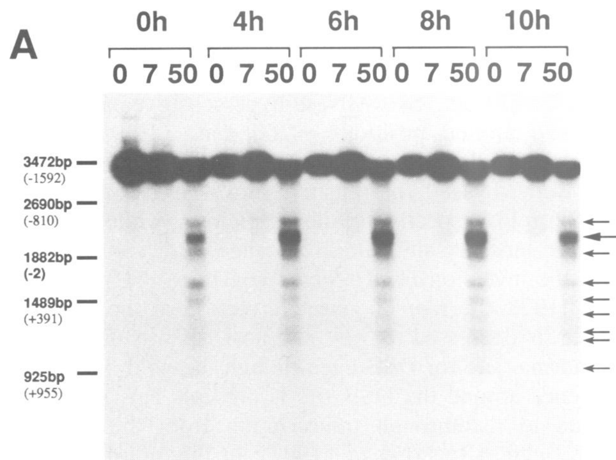
Increase of MNase sensitivity at the ARG4 DSB site occurs before DSB formation

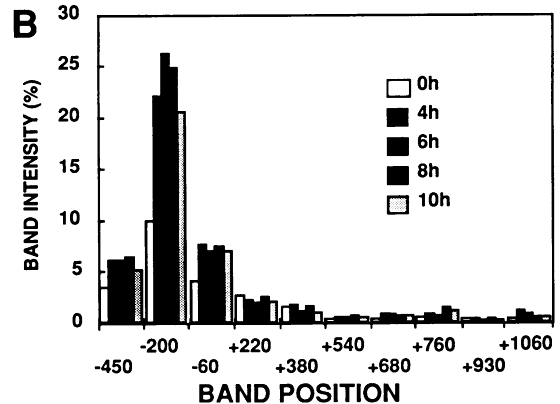
The increase in MNase sensitivity at the *ARG4* −200-hypersensitive site had mostly occurred at t = 4 h after transferring the cells into the sporulation medium (Figure 3A). Thereafter, MNase sensitivity reaches a slightly higher level (t = 6 h and t = 10 h) (Figure 3A) and then slightly decreases at t = 26 h (data not shown). To establish rigorously the temporal appearance of this chromatin change with respect to recombination, we have examined two landmarks of meiotic recombination: the physical appearance of transient DSBs (Sun *et al.*, 1989) and that of mature recombined molecules (Padmore *et al.*, 1991; Goyon and Lichten, 1993) using the chromosomal DNA from the same meiotic cell samples as those for chromatin preparation. For the detection of recombinant molecules, the strain ORD149 is appropriately marked by the *arg4-RV* and *arg4-Bg* heteroalleles, giving rise to novel DNA restriction fragments upon recombination (Materials and methods). In this strain, wild type for the *RAD50* gene, we observed that little smear product of the transient DSB is detected before t = 6 h; then the DSBs fragments reach their maximal level at t = 8 h and begin to disappear at t = 10 h (Figure 3B). Accordingly, the first recombined molecules appear at later time (t = 8 h) (Padmore *et al.*, 1991; Goyon and Lichten, 1993) and then accumulate to their maximal level reached at t = 26 h (Figure 3B). These results demonstrate that the increase of MNase hypersensitivity at the −200 site which is already detected at t = 4 h occurs before the physical landmarks of meiotic recombination, in particular before the formation of initiating meiotic DSBs.

Correlation of meiotic DSBs and MNase-hypersensitive sites in hyper- and hypo-recombinogenic ARG4 constructs

To examine the significance of the MNase-hypersensitive sites in the initiation of meiotic recombination, we then studied the MNase-hypersensitive sites in several constructs that either increase or decrease the activity of the *ARG4* initiation site.

The *arg4−poly1* construct carries a replacement of the −316 to −140 (*Hpa*I−*Afl*III) region, including the normal site for DSB, by an unrelated polylinker sequence. In the *arg4−poly1* strain, the DSB is formed at a position similar to the wild type position, and its amount is 2.3-fold higher than in the wild type (de Massy and Nicolas, 1993). In chromatin extracts of *arg4−poly1* meiotic cells (ORD887), we observed a MNase-hypersensitive site located around position −240, close to the DSB site (Figure 4A). MNase sensitivity increases by 2.1 ± 0.2-fold (n = two experi-





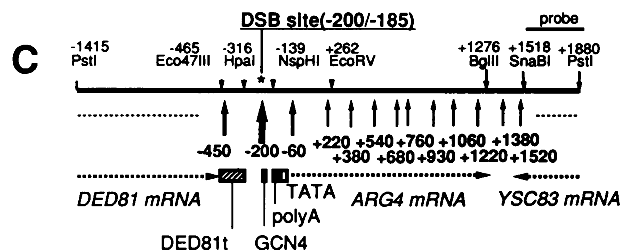


Fig. 2. Increase in MNase sensitivity around DSB sites in *ARG4* during meiosis in the wild type strain (ORD149). (**A**) Chromatin was prepared at various time points during meiosis (lanes 0, 4, 6, 8 and 10 h), treated with 0, 7 or 50 U/ml of MNase (lanes 0, 7 and 50 respectively), and analyzed as in Figure 1A. DNA fragments were separated in 1.2% agarose gels. Small and large arrows indicate MNase-sensitive and MNase-hypersensitive sites, respectively. Molecular size standards are fragments by *Eco*T14I digestion of phage λ DNA. (**B**) The intensity of each MNase band was quantified and expressed as a percentage of the total band intensity in the lane. (**C**) Restriction maps of the *ARG4* region. Horizontal broken arrows indicate the orientation of transcripts. Vertical arrows above a line indicating DNA and those below the line show sites for restriction enzymes and estimated MNase-sensitive sites, respectively. Positions for restriction sites and estimated MNase-sensitive sites are indicated respective to position +1, the first base of the *ARG4* coding region. The numbers for restriction sites give the position of the nucleotide 3′ of the cut site on the top strand. Asterisks show the position of the meiotic DSB. Boxes indicate: terminator of the *DED81* gene (DED81t), a GCN4p binding site (GCN4), poly(A) tracts (polyA) and a TATA element (TATA).

