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Induction of Early Transcription in One-Cell Mouse Embryos by Microinjection of the Nonhistone Chromosomal Protein HMG-I

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In the mouse embryo, the onset of zygotic transcription occurs at the end of the first cell cycle, upon completion of DNA replication. We show that the nonhistone chromosomal protein HMG-I, whose translocation into the pronuclei of one-cell embryos is linked to this first round of DNA synthesis, plays a critical role in the activation of zygotic transcription. Indeed, microinjection of purified HMG-I results in a higher nuclear accumulation of the protein and triggers an earlier activation of zygotic transcription, an effect which is abolished by the preincubation of the protein with a specific antibody directed against its AT-hook DNA-binding motifs. Significantly, microinjection of this antibody also prevents the normal onset of transcription in the embryo, suggesting that endogenous HMG-I is similarly involved in this process. Finally, microinjection of the exogenous protein modifies chromatin structure as measured by *in situ* accessibility to DNase I. We propose that general chromosomal architectural factors such as HMG-I can modulate the accessibility of chromatin to specialized regulatory factors, thereby promoting a transcriptionally competent state. © 2000 Academic Press

Key Words: **colocalization software image analysis; early embryonic transcription; HMG-I; nuclear compartments; AT hook; scaffold-associated regions.**

INTRODUCTION

HMG-I/Y belongs to a subclass of the high-mobility-group (HMG) proteins, a family of abundant low-molecular-weight mammalian chromosomal proteins (Bustin and Reeves, 1996). Three members of this subclass are known: HMG-I; HMG-Y, which differs by a deletion of 11 amino acids generated by alternative splicing of the HMG-I transcript; and HMG-IC, which is encoded by a distinct gene. HMG-I/Y and HMG-IC bind with high affinity to the minor groove of AT-rich DNA, an interaction mediated by a specific DNA-binding domain, the "AT-hook," which is distinct from and unrelated to the "HMG box" present in other HMG proteins (Reeves and Nissen, 1990). HMG-I, which contains three tandem repetitions of the AT-hook motif, was first isolated as a satellite-binding protein (Strauss and Varshavsky, 1984) and was later shown to bind without specificity to any stretch of five to six dA · dT base pairs (Solomon *et al.*, 1986). It has a particularly high affinity *in vitro* for the AT-rich sequences found in SARs/MARs (scaffold or matrix-associated regions), from which it is able to displace histone H1 (Zhao *et al.*, 1993), and could thus play a role in the modulation of chromatin structure and accessibility (Käs *et al.*, 1993). Indeed, MATH-20, an artificial multi-AT-hook protein derived from HMG-I, has been shown to suppress the variegation of a *white* gene

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located close to AT-rich centromeric heterochromatin in *Drosophila* (Girard *et al.,* 1998). This activity is consistent with the hypothesis that proteins related to HMG-I can effect changes in chromatin structure and, in this case, affect the functional properties of heterochromatin.

HMG-I is involved in the transcriptional regulation of specific genes, such as the α-chain of the interleukin-2 (IL-2) receptor (John *et al.,* 1995) and the IL-4 (Klein-Hessling *et al.,* 1996), human interferon β (Thanos and Maniatis, 1992; Yie *et al.,* 1997), and germ-line ϵ heavy-chain immunoglobulin (Kim *et al.,* 1995) genes; it is also required for the activity of the herpes simplex virus latency-active promoter 2 (French *et al.,* 1996). In all these cases, HMG-I could exert its biological function(s) through both binding-induced structural modifications of DNA and direct protein/protein interactions. More generally, HMG-I seems to play the role of an "architectural transcriptional factor" as it seems to bind with high affinity to noncanonical and/or distorted DNA structures and can influence DNA bending (Nissen and Reeves, 1995; Reeves and Wolffe, 1996); these properties might be utilized to facilitate the assembly of multiprotein complexes ("enhanceosomes") onto specific DNA sequences (Falvo *et al.,* 1995; Thanos and Maniatis, 1995).

Several studies have shown that HMG-I is specifically expressed at high levels in rapidly proliferating cells that are either normal or malignant (Johnson *et al.,* 1990; Chiappetta *et al.,* 1996; Vartiainen *et al.,* 1988; Giancotti *et al.,* 1989). However, the correlation between high levels of expression and cellular proliferation with any well-defined biological function of the protein, and the extent to which any such function depends on the DNA-binding properties of HMG-I, remains unclear at present. One important clue to a possible role of this family of proteins comes from studies of *pygmy* mice: the loss of function of the HMG-IC gene results in adult homozygous mutant mice markedly smaller than normal, a phenotype which appears related to a significant decrease in the rate of cellular proliferation (Zhou *et al.,* 1995), suggesting a general role for HMG-I in the control of gene expression.

While the gene-specific roles of HMG-I/Y have previously been studied in tissue culture cells or in *ex vivo* systems, early embryonic development, in which the transition from a transcriptionally inactive to an active state is part of a tightly regulated program, provides a model system in which a more general role might be assayed directly in individual cells. In very early preimplantation stages, one- to eight-cell mouse embryos are characterized, in addition to their totipotent character, by a programmed regulation of polymerase II-dependent transcriptional activity. After a period of inactivity, transcription resumes at the end of the first cell cycle in an unregulated manner (Latham *et al.,* 1992; Christians *et al.,* 1995; Bouniol *et al.,* 1995), the need for specific transcriptional enhancers appearing only after the first cleavage division (Martinez-Salas *et al.,* 1989; Wiekowski *et al.,* 1991). This pattern of transcriptional activation might reflect the ordered modification of chromatin structure by a limited number of effector proteins (Worrad *et al.,* 1995; Wiekowski *et al.,* 1997). The implication of HMG-I in general as well as gene-specific transcriptional regulation (see above) suggests that it might play a similar role in the activation of zygotic transcription in the embryo.

A previous study by Thompson *et al.* (1995) showed a punctate nuclear distribution of HMG-I throughout the first cell cycles of early mouse embryos, with HMG-I mRNA levels decreasing dramatically at the eight-cell stage. The localization of the protein and the variations in its expression levels suggest that it might play a role in the modifications of chromatin structure that most likely precede or accompany early events in embryogenesis. We recently showed that, in different human and mouse cell lines, HMG-I is distributed in three nuclear subpopulations that differ in their susceptibility to nuclease digestion and *in situ* competition by Hoechst 33342 (Amirand *et al.,* 1998). We proposed that these characteristics might be related to the association of HMG-I with distinct regions of chromatin, further suggesting a role for the protein in the modulation of chromatin structure and function.

We have now addressed this question in more detail by focusing on the possible involvement of HMG-I in the establishment of a transcriptionally competent chromatin conformation in one-cell mouse embryos. We find that the nuclear translocation of endogenous HMG-I is temporally regulated and depends on the initiation of the first round of zygotic DNA replication. Results described below show that it is possible to alter selectively the timing of the onset of zygotic transcription by modulating the nuclear concentration of HMG-I and/or its DNA binding capacity. Conversely, microinjection of histone H1, which competes with HMG-I for binding to AT-rich DNA (Käs *et al.,* 1993; Zhao *et al.,* 1993), has an inhibitory effect on zygotic transcription. These results are the first describing a general role for HMG-I in the activation of transcription *in vivo,* an activity that might be linked to the displacement of one or several factors interacting with AT-rich sequences, thereby promoting a transcriptionally competent state.

MATERIALS AND METHODS

Collection of Oocytes and Embryos

Ovarian oocytes at the germinal vesicle (GV) stage were collected from 4-week-old C57/CBA mice as already described (Debey *et al.,* 1993) and cultured at 37°C, under paraffin oil, in M2 medium supplemented with dibutyryl cyclic AMP (100 μg/ml) to prevent spontaneous meiotic maturation. Superovulation was induced in C57/CBA female mice by injection of pregnant mare serum gonadotropin (Intervet, France), followed 48 h later by injection of human chorionic gonadotropin (hCG; Intervet). Females were then mated with C57/CBA males. Mating was assessed by the presence of plugs 17 h after hCG injection (hphCG). One-cell embryos were then collected and cultured in Whitten's medium as previously described (Bouniol-Baly *et al.,* 1997). To take into account the asynchrony of fertilization, all experimental lots contained embryos collected from three to five mice. Two- to eight-cell embryos

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were obtained from one-cell embryos cultured *in vitro*. In experiments in which two-cell embryos had to be analyzed just after the first cleavage, one-cell embryos were checked every 30 min between 28 and 33 hphCG. Those undergoing the first cleavage division were selected and cultured in Whitten's medium for 15 to 60 min before being fixed and processed for immunolabeling.

HMG-I Protein, AT-Hook Peptide, and Antibodies

Recombinant human HMG-I protein was produced in *Escherichia coli* and purified by HPLC (Elton and Reeves, 1986). An 18-mer AT-hook peptide (Reeves and Nissen, 1990), ATX, was synthesized and purified by HPLC. Its sequence (YGRPKKPRGRPKKGRPKK, 2.1 kDa) corresponds to a consensus AT-hook sequence flanked by repetitions of the GRP core motif. An aliquot of the peptide was coupled to ovalbumin (OVA) via the N-terminal tyrosine to yield the hapten-carrier conjugate used to raise anti-ATX antibodies. Polyclonal antibodies against HMG-I and the AT-hook were obtained from rabbits immunized with purified HMG-I or ATX-OVA, respectively (Eurogentec). High-titer serum fractions were affinity purified against HMG-I protein bound to activated CNBr-Sepharose (Pharmacia). Eluted fractions were tested by Western blotting and those that reacted specifically with HMG-I in extracts prepared from whole HeLa cells were pooled and used in our experiments. The identity of cross-reacting material as HMG-I/Y was confirmed by electrophoresis on acid/urea gels alongside purified recombinant HMG-I, HMG-Y, HMG-14, HMG17, and HMG-1 proteins, followed by Western blotting. Western blots were performed according to standard procedures. For the depletion experiments shown in Fig. 1B, the serum was incubated with the samples in the absence or presence of purified HMG-I protein.

