Alteration of nuclear architecture in male germ cells of chromosomally derived subfertile mice

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SUMMARY

The mammalian cell nucleus consists of numerous compartments involved in the regular unfolding of processes such as DNA replication and transcription, RNA maturation, protein synthesis and cell division. Knowledge is increasing of the relationships between high-order levels of chromatin organization and its spatial organization, and of how these relationships contribute to the various functions carried out in the nucleus. We have studied the spatial arrangement of mouse telocentric chromosomes 5, 11, 13, 15, 16 and 17, some of their metacentric Robertsonian derivatives, and X and Y chromosomes by whole chromosome painting in male germ (spermatogonia, pachytene spermatocytes and spermatids) and Sertoli cells of homozygous and heterozygous individuals. Using dual-colour fluorescence in situ hybridization we found that these chromosomes occupy specific nuclear territories in each cell type analysed. When chromosomes are present as Robertsonian metacentrics in the heterozygous state, that is, as Robertsonian metacentrics and their homologous telocentrics, differences in their nuclear positions are detectable: heterozygosity regularly produces a change in the nuclear position of one of the two homologous telocentrics in all the cell types studied. In the Robertsonian heterozygotes, the vast majority of the Sertoli cells show the sex chromosomes in a condensed state, whereas they appear decondensed in the Robertsonian homozygotes. As the Robertsonian heterozygosities we studied produce a chromosomally derived impairment of male germ-cell differentiation, we discuss the possibility that changes in chromosome spatial territories may alter some nuclear machinery (e.g., synapsis, differential gene expression) important for the correct unfolding of the meiotic process and for the proper functioning of Sertoli cells.

Key words: Nuclear architecture, Germ cells, Chromosome translocation, Subfertile mice, Sertoli cell, X and Y chromosomes

INTRODUCTION

The anticipatory idea of Rabl and Boveri that individual chromosomes in interphase nuclei tend to occupy exclusive territories rather than to intermingle is now well supported by studies of the relationships between nuclear compartmentalization and functional states of the cells (Manuelidis, 1990; De Boni, 1994; Lamond and Earnshaw, 1998). More recently, the application of cytochemical and computer imaging techniques (van der Ploeg, 2000) to reveal specific DNA and protein components of chromatin has clarified the role of chromatin dynamics in nuclear structuring (Abney et al., 1997). Nuclear compartmentalization changes during the cell cycle (Manuelidis and Borden, 1988; Ferguson and Ward, 1992) and during cell differentiation (Manuelidis, 1997) demonstrate the correlation between nuclear architecture, gene expression and cell function. Such compartmentalization is highly flexible and reversible, as is clearly shown by the successful mammalian cloning experiments (Wakayama et al., 1998). Indeed, the nucleus and its architecture are now seen by molecular morphologists as the structure that supports the global genomic coordination and regulation of cellular processes (Boyle et al., 2001; Cremer and Cremer, 2001). The molecular mechanisms underlying these levels of genome structuring are known to be operative in all eukaryotic cells, ranging from yeast to flies to mammals (van Driel and Otte, 1997; Leger-Silvestre et al., 1999).

Our knowledge, however, of large-scale chromosomal and nuclear architecture is limited and little is known about the influence of karyotype restructuring on nuclear spatial organization and on the regulation of gene activity. Making use of dual-colour FISH (fluorescence in situ hybridisation) for whole-chromosome painting, we have analysed the spatial arrangements of chromosomes 5, 11, 13, 15, 16, 17, X and Y in the nuclei of Sertoli, spermatogonia, pachytene spermatocytes and spermatid cells in mice exhibiting karyotype variation owing to the presence or absence of Robertsonian (Rb) fusion chromosomes. The analysis of male germ-cell differentiation throughout the cycle of the seminiferous epithelium made it possible: (1) to follow the changes in the nuclear territories

occupied by each single telocentric chromosome when in an 'all-telocentric' karyotype or when that same chromosome fuses with another to form an Rb metacentric in either a homozygous or a heterozygous state; and (2) to try and relate these changes of chromosome localisation (nuclear architecture) to the associated changes in spermatogenesis. We show that the presence of Rb heterozygosities in the nucleus has a marked impact on the spatial arrangements of chromosomes, producing large-scale changes in chromosome territories, which correlate with detrimental effects on male germ-cell differentiation. Interestingly, the Sertoli cells of subfertile mice show X and Y chromosome FISH-signals condensed as in human patients with the 'Sertoli-cell-only' syndrome.

