Meiotic double-strand breaks in yeast artificial chromosomes containing human DNA

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ABSTRACT

Meiotic recombination in the yeast Saccharomyces cerevisiae is initiated by double-strand breaks (DSB) in chromosomal DNA. These DSB, which can be mapped in the rad 50S mutant yeast strain, are caused by a topoisomerase II-like enzyme, the protein Spo11. Evidence suggests that this protein is located in the axial element of the meiotic chromosome which implies that the DSB are located in these chromosomes in the vicinity of the bases of the DNA loops. We have found that in the yeast artificial chromosomes carrying human DNA, at the level of resolution obtained by pulsed field gel electrophoresis (PFGE), the meiotic DSB in the diploid yeast are co-localized with the DNase I hypersensitive sites (HS) in a haploid strain of yeast. These HS are located close to sequences which, under stress, have the potential to form secondary structures containing unpaired nucleotides. Clusters of such sequences could be a hallmark of the bases of the chromatin loops.

INTRODUCTION

The packaging of DNA in the nucleus and in mitotic or meiotic chromosomes necessitates several levels of folding. High level folding involves the formation of DNA loops attached to the chromosomal scaffold during mitosis, to the axial element of the chromosome during meiosis, or to the nuclear matrix during interphase. Several lines of evidence support the idea that the size and position of the loops and the extent of chromatin condensation are dictated by cis sites in the DNA (for review see 1). In an attempt to identify these sites it was assumed that the DNA segments at the bases of loops form strong complexes with the scaffold proteins. An experimental approach based on this assumption led to the discovery of scaffold-associated regions (SAR) and matrix attachment regions (MAR) (2,3). However, the possibility that the strong complexes formed between some of the SAR/MAR regions and the matrix represent experimental artefacts has not been ruled out (1,4–6).

On the other hand, it has been shown that early during apoptosis or as a result of the treatment of cells with topoisomerase II inhibitors the DNA is fragmented into segments of about 50 kb each (7). Pronounced DNase I hypersensitive sites (HS) showing chromatin loop-size spacings are apparently present in the chromatin. Evidence that HS are close to the bases of DNA loops has accumulated during recent years (8–11).

Most of the HS studied in various laboratories have been located in the chromosomal regions involved in transcription and containing promoters or enhancers (12). One may also ask whether untranscribed DNA is organized into loops, whether its folding also involves HS and if so, what are the features that make some DNA sequences, and not others, function as bases of loops. The study of the chromosome during meiosis may help to answer these questions.

The mammalian meiotic chromosome is built of loops that each contain ~120 kb of DNA. The 40 head-to-tail tandem repeats of λ phage DNA, however, when integrated into the mouse chromosome, form one single giant loop (13). It seems that the λ DNA lacks the sequences that are able to serve in mammalian cells as loop bases. On the other hand, the loops in the meiotic YACs carrying human DNA have a length of ~20 kb (14,15).

We chose this latter model, the YAC carrying a segment of human DNA, to study the bases of the loops in non-transcribed DNA. First, we were able to demonstrate that the HS in the chromatin in various YACs show spacings that are compatible with the loop size (16,17). In the present work we show that the HS in YACs are located close to, or are co-localised with, the sites of DSB appearing in the diploid yeast strain at the onset of meiosis. The DSB are caused by the Spo11 protein which is involved in the formation of the meiotic chromosome axial core (18). This implies that the HS/DSB sites, at which this protein interacts with the chromatin fiber, are the hallmarks of the attachment of DNA to the scaffold.