In Vitro HMG-I/DNA Binding Experiments

Gel-shift experiments were performed using purified HMG-I protein and a 657-bp DNA fragment corresponding to the SAR region located in the *Drosophila* histone gene repeat that binds HMG-I highly selectively (Zhao *et al.*, 1993). Twenty-five-microliter reactions containing about 20,000 cpm (2 ng) labeled DNA and 250 ng salmon sperm competitor DNA in binding buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5% glycerol) were incubated with 2.5–25 ng purified protein for 20 min at room temperature before loading on a 6% acrylamide gel in 0.25× TBE buffer. After electrophoresis at 200 V for 18 h at 4°C with buffer recirculation, the gel was dried and exposed. Preincubation of the protein (25 ng) with antibodies (10 ng of affinity-purified anti-HMG-I or 10 and 50 ng of anti-ATX) was performed for 1 h at room temperature in 10 μl binding buffer. The antibody/protein complexes were then mixed with labeled probe and competitor DNA in a 25-μl final volume of binding buffer and treated as above. Co-incubation experiments were performed by incubating DNA and protein (25 ng) in a 25-μl volume of binding buffer for 10 min at room temperature before addition of antibodies (10 ng of anti-HMG-I or 10 and 50 ng of anti-ATX) and continued incubation for an additional 20 min at room temperature. Here and below, the antibody amounts indicated correspond to IgG heavy and light chains as quantitated by SDS–PAGE analysis of the sera followed by silver staining.

Microinjection of One-Cell Embryos

Embryos were microinjected in the cytoplasm with 1 ± 0.5 pl of the solutions described below, yielding an approximate dilution of 1/2000. All microinjections were performed on a Nikon inverted microscope using Narishige micromanipulators and an Eppendorf microinjector. To detect transcription sites, 100 mM BrUTP in 2 mM Pipes, pH 7.4, 140 mM KCl were microinjected. To analyze the effect of HMG-I on the onset of transcription, HMG-I (70, 50, or 30 μM initial concentrations) or the AT-hook peptide (800 or 80 μM) were comicroinjected with BrUTP (100 mM) in the same buffer. In some experiments, HMG-I was incubated before microinjection for 1 h at room temperature with the anti-ATX antibody at a weight ratio of 5/1 and then mixed with BrUTP to yield concentrations of 70 μM HMG-I and 100 mM BrUTP, respectively. In two separate experiments, the anti-ATX antibody (1.6 μg/μl) was comicroinjected with BrUTP as above. As a control, the preimmune serum was comicroinjected with BrUTP under the same conditions. The histone-like HU protein (a generous gift from J. Rouvière-Yaniv, URA CNRS 1139, Paris) and histone H1 (Boehringer Mannheim) were similarly comicroinjected with BrUTP at initial concentrations of 88 and 70 μM, respectively. All microinjections were performed at 19–20 hphCG and all experiments included a control set of embryos microinjected with BrUTP alone at the same time. Incubation of embryos was then continued in Whitten's culture medium until fixation.

Drug Treatments

To block protein synthesis, embryos were collected and cultured in the presence of cycloheximide (10 μg/ml) or puromycin (20 μg/ml). To prevent DNA replication, embryos were cultured in the presence of aphidicolin (4 μg/ml) until fixation. To block RNA polymerase II (Pol II)-dependent transcription, embryos were collected and cultured in the presence of α-amanitin (10 μg/ml). In this case, α-amanitin (50 μg/ml) was also included in the injection buffer (intracellular concentration of 25 ± 12.5 ng/ml). These treatments did not give rise to any detectable lethality over the time course of the experiments.

Immunocytochemistry

Oocytes and embryos were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, permeabilized with 0.2% Triton X-100 (15 min), and then blocked for 1 h in PBS containing 2% bovine serum albumin (BSA). Incubation with the primary antibodies was allowed to proceed overnight at 4°C before two washes with PBS (5 min each) and incubation with the secondary antibody (1 h, room temperature). Chromatin was then stained for 20 min with either propidium iodide (50 μg/ml) followed by a ribonuclease A treatment (400 μg/ml, 30 min at 37°C) or 2 μg/ml Hoechst 33342. For fluorescence observation, oocytes and embryos were mounted on glass microscope slides in Citifluor (Citifluor Company). Transcription sites were detected with a monoclonal mouse anti-BrdU antibody which also recognizes BrU (IgG, Caltag Laboratories, 1:400 dilution), using a fluorescein (FITC)-conjugated donkey anti-mouse secondary antibody (IgG(H+L); Jackson ImmunoResearch Laboratories; 1:500 dilution). HMG-I was detected with the specific anti-HMG-I rabbit polyclonal serum purified as described above (1:100 dilution), using a lissamine–rhodamine- or FITC-conjugated donkey anti-rabbit secondary antibody (IgG(H+L); Jackson ImmunoResearch Laboratories; 1:100 dilution). Depletion of the anti-HMG-I antibody with

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the purified protein (Fig. 2B) was performed as described (Amirand *et al.*, 1998), except that centrifugation was for 1 h. For double immunostaining, oocytes and embryos were incubated with antibodies mixed together at the same final dilutions as above.

Micrococcal Nuclease Treatment

Micrococcal nuclease (MNase; Sigma Chemical; N 5386) was prepared freshly before use in PBS containing 1 mM $CaCl_2$. After fixation and permeabilization, embryos were rinsed in PBS for 10 min and treated for 30 min at 37°C with different concentrations of enzyme. DNA digestion was stopped by incubating embryos for 10 min at 4°C in PBS containing 20 mM EDTA.

Fluorescence Detection of DNase I Digestion Sites

Embryos microinjected with the HMG-I and HU proteins, with the ATX peptide, or with dilution buffer alone (2 mM Pipes, pH 7.4, 140 mM KCl) were fixed, permeabilized, and kept at 4°C overnight in PBS containing 2% BSA. After a 15-min incubation in digestion buffer (40 mM Tris–HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$ and 10 mM $CaCl_2$), embryos were treated for 5 min with 0.05 μg/ml deoxyribonuclease I (DNase I; Amersham Life Science). They were then washed once with water (5 min) and twice with PBS (10 min each). *In situ* 3′-end labeling reactions were performed for 1 h at 37°C in 25 μl of 1× terminal deoxynucleotide transferase (TdT) reaction buffer (0.2 M potassium cacodylate, 25 mM Tris–HCl, pH 6.6, 0.25 mg/ml of BSA) containing 2.5 mM $CoCl_2$, 100 μM BrdUTP (Sigma) and 12.5 units of TdT (Boehringer Mannheim). Reactions were stopped by incubating the embryos in 2× SSC for 15 min at room temperature, followed by two washes in PBS (20 min each). For immunodetection of BrdU incorporation sites, embryos were blocked for 1 h in PBS containing 2% BSA before incubation with the monoclonal mouse anti-BrdU antibody (1 h at room temperature, 1:100 dilution). Subsequent steps were performed as described above except that the FITC-conjugated donkey anti-mouse secondary antibody was used at a 1:100 dilution.

Image Capture and Analysis

Fluorescence microscopy was performed on a Zeiss inverted microscope (Axiovert 35), using a filter wheel equipped with standard filters for FITC, rhodamine, and Hoechst emissions. Images were captured through a Zeiss plan Neofluor ×100 (NA 1.3) oil-immersion objective by a cooled CCD camera (Photometrics Type KAF 1400, 12-bit dynamic range) coupled to the IPLAB Spectrum imaging software (Amirand *et al.*, 1998). A Zeiss ×20 objective (NA 0.5) was used for quantification of total pronuclear fluorescence intensities. Confocal microscopy was performed on an upright Nikon microscope equipped with the Bio-Rad Laser-Sharp MRC-1024 confocal laser scanning software, using a Nikon Fluor 100× (NA 1.3) oil-immersion objective and the 488- and 568-nm excitation wavelengths of the laser. The colocalization image analysis software (SPIMAC) developed in our laboratory (Amirand *et al.*, 1998) was used to analyze in more detail the spatial relationship between HMG-I, chromatin, and transcription sites. Briefly, from two images of the same cell taken at two different wavelengths, SPIMAC constructs a 2D histogram in which x and y coordinates represent the gray levels of the same pixel in the two images. Signal intensities are normalized by setting the highest x and y values in each image to 100. A color associated with each point of the 2D histogram represents the number of pixels with the same gray levels. Subpopulations appearing on the 2D histogram can then be selected and interactively localized on the respective original images. In addition, the software measures the number of pixels of a given population and their relative contribution to the fluorescence intensity.

RESULTS

In order to monitor the nuclear localization of HMG-I and to analyze its possible role in early embryonic development, we first raised rabbit polyclonal sera against the human recombinant HMG-I protein and selected affinity-purified fractions suitable for immunolocalization studies (Amirand *et al.*, 1998). Western blots of total HeLa nuclei show that these antibody fractions react highly specifically with human HMG-I (Fig. 1A, lanes 1–3). They also react specifically, but less tightly, with an unknown 120-kDa protein (Fig. 1A, compare lanes 1 and 2, which correspond to a fivefold difference in serum dilution). This polyclonal serum also cross-reacts with HMG-I in mouse extracts with a similar specificity (data not shown).

We verified the specificity of the serum for HMG-I by performing the depletion experiment shown in Fig. 1B. Western blots were performed against HeLa nuclear extracts and purified HMG-I protein as described above, except that incubation with the serum was performed in the absence or presence of increasing concentrations of purified HMG-I protein. Comparison of the controls in lanes 1 and 2 (no depletion) with lanes 3–6 (depletion with increasing amounts of purified protein) shows that reaction of the depleted serum with HMG-I present in HeLa nuclei is selectively abolished relative to p120. These results demonstrate that the serum raised against HMG-I is specific for that protein and that the limited cross-reactivity observed with p120 is most likely due to recognition of a distinct epitope absent in HMG-I.