MATERIALS AND METHODS

Mice

Two ten-week-old male mice of each of the following karyotypes were employed: laboratory strain C3H (Charles River, Italy; 2n=40, all-telocentrics), Cremona (CR) race (Gropp et al., 1982) (2n=22, nine pairs of Rb metacentrics in a homozygous state, that is Rb(1.6), Rb(2.8), Rb(3.4), Rb(5.15), Rb(7.18), Rb(9.14), Rb(10.12), Rb(11.13), Rb(16.17) and F_1 hybrids C3H × CR (F1, 2n=31), which are heterozygous for nine independent Rb metacentrics (the CR Rb metacentrics, which will form trivalents at the pachytene stage).

Testicular histology, isolation and countings of germ cells

One testis from each animal was used for histology; the other was minced to separate the germ cells. Isolated germ cells were released from the seminiferous epithelium in 2.2% sodium citrate, pelleted by centrifugation and resuspended in 0.56% KCl. After the hypotonic treatment, cells were fixed in 3:1 (v/v) methanol:acetic acid, dropped onto slides and air-dried. The criteria to assess the dividing line between diakinesis and MI-spermatocytes [degree of condensation and 'opening out' of the bivalents and trivalents, as in (Odorisio et al., 1998)] were established before the blind scoring by two operators on two Giemsa-stained slides per animal. Using a low-power objective, the number of diakinesis, MI and MII-spermatocytes was assessed for 100 microscopic fields per slide by each operator. Analysis of variance was performed to determine the significance of the differences between the means.

Testes were fixed in Bouin's fixative for 24 hours, embedded in paraffin wax (Paraplast) and sectioned (6 μm). Ten sections per slide were prepared, five sections being discarded of each of those collected. Four slides were prepared for each mouse. The twelve stages of the seminiferous epithelium cycle were scored after periodic acid-Schiff and haematoxylin staining according to (Oakberg, 1956). The number of primary spermatocytes and spermatids, evaluated by three operators, was corrected according to Abercrombie (Abercrombie, 1946); no significant intra- or inter-animal variation was recorded by the three operators.

In situ hybridization and hybridization probes

Mouse whole-chromosome specific probes (CAMBIO, UK) for chromosomes 5, 11, 13, 15, 16, 17, X and Y were employed for FISH on fixed isolated germ cells. Probes for chromosomes 5, 11, 16 and X were fluorescein-isothiocyanate-(FITC)-labelled; those for chromosomes 13 and 17 were biotin-labelled, and those for chromosomes 15 and Y were Cy3 labelled. For each of the two animals employed, three slides for each karyotype (CR, F_1 and C3H) were subjected to chromosome painting (for chromosome pairs 5/15, 11/13, 16/17 and X/Y) according to the manufacturer's instructions. Stringency washes were in 50% formamide in 0.5×SSC at 42°C. FITC signals were amplified by the use of the FITC Amplification kit (CAMBIO catalogue number 1084-KF); biotin-labelled probes with the Biotin (Texas Red) painting kit (CAMBIO, catalogue number 1082-KT). Slides were counterstained with 4′-6-diamino-2-phenylindole (DAPI, 0.05 μg/ml) and mounted with an antifade (DABCO, 2%).