ments) from $t = 0$ to 8 h. This is similar to the 2.6 ± 0.9-fold (n = four experiments) increase observed in the wild type strain. The inducible site in *arg4−polyl* is 1.9 ± 0.09-fold (n = three experiments) more sensitive than the wild type site, in good correlation with the enhancement of DSB formation; i.e. 4.5% of meiotic DNA in *arg4−polyl* versus 2% in the wild type site in the rad50S background (de Massy and Nicolas, 1993). The overall similarity in MNase pattern obtained in the wild type and the *arg4−polyl* constructs demonstrates that the local chromatin structure is not determined by a specific short sequence surrounding the site of the DSB but more likely by external *cis*-acting chromosomal sequences or structures.

Δ5 is a deletion of the −316 to −140 (*Hpa*I−*Nsp*HI) region including the normal site for DSB (Nicolas *et al.*, 1989; Sun *et al.*, 1991). In *Δ5*, a weak meiosis-specific DSB appears in a novel site (position −350 in the wild type sequence) and the gene conversion frequency of the *arg4-RV* marker is reduced to 38% of the wild type level (Nicolas *et al.*, 1989; de Massy and Nicolas, 1993). As in the wild type and the *arg4−polyl* construct, we observed in the chromatin extract of *Δ5* cells (ORD805) a MNase-hypersensitive site in the 5′ region of *ARG4* (Figure 4A). Quantitatively, the sensitivity at this MNase site is intermediate [0.57 ± 0.09 (n = two experiments) of the wild type level at $t = 8$ h] and does not seem to be significantly altered during meiosis. Its position correlates with the position of the DSB site (de Massy and Nicolas, 1993).

ΔEA is an extensive deletion of the −465 to −2 (*Eco*47III−*Afl*III) intergenic region containing the transcription terminator of the upstream gene (*DED81*), the DSB site and all the *ARG4* promoter elements. In *ΔEA*, no DSB can be detected in the 5′ region of *ARG4*, and gene conversion frequency is very low, <1% (de Massy and Nicolas, 1993). In accordance with these data, we did not detect a MNase-hypersensitive site in the 5′ region of *ARG4* at either $t = 0$ h or $t = 8$ h of meiosis (Figure 4A). Figure 4B summarizes the results obtained with the wild type, *arg4-polyl*, *Δ5* and *ΔEA* strains.

Correlation of meiotic DSBs and MNase-hypersensitive sites in transplaced ARG4 constructs

To test further the correlation between MNase-hypersensitive sites and DSB sites, we also examined two transplacement constructs including the *ARG4* locus. We chose two inversions, diagrammed in Figure 5B, in which the efficiency of DSB formation and the rate of meiotic gene conversion in *ARG4* are very different due to transcriptional interference (Rocco *et al.*, 1992). In the

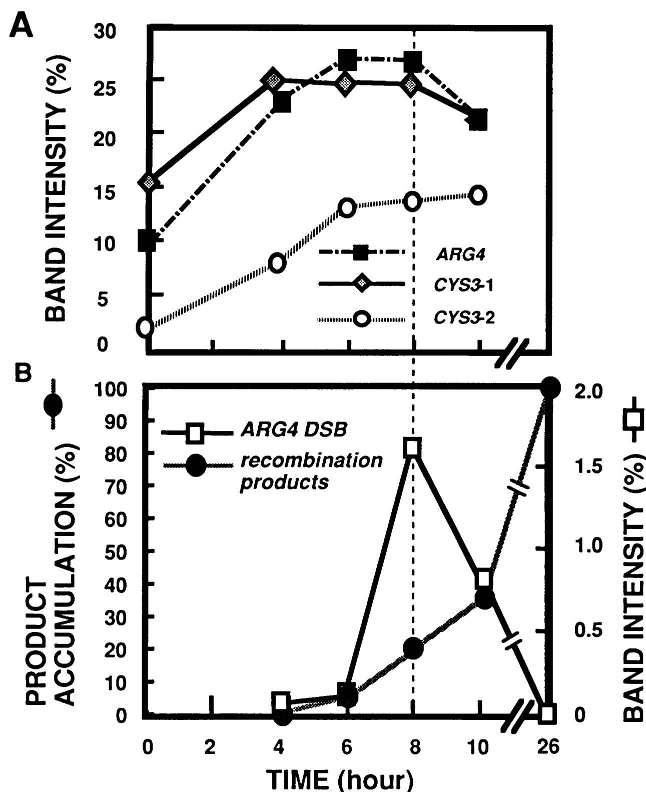


Fig. 3. Timing of the transition of chromatin structure at DSB sites. The cells used for each experiment were harvested from the same sporulation culture. (**A**) Intensity of bands corresponding to MNase-hypersensitive sites in *ARG4* (site −200) and *CYS3* (CYS3-I, site −270; CYS3-II, site −160; see Figure 6) was plotted versus time (h) after transferring the cells into the sporulation medium. (**B**) Amounts of meiotic DSB and recombination products in *ARG4* were quantified as described in Materials and methods. The amount of the meiotic DSB is indicated by the intensity of the corresponding band compared with total band intensity (%). The recombination product is expressed as a percentage of the maximum value observed at t = 26 h (100%).

