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HMGA1 and HMGA2 protein expression in mouse spermatogenesis

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The high-mobility group A (HMGA) nonhistone chromosomal proteins HMGA1 and HMGA2 play a role in determining chromatin structure and in regulating the transcription of several genes. High levels of these proteins are characteristic of rapidly dividing cells in embryonic tissue and in tumors. The aim of this study was to determine the role of HMGA1 and HMGA2 throughout mouse spermatogenesis. Northern blot analysis and immunocytochemistry showed HMGA1 and HMGA2 expression during the progression from spermatocyte to spermatid. Interestingly, Western blot analysis with antibodies against the HMGA1 gene product revealed only the HMG1c isoform (27 kDa) in the testis; HMGA1a and HMGA1b were undetectable. These three isoforms are encoded by the HMGA1 gene through alternative splicing. Finally, few spermatids and complete absence of spermatozoa were observed in the testes of HMGA2-null mice, which suggests that the HMGA2 gene plays a critical role in male fertility.
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Introduction

Spermatogenesis, the multistep process by which spermatogonial stem cells give rise to mature spermatozoa, takes place within the seminiferous tubules of mammalian testes. Seminiferous tubules contain three main cell types: peritubular cells, which form the exterior wall of the seminiferous tubule, germ cells at various stages of development, and Sertoli cells, which relay external signals and provide factors required for the differentiation and proliferation of germ cells. Hormones and growth factors are involved in this unique and complex process (Sharpe, 1994; De Kretser *et al*., 1995). In addition, a number of cytokines, among which interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α), are paracrine regulators of testis gene expression (Kishimoto *et al*., 1992; Okuda and Morris, 1994; Gerard *et al*., 1991; Boockfor and Schwarz, 1991; Chuvpilo *et al*., 1993; John *et al*., 1995).

The mammalian high mobility group A (HMGA) family of chromosomal proteins includes HMGA1a and HMGA1b which are encoded by the same gene, HMGA1, through alternative splicing (Johnson *et al*., 1989), and the closely related HMGA2 (Manfioletti *et al*., 1991; reviewed by Fedele *et al*., 2001; Grosschedl *et al*., 1994). They are small, non-histone, chromatin-associated proteins that bind DNA in AT-rich regions through three basic domains called 'AT-hooks'. Members of the HMGA protein family have no intrinsic transcriptional activity, but they can regulate transcription by altering the architecture of chromatin so furthering the assembly of multiprotein complexes of transcriptional factors (Du and Maniatis, 1994; Leger *et al*., 1995; Falvo *et al*., 1995; Thanos and Maniatis, 1992). The HMGA2 gene is not expressed in any adult mouse (Manfioletti *et al*., 1991) or human tissues tested except for very low expression in CD34-positive hematopoietic stem cells (Rommel *et al*., 1997), and in mouse preadipocytic proliferating cells (Anand and Chada, 2000). HMGA1 gene expression is low in adult murine and human tissues, and somewhat higher in adult testis, skeletal muscle, and thymus (Chiappetta *et al*., 1996). Conversely, both genes are widely expressed during embryogenesis (Zhou *et al*., 1995; Chiappetta *et al*., 1996).

Disruption of the HMGA2 gene results in a pygmy phenotype with a drastic reduction in fat tissue and is associated with a longer cell cycle of embryonic fibroblasts (Zhou *et al*., 1995). Transgenic mice carrying an activated HMGA2 gene show a giant phenotype associated with pelvic/abdominal lipomatosis (Ashar *et al*., 1995; Battista *et al*., 1999; Arlotta *et al*., 2000). On the other hand, mice carrying one

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disrupted HMGA1 allele are smaller and have abundant fat pads in the abdominal/pelvic region (Fedele *et al*., manuscript in preparation). These results suggest that HMGA proteins play a critical role in embryonic development and fat tissue growth.