Scoring of Sertoli and germ cells hybridization patterns

Thirty spermatogonia, spermatocytes, spermatids and Sertoli cells from each of the three DAPI-stained slides for each of the two animals (to give a total number of 540 scorings per germ-cell type) were independently analysed by the three observers. Before the blind scoring, common criteria were adopted to identify and stage the cells on the basis of the nuclear and the cellular characteristics of the mouse testis cells as described (Meistrich et al., 1973). The hybridization patterns were evaluated after focusing on the brightest area of the fluorescent signal. A signal was regarded as decondensed, as opposed to condensed, if expanded over a relatively large area. The nuclear position occupied by a probe signal was seen to vary from being close to the nuclear membrane (peripheral) or spread across the nucleus (central). The proximity between chromosomes, overlapping versus opposite ends, was recorded as well. During pilot runs, we performed image analysis of the signals on selected cells both by printing photos and converting to μm the area and linear distances of the signals and by using the NIH Image program (http://rsb.info.nih.gov/nih-image/about.html). It was obvious that for our purposes the signals can be classified equally well by the subjective evaluation of the observer. It must, however, be recalled that caution must be exercised when drawing conclusions from 2D FISH analysis because, when air-dried onto a slide, a nucleus with a peripheral signal might become one with a central signal and vice versa. Regarding the reliability of the conclusions we draw, it should be considered that several systematic comparisons of 2D versus 3D FISH analysis support the validity of the assumption that the relative organization of the nucleus is not significantly altered by the hypotonic treatment of the cells and its subsequent squashing [(Csink and Henikoff, 1998) in *Drosophila*; (Croft et al., 1999) in humans; (Mayer et al., 2000), in mouse; (Metzler-Guillemain et al., 2000), in man, chimpanzee and mouse; (Nikiforova et al., 2000) in man; (Cremer and Cremer, 2001) in mouse]. The analysis of the frequency of the detectable hybridization patterns theoretically expected (on the basis of the combined possibilities of the three categories we employed to classify the signals: position, condensation and proximity) showed one pattern remarkably being 15-20 times commoner than any other (Fig. 2). This 'most frequent' pattern showed negligible variations among observers (the greatest variation ranged from 76-82% for observers 2 and 3 in the pachytene cells of testes from Rb(16.17)/+ mice to 81-88% for observers 3 and 1 in the spermatids of C3H mice with chromosome pairs 11 and 13 in the homozygous state), and therefore we called it 'typical'. Images were captured using an Olympus (Provis) epifluorescence microscope equipped with a thermoelectronically cooled charge-coupled device camera (Photometrics CH350). Grey-scale images were recorded separately for each fluorescent dye (viewed with the appropriate filters) using the IPLab Spectrum Imaging software (Signal Analytics Corporation) and then pseudocoloured (red for Cy3 and Texas Red, yellow-green for FITC and blue for DAPI) and superimposed.

RESULTS

For each of the three karyotypes studied, Fig. 1 shows the typical (see Materials and Methods) nuclear distributions for each chromosome analysed in spermatogonia, pachytene spermatocytes, spermatids and Sertoli cells. Fig. 2 shows, as a pie chart, the frequency distribution of the distinguishable different hybridization patterns. The frequency of the typical

