A developmentally regulated GAGA box-binding factor and Sp1 are required for transcription of the *hsp70.1* gene at the onset of mouse zygotic genome activation

Arturo Bevilacqua*, Maria Teresa Fiorenza* and Franco Mangia‡

Department of Psychology and Department of Histology and Medical Embryology, La Sapienza University of Rome, Via Borelli 50, 00161 Rome, Italy

*These authors contributed equally to this study

‡Author for correspondence (e-mail: mangia@uniroma1.it)

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SUMMARY

We have investigated the onset of zygotic genome transcription in early two-cell mouse embryos by analyzing the regulation of *hsp70.1*, one of the first genes expressed after fertilization. The transcriptional activation of both an episomic *hsp70* promoter and the endogenous *hsp70.1* gene requires the contiguity of the GC box proximal to the TATA box with a GAGA box and involves GC box- and GAGA box-binding factors. In vivo transcription factor titrations with double-stranded oligodeoxyribonucleotides and antibodies pinpoint these factors as Sp1 and a novel murine GAGA box-binding factor, which is structurally related to the *Drosophila* GAGA factor and acts as transcriptional coactivator/potentiator of Sp1. Mouse unfertilized eggs and one-cell and two-cell embryos display a GAGA box-binding activity of maternal origin that disappears at the four-cell stage and is also abundant in the gonads, but is barely detectable in other adult tissues. In light of the well-established nucleosome-disruption role of the *Drosophila* GAGA factor, these findings suggest a novel mechanism of enhancer-independent gene derepression in early mouse embryos.

Key words: *Hsp70.1*, GAGA factor, Sp1, HSF1, Preimplantation development, Zygotic transcription, Mouse

INTRODUCTION

In the mouse, new embryonic transcription appears after fertilization during the S phase of the first cell cycle in the male pronucleus, while the female pronucleus is largely repressed (Bouniol et al., 1995; Christians et al., 1995; Majumder et al., 1993; Ram and Schultz, 1993). Both pronuclei then become equally repressed during the subsequent G2 phase, concomitant with de novo synthesis of histones H2A, H2B and H1 (Wiekowski et al., 1997) and extensive histone H4 deacetylation (Adenot et al., 1997).

The first intense burst of translation-coupled zygotic transcription (zygotic genome activation, ZGA) appears at the early G1 phase of the two-cell stage with the activation of a few genes (Conover et al., 1991; Latham et al., 1991) that include, among others, the heat shock gene *hsp70.1* (Bensaude et al., 1983; Christians et al., 1995). This transcriptional wave is marked by a number of features, as replacement of the oocyte-derived largest subunit of RNA polymerase II with an embryo-specific form (Bellier et al., 1997) and synthesis of proteins relevant to RNA synthesis, maturation and translation (Davis et al., 1996; Latham et al., 1995; Worrad et al., 1994). These result in a substantial change in the embryo’s transcriptional and translational controls with respect to those acting at the one-cell stage. The increase in the overall efficiency of embryo’s transcription/translation machinery is accompanied by the appearance of diacetylated histone H4, colocalizing with RNA polymerase II at the periphery of blastomere nuclei (Worrad et al., 1995). This suggests that at the time of ZGA, the mouse zygotic genome is largely assembled into compact chromatin and that only a few chromosomal domains, preferentially located near the nuclear membrane, are engaged in transcription. These features imply that a locally acting mechanism(s) of chromatin relief newly appears at ZGA. Information on this issue is so far only indirect and mostly based on evidence derived from transient expression experiments, in which enhancer-less or enhancer-provided DNA constructs were microinjected into either one-cell or two-cell mouse embryos (Majumder et al., 1993). It appeared that, whilst mid one-cell embryos efficiently express both types of DNA constructs, two-cell embryos can only transcribe the enhancer-provided ones, unless they are treated with a histone deacetylase inhibitor (Majumder et al., 1993). Enhancers are thus dispensable for transcription of injected constructs at the one-cell stage, but are required at the early two-cell stage to relieve chromatin-dependent repression. This model of enhancer function at ZGA has recently been
MATERIALS AND METHODS

DNA constructs and mutagenesis

Construct phspPTlacZpA (phsplacZ) was previously described (Kothary et al., 1989). Constructs pmutGAGAhsplacZ (Bivalacqua et al., 1997) and pinsGAGAhsplacZ (Fig. 1A) were made by overlapping recombinant PCR using Vent DNA polymerase (New England Biolabs, Beverly, MA, USA). For microinjections, circular constructs were dissolved in 10 mM Tris, 0.1 mM EDTA, pH 7.4 (TE) at a final concentration of 0.5 ng/µl, corresponding to approximately 250 construct copies/pl.

Transient construct expression in L-cells

L-cell monolayers were transfected, heat-shocked and processed for transient expression assays as described (Bivalacqua et al., 1997). Transfection efficiency was monitored by cotransfection with pCAT-Control Vector (Promega, Madison, WI, USA).

