DNase I nick translation *in situ* on meiotic chromosomes of the mouse, *Mus musculus**

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* We dedicate this paper to the memory of the late Menashe Marcus, who contributed greatly to the development of the *in situ* DNase I nick-translation method, which enabled us to carry out the present investigation

Summary

DNase-I-sensitive sites have been located on the meiotic chromosomes of the mouse, *Mus musculus*, by the *in situ* DNase I nick-translation method. We find that: (1) of all the cell types studied, pachytene nuclei are the most sensitive to DNase I; (2) in diplotene the nicks occur preferentially in the vicinity of chiasmata; (3) the sex chromosomes are also sensitive to the enzyme despite their transcriptional quiescence; and (4) in the sex bivalent the nicks are primarily observed in the putative region of recombination. We conclude that, in addition to discriminating between the transcriptionally active and inactive states of chromatin, DNase I identifies recombination-specific chromatin changes in meiotic prophase.

Key words: DNase-I-sensitive sites, meiotic chromosomes, *Mus musculus*.

Introduction

It is well established that DNase I preferentially cleaves transcriptionally active regions of chromatin (Garel & Axel, 1976; Weintraub & Groudine, 1976; Elgin, 1981). Nick translation with this endonuclease thus provides a sensitive method for molecular analysis of functionally distinct fractions of chromatin. In an apparently surprising discovery, Gazit *et al.* (1982) found that the genes potentially active during interphase of the cell cycle retain their DNase I sensitivity at the following metaphase, the stage at which chromatin is totally inactive but cytologically visible as discrete chromosomes. This discovery paved the way for developing methods for cytological mapping of active regions of chromosomes by *in situ* DNase I nick translation (Kerem *et al.* 1983; Kuo & Plunkett, 1985). The efficiency of the method has been demonstrated in several studies (Kerem *et al.* 1984; Sperling *et al.* 1985). While data have been accumulating on the sensitivity of somatic cell chromatin, information on meiotic cell chromatin is rather sporadic, in spite of the fact that the vital function of recombination of genes and segregation of chromosomes takes place during this division. Of the three detailed studies hitherto published, one on human spermatocytes shows hypersensitivity of the XY bivalent at the pairing tips, the putative zone of recombination (Chandley & McBeath, 1987). With mouse, Richler *et al.* (1987) have made similar observations to these made with human spermatocytes, but Separovic & Chandley (1987) have found the opposite.

In an independent study on the DNase I nick-translated mouse spermatocytes (*in situ*), we too have found hypersensitivity at the pairing tips of the XY bivalents. We have also noticed preferential sensitivity to the enzyme of autosomal chiasmata regions, which suggests a coincidence between the recombinogenic activity of chromatin and its DNase I sensitivity. The results are presented in this paper.

Materials and methods

Chromosome preparation

Three mice of Park's strain, originally obtained from Central Drug Research Institute, India, were used for the study. Testis tubules were minced in Eagle's minimal essential medium (MEM) to release cells, which were then transferred to 0·56% KCl for 5 min. The cells were then fixed in methanol:acetic acid (3:1, v/v) fixative for 2–3 min. The

hypotonic and fixation treatments were kept to a minimum in order to retain chromosome organization as close as possible to their pre-fixed state, and especially to avoid alteration or removal of chromosomal proteins that result from prolonged fixation. A few drops of the suspension were air-dried on clean, chilled slides. They were immediately used for the nick-translation reaction, but for the intervening period (maximum 30 min) they were kept in a refrigerator at 10°C.

DNase I nick translation

The procedure for the nick translation was the same as developed by Kerem *et al.* (1983). Briefly, each slide was covered with 20 μl of the chilled nick-translation mixture (50 mM-Tris·HCl, pH 7·9, 5 mM-$MgCl_2$, 50 μg ml^{-1} BSA (bovine serum albumin), 0·1 mM-DTT (dithiothreitol), 10 units ml^{-1} of DNase-free DNA Pol I; 4 μM each of dATP, dGTP, dCTP; 0·3 μM-[^{3}H]TTP (sp. act. 53·5 Ci mmol^{-1})) and different concentrations of DNase I (0·1, 0·5, 1·0 ng ml^{-1}). No DNase I was added to control slides. The nick-translation reaction was allowed to proceed for 5 min, following which slides were rinsed in two changes of 2×SSC (SSC is 0·15 M-NaCl, 0·015 M-sodium citrate) containing 1 mM-EDTA. The dried slides were briefly refixed in methanol:acetic acid fixative.