HMGA proteins are overexpressed in carcinomas of the thyroid (Chiappetta *et al*., 1995, 1998), pancreas (Abe *et al*., 2000), uterus (Bandiera *et al*., 1998), ovary (Masciullo *et al*., manuscript in preparation), prostate (Tamimi *et al*., 1993) and colon (Fedele *et al*., 1996; Abe *et al*., 1999; Chiappetta *et al*., 2001). Overexpression is not causally related to malignancy; indeed, suppression of HMGA synthesis prevents thyroid cell transformation by murine acute retroviruses (Berlingieri *et al*., 1995). Similarly, HMGA1 and HMGA2 gene rearrangements have been identified in several benign tumors. These rearrangements are generated subsequent to chromosomal translocations of regions 12q and 6p21 that harbor HMGA1 and HMGA2, respectively (reviewed by Hess, 1998).

We have analysed the expression of proteins HMGA1 and HMGA2 during the progression of spermatocytes to spermatids. Interestingly, using antibodies against the HMGA1 gene product revealed only the HMG1c isoform in the testis. In addition, we found that spermatogenesis is disrupted in HMGA2-null mice, which indicates that the gene plays an essential role in male germ cell development.

Results

Immunohistochemical analysis of HMGA1 and HMGA2 proteins in mouse testis

Immunocytochemical analysis was performed on serial testis sections using antibodies against the N-terminal region of the HMGA1 protein (Figure 1a) or against the recombinant HMGA2 protein (Figure 2a). Both proteins were found in the germinal epithelium in the nuclei of few spermatogonia and in nuclei of spermatocytes, spermatids and in Sertoli cells. The antiserum used in this study fulfils the criteria of specificity. In particular, immunoadsorption tests revealed that the labeling was totally blocked preincubating antibodies with 10^{-6} M of the cognate peptide (Figure 1b; Figure 2b). In addition, to demonstrate the specificity of immunoreactivity of HMGA2 antibodies we performed immunocytochemical analysis on testes sections of HMGA2 $-/-$ (Zhou *et al*., 1995), in which no immunostaining was detected (Figure 2c).

HMGA1 and HMGA2 expression in testicular cells

We evaluated HMGA expression in various testis cells using Northern blot hybridization with HMGA1 and HMGA2 specific probes. HMGA2 mRNA was expressed as a single band of about 4.5 kb in germ cells during the progression of spermatocytes to spermatids (Figure 3a). The transcript was more abundant in spermatids than in spermatocytes. It was expressed also in the interstitium, but very low in spermatogonia. HMGA1 expression was similar to that of HMGA2. In fact, a 1.8 (or 2.0 kb) band corresponding to the HMGA1 specific transcript was detected in spermatocytes, spermatids and interstitium, and a weak band was observed in spermatogonia (Figure 4a). However, HMGA1 expression was higher in spermatocytes than in spermatids. With Western blotting only one band of 27 kDa was identified (Figure 5), which probably corresponds to the recently described HMGA1c isoform (Nagpal *et al*., 1999) that derives from alternative splicing of the HMGA1 gene. Differently, HMGA1a and HMGA1b were detected in the malignantly transformed rat thyroid cells that served as a control.

HMGA1 and HMGA2 expression in testes of young mice

We next evaluated HMGA1 and HMGA2 expression during spermatogenesis using total RNA from testes of mice of different ages (6, 13, 34 days). At day six after birth the mouse testis contains mainly spermatogonia, within day 13 mainly spermatocytes, and a 34 day spermatocytes and spermatids. HMGA1 and HMGA2 specific transcripts were absent (HMGA2, Figure 3b) or very low (HMGA1, Figure 4b) in testes of 6-day-old

