The meiotic recombination hot spot created by the single-base substitution ade6-M26 results in remodeling of chromatin structure in fission yeast

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The G → T transversion mutation, ade6-M26, creates the heptanucleotide sequence ATGACTG, which lies close to the 5' end of the open reading frame of the ade6 gene in Schizosaccharomyces pombe. The mutation generates a meiosis-specific recombination hot spot and a binding site for the Mts1/Mts2 protein. We examined the chromatin structure at the ade6 locus in the M26 strain and compared it to that of the wild-type and hot spot-negative control M375. Micrococcal nuclease (MNase) digestion and indirect end-labeling methods were applied. In the M26 strain, we detected a new MNase-hypersensitive site at the position of the M26 mutation and no longer observed the phasing of nucleosomes seen in the wild-type and the M375 strains. Quantitative comparison of MNase sensitivity of the chromatin in premeiotic and meiotic cultures revealed a small meiotic induction of MNase hypersensitivity in the ade6 promoter region of the wild-type and M375 strains. The meiotic induction of MNase hypersensitivity was enhanced significantly in the ade6 promoter region of the M26 strain and also occurred at the M26 mutation site. The formation of the MNase-sensitive region around the heptamer sequence was abolished by the introduction of single-nucleotide substitutions in the heptamer sequence, which also abolish hot spot activity and binding of Mts1/Mts2. These data suggest that Mts1/Mts2 binding to the heptamer sequence results in a chromatin structure suitable for the recruitment of a meiosis-specific recombination function or functions.

[Key Words: recombination; meiosis; yeast; hot spot; chromatin]

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It has been demonstrated that meiotic recombination occurs preferentially at defined sites called hot spots on meiotic chromosomes in various eukaryotic organisms [Shiroishi et al. 1993; Smith 1994; Lichten and Goldman 1995]. In the budding yeast Saccharomyces cerevisiae, most of the meiotic recombination is initiated by the formation of meiosis-specific DNA-double strand breaks (DSBs) at hot spots [Nicolas et al. 1989; Sun et al. 1989; Cao et al. 1990; Zenzvirh et al. 1992; de Massy and Nicolas 1993; Goyon and Lichten 1993; Nag and Petes 1993; Wu and Lichten 1994]. Meiotic DSBs on chromosomes are preferentially introduced at nucleosome-free regions that show hypersensitivity to nucleases [Ohta et al. 1994; Wu and Lichten 1994; Fan and Petes 1996]. Therefore, accessibility of DNA is a primary requirement for hot spot activity. In addition, hypersensitivity to micrococcal nuclease (MNase) becomes more specific and quantitatively more important at hot spots during early meiotic prophase [Ohta et al. 1994]. This implies the action of meiosis-specific functions for chromatin changes at hot spots. However, the existence of meiotic DSBs and the role of chromatin structure at hot spots have not been established in organisms other than S. cerevisiae.

In the fission yeast Schizosaccharomyces pombe, the meiotic recombination hot spot ade6-M26 [M26] (Gutz 1971) is created by a G → T transversion in the open reading frame close to the 5' region of the ade6 gene [Ponticelli et al. 1988; Szankasi et al. 1988]. The mutation creates a nonsense codon and causes up to a 15-fold, meiosis-specific elevation of recombination frequency compared to the ade6-M375 mutation [Gutz 1971; Ponticelli et al. 1988; Schuchert and Kohli 1988]. The mutation ade6-M375 [M375] is a G → T base substitution that creates a nonsense mutation in the codon preceding M26 [Ponticelli et al. 1988; Szankasi et al. 1988], but it does not show hot spot activity [Gutz 1971], thus pro-
providing an excellent negative control for M26. M26 has features similar to hot spots in *S. cerevisiae*. (1) The M26-carrying chromatid is a preferential recipient of genetic information: It is preferentially converted to wild-type in one-factor crosses (Gutz 1971). This is a typical and predicted feature of the DSB repair model (Szostak et al. 1983). (2) The conversion rates of markers upstream of M26 increase steadily as their distance from M26 decreases. This is concluded from the combination of tetrad data (Gutz 1971) with the physical analysis of conversion tracts (Grimm et al. 1994), suggesting the existence of an initiation site in the 5' region of the ade6–M26 gene. (3) The activity of M26 is dependent on the chromosomal context. Transplacement of the whole ade6–M26 gene to other sites in the genome often abolishes the hot spot activity (Ponticelli and Smith 1992; Virgin et al. 1995), whereas the transplacement of the ade6 gene with M26 sometimes leaves the hot spot active. In addition, M26 enhances recombination between plasmid versus chromosome, only when it is located on the chromosome (Ponticelli and Smith 1992; Virgin et al. 1995). These results imply a role for chromatin structure or higher-order chromosome structure in the control of the M26 hotspot.