strain ORD195 containing the *Eco*47III−*Sna*B1 (−465 to +1519) inversion, gene conversion at the *arg4-RV* marker is very high (21% compared with 9.6% in the wild type). In this construct, two MNase-hypersensitive sites were observed around positions −260 and −190, in close proximity to the DSBs that formed in this region (Figure 5A). Both MNase-hypersensitive sites were inducible (2.4- and 2.6-fold, respectively) during meiosis, while the level at other sensitive sites did not change.

In the inversion *Hpa*I−*Sna*BI (−316 to +1519; strain ORD1132), the rate of gene conversion at the *arg4-RV* marker is depressed (0.5%), and no DSB was detected at the normal site for DSB even though the wild type DNA sequence around the DSB site is present. This inhibition is due to readthrough transcription from the adjacent *YSC83* gene (Rocco *et al.*, 1992). In this non-functional inversion, no MNase-hypersensitive site could be detected around the normal site of DSB (Figure 5A). Comparison with the digestion of naked DNA suggests that the expected DSB site was overlapped by a phased nucleosome (Figure 5B).

In summary, the study of the five strains affected in meiotic recombination confirms the correlation between the position of MNase-hypersensitive sites and DSB sites observed in the wild type strain and establishes a correlation between MNase sensitivity, DSB formation and the rate of meiotic gene conversion (Figures 4B and 5B). In all constructs, no MNase-hypersensitive sites were observed at the corresponding sites in naked DNA indicating that the functionality of the *cis*-acting elements for the initiation of recombination depends on the conformation of the local chromatin structure.

MNase-hypersensitive sites in CYS3, another hot spot of meiotic gene conversion

To generalize the results obtained at the *ARG4* locus, we then examined the chromatin structure and its dynamics

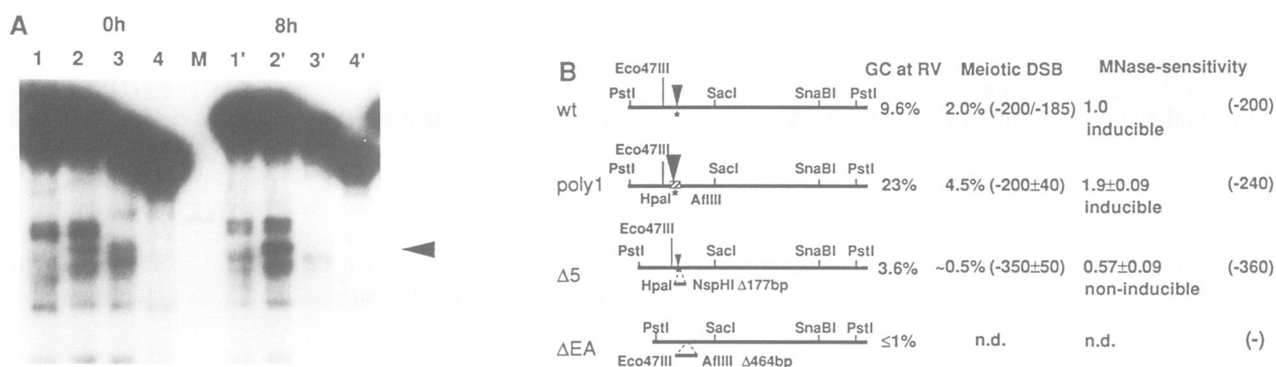


Fig. 4. Effects of modification in the *cis*-acting sequences of *ARG4* on MNase-hypersensitive sites. (**A**) Chromatin was prepared from the following strains: wild type ORD149 (lanes 1 and 1′), *poly1* ORD887 (2 and 2′), *Δ5* ORD805 (3 and 3′), and *ΔEA* ORD392 (4 and 4′) at both 0 h (1–4) and 8 h (1′−4′) time points in meiosis. MNase-sensitive sites were analyzed as in Figure 1 using the *Sna*BI−*Pst*I fragment as a probe. The arrowhead indicates the position of a MNase-hypersensitive site (at −240) in *poly1*. A band in lane M is the 2345 bp *Eco*47III−*Pst*I fragment of the wild type *ARG4* locus (see Figure 4B, wt). (**B**) Restriction maps of the *ARG4* regions of the wild type (wt), *poly1*, *Δ5* and *ΔEA* strains. Deleted regions in *Δ5* and *ΔEA* are indicated with their lengths. The hatched box in *poly1* represents the region of the replacement. Arrowheads show the position of the major MNase-hypersensitive sites. Asterisks show the position of the meiotic DSBs. Data in 'GC at RV' indicate the frequency of meiotic gene conversion at the *arg4-RV* marker, reported by Nicolas *et al.* (1989) and de Massy and Nicolas (1993). Data in 'Meiotic DSB' represent the amount of DSB expressed as a percentage of the unbroken parental fragments and their positions (numbers in parentheses) in the *rad50S* derivative of each construct (de Massy and Nicolas, 1993). Data in 'MNase sensitivity' show the relative sensitivity compared with the wild type level and the positions (number in parentheses) of the major MNase-hypersensitive sites at t = 8 h with indications of their inducibility. 'n.d.' means not detected.

