Acta Biochimica Polonica

Vol. 41 No. 4/1994

QUARTERLY

MspI8, the repetitive sequence specifically interacting with nuclear matrix of rat testis cells*

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Received: 12 August, 1994

Key words: rat repetitive sequence, nuclear matrix, protein: DNA interaction

The nuclear matrix bound DNA fraction of rat testis showed enrichment in repetitive sequences found in the 450 bp band after gel electrophoresis of the *Msp*I digested rat DNA. DNA fragments isolated from this band were cloned. DNA of the clone pMspI8 showed homology to some representatives of rat LINE sequence family, and complexed *in vitro* more efficiently with testes nuclear matrix proteins than with yeast ARS1 sequence containing the matrix association region (MAR) or DNA from an other clone, MspI19. Western blot analysis showed that MspI8 sequence interacts with testes matrix protein of about 120 kDa.

DNA of somatic cells is attached to nuclear matrix at approximately every 60000 base pairs. The interactions with nuclear matrix are responsible for the organization of DNA into loop domains and are related to different nuclear functions [1]. It was shown that nuclear matrices originating from different tissues contained different sets of proteins [2] and some of matrix proteins interacted with specific regions of genes [3, 4]. Matrix associated regions (MAR)[1] share some sequence motifs with origins of replication, enhancers and homeotic protein binding sites [5] and they were shown to interact highly preferentially with various nuclear proteins [6–11].

Mammalian spermatozoa also contain a structure closely resembling the nuclear matrix and like in somatic cells, sperm DNA is organized into loops. The mean size of the sperm DNA loops was, however, about half the size of loops found in other tissues [12]. Shortening of loop sizes may suggest that new sites of DNA attachment appeared at some stage of spermato- or spermiogenesis. We wondered whether the interactions of the repetitive sequences with matrix proteins of reproductive tissue could be responsible for creation of these new attachment sites. Rat testis was chosen as a model tissue for the studies.

MATERIALS AND METHODS

Molecular cloning of DNA fragments in PUC19 vector and DNA sequencing were performed according to protocols described in Sambrook's *et al.* manual [13].

Analysis of DNA sequences was done with the help of PC/Gene computer program (IntelliGenetics Inc. & Genofit SA).

Nuclear matrix was isolated and complexes between DNA probes and nuclear matrix pro-

*The work was supported by the Committee for Scientific Research grant 6P203 028 05 and Foundation for Polish Science BIMOL 76/93.

[1] Abbreviations: MAR, matrix associated regions.

teins were performed *in vitro* as described by Cockerill & Garrard [14]. Complexes of the *Msp*I digested total rat DNA and rat testis matrix proteins were formed in the presence of matrices from aproximately 5×10^7 cells and 250 µg of unlabeled rat DNA. Complexes of labeled probes (plasmids pMspI19, pMspI8, pYRP7) were obtained in the volume of 100 µl of assay buffer (50 mM NaCl, 10 mM Tris/HCl, pH 7.4, 2 mM EDTA, 0.25 M sucrose, 0.25 mg/ml bovine serum albumin). The reaction mixture contained labeled DNA probes at the concentration of 20 ng/ml and sonicated *E. coli* DNA (200–400 µg/ml).

Plasmids pMspI8 and pMspI19 were obtained after molecular cloning of 450 bp and 280 bp DNA fragments in PUC19. The fragments were isolated from bands seen in the agarose gel after electrophoresis of total DNA digested with *Msp*I. The plasmid pYRP7 containing the yeast genomic ARS1 element was a kind gift from Dr K. Spirin.

Testis nuclear matrix proteins were fractionated by SDS/PAGE method and electrophoretically transferred to nitrocellulose filter (Schleicher & Schuell). Filter bound proteins were renatured by incubation for 24 h at 4°C in 50 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% skim milk. Following renaturation, the filters were rinsed twice for 15 min with binding buffer (the same as renaturation buffer except that 0.25% milk was used). DNA binding was performed in a bath containing binding buffer supplemented with 1 µg/ml of poly(dG-dC) and 1×10^6 c.p.m./ml of ^{35}S-labeled probe. This mixture was shaken gently at 4°C for 10 h, and then washed three times over a period of 45 min each in binding buffer without DNA and poly(dG-dC). Filters were air dried and exposed to X-ray film.