[^{3}H]uridine labelling

Cell suspensions of two somatic tissues (liver and bone marrow) and testis in Eagle's MEM were labelled *in vitro* with tritiated uridine (sp. act. 13·5 Ci mmol^{-1}). While the somatic cells were labelled for 15 min in 10 μCi ml^{-1}, those of testis were incubated for 2 h in 25 μCi ml^{-1}. The cells were fixed directly after labelling.

Autoradiography

Both the nick-translated and [^{3}H]uridine-labelled slides were exposed for autoradiography. They were treated with chilled trichloroacetic acid before applying L4-nuclear emulsion (Ilford). All the nick-translated and uridine-labelled slides from liver and bone marrow were exposed for 10–12 days. The uridine-labelled testis slides, however, were kept in the dark for 8 months. The slides were developed in D19b developer for 4 min (12°C) and fixed in acid fixer. They were stained with Giemsa after developing. Silver grains were removed after examination of the autoradiograms and the preparations were restained with Giemsa.

Results

Three experiments were carried out using different concentrations of DNase I. Adequate labelling was obtained even with the lowest concentration (0·1 ng ml^{-1}) and, since there was no remarkable difference in the labelling pattern among different concentrations, 0·1 ng appears to be adequate for nick translation in meiocytes. The results presented here are generally pooled from experiments with 1·0 and 0·1 ng DNase I. In the control slides (without DNase I), meiocytes were not labelled. Some labelling, 4–10 grains, was recorded in a few gonial interphases, presumably due to the utilization of the exogenously supplied enzymes and precursors by the already replicating S-phase cells. Among the experiments, all but a few spermatids and spermatozoa were labelled.

Pachytene chromosomes are the most DNase-I-sensitive

Grain counts in cells occupying 20 randomly chosen areas in two slides revealed that the extent of labelling in zygotene/pachytene nuclei was considerably higher than in all the other cell types studied (Table 1). Pachytene grain counts were also higher than in metaphase I (Table 1) and bone marrow cells (data not shown). This picture contrasted with the actual states of transcription in the cell types studied. We confirmed it by studying [^{3}H]uridine incorporation in testis and two somatic tissues (liver and bone marrow). While a 15-day exposure revealed intense labelling of nuclei of somatic tissues, it took 8 months to achieve comparable grain density on spermatogonials in testis. Even after such prolonged exposure, only 30% of the pachytenes were sufficiently labelled but their grain count was poorer than those of the gonials. Post-pachytene cells were generally unlabelled and the sex bivalent did not incorporate uridine at any stage of meiosis, including pachytene (Fig. 1). Thus, despite their low transcription, the pachytene chromosomes were hypersensitive to DNase I.

Table 1. *Pooled grain counts from 20 randomly chosen areas from two slides*

Cell types	No. of cells	Total grain count	Mean grain count/cell ±S.D.
Interphases	140	6031	43·1 ± 6·3
Pachytenes	86	7109	82·7 ± 9·1
Spermatids	62	1067	17·2 ± 4·1
Sperm heads	75	676	9·0 ± 3·0
Metaphases*	50	1493	29·9 ± 3·0

Ten areas from a slide, each with 0·1 and 1·0 ng ml^{-1} DNase I, were chosen.
* Cells counted independently of the 20 areas mentioned above.