MATERIALS AND METHODS

Preparation of agarose blocks containing nuclei or naked DNA

The YAC 881D2-containing segment of human chromosome 21 was obtained from the CEPH (Centre d’Etudes du Polymorphisme Humain, Paris). We used two YACs carrying the β-globin gene cluster: the pYAC A85D10 was a kind gift from K.R.Peterson; and the pYAC A201F4 was purchased from Research Genetics (Huntsville, AL). Yeast spheroplasts, prepared as described...
previously (17), were suspended in ice cold hypotonic ‘nuclear’ buffer (40 mM K$_2$HPO$_4$–KH$_2$PO$_4$ buffer pH 6.4, 5 mM MgCl$_2$, 5 mM EGTA, 0.1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM DTT, 0.2 mM PMSF) and briefly centrifuged in an Eppendorf microfuge. The washing was repeated three times. The resulting crude preparation of nuclei was mixed with an equal volume of 1.8% LMP agarose at 37°C, poured into moulds and left to gel.

Naked DNA was prepared from yeast nuclei embedded in agarose blocks by lysis with proK/SDS buffer (0.1% sodium dodecyl sulphate, 0.25 M EDTA, 1 mg/ml protease K, 0.9% sodium sarcosylate, overnight incubation at 55°C).

**Low resolution mapping of HS in YACs**

The HS were mapped in the yeast nuclei embedded in the agarose blocks, at a concentration equivalent to ~2–5 µg of DNA per block, equilibrated at 0°C in a buffer: 15 mM Tris pH 7.5, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.15 mM spermine, 0.5 mM spermidine and DNase I at concentration as indicated. The reaction was initiated by adding MgCl$_2$ to a final concentration of 10 mM and CaCl$_2$ to a final concentration of 1 mM and then by transferring the blocks to 37°C for 20 min. The mapping experiments were also performed in low pH buffer (100 mM sodium acetate pH 5.2, 5 mM MgCl$_2$) in order to minimize the background autodigestion that occurs in the pH 7.5 buffer. The blocks were incubated at 30°C for 20 min with up to 25 U/ml DNase I (Sigma) after preincubation on ice for 30 min. In some experiments we used 10 mM MnCl$_2$ instead of MgCl$_2$ which made no apparent difference to the pattern of the YAC digestion products. In control experiments, naked DNA embedded in agarose blocks was digested in the same buffer with ~1% of the concentration of DNase I used for digestion of DNA in nuclei (up to 0.075 U/ml). After isolation of DNA by lysis of the nuclei embedded in blocks in the proK/SDS buffer, pulsed field gel electrophoresis and blotting, the pattern of HS in the YAC was revealed by hybridization with telomeric probes.

**Mapping sites sensitive to nuclease S1**

Yeast nuclei were embedded in the agarose blocks, washed with S1 nuclease buffer (33 mM sodium acetate pH 4.5, 50 mM NaCl, 0.5 mM ZnSO$_4$) and incubated for 30 min at 37°C, with S1 nuclease (Eurogentec) after preincubation on ice for 30 min.

**Mapping of toposomerase II inhibitor VM26**

The blocks of agarose with embedded nuclei were washed three times for 30 min on ice in KCl–MgCl$_2$ buffer (30 mM KCl, 10 mM MgCl$_2$, 25 mM Tris–HCl pH 7.5, 0.1 mM EDTA, 20 mM ATP, 0.1 mg/ml BSA), supplemented with 70 µg/ml VM26 and incubated for an additional 20 min on ice. The reaction lasted 12 min at 30°C. The blocks were then transferred to proK/SDS buffer.

**Analysis of DNA fragments with 5–10 kb resolution**

The blocks containing the products of partial DNA digestion were embedded into agarose slabs. The DNA was then analyzed by pulsed field gel electrophoresis and hybridization with one of the $^{32}$P-labelled telomeric probes. We ran the pulsed field gel electrophoresis at 14°C in 0.5× TBE buffer using 1% Sea-Kem agarose and a CHEF II apparatus (Bio-Rad Laboratories) in the conditions recommended by the manufacturer. After electrophoresis the DNA was transferred from the gel to a nylon membrane (Hybond plus, Amersham) according to the alkaline technique recommended by the manufacturer. We used rapid hybridization buffer (Amersham) to hybridise the filters to a $^{32}$P-labelled probe, and autoradiographed them using Fuji X-ray film. Probe ‘t’ was a BamHI–PvuII fragment and probe ‘c’ a PvuII–PstI fragment of pBR322 recognizing the YAC telomeres ‘T’ and ‘C’ (distal and proximal to the centromere), respectively. As a length standard a ‘lambda ladder’ (a mixture of $\lambda$ phage DNA oligomers, Biolabs) was electrophoresed along with the sample and was revealed by addition of $^{32}$P-labelled $\lambda$ phage DNA to the hybridization solution. The resolution of the pulsed field gel electrophoresis was within the range of ±5–10 kb.