We also raised an antibody against ATX, a synthetic peptide corresponding to the AT-hook DNA-binding domain of HMG-I (Reeves and Nissen, 1990). Western blots of HeLa nuclei using an affinity-purified fraction of the anti-ATX serum are shown in Fig. 1A, lanes 4–6. By this assay, anti-ATX has a selectivity identical to that of anti-HMG-I and, at lower dilutions, also cross-reacts with the 120-kDa band, which must therefore correspond to a novel human AT-hook-containing protein. Anti-ATX is indeed highly specific for the AT-hooks of the HMG-I protein. Gel-shift experiments can be used to demonstrate the selective binding of HMG-I to a DNA fragment containing a SAR (Fig. 1C, lanes 2–6). This binding was diminished by preincubation of anti-ATX with HMG-I prior to addition of the SAR DNA (lanes 7 and 8). By comparison to the ladders generated by incubation of the DNA with different amounts of HMG-I, one can estimate that binding was inhibited by about 50% at the highest antibody concentration tested (compare lanes 4 and 8). Higher amounts of anti-ATX could not be used as they gave rise to smearing of the DNA bands (see Fig. 1C, lane 10, in which DNA alone

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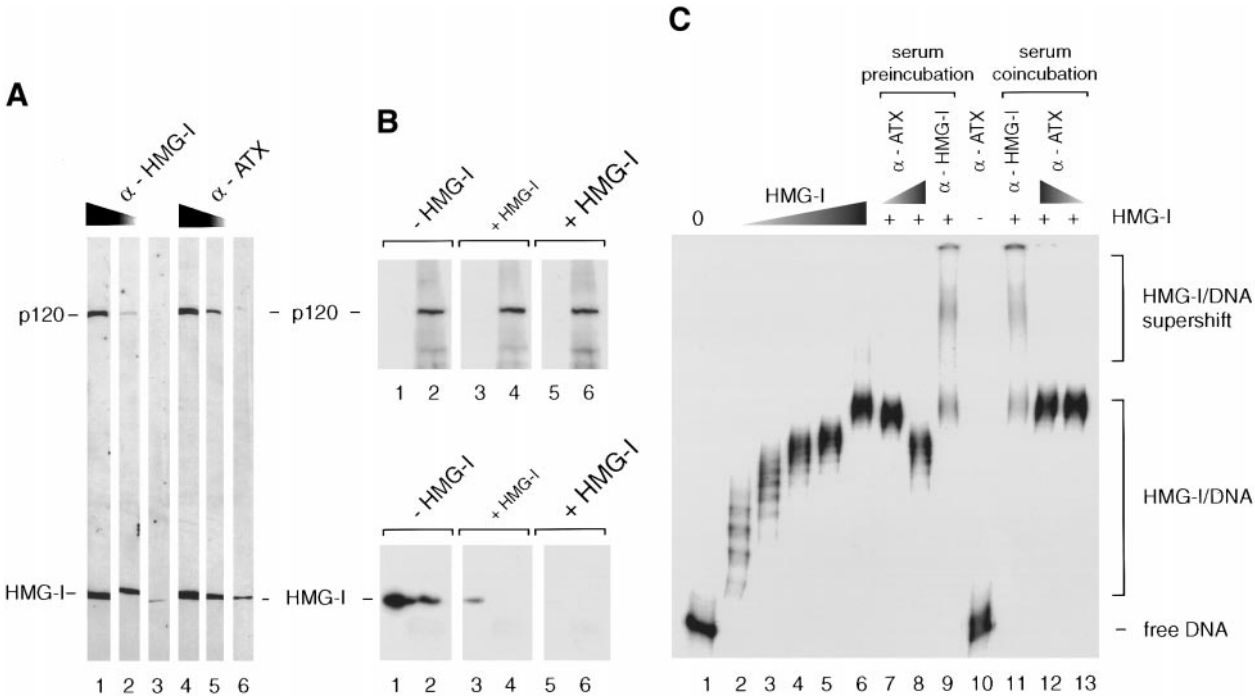

FIG. 1. Specificity of the anti HMG-I and anti-ATX sera. (A) Affinity-purified rabbit polyclonal sera raised against HMG-I and ATX (see Materials and Methods) were tested against total HeLa nuclei (0.5 A_{260} unit of whole nuclei per lane) electrophoresed on 7.5–15% SDS–acrylamide gels. Increasing dilutions of the purified anti HMG-I or anti-ATX sera were used in lanes 1–3 and 4–6 (10^{-2}, 2×10^{-3}, 4×10^{-4}, respectively). The HMG-I and p120 signals were not detected using the corresponding preimmune sera used at a 1:100 dilution (data not shown). (B) The affinity-purified anti-HMG-I serum (10^{-2} dilution) was tested as above against purified HMG-I protein (lanes 1, 3, and 5) and HeLa nuclear extracts (lanes 2, 4, and 6) in the presence of increasing concentrations of HMG-I protein (0, 0.5, and 2 μg/ml final concentration in lanes 1 and 2, 3 and 4, and 5 and 6, respectively), resulting in the specific loss of the HMG-I signal. Depletion was without effect on the p120 band. (C) For gel-shift experiments, a 657-bp labeled SAR DNA fragment was incubated with increasing amounts of HMG-I in the absence (lanes 2–6) or presence of affinity-purified antibodies raised against the ATX peptide (lanes 7–8 and 12 and 13) or against the full-length HMG-I protein (lanes 9 and 11). HMG-I concentrations were 0 (lanes 1 and 10), 2.5 (lane 2), 5 (lane 3), 10 (lane 4), 15 (lane 5), and 25 ng HMG-I (lanes 6–9 and 11–13). Antibodies were incubated with 25 ng of HMG-I before addition of DNA (lanes 7–9) or after preincubation of HMG-I with DNA (lanes 11–13). Antibodies were added in the following amounts: approximately 10 ng of anti-ATX or anti-HMG-I (lanes 7 and 9, respectively) or 50 ng of anti-ATX (lane 8), 10 ng of anti-HMG-I or anti-ATX (lanes 11 and 13, respectively), or 50 ng of purified anti-ATX (lane 12). Lane 10 contained DNA alone incubated with 100 ng of the purified anti-ATX serum.

was incubated with 100 ng anti-ATX before electrophoresis). In contrast, binding was not affected when HMG-I was incubated with the SAR-containing DNA before addition of the antibody (compare lanes 12 and 13 with lane 6). In addition, no supershift could be observed under these conditions, indicating that DNA-bound AT-hooks are no longer accessible to anti-ATX, whereas the anti-HMG-I serum could indeed induce a supershift of DNA/HMG-I complexes when added before (lane 9) or after (lane 11) incubation with DNA.

In summary, although they exhibit an identical selectivity in Western blots, our antibodies clearly recognize different epitopes present in HMG-I. The serum directed against HMG-I allows us to detect the protein whether bound to DNA or not. Conversely, the anti-ATX antibody provides us with the means to distinguish between free and DNA-bound HMG-I protein and, in addition, is capable of inhibiting DNA binding by HMG-I.

Dynamic Distribution of HMG-I in Pronuclei of One-Cell Embryos

While HMG-I has been shown to be present in the nuclei of mouse oocytes and early embryos (Thompson *et al.*, 1995; Amirand *et al.*, 1998), its intranuclear localization has not been examined in detail during the first embryonic cell cycle. As a role for HMG-I in early embryonic development might be reflected by changes in the distribution of the protein, we examined the localization of HMG-I in embryos fixed at various times after fertilization using the anti-HMG-I antibody described above. Up until 19 hphCG, no signal was detectable in the pronuclei of one-cell embryos

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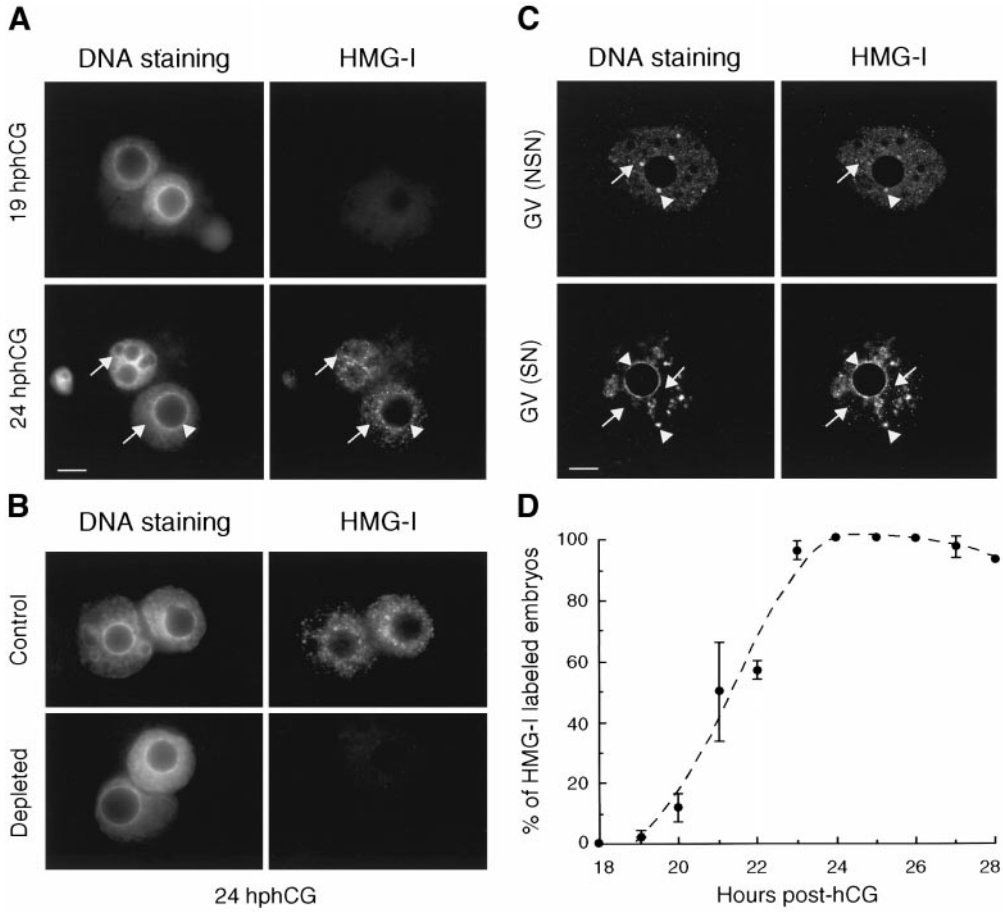