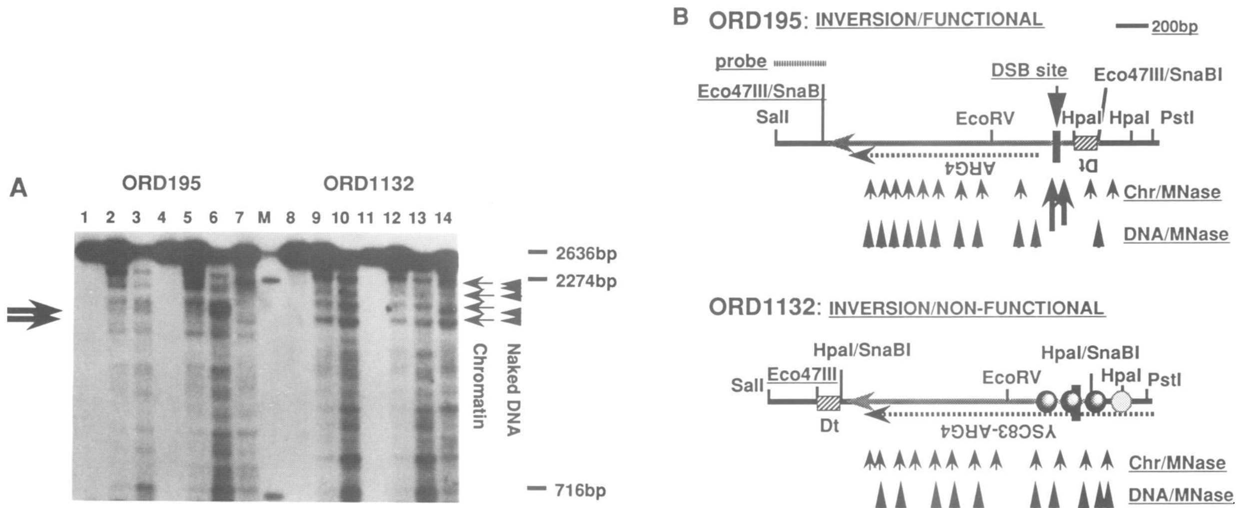


Fig. 5. MNase-sensitive sites in inversions of the *ARG4* locus. (**A**) Chromatin was prepared at two time points during meiosis (lanes 1–3, 8–10, at 0 h; lanes 4–6, 11–13, at 8 h), treated with 0 U/ml (lanes 1, 4, 8 and 11), 7 U/ml (lanes 2, 5, 9 and 12) and 50 U/ml (lanes 3, 6, 10 and 13) of MNase from the inversion constructs ORD195 (lanes 1–7) and ORD1132 (lanes 8–14). Naked DNA (lanes 7 and 14) was digested with 5 U/ml of MNase followed by digestion with *Pst*I. Bands in lane M indicate size markers of chromosome DNA of the wild type strain which was digested with various restriction enzymes: upper band (same as the parental band), 2636 bp *Sal*I–*Pst*I fragment; middle, 2274 bp *Sal*I–*Sna*BI fragment; lower, 291bp *Sal*I–*Eco*47III fragment. The large arrows on the left indicate the position of MNase-hypersensitive sites in ORD195. Arrows and arrowheads on the right side of the panel represent the position of MNase-sensitive sites in chromatin and naked DNA of ORD1132, respectively. Molecular size standards are indicated on the right (bp). (**B**) Schematic and restriction maps of inversion constructs. The vertical arrows and arrowheads represent the position of MNase-sensitive sites in chromatin (Chr/MNase) and naked DNA (DNA/MNase), respectively. The position of the DSB site observed in the wild type orientation is indicated by a black box. The thin arrow labeled 'DSB site' on the ORD195 diagram indicates the position of the DSB observed in this construct (Rocco *et al.*, 1992). The hatched boxes represent the transcription terminator of the upstream *DED81* gene. The horizontal broken arrows show the direction of transcripts of *ARG4* and *YSC83–ARG4* (readthrough transcript; Rocco *et al.*, 1992). Circles on the scheme of ORD1132 represent the position of the phased nucleosomes estimated by comparison of the sensitive sites in chromatin and naked DNA.

during meiosis at the *CYS3* locus, another hot spot for meiotic gene conversion (Cherest and Surdin-Kerjan, 1992). We detected two MNase-hypersensitive sites (hereafter named CYS3-I and CYS3-II) located in the *CYS3* promoter region, at positions −270 and −160, respectively (Figure 6A). As in *ARG4*, the position of these MNase-hypersensitive sites correlate with those of two adjacent meiotic DSBs (M.Vedel and A.Nicolas, unpublished data), and their hypersensitivity increases in meiosis (Figure 6B). However, more subtle features of these MNase sites are different in four respects. First, only the CYS3-II site is chromatin specific; no MNase-hypersensitive site is detected at this site in naked DNA whereas hypersensitivity at the CYS3-I site is detectable (data not shown). Second, the CYS3-II site is more inducible (4.1 ± 1.3-fold increase, n = four experiments) than CYS3-I (2.0 ± 0.7-fold increase, n = four experiments; Figure 6B). Third, the kinetics of the increased sensivity during meiosis is as rapid at the CYS3-I site as in *ARG4*, reaching its maximal level at t = 4 h but is slower at the CYS3-II site (Figure 3A) although no difference in the timing of appearance of the *CYS3* DSBs has been observed (M.Vedel and A.Nicolas unpublished data). Fourth, we observed that the increase in MNase sensitivity in the *CYS3* locus occurs at other sensitive sites adjacent to the DSB sites (Figure 6A). It occurs to a lower extent and with different kinetics to those of hypersensitive sites CYS3-I and CYS3-II.