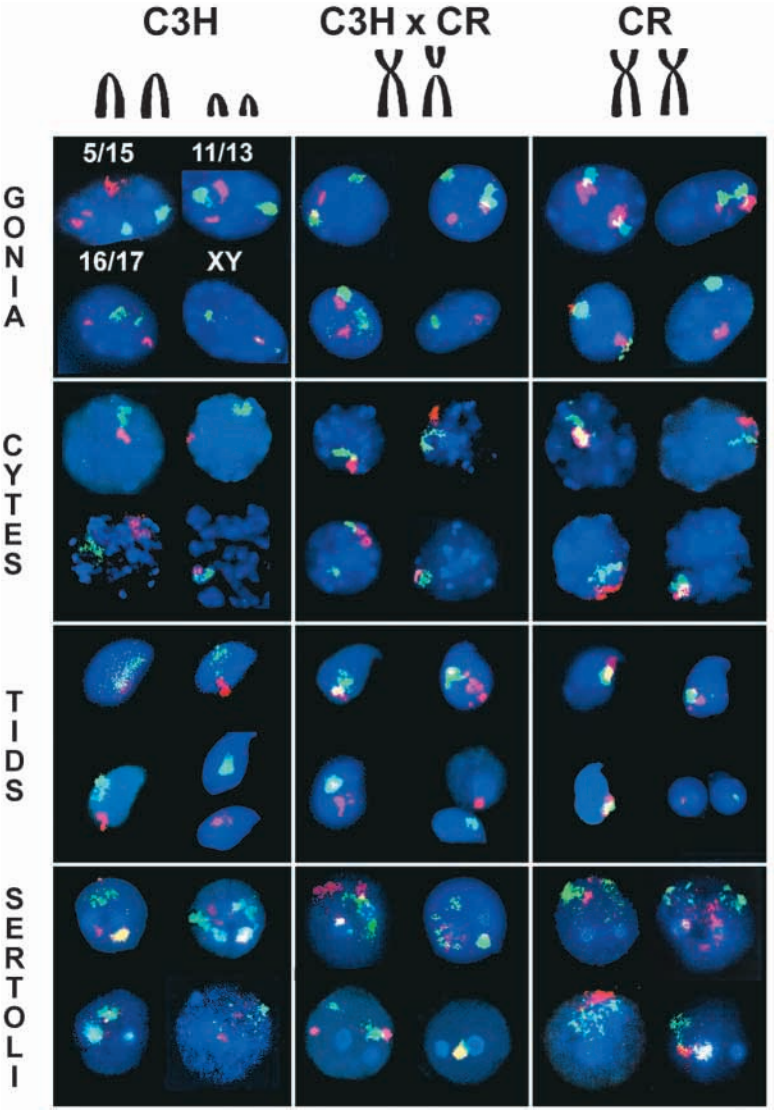

Fig. 1. Chromosome painting with probes for chromosomes 5, 11, 16, X (green) and 13, 15, 17 and Y (red). The typical hybridisation patterns (see also Materials and Methods) of germ and Sertoli cell nuclei of the three karyotypes studied are shown.

pattern is clearly 15-20 times greater than that of any other. In the all-telocentric karyotype, FISH signals in spermatogonia always show distinct, non-overlapping spatial domains; chromosomes 5, 11, 15 and 17 are confined more towards the nuclear periphery than are chromosomes 13 and 16; X and Y are positioned apart from each other near the periphery. At the pachytene stage, the synapsis of the homologues is clearly detectable, with each autosome pair maintaining a distinct position within the nucleus; the X and Y are confined to the 'sex vesicle'. As expected, in the haploid (step 14-15) spermatids, only one signal is detected for each of the chromosomes: chromosome 5 shows a diffuse granular signal indicating decondensation of its chromatin, whereas all the other chromosomes show various degrees of condensation. The Sertoli nuclei constantly exhibit diffuse X and Y FISH signals, with the Y signal preferentially confined near one of the two heterochromatin blocks adjacent to the prominent nucleolus; the signals on the autosomes are diffuse and dispersed.

In the Rb homozygotes, at the spermatogonia stage, one each of the Rb chromosomes 5.15 and 16.17 are located at opposite poles of the nuclear periphery, with the signals of the metacentric chromosomal arms (i.e. the signals corresponding to the telocentric chromosomes) fused and partially overlapping. Rb 11.13 homologues were both positioned on one side of the nuclear periphery. The pachytene spermatocytes show synapsed bivalents at the periphery of the nucleus. The fusion between telocentric pairs that gives rise to the Rb metacentrics is clearly detectable in spermatids, as is revealed by the partial overlapping of the in situ hybridization signals. Sertoli nuclei show chromosome-localization patterns similar to those described in the all-telocentric homozygotes with diffuse FISH signals.