Animals, embryo culture and construct microinjection

Oocytes and preimplantation embryos were obtained from hormonally primed 40- to 60-day-old, female B6D2F1 mice (Charles River Italia, Calco, Italy), taking midnight after mating as fertilization time. One-cell embryos were collected 10-12 hours post fertilization (p.f.) and cultured in vitro as needed. Construct injection (approximately 2 pl DNA solution/nucleus) was performed into the male pronucleus of one-cell embryos (13-14 hours p.f.), or the nucleus of a single blastomere of two-cell embryos (32-34 hours p.f.). The amount of construct copies injected/embryo (approximately 500) was selected because, while not oversaturating embryo’s transcription/translation machinery (not shown), it guarantees a high level of β-galactosidase synthesis, thus providing maximal sensitivity over the experimental inhibition of construct expression (Bivalacqua et al., 1997). Following the injection, embryos were cultured in vitro for 20 hours (one-cell embryos) or 5 hours (two-cell embryos), and eventually assayed for β-galactosidase activity (Bivalacqua et al., 1995). Routinely, 60-70% of one-cell embryos survived the injection of DNA constructs and/or oligodeoxyribonucleotides and then developed to the two-cell stage with no apparent damage at the time of the assay. Further embryo development was not investigated. Survival and development to the two-cell stage of injected one-cell embryos decreased to approximately 40% with injection of antibodies. The lowest rate of embryo survival was observed when embryos received the antibodies shortly before their first cleavage, as in the experiments focused on the endogenous gene. Similar frequencies of embryo survival (70-80% with oligodeoxyribonucleotides and 40-50% with antibodies) were observed when the injection was performed at the two-cell stage. Damaged embryos were immediately discarded and not considered for subsequent assays.

In vivo titration assays with double-stranded oligodeoxyribonucleotides

Complementary oligodeoxyribonucleotide pairs carried consensus sequences for GC box, HSE and mutated GC box as previously described (Bivalacqua et al., 1997). Following annealing, these oligodeoxyribonucleotides had 5'-protruding GATC termini. The GAGA box oligodeoxyribonucleotide pairs were used as follows: GAGA box (sense), 5'-AGAGAGAGAGAGAGAAAAGAGAGAG-3'; GAGA box (antisense), 5'-CTCTCTCTCTCTCTCTCTCT-3'; mutated GAGA box (sense), 5'-ACACACACAGTCAGAAAAGAGTCAG-3'; mutated GAGA box (antisense), 5'-CAGACTCTTCTCTGACTCT-3'. Oligodeoxyribonucleotides were annealed at a concentration of 0.2 mg/ml in TE containing 0.025 M NaCl. Routinely, GAGA box and mutated GAGA box oligodeoxyribonucleotides were blunt-ended by fill-in reaction with Klenow enzyme, while the GC box and HSE oligodeoxyribonucleotides were used without modification. In control experiments in which the activities of oligodeoxyribonucleotides with blunt and sticky termini were compared, the following GAGA box oligodeoxyribonucleotides were used: sense, 5'-GATCACAGAGAGAGAGAGAGAGAGGAGAGAG-3'; antisense, 5'-GATCTCTCTCTTCTCTCTCTCTCTCTCTCTCTCTCT-3'. For titration experiments on the episomal hsp70.1 promoter, appropriate oligodeoxyribonucleotide amounts were mixed 1:1 with a 2× phsplacZ solution, and immediately used for microinjections. Injected one-cell and two-cell embryos were cultured in vitro and processed for β-galactosidase activity, as described above. For titration experiments on endogenous hsp70.1, oligodeoxyribonucleotides were diluted in TE at a final concentration of 0.5 ng/µl and injected into the male pronucleus of one-cell embryos 20-22 hours p.f. Injected embryos were cultured in vitro for 12 hours and eventually processed for RT-PCR assay.

In vivo titration assays with antibodies

Affinity-purified mouse monoclonal IgG1 reacting with amino acids 520-538 of human Sp1 (cat. # sc-420 X) and rabbit polyclonal IgG reacting with murine and human carboxy terminus of Sp3 (cat. # sc-644), but not crossreacting with other members of the Sp family, were from Santa Cruz Biotechnology (Heidelberg, Germany). Rabbit
polyclonal antisera raised to recombinant murine HSF1 and HSF2 and preimmune sera (Fiorenza et al., 1995) were from Dr V. Zimarino. Rabbit polyclonal antiserum raised to recombinant dGAGA factor and preimmune serum (Tsukiyama et al., 1994) were from Dr C. Wu. Sp1 and Sp3 antibodies were microdialyzed against TE through MF filters (Millipore, Rome, Italy) and diluted to a final IgG concentration of approximately 250 ng/μl. As needed, Sp1 antibodies were preabsorbed with the immunogenic peptide (Santa Cruz Biotechnology) at a molar ratio of 1:10. Sera were reconstituted in H2O, microdialyzed against TE, and diluted to comparable IgG concentrations. For titration experiments on the episomal hsp70 promoter, dialyzed antibodies and sera were mixed with phsplacZ and immediately used for microinjection. Injection-assay protocols were as described above. For titration experiments on endogenous hsp70.1, antibodies were diluted fivefold and microinjected as described above.

**RT-PCR analysis of hsp70.1 expression in one- and two-cell embryos**

At appropriate developmental times, groups of five control or injected embryos were transferred to a tube containing 2 μl of embryo-quality H2O (Sigma-Aldrich, Milano, Italy) containing 1 U/μl RNasin Ribonuclease Inhibitor (Promega) and immediately frozen on dry ice. Genomic DNA digestion with DNase I and quantitative RT-PCR amplification of HSP70.1 and S16 ribosomal protein mRNAs were performed with rTth Reverse Transcriptase (Perkin-Elmer Italia, Monza, Italy) as described (Fiorenza and Mangia, 1998), using a MiniCycle™ (MJ Research, Watertown, MA, USA), with a first denaturation step at 94°C for 1 minute, followed by 34 cycles of denaturation at 94°C for 1 minute and annealing-elongation at 62°C for 1 minute. 5 μCi (α-32P)dCTP (DuPont Italiana, Cologno Monzese, Italy) were added during PCR steps as tracer. 32P incorporation into DNA amplification bands was measured with a Model A2024 InstantImager™ (Canberra Packard, Milano, Italy). Primer pairs: HSP70.1 (amplification fragment, 239 bp), 5'-GAAGGTGCCTG-GACAAGTGCT-3' (2509-2527 nucleotides (nt), sense), 5'-GCCAGCGAGGCTCTATTCTA-3' (2729-2748 nt, antisense); S16 (amplification fragment, 103 bp), 5'-AGGAGGCTTGTTCGTGTTGGA-3' (1451-1471 nt, sense), 5'-GCTACTACGCGCTGTGATGG-3' (1621-1641 nt, antisense). S16 mRNA was used as the internal standard in this study, because it does not significantly vary in amount during early mouse embryo development. Under our RT-PCR amplification conditions, S16 mRNA was amplified linearly for at least 40 PCR cycles (Fiorenza and Mangia, 1998).