FIG. 2. Nuclear distribution of endogenous HMG-I and chromatin in one-cell mouse embryos and ovarian GV oocytes. Embryos and oocytes were processed for immunodetection of HMG-I. DNA staining is shown in the photographs on the left and HMG-I labeling on the right. (A) Conventional microscopy of one-cell embryos fixed at 19 or at 24 hphCG. DNA is labeled with Hoechst. HMG-I labeling is absent in the pronuclei of 19-hphCG embryos. At 24 hphCG, HMG-I accumulates in both pronuclei as discrete bright dots localized in regions of relatively low chromatin density (arrows), superimposed over a diffuse staining pattern of lower intensity. The perinucleolar condensed chromatin ring is not labeled (arrowhead). (B) A similar experiment performed with the HMG-I antibody depleted with purified HMG-I protein (2 μg/μl final concentration) before immunolabeling. These conditions correspond to those that result in the specific extinction of the HMG-I signal in Western blots of HeLa nuclei (Fig. 1B, lanes 4 and 6). Embryos were fixed and processed at 24 hphCG. (C) Confocal images of ovarian GV oocytes with NSN or SN chromatin configurations (see Debey *et al.*, 1993). DNA is labeled with propidium iodide. Both types of oocytes are heavily labeled for HMG-I. In addition to small dots dispersed in areas of low chromatin density (arrows), condensed chromatin is also labeled (arrowheads). Bar, 10 μm. (D) In the time course of HMG-I accumulation in the pronuclei of one-cell embryos, each point represents the mean value of the percentage of HMG-I-labeled embryos averaged from results of several experiments. Error bars represent the standard errors between values obtained in different experiments. Embryos are scored as positive for HMG-I nuclear labeling relative to the background observed at 19 hphCG (A, top right photograph). Each time point represents over 30 embryos from at least four different experiments.

(Fig. 2A, top row), even when the antibody concentration was increased (data not shown). By 24 hphCG, HMG-I immunolabeling was detectable in all embryos in the form of small discrete dots of 0.3 μm average diameter, dispersed throughout the nucleoplasm except for the area occupied by the nucleolus-like bodies (NLBs; see Fig. 2A, second row). These bright dots, which are clearly localized in regions of low chromatin density indicated by arrows, represent only 3 to 7% of the total nuclear fluorescence intensity and 2 to 5% of the stained pixels (quantitative data provided from the SPIMAC analysis of 14 embryos, see Materials and Methods). They are superimposed over a diffuse staining pattern of lower intensity which accounts for most of the immunofluorescence signal.

As we were concerned that at least part of the labeling could be due to the p120 protein which cross-reacts with

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the anti-HMG-I antibody, we incubated the serum with increasing concentrations of purified recombinant HMG-I protein. Depletion of the serum led to the complete extinction of the signal at the highest protein/serum ratio tested (Fig. 2B). This demonstrates that the immunostaining pattern we observe indeed represents HMG-I labeling. Finally, together with the results of the Western blots shown in Fig. 1B, these experiments establish that, despite a limited cross-reactivity with the anti-HMG-I serum, there is no contribution of p120 to the signal we observe in one-cell embryos.

The punctate distribution of HMG-I and the relative contribution of the dots to the overall staining pattern are quite similar to those previously observed in mouse and human cultured cell lines and in young mouse oocytes (Amirand *et al.*, 1998). The same labeling, indicated by arrows, is also seen in both populations of fully grown oocytes that can be distinguished at the GV stage before ovulation (Fig. 2C; see also Debey *et al.*, 1993). In contrast, the labeling of condensed chromatin found in oocytes could not be detected in one-cell embryos, particularly at the periphery of NLBs (see arrowheads in Figs. 2A and 2C). Finally, HMG-I could not be detected on metaphase chromosomes from the second meiotic metaphase to the fourth embryonic division. This is again different from the situation observed in somatic metaphases (Disney *et al.*, 1989, and our unpublished observations).

We then tested whether the distinct subpopulations of HMG-I we detect in one-cell embryos are indeed associated with DNA. Digestion of one-cell embryos fixed at 24 hphCG with 5 or 10 units/ml of MNase resulted in the loss of the HMG-I signal in most of the embryos (92%, $n = 13$, and 87%, $n = 15$, respectively). Under these conditions, chromatin could no longer be detected by Hoechst staining, showing that it had been extensively digested (data not shown). Therefore, the different populations of HMG-I we detect in the pronuclei of one-cell embryos are most likely DNA-bound.

From 19 hphCG on, the proportion of embryos positive for HMG-I labeling increases regularly with time, reaching 100% at 24 hphCG and remaining stable after that time to the end of the first cell cycle (Fig. 2D). The HMG-I localization pattern also persists throughout this interphase, except that the number of HMG-I dots increases between 19 and 24 hphCG. This time-dependent increase in the accumulation of HMG-I in the pronuclei of one-cell embryos might result from the translation of stored HMG-I messenger RNAs or from a regulated translocation of HMG-I protein already present in the cytoplasm. To distinguish between these or other possibilities, we treated 19-hphCG embryos with 10 μg/ml cycloheximide or 10 μg/ml puromycin for 5 h to inhibit protein synthesis. All treated embryos analyzed at 24 hphCG ($n = 12$ for each drug) displayed detectable pronuclear HMG-I and the labeling intensity was not decreased by the presence of these inhibitors (data not shown). Thus, the time-dependent accumulation of HMG-I in pronuclei does not require *de novo* protein synthesis. In addition, the distribution of the dots relative to the diffuse component was not changed by drug treatment, as assessed by quantitative SPIMAC analysis of 13 treated embryos. This result, together with the fact that HMG-I is present in nuclei of preovulatory oocytes, suggests that, although not detected by immunofluorescence, the protein is stored in the cytoplasm and that its translocation in pronuclei is somehow regulated.

Interestingly, the earliest detection of HMG-I in pronuclei coincides with the beginning of the first round of DNA replication at approximately 20–21 hphCG (Bouniol-Baly *et al.*, 1997). To determine whether the pronuclear accumulation of HMG-I might be linked to this event, we cultured embryos in the presence of 4 μg/ml aphidicolin. Remarkably, we could detect no HMG-I in the pronuclei of one-cell embryos in which DNA synthesis was inhibited, at 24 hphCG ($n = 33$) and as late as 27 hphCG ($n = 71$), when all pronuclei from control embryos were labeled (Fig. 3). This result strongly suggests that the localization of HMG-I in pronuclei requires DNA replication.

We then asked whether a similar nuclear localization and time-dependence could be observed in subsequent cell cycles. We selected embryos undergoing the first cleavage division and cultured them in Whitten's medium for between 15 and 60 min before fixing and processing them for immunolabeling. Their HMG-I labeling pattern was then analyzed as a function of the cleavage state, from anaphase to complete reassembly of nuclei, as assessed by the presence of visible nucleoli. In two different experiments, none of the embryos in anaphase of the first division ($n = 19$) were labeled, while 40% of those in telophase ($n = 15$) and 92% of those with reformed nuclei ($n = 66$) showed nuclear HMG-I dots (data not shown). This indicates that, at the two-cell stage and in contrast to the results shown for one-cell embryos, HMG-I localizes to nuclei as soon as they are formed and before the next S phase. Furthermore, in two- to eight-cell embryos, the HMG-I labeling pattern was similar to that seen at the one-cell stage, with discrete intense dots superimposed over a diffuse lower-intensity staining pattern. HMG-I labeling over condensed chromatin could not be seen in these cases (data not shown).

Microinjection of Exogenous HMG-I Advances the Onset of Transcription in One-Cell Embryos

The results presented above demonstrate that the nuclear localization of HMG-I varies dynamically through the first cell cycle, all embryos being positive for nuclear HMG-I at 24 hphCG. This is only a few hours before the activation of transcription that occurs at 27 hphCG in the mouse (Bouniol *et al.*, 1995) and we next asked whether the timed nuclear translocation of HMG-I might be linked to the onset of transcription. HMG-I was already suggested to play a role in establishing a more "open" chromatin structure with an increased accessibility to the transcriptional machinery (Laemmli *et al.*, 1992; Käs *et al.*, 1993; Zhao *et al.*, 1993). According to this model, the regulated entry of

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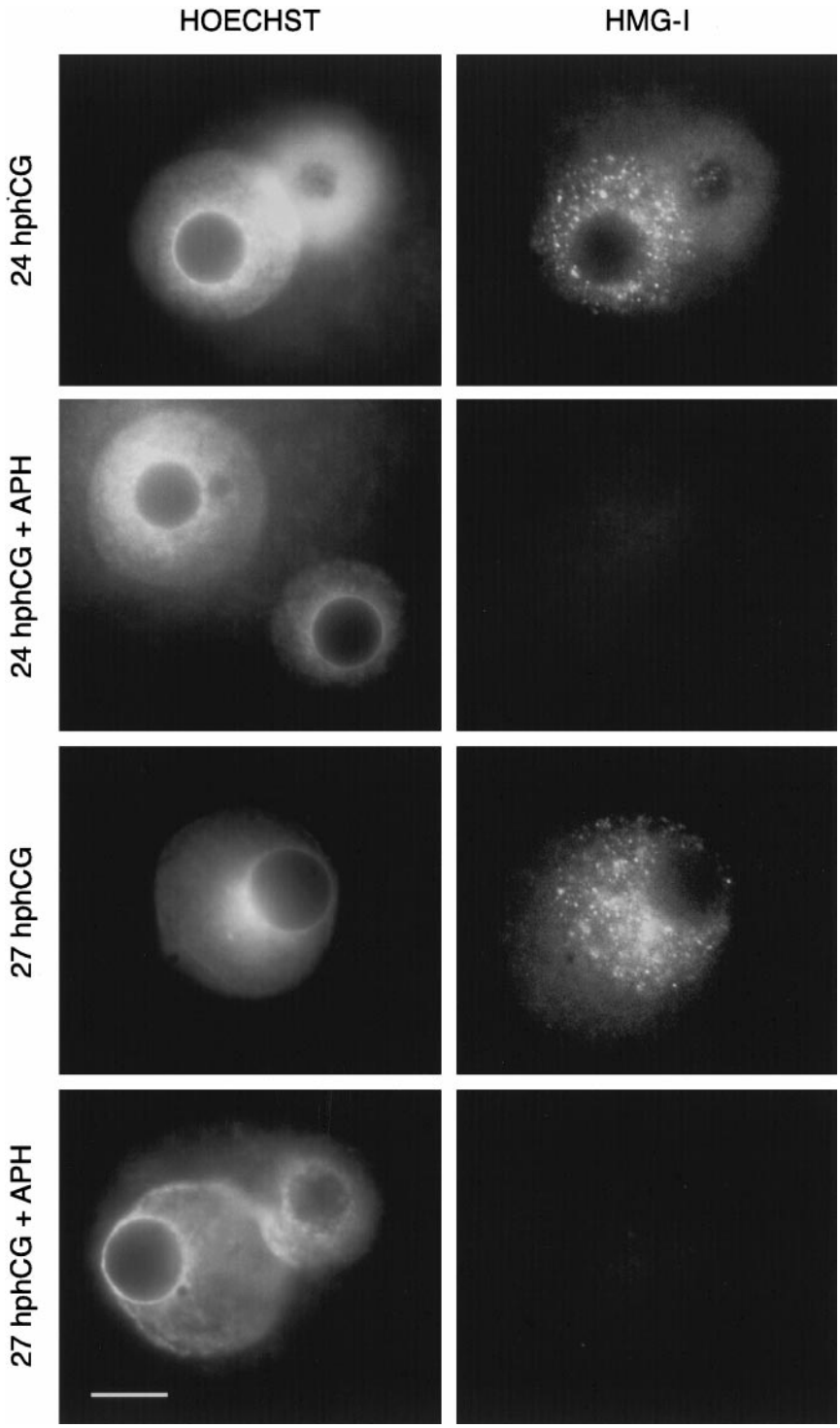