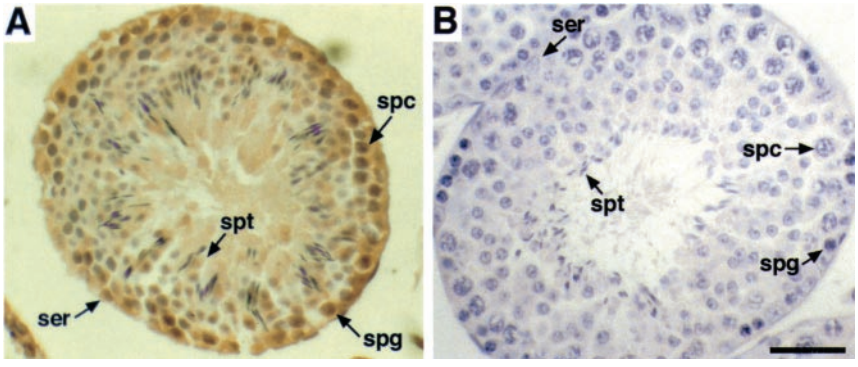


Figure 1 (**a**) Localization of the HMGA1 protein in sections of adult mouse testis by immunocytochemistry. A representative seminiferous tubule showing staining in the nuclei of spermatogonia (spg), spermatocytes (spc), spermatids (spt), and Sertoli cells (ser). (**b**) Control section using the antibody preadsorbed with the cognate peptide (10^{-6} M); symbols are as indicated above. Bar = 50 μm

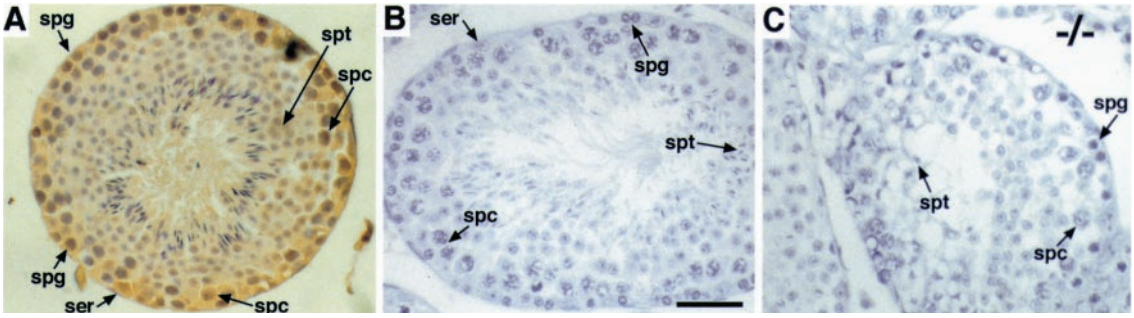
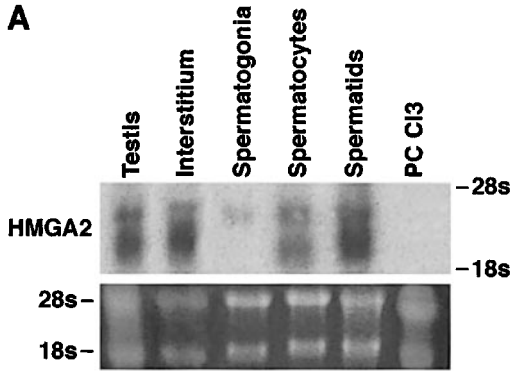


Figure 2 (**a**) Localization of HMGA2 protein in sections of adult mouse testis by immunocytochemistry. A representative seminiferous tubule showing staining in the nuclei of spermatogonia (spg), spermatocytes (spc), spermatids (spt), and Sertoli cells (ser). (**b**) Control section using the antibody preadsorbed with the cognate peptide (10^{-6} M); symbols are as indicted above. (**c**) Immunocytochemical analysis of HMGA2 protein in sections of adult mouse testis of HMGA2 −/−. A representative seminiferous tubule showing no immunostainings in any cellular type; symbols are as indicated above. Bar = 50 μm