**Electrophoretic mobility-shift assays**

Mouse eggs and preimplantation embryos were pooled and frozen in 5-10 μl of 10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT (DIGNAM A) (Dignam et al., 1983) supplemented with 0.2 mM PMSF (Sigma-Aldrich), and extracted at 4-6°C for 30 minutes with DIGNAM A containing 5% glycerol, 0.4 mg/ml bovine serum albumin, complete protease inhibitor cocktail (Boehringer Mannheim, Germany) and 0.35 M NaCl. Embryo lysates were then centrifuged in a table top centrifuge at 4°C for 5 minutes, and immediately used for the assay. Adult mouse tissues and D. melanogaster embryos were homogenized and extracted as described (Zimario et al., 1990). Double-stranded GAGA box and mutated GAGA box oligodeoxyribonucleotides, having the sequence of those used for in vivo titration assays, were annealed as described above and labeled by fill-in reaction with (α-32P)dCTP (DuPont Italiana). For binding reactions, 10 μl of protein extract were mixed with 2 ng of 32P-labeled GAGA box oligodeoxyribonucleotides and 1 mg of sonicated calf thymus DNA in binding buffer, and incubated at 4°C for 20 minutes. Final concentrations of buffer components were: 20 mM Tris, pH 8.0, 12% glycerol, 0.04% NP40, 0.24 mM MgCl2, 8 mM EDTA and 2.5 mM DTT. As needed, a 40-fold excess of unlabeled competitor was added to the mixture.

**RESULTS**

**Transcriptional activation of the episomal hsp70 promoter in early two-cell mouse embryos requires a proximal tandem array of GC box and GAGA box**

Transcriptional relevance of the GC box and adjacent GAGA box for hsp70.1 activation was first investigated by transient expression analysis of phsplacZ and its point-mutated derivatives (Fig. 1A). pHSplacZ is driven by the promoter of the murine hsp70.3 gene (Ferry and Moran, 1987), which shares full identity with hsp70.1 in the region spanning from the transcription start site to position −195, and 99% similarity from −195 to −340. The phsplacZ promoter is hereafter referred to as episomal hsp70 promoter. Point mutations included the inactivation of either the GC box or the GAGA box by base substitution, and a half-helical-turn displacement of GAGA box from GC box by a 5 bp insertion.

Transcriptional activity of wild type/point-mutated phsplacZ was first investigated in transfected L-cells (Fig. 1B). In these cells, mutation of the GC box significantly decreased transient construct expression under both normal and stress conditions, as described (Bevilaqua et al., 1997). In contrast, no significant effect was observed with GAGA box mutations, indicating that somatic cells require the GC box for episomic promoter activation, but fully dispense with the GAGA box. Transient expression of wild-type and mutated DNA constructs was then analyzed in embryos injected at either the one-cell or the two-cell stage (Fig. 1C). Mutation of the GC box severely depressed construct activity regardless of the injection protocol (Bevilaqua et al., 1997). In contrast to somatic cells, GAGA box mutations strongly inhibited construct activity to an extent similar to each other, and milder than that of GC box mutation. These findings indicate that episomal hsp70 promoter activation in early two-cell embryos requires both the GC box and the GAGA box, as well as the contiguity and/or spatial alignment of these two nucleotide sequences.

Trans-acting factors that bind the GC box and GAGA box were then titrated by injection of increasing amounts of double-stranded oligodeoxyribonucleotides with GC box or GAGA box repeats (Bevilaqua et al., 1997). GC box

<table>
<thead>
<tr>
<th>Oligodeoxyribonucleotide</th>
<th>Blunt termini</th>
<th>Sticky termini</th>
<th>P</th>
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<tbody>
<tr>
<td>GC box 23±4±2</td>
<td>27±4±4</td>
<td>&gt;0.5</td>
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<tr>
<td>GAGA box 51.8±5.5</td>
<td>52.2±5.4</td>
<td>&gt;0.5</td>
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aOligodeoxyribonucleotides were blunt-ended by fill-in reaction with Klenow enzyme.

bBoth sticky-ended GC box and GAGA box oligodeoxyribonucleotides had a GATC 5’ protruding sequence.

cP calculated by ANOVA.

dValues represent the mean ± s.e.m. of β-galactosidase activity expressed as a percentage of the activity of control embryos injected with phsplacZ alone (not shown).

eValues in parentheses indicate the number of individually assayed embryos, pooled from two independent experiments.