The M26 mutation generates the heptanucleotide sequence [5'–ATGACGT-3', M26 mutation underlined]. All base substitutions at any of the seven positions in the heptamer abolish hot spot activity (Schuchert et al. 1991). The heterodimeric protein Mts1/Mts2 was identified as a factor binding specifically to DNA carrying the heptamer sequence (Wahls and Smith 1994). This binding was abolished when single-base substitutions were introduced into the heptamer sequences, showing that there is perfect correlation between the sequence requirements of the heptamer sequence for hot spot activity in vivo and Mts1/Mts2-binding activity (Schuchert et al. 1991). Thus, the Mts1/Mts2 protein could have a crucial role in the establishment of the M26 hot spot. However, the reason for the meiosis specificity of M26 still remains unexplained, as the activity of the Mts1/Mts2 protein is present both in mitotic and meiotic cell extracts (Wahls and Smith 1994). One possibility is that meiotic alteration of chromatin structure may contribute to the establishment of the M26 hot spot.

In this study we have examined the chromatin structure at the ade6 gene before and during meiosis of diploid strains homozygous for M26, M375, or wild-type sequences. We found that the ade6 chromatin is remodeled strikingly in M26 diploids but not in wild-type or M375 diploids.

**Results**

**M26-dependent remodeling of chromatin structure**

We examined the meiotic chromatin structure at the ade6 region by an indirect end-labeling method. Chromatin was isolated from diploid strains homozygous for either wild-type, M375, or M26 at various time points (t = 0, 2, 3, 4, 6 hr) after transfer of cells into a sporulation medium. The isolated chromatin was digested with various concentrations of MNase. DNA was purified and examined by agarose gel electrophoresis, followed by ethidium bromide staining. A ladder of several broad bands indicated integrity of nucleosomal repeats in the chromatin fraction [data not shown]. The MNase-digested DNA was then cleaved by appropriate restriction endonucleases, and the DNA fragments were analyzed by Southern hybridization using short probes for sequences adjacent to one of the restriction sites.

Figure 1 illustrates the MNase-sensitive sites in chromatin of the ade6 locus in wild-type, M375, and M26 mutations at t = 3 hr after transfer of the cells into a sporulation medium. The MNase-digestion patterns of wild-type and M375 were similar. We observed two hypersensitive sites (–210 and –80) in a presumed promoter region that lies in the 5' intergenic region of the ade6 locus and a ladder of sensitive sites in the open reading frame of ade6. The regular spacing of average 144 bp [seven nucleosomes at positions 90–1100] represents a phasing of nucleosomes (Bernardi et al. 1991) as revealed by comparison with the digestion patterns of naked DNA. In contrast, the chromatin of M26 was significantly different. In addition to the two hypersensitive sites (–210 and –80) in the presumed promoter region, we detected a novel hypersensitive site at the position of the M26 mutation (170) that is covered by a phased nucleosome in wild-type and M375 mutations. Furthermore, the regular spacing of –150 bp was not observed and some new MNase-sensitive sites appeared at positions corresponding to MNase-sensitive sites in the naked DNA of the coding region [e.g., bands at 340, 480, and 670].

**M26-dependent enhancement of meiotic induction of MNase hypersensitivity**

Next, we compared the chromatin structure at the ade6 locus in premeiotic and meiotic cells [Figs. 2 and 3]. To allow comparison, the radioactivity of each band was quantified. The MNase sensitivity at each site was expressed relative to the sum of all band intensities in the lane. For M375, we found that MNase sensitivity at the presumed promoter increased slightly during meiosis (2.4 ± 1.3-fold at site –210, 3.9 ± 2.2-fold at site –80, n = 3; see Table 1). We observed the same features for the wild type [Table 1]. On the other hand, the sensitivity at other sites did not change significantly, for example, 1.3 ± 0.4-fold (n = 3) and 1.2 ± 0.1-fold (n = 2) at site –80 in M375 and wild type, respectively. This phenomenon is similar to the observations made at hot spots for meiotic recombination in budding yeast (Ohta et al. 1994).

