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Interactions of rat repetitive sequence MspI8 with nuclear matrix proteins during spermatogenesis*

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Using the Southwestern blot analysis we have studied the interactions between rat repetitive sequence MspI8 and the nuclear matrix proteins of rat testis cells. Starting from 2 weeks the young to adult animals showed differences in type of testis nuclear matrix proteins recognizing the MspI8 sequence. The same sets of nuclear matrix proteins were detected in some fractions enriched in spermatocytes and spermatides and obtained after fractionation of testis cells of adult animals by the velocity sedimentation technique.

Eukaryotic interphase chromatin as well as metaphase chromosomes are thought to be organized into loop domains attached at their basis to proteinaceous structure called nuclear matrix or chromosomal skeleton. This loop organization is probably important not only for compaction of the chromatin but also for the regulation of gene expression and replication [1]. Sequences delimiting the domains called MARs or SARs (matrix/scaffold attachment regions) are operationally defined by high binding affinity to the nuclear matrix [2, 3]. Very often the regulatory sequences including enhancer sequences were found in close vicinity or inside of MAR sequences [2, 4]. DNA-nuclear matrix interactions change during different nuclear processes and during the differentiation of cells. It was shown that on actively transcribed sequences additional attachments to nuclear matrix appear [5]. This probably correlates with changes of loop sizes observed in some pathological states and during the differentiation of cells [6].

Previously we have isolated a 454 bp long fragment of rat repetitive sequence belonging to rat LINE family. The sequence called MspI8 complexed efficiently to rat testis nuclear matrix proteins [7]. Mammalian spermatogenesis, a complex differentiation process, requires the coordinated expression of numerous genes and very deep reorganization of genetic material [8, 9]. To study the possible role of MspI8 repetitive sequence during the process of spermatogenesis we looked for the nuclear matrix proteins recognizing this sequence in testis cells of young animals of different age in which the spermatogenesis process was not completed or in the testis cells of adult animals fractionated by the sedimentation method. Changes in nuclear matrix-MspI8 repetitive sequence during the differentiation were observed.

MATERIALS AND METHODS

DNA. Rat repetitive sequence MspI8 was isolated and cloned into pUC19 vector as described before [7]. Plasmid DNA was isolated according to standard lysozyme and alkali lysis method [10]. MspI8 fragments were cut from

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Abbreviations: BSA, bovine serum albumin; LINE, long interspersed repeated sequences; MAR, matrix attachement region; SAR, scaffold attachement region.

plasmid with *Hin*dIII and *Eco*RI enzymes, separated from vector sequences on agarose gel and end-labeled with ^{32}P using polynucleotide kinase or Klenow polymerase [10].

Nuclear matrices. Proteins of the nuclear matrices from rat testis were isolated according to the modified "high-salt" method [11]. The nuclei were purified by centrifugation in 2.2 M sucrose and washed with 1% Triton X-100 in STM buffer (0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, 3 mM $MgCl_2$). The nuclear pellet was stabilized (10 min incubation in STM buffer completed with 5 mM $CuSO_4$) and washed with STM. Then the nuclei were treated with DNase I at 100–200 μg/ml in 0.1 M NaCl/STM for 60 min at 20°C. The remnant nuclei were extracted with 0.5 M NaCl/STM followed by 2 M NaCl in STM to obtain the matrices. On all stages the 0.5 mM PMSF/phenylmethylsulfonyl fluoride (Sigma) was present in the buffers. The final nuclear matrix protein preparations were suspended in 0.1 M NaCl/STM with 50% glycerol and stored at –20°C. Isolation of proteins of the nuclear matrices from spermatozoa and cells separated in sedimentation apparatus was performed as described above omitting centrifugation in 2.2 M sucrose.

Southwestern blot analysis. The nuclear matrix proteins (about 100 μg per slot) were resolved in 10% polyacrylamide/SDS gel and electrotransferred onto filter (Hybond C extra or Immobilon P) in 25 mM Tris, 190 mM glycine and 20% methanol. After transfer these filter-bound proteins were incubated for 5 h at 25°C in renaturation buffer containing 50 mM Tris (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 2.5% BSA in the hybridization oven. After washing with the binding buffer (the same composition as above except that 0.25% BSA was added) filters were incubated for 5 h at 20°C or 37°C in the binding buffer supplemented with ^{32}P-end-labeled DNA probe and one of different competitor DNA. Then filters were washed, dried and autoradiographed. To determine molecular weight (M_r) of investigated proteins we used two kits of Prestained SDS Molecular Weight Markers (Sigma SDS-7B).