**Table 1. Effect of blunt and sticky termini on double-stranded oligodeoxyribonucleotide ability to inhibit phsplacZ expression in two-cell mouse embryos**
oligodeoxyribonucleotides progressively inhibited *hsplacZ* expression (Fig. 2A,B), as observed previously (Bevilacqua et al., 1997). GAGA box oligodeoxyribonucleotides were also inhibitory, although with an effect milder than that of GC box oligodeoxyribonucleotides. Mutated GC box or GAGA box oligodeoxyribonucleotides were ineffective, except for a mild inhibition of construct expression at the highest concentration, suggesting non-specific toxicity. Differential effectiveness of GC box and GAGA box oligodeoxyribonucleotides was then analyzed in more detail in the two-cell embryos, by determining whether it was related to: (1) different oligodeoxyribonucleotide stability following the injection; and (2) the presence of blunt or sticky oligodeoxyribonucleotide termini. The first question was addressed by the following experiment: a constant amount of radioactively labeled, blunt-ended GC box or GAGA box oligodeoxyribonucleotides was injected in a blastomere nucleus of two-cell embryos. The embryos were then processed for extraction and analysis of injected oligodeoxyribonucleotides by polyacrylamide gel electrophoresis at increasing times post-injection (p.i.). Virtually all injected GC box or GAGA box oligodeoxyribonucleotides were still in a non-degraded and non-concatemerized form at 2 and 4 hours p.i., their amount beginning to slightly decrease only at 6 hours p.i. (Fig. 2C). The second issue was addressed by matched experiments, in which the effect of GAGA box and GC box oligodeoxyribonucleotides on *hsplacZ* expression was determined as a function of the oligodeoxyribonucleotide’s sticky or blunt termini (Table 1). In light of the previous finding that *hsplacZ* transcription/translation in two-cell mouse embryos is virtually completed by 2 hours p.i. (Bevilacqua et al., 1995), and since the presence of blunt or sticky termini did not affect oligodeoxyribonucleotides’ biological potencies, we conclude that differential activities of GC box and GAGA box oligodeoxyribonucleotides exclusively depend on the specific consensus sequence they carry.

To better analyze differential effects of GC box and GAGA box oligodeoxyribonucleotides, values in Fig. 2B were expressed as inhibition of reporter construct expression (net of the mean inhibition value observed with mutated GC box/GAGA box oligodeoxyribonucleotides at each concentration, representing the non-specific effect of injection), and plotted in the graph of Fig. 3A. As shown by half-inhibition values (I50) and maximum inhibition levels, the inhibitory potency of GC box oligodeoxyribonucleotides was almost double that of GAGA box oligodeoxyribonucleotides, in agreement with results obtained with point-mutated constructs. We therefore conclude that episomic *hsp70* promoter activation in early two-cell embryos depends on trans-acting factors that bind the GC box-GAGA box tandem array, and that these factors play distinct transcriptional roles. Do the GC box- and GAGA box-binding factors functionally depend on each other? This question was addressed by determining whether the inhibitory effects of GC box and GAGA box...
A typical experiment of 32 P-labeled GAGA box = - box, r - box experiments. Effect of oligodeoxyribonucleotides: GC box, 20-30 individual embryos, pooled from at least three independent experiments. Values are the mean ± s.e.m. of at least three independent experiments. Effect of oligodeoxyribonucleotides: GC box, r = 0.815, P < 0.0001; GAGA box, r = 0.547, P < 0.0001; mutated GC box, r = 0.075, P > 0.05; mutated GAGA box, r = 0.155, P > 0.2. (B) Two-cell embryos were treated as indicated above for one-cell embryos (symbols as in A). Values are the mean ± s.e.m. of 20-25 individual embryos, pooled from at least three independent experiments. Effect of oligodeoxyribonucleotides: GC box, r = 0.776, P < 0.0001; GAGA box, r = 0.594, P < 0.0001; mutated GC box, r = 0.134, P > 0.05; mutated GAGA box, r = 0.232, P > 0.07. (C) A typical experiment of 32P-labeled GAGA box oligodeoxyribonucleotide injection and recovery from two-cell mouse embryos. Numbers above the figure indicate hours post-injection of embryo processing for the assay. Base pair (bp) standards and a sample of labeled reference oligodeoxyribonucleotide were loaded on lanes MW and st., respectively. Radioactivity values, determined by InstantImageMT and expressed as percentage of lane 0, were as follows: lane 2, 102.1%; lane 4, 95.7%; lane 6, 90.5%. Migration of standards (bp) is indicated at the right.

Fig. 2. Titration of GC box- and GAGA box-binding factors by intranuclear injection of increasing amounts of double-stranded oligodeoxyribonucleotides. (A) One-cell embryos received the injection of a mixture of 500 phspLacZ copies and increasing amounts of GC box (■), GAGA box (●), mutated GC box (□), or mutated GAGA box (□) oligodeoxyribonucleotides. Values are means ± s.e.m. of 20-30 individual embryos, pooled from at least three independent experiments. Effect of oligodeoxyribonucleotides: GC box, r = 0.815, P < 0.0001; GAGA box, r = 0.547, P < 0.0001; mutated GC box, r = 0.075, P > 0.05; mutated GAGA box, r = 0.155, P > 0.2. (B) Two-cell embryos were treated as indicated above for one-cell embryos (symbols as in A). Values are the mean ± s.e.m. of 20-25 individual embryos, pooled from at least three independent experiments. Effect of oligodeoxyribonucleotides: GC box, r = 0.776, P < 0.0001; GAGA box, r = 0.594, P < 0.0001; mutated GC box, r = 0.134, P > 0.05; mutated GAGA box, r = 0.232, P > 0.07. (C) A typical experiment of 32P-labeled GAGA box oligodeoxyribonucleotide injection and recovery from two-cell mouse embryos. Numbers above the figure indicate hours post-injection of embryo processing for the assay. Base pair (bp) standards and a sample of labeled reference oligodeoxyribonucleotide were loaded on lanes MW and st., respectively. Radioactivity values, determined by InstantImageMT and expressed as percentage of lane 0, were as follows: lane 2, 102.1%; lane 4, 95.7%; lane 6, 90.5%. Migration of standards (bp) is indicated at the right.