RESULTS

To study specific interactions of highly reiterated sequences and nuclear matrix proteins of rat testes, we performed the reaction of complex formation *in vitro* between total DNA digested with different restriction enzymes and matrix isolated according to the method of Cockerill & Garrard [14]. Complexes were separated by centrifugation and DNA from both pellet and supernatant fractions was analysed

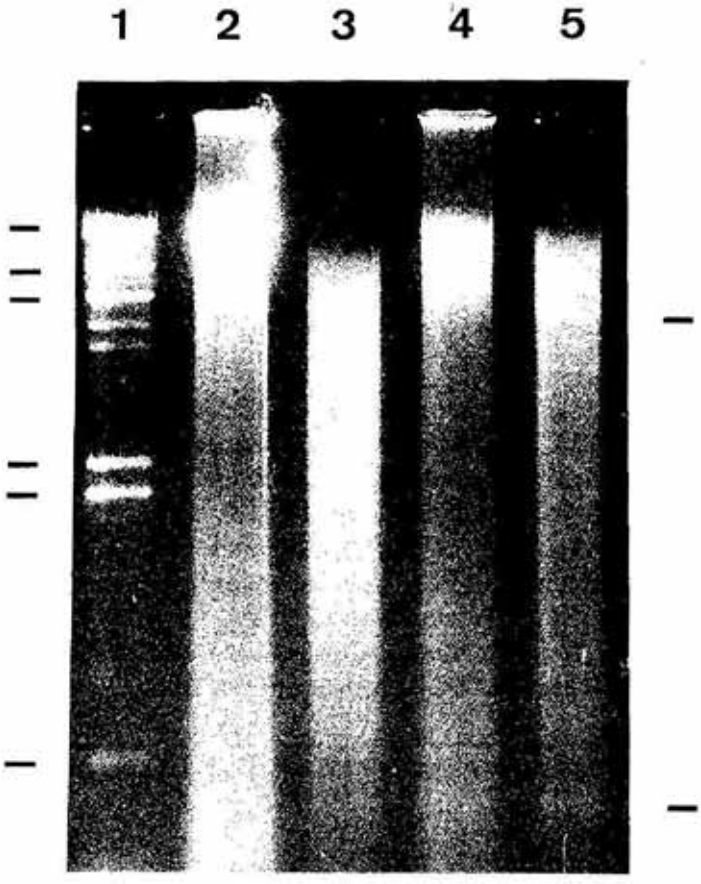

Fig. 1. Electrophoresis of DNA isolated from the complex of MspI digested rat DNA and nuclear matrix from testes.
Lane 1, size markers: phage λ DNA digested with *Hin*dIII; lane 2, exogenous nuclear matrix attached DNA; lane 3, DNA isolated from supernatant fraction; lane 4, DNA isolated from pellet containing the complex formed by *Msp*I digested DNA and nuclear matrix; lane 5, total nonfractionated DNA digested with *Msp*I.