Absence of increased MNase sensitivity at the DSBs sites in non-sporulating cells

To test the possibility that the increase in MNase sensitivity was due to a metabolic effect induced by the transfer of the cells to the sporulation medium (1% KAc) rather than their entry into meiosis, we examined the pattern of MNase sensitivity of a haploid strain (YKN1419) upon transfer in the sporulation medium. No increase in MNase sensitivity at the *CYS3* locus (Figure 7) or at the *ARG4* locus (data not shown) was observed during 8 h of incubation in the sporulation medium.

In conclusion, the results at the *ARG4* and *CYS3* loci strongly suggest that the colocalization of MNase-hypersensitive sites and DSB sites as well as the increase of hypersensitivity at an earlier time are a general feature of yeast meiosis. The more subtle comparison between the *ARG4* and *CYS3* regions indicates that the potentiation of MNase-hypersensitive sites in early meiotic prophase could be differentially and finely regulated from one site to another.

Discussion

The main results of this report are that (i) DSB sites at hot spots of meiotic recombination colocalize with sites hypersensitive to MNase, in confirmation of the previous report of DNase I sensitivity (Wu and Lichten, 1994), (ii) MNase hypersensitivity around DSB sites reproducibly and significantly increased 2- to 4-fold during early meiosis, in contrast to neighboring sites, which are less affected or unchanged and (iii) increased hypersensitivity occurs early in meiosis, before the formation of DSBs.

Correlation between MNase-hypersensitive sites and DSB sites

We have shown that all DSB sites in *ARG4* (wild type, hypo- and hyper-recombinogenic constructs) and *CYS3*