Fractionation of rat testis cells. The procedure was described by Wolgemuth *et al.* [12] with small modifications. Briefly: after the removal of tunica albuginea, testes of 3 rats were digested successively with collagenase in 1 mg/ml of Dulbecco modified phosphate buffered saline (DPBS) at 33°C for 20 min. Then digestion with trypsin and DNase I at concentrations of 0.25 and 1 mg/ml, respectively, was performed at 33°C for 5 min. Cell suspension was homogenized using Pasteur pipette and centrifuged 200 × *g* through a cushion of 0.5% bovine serum albumin (BSA) containing 1 μg/ml DNase for 10 min at 10°C. The pellet was resuspended in 0.5% BSA with 1 μg/ml DNase, filtered through gauze and layered on the top of 2%–4% bovine serum albumin gradient preformed earlier in sedimentation apparatus. Total volume of the gradient was 600 ml. On the bottom the 150 ml cushion of 10% BSA was placed. Cells were layered on the top of gradient in volume of 25 ml and the top of the gradient was covered with 50 ml of 0.2% BSA. The gravitational sedimentation of cells was carried for 90 min at 4°C and the material from the gradient was collected in 10 fractions. The amount of cells in each fraction was counted.

RESULTS

To study the interactions between nuclear matrix proteins and MspI8 sequence we used the Southwestern blot technique. The nuclear matrix proteins of rat testis, brain, liver and kidney cells separated in SDS/polyacrylamide gel electrophoresis and transferred to membrane were complexed to labeled MspI8 sequence in the presence of 1 μg poly dAdT/ml as a competitor at 20°C. Fig. 1 demonstrates an observation, that in nuclear matrices isolated from different tissues there exist sets of proteins, of different M_r, interacting with MspI8 sequence. The electrophoregrams of proteins isolated from various sources (Fig. 1A) were similar, but in Southwestern blot analysis these proteins gave different, tissue-specific patterns.

In the next experiments we investigated the binding of MspI8 to rat testis nuclear matrix proteins at different temperatures and in the presence of different competitors. Fig. 2 compares the results obtained in the presence of sonicated *E. coli* DNA, poly dAdT and poly dIdC as competitors and at two different temperatures. The amount of the protein bands complexing with the MspI8 was dependent on the conditions, i.e. the type and amount of competing sequences and the temperature at which the reaction took place. The most prominent

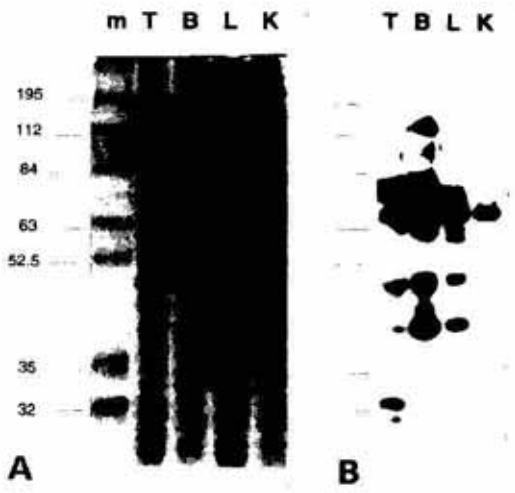

Fig. 1. Southwestern analysis of complexes formed by MspI8 with nuclear matrix proteins isolated from different rat tissues: testis (denoted T), brain (B), liver (L) and kidney (K).

Molecular weight markers of prestained proteins (α_2-macroglobulin from human plasma, β-galactosidase from *E. coli*, fructose-6-phosphate kinase from rabbit muscle, pyruvate kinase from chicken muscle, fumarase from porcine heart, lactic dehydrogenase and triosephosphate isomerase from rabbit muscle) were bought in Sigma and had apparent molecular weight given by producer; on the figures $M_r \times 10^{-3}$ are given. A. Coomassie Blue stained nuclear matrix proteins of different tissues resolved on 10% polyacrylamide gel electrophoresis. B. Binding of MspI8 to nuclear matrix proteins of different tissues. The reaction was performed at 20°C in the presence of 1 μg/ml poly dIdC as a competitor.