The meiotic induction of MNase sensitivity was enhanced by up to 9.3 ± 0.6-fold as a result of M26 at site –80 in the presumed promoter region (n = 3; see Fig. 3; Table 1), whereas the sensitivity at site 80 was not enhanced at all (1.0 ± 0.1-fold, n = 3). MNase sensitivity at the M26 mutation site also increased significantly during meiosis (5.4 ± 2.2-fold, n = 3; see Fig. 3 and Table 1). The increase in MNase hypersensitivity was first de-
Figure 1. Comparison of chromatin structures at the ade6 locus in wild-type, M375, and M26 strains. The yeast strains were harvested at t = 3 hr after transfer of cells into the sporulation medium. Chromatin and naked genomic DNA (from the M26 strain) were isolated, treated with MNase (50 and 32 U/ml, respectively), and analyzed by indirect end-labeling, as described in Materials and Methods. DNA was digested with XhoI (A) and Stul-EcoRI (B), and subjected to Southern hybridization with probes for the sequence adjacent to the XhoI site at +1469 bp from the ATG of the ade6 coding region (probe B in A), or for the sequence adjacent to the Stul site at −785 bp (probe C in B). An interpretation of the nucleosome positions is shown. [Shaded shapes] Nucleosomes; [horizontal arrows] MNase-sensitive sites; [open triangles] the M26 mutation site. Vertical open arrows indicate the position of the coding region for the ade6 locus. The numbers beside the horizontal arrows indicate the distance from the A of the ATG at the beginning of the ade6 coding sequence. Sequences recognized by the hybridization probes are indicated by vertical open rectangles. The DNA size markers are XhoI (1953 bp), XhoI-HindIII (627 bp), and XhoI-BamHI (1443 bp) for A, and Stul-EcoRI (2015 bp), Stul-HindIII (1626 bp), and Stul-BamHI (810 bp) for B. Note that the M375 and M26 sequences differ only by single-base substitutions from the wild-type ade6 sequence.

Figure 2. Quantitative comparison of pre-meiotic and meiotic chromatin structures at the ade6 locus in the M375 strain. Chromatin [0 hr premeiotic; 3 hr meiotic] of the M375 strain was digested with 0, 7, 50, and 225 U/ml of MNase and examined as described in Fig. 1. DNA was digested with XhoI and hybridized with probe B (see Fig. 1A). The radioactivity of each band was quantified by a Fuji BAS2000 system and Bio Image analyzer as described in Materials and Methods. Band intensities in lanes containing samples digested with 50 U/ml of MNase were expressed as percentage of the sum of all band intensities. For further explanations, see Fig. 1. Intensity of a faint band corresponding to the band at 0 in Fig. 1 was not quantified in this study, as the intensity was too low for accurate quantification. [Open bars] Data of t = 0 hr; [hatched bars] data of t = 3 hr. The numbers beside the horizontal arrows indicate the distance from the A of the ATG at the beginning of the ade6 coding sequence.
Chromatin remodeling in ade6-M26 hot spot

Figure 3. Quantitative comparison of premeiotic and meiotic chromatin structures at the ade6 locus in the M26 strain. Chromatin isolated from the M26 strain was digested with 7, 50, and 225 U/ml of MNase and examined as described in Figs. 1 and 2. For further explanations see, Fig. 2. Open triangles show the position of the M26 mutation. Bars with horizontal arrows represent the sites of meiotic enhancement of MNase hypersensitivity. In this experiment the band intensity of some faint bands (bands corresponding to those at 450, 590, and 630 in Fig. 1) was not quantified, as the intensity was too low for accurate quantification. The numbers beside the horizontal arrows indicate the distance from the A of the ATG at the beginning of the ade6 coding sequence.