In the spermatogonia of heterozygous mice, the Rb metacentric FISH signals are clearly well apart from the homologous telocentrics, the latter being localized in separate nuclear domains. The telocentrics showed a location similar to that described for the all-telocentric homozygous condition; on the contrary, the signals pertaining to the Rb metacentrics show that one chromosomal arm is positioned near the spatial domain of the other, being 'sequestered' there (when compared to the all-telocentric pattern). The patterns of chromosome spatial location described are similar for all the chromosome pairs analysed and in each of the cell types studied, and are clearly visible in gonia and pachytene nuclei. In the Rb heterozygotes, the spatial relationship of both chromosome arms of the Rb metacentric and that of its telocentric homologues differ from that of the homozygotes. This suggests that the Rb fusion leaves the position of one homologue of each chromosome pair involved in the translocation unchanged, inducing a change in the position of the other homologue. The typical pachytene spermatocyte FISHed for chromosomes 16/17 (second column, Fig. 1) shows one signal well separated from the other three, suggesting complete asynapsis of chromosome 16 in over 75% of pachytene cells. This is contrary to synaptonemal complex analyses of other heterozygotes of this genotype, which failed to detect such asynapsis, although the Rb-telocentric-telocentric trivalent associations did have limited, localised asynapsis (Redi and Capanna, 1988). Further work is needed to resolve this discrepancy. The typical (76%) hybridization pattern of the diploid interphase nuclei of the Sertoli cells is characterized by condensed and overlapping X and Y chromosomes located near one of the two heterochromatic blocks at the sides of the prominent nucleolus. In total, the hybridization patterns showing condensed signals for X and Y amount to 84% of the total number of cells.

As there are possible links between the changed chromatin domains in the Rb heterozygotes and their known spermatogenic impairment (Redi and Capanna, 1988), we have characterised the spermatogenic impairment in detail. A major feature of the impairment is that at least 80% of tubule cross-sections show such severe damage to the spermatogenic epithelium (Fig. 3, inserts on the right: top, fertile homozygotes; bottom, subfertile heterozygotes) that the