FIG. 3. Inhibition of DNA synthesis prevents accumulation of HMG-I in the pronuclei of one-cell embryos. Control embryos are shown in the first and third rows, one-cell embryos treated from the time of their collection on with 4 μg/ml aphidicolin (APH) in the second and fourth rows. Samples were fixed and stained at 24 or at 27 hphCG. Images were captured in the focal plane of the male pronucleus. Hoechst DNA staining is shown in the photographs on the left, HMG-I labeling on the right. Treated embryos do not show any HMG-I labeling at 24 hphCG or as late as 27 hphCG, in contrast to controls fixed and stained at the same time. Bar, 10 μm.

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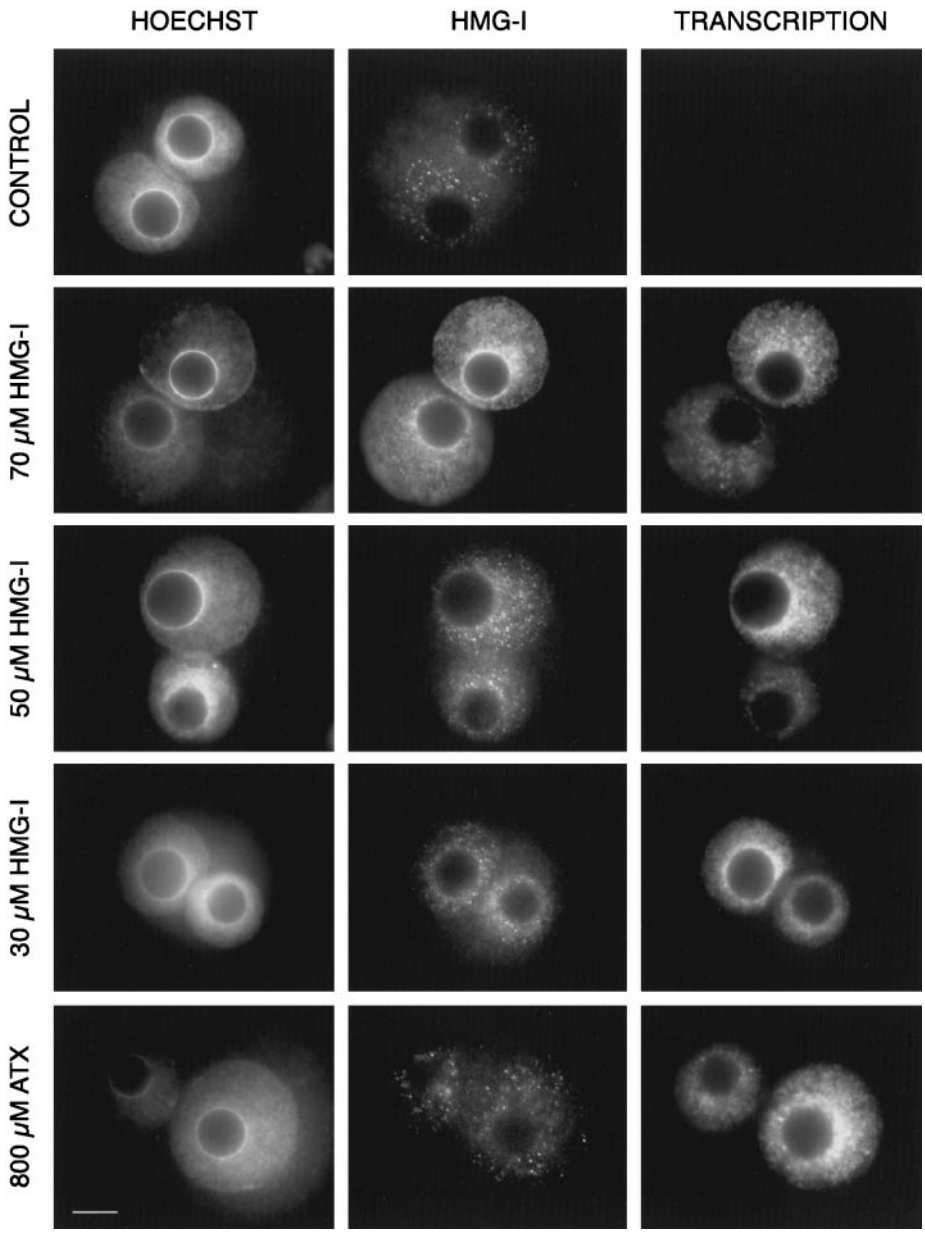

FIG. 4. Early onset of transcription induced by microinjection of HMG-I. One-cell embryos were microinjected with 30–70 μM HMG-I or with 800 μM ATX at 19–20 hphCG. Photographs on the left show DNA staining by Hoechst 33342; photographs in the center show the immunodetection of nuclear HMG-I; transcription sites detected by incorporation of BrUTP are shown on the right. Embryos were fixed at 25 hphCG in all cases. Control embryos microinjected with BrUTP alone are transcriptionally inactive at that time (top row), while some embryos that were comicroinjected with BrUTP and 70, 50, or 30 μM HMG-I are transcriptionally active, as summarized in Fig. 5A and Table 1. Microinjection of the ATX peptide (800 μM) also activates the onset of transcription. Bar, 10 μm.

HMG-I into pronuclei could lead to an increase in chromatin accessibility that precedes or signals the beginning of transcription.

We therefore hypothesized that an earlier accumulation of HMG-I in pronuclei might advance the onset of transcription. To test this possibility, we microinjected HMG-I (70 μM initial concentration) in embryos at 19–20 hphCG (before endogenous HMG-I accumulates in pronuclei) together with BrUTP. The embryos were then fixed at selected times after hCG injection, from 24 to 27 hphCG. Results of these experiments are shown in Fig. 4 and summarized in Fig. 5A and Table 1. In contrast to the

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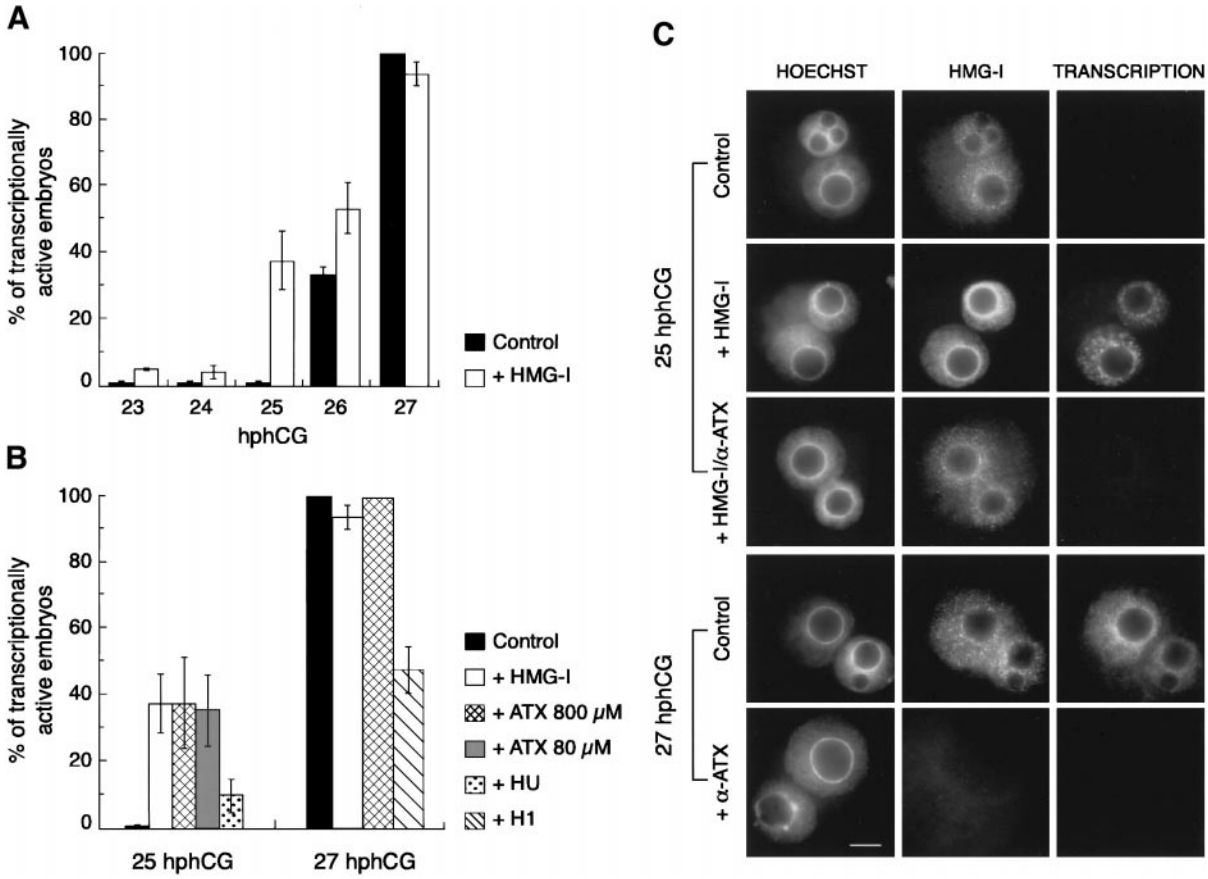