Fig. 3. (A) The experimental values of Fig. 2B, relative to GC box (■) and GAGA box (●) oligodeoxyribonucleotides, were expressed as inhibition percentages and then graphically interpolated as described in the text. Maximum inhibition levels: GC box, 62%; GAGA box, 39%. I50 (arrows): GC box, 19-fold excess; GAGA box, 33-fold excess. (B) Two-cell embryos received 500 copies of phspLacZ, or a mixture of 500 phspLacZ copies and one of the following oligodeoxyribonucleotides, each having a final concentration corresponding to a 25-fold excess (25x) or a 50-fold excess (50x): an excess of GAGA box oligodeoxyribonucleotides (empty bars); an excess of GC box oligodeoxyribonucleotides (hatched bars); a mixture of an excess of GC box oligodeoxyribonucleotides and a similar excess of GAGA box oligodeoxyribonucleotides (filled bars). Each histogram represents the mean ± s.e.m. of 20-25 individual embryos, pooled from three independent experiments. Symbols above bars indicate a significant difference from GC box oligodeoxyribonucleotides (calculated by ANOVA: †, P > 0.2; *, P < 0.05; **, P < 0.001). The non-specific effect of 50-fold excess oligodeoxyribonucleotides was determined with similar amounts of mutated GC box and GAGA box oligodeoxyribonucleotides.

Transcriptional activation of the episomic hsp70 promoter in early two-cell mouse embryos requires Sp1 and a GAGA box-binding factor structurally related to Drosophila GAGA factor

In these experiments, the GC box-binding factor(s) required for episomic hsp70 promoter activation in two-cell mouse embryos was identified by monitoring the inhibitory effect exerted on phspLacZ expression by co-injection of antibodies to Sp1 (Fig. 4A,B), as described (Bevilacqua et al., 1997). Antibodies to Sp3, a negative transcriptional regulator that competes with Sp1 for the GC box (Hagen et al., 1994), had no effect either when injected alone or in combination with Sp1 antibodies, ruling out the possibility that Sp3 plays a significant regulatory role on the hsp70 promoter.

As for the GAGA box-binding factor, we addressed the question of whether this factor was structurally related to the Drosophila GAGA (dGAGA) factor, by using polyclonal antibodies raised to the dGAGA factor (Tsukiyama et al., 1994) (Fig. 4A,B). Increasing amounts of dGAGA factor antisem progressively inhibited construct expression, whereas comparable amounts of preimmune serum had no effect. Interestingly, the inhibitory effect of dGAGA factor antibodies was less than that of Sp1 antibodies, in agreement with differential inhibition levels observed with GC box/GAGA box point mutations and GC box/GAGA box oligodeoxyribonucleotides. The finding that a factor(s) needed for episomic hsp70 promoter activation in two-cell mouse...
Embryos is recognized by polyclonal antibodies raised to dGAGA factor indicates that murine and Drosophila factors share common epitopes and therefore are, at least in part, structurally related.

Transcriptional activation of endogenous hsp70.1 in early two-cell mouse embryos requires Sp1, the GAGA box-binding factor, and HSF1

Do mechanisms acting on the episomic hsp70 promoter also regulate the endogenous hsp70.1 gene? We addressed this issue by determining the hsp70.1 mRNA content (relative to S16 mRNA) of embryos that had received an oligodeoxyribonucleotide/antibody injection, using a sensitive whole-cell, reverse transcription (RT)-PCR amplification method (Fiorenza and Mangia, 1998). RT-PCR analysis of normally developing embryos at increasing times p.f. revealed a consistent and transient increase in hsp70.1 mRNA amount.

**Fig. 4.** Titration of GC box- and GAGA box-binding factors by intranuclear injection of increasing amounts of antibodies to transcription factors. (A) One-cell embryos received a mixture of 500 phsplacZ copies and increasing amounts of antibodies, giving an approximate final IgG concentration of 12.5 ng/ml (1/20 dilution), 25 ng/ml (1/10 dilution) and 50 ng/ml (1/5 dilution). Each value represents the mean ± s.e.m. of 25-40 individual embryos, pooled from at least three independent experiments. Antibodies injected were: (●) anti-Sp1; (○) anti-Sp1 pre-adsorbed with the antigenic peptide; (△) anti-Sp3; (●) anti-GAGA factor; (□) preimmune serum of dGAGA factor antibodies. Effect of antibodies: anti-Sp1, r=−0.698, P<0.0001; pre-adsorbed anti-Sp1, r=0.016, P>0.9; anti-Sp3, r=0.051, P>0.6; mixture of anti-Sp1 and anti-Sp3, r=−0.648, P<0.0001; anti-dGAGA factor, r=−0.478, P<0.0001; preimmune serum, r=−0.021, P>0.8. (B) Two-cell embryos received a mixture of phsplacZ and antibodies (symbols as in A). Each value represents the mean ± s.e.m. of 20-40 individual embryos, pooled from at least three independent experiments. Effect of antibodies: anti-Sp1, r=−0.620, P<0.0001; pre-adsorbed anti-Sp1, r=−0.079, P>0.5; anti-Sp3, r=−0.063, P>0.6; mixture of anti-Sp1 and anti-Sp3, r=−0.648, P<0.0001; anti-dGAGA factor, r=−0.442, P<0.0001; preimmune serum, r=−0.124, P>0.3.