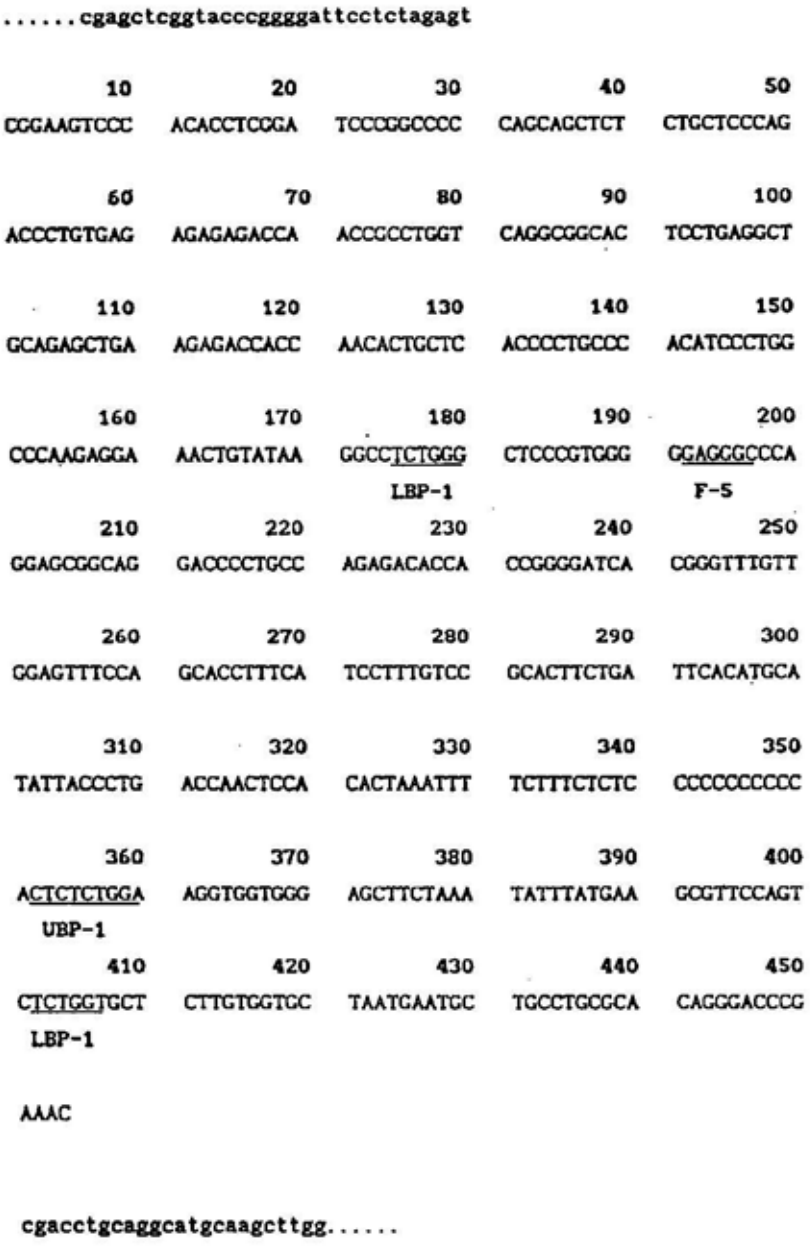

Fig. 2. Nucleotide sequence of the DNA fragment found in pMspI8 clone.

electrophoretically (Fig. 1). Agarose gel electrophoresis of total DNA digested with *MspI* showed the presence of two bands containing repetitive sequences. One of the bands was in the region of about 450 bp. The second contained DNA fragments of about 7 kb. Either bands contained a similar amount of DNA. On the electrophoretic pattern obtained for DNA isolated from supernatant (fraction which was not complexed with proteins) only the high molecular mass band was clearly seen. In this DNA fraction the fragments forming the band of 450 bp were present in much lower concentration than in total DNA. On the other hand, in DNA isolated from the pellet the fragments of 7 kb band were underrepresented whereas the fragments of 450 bp seemed to be enriched (Fig. 1, lane 4).

The DNA fragments present in the 450 bp band (Fig. 1, lane 5) were eluted and cloned in *AccI* site of pUC19. One of the positive clones designated as pMspI8 contained the repetitive sequences. The DNA fragment of pMspI8 was sequenced (Fig. 2). This sequence was analysed by the PC/Gene computer data program and 85% homology was found between pMspI8

Table 1

The examples of sequences showing high degree of homology with the DNA sequence of MspI8 clone

No.	Sequence name	Sequence origin	Homology with MspI8
1	RNL1RTP5F	1826 bp *R. norvegicus* retroposon/pseudogene 5′ flank and ORF1 homology in 720–1181	70%
2	RNLINED	6335 bp *R. norvegicus* long interspersed repetitive DNA containing 7 ORF'S homology in 1–448	69.8%
3	RSLIN3A	8048 bp rat rat long interspersed repetitive DNA sequence LINE3 (L1 Rn) homology in 443–899	69.4%
4	RNLB6	1857 bp rat L1 Rn B6 repetitive DNA element homology in 605–1051	67.7%
5	RNLB7	2288 bp rat L1 Rn B7 repetitive DNA element homology in 572–1032	66.9%
6	RREF1AA	3047 bp rat transcription factor EF-1 (A) gene homology in 2377–2765	57.8%
7	HSPP14B	8076 bp human placental protein 14 (PP14) gene homology in 4793–5238	52%
8	OCAMYA	5651 bp rabbit serum amyloid A gene exons 1, 2, 3, and 4 homology in 1082–1543	51.9%
9	SSTRNP2B	1863 bp Sus scrofa transition protein 2 gene exons 1 and 2 homology in 455–917	51.2%
10	HSADPRF2	2558 bp human ADP-ribosylation factor 1 gene exons 2–5 homology in 630–1072	50.3%

and rat DNA fragment containing long interspersed repeat sequence (RNL1NH1). The homology of MspI8 sequence to other representatives registered in PC/Gene Data Bank did not exceed 70% (Table 1). Sequences with 50–70% homology to MspI8 were present in many different places of rat and other genomes in noncoding as well as in coding parts of the DNA.