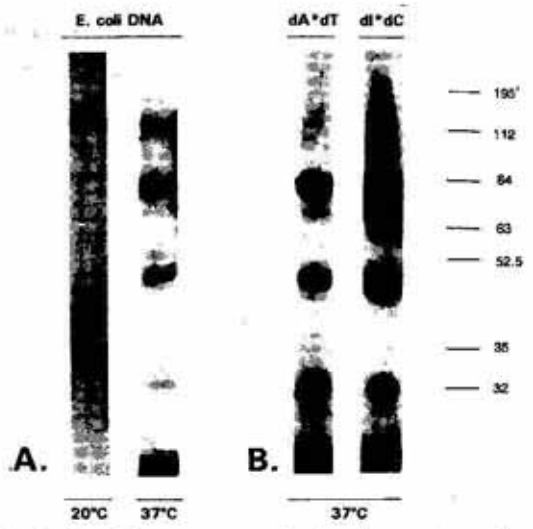

Fig. 2. Southwestern analysis of complexes formed by MspI8 with nuclear matrix proteins of rat testis in the presence of different competitors and at different temperatures.

A. Binding of MspI8 to rat testis nuclear matrix proteins in the presence of 10 μg/ml sonicated *E. coli* DNA as a competitor at 20°C (left lane) and 37°C (right lane). B. Binding of MspI8 to rat testis nuclear matrix proteins at 37°C in the presence of different competitors in concentration of 2 μg/ml (poly dAdT, left lane, and poly dIdC, right lane).

signals were always obtained from bands containing proteins of about 32000, 46000–48000, 76000–84000 and 120000 (Fig. 2). Comparison of the results obtained after the reaction performed in different temperatures shows that this parameter has great influence on the efficiency and quality of DNA-nuclear matrix proteins interactions. Increasing the temperature generally improved the efficiency of the complex formation in presence of all types of competitors. The formation of complex between MspI8 and high M_r proteins of about 120000, however was strongly inhibited at temperatures higher than 37°C (Fig. 2A). In our experiments poly dAdT was more efficient competitor than poly dIdC and *E. coli* DNA (Fig. 2B).

The proteins found in different electrophoretic bands differed in their affinity to subfragments of MspI8 molecule. Figure 3 compares the interactions of two parts of MspI8 molecule and nuclear matrix proteins of rat testis. The studies performed by the Southwestern method show that subfragments differ in their affinity to low and high M_r proteins. The prominent signal on the blot probed with labeled 322 bp subfragment (5'-subfragment of MspI8) was obtained from the protein with M_r of about 120000 whereas the shorter, 183 bp subfragment (3'-part) interacted most efficiently with proteins of about 84000.

Searching for the role of MspI8-protein interactions in the process of spermatogenesis we performed the experiments using the proteins

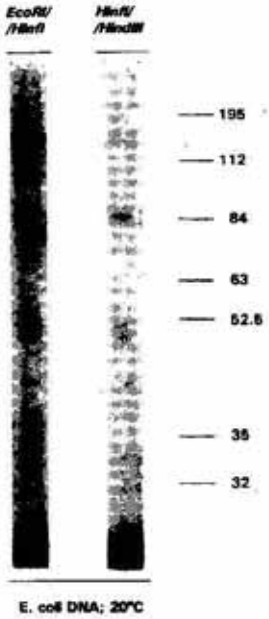

Fig. 3. Binding of subfragments of MspI8 to rat testis nuclear matrix proteins; 5' fragment of 322 bp (denoted EcoRI/HinfI) and to 3' fragment of 183 bp (HinfI/HindIII).

Southwestern analysis of proteins recognizing the MspI8 subfragments was performed in presence of 10 μg sonicated DNA *E. coli* as competitor.

isolated from testis of young animals in which the spermatogenesis was not completed. Figure 4 presents the results of Southwestern blot analysis performed with proteins of animals differing in age. Nuclear matrix proteins obtained from testis of 2 to 8 weeks old rats were probed with MspI8 at 37°C in the presence of 2 μg/ml of poly dIdC. The pattern of proteins recognizing the MspI8 sequence seems to change during spermatogenesis. In the conditions studied the most prominent in interaction with MspI8 DNA were bands containing proteins of about: 20000–36000, 48000, 56000, 66000 and 76000. The relative amount of DNA binding proteins with M_r in the range 20000–48000 and 66000–76000 is rather low in preparations from animals younger than 4 and elder than 6 weeks (Fig. 4). The last slot on the Fig. 4 contained proteins isolated from rat spermatozoa. We did not detect any proteins recognizing the MspI8 sequence in spermatozoa in these conditions.