FIG. 5. Specificity of the activation of transcription by HMG-I. The bar graphs presented in A and B show results expressed as a percentage of embryos positive for BrUTP incorporation as a function of the microinjected protein relative to control embryos microinjected with BrUTP alone. Errors bars represent the standard errors between values obtained in different experiments. (A) Embryos were microinjected at 19–20 hphCG with BrUTP alone or with BrUTP plus HMG-I (70 μM initial concentration), fixed at the times shown (23 to 27 hphCG), and scored for transcriptional activity. Statistical analysis of these results was performed with the χ^2 test, the difference being significant at 25 hphCG ($P < 0.01$). (B) Embryos were microinjected at 19–20 hphCG with BrUTP plus HMG-I (70 μM), the ATX peptide (800 or 80 μM), HU (88 μM), or histone H1 (70 μM) and fixed at 25 or 27 hphCG. (C) One-cell embryos were microinjected at 19–20 hphCG with BrUTP plus 70 μM HMG-I, with BrUTP plus HMG-I preincubated with the anti-ATX antibody, or with BrUTP together with the anti-ATX antibody. Control embryos were microinjected with BrUTP alone. Immunodetection of both HMG-I and transcription sites was performed after fixation at 25 or 27 hphCG, respectively. The corresponding samples are identified by the legends next to the photographs. Bar, 10 μm.

control population microinjected with BrUTP alone (n = 133), which yielded no positive embryos at 25 hphCG and in which transcription was first observed at 26 hphCG, microinjection of HMG-I resulted in a significant fraction (40%, n = 134) of transcriptionally active embryos as early as 25 hphCG, with the proportion of active embryos increasing until 27 hphCG (Figs. 4 and 5A; Table 1). As expected, microinjection of HMG-I led to more intense pronuclear HMG-I immunolabeling than in controls microinjected with BrUTP alone. This is particularly evident in regions of condensed chromatin such as the periphery of the NLBs (compare the DNA and HMG-I images in the first two rows of Fig. 4; see also Fig. 2A, second row). We verified that the transcription detected in both microinjected and control embryos was indeed due to RNA Pol II activity since addition of α-amanitin resulted in the complete inhibition of BrUTP incorporation (data not shown). Finally, microinjection of HMG-I alone did not disturb normal development as 90% of microinjected and control embryos reached blastocyst stage *in vitro* (n = 30 for each).

Does this early activation of transcription require high concentrations of exogenous HMG-I? To answer this question, we chose to analyze embryos at 25 hphCG, when the activating effect driven by HMG-I described above is readily measurable as control embryos are never transcriptionally active at that time (see above). Decreasing the initial concentration of microinjected HMG-I did not change significantly the percentage of embryos transcriptionally ac-

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TABLE 1
Transcriptional Activation in One-Cell Mouse Embryos

	Hours post-hCG				
	23	24	25	26	27
BrUTP (controls)	0% (n = 20)	0% (n = 52)	0% (n = 133)	33% (n = 24)	95% (n = 79)
+HMG-I					
70 μM	5%* (n = 20)	4%* (n = 27)	40%# (n = 134)	48%* (n = 65)	94%* (n = 20)
50 μM			41%# (n = 34)		
30 μM			38%# (n = 21)		
+HU, 88 μM			5%§ (n = 18)		
+ATX					
800 μM			39%# (n = 66)		100%* (n = 20)
80 μM			40%# (n = 25)		
+HMG-I/α-ATX			4%§ (n = 51)		
+α-ATX					5%# (n = 20)
+Preimmune serum					100%* (n = 15)
+H1, 70 μM					28%# (n = 57)

Note. BrUTP immunofluorescence results from 23 to 27 hphCG are summarized for the different experimental conditions detailed in the text. Results are expressed as percentages of embryos positive for BrUTP incorporation at the times shown. Sample sizes (n) are indicated in parentheses. The # symbol denotes values that are statistically different from the corresponding controls assayed at the same time ($P <$ 0.005), while those that do not significantly differ are denoted by an asterisk (*). The § symbol indicates values that are statistically different from the samples microinjected with HMG-I and analyzed at 25 hphCG. Statistical analysis was performed using the χ^2 test.

tive at 25 hphCG (41%, n = 34, and 38%, n = 21, for initial HMG-I concentrations of 50 and 30 μM, respectively; see Table 1). In parallel, the HMG-I staining of condensed perinucleolar chromatin was abolished, although the HMG-I signal, while decreasing as a function of the concentration of microinjected protein, remained higher than in controls. This suggests that the strong accumulation of HMG-I in regions of condensed chromatin observed at higher concentrations of the exogenous protein bears little or no relation to the early activation of transcription. Microinjection of HMG-I at initial concentrations below 30 μM did not change the percentage of active embryos relative to controls.

AT-Hook-Mediated DNA Binding Is Necessary for the Early Activation of Transcription Driven by HMG-I

The striking effect of an early accumulation of HMG-I in pronuclei on the onset of transcription raises the question of its specificity. If this effect is unique to HMG-I, other DNA-binding proteins should have no effect on transcription. To verify this, we microinjected, under the same conditions, the bacterial HU protein which, in addition to being comparable in size and charge to HMG-I, also shares some of the general properties of HMG proteins (see Oberto *et al.*, 1994, for review). Only 1 (5%) of the 18 embryos microinjected with HU was positive for transcription at 25 hphCG (Fig. 5B and Table 1), whereas none of the untreated control embryos were positive. This small percentage of active embryos observed after microinjection of HU was statistically different from that obtained at the same time after microinjection of HMG-I (Table 1).

Another important question relating to the specificity of the effect of HMG-I in one-cell embryos is whether it requires DNA binding. If so, a similar advance in the onset of transcription might be induced by microinjecting a ligand that interacts with DNA in the same way as HMG-I. This is the case of the synthetic AT-hook (ATX) peptide, which recognizes *in vitro* the same AT-rich DNA sequences as HMG-I with an approximately 10-fold lower affinity (unpublished results). We microinjected the ATX peptide at 19–20 hphCG at two different initial concentrations, 800 and 80 μM, and transcription was again analyzed at 25 hphCG. As shown in Figs. 4 and 5B and summarized in Table 1, microinjection of the ATX peptide led, in both cases, to an advance in the activation of transcription in 39% (n = 66) and 40% (n = 25) of the embryos, respectively, an effect similar to that obtained after microinjection of full-length HMG-I. Thus, the DNA-binding properties of the AT-hook appear to be sufficient to advance the onset of transcription. As our anti-HMG-I antibody does not recognize the ATX peptide, it is possible to examine the localization of the endogenous nuclear HMG-I in these embryos. Interestingly, microinjection of the ATX peptide did not modify the dot-like pattern of the endogenous protein (Fig. 4). As the effect of the ATX peptide is most likely exerted at sites that normally interact with endogenous HMG-I, this result suggests that the population of HMG-I that corresponds to the intense dots does not play a major role in transcriptional activation, in agreement with

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our previous findings in nuclei of HeLa or 3T3 cells (Amirand *et al.*, 1998).

A second prediction is that the effect of HMG-I on transcription should not be seen if the microinjected protein cannot interact with DNA and that the effect of endogenous HMG-I on transcription should be similarly sensitive to the loss of DNA binding. We thus took advantage of our polyclonal antibody raised against the ATX peptide. This antibody recognizes HMG-I highly effectively in Western blots (see Fig. 1A, lanes 4–6), inhibits DNA binding by HMG-I, but fails to recognize the DNA-bound protein (Fig. 1C). Indeed, microinjection at 19–20 hphCG of the purified HMG-I preincubated with this antibody abolished the activation of transcription by the exogenous protein: only 2 (4%) of the 51 embryos microinjected with the protein/antibody complex were transcriptionally active at 25 hphCG. Significantly, HMG-I immunostaining returned to levels similar to those observed in control embryos (Fig. 5C, compare the HMG-I images of the first and third rows).

We then examined the sensitivity of the endogenous protein to this antibody. At 19–20 hphCG, when essentially no endogenous HMG-I is detected in the pronuclei and the protein is most likely in the cytoplasm (see Discussion), the anti-ATX antibody might titrate the AT-hooks of endogenous HMG-I as it does *in vitro*, resulting in a comparable inhibitory effect. Indeed, in two experiments in which the anti-ATX antibody was microinjected alone at 19–20 hphCG, 19 (95%) of the 20 embryos analyzed were not transcribing at 27 hphCG (Fig. 5C and Table 1), while all the control embryos microinjected with BrUTP added to preimmune serum were active by that time (Table 1). In addition, HMG-I immunolabeling was significantly decreased in these transcriptionally inactive embryos (see the HMG-I image in the bottom row of Fig. 5C). This shows that the interaction of the anti-ATX antibody with endogenous or exogenous HMG-I protein can suppress both the normal and the advanced onset of transcription, by inhibiting the DNA-binding capacity of HMG-I and/or by preventing its nuclear accumulation. Alternatively, anti-ATX could exert these effects by interfering with other AT-hook-containing proteins.