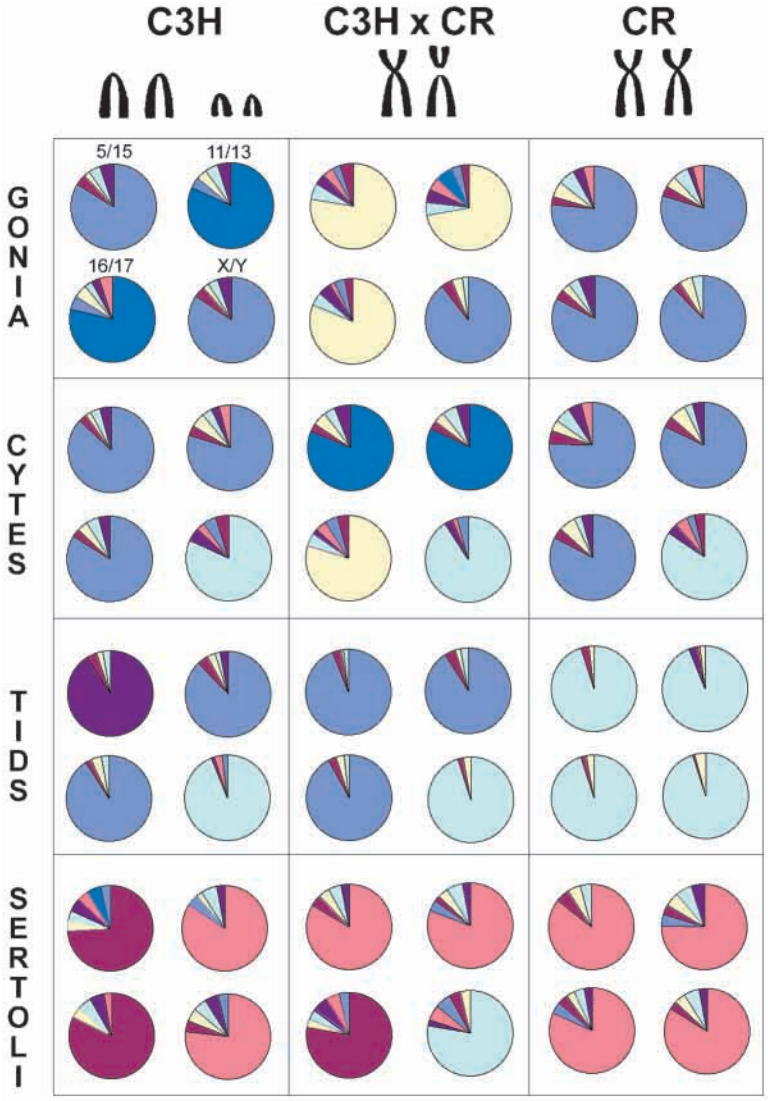

Fig. 2. The frequency distributions, shown as pie charts, of the 'typical' pattern (those shown in Fig. 1) are 15-20 times more frequent than some of the other patterns detected among those expected, on the basis of the combined possibilities of the three categories (condensation, proximity and position) in which we placed the fluorescent signals. Blue represents condensed, overlapping signals, either central or peripheral; dark blue and purple represent condensed, opposite ends, either central or peripheral, depending on the number of hybridization signals; pink, brown and light brown represent decondensed, opposite ends, both central and peripheral, depending on the number of hybridization signals. In the Rb heterozygotes, the spatial relationship between the Rb metacentric chromosome arms and their telocentric homologues differs from that of the homozygotes (yellow).

tubules can not be staged using the Oakberg criteria. The quantitative assessment of the the remaining 20% of tubules shows that even in these tubules the spermatogenetic process is impaired, with the production of fewer spermatids at stage I (Fig. 3). The pachytene spermatocytes/spermatids ratio is 1:2.2 in C3H × CR males, which is far from the theoretical 1:4 ratio expected for fertile animals or the observed ratios of 1:3.7 and 1:3.8 for the all-telocentrics and Rb homozygotes, respectively. In order to pinpoint the stage of loss, we have scored the relative numbers of diakinesis, MI- and MII-spermatocytes for the fertile homozygotes and the Rb heterozygote (Table 1). The mean values, and the ratio of MI to diakinesis spermatocytes, do not differ significantly among the three karyotypes, whereas MII-spermatocytes are significantly fewer ($P<0.05$) in the Rb heterozygote compared to the other two karyotypes. This suggests that the germ cell losses in the 20% of relatively normal tubules of C3H × CR Rb heterozygous mice occur during the transition from MI to round spermatids, probably at the spermatocyte II stage (as the reduced numbers of MII-spermatocytes corroborate, Table 1). Those round spermatids that reach stage I are able to differentiate until stage VII–VIII (Fig. 3).

DISCUSSION

The visualisation of nuclear architecture and chromosome territories provides a great cytological tool to understand genome sequences and protein function (Berezney and Wei, 1998; Boyle et al., 2001; Cremer and Cremer, 2001). Chromosome territories are not static, pre-assembled structures, rather, their in vivo spatial organisation (Zink et al., 1998) has a determinant role in the regular functioning of various cell machineries (Hamkalo and Elgin, 1991). Clear causative associations have been shown between changes in the spatial arrangement of some chromosome territories and the triggering of phenomena such as the pathogenesis of human leukemias, owing to t(9,22) translocation (Kozubek et al., 1999), and the induction of trisomy 21 (Nagele et al., 1998). Nagele and coworkers, studying the position of human chromosomes in normal cells and cells trisomic for chromosome 21, showed that the spatial order of chromosomes is established early and stably propagated through mitosis, as the 23 chromosomes of each haploid set are inherited from parents in a specific spatial order (Nagele et al., 1998). In the mouse, the separation of chromatin according to parental origin is preserved up until the four-cell embryo stage (Mayer et al., 2000), and, interestingly, during this period chromatin remodelling organises zygotic genes expression (Schultz et al., 1999). The spatial relationships of the chromosome pairs studied here indicate that they occupy a specific territory of the cell nucleus at each of the cell-cycle stages analysed (G_0 for the non-cycling Sertoli cell, G_1 for the long-cycling spermatogonia, M for the pachytene cells; the same is true of the haploid spermatids), providing further evidence for regular structuring of the mouse genome (Katsumata and Lo, 1988; Cerda et al., 1999). Large-scale spatial relationships of the chromosomal arms in Rb heterozygotes differ from those detectable in the all-telocentric pattern, with one homologue of each chromosome pair 'sequestered' from its original position, as occurs in human chromosomes 18 and 19 when involved in reciprocal translocation (Croft et al., 1999). Our findings suggest that the changes in the chromosomal nuclear territories owing to the