**Mapping of DNase I HS in YAC chromatin with 0.2–0.6 kb resolution**

Cell lysates were prepared and DNase I digestion was carried out according to the protocol first described by Wu and Lichten (19) and then modified by Fan and Petes (20). The chromatin was digested for 5 min on ice with either 2.5 or 0.25 U/ml DNase I. The isolated DNA was subsequently digested with the BamHI restriction enzyme, electrophoresed using the classical gel electrophoresis, blotted and hybridized to ‘is2’ probe corresponding to the BamHI–EcoRI segment of intron 2 of the $\beta$-globin gene.

**Mapping of the meiotic double-strand breaks**

We transferred the YACs 881D2 and A85D10 from the yeast strain AB1380 (MATa ura3 trpl1 ade2-1 can1-100 lys2-1 his5 Ile‘ Thr‘ [YAC (URA3 TRIP1)]) to the haploid strain 2850 (MATa ho ura3 lys2 leu2 trpl1 rad50S::ura3 can1) by using the procedure developed by Hugerat et al. (21). The transfer was performed using intermediary strain 2474 (MATa ho ura3 lys2-101 his5Δ200 trp1 Δ1 leu2Δ1 cyh r kar1Δ15). The final YAC-carrying haploid strain 2850 was mated with the strain 2851 (MATa ho ura3 lys2 leu2 trpl1 rad50S::ura3 cyh2). The rad50S and kar strains were kindly provided by G.Simchen and D.Zenvirith. The breaks were mapped as described by Klein et al. (22).

**RESULTS**

In order to map the HS, the S1 sensitive sites and the toposites in yeast artificial chromosomes, the nuclei isolated as indicated in Materials and Methods, and embedded in agarose blocks, were treated with one of the following: the DNase I, nuclease S1 or toposomerase II inhibitor VM-26. After treatment, the nuclei-containing agarose blocks were lysed in protease K/SDS buffer, washed and incorporated into agarose slabs. The DNA present there was analyzed by pulsed field gel electrophoresis (PFGE) followed by hybridization with the $^{32}$P-labelled telomeric probes specific to either the left or the right end of YAC.

An experiment of this type, in which we used the yeast strain AB1380 containing either the YAC A85D10 or the YAC A201F4 (23) both carrying the human $\beta$-globin gene cluster (see Fig. 1A for their respective maps) is shown in Figure 1B. The two YACs display the same polarities and in both the artificial telomere ‘C’ was fused with the human genomic DNA at 39 kb 5’ from the nucleotide 1 (Nt 1) (the beginning of the sequenced part of the $\beta$-globin gene cluster, retrieved from GenBank where it was
Comparison of the hypersensitive sites (HS) and meiotic double strand breaks (DSB) observed in YAC A85D10 in haploid RAD 50 wild type and diploid rad 50S mutant strains, respectively. (A) Map of the relevant region of the YAC. The genes are shown as rectangles and the sequences around the centromere are proximal and distal, respectively, to the centromere. The arrows and arrowheads show the position of the strong and weak HS, respectively, in the YAC. The numerals show the positions of the HS determined with medium resolution by classical gel electrophoresis in ref. 16 and in this work. (B) Map of the main DSB in the same YAC. Upper numbers show the Mw scale, lower numerals show the positions of the DSB. (C) Lane 1, mapping of DSB in YAC A85 D10 in the rad 50S mutant strain, appearing after 6 h incubation in the sporulation medium; lane 2, partial digestion of the YAC chromatin, isolated from the AB1380 strain with 2 U DNase I; below a schematic representation of the YAC, see Figure 1 for description.