Microinjection of Somatic Histone H1 Inhibits the Normal Onset of Transcription in One-Cell Embryos

If DNA binding of HMG-I is important for transcriptional activation, proteins that can compete with HMG-I for binding to its genomic targets should have an effect similar to that of the anti-ATX antibody, i.e., opposite to that of HMG-I. Histone H1 has been shown to be able to compete *in vitro* with HMG-I for binding to AT-rich DNA (Zhao *et al.*, 1993). Indeed, after microinjection of somatic histone H1 (70 μM) together with BrUTP at 19–20 hphCG, only 28% ($n = 57$) of the microinjected embryos were transcriptionally active at 27 hphCG, in contrast to control embryos microinjected with BrUTP alone (Figs. 5B and Table 1). This result is consistent with the hypothesis that the activation of transcription by HMG-I could be effected by a redistribution of the embryonic linker histone resulting from specific interactions between HMG-I and AT-rich regions of the genome (Laemmli *et al.*, 1992; Käs *et al.*, 1993; Zhao *et al.*, 1993). If this were the case, one would expect such a displacement to be reflected by general changes in chromatin structure and organization.

Microinjection of HMG-I Increases Accessibility of Chromatin to DNase I

If HMG-I activates transcription by inducing changes in chromatin structure, for instance by promoting "chromatin opening" (Käs *et al.*, 1993), such an unfolding of the chromatin fiber might be accompanied by detectable changes in its accessibility to nucleases. To test this hypothesis, we compared the overall sensitivity of pronuclear chromatin to limited *in situ* digestion by DNase I in control and HMG-I-microinjected one-cell embryos. Cleavage sites were then mapped by immunofluorescence detection of modified nucleotides (BrdU) incorporated by TdT in embryos fixed at 22 or 25 hphCG. Confocal images of the signal detected in control and HMG-I-microinjected one-cell embryos are shown in Fig. 6A. In both cases, DNase I cleavage sites are distributed fairly uniformly throughout the nucleoplasm of the pronuclei. This distribution is similar to that previously observed in nuclei of highly proliferating cells such as undifferentiated PC12 or malignant glioma cells (Puck *et al.*, 1991; Park and De Boni, 1998). Significantly, microinjection of HMG-I (70 μM) at 19 hphCG markedly increased the average number of DNase I cleavage sites in the pronuclei of most embryos relative to controls (compare the two photographs in Fig. 6A). When the overall nuclear fluorescence intensity was quantified using a 20× objective (thus allowing collection of the fluorescence signal emitted by the whole pronuclei), it was found to be increased by an average of 40% in HMG-I-microinjected embryos fixed either at 22 or at 25 hphCG, a statistically significant difference (Fig. 6B; $n \geq 20$ for each, $P < 0.01$). However, it is important to note that microinjection of HMG-I at a lower concentration (30 μM) and of the ATX peptide (800 μM), although activating transcription, did not lead to a significant increase in the overall nuclear fluorescence intensity (data not shown and see Discussion). Microinjection of HU (88 μM) under the same conditions had no effect on the accessibility of chromatin to DNase I.

Relationship between the Nuclear Distribution of Endogenous HMG-I and Transcription Sites in One-Cell Embryos

Because of the apparent relationship between HMG-I (or AT-hook) nuclear content and transcriptional activation, we asked whether HMG-I-rich sites might indeed corre-

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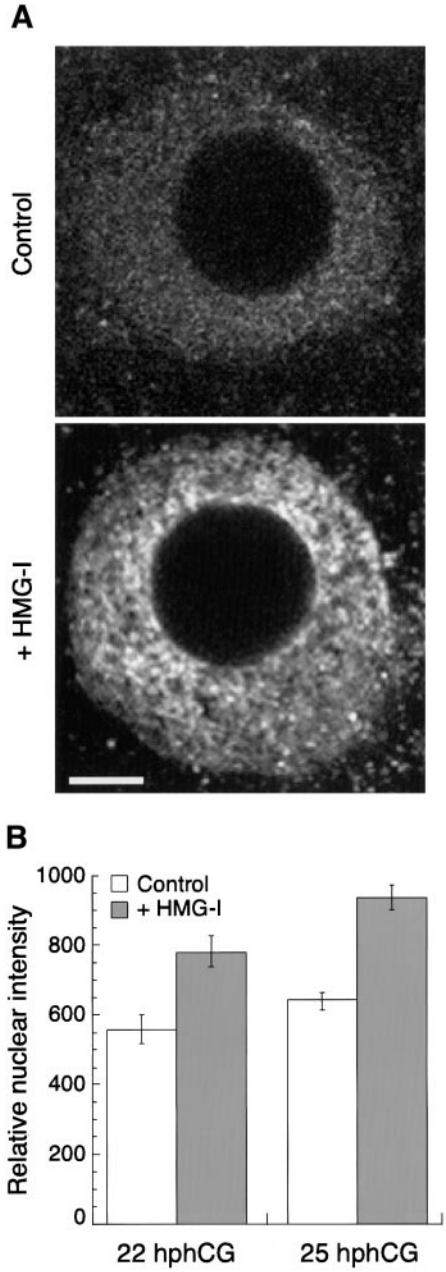

FIG. 6. Microinjection of HMG-I affects DNase I sensitivity of chromatin in one-cell embryos. *In situ* DNase I cleavage sites were detected in embryos microinjected at 19–20 hphCG with dilution buffer alone or with 70 μM HMG-I and fixed at 22 or 25 hphCG. (A) Confocal images of the male pronucleus are shown for control and HMG-I-microinjected embryos fixed at 25 hphCG. Bar, 5 μm. (B) The total nuclear fluorescence intensities detected in control and HMG-I-microinjected embryos were quantified using a 20× objective.

spond to transcription sites. Using the SPIMAC colocalization program (Amirand *et al.,* 1998), we compared endogenous HMG-I and BrUTP immunofluorescence images obtained by confocal microscopy of embryos fixed at 27 hphCG. The 2D histogram generated by SPIMAC (Fig. 7B) shows that the population of pixels with high HMG-I content (white frame) corresponding to the small intense dots, which represent 3.4% of the stained pixels, and sites of high BrUTP incorporation (red frame), which account for 9.3% of the transcription signal, do not colocalize (compare the SPIMAC images in Fig. 7A). In keeping with our observation that HMG-I dots are not displaced by microinjection of the ATX peptide (see Fig. 4), this result again suggests that they most likely do not play a role in transcriptional activation. A broad correlation between the intensity of HMG-I labeling and of the BrUTP signal is, however, obvious for the rest of the pixels, which correspond to the population of HMG-I revealed as a diffuse staining pattern of lower intensity (Fig. 7B), a result similar to that obtained in somatic cells (Amirand *et al.,* 1998).

DISCUSSION

All of the known functions of HMG-I, an architectural component of mammalian chromosomes, must be carried out through DNA binding mediated by the AT-hook domains of the protein. This sequence/structure recognition motif interacts with a very broad family of potential binding sites as well as with specialized DNA structures. These targets can be found in a variety of contexts: sequences located in scaffold/matrix-associated regions, in different satellite DNA repeats found in heterochromatin, in generally AT-rich boundary elements (Hart *et al.,* 1997; Cuvier *et al.,* 1998), or dispersed in the genome next to gene-regulatory regions, where they can be clustered or unique. Given this very broad specificity for DNA binding, it is far from clear how specific functions might be carried out by HMG-I or related multi-AT-hook proteins. In some cases, HMG-I might act predominantly in general gene-regulatory pathways, while gene-specific functions might involve, in addition to DNA binding, specific interactions with direct regulators.

While tissue culture or *ex vivo* systems have been widely used to analyze specific functions for HMG-I (see Introduction and references therein), the hypothetical role proposed several years ago for this protein as a general modulator of chromatin structure (Laemmli *et al.,* 1992) has not been directly studied. We chose to address this question using the one-cell mouse embryo as a model system in which the first burst of transcription does not require specific transcription factors or coactivators and thus most likely reflects a general genome-wide activation or derepression. In this system, we show that nuclear accumulation of exogenous HMG-I advances the onset of transcription and that inhibition of DNA binding by HMG-I leads to the loss of transcriptional activation. While the exact mechanism whereby HMG-I activates transcription remains to be determined, our results are consistent with the hypothesis that one of the roles of HMG-I is to effect the modifications

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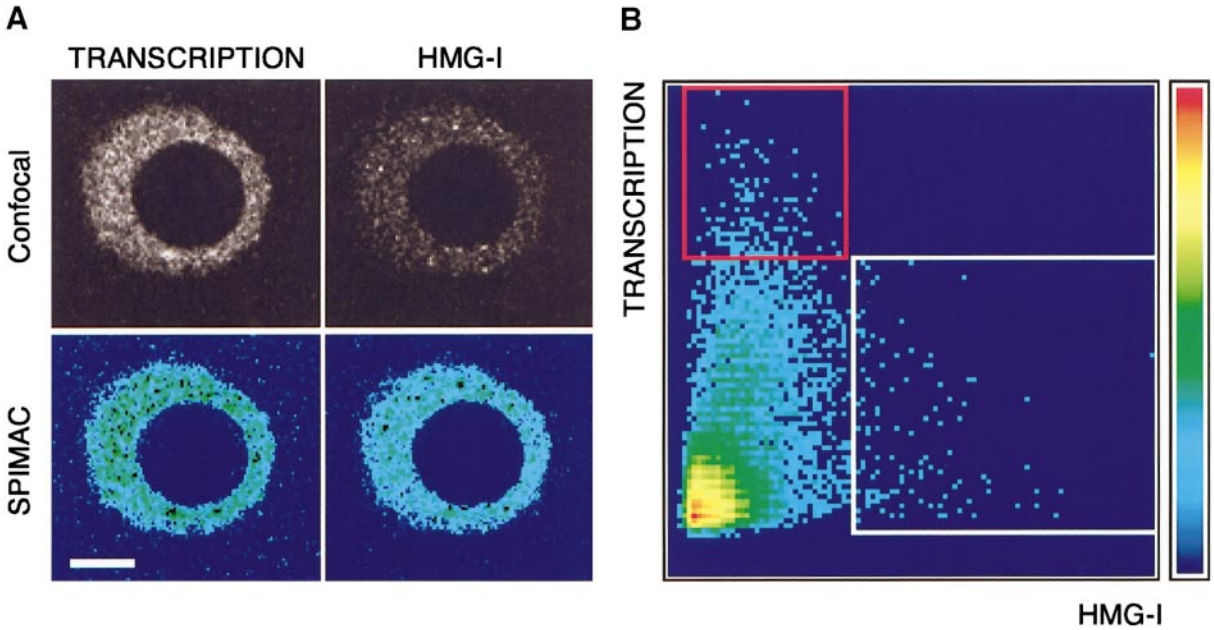

FIG. 7. Nuclear localization of HMG-I and transcription sites. The localization of endogenous HMG-I and of transcription sites was compared by SPIMAC analysis in one-cell embryos fixed at 27 hphCG after BrUTP microinjection. Confocal images of BrUTP (transcription) and HMG-I immunodetection in the female pronucleus were processed to yield the corresponding SPIMAC images (A) and the 2D histogram generated from the comparison of these two images (B). The black pixels in the SPIMAC image of transcription sites correspond to the points selected in the red frame on the 2D histogram. In the SPIMAC image of HMG-I localization sites, the black pixels correspond to the points selected in the white frame on the 2D histogram. SPIMAC analysis of the male pronucleus yielded identical results. Bar, 5 μm.

of chromatin structure that most likely precede or accompany the activation of transcription, possibly through its interaction with the AT-rich SAR/MAR sequences (Zhao *et al.*, 1993) that have been implicated in general transcriptional control in the preimplantation mouse embryo (Thompson *et al.*, 1994).