**Fig. 5.** RT-PCR amplification assay of HSP70.1 mRNA of two-cell mouse embryos during normal development and following the injection of a competing amount of oligodeoxyribonucleotides. (A) One-cell embryos were allowed to develop in vitro and processed for RT-PCR assay at the times p.f. indicated above lanes. The panel shows a typical experiment. Numbers below lanes indicate HSP70.1/S16 incorporation ratios. Migration of standards (bp) is indicated on the left. (B) Histograms represent the mean ± s.e.m. of HSP70.1/S16 ratios obtained in three independent experiments. Open bars: one-cell embryos; tinted bars: two-cell embryos. (C) Late one-cell embryos (20-22 hours p.f.) received the injection of plain buffer (buffer), or approximately 37500 copies of GAGA box, HSE, GC box, mutated GAGA box (mutGAGA box), or mutated GC box (mutGC box) oligodeoxyribonucleotides. Embryos were then cultured for 12 hours and eventually processed for RT-PCR assay. Non-injected embryos were either immediately processed for RT-PCR assay (t0) or cultured for 12 hours and then processed for RT-PCR assay (non-inj.). The panel shows a typical experiment. Numbers below lanes indicate HSP70.1/S16 incorporation ratios. (D) Histograms represent the mean ± s.e.m. of HSP70.1/S16 incorporation ratios, relative to those of non-injected embryos, obtained in three independent experiments. Double asterisks above bars indicate a significant difference (P<0.001, ANOVA) from non-injected embryos.
1547Hsp70.1 regulation in early mouse embryos after the first embryo cleavage (Fig. 5A,B), as previously described (Christians et al., 1995). This increase was fully suppressed by embryo exposure to α-amanitin (not shown).

Experiments on endogenous hsp70.1 also analyzed the regulatory role of HSEs and their binding factors Heat Shock Factor 1 and 2 (HSF1, HSF2), in light of the previous finding that HSF1 is involved in the activation of an hsp70.1 promoter-driven transgene in two-cell mouse embryos (Christians et al., 1995, 1997). Regulation of the endogenous hsp70.1 was first probed with oligodeoxyribonucleotides (Fig. 5C,D). When injected just prior to occurrence of the first mitosis, GC box and GAGA box oligodeoxyribonucleotides reduced the embryo’s content in hsp70.1 mRNA to approximately 20% and 45% of that of control embryos, respectively. Mutated GC box or GAGA box oligodeoxyribonucleotides had no effect, ruling out the possibility of increased mRNA degradation in consequence of the injection per se. The injection of HSE oligodeoxyribonucleotides significantly decreased the hsp70.1 mRNA content, showing that endogenous hsp70.1 was also positively regulated by an HSE-binding factor(s).

Fig. 6. RT-PCR amplification assay of HSP70.1 mRNA carried by two-cell embryos following intranuclear injection of a competing amount of antibodies. (A) Late one-cell embryos (20-22 hours p.f.) received the injection of plain buffer (buffer), or a 1/5-diluted solution of antibodies to HSF1 (aHSF1), HSF2 (aHSF2), Sp1 (aSp1), dGAGA factor (aGAGA), pre-immune serum of dGAGA factor antibodies (pre-immune) or Sp1 pre-adsorbed with the antigenic peptide (pre-ads. αSp1). Embryos were cultured and assayed as described in the legend to Fig. 5C. Non-injected embryos were either immediately processed for RT-PCR assay (t0) or cultured for 12 hours and then processed for RT-PCR assay (non-inj.). The panel shows a typical experiment. Numbers below lanes indicate HSP70.1/S16 incorporation ratios. (B) Histograms represent the mean ± s.e.m. of HSP70.1/S16 incorporation ratios, relative to those of non-injected embryos, obtained in three independent experiments. A double asterisk above bars indicates a significant difference (P<0.001, ANOVA) from non-injected embryos.

Identification of hsp70.1 regulators was achieved by injection of saturating amounts of antibodies to specific factors, including HSF1, HSF2, Sp1, Sp3, and dGAGA factor (Fig. 6A,B). Sp1 antibodies reduced the hsp70.1 mRNA amount to...
approximately 25% of that of control embryos, losing their effect when pre-adsorbed with the antigenic peptide. A significant decrease in hsp70.1 mRNA content to approximately 70% of that of control embryos was also observed with HSF1 antibodies. Unexpectedly, dGAGA factor antibodies had no significant effect. Other antibodies tested, including those to HSF2 and Sp3 (not shown) and preimmune rabbit serum, were also ineffective. Except for ineffectiveness of dGAGA factor antibodies and for the role played by HSF1, results obtained on the endogenous hsp70.1 gene confirmed our conclusions on the episomic hsp70 promoter.

One-cell and two-cell mouse embryos, but not other stages of preimplantation development, contain a GAGA box-binding activity of maternal origin, also expressed at high levels in adult gonads

Results reported above predicted the presence of a GAGA box-binding activity in early mouse embryos. Extracts of two-cell mouse embryos were thus analyzed by electrophoretic mobility-shift assays and compared to those of Drosophila embryos. Mouse embryos displayed a GAGA box-binding activity, as shown by a sharp protein-DNA complex having an apparent electrophoretic mobility a little slower than that of Drosophila embryos (Fig. 7A). A detailed analysis through mouse preimplantation development (Fig. 7B) showed the presence of comparable amounts of GAGA box-binding activity in metaphase II oocytes, one-cell and two-cell embryos, and ovary extracts as well, suggesting that this activity was of maternal origin. In contrast, no GAGA box-binding activity was detected in embryos ranging from the four-cell stage to blastocyst, showing that the GAGA box-binding activity is specifically lost sometime from the mid two-cell to the four-cell stage. Post-implantation stages of mouse development were not investigated. The analysis of several adult mouse tissues (Fig. 7C) showed that this activity is abundantly expressed in the gonads of both sexes, but is barely detectable in other tissues examined. The GAGA box-binding activity thus represents a prominent and specific feature of mouse spermatogenesis, oogenesis and post-fertilization embryo development up to the two-cell stage, suggesting that it plays a major and specific role(s) in germ cell differentiation and early embryonic development.