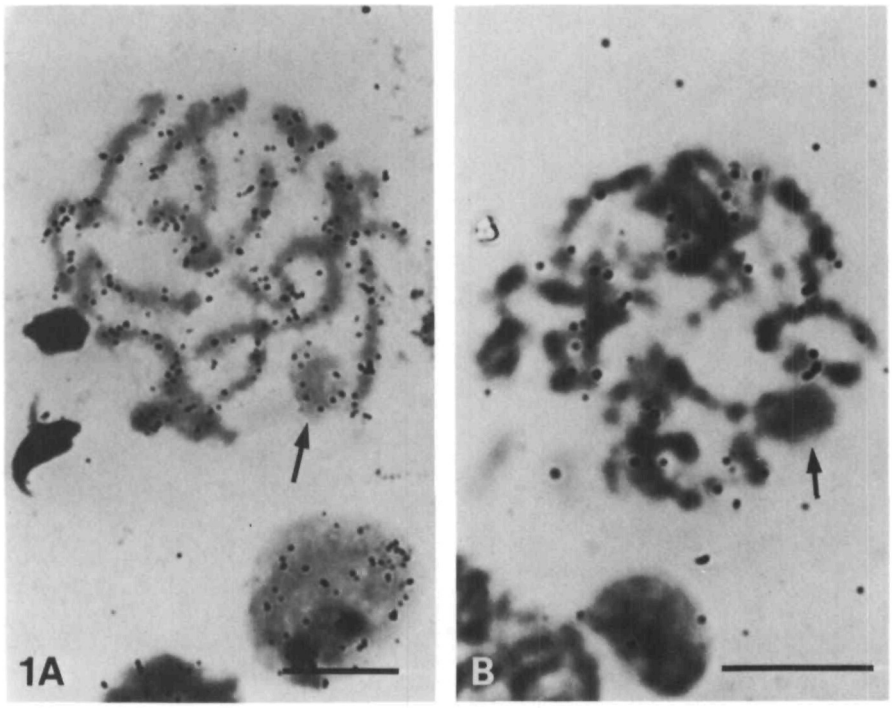

Fig. 1. Autoradiograms of (A) DNase-I nick-translated and (B) [^{3}H]uridine-labelled meiotic cells. While the XY bivalent (arrows) is labelled in the nick-translated pachytene, it is unlabelled in the [^{3}H]uridine-labelled one. Note labelling on the nick-translated interphase nucleus. The few grains on the sperm heads have been obscured by heavy staining. Bars, 10 μm.

Sex bivalent is DNase-I-sensitive

The sex bivalent was the main point of reference for DNase I sensitivity because of its easy discrimination and transcriptional quiescence. Following the nick translation, however, it was labelled in all the 200 pachytenes and 100 metaphase Is analysed (Figs 1A,2). The average grain count on the sex vesicle in pachytenes was 6·3 per cell. Owing to their diffuse nature it was difficult to identify the labelled region at pachytene. At metaphase I, where the overall grain count was much lower (Table 1), the sex bivalent had 1–5 grains (Fig. 2A,B). In an attempt to map the distribution of grains, the sex bivalent was divided into four segments; namely, the terminally aligned regions of the X and Y, the rest of the Y, the region proximal to the centromere of the X and the interstitial segment of the X. As shown in Fig. 2C, more than half the grains were located on the region of X–Y terminal alignment, the putative site of their recombination.

Chiasmata regions are also DNase-I-sensitive

Following this indication of preferential labelling of the putative recombination zone of the sex chromosomes, we scrutinized the chiasmata of autosomal bivalents in diplotenes. Although, owing to extremely brief hypotonic and fixative treatments, the morphology of the bivalents was not optimal, four diplotene plates were karyotyped. There were indications of preferential distribution of grains on or around chiasmata.

In order to determine whether the preferential occurrence of grains on chiasmata was statistically significant, a χ^2 test was done to compare chiasmatic and non-chiasmatic regions and terminal and interstitial chiasmata. For this purpose, photographs of four Giemsa-stained diplotene plates were given to a person other than the authors, after removing the autoradiographic grains, and he was asked to measure lengths in millimetres or parts thereof of centromeric, interstitial and terminal chiasmata, as well as non-chiasmatic regions. The grains were then mapped on these regions on their autoradiograms. However, there were inherent limitations in this approach: (1) certain bivalents did not reveal unambiguous chiasmata through their length and had to be omitted from consideration (e.g. bivalents boxed in Fig. 3); and (2) since telomeric ends were not always distinguishable in bivalents having terminal or subterminal chiasmata, each such area was termed a telomeric chiasma (see, for instance, arrowheaded chiasmata in Fig. 3). As a result, particularly in small bivalents, the total chiasmatic area measured was more than the non-chiasmatic area. It was not always possible to distinguish between pericentromeric and terminal regions and, since their grain counts were similar, we pooled the two. Thus only three classes, namely terminal chiasmata, interstitial chiasmata and non-chiasmate regions, were tabulated, and a total of 46 bivalents were analysed. Differences in grain frequency in different regions were tested statistically. Within the limitations cited above, the difference in grain frequency between different chiasmata regions was not significant but that between chiasmate and non-chiasmate regions was significant in favour of the former (Table 2). Five pachytene plates were also karyotyped to map grains on autosomal bivalents, but they did not reveal any strikingly sensitive or insensitive regions. Owing to excessive condensation, as well as