Table 1. Quantification of MNase hypersensitivity

| Strains  | Site -210 | Site -80  | Site M26 |
|----------|-----------|-----------|----------|
| Wild type| 2.9 ± 0.3 [2.7, 3.1] | 4.1 ± 2.4 [2.4, 5.8] | —        |
| M375     | 2.4 ± 1.3 [2.2, 3.7, 1.2] | 3.9 ± 2.2 [2.1, 6.3, 3.2] | —        |
| M26      | 2.6 ± 0.7 [3.1, 3.0, 1.8] | 9.3 ± 0.6 [8.7, 9.5, 9.8] | 5.4 ± 2.2 [7.9, 3.5, 4.8] |
| 7T       | 2.6 ± 1.3 [3.6, 1.7] | 10.2 ± 2.0 [11.6, 8.8] | 4.7 ± 0.5 [5.1, 4.4] |
| 13G      | 1.9 ± 0.4 [2.1, 1.6] | 4.1 ± 2.0 [5.5, 2.7] | —        |
| 14C      | 1.9 ± 0.4 [2.2, 1.6] | 3.0 ± 1.7 [1.8, 4.2] | —        |
| 16C      | 2.4 ± 0.2 [2.5, 2.3] | 11.5 ± 1.6 [12.6, 10.4] | 3.6 ± 0.2 [3.7, 3.4] |

The MNase hypersensitivity in each strain was quantified by a Fuji BAS2000 system and a Bio Image analyzer as described in Materials and Methods and Figs. 2 and 3. Meiotic induction of MNase hypersensitivity is expressed as the ratio of the meiotic hypersensitivity to the premeiotic hypersensitivity at each site including standard deviations. Numbers after ± represent standard deviations of the measurement. Numbers in parenthesis show the data for each experiment. The experiments were repeated three times with the M26 and M375 strains and twice with the wild-type, the 7T, 13G, 14C, and 16C strains. The ratio of the MNase hypersensitivity is basically independent from the concentration of MNase up to 50 U/ml.
different from those in 7T or 16C. As expected, they were almost identical with those of the wild type and M375 cells; that is, the MNase-hypersensitive site at the heptamer was absent and meiotic induction of MNase hypersensitivity at site -80 was reduced. The difference in the band patterns in these strains is not attributable to the difference in MNase-sensitive sites on naked DNA, as the heptamer mutations did not affect the band patterns on naked DNA [data not shown]. Therefore, there is positive correlation between hot spot activity, binding of Mts1/Mts2 to the heptamer, formation of an accessible DNA region around the heptamer, and enhanced meiotic induction of MNase sensitivity. Interestingly, the 16C construct yielded a novel pattern; that is, the three positioned nucleosomes present in wild type and absent in M26 reappeared in 16C but were displaced in the 5' direction. The novel phasing of these three nucleosomes seems to be directed by the MNase-hypersensitive site at M26 and the additional base substitution 16C (Figs. 5 and 6).

Discussion
Chromatin remodeling by the heptamer sequence and the Mts1/Mts2 protein
The results presented here indicate that the heptamer sequence ATGACGT affects nucleosome positioning, possibly by facilitating the binding of the Mts1/Mts2 heterodimeric protein. In M26 chromatin, the phasing of nucleosomes was affected around the heptamer and downstream in the open reading frame of the ade6 gene. The MNase-sensitive sites characteristic of naked DNA were observed in the chromatin digestion, and a novel MNase-hypersensitive site also appeared at the heptamer. All of these changes in DNA accessibility were the consequence of the single G → T base substitution that created M26 from the wild-type sequence. Our results seem to be inconsistent with the data of Bernardi and coworkers [1991], who found the chromatin in M26 haploid vegetative cells to be very similar to that in wild-type cells. We have confirmed their results by showing that the nucleosome positioning in the ade6 region in the haploid M26 strain during vegetative growth was very similar to the patterns seen in diploid wild-type and M375 strains [K. Mizuno, J. Kohli, and K. Ohta, unpubl.]. Therefore, the remodeling of chromatin structure found in the mitotic cells (i.e., premeiotic cells at t = 0 in the meiotic time course in this study) seems to be related closely to ploidy or some other factors. We are currently analyzing this phenomenon.

We found that chromatin remodeling by M26 requires the same integrity of the heptamer sequence as that required for the binding of the Mts1/Mts2 protein [Wahls and Smith 1994]. Altered chromatin structure as a consequence of sequence-specific DNA-binding proteins has been reported for transcription factors. The α2 repressor in S. cerevisiae was shown to affect the positioning of nucleosomes at the promoter through binding to its target DNA sequence in vivo [Shimizu et al. 1991]. The GAGA factor in Drosophila was shown to be involved in formation of nuclease hypersensitive sites and remodeling of chromatin at the heat shock-responsive element in vitro [Tsukiyama et al. 1994]. Probably, the binding of Mts1/Mts2 to the heptamer influences the binding of nucleosomes to this region, thereby altering nucleosome positioning [Fig. 7]. In this respect, it would be interesting to analyze chromatin structure in M26 strains disrupted for the genes coding for Mts1 and Mts2.