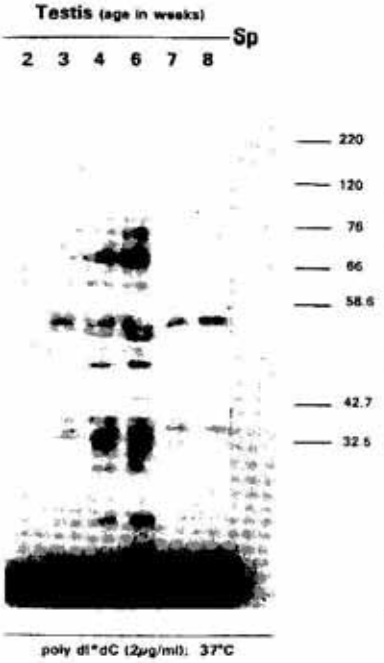

Fig. 4. Southwestern analysis of complexes formed by MspI8 with nuclear matrix proteins of rat testis obtained from animals differing in age (2 to 8 weeks old) and nuclear matrix proteins isolated from spermatozoa (Sp).

In the next experiment we separated the testis cells of adult rats using the method of sedimentation in albumin gradient. This method enables the fractionation seminiferous epithelial cells differing in shapes and densities and to obtain the fractions enriched in cells from different stages of spermatogenesis [12–14]. The separation is not complete and cross-contaminations of all fractions with cells from different stages of spermatogenesis were observed. Some fractions, however, are enriched in cells of defined phases of spermatogenesis. According to our own microscopical observations and to the literature data [15] the spermatogonia, spermatocytes and early spermatides are present in the very quickly sedimenting fractions near the bottom and in the middle parts of the gradient. Figure 5A shows the sedimentation profile of testis cells separated in the sedimentation apparatus. Figure 5B presents the results of Southwestern blot analysis of interactions between nuclear matrix proteins isolated from separated fractions of cells and labeled sequence MspI8. Fractions number 2, 4, 5 and 6 contained the highest levels of proteins recognizing the MspI8 sequence in the reaction *in vitro*. The same fractions were enriched (in comparison to other fractions) in spermatogonia, spermatocytes and early spermatides. In fractions containing slowly sedimenting cells and present on the top of the gradient we identified mainly cells at the stage of late spermatid and spermatozoa. In Southwestern blot analysis in this fractions rather low concentrations of proteins interacting with MspI8 were detected.

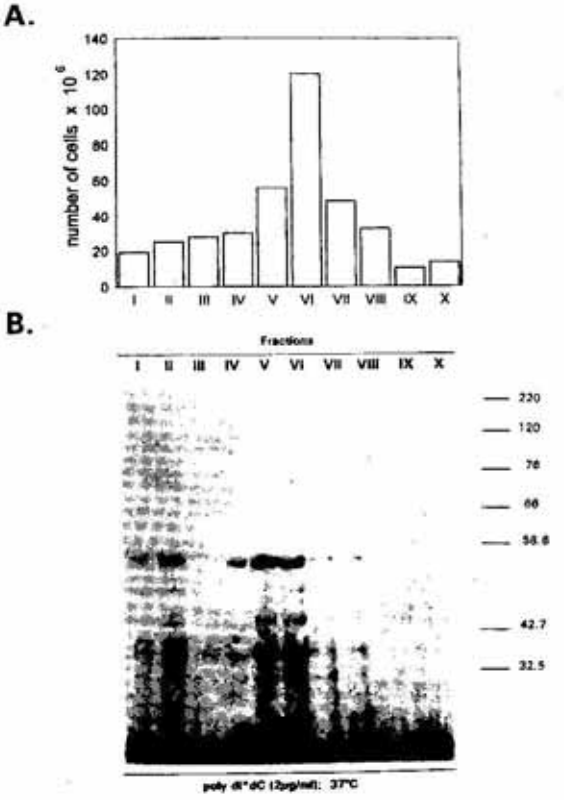

Fig. 5. Nuclear matrix proteins recognizing MspI8 sequence in testis cells fractionated by sedimentation technique (fractions I–X).
A. Fractionation profile of testis cells separated on sedimentation apparatus. B. Southwestern analysis of complexes formed by MspI8 with nuclear matrix proteins of separated cells.