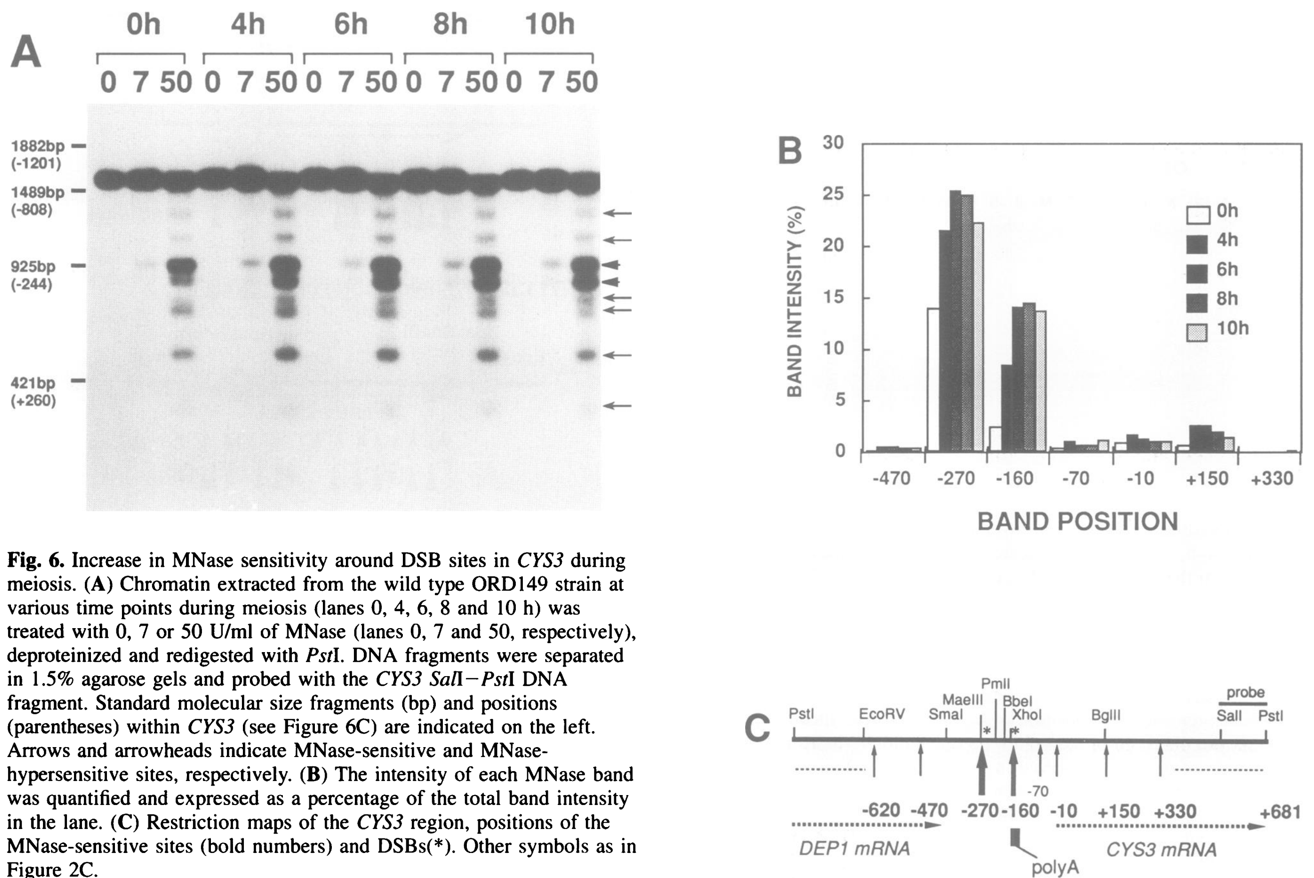


Fig. 6. Increase in MNase sensitivity around DSB sites in *CYS3* during meiosis. (**A**) Chromatin extracted from the wild type ORD149 strain at various time points during meiosis (lanes 0, 4, 6, 8 and 10 h) was treated with 0, 7 or 50 U/ml of MNase (lanes 0, 7 and 50, respectively), deproteinized and redigested with *Pst*I. DNA fragments were separated in 1.5% agarose gels and probed with the *CYS3 Sal*I–*Pst*I DNA fragment. Standard molecular size fragments (bp) and positions (parentheses) within *CYS3* (see Figure 6C) are indicated on the left. Arrows and arrowheads indicate MNase-sensitive and MNase-hypersensitive sites, respectively. (**B**) The intensity of each MNase band was quantified and expressed as a percentage of the total band intensity in the lane. (**C**) Restriction maps of the *CYS3* region, positions of the MNase-sensitive sites (bold numbers) and DSBs(*). Other symbols as in Figure 2C.

(wild type) hot spots of meiotic recombination, colocalize with meiotic MNase hypersensitive sites. This correlation, observed at higher resolution (<100 bp, see Figures 2A and 6A) than in the previous DNase I study of Wu and Lichten (1994), confirms that the positions of the meiotic DSBs are at least partly determined at the level of chromatin structure. Such structure can be the local 'openness' of chromatin as proposed by Wu and Lichten (1994). It would explain why DSBs can occur in insertions of heterologous DNA into yeast chromosomes, without apparent promoter activity but possibly generating various structural changes (Cao *et al.*, 1990; Wu and Lichten, 1993; Goyon and Lichten, 1993), or be induced in the *PHO5* promoter region upon induction-associated nucleosome disruption (Wu and Lichten, 1994). However, this idea may be oversimplified because all MNase-hypersensitive sites are not necessarily meiotic DSB sites; for instance, the MNase-hypersensitive site located at −450 in the *ARG4* upstream intergenic region is not a site for meiotic DSB, suggesting that the position and efficiency of meiotic DSBs are determined by additional factors other than the local 'openness' of chromatin.

Local change in chromatin structure at the DSB sites

At *ARG4*, we observed that MNase-hypersensitive sites around the DSB sites significantly increased 2- to 4-fold during early meiosis (except *arg4-Δ5*, discussed below) whereas the sensitivity of neighboring sites is unchanged. For example, the average increase of MNase hypersensitivity at the −200 *ARG4* site is 2.6 ± 0.9-fold (measured in four independent experiments) in contrast to 0.9 ± 0.3-fold at the +220 sensitive site (measured from five independent experiments). Such increase of MNase sensitivity during meiosis was not mentioned in the similar experiment performed with DNase I by Wu and Lichten (1994). The possibility that DNase I and MNase I assays give different results and therefore allow the detection of different states of chromatin structure warrants further examination. DNase I preferentially attacks double-stranded rather than single-stranded DNA. On the other hand, MNase prefers single-stranded DNA to double-stranded DNA. Thus, it is likely that the MNase probes the changes in the higher order structure of DNA at the site, in addition to the accessibility of the DNA region to the enzyme. The use of single-stranded DNA-specific endonucleases will test this hypothesis.

The temporal, quantitative and positional relationships between induced MNase-hypersensitive sites and DSBs suggest the attractive hypothesis that a genome-wide change of chromatin conformation during early meiosis is the prerequisite for DSB formation. However, because in one construct (*arg4-Δ5*) the DSB site seems to correspond to a non-induced MNase site (Figure 4A) and slightly induced MNase sensitivity sites are not DSB sites in *CYS3* (for example at position −70, Figure 6A), a more accurate view might be that DSBs generally occur at the subset of accessible chromatin sites potentiated in early meiosis but can also occur at a lower level in the absence of chromatin changes at the most accessible promoter site (alike in *arg4-Δ5*). The developmentally regulated chromatin remodeling will therefore play a role in the selectivity of the position of the DSBs and contributes to the efficiency of their formation. The present study indi-

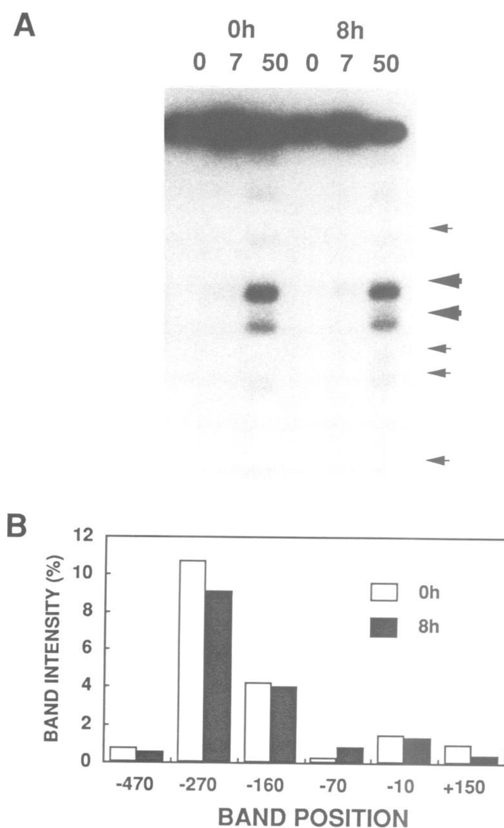


Fig. 7. MNase sensitivity around DSB sites in *CYS3* in non-sporulating cells. (**A**) Chromatin extracted from the NKY1419 strain at t = 0 h (lanes 0 h) and t = 8 h (lanes 8 h) incubation in the sporulation medium was treated with 0, 7 or 50 U/ml of MNase (lanes 0, 7 and 50), and analysed as described in Figure 6A. Arrows and arrowheads indicate MNase-sensitive and MNase-hypersensitive sites, respectively. (**B**) Quantification of MNase band intensity expressed as a percentage to the total band intensity in the lane.