It is intriguing that a pattern of nucleosome phasing downstream of M26 is restored, when the single-base substitution 16C is added to the heptanucleotide. The resulting chromatin structure, however, differs from the wild type in that the nucleosomes are shifted toward the 5' end. We propose that the 16C sequence forms a boundary for nucleosome positioning. Although the mecha-
should be noted that the 16C mutation also enhances the binding of Mts1/Mts2 (Wahls and Smith 1994) and increases meiotic recombination frequency beyond the M26 level (Schuchert et al. 1991).

Meiotic induction and M26 enhancement of MNase hypersensitivity: implication for the meiosis specificity of the M26 hot spot and a general model for the initiation of meiotic recombination at ade6

The mechanism that creates the meiosis specificity of the M26 recombination hot spot (Ponticelli et al. 1988; Schuchert and Kohli 1988) is not yet fully understood. The Mts1/Mts2 protein and its heptamer-binding activity are present in extracts of both mitotic and meiotic cells (Wahls and Smith 1994). In addition, the MNase-sensitive site at M26 is detected in mitotic and meiotic diploid cells, suggesting the constitutive interaction of Mts1/Mts2 with the heptamer in vivo and the necessity to invoke the existence of additional factors to explain M26 meiosis specificity.

We have demonstrated here that MNase hypersensitivity increases significantly at the presumed promoter and the heptamer sequence during early meiosis (Table 1). The increase in MNase sensitivity was observed from two aspects: [1] Meiotic induction of MNase hypersensitivity is observed in all strains (wild type, M375, and M26) at the two sites, −210 and −80, in the presumed promoter, and [2] in M26, meiotic induction of MNase hypersensitivity is significantly stronger at the −80 promoter site and at the M26 site when compared to those in wild-type and M375 strains. These are the first observations suggesting the interaction of meiosis-specific functions with accessible DNA regions during S. pombe meiosis. We put forward three explanations for the meiosis-specific functions that cause the meiotic increase in the MNase sensitivity of the M26 hot spot.

The first possibility is that enhanced binding of Mts1/Mts2 to the heptamer during meiosis increases DNA accessibility at M26. This is supported by the observation that the MNase hypersensitivity at the heptamer increased about fivefold during meiosis. Although this possibility is likely, it should be noted that the MNase-hypersensitive site at M26 is already present in mitotic cells, in which M26 enhanced homologous recombination could not be detected at all (Ponticelli et al. 1988; Schuchert and Kohli 1988) unless a DSB is supplied at the ade6 region by introduction of HO endonuclease (Osman et al. 1996). This observation implies that meiotic activation of the M26 hot spot might include other steps such as recruitment of a meiosis-specific nuclease or a recombination protein to accessible DNA regions created by Mts1/Mts2 (see below).

The second possibility is that changes in DNA structure occur at the heptamer and in the presumed promoter region during meiosis (e.g., melting of DNA yielding single-stranded regions). However, in the case of budding yeast, we could not detect a significant change in DNA structure during meiosis at the nucleosome-free regions of the ARG4 hot spot by genomic footprinting analysis using single
stranded DNA (ssDNA)-specific probes such as KMnO₄, mung bean, and P1 nucleases (K. Ohta, A. Nicolas, and T. Shibata, unpubl.). Thus, changes in DNA structure might be too subtle to cause the increase in MNase sensitivity. Assuming that the same mechanism for meiotic enhancement of MNase hypersensitivity is operative in both yeasts, changes in DNA structure, at least formation of ssDNA, is unlikely to explain the meiotic increase in MNase sensitivity of the M26 hot spot.

