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Stage-Specific Regulation of Murine *Hsp68* Gene Promoter in Preimplantation Mouse Embryos

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In early mouse embryos, the major inducible heat shock gene, *hsp68*, is spontaneously and transiently activated at the two-cell stage and becomes heat-inducible around blastocyst stage. We have probed mouse embryo's ability to activate the promoter of this gene during preimplantation development by expression analysis of DNA constructs containing a reporter *lacZ* gene driven by *hsp68* (*hsp70A1*) 5′-regulatory sequences of various length: (i) a full-length promoter (construct p*hsplacZ*); (ii) a heat shock element (HSE)-deleted promoter (pΔ*1hsplacZ*); and (iii) a minimal, proximal promoter (pΔ*2hsplacZ*). When analyzed in transfected L-cells, p*hsplacZ* was heat-inducible, while neither pΔ*1hsplacZ* nor pΔ*2hsplacZ* was. Developmental activity of the full-length construct was first analyzed after genome integration in transgenic embryos and found to follow endogenous *hsp68* expression in terms of spontaneous activation at the 2-cell stage, down-regulation at the 4-cell stage, and acquisition of heat inducibility at the 16/32-cell stage. In transient expression experiments, injected p*hsplacZ*, pΔ*1hsplacZ*, and pΔ*2hsplacZ* were expressed at similar levels by 2-cell embryos, independently of construct topology and injection stage. At the 4-cell stage, however, p*hsplacZ* and pΔ*1hsplacZ* were expressed at similar levels, while pΔ*2hsplacZ* was inactive. Only p*hsplacZ* became heat-inducible in late morulas. We conclude that in early mouse embryos, developmental activity of episomic *hsp68* promoter depends on proximal sequences at the 2-cell stage and on putative enhancer sequences at the 4-cell stage, while HSEs appear dispensable during early cleavage. © 1995 Academic Press, Inc.

INTRODUCTION

During mouse preimplantation development, the zygotic genome is first expressed at the G1 phase of the two-cell stage (Clegg and Pikó, 1982; Flach *et al.*, 1982; Telford *et al.*, 1990) with activation of a number of genes, including those coding for heat shock cognate 70 (Bensaude *et al.*, 1983; Howlett and Bolton, 1985), heat shock protein 68 (*hsp68*) (Bensaude *et al.*, 1983; Christians *et al.*, 1995), heat shock protein 80 (Latham *et al.*, 1991), α-tubulin (Latham *et al.*, 1991), a complex of nuclear-associated proteins of 70 × 10^3 M_r (TRC) (Conover *et al.*, 1991), and other polypeptides so far identified according to their migration on two-dimensional gel electrophoresis (Latham *et al.*, 1991). Several of these genes are expressed transiently (Barnes *et al.*, 1987; Howlett *et al.*, 1987; Latham *et al.*, 1991; Temeles *et al.*, 1994), while other genes are transcribed later, at the mid/late two-cell stage, and during the four-cell stage (Latham *et al.*, 1991). The *β-actin* gene is paradigmatic of such "late" transcriptional activation. In fact β-actin synthesis, while taking place on maternal messages at the one- and two-cell stages (Bachvarova *et al.*, 1989; Taylor and Pikó, 1990), significantly increases at the four- and eight-cell stages in consequence of *de novo* transcription (Taylor and Pikó, 1990; Rothstein *et al.*, 1992; Temeles *et al.*, 1994).

The onset of gene expression in two-cell mouse embryos has been shown to be independent of DNA synthesis (Howlett, 1986; Poueymirou and Schultz, 1987), cell division (Petzoldt, 1984; Poueymirou and Schultz, 1987, 1989), and nucleoplasmic/cytoplasmic ratio (Petzoldt and Muggleton-Harris, 1987) and appears to be triggered by a fertilization-activated "zygotic clock" (Wiekowski *et al.*, 1991) controlled by cytoplasmic factors (Barnes *et al.*, 1987; Howlett *et al.*, 1987; Poueymirou and Schultz, 1989; Manejwala *et al.*, 1991; Schwartz and Schultz, 1992). Information on control of transcriptional activation in mouse embryos has been widely obtained by transient expression analysis of intranuclearly microinjected DNA constructs carrying the coding region of a reporter gene driven by an appropriate 5′-regulatory region (Ueno *et al.*, 1987; Stevens *et al.*, 1989; Dooley *et al.*, 1989; Martínez-Salas *et al.*, 1989; Schwartz and Schultz, 1992; Wiekowski *et al.*, 1991, 1993; Bevilacqua and Mangia, 1993). However, most studies performed so

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far by this approach have used constructs driven by viral promoters and/or enhancers (Majumder *et al.*, 1993; Wiekowski *et al.*, 1993; Mélin *et al.*, 1993) and metabolic inhibitors such as aphidicolin, while information on regulation of promoters physiologically active in normally developing embryos needs to be implemented.