cates that the local remodeling of chromatin structure potentiates the most prominent MNases-sensitive sites pre-existing in presporulation time (t = 0 h) at *ARG4* [−200 site (Figure 2)] and *CYS3* [−270 CYS3-I site (Figure 6)] but can also potentiate a weaker one [(−160 CYS3-II site (Figure 6)]. Additional molecular factors are also likely required for the formation of DSBs. This is suggested by two observations. First, the delay observed between the formation of DSBs molecules, first detected 2–4 h later after the appearance of the local increase in MNase sensitivity at *ARG4* (see Figure 3) indicates that the change in chromatin structure is not sufficient for the immediate formation of meiotic DSBs. Second, the great difference in the degree of induction between meiotic recombination (10^3-fold) and MNase sensitivity of DSB sites (2- to 4-fold) suggests that the change of chromatin structure as measured by MNase sensitivity may be only one component of a multi-step process that remains to be elucidated. One of the additional factors might be the induction of a recombination-specific endonuclease(s).

Hypothetical events that may be the source of chromatin alteration leading to increased MNase sensitivity are for example: transcription (discussed below), unwinding of DNA stretches or association of sequence-specific DNA binding proteins, bending of DNA, negative supercoiling, progression of chromosome condensation, etc. Such events or their combination can also be the source of the subtle differences observed from site to site (see *CYS3* in Results). Globally, these differences concerning the selectivity of induction among neighboring MNase-hypersensitive sites (a single site at *ARG4* versus two sites at *CYS3*), the amount of induction (CYS3-I versus CYS3-II) and the kinetics of induction (CYS3-I versus CYS3-II) strongly suggest that the potentiation of MNase-hypersensitive sites in early meiotic prophase could be differentially and finely regulated from one site to another and from one locus to another.

Formation of DSBs: cis- and trans-acting factors

Once we consider the role of chromatin structure in the control of the initiation of recombination, a crucial question concerning the formation of DSBs is the relative role of the *cis*-acting sequences versus nucleosome positioning. This issue is raised in the non-functional inversion construct examined in this report (ORD1132) which exhibits a strong positional effect (Figure 5B) although the *cis*-acting element for DSB formation and positioning, mapped between position −139 and +3 of the *ARG4* promoter (de Massy and Nicolas, 1993), is present. In this construct, where no DSB is detected, Rocco *et al.* (1992) demonstrated that a readthrough *YSC83–ARG4* transcript traverses the DSB site, thus inactivating the *ARG4* recombination initiation site. At the chromatin level, we now observe that the −200 region containing the normal site of DSB is included in a region protected by a phased nucleosome (Thoma *et al.*, 1984; Fedor *et al.*,1988; Roth *et al.*, 1990) carried over from the adjacent gene context (illustrated in Figure 5B). This indicates that neither the suggested genome-wide chromatin change in early meiosis nor the *cis*-acting control element(s) for the formation of DSBs can overcome the pre-existing nucleosomes. Randomly distributed or poorly positioned nucleosomes are common features of the 5′ intergenic region where active recombination initiation sites are often localized, as in the upstream *ARG4* intergenic region (Figures 2B and 5B).

Trans-acting factors affecting chromatin structure at DSB sites are not known. All natural DSB sites identified so far are localized in promotor regions (Sun *et al.*, 1989; Zenvirth *et al.*, 1992; Nag and Petes, 1993; Wu and Lichten, 1994). It is therefore possible that general *trans*-acting factors, such as transcription factors, play an important role in the positioning of DSBs. Indeed, in *HIS4*, it was shown that the BAS1, BAS2 and RAP1 transcription factors were needed for wild type levels of recombination perhaps to create the appropriate chromatin structure for the yet unidentified nuclease (White *et al.*, 1993). In *ARG4*, since the increase in the MNase sensitivity was also observed in the *arg4–polyl* construct where the binding sequence for the GCN4 protein (Hill *et al.*, 1986) was eliminated, GCN4p is not involved. This is consistent with the previous observation that deletion of the GCN4p binding site (*arg4–Δ13*) or disruption of the *GNC4* locus (*gcn4Δ*) display wild type levels of gene conversion at the *ARG4* locus (Schultes and Szostak, 1991). Another possibility is that the gene products required for the formation of DSBs bind to the *cis*-acting sequences altering chromatin structure to recruit the putative recombinational endonuclease.

Further characterization of the chromatin structure of recombination initiation sites and its developmentally regulated changes of state during prophase I of meiosis should help to identify such *trans*-acting factors and thus characterize the probable interplay between genome-wide and local *cis*- and *trans*-regulatory elements that enhance homologous recombination in meiotic cells.