The third explanation is that the meiotic increase in MNase sensitivity at M26 and then hot spot activity is caused by the recruitment of other proteins to the ade6 gene that, unlike Mts1/Mts2, would be specific for meiotic recombination (Fig. 7). Such recombination proteins would be recruited to the accessible DNA regions formed by the binding of Mts1/Mts2 protein and also probably by transcription factors bound to the promoter. This model is based on our results showing that MNase hypersensitivity at hot spots increases significantly during meiosis prior to the formation of meiotic dsDNA breaks. The appearance of meiosis-specific footprints was not detected in a subset of meiotic DSB-deficient mutants such as mre11. Therefore, we speculate that the meiotic induction of MNase sensitivity at hot spots in budding yeast reflects an essential reaction caused by the interaction of recombination proteins with DNA prior to the DSB formation. It is also possible that the meiotic enhancement of MNase hypersensitivity at the ade6–M26 locus may reflect the action of meiotic recombination enzymes. If meiotic recombination mechanisms are conserved between the two yeast species, rad32⁺, the fission yeast homolog of the MRE11 gene (Tavassoli et al. 1995), may be involved in the meiotic enhancement of MNase hypersensitivity. The study of the chromatin structure in yeast cells with a disruption of the rad32* or other recombination genes will be a test of this model.

Is the ATGACGT sequence enhancing a preexisting recombination initiation site?

In budding yeast, hot spots often coincide with accessible promoter regions that show meiotic induction of MNase hypersensitivity (Ohta et al. 1994). The presence of meiotically enhanced chromatin alterations in the

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**Figure 6.** A schematic diagram showing nucleosome positions in different ade6 strains. The positions of nucleosomes were estimated from the data on MNase-digested chromatin and digests of naked DNA in Fig. 1. The nucleosome patterns were classified into three types: Type A includes wild-type, M375, 13G, and 14C; type B includes M26 and 7T; and type C includes 16C. Vertical arrows indicate the positions of MNase-sensitive sites. Hatched horizontal arrows represent the ade6 coding region. The numbers in parenthesis give the distances from the first A of the initiation codon of the ade6 coding sequence. The positions of the heptamer sequences are indicated by thin solid boxes; the nucleosomes are indicated by hatched circles; the Mts1/Mts2 protein is shown as two kinked ovals. A shaded oval with a question mark in type C represents a putative third factor that may be involved in the boundary formation by the 16C sequence.
The hatched hexagon indicates putative initiation site. The presumed promoter region (hatched rectangle) is constitutively open and can act as a natural recombinational site. According to this model, the M26 heptamer would play the double role of an enhancer of the background MNase hypersensitivity and meiotic initiation site in the presumed promoter and of a novel hot spot of initiation at the heptamer.

In Figure 7 we propose an alternative idea that both the presumed promoter and the heptamer regions serve as initiation sites for meiotic recombination at the M26 locus. Assuming that meiotic induction of MNase sensitivity is an obligatory signal for initiation, as is the case in *S. cerevisiae*, the presence of the induction in MNase hypersensitivity after meiotic induction at the presumed promoter sites and the M26 heptamer is consistent with the notion that both sites are initiation sites. According to this model, the M26 heptamer would play the double role of an enhancer of the background MNase hypersensitivity and meiotic initiation site in the presumed promoter and of a novel hot spot of initiation at the heptamer. If the heptanucleotide is contributing to the enhancement of the initiation site in the presumed promoter, then position effects of the heptanucleotide would be expected. This could be examined by varying the position of the heptanucleotide with respect to the presumed promoter and analyzing for any changes in chromatin structure. In relation to this, it is worthwhile noting that the heptamer still functions as a hot spot when it is moved to downstream positions in the *ade6* gene (G. Smith, pers. comm.).

**Materials and methods**

**DNA and strains**

The plasmid containing the *ade6-M26* sequence (pade6-M26) is a derivative of pAS1 (Szankasi et al. 1988). The plasmid pade6-M26 was digested with *XhoI-EcoRl* or *XhoI-Stul* to form 240- and 300-bp fragments. The fragments were subcloned into the *pBluescript(KS+)*, respectively. Probes for indirect end-labeling were prepared from pAS1-1 and pAS1-2 by digesting the plasmids with *XhoI-EcoRl* followed by the purification of DNA fragments in agarose gels. All strains are from the collection at the University of Bern (Switzerland) as summarized in Table 2.