**DISCUSSION**

**Transcriptional regulation of the episomic hsp70 promoter**

The different experimental approaches used in this study to probe the regulation of episomic hsp70 promoter (Fig. 8) have provided converging evidence of the transcriptional relevance of the GC box and other genetic elements proximal to the TATA box. This is a typical feature of mammalian hsp70 genes, in which these sequences are bound by transcription factors that either directly stimulate transcription under a variety of normal cell growth conditions, or allow HSF1 to interact with basal transcription complex during stress-induction (Williams and Morimoto, 1990). In line with these features, we found that phspPlacZ expression depends on the CCAAT box and GC box in L-cells, and on the GC box, but not CCAAT box, in two-cell embryos (Bevilacqua et al., 1997; and present results). Among trans-acting factors that putatively bind the GC box in the embryos, results with antibodies have conclusively pinpointed Sp1 as the major regulator of episomic hsp70 activation. While not ruling out the possibility of a more complex regulation, we conclude that Sp1 binds the GC box and consequently interacts with the basal transcription complex, according to a recently described mechanism (Ryu et al., 1999).

This study also demonstrates that, besides the GC box, the GAGA box is required for episomic hsp70 activation in two-cell mouse embryos, while it is fully dispensable in L-cells. This suggests that the GAGA box-binding factor plays a role relevant to early mouse embryos, rather than adult somatic tissues. Interestingly, both types of GAGA box mutations tested have produced similar levels of hsp70 promoter inhibition (Fig. 8A), indicating that the functional role of GAGA box-binding factor strictly depends on GAGA box contiguity/rotational alignment with adjacent GC box. Formal evidence that the GAGA box is the binding site of a specific trans-acting factor(s) has been provided by competition experiments with oligodeoxyribonucleotides (Fig. 8B), which
have also given a first insight on how the GAGA box-binding factor is functionally linked to Sp1. First, different transcriptional roles of the two factors are suggested by different inhibitory potencies and titration kinetics of GC box and GAGA box oligodeoxyribonucleotides. Second, GAGA box-binding factor dependence on Sp1 is demonstrated by the finding that GAGA box oligodeoxyribonucleotides had no effect when co-injected with GC box oligodeoxyribonucleotides. Taken together, results obtained with point-mutated constructs and with oligodeoxyribonucleotide titrations allow us to conclude that: (1) the GC box and the GAGA box are the binding sites of specific trans-acting factors; (2) these factors stericly interact with each other on the hsp70 promoter, their tandem array being an absolute requirement for activity of GAGA box-binding factor; and (3) owing to dependence on Sp1, the GAGA box-binding factor resembles a transcriptional potentiator/coactivator of Sp1, more than a canonical transcription factor.

The molecular nature of the GAGA box-binding factor has been investigated by using antibodies raised to the dGAGA factor. This strategy was pursued since a mammalian GAGA factor has, to our knowledge, not yet been cloned or isolated, and in light of the possibility that the GAGA box-binding factor(s) of mouse embryos is a transcriptional co-activator similar to the dGAGA factor (Wilkins and Lis, 1997).

Effectiveness of dGAGA factor antibodies indicates that the murine GAGA box-binding factor is, at least in part, structurally related to the dGAGA factor.

**Transcriptional regulation of endogenous hsp70.1**

We have probed the transcriptional regulation of endogenous hsp70.1 in two-cell embryos by coupling oligodeoxyribonucleotide/antibody titrations with quantitative mRNA amplification by RT-PCR (Fiorenza and Mangia, 1998). To our knowledge, this is the first time that the molecular control of an endogenous gene expressed at ZGA is directly investigated in a mammalian embryo. Oligodeoxyribonucleotide titrations of GC box- and GAGA box-binding factors demonstrate that both factors represent a major requirement for transcription of endogenous hsp70.1 in the embryos, as found for the epitomic promoter. Whilst this conclusion was also validated by titration experiments with antibodies, as for Sp1, it was not for GAGA box-binding factor, the dGAGA factor antibodies having not displayed a significant inhibitory effect. Differential effectiveness of dGAGA factor antibodies on the episomic hsp70 and the endogenous gene is likely to reflect the different copy numbers of the two kinds of genes (500 versus 2, respectively). In fact, while in the case of the episomic genes a significant inhibition would result from the sequestration of the majority, and not necessarily the totality, of the GAGA box-binding factor, the inhibition of the endogenous genes would require that the factor is totally sequestered by the antibody. Alternative explanations, not excluding the first one, are that the endogenous hsp70.1 promoter is partially inaccessible to injected antibodies; and/or, more likely, that the final antibody titer is insufficient to fully sequester a factor that may be abundant and/or already resident on the promoter. Therefore, considering the results obtained with oligodeoxyribonucleotides, we conclude that endogenous hsp70.1 transcription is controlled by both Sp1 and GAGA box-binding factor, and that these factors likely interact with each other and with the basal transcription complex according to regulatory mechanisms similar to those acting on the episomic promoter.