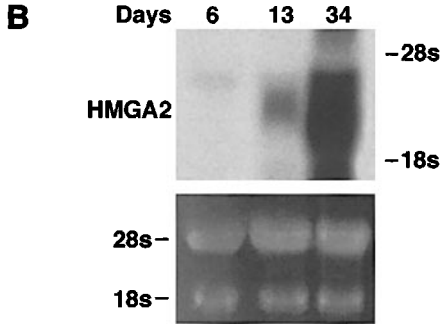
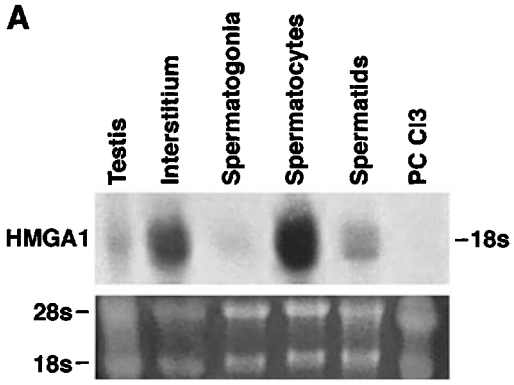


Figure 3 (**a**) Expression of HMGA2 mRNA in mouse testis. Northern blot analysis of HMGA2 mRNA in adult mouse testis (lane 1), interstitial tissue (lane 2), in normal freshly isolated testicular cell populations (lanes 3–5), in PC Cl3 as negative control (land 6), and (**b**) in testes of 6, 13, 34-day old mice (lanes 1–3). Each lane contained 20 μg of total RNA. All blots were probed with HMGA2-cDNA. The integrity and relative abundance of RNA samples were determined by ethidium bromide staining of the filter (lower frame)



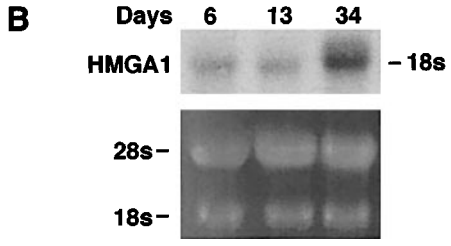


Figure 4 (**a**) HMGA1 mRNA expression in mouse testis. Northern blot analysis of HMGA1 mRNA in adult mouse testis (lane 1), interstitial tissue (lane 2), in normal freshly isolated testicular cell populations (lanes 3–5), in PC Cl3 as negative control (lane 6), and (**b**) in testes of 6-, 13- and 34-day-old mice (lanes 1–3). Each lane contained 20 μg of total RNA. All blots were probed with HMGA1-cDNA. The integrity and relative abundance of RNA samples were determined by ethidium bromide staining of the filter (lower frame)

mice, present in those of 13-day-old mice, and very abundant (particularly HMGA2) in testes from 34-day-old mice when germinal cells are rich in spermatocytes and spermatids. This is consistent with the observation that HMGA gene expression occurs in the late stages of spermatogenesis.

Disruption of the HMGA2 gene results in a block of spermatogenesis

To assess the role of the HMGA2 gene in the process of spermatogenesis we analysed the structure of the testis of HMGA2 −/− mice. These mice, which were generated by Zhou *et al*. (1995), are characterized by a pygmy phenotype and a drastic reduction in fat tissue;

nothing is known about the fertility of these mice. Histological examination showed that germ cell maturation was blocked in the testes of homozygous mice: degenerating spermatocytes and few spermatids were identified, and there were no spermatozoa (Figure 6b). Moreover, spermatogonia had large clear cytoplasms, which is a sign of cellular damage. These mice were not fertile at the homozygous state.

In addition, we have analysed the testes of the prepubescent HMGA2 −/− mice at different ages (8, 14, 30 days) in which no alterations compared with the 'wild type' mice were observed (data not shown).

Discussion

Spermatogenesis in mammals is characterized by a well-defined sequence of mitotic and meiotic divisions that leads to the production of mature spermatozoa. However, testicular development and normal spermatogenesis require specialized transcriptional mechanisms that ensure stringent stage-specific gene expression (McCarrey, 1993).