**Enzymes and chemicals**

Restriction endonucleases were purchased from New England Biolabs and Takara Shuzo Co. Ltd. MNase and Ficoll 400 were from Pharmacia. Zymolyase 100T was from Seikagaku Kogyo.
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Table 2. Genotypes of strains

| Diploid     | Haploid  | mat | ade6   | ura1  | leu3 |
|-------------|----------|-----|--------|-------|------|
| ELD201      | ELH101   | h*  | +      | ura1–61 | +    |
| (wild-type) | ELH102   | h*  | +      | ura1–61 | +    |
| ELD203      | ELH103   | h*  | ade6–M375 | ura1–61 | +    |
| (M375)      | ELH104   | h*  | ade6–M375 |          | leu3–155 |
| ELD205      | ELH105   | h*  | ade6–M26 | ura1–61 | +    |
| (M26)       | ELH106   | h*  | ade6–M26 |          | leu3–155 |
| ELD207      | ELH107   | h*  | ade6–M26/7T | ura1–61 | +    |
| [7T]        | ELH108   | h*  | ade6–M26/7T |          | leu3–155 |
| (13G)       | ELH110   | h*  | ade6–M26/13G | ura1–61 | +    |
| ELD211      | ELH111   | h*  | ade6–M26/13G |          | leu3–155 |
| [14C]       | ELH112   | h*  | ade6–M26/14C |          | leu3–155 |
| ELD213      | ELH113   | h*  | ade6–M26/16C | ura1–61 | +    |
| [16C]       | ELH114   | h*  | ade6–M26/16C |          | leu3–155 |

All strains are from the Bern collection. The construction of the heptamer mutants was described by Schuchert et al. (1991).

Co. Ltd. Proteinase K in solution was from Boehringer Mannheim. RNase A was from Sigma.

Cell culture and sporulation

Standard media, preporulation, and sporulation were as described [Bahler et al. 1991, 1993]. Briefly, diploid colonies were selected on minimal medium plates containing phloxin B. Colonies showing efficient and synchronous sporulation were further selected. Cells were grown in 1 liter of PM (Watanabe et al. 1988) containing supplements with vigorous aeration at 30°C to a cell density of 0.8 × 10^6 to 0.9 × 10^7, washed once in PM-N (Watanabe et al. 1988). Half of the cells were precipitated and frozen in liquid nitrogen, and then stored at -85°C. The result-

Preparation of chromatin and digestion of chromatin DNA by MNase

The preparation of crude chromatin fractions from S. pombe cells was done according to the method of Bernardi et al. [1991], with some modifications. For the quantitative comparison of MNase sensitivity, chromatin samples were prepared from a fixed amount of cells (0.6–1.0 gram wet weight) in each experiment. Briefly, 1 gram of cells was incubated in 2 ml of preincuba-

Digestion of naked DNA

Naked DNA samples were prepared as described above except that addition of MNase was omitted. Nine hundred microliters of buffer A was added to 100 µl of naked DNA (20–30 µg). The diluted DNA was incubated with various amounts of MNase [5–60 U/ml] in the presence of 5 mM 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/isoamyl alcohol, and precipitated by ethanol. The final precipitate was resuspended in 100 µl of TE buffer [10 mM Tris-HCl, 1 mM EDTA] at pH 8.0. The typical yield of DNA from 1 gram cells was ~200 µg.

Indirect end-labeling

Indirect end-labeling was performed as described [Bernardi et al. 1991; Ohta et al. 1994]. Briefly, 10 µl of DNA (2–3 µg) was digested completely by restriction endonucleases. The digested
DNA was ethanol-precipitated, separated by electrophoresis on a 1.2% agarose gel (40 cm long) containing 0.5 μg/ml of ethidium bromide in TAE buffer (0.04 M Tris/acetate, 1 mM EDTA) at pH 8.0) at 80 V for 15-17 hr and alkali-transferred to nylon membranes (Hybond N+, Amersham) under vacuum using a Vacugene (LKB) apparatus. Membranes were prehybridized for 1 hr and hybridized for 24 hr 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 [Pharmacia] using 3.7 MBq (100 μCi) of [α-32P]dCTP (sp. act. 3000 Ci/mmol) for 100 ng of DNA fragments. Radioactive DNA fragments were quantified using the imaging plates for Fuji BAS2000 Image Analyzer combined with the whole-band program of Bio Image (Bio Image Co. Ltd). Band intensity was expressed as a percentage of the total of all the band intensities of a lane including the unbroken parental fragment. For comparison, the same number of bands in lanes for each sample was quantified by the whole band program. The nucleotide positions of the MNase-sensitive sites were measured according to the auto-band detection algorithm of Bio Image. The nucleotide numbers were estimated from the mean values of at least four independent measurements.

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