Present experiments demonstrate that the endogenous hsp70.1 gene is also regulated by HSF1. This is not surprising, since this factor has already been proposed to activate transcription of an hsp70.1 promoter-driven transgene in two-cell mouse embryos (Christians et al., 1997). This conclusion was based on the finding that transgene expression was successfully competed by the intranuclear injection of an excess amount of the entire hsp70.1 promoter, spanning from downstream of the TATA box to upstream of the HSEs, whereas an equal amount of hsp70.1 promoter with mutated HSEs was ineffective (Christians et al., 1997). Unfortunately, competition experiments using long promoter sequences with several genetic elements are difficult to interpret, because of the very large number of factors that may bind the competitor DNA. In those experiments, however, the amount of the injected DNA was sufficient to titrate HSF1, but not other factors such as Sp1, GAGA box-binding factor, TBP, etc. (as shown by the transgene expression in the presence of the HSE-mutated competitor), suggesting that two-cell mouse embryos carry an HSF1 amount that is substantially lower than that of other transcription factors. A limited HSF1 availability may also explain why hsp7lacZ expression is independent of HSEs in two-cell mouse embryos (Bevilacqua et al., 1995, 1997). If HSF1 is rare, in fact, it will rapidly be sequestered by a few of the hundreds of construct molecules injected in transient expression experiments, allowing the remaining majority of injected DNA to be solely regulated by the more abundant Sp1 and GAGA box-binding factor.

What is the functional meaning of such HSF1 involvement in hsp70.1 activation in mouse embryos? Among various HSFs cloned in mammals, HSF1 mediates stress-activation of heat shock genes (Morimoto et al., 1996). When cultured in vitro, two-cell mouse embryos display an amount of hsp70.1 mRNA significantly higher than that of in vivo developing embryos (Christians et al., 1995). Such hsp70.1 overexpression is partially reverted when in vitro cultured embryos are maintained in the presence of CuZn-superoxide dismutase (Christians et al., 1995), suggesting that isolation from the oviducts gives the embryo an oxidative stress (Nasr-Esfahani et al., 1990) that elicits a stress response. We therefore conclude that: (1) mouse embryos spontaneously and transiently transcribe hsp70.1 at the early two-cell stage according to a developmental schedule of specific gene derepression at ZGA; and (2) endogenous hsp70.1 expression of in vitro cultured embryos is triggered by the concerted action of Sp1, GAGA box-binding factor and HSF1, the last factor likely mediating a stress-elicted hyperactivation of hsp70.1 in vitro isolated embryos. If this is true, it is reasonable to hypothesize that hsp70.1 activation in in vivo developing embryos is predominantly mediated by Sp1 and GAGA box-binding factor, while HSF1 has no relevant role.

**GAGA box-binding activity of mouse preimplantation embryos and adult tissues**

A striking feature of GAGA box-binding activity revealed by our gel retardation assays is its presence in unfertilized eggs and one-cell and two-cell embryos, and complete absence from the four-cell stage on. Since in these experiments, extracts from
equal numbers of unfertilized and fertilized eggs gave identical signals, the amount of GAGA box-binding activity/egg does not change across fertilization, making unlikely the possibilities of a significant contribution by the sperm, and of extensive de novo synthesis after fertilization. We therefore conclude that the GAGA box-binding activity is synthesized and accumulated during oogenesis, then inherited by the zygote, and eventually destroyed sometime between the mid two-cell and four-cell stages. The exact timing and mechanism(s) of the loss of this activity during preimplantation development still remain to be elucidated. The loss of the activity at the four-cell stage strongly argues in favor of a specific role played at ZGA. At the four-cell stage, in fact, the process of progressive embryo genome repression by chromatin remodeling, initiated in late one-cell embryos, is almost completed (Thompson et al., 1995; Wiekowski et al., 1997; Kaneko and DePamphilis, 1998).

The abundance of GAGA box-binding activity in the gonads, but not in other adult tissues examined, is also intriguing. A minimal explanation for the presence of GAGA box-binding factor in the gonads is required to identify the gonadal cells in which the activity is synthesized by oocytes to be transferred to zygotes. Further study is needed for a chromatin-remodeling activity(ies) during gametogenesis may also justify the presence of GAGA box-binding factor in the gonads.

Conclusions

Similarity of the GAGA box-binding factor of early mouse embryos with the dGAGA factor is suggested by different kinds of evidence: (1) functional dependence on Sp1 points a role of transcriptional activator/potentiator more than canonical transcription factor; and (2) the ability of polyclonal antibodies raised to the Drosophila factor to inhibit hsp70 promoter activity indicates that a murine factor structurally related to dGAGA factor is required for such activity. Even though the possibility that the antiserum tested recognized other unrelated proteins having similar domains cannot be ruled out at the present, it is reasonable to conclude that the murine GAGA box-binding factor shares similar functional and structural features with the dGAGA factor. A GAGA factor acting as chromatin remodeler was recently described on the serine protease inhibitor 2.1 gene promoter in the rat (Simar-Blanchet et al., 1998).

The function of dGAGA factor has particularly been studied on heat shock genes. This factor relieves the inhibitory effect of H1 and, in conjunction with the NURF remodeling complex (Tsukiyama et al., 1994), restructures chromatin at specific promoter regions, making them accessible to transcription factors, as HSF (Wilkins and Lis, 1997). It is thus tempting to hypothesize that the GAGA box-binding factor of mouse embryos acts at the early two-cell stage to relieve the chromatin assembly repression that the zygotic genome has undergone from the late one-cell stage. If proved true by further study, this would represent a novel mechanism of gene derepression at ZGA, acting at the proximal promoter level by an enhancer-independent fashion. The need for a chromatin-remodeling activity(ies) during gametogenesis may also justify the presence of GAGA box-binding factor in the gonads.

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REFERENCES