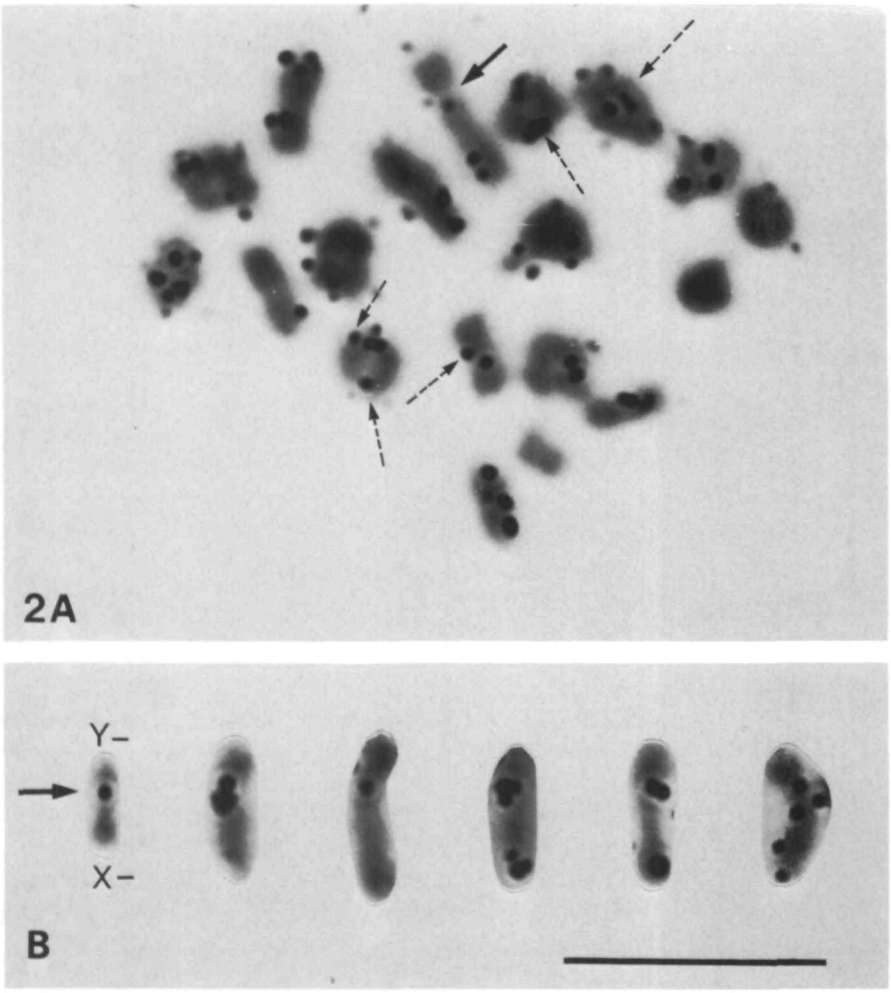

Fig. 2. Nick-translated (A) metaphase I plate and (B) cut-outs of XY bivalents from different metaphases showing preferential localization of grains on their attachment site (arrows). In certain autosomal bivalents also, their chiasmatic regions are labelled (broken arrows). Bar, 10 μm. C. A diagrammatic representation of distribution of grains on different regions of the XY bivalent computed from 50 plates. The numbers beside the horizontal bars indicate the total number of grains scored.