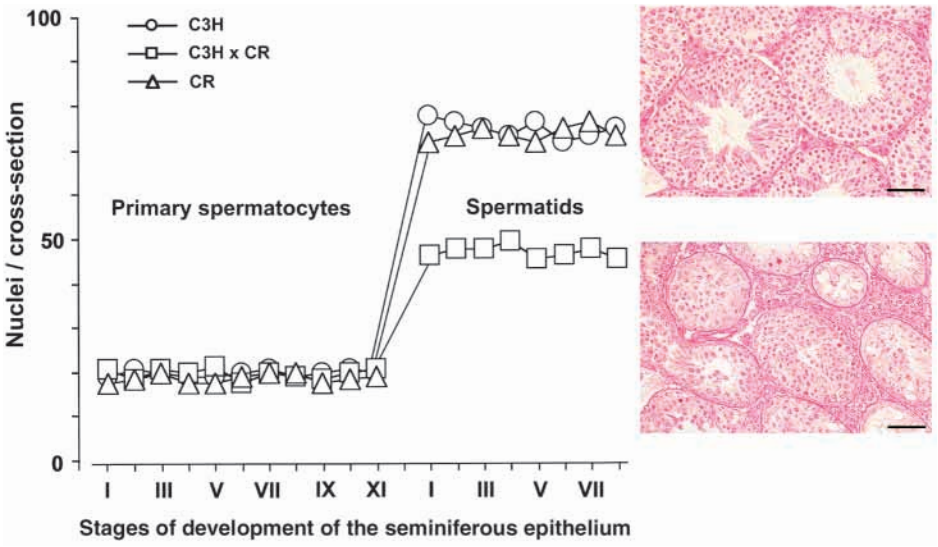

Fig. 3. Kinetics of pachytene/spermatid stages of spermatogenesis and spermiogenesis in homozygotes (all-telocentrics, C3H standard karyotype shown by O; all-Rb metacentrics, CR mice shown by Δ) and heterozygotes (seven trivalents in diakinesis, C3H × CR shown by □). The pachytene/spermatid ratios reach the expected 1:4 value only for the homozygous mice, whereas the C3H × CR heterozygotes show a decreased value because of germ-cell losses. On the right, the inserts show seminiferous tubules of a fully fertile (tubules with normal histoarchitectural germ cell arrangement) C3H (top) and a subfertile (tubules with different degrees of cell depletion) C3H × CR (bottom) mouse from histological sections of testes. The testicular picture of the fertile Rb homozygous CR mice is not reported as it looks like that of the C3H mice. The bar represents 100 μm.