Figure 2. Comparison of the hypersensitive sites (HS) and meiotic double strand breaks (DSB) observed in YAC A85D10 in haploid RAD 50 wild type and diploid rad 50S mutant strains, respectively. (A) Map of the relevant region of the YAC. The genes are shown as rectangles and the sequences around the centromere are proximal and distal, respectively, to the centromere. The arrows and arrowheads show the position of the strong and weak HS, respectively, in the YAC. The numerals show the positions of the HS determined with medium resolution by classical gel electrophoresis in ref. 16 and in this work. (B) Map of the main DSB in the same YAC. Upper numbers show the Mw scale, lower numerals show the positions of the DSB. (C) Lane 1, mapping of DSB in YAC A85 D10 in the rad 50S mutant strain, appearing after 6 h incubation in the sporulation medium; lane 2, partial digestion of the YAC chromatin, isolated from the AB1380 strain with 2 U DNase I; below a schematic representation of the YAC, see Figure 1 for description.

Figure 1. Indirect end-labelling of the partial DNase I digest of β-globin YACs. (A) Schematic representation of the YACs. C' and T', artificial telomers proximal and distal to the centromere, respectively; rectangle, the sequenced part of the gene cluster; filled part of the rectangle, the locus control region (LCR); hatched rectangle, the position of the probe ‘c’. (B) Autoradiograms obtained by hybridization of DNA isolated from the YACs chromatin partially digested with DNase I, fractionated to nylon filters and hybridized to the probe ‘c’. Lanes 1–3, YAC A85D10; lanes 4–6, YAC A201F4. The numbers on the right show the positions of the sites measured from the beginning of the sequenced portion of the β-globin gene cluster. Lanes 1 and 4, no DNase; lanes 2 and 5, 1 U DNase I; lanes 3 and 6, 2 U DNase I.

The results of this experiment are shown in Figure 2. The bands corresponding to the meiotic DSB (Fig. 2C, lane 1), are coincident with the bands corresponding to the HS in the YAC A85D10 and with the HS (Fig. 3, lane 2), S1 sites (Fig. 3, lane 1) and diploid YAC 881D2 transferred into the diploid strain 881D2 wild type with the specific endonuclease R 50S background (Fig 3, lane 6) with the HS determined in vitro in this YAC in the haploid rad 50S mutant strain (Fig. 3, lane 5) and with the HS (Fig. 3, lane 2), S1 sites (Fig. 3, lane 1) and toposites (Fig. 3, lanes 3 and 4) appearing in the YAC in the haploid AB1380 strain carrying the RAD 50 wild type allele. All the patterns are very similar.

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6 h in the sporulation medium. Their DNA was then analyzed by PFGE and indirect end-labelling.

The results of this experiment are shown in Figure 2. The bands corresponding to the meiotic DSB (Fig. 2C, lane 1), are coincident with the bands corresponding to the HS in the AB1380 strain (Fig. 2C, lane 2). It should be stressed that the AB1380 strain contains the wild type allele of RAD 50 gene, is haploid, does not enter meiosis and its DNA does not break spontaneously upon incubation in the sporulation medium (not shown). The main premeiotic DSB are located at positions 29, 63 and 96 kb. In addition, somewhat ‘weaker’ sites were found at 41, 21 and 16 kb. One DSB was located within the LCR at approximately 96 kb and another at about –4 kb. Although qualitatively similar, the patterns of the DSB and that of the HS show some quantitative differences. In particular the DSB present at 96 kb is ‘strong’, whereas the HS present there is rather ‘weak’.

The coincidence between DSB occurring in vivo in the diploid strains, and the HS observed in the YAC chromatin in isolated nuclei is not peculiar to the β-globin YAC. We compared the meiotic DSB in the YAC 881D2 transferred into the diploid rad 50S background (Fig 3, lane 6) with the HS determined in vitro in this YAC in the haploid rad 50S mutant strain (Fig. 3, lane 5) and with the HS (Fig. 3, lane 2), S1 sites (Fig. 3, lane 1) and toposites (Fig. 3, lanes 3 and 4) appearing in the YAC in the haploid AB1380 strain carrying the RAD 50 wild type allele. All the patterns are very similar.