The MspI8 sequence contains four sites identified as protein binding sites in other types of cells. There are two TCTGG sites and moreover, one CTCTCTGG site identified in HeLa cell line as binding the LBP-1 and UBP-1 proteins, and a GAGGG fragment which was shown to bind a specific F5 protein factor in chicken cells [15–17].

The efficiency of complex formation between rat testes nuclear matrix proteins and MspI8 and also two other reference sequences (matrix associated region of yeast ARS1 sequence, and repetitive sequence of MspI19 clone) was compared. All three fragments were labeled with radioactive thymidine. The same amounts of DNA probes were added to standard amounts of matrix proteins and the complexes were formed in *in vitro* according to Cockerill & Garrard [14]. The amount of DNA in complexes was estimated on the basis of radioactivity. Figure 3 shows the relative amounts of DNA found in complex in two independent experiments.

The sequence present in clone pMspI8 showed higher efficiency in formation of complexes with nuclear matrix of testes than the specific matrix attachment region of ARS1 yeast sequence or the repetitive sequence found in clone pMspI19.

We wondered whether the nuclear matrices of some cells in testes contain proteins specifically

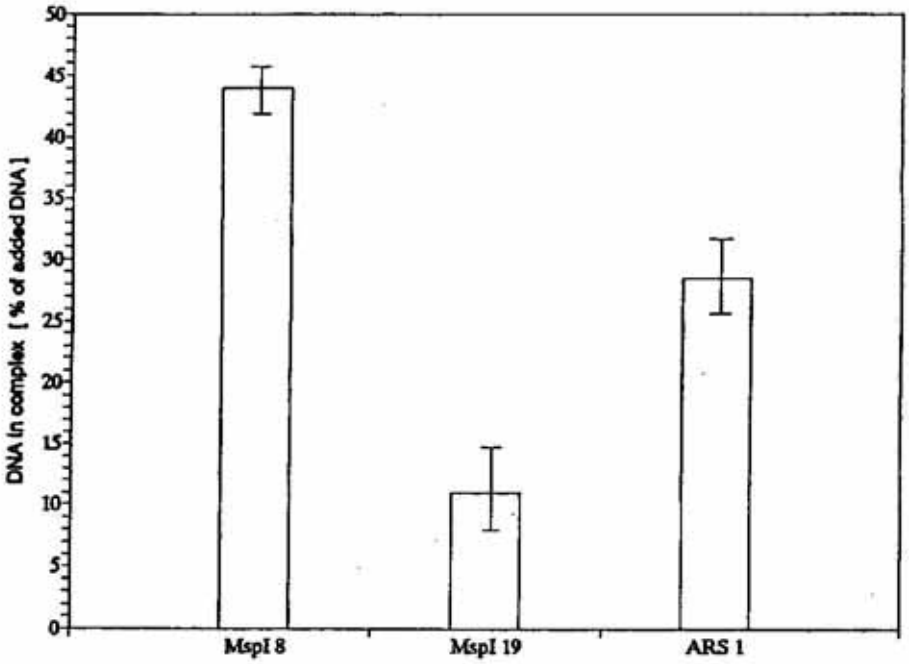

Fig. 3. In vitro DNA-matrix interaction of MspI8, MspI19 and ARS1 sequences with nuclear matrix of rat testis.
The bars mark the range in two independent experiments.

interacting with this sequence. To test for this possibility Western blot of rat testes nuclear matrix proteins was prepared and the reaction of radioactively labeled MspI8 sequence with proteins immobilized on nitrocellulose was performed as described in Materials and Methods. Figure 4 shows the result of this reaction. The most prominent band interacting with MspI8 sequence contained proteins of about 120 kDa (Fig. 4A). Under the same conditions the control DNA sequence of pUC19 did not complex to any of the nuclear matrix proteins (Fig. 4B, lane 1). In contrast, pUC19 complexed with histones and low molecular mass proteins when interaction of total nuclear proteins from testes was tested (Fig. 4B, lane 2). Similar nuclear low molecular mass proteins from rat liver cells interacted with MspI8 (Fig. 4C, lane 1). Liver nuclear matrix did not contain proteins efficiently interacting with MspI8 under the same conditions (Fig. 4C, lane 2). Thus the MspI8-120 kDa protein interaction seems to be tissue specific.