In the present study, we have used normally cleaving embryos to investigate the developmental activity of promoter sequences derived from a gene, the murine *hsp68*, which is spontaneously and transiently expressed with the onset of embryonic transcription (Bensaude *et al.*, 1983; Christians *et al.*, 1995). We show that an *hsp68-lacZ* fusion gene, which behaves as a typically inducible heat shock gene in transfected mouse L-cells, is developmentally regulated according to expression of endogenous *hsp68* when genome-integrated in transgenic preimplantation embryos. By analyzing transient expression of injected *hsp68-lacZ* constructs, we further show that activity of such fusion genes is regulated at the level of the proximal promoter at the two-cell stage and depends on the presence of distal 5′-regulatory sequences, but not heat shock elements (HSEs), at the four-cell stage, while HSEs are needed for heat inducibility at late preimplantation stages.

MATERIALS AND METHODS

DNA Constructs

Construct p*hspPTlacZpA* (p*hsplacZ*) consists of a murine *hsp68-Escherichia coli lacZ* fusion gene ligated to pUC19 (Kothary *et al.*, 1989). Construct pΔ*1hspPTlacZpA* (pΔ*1hsplacZ*) was derived from p*hsplacZ* by deleting the *hsp68* region of a 209-bp *Stu*I fragment containing two tetrameric HSEs and one SP1 binding site (Bevilacqua and Mangia, 1993). Construct pΔ*2hspPTlacZpA* (pΔ*2hsplacZ*) was also derived from p*hsplacZ* by *Stu*I-*Bam*HI deletion of all 5′-regulatory sequences upstream of nucleotide -87 from the transcription start site and subsequent ligation of *hsp68-lacZ* sequences to a *Sma*I-*Bam*HI-digested pUC19. Constructs p*hsplacZ*, pΔ*1hsplacZ*, and pΔ*2hsplacZ* are outlined in Fig. 1. Construct p*βactinPSDKlacZpA* (p*βactinlacZ*) contains a *lacZ* coding sequence/SV40 polyadenylation site driven by a 500-bp-long fragment of the human *β-actin* promoter, excluding the first intron. In present experiments, p*hsplacZ*, pΔ*1hsplacZ*, pΔ*2hsplacZ*, and p*βactinlacZ* were used either as supercoiled DNAs or following linearization with *Sca*I. Before microinjection, constructs were dissolved in 10 m*M* Tris, 0.1 m*M* EDTA, pH 7.4 (TE), at a final concentration of 250 construct copies/pl (1.2–1.6 ng/μl, depending on the construct). Construct pCAT-control vector (pCAT-CV, Promega, Madison, WI) was used to monitor transfection efficiency in cotransfection experiments carried out on L-cells.

Construct Transient Expression Assays in L-Cells

L-cells were grown in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum. Cells were seeded on 17-mm multiwell plates at a density of 2×10^5 cells/well, cultured for 24 hr, and then cotransfected with 0.6 μg of circular pCAT-CV and 0.6 μg of circular p*hsplacZ*, pΔ*1hsplacZ*, or pΔ*2hsplacZ* by standard Ca phosphate precipitation for 6 hr, followed by a 15% glycerol shock in Hepes-buffered Na phosphate medium for 3 min (Davis *et al.*, 1986). After an additional culture for 40 hr, cells were either directly processed for biochemical determinations of β-galactosidase and chloramphenicol acethyltransferase (CAT) activities or heat-shocked at 44 ± 0.1°C for 20 min in a precision water bath as described (Curci *et al.*, 1987), then allowed to recover at 37°C for 3 hr, and eventually processed for the assays. Biochemical determinations of CAT and β-galactosidase activities were performed on different aliquots of the same cell lysate, by phase extraction assay (Ausubel *et al.*, 1994) and as described below for embryos with minor modifications, respectively.