The increase in MNase sensibility of the DSBs sites in early meiosis provides a novel landmark to refine the temporal and functional dissection of the complex developmental pattern of meiosis (Padmore *et al.*,1991; Kleckner and Weiner, 1993).

Materials and methods

Yeast strains, media and genetic analyses

The origin and relevant genotypes of the strains used are listed in Table I. Standard media and growth conditions were used (Nakagawa *et al.*, 1992). Presporulation and sporulation were as described (Rocco *et al.*, 1992; de Massy and Nicolas, 1993). Briefly, cells were grown in a presporulation medium with vigorous aeration at 30°C to a cell density of $2\text{–}5\times10^7$, washed once in water and incubated with vigorous aeration at 30°C in sporulation media (1% KAc). Commitment to meiosis was monitored by counting ARG^+ recombinants in return to growth experiments of the heteroallelic *arg4-RV /arg4-Bg* diploids, as previously described (Rocco *et al.*, 1992; de Massy and Nicolas, 1993). In these diploids, commitment to meiotic recombination occurs 8–10 h after transfer of the cell into the sporulation medium (t = 0 h), and 50–60% of the cells complete sporulation after 24 h. Strain YKN1419 (*Mat*α *ura3-52 leu2 met3*) was used for a control experiment as a haploid wild type.

Enzymes and chemicals

Restriction endonucleases were from New England Biolabs and Takara Shuzo Co. Ltd. MNase and Ficoll 400 were purchased from Pharmacia. Zymolyase 100T was from Seikagaku Kogyo Co. Ltd. Proteinase K in solution was from Boehringer Mannheim. RNase A was from Sigma and Boehringer Mannheim.

Preparation of chromatin and digestion of chromatin DNA by nucleases

Preparation of crude chromatin fraction from *S.cerevisiae* cells was carried out according to the method of Bernardi *et al.* (1991). For the quantitative comparison of MNase sensitivity, samples of chromatin were prepared from a fixed amount of cells (1–2 g wet weight) in each experiment. Spheroplasts were prepared by an incubation with zymolyase for 15 min at 30°C. One ml aliquots of the crude chromatin suspension were digested with different amounts of MNase (0, 7 or 50 U/ml) for 5 min at 37°C in the presence of 5 mM $CaCl_2$. The reaction was terminated by adding 20 mM EDTA, 1% (w/v) SDS and 20 μg of proteinase K, and incubated at 50°C for 2 h. Insoluble material was removed by microcentrifugation at room temperature and 15 000 r.p.m. for 10 min. The supernatants were extracted twice by phenol/chloroform/isoamylalcohol, digested by RNase A, extracted once in phenol/chloroform/isoamylalcohol. Extracted DNA was precipitated by ethanol, rinsed in 70% ethanol, and resuspended in 100 μl of TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Amounts of DNA are expressed as molecules of nucleotides.

Digestion of naked DNA

Naked DNA samples were prepared as described above except that addition of MNase was omitted. 900 μl of Buffer A were added to 100 μl of naked DNA (70–140 nmol). The diluted DNA was incubated with various amounts of MNase (1–5 U/ml) in the presence of 5 mM of $CaCl_2$ at 37°C for 5 min. The reaction was stopped as described above. The mixture was treated with 5 μg of Proteinase K at 50°C for 30 min, extracted with phenol/chloroform/isoamylalcohol and precipitated by ethanol. The final precipitate was resuspended in 100 μl of TE.

Detection of nuclease hypersensitive site by indirect end-labeling, meiotic double-strand break and recombination products

Indirect end-labeling (Wu, 1980) was performed as described (Bernardi *et al.*, 1991; Thoma *et al.*, 1984). 10 μl of DNA (7–14 nmol) were completely digested by restriction endonucleases. The digested DNA was ethanol-precipitated, separated by electrophoresis on a 1.2–1.5% agarose gel (20 or 40 cm long) containing 0.5 μg/ml of ethidium bromide in TAE (0.04 M Tris–acetate, 1 mM EDTA, pH 8.0) at 80 V for 15–17 h, and alkali-transferred to nylon membranes (Hybond N^+, Amersham) under vacuum using a VACUGENE (LKB) apparatus. Membranes were prehybridized for 1 h and hybridized for 24 h according to Church and Gilbert (1984) with 0.5–1 ng/ml of labeled probes. Probes were labeled by the random priming method according to the manufacturer (Amersham or Pharmacia) using 3.7 MBq (100 μCi) of [α-^{32}P]dCTP (specific activity: 3000 Ci/mmol) for 100 ng of DNA fragments. Labeled DNA fragments were visualized by autoradiography and quantified by the camera-input-densitometry program (whole band) of Milligen BioImage. Otherwise, membranes were directly quantified using the Imaging Plates for Fuji BAS2000 Image Analyzer combined with the whole band program of Milligen BioImage. Both methods gave the same results. Band intensity was expressed as a percentage of the total band intensity including the unbroken parental fragment. For comparison, the same number of bands in lanes for each sample was quantified in the whole band program. Meiotic DSBs in the diploid strain ORD307 were detected as previously described (Rocco *et al.*, 1992; de Massy and Nicolas, 1993). For the detection of meiotic recombination products, DNA of strain ORD149, heteroallelic for the *arg4-RV/arg4-Bg* mutations was digested by *Pst*I, *Eco*RV and *Bgl*II. DNA fragments were separated as described above, and probed with the *ARG4 Eco*RV–*Bgl*II fragment. The ARG^+ recombinant product is revealed by the appearance of a 1014 bp band.

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