DISCUSSION

The mean size of DNA loops in somatic cells correlates with the size of the replicons and is nearly twice as high as the size of loops found in mammalian spermatozoa [1,12]. The amount of nuclear matrix attachment sites nearly doubles during spermato- or spermiogenesis. Creation of these new matrix attachment sites results probably from the appearance of proteins specifically and firmly interacting with some DNA sequences. It seemed reasonable to postulate that some of the repetitive sequences might play a role in this process. Some examples of repetitive sequences localized in the vicinity of genes and playing the role of nuclear matrix attachment sites are already known. An example may be the permanent attachment site to nuclear matrix of the kappa immunoglobulin gene domain occuring in evolutionarily conserved, repetitive sequence [14] or MAR sequence lying at the 3′ end of α-globin gene domain in avian erythroid cells [18]. Other examples of different classes of repetitive sequences enriched in nuclear matrix fraction were also reported [19, 20].

The repetitive MspI8 sequence isolated by us from rat total DNA on the basis of affinity to nuclear matrix seemed to bind specifically to single protein present in rat male reproductive tissue. The specificity of this binding was stated on the basis of the following observations: a) the binding to matrix proteins occurred in the

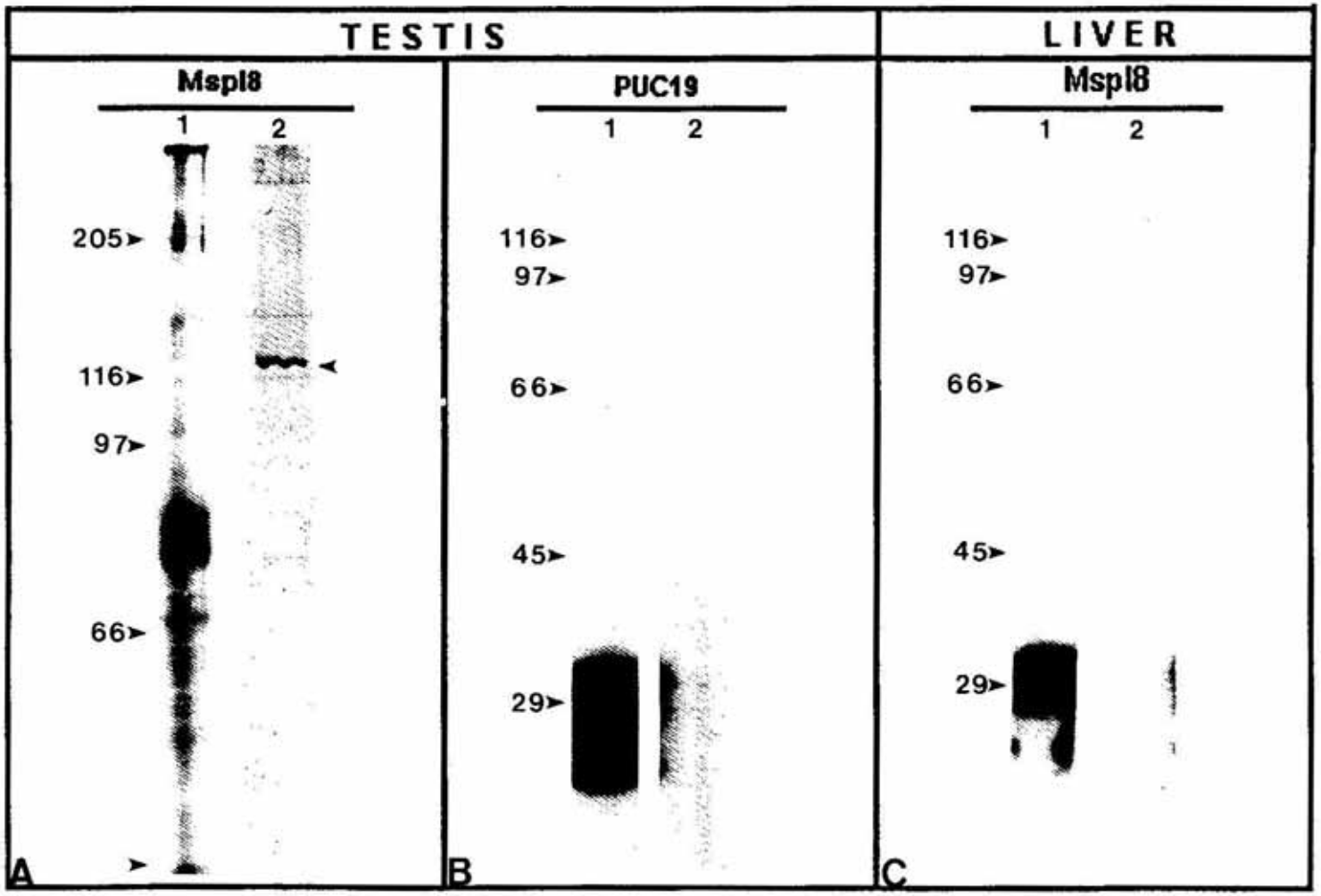

Fig. 4. Southwestern analysis of complexes formed by MspI8 and control PUC19 DNA sequences with nuclear matrix proteins of rat testis and liver.
A). Binding of MspI8 to rat testis nuclear matrix proteins. Lane 1, Coomassie Blue stained rat testis nuclear matrix proteins; lane 2, autoradiography of complex formed by the same proteins immobilized on nitrocellulose membrane with labeled MspI8 DNA sequence. B). Binding of control PUC19 DNA fragment to total nuclear and nuclear matrix proteins of rat testis. Lane 1, interaction with total nuclear proteins; lane 2, interaction with nuclear matrix proteins. C). Binding of labeled MspI8 sequence to nuclear proteins of rat liver cells: lane 1, binding to total nuclear proteins; lane 2, binding to nuclear matrix proteins.

presence of excess competitor poly(dG-dC) or *E. coli* DNA; b) only one band from many present on electrophoretogram of matrix proteins bound to MspI8 sequence; c) under the same conditions the end-labeled control DNA of PUC19 did not complex to any of the testis nuclear matrix proteins.