Dynamic Localization of HMG-I in the Pronuclei of One-Cell Embryos

Although highly abundant in preovulatory GV oocytes, HMG-I cannot be detected by immunocytochemistry in one-cell pronuclei before 20 hphCG, that is, approximately 8 h postfertilization. This is well after the formation of the pronuclei, which takes place around 5 h postfertilization. HMG-I behaves in this respect as several other nuclear proteins, such as SC-35 (Vautier, 1994), snRNPs (Dean *et al.*, 1989; Vautier *et al.*, 1994), hnRNP A (Ferreira *et al.*, 1995), Sp1 and TBP (Worrad *et al.*, 1994), or Pol II (Bellier *et al.*, 1997), which all concentrate only progressively in the pronuclei of early embryos. The fact that *de novo* protein synthesis is not required for the nuclear detection of HMG-I indicates that the protein is present at fertilization, despite the lack of detection on metaphase II chromosomes, and that the time dependence of its nuclear immunodetection at the one-cell stage reflects its nuclear translocation. When not detected, we can suppose that HMG-I is too highly diluted or lost due to detergent treatment. Finally, the effect of depleting the anti-HMG-I serum with the purified protein shows that the immunofluorescence signal we detect is indeed due to HMG-I and not to other AT-hook-bearing proteins such as the p120 band which exhibits limited cross-reactivity with our antibodies (Figs. 1B and 2B).

These particular kinetics of HMG-I nuclear localization are specific to the first cell cycle, as we found the protein in the nuclei of two-cell embryos as soon as they reform. Taking into account the low molecular weight of the protein (11.7 kDa), which in principle should allow its free shuttling from the cytoplasm to the nucleus, several hypotheses can be proposed: this apparent initial cytoplasmic retention could reflect a modification of transport mechanisms in one-cell mouse embryos relative to later embryonic stages and somatic cells. Alternatively, the replication-dependent accumulation of HMG-I in the nucleus could result from the increased availability or unmasking of DNA binding sites during S phase, resulting in a shift in the equilibrium between cytoplasmic and nuclear HMG-I concentrations. Finally, HMG-I nuclear translocation could require posttranslational modifications, such as phosphor-

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ylation, as already shown in the case of Pol II during the first embryonic cell cycle (Bellier *et al.*, 1997).

We also note that the nuclear distribution of HMG-I up to the eight-cell stage differs from that found in somatic cells or in GV oocytes, particularly through the absence of labeling of condensed chromatin. These regions nevertheless became labeled when the cellular concentration of HMG-I was increased through microinjection of the protein, in a process which clearly depends on the concentration of exogenously added HMG-I (see Fig. 4). This suggests either that condensed chromatin of one-cell embryos has a lower affinity for HMG-I or that HMG-I is less concentrated in these cells than in the tissue-culture lines analyzed so far. Indeed, we previously showed that, in cultured somatic cells and in young mouse oocytes, the population of HMG-I bound to condensed chromatin is the most easily displaced by competition with Hoechst 33342, a minor groove-specific ligand (Amirand *et al.*, 1998). Another difference with observations made in somatic cells is the absence of labeling of metaphase chromosomes, whether from MII oocytes or from subsequent embryonic divisions.

The Accumulation of HMG-I in Pronuclei Determines the Onset of Transcription

The most striking result of our studies is the 2-h advance in the onset of transcription that can be induced in one-cell embryos by increasing the cellular concentration of HMG-I. This effect is specific, as it is not obtained with the bacterial HU protein. It requires the binding of HMG-I to DNA, since a similar advance is obtained by microinjection of the ATX peptide, which corresponds to the DNA-binding domain of the protein. Finally, this effect is abolished by preincubation of HMG-I with the anti-ATX antibody before microinjection. Similarly, microinjection of anti-ATX inhibits the normal onset of transcription in one-cell embryos. These results suggest that the transcription-activating effect of HMG-I is linked to its DNA-binding activity, although we cannot completely exclude that anti-ATX could exert its inhibitory effect by retaining the protein in the cytoplasm or by binding to other AT-hook-containing proteins. However, the strong effect of the anti-ATX antibody on HMG-I labeling argues against this hypothesis and suggests that it primarily interacts with the exogenously added or cytoplasmic endogenous HMG-I (Fig. 5C).

While we cannot, in this system, clearly distinguish between an effect on specific and/or abundant transcripts and a general mechanism affecting all transcription, it is important to note the following: (1) the positive modulation we observe reflects an all-or-none phenomenon and (2) microinjection of our specific anti-ATX antibody leads to the inhibition of transcription rather than a redistribution of transcription sites. These results argue for a general—rather than gene-specific—regulatory function. As a case in point, the ATX peptide can also induce an early onset of transcription. DNA binding by AT-hook motifs is thus sufficient for transcriptional activation in the one-cell mouse embryo, whereas gene-specific activation by HMG-I in somatic cells has been shown to require the presence of domains other than the AT hooks (see for instance Yie *et al.*, 1997).

The ATX peptide, microinjected at concentrations that activate transcription, does not change the dot-like localization of the endogenous HMG-I protein, strongly suggesting that it does not displace the protein from these sites. We favor the interpretation that the intense dots do not represent sites where HMG-I exerts its activating effect on transcription, although they are located in regions of low DNA density. This is further confirmed by the SPIMAC analysis of the transcription sites relative to the distribution of the endogenous HMG-I protein, which shows that regions of high HMG-I signal intensities do not correspond to sites of high BrUTP incorporation (Fig. 7), while the diffuse component of lower intensity shows a better correlation with transcription sites. A similar result was already obtained in somatic cells (Amirand *et al.*, 1998).

Microinjected HMG-I Modifies the Accessibility of Chromatin to DNase I

What is the mechanism whereby HMG-I affects the onset of transcription? Studies performed in *Xenopus* have shown that the transcriptional block at the beginning of embryonic life is essentially due to the general inhibitory effect of histone H1 or H1-like proteins (Wolffe, 1989; Bouvet *et al.*, 1994). These proteins might play a similar role in the mouse embryo. Lin and Clarke (1996) have shown that somatic histone H1 is transported efficiently in the pronuclei of late one-cell embryos when microinjected in the cytoplasm at the beginning of the first cell cycle. Using the same experimental conditions, we indeed found that the microinjection of H1 inhibits the normal onset of transcription at 27 hphCG in more than 50% of the embryos. If histone H1 or H1-like proteins were responsible, at least in part, for the repression of transcription up to 27 hphCG, the activating effect of HMG-I in one-cell mouse embryos could be mediated by selective displacement of histone H1 or other general repressors, rendering it accessible to the transcription machinery (Laemmli *et al.*, 1992; Käs *et al.*, 1993; Zhao *et al.*, 1993; Poljak *et al.*, 1994).

If this were the case, one could expect that both the normal and the early onsets of transcription that require HMG-I would be accompanied by measurable changes in chromatin structure. The results of our experiments based on the *in situ* detection of chromatin regions accessible to DNase I (Fig. 6) indeed support the idea that the effect of HMG-I on early zygotic transcription is mediated by measurable changes in chromatin structure and/or accessibility. However, it is important to note that microinjected HMG-I can enhance the DNase I-sensitivity of chromatin as early as 22 hphCG, when no transcriptionally active embryos can be detected, and affects practically all embryos at 25 hphCG, when only 40% of them undergo advanced transcription. These observations suggest that the changes in

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chromatin accessibility that can be induced by microinjected HMG-I most likely precede the activation of transcription.

Nevertheless, DNase I cleavage sites clearly preexist in the pronuclei of one-cell embryos and microinjection of the ATX peptide or of a lower HMG-I concentration, which both induce an earlier onset of transcription (Fig. 4), does not lead to a significant increase in the average DNase sensitivity of chromatin. One possible explanation for this behavior is that, in the latter cases, the increase in DNase I sensitivity induced in each individual embryo is statistically too small to be distinguishable from the mean basal level, given the natural asynchrony among embryos (see Fig. 2D) and the inherent variability in the amount of microinjected HMG-I. Finally, it is important to note that it is difficult to determine whether the DNase cleavage sites detected *in situ* correspond to specific hypersensitive sites or to broader DNase I-sensitive chromatin regions. We are currently investigating this important question.

We conclude from these studies that HMG-I is the first chromosomal protein described so far that appears to be required for general transcriptional activation and whose presence in the nucleus can be shown to advance the onset of transcription in one-cell mouse embryos. This activity requires only DNA binding, suggesting that it is based on a simple competition with other proteins that bind to the same broad family of target sites, a result which bears on how titration of highly reiterated AT-rich sequences might play a critical role in early steps of transcriptional activation as well as in other aspects of genome dynamics (Strick and Laemmli, 1995; Girard *et al.*, 1998). This role might be superseded by more specific functions as the concentration of HMG-I protein decreases during development and differentiation (Thompson *et al.*, 1994). This experimental system should prove useful to analyze in more detail the precise relationships that exist between DNA synthesis, nuclear import of HMG-I, and the onset of zygotic transcription and how they correlate with the changes in chromatin structure that precede or accompany these different events of early embryonic development.

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