presence of Rb heterozygosities do not affect long-cycling mitotic cells (i.e., spermatogonia) as these produce a regular number of pachytene spermatocytes. Instead, they suggest that the damage to spermatogenesis occurs at the meiotic stages, as is shown by the few spermatids produced at the end of meiosis II by the Rb heterozygotes (1:2.4 ratio between pachytene spermatocytes and spermatids versus the theoretical 1:4; Fig. 3). More precisely, considering the similar quantitative figures for pachytene, diakinesis and MI-spermatocytes of the three karyotypes (Table 1), the detrimental effects on spermatogenesis occur at later stages of male germ-cell cytodifferentiation when correct nuclear architecture at prophase I is crucial for synapsis, recombination and segregation. It seems likely that the possible adverse consequences to spermatogenesis due to the spatial alterations of the nuclear architecture are triggered at prophase I (e.g., by the failure to saturate pairing sites or by the expression of non-permissible genes) and become evident at the MI-MII transition (as is shown by the reduced number of MII-spermatocytes of Rb heterozygotes, Table 1). Interestingly, primary spermatocytes of structural heterozygotes show deregulated enzymatic activities (Hotta and Chandley, 1982; Redi et al., 1983), qualitative differences in protein patterns (Forejt, 1982) and have a longer prophase I (Redi et al., 1985), as if to resolve tangles or interlocks between non-homologues (Rose and Holm, 1993). The complex cellular phenomena occurring at meiotic prophase I, and particularly the need for homologues to pair, are probably governed by checkpoints (Handel et al., 1999) that are evolutionarily selected for controlling the presence of 3D irregularities. The existence of one such checkpoint in mammals was clearly shown by Burgoyne and co-workers, proving the existence of a meiotic checkpoint monitoring synapsis ('synapsis checkpoint', a modification of the yeast recombination checkpoint) able to trigger apoptotic elimination of spermatocytes with synaptic errors in a p53-independent manner (Odorisio et al., 1998).

The Sertoli cells of Rb homozygous fertile animals always show diffuse X and Y FISH signals, suggesting a decondensed chromatin organisation correlated with an active gene expression. It is well known that the condensation of the X and Y chromosomes in Sertoli cells of man and mouse is dynamic. For example, the X and Y chromosomes are condensed in pre-pubertal fertile individuals and infertile men with the Sertoli-cell-only syndrome, although they are expanded in adult fertile individuals [(Guttenbach et al., 1996; Chandley and Speed, 1995) in the house mouse; (Speed et al., 1993; Kofman-Alfaro et al., 1994) in man]. Our finding that the X and Y signals are condensed in the majority (84%) of the Sertoli nuclei of Rb subfertile mice, parallels similar findings in humans with the Sertoli-cell-only syndrome (Kofman-Alfaro et al., 1994). On the one hand this supports the view that at least some facets of the spermatogenesis process may not be autonomous to the germ cells, instead it is controlled by the Sertoli cells (Speed et al., 1993). On the other hand, it suggests that the subfertile Robertsonian mouse might be a useful model of human impaired spermatogenesis.

We suggest that large-scale changes in chromosome territories by changing the physical interactions of specific genome portions act as an epigenetic factor in controlling gene expression in somatic and germ cells. Future studies of gene

Table 1. The means (± σ) of diakinesis, MI-, MII-spermatocytes and MI/dia ratios

Karyotype	Diakinesis	MI	MII	MI/dia ratio
C3H	16.5±5.8 (132)	14.8±4.9 (119)	23.2±4.7 (186)	0.9±0.27
C3H × CR	13.1±4.2 (105)	15.3±4.8 (122)	16.2±3.8 (130)	1.2±0.34
CR	13.6±5.1 (108)	14.4±4.0 (115)	25.5±4.3 (204)	1.1±0.23

Means (± standard deviation) of diakinesis, MI and MII-spermatocytes in C3H, C3H x CR and CR mice. In parenthesis the total number of cells counted are shown. MII-spermatocytes in C3H × CR mice are significantly (P<0.05) fewer than those counted in both C3H and CR mice. The MI/dia ratios do not differ among the three karyotypes.

expression in Sertoli cells, as well as in diploid and haploid germ cells of Rb heterozygotes, could help to highlight the cell-specific orchestration of gene activity that governs cell differentiation – one of the crucial themes of present-day biomedical researches.

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