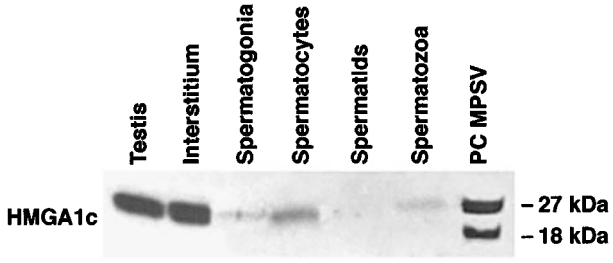


Figure 5 Distribution of HMGA1 protein in mouse testis. Western blot analysis of HMGA1 protein in mouse adult testis (lane 1), interstitium (lane 2), in normal mouse testis germ cells (lanes 3–6), and in PC MPSV cells as positive control (lane 7) (40 μg/lane). Whole lysates were detected by Western blotting with anti- HMGA1 polyclonal serum. The specific band of about 27 kDa was identified by comparison with comigrating size markers (Bio-Rad, Melville, NY, USA)

Because HMGA1 and HMGA2 interact and modulate the activity of several transcription factors, these non-histonic chromatin-associated proteins are primary factors in the formation of multimeric transcriptional complexes during the maturation of germ cells. For example, HMGA1 interacts through its AT-hook-binding domain with PATZ (Pierantoni *et al*., unpublished observation), a novel POZ-AT hook-zinc finger protein containing transcriptional repressor (Fedele *et al*., 2000), and with the RING-finger protein RNF4 (Chiariotti *et al*., 1998), both of which are highly expressed in the testis (Pero *et al*., 2001).

Here we show that both HMGA1 and HMGA2 are expressed at relatively high levels in mouse testes. In wild type mice HMGA1 expression was highest in spermatocytes, whereas HMGA2 expression was highest in spermatids. In this context, it is noteworthy that a stage-specific gene has been described for pachytene-specific expression (reviewed by Eddy, 1998; Wisniewski *et al*., 1993; vanWert *et al*., 1998), and for spermatid-specific expression (Albanesi *et al*., 1996; Tamura *et al*., 1992). In addition, since the DNA of late spermatids is highly condensed to a transcriptionally inactive state (Bellve, 1979), these results suggest that HMGA proteins are associated with functional nucleosomal chromatin. The enormous increase of testis HMGA2 isoform is associated with meiosis and not proliferation. Possible functions for high HMGA2 levels may include role in testis-specific events such as genetic recombination, meiotic divisions, or replacement of histones by late spermatidal basic proteins during nuclear condensation. Conversely, high levels of HMGA2 in germ cells may only reflect an increased need of germ cells for a function common to all cells, such as developmentally controlled gene expression, since pachytene spermatocytes initiate synthesis of many germ-cell-specific proteins, including enzymes and surface antigens (Bellve, 1979).

To establish whether HMGA gene expression is crucial for testis maturation, we also analysed the testes of HMGA2-null mice, which do not produce mature