The molecular mass of the protein recognized by the MspI8 sequence was estimated to be 120 kDa that is however, only the mean value from one dimensional gel electrophoresis experiments done so far. The distribution of the molecular mass estimations was in the range of ± 10 kDa and we cannot exclude that the real value was 115 kDa or 130 kDa.

The nuclear matrix proteins specifically binding to MAR sequences and having the molecular mass of 120 kDa were detected in chicken oviduct (p120), human HeLa (SAF-A) and rat brain cells (SP120) [7, 21, 11]. The nuclear matrix p120 was shown to be identical with the hnRNP binding U protein. Some properties (i.e. aggregation with MAR sequences into looped structures) of p120/hnRNPU and the SAF-A protein were similar, suggesting for both of them a similar role. The third SP120 protein detected by Tsutsui *et al.* in rat brain cells [11] is probably the rat homolog of p120/hnRNPU protein because their amino-acid sequences are homologous [7]. At this moment it is too early to make any speculations on the relation between the rat testis protein detecting our MspI8 sequence and those three proteins. All of them are able to interact with MAR sequences originating from different places in the genome and even from different species. The MspI8 sequence differs from other typical MAR sequences. It contains only 41% of AT base pairs. On the other hand, it contains short ATTA, TAAAT, ATTTA motifs which often are the cores of the consensus sequences recognized by numerous, also nuclear matrix, protein factors [5].

It is also too early for speculations on the role of the MspI8 recognizing protein in creation of new matrix attachment sites appearing at some point of development of sperm cells. For our studies we used nonfractionated cells of whole rat testes. The germ cells which undergo several developmental changes in the process of spermato- and spermiogenesis are the main component of the testis. However, the male gonad is composed of different types of cells derived from four lineages: supporting cells, steroid producing cells, connective tissue cells and germ cells [22]. Thus, further studies elucidating the origin of the protein recognizing MspI8 sequence, its presence in germ cells at different stages of development and in cells of other tissues, as well as its interactions with other sequences will be essential.

We thank to Prof. S.V. Razin and Dr Z. Krawczyk for helpfull discussion and to K. Klyszcz for technical assistance.

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