The results of this experiment are shown in Figure 2. The bands corresponding to the meiotic DSB (Fig. 2C, lane 1), are coincident with the bands corresponding to the HS in the YAC A85D10 and the nuclease S1 HS in the YAC A201F4. The pattern of the HS initially determined with low resolution by PFGE were subsequently mapped with medium resolution (~0.5 kb) by classic gel electrophoresis. Under these conditions the 8 kb site split to two sites located at 7.2 and 8.8 kb, the 29 kb site corresponding to a unique 29.2 kb site (16) and the 63 kb site produced a triplet located at 64.8, 66.3 and 67.7 kb (this work). These latter sites were mapped between the two BamHI sites, located at 62 613 and 72 015, with the ‘is2’ probe (see Materials and Methods).

In order to analyze the meiotic DSB we constructed a diploid strain containing the A85D10 YAC in a rad 50S background, using the technique developed by Hugerat et al. (21). The diploid cells were grown successively for 18 h in the YPD medium, then for 24 h in pre-spore medium and were finally incubated for
with, the HS revealed occurring spontaneously at the onset of meiosis in Y ACs in the
The main finding of this study is that the double-strand breaks, DISCUSSION
in the haploid yeast carrying either HS5, the 8 kb site is close to HS2 and the 85 kb site is close to
error with the HS/DSB observed in yeast. The –4 kb site is close
found that these sites are also coincident within an experimental
toposites and S1 sensitive sites (Figs 2 A and 3 ) suggests that these
and incubation in buffers containing components normally absent
was fractionated by PFGE, transferred to nylon membrane, and hybridized to
the probe 't'. Lane 1, crude preparation of the nuclei incubated in the nuclease
S1 buffer in the presence of 3000 U of the enzyme; lane 2, nuclei treated with
2 U DNase I in the pH 5.2 buffer; lanes 3 and 4, nuclei incubated in the
KCl–MgCl₂ buffer in the absence and in the presence of topoisomerase
in vitro in the chromatin of the same YACs
in the haploid yeast carrying either RAD50 wild type or rad50S mutant allele. The HS were revealed following isolation of nuclei
and incubation in buffers containing components normally absent
from a yeast nucleus, whereas in vivo meiotic DSB appear in the
chromosomes. The co-localization of the DSB with the HS,
toposites and S1 sensitive sites (Figs 2A and 3) suggests that these
three latter types of sites are not experimental artefacts but are
indeed features of the chromatin of functionally important
segments of the chromosome.
A coincidence between HS and DSB has been reported for
natural yeast chromosomes (19,24) and they were almost always
located near the strong promoters. In yeast cells, which are the
‘natural hosts’ of these chromosomes, the regulatory proteins
provoke a transcription-related folding of the chromatin and
hypersensitivity of the sites of interaction with DNA. This is not
the case with YACs.
The human β-globin gene cluster is flanked by six major HS
(labelled from 1 to 6) (25). The sites corresponding to the HS1,
HS3 and HS4 in human cells are not hypersensitive in the
β-globin YACs. Their hypersensitivity in human cells is probably
cauised by specific regulatory proteins not present in yeast. Three
of the sites HS2, HS5 and HS6, are hypersensitive in erythroid,
non-erythroid and non-hematopoietic tissues (26). We have
found that these sites are also coincident within an experimental
error with the HS/DSB observed in yeast. The –4 kb site is close
to HS5, the 8 kb site is close to HS2 and the 85 kb site is close
to HS6. The hypersensitivity of these HS/DSB sites is probably
caused by a protein specific to the human regulatory sequences.
Another HS/DSB site (63 kb) observed in the YAC is located
close to the site of termination of transcription of the β-globin
gene, while the HS/DSB sites located at 29 and 96 kb are far from
any known β-globin regulatory regions. We propose that these
sites are all involved in the folding of the chromatin fibre in the
β-globin YACs.
The second important point is that one of the sites identified
here (96 ± 5 kb) as a strong meiotic DSB and a weak HS in yeast
is co-localized within experimental error with the chromosomal