Table 2. χ^2 *Test for evaluating randomness of grain distribution in chiasmatic and non-chiasmatic regions of the bivalents*

Chiasmata regions	Length (mm)	Number of grains		$\Sigma\chi^2$
		Observed	Expected	
Telomeric/centromeric	281·3	136	134·0	0·168 $P > 0·95$
Interstitial	69·5	31	33·1	
Chiasmata	350·8	167	111·86	53·40 $P < 0·005$
Non-chiasmatic	364·9	61	116·36	

the possibility of terminalization of chiasmata, grains were not mapped on metaphase I cells.

Briefly, the results showed the following: (1) maximal sensitivity of pachytene chromatin; (2) preferential nicking in the chiasmata regions; and (3) sensitivity to DNase I of the sex bivalent in the putative zone of recombination.

Discussion

Mus musculus was chosen for this study because its spermatogenesis has been studied in some detail. It is known that transcriptional activity declines with the progression of meiosis and that few transcripts are released by post-pachytene cells, that the sex chromosomes do not transcribe throughout meiosis (Monesi *et al.* 1978) and the molecular evidence for X–Y recombination, despite the lack of a chiasma, is unequivocal (Keitges *et al.* 1985).

Our observation on the preferential sensitivity of the recombination zone of the XY bivalent is in agreement with that of Richler *et al.* (1987) but contrasts with