Embryo Culture and Microinjection

(C57BL/6J × SJL/J) F1 hybrid mice (The Jackson Laboratory, Bar Harbor, ME) were used in all experiments. Fertilized eggs were obtained from hormonally primed, 30- to 60-day-old females, taking midnight after mating as fertilization time. Eggs were collected 10–12 hr postfertilization (pf) in medium M2 (Hogan *et al.*, 1986) supplemented with 0.5 mg/ml hyaluronidase, washed with plain M2, transferred to drops of M16 (Hogan *et al.*, 1986) under liquid paraffin, and cultured at 37°C under a humidified atmosphere of 5% CO_2 in air. At 14, 36, and 46 hr pf, one-, two-, and four-cell embryos, respectively, were transferred to 50-μl drops of M2 under liquid paraffin and subjected to microinjection using a Nikon invertoscope (Nikon Corp., Tokyo, Japan) equipped with Hoffman modulation contrast optics (Modulation Optics, Greenvale, NY) and two Leitz mechanical micromanipulators (Wild Leica, Heerbrugg, Switzerland). Approximately 2 pl of the appropriate DNA solution was routinely injected into the male pronucleus of one-cell embryos or the nucleus of a single blastomere of two- or four-cell embryos. Overall embryo survival to microinjection was 60 to 80%, regardless of the stage of injection and construct used. Following injection, embryos were cultured at 37°C in M16 as needed and eventually assayed for β-galactosidase activity. The developmental rate of microinjected embryos (see dashed curves in Figs. 4, 5, and 6) was similar to that of uninjected embryos.

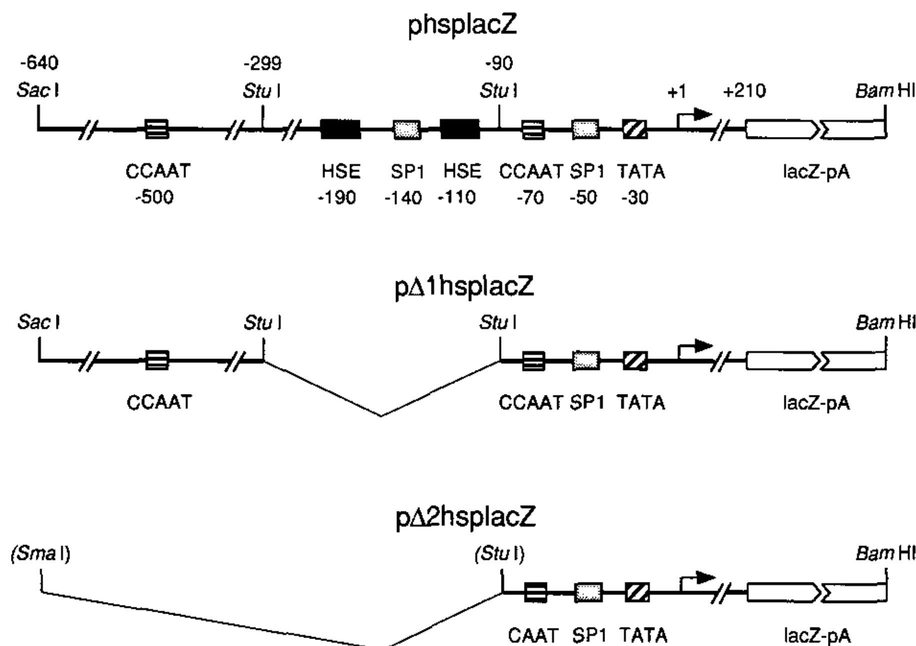

FIG. 1. *Hsp68-lacZ* fusion genes used in the present study. Boxes represent genetic elements identified according to their consensus sequence. 5'-Regulatory regions and sequences downstream of the transcription start site, including Kozak consensus sequences for translational initiation, were derived from the murine *hsp68* gene (Kothary *et al.*, 1989). Polyadenylation signal (pA) was derived from simian virus 40. Numbers indicate nucleotide positions relative to the transcription start site (arrow). Vector sequences (pUC19) are not represented.