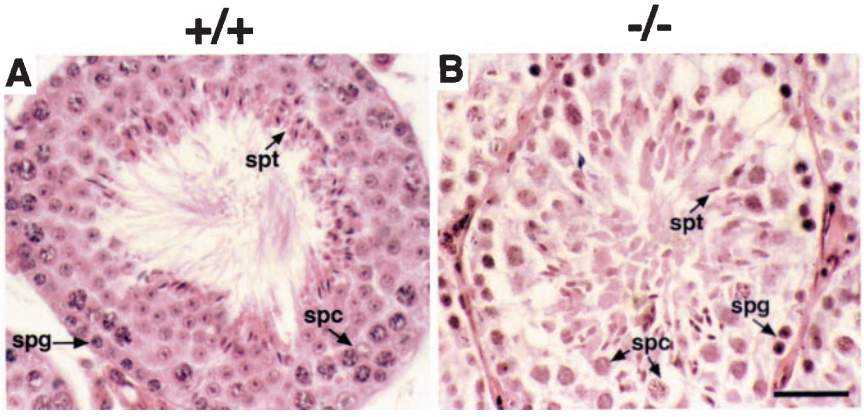


Figure 6 Hematoxylin and eosin staining for testicular morphology. (**a**) Testis morphology was normal in all four 1-year-old wild type animals. (**b**) Major disruptions to spermatogenesis were evident in the testes of 1-year-old HMGA2 −/−, the site of disruption appearing to be early meiosis during which degenerating spermatogonia (spg), spermatocytes (spc) and spermatids (spt) were observed (bar = 25 μm)

sperm and in which most spermatogonia and spermatocytes have an abnormal morphology. In sterile mice, few spermatogonia exited from the mitotic cycle. Moreover, residual postmitotic spermatocytes did not undergo correctly the meiotic divisions required to generate spermatids and, consequently, haploid gametes. Therefore, HMGA2 seems to be involved in the network that regulates the development of the germinal epithelium. It is noteworthy to observe that in contrast with the pigmy HMGA2 null mice the dwarf mice, carrying Pit-1 mutation, show a normal testis cytoarchitecture (Chubb and Nolan, 1985). Because the HMGA1 homozygous null phenotype is lethal, we were not able to analyse the testes of these mice. However, we have identified testis modifications in chimeric mice generated from embryonic stem cells double knocked out for the HMGA1 gene (Fedele *et al*., unpublished observation), which suggests that also the HMGA1 gene plays a critical role in spermatogenesis. It is interesting to note that Western blot analysis with antibodies against the HMGA1 gene product revealed only the HMGA1c in the testis; this isoform is encoded by the HMGA1 gene through alternative splicing that, however, appears to be the only isoform present in the normal mouse testis, suggesting a specific role of this isoform in the spermatogenesis.

We hypothesize that HMGA proteins interact with such other nuclear proteins as PATZ and RNF4 to regulate the expression of testis-specific proteins that are required for spermatogenic differentiation. The analysis of the genes expressed in normal and HMGA2-null mice testes might lead to the identification of other factors involved in the process of fertilization.

In conclusion, here we demonstrate that HMGA1 and HMGA2 are expressed in a stage-specific manner in the mouse germinal epithelium, and that impairment of HMGA2 gene function results in disruption of the testis cytoarchitecture and block of spermatogenesis.

Materials and methods

Preparation of testicular cells

Germ cells were prepared from testes of adult CD1 mice (Charles River Italia). Testes were freed from the albuginea membrane, and digested for 15 min in 0.25% (w/v) collagenase (type IX, Sigma) at room temperature under constant shaking. They were then washed twice in minimum essential medium (Life Technologies, Inc.), seminiferous tubules were cut into pieces with a sterile blade and further digested in minimum essential medium containing 1 mg/ml trypsin for 30 min at 30°C. Digestion was stopped by adding 10% fetal calf serum and the germ cells released were collected after sedimentation (10 min at room temperature) of tissue debris. Germ cells were centrifuged for 13 min at 1500 r.p.m. at 4°C and the pellet resuspended in 20 ml of elutriation medium (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM MgS_4 ($7H_2O$), 1.3 mM $CaCl_2$, 11 mM glucose, 1 X essential amino acid (Life Technologies, Inc.), penicillin, streptomycin, 0.5% bovine serum albumin). Pachytene spermatocyte and spermatid germ cells were obtained by elutriation of the unfractionated single cell suspension as described elsewhere (Meistrich, 1977). Homogeneity of cell populations ranged between 80 and 85% (pachytene spermatocytes) and 95% (spermatids), was routinely monitored morphologically. Mature spermatozoa were obtained from the cauda of the epidydmus of mature mice as described previously (Sette *et al*., 1997). Spermatogonia were obtained from prepuberal mice as previously described (Rossi *et al*., 1993; Grimaldi *et al*., 1993). PC MPSV cells are rat thyroid cells (PC Cl 3) infected with the myeloproliferative sarcoma virus (Berlingieri *et al*., 1995).