DISCUSSION

The repetitive sequence MspI8 interacts with nuclear matrix proteins of different tissues. Electrophoretic patterns of proteins recognizing MspI8 in Southwestern reaction show tissue specificity. The highest amounts of different proteins complexing with MspI8 were detected in nuclear matrices of testis and brain cells. However, on the basis of one dimensional electrophoresis we have not been able to distinguish differences resulting from the presence of tissue-specific proteins from differences pertaining to different relative levels of those same nuclear matrix proteins only present in tissues in different concentrations.

In our previous studies we found that in Southwestern blot analysis MspI8 repetitive sequence interacts with only one protein band when complexed to testis nuclear matrix proteins. The reaction was performed at low temperature of about 4°C [7]. The experiments presented in this paper show that incubation of DNA and matrix proteins at more "physiological" temperatures (i.e. about 37°C) makes the pattern of proteins recognizing the MspI8 sequence more complexed. The stabilizing effect of higher temperature and metal ions on DNA-nuclear matrix interactions was shown in many experiments [16, 17, 18]. In our studies we have observed that the temperature increase stabilizes some MspI8 interactions with proteins of lower M_r and has a destabilizing effect on MspI8 interactions with higher M_r proteins. The reaction was very sensitive to temperature and presence of different competitors. It seems that most of the nuclear matrix proteins interacted with AT tracts present in MspI8 sequence as the poly dAdT proved the strongest competitor of all which we investigated.

The change in affinity of nuclear matrix proteins to some DNA sequences coupled to temperature change and observed *in vitro* may reflect phenomena taking place in the nucleus during the heat shock, when some sets of genes are turned off and others are activated [19]. It seems quite possible that temperature-induced change in DNA-protein affinity plays some role in these processes. The elucidation of which particular proteins change their affinity to DNA, depending on temperature, needs further studies.

The development of spermatozoa in seminiferous epithelium includes three main phases: spermatogonial multiplication, meiosis and spermiogenesis. Cells in these phases are called spermatogonia, spermatocytes and spermatids. Mammalian spermatozoa contain a structure closely resembling the nuclear matrix and DNA organized in loops attached to it. The mean size of the DNA loop in spermatozoon was, however, about half of the size of the loop found in other tissues [9, 20]. It seems probable that responsible for this diminishing of the loop size may be interactions of some repetitive sequence with nuclear matrix proteins. To test this hypothesis we chose the MspI8 DNA sequence which is highly repetitive and showed higher affinity to rat testis nuclear matrix proteins compared to other sequences. An experiment in which rat spermatozoa proteins were complexed to MspI8 showed, however, that in nuclear matrices of these cells any protein bands recognizing MspI8 could be detected. This finding rather suggests a different role for nuclear matrix-MspI8 interactions than final compaction of chromatin in spermatozoa.

The relative amount of nuclear matrix proteins recognizing the MspI8 was highest in the testis of 4–6 weeks old animals. In the seminiferous epithelium of these animals process of spermatogenesis was not completed. Clermont and Perey [21] have shown that the spermatogenesis reaches the stage of very early spermatides in 4 weeks-old rats and XIV stage of spermatides in 6 weeks-old animals. In testis of 4–6 week old animals we observed the highest level of proteins interacting with MspI8. Later on, the relative amount of proteins recognizing the MspI8 sequence seemed to drop down. This finding could suggest that some of the MspI8 recognizing nuclear matrix proteins could be the transition proteins appearing in the mid-spermatides.

Studies performed on the nuclear matrix proteins isolated from fractionated testis cells of adult rats show that the proteins recognizing MspI8 sequence are concentrated in fractions II, V and VI of velocity sedimentation procedure. According to literature data [12, 15] and our own light microscope observations, these fractions contained spermatocytes and cells at the stage of the mid-spermatides. Thus the results obtained with proteins isolated from fractionated cells are not contradictory to the results

shown above. These results may also suggest that at some stages of spermatogenesis there is an increase in the amount of proteins recognizing some subsequences present in MspI8.

The lack of proteins recognizing MspI8 in spermatozoa testifies against the hypothetical role of MspI8 in organization of chromatin loops in spermatozoa. We can not, however, rule out that this sequence could be engaged in one of the two processes taking place in pachytene and mid spermatid stage: 1) the reorganization of genetic material involving participation of transition proteins, and 2) the regulation of RNA transcription at the stage of pachytene and mid spermatides.