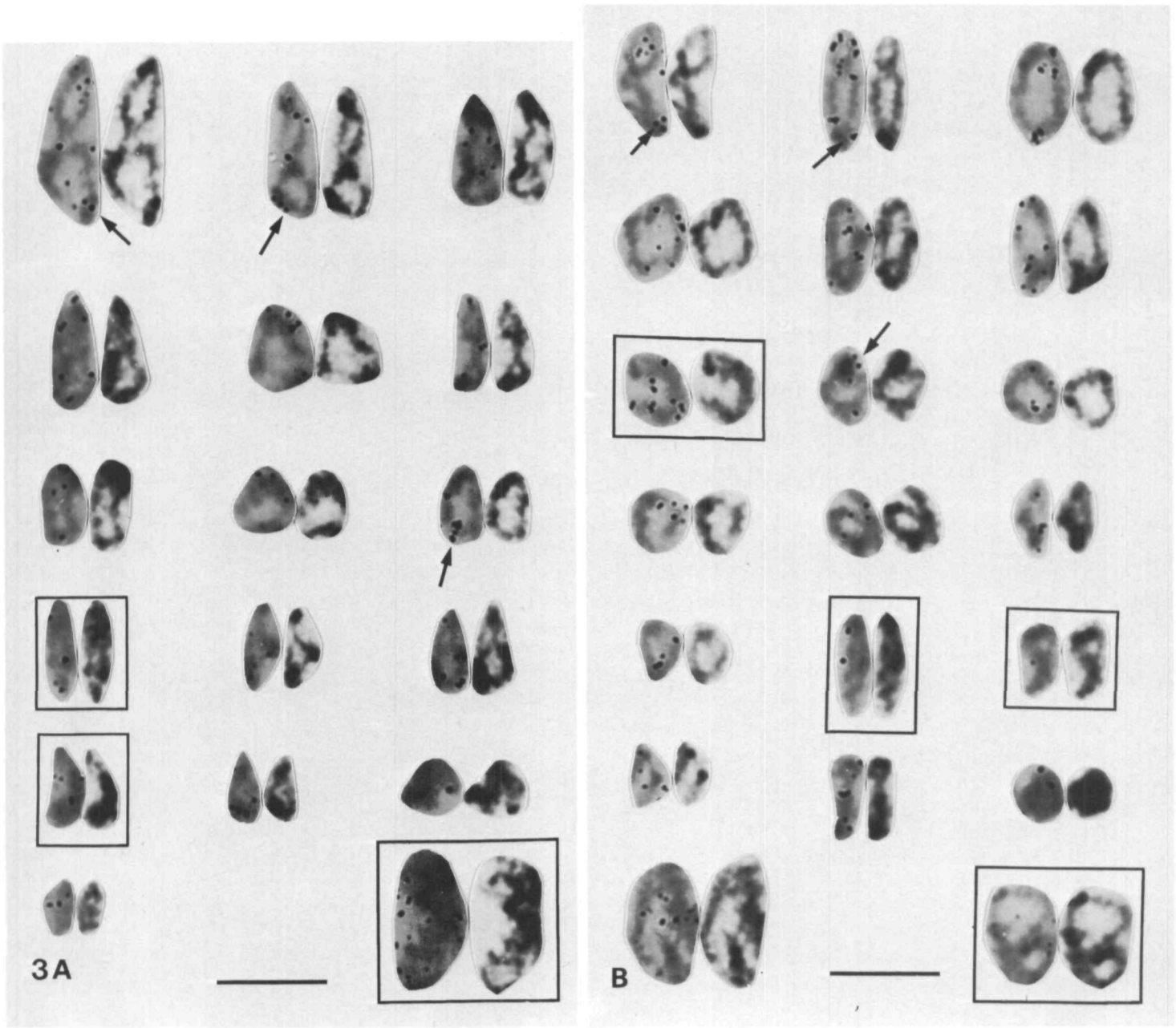

Fig. 3. Karyotypical arrangement of two nick-translated diplotenes. Bivalents with and without grains have been arranged side-by-side. These two plates were among the four diplotenes that provided data for Table 2. Boxed bivalents were omitted from the calculations for grain distribution. Arrowheads indicate chiasmata whose location cannot be distinguished between terminal and subterminal category. For details see the text and Table 2. Bars, 10 μm.

another study on the mouse (Separovic & Chandley, 1987), which identifies instead the early replicating X-inactivation centre (Xce) as the most sensitive site. Though the interstitial region of the X, which included the Xce, was also occasionally labelled in our preparations it was not possible for us to identify any specific region, perhaps due to low grain count. We cannot explain the difference between our observations and those of Separovic & Chandley (1987). Incidentally, in two other mammals, man (Chandley & McBeath, 1987) and the musk shrew, *Suncus murinus* (Raman & Nanda, 1985), studied by this method hypersensitivity of the X–Y recombination zone to DNase I has also been indicated.

The observation that pachytene may have possibly the most sensitive chromatin of all the somatic and germ cell types is consistent with the biochemically analysed DNase I sensitivity in the rat (Rao *et al.* 1985) and in microsporocytes of the lily plant (Hotta *et al.* 1985). It is believed that DNase I identifies active or potentially active domains of transcription in chromatin, but the hypersensitivity of pachytene chromatin does not seem to imply this. Compared with the pre-meiotic germ cells and proliferating somatic cells,

much less transcription occurs in pachytene and it continues to decline in post-pachytene cell types right up to zygote formation. This implies, therefore, that the pachytene sensitivity is quite disproportionate to its transcriptional potential. It should therefore be reasonable to assume that factor(s) besides transcriptional potential contribute to the hypersensitivity of the pachytene chromatin. Considering that recombination, which takes place during pachytene, requires intense nicking and repairing activity, it is likely that an altered chromatin organization is required to facilitate nick formation. It is most plausible that the same alterations must also make the chromatin DNase-I-sensitive. That this could indeed be so has been demonstrated in the lily, where the *zyg* and *psn* DNA, the DNAs synthesized during zygotene and pachytene, respectively, are the most sensitive to DNase I during the stage of their synthesis (Hotta *et al.* 1985). The unique feature of the *psn* DNA, which flanks the P-DNA domains in meiocytes, is that it provides sites for the endogenous meiotic endonuclease for recombinational nicks. This property is achieved by a qualitative change in chromatin organization of *psn* DNA brought about by the removal of histones and binding with *psn* RNA and *psn* protein (Stern & Hotta, 1983). It is most likely that the same region is hypersensitive to DNase I in lily. Although equally exhaustive studies on mouse meiosis are lacking, since most molecular events of meiosis seem to be evolutionarily conserved, we suggest that the hypersensitivity of pachytene in the mouse is caused by the chromatin changes brought about for recombination as well as transcription. We further suggest that the altered conformation of recombinogenic regions of chromatin is retained till later in cell division and thus leads to preferential sensitivity of perichiasmate regions in diplotene, the stage closest to pachytene. The sensitivity of what appears to be the recombination zone of the XY bivalent even at metaphase I goes to strengthen the suggestion that DNase I identifies recombinogenic chromatin for creating nicks.

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