breakpoint in several unrelated cases of thalassemia in humans
(94 kb) (27). This finding strengthens the hypothesis put forward by
Klein et al. (22) that the recombination hotspots in human DNA are
coincident with the meiotic DSB if the DNA segments carrying
these sites are propagated in yeast. There is apparently a basic
similarity between the structures of yeast and human chromatin.
Finally, low and medium resolution mapping revealed that the
HS/DSB are close to the sequences that have the potential to
release stress in DNA by changing the latter’s secondary
structures. The sites 7.2, 8.8, 67.7 and 96 ± 5 kb are located close
to the direct and/or inverted repeats at Nt 7699, 8781, 67 042 and
at 94 kb, respectively, and the 29.2, 64.8 and 66.3 sites are close
to the regions containing multiple copies of the ATATTT
hexanucleotide, which is the core of an unwinding sequence (8).
The HS/DSB sites are also close to either one of the integrated
replicative sequences, Alu or Line I.
The stress releasing potential and the presence of integrated
replicative sequences make these sites similar to the viral
integration sites characterized by Mielke et al. (28). A possible
reason for this similarity could be that both recombination in yeast
and viral integration in mammalian cells are mediated by
topoisomerase-like proteins : the Spo11 protein in yeast (29,30)
and the IN protein in the case of viral integration (31). These
proteins possibly use similar ‘unusual’ DNA structures as targets
(32,33). Another, not exclusive, reason for this similarity could be
that the viral integration sites studied by Mielke et al. (28), and
the sites of meiotic DSB determined here, are all probably located
close to the bases of DNA loops. The ‘highly expressing sites’ of viral
integration displayed properties of SARs. As for the meiotic DSB,
evidence suggests that the protein responsible for their appearance,
Spo11, is also located in the the axial element (18) adjacent to or
merged with the scaffold of the meiotic yeast chromosome.
It has been proposed (28) that the loop anchorage sites contain
unpaired DNA segments generated by the build-up of negative
superhelical tension. Such tension causes the cruciform extrusion
and formation of other nuclease sensitive structures. Recently
Kimura and Hirano (34) have studied a protein complex, condensin,
containing a component belonging to the SMC family of proteins,
which actively condenses the chromatin. In the presence of ATP,
the complex wound up positive coils in one region of a circular
DNA molecule and generated negative supercoiling in the rest of
it. The condensin has a special affinity for the cruciform
structures, probably because the DNA is bent in these sites
forming two angles of 60° and two of 120°. Certain enzymes
involved in recombination are able to measure these angles (35).
The cruciform extrusion caused by the negative supercoiling and
the affinity of the condensin for the cruciforms causes a positive
feedback loop: the more the chromatin condenses, the more it
produces cruciforms and the newly formed cruciforms attract more
molecules of condensin. The condensin eventually accumulates in
the regions that possess a high potential for cruciform formation
(or containing other bendable DNAs). We think that these

Figure 3. Comparison of the meiotic DSB with the DNase I HS, toposites and
S1 sensitive sites in the YAC811D2. DNA isolated from the nuclei of the RAD
50 wt strain (lanes 1–4), from the nuclei of the haploid rad 50S mutant strain
with 2 U DNase I (lane 5) or from the spheroplasts of the diploid rad 50S
mutant strain isolated after 6 h incubation in the sporulation medium (lane 6)
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molecules of condensin. The condensin eventually accumulates in
the regions that possess a high potential for cruciform formation
(or containing other bendable DNAs). We think that these
idiosyncratic, stress-relieving segments of the chromosome constitute the bases of the chromatin loops (36), where the clusters of hypersensitive sites appear and the recombination-initiating Spo11 protein interacts with the DNA (Fig. 4).

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