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REFERENCES

1. Gasser, S.M., Amati, B.B., Cardenas, M.E. & Hoffmann, J.F.-X. (1989) Studies on scaffold attachment sites and their relation to genome function. *Int. Rev. Cytol.* **119**, 57–96.
2. Cockerill, P.N. & Garrard, W.T. (1986) Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell* **44**, 273–282.
3. Boulikas, T. (1993) Nature of DNA sequences at the attachment regions of genes to the nuclear matrix. *J. Cell. Biochem.* **52**, 14–22.
4. Cockerill, P.N., Yuen, M.H. & Garrard, W.T. (1987) The enhancer of the immunoglobulin heavy chain locus is flanked by presumptive chromosomal loop anchorage elements. *J. Biol. Chem.* **262**, 5394–5397.
5. Farache, G., Razin, S.V., Rzeszowska-Wolny, J., Moreau, J., Recillas Targa, F. & Scherrer, K. (1990) Mapping of structural and transcription-related matrix attachment sites in the α-globin gene domain of avian erythroblasts and erythrocytes. *Mol. Cell. Biol.* **10**, 5349–5358.
6. Gerdes, M.G., Carter, K.C., Moen, P.T., Jr. & Lawrence, J.B. (1994) Dynamic changes in the higher-level chromatin organization of specific sequences revealed by in situ hybridization to nuclear halos. *J. Cell Biol.* **126**, 289–304.
7. Rzeszowska-Wolny, J. & Rogoliński, J. (1994) MspI8, the repetitive sequence specifically interacting with nuclear matrix of rat testis cells. *Acta Biochim. Polon.* **41**, 459–471.
8. *Molecular Biology of the Male Reproductive System* (1993) (de Kretser, D., ed.) Academic Press Inc., New York.
9. Ward, W.S., Partin, A.W. & Coffey, D.S. (1989) DNA loop domains in mammalian spermatozoa. *Chromosoma* **98**, 153–159.
10. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*; 2nd edn., Cold Spring Harbor Laboratory Press.
11. Berezney, R. & Coffey, D.S. (1974) Identification of nuclear protein matrix. *Biochem. Biophys. Res. Commun.* **60**, 1410–1417.
12. Wolgemuth, D.J., Gizang-Ginsberg, E., Engelmyer, E., Gavin, B.J. & Ponzetto, C. (1985) Separation of mouse testis cells on a Celsep™ apparatus and their usefulness as a source of high molecular weight DNA or RNA. *Gamete Res.* **12**, 1–10.
13. Meistrich, M.L. (1977) Separation of spermatogenic cells and nuclei from rodent testes. *Meth. Cell Biol.* **15**, 15–54.
14. Bellve', A.R. (1993) Purification, culture and fractionation of spermatogenic cells. *Meth. Enzymol.* **225**, 84–113.
15. Lam, D.M., Furrer, R. & Bruce, W.R. (1970) The separation, physical characterization and differentiation kinetics of spermatogonial cells of the mouse. *Proc. Natl. Acad. Sci. U.S.A.* **65**, 192–199.
16. Mirkovitch, J., Gasser, S.M. & Laemmli, U.K. (1988) Scaffold attachment of DNA loops in metaphase chromosomes. *J. Mol. Biol.* **200**, 101–109.
17. Rzeszowska-Wolny, J., Razin, S., Puvion, E., Moreau, J. & Scherrer, K. (1988) Isolation and characterization of stable nuclear matrix preparations and associated DNA from avian erythroblasts. *Biol. Cell* **64**, 13–22.
18. Ivanchenko, M. & Avramova, Z. (1992) Interaction of MAR-sequences with nuclear matrix proteins. *J. Cell. Biochem.* **50**, 190–200.
19. Morimoto, R.I. (1993) Cell in stress: Transcriptional activation of heat shock genes. *Science* **259**, 1409–1410.
20. Ward, W.S. (1994) The structure of the sleeping genome: Implication of sperm DNA organization for somatic cells. *J. Cell. Biochem.* **55**, 77–82.
21. Clermont, Y. & Perey, B. (1957) Quantitative study of the cell population of the seminiferous tubules in immature rats. *Am. J. Anat.* **100**, 241–268.