Histologic analysis and immunohistochemistry

For light microscopy, tissues were fixed by immersion in 10% formalin and embedded in paraffin by standard procedures. Five micrometer sections were stained with hematoxylin and eosin or processed for immunohistochemistry. For each paraffin-embedded sample a 4-μm serial section mounted on slides pretreated for immunohistochemistry were dewaxed in xylene and brought through ethanols to deionized distilled water. Before staining for immunohistochemistry, sections were incubated in a 750 W microwave oven for 15 min in 10 mM, 6.0 pH buffered citrate to complete antigen unmasking. The classical Avidin-Biotin peroxidase Complex (ABC) procedure was used for immunohistochemistry. In the ABC system, endogenous peroxidase was quenched by incubation of the sections in 0.1% sodium azide with 0.3% hydrogen peroxide for 30 min at room temperature. Non-specific binding was blocked by incubation with non-immune serum (1% TRIS-bovine albumin for 15 min at room temperature). Sections were incubated overnight with antibodies against HMGA1 and HMGA2 at a dilution 1 : 200. Negative controls were run with normal mouse serum instead of the primary antibody or the antibody was preadsorbed with the cognate peptide (10^{-6} M). Peroxidase activity was developed with the use of a filtered solution of 5 mg of 3-3′-diaminobenzideine tetrahydrochloride (dissolved in 10 ml of 0.05 M tris buffer, pH 7.6) and 0.03% H_2O_2. We used Mayer's hematoxylin for nuclear counterstaining. Sections were mounted with a synthetic medium. The antibodies versus the HMGA1 and HMGA2 antibodies are described elsewhere (Chiappetta *et al*., 1996).

RNA extraction and Northern blot analysis

Total RNA was extracted from cells and tissue using the RNAzol kit (Tel-Test, Inc., Friendswood, TX, USA) according to standard procedures. Twenty micrograms of total RNA were fractionated on a 1.2% agarose/formaldehyde gel and blotted onto a nylon membrane (Hybond-N, Amersham, UK). Northern blot hybridization was performed as described previously (Sambrook *et al*., 1989). Hybridization was carried out overnight at 42°C with 2×10^6 c.p.m./ml of hybridization solution. The HMGA1 and HMGA2 cDNA (Chiappetta *et al*., 1996) were radiolabeled with a random prime synthesis kit (Amersham Pharmacia Biotech, Italy).

Protein extraction and Western blot analysis

Mouse testes were homogenized directly into lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg aprotinin, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate). The lysates were clarified by centrifugation at $14\,000 \times 10$ min. Protein concentrations

were estimated by a Bio-Rad assay (Bio-Rad, München, Germany), and boiled in Laemmli buffer (Tris-HCl pH 6.8 0.125 M, SDS 4%, glycerol 20%, 2-mercaptoethanol 10%, bromophenol blue 0.002%) for 5 min before electrophoresis. Proteins were subjected to SDS–PAGE (14% polyacrylamide) under reducing condition. After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon Millipore Corporation); complete transfer was assessed using prestained protein standards (Bio-Rad, Hercules, CA, USA). After blocking with TBS-BSA (25 mM Tris, pH 7.4, 200 mM NaCl, 5% bovine serum albumin), the membrane was incubated with the primary antibody against HMGA1 (1 : 400), for 1 h (at room temperature). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1 : 10000) for 45 min (at room temperature) and the reaction was detected with an ECL system (Amersham Life Science, UK).

Note added in proof

The nomenclature of the high mobility group (HMG) proteins has been recently revised; see Chromosomal Proteins Nomenclature Home Page: http://www.informatics.jax.org/mgihome/nomen/genefamilies/hmgfamily.shtml. In this study we use the new nomenclature.

Acknowledgments

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