Generation of Transgenic Mice and Analysis of Transgene Expression in Preimplantation Embryos

Fertilized eggs, subjected to microinjection of the *Bam*HI insert of p*hsplacZ* (1.0 ng/μl in TE) into the male pronucleus at 14 hr pf, were cultured *in vitro* to the two-cell stage and transferred into pseudopregnant females (Hogan *et al.*, 1986) of the Swiss CD-1 strain (Charles River Italia, Calco, Italy). Offspring were subjected to tail biopsy for DNA extraction, Southern analysis, and β-galactosidase histochemistry (Kothary *et al.*, 1989) as follows. Tail biopsies were fixed with glutaraldehyde and stained with the substrate 4-chloro-5-bromo-3-indolyl-β-D-galactopyranoside (X-gal) or heat-shocked at 43°C for 30 min in phosphate-buffered saline (PBS), allowed to express the transgene at 37°C for 3 hr, and eventually processed for X-gal assay. Reporter enzyme activity in tail tissues of transgenic animals was consistently observed at a high level upon heat shock, but only occasionally and at a low level in the absence of heat shock. No β-galactosidase activity was ever detected in tissues of nontransgenic animals.

Transgenic lines were established by breeding founder animals with (C57BL/6J × SJL/J) F1 mice. Analysis of transgene expression in preimplantation embryos was performed by mating hemizygous transgenic males with hormonally primed, wild-type F1 females. Fertilized eggs obtained from these crosses were cultured *in vitro* in M16 and stained with X-gal at various developmental times as described below for embryos subjected to microinjection.

Transient Expression Assays in Preimplantation Embryos

Embryos that had developed normally following construct microinjection were processed for either cytochemical or biochemical assay of reporter enzyme activity. Cytochemical determination of β-galactosidase activity was carried out by using X-gal as previously described (Bevilacqua *et al.*, 1992). Briefly, embryos were fixed with glutaraldehyde, washed with phosphate buffer containing Na deoxycholate, incubated for 12 hr at 37°C in the presence of X-gal, and eventually scored for the presence and intensity of blue staining. Results were expressed in terms of the percentage of assayed embryos that were positively stained (Bevilacqua *et al.*, 1992; Bevilacqua and Mangia, 1993).

Biochemical assay of β-galactosidase activity was performed according to An *et al.* (1982) on single embryos as follows. Embryos were washed in PBS supplemented with 2% polyvinyl pyrrolidone and individually frozen and thawed twice in 10 μl H_2O. Fifty microliters of 0.3 m*M* 4-methylumbelliferyl-β-D-ga-

lactoside in 25 m*M* Tris-HCl, pH 7.5, 125 m*M* NaCl, 2 m*M* $MgCl_2$, 12 m*M* 2-mercaptoethanol was then added to each embryo lysate and samples were incubated at 37°C for 30 min. The enzymatic reaction was stopped by ice-cold 5% trichloroacetic acid. Clarified supernatants were mixed with 1.5 ml of 133 m*M* glycine, 83 m*M* Na_2CO_3, pH 10.7, and concentration of the reaction product, 4-methylumbelliferone (4-MU), was immediately determined using a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Net fluorescence values were calculated by subtracting a blank value obtained with uninjected embryos at the appropriate stage of development. Reference calibration curves were obtained with increasing concentrations of the reaction product and were linear up to 75 pmole 4-MU.

When needed, injected construct expression was also analyzed after heat shock. To this purpose, injected embryos were allowed to develop to the appropriate stage under normal temperature conditions. They were then heat-shocked at 43°C for 30 min and subsequently incubated at 37°C for 3 hr prior to the enzymatic assay, as previously described (Bevilacqua *et al.*, 1992; Bevilacqua and Mangia, 1993).

Chemicals

Restriction enzymes were purchased from BRL (Gaithersburg, Germany), Pharmacia (Milano, Italy), and Promega (Madison, WI); hyaluronidase from Boehringer-Mannheim Italia (Milano, Italy); and hormones and other chemicals from Sigma Chemical Co. (St. Louis, MO).

RESULTS

Construct Transient Expression in L-cells

Proper regulation of constructs p*hsplacZ*, pΔ*1hsplacZ*, and pΔ*2hsplacZ* was first analyzed by transient expression assays on transfected L-cells. These cells were selected for this purpose because they express *hsp68* after heat shock and were originally used to clone this gene (Lowe and Moran, 1986). Transfection efficiency was monitored by cotransfection with pCAT-CV, and β-galactosidase activity produced by different constructs was expressed in terms of ratio to CAT activity (Fig. 2).

Basal activities of p*hsplacZ* and its derivatives were similar. When L-cells were heat-shocked, however, β-galactosidase activity produced by p*hsplacZ*, but not other constructs, increased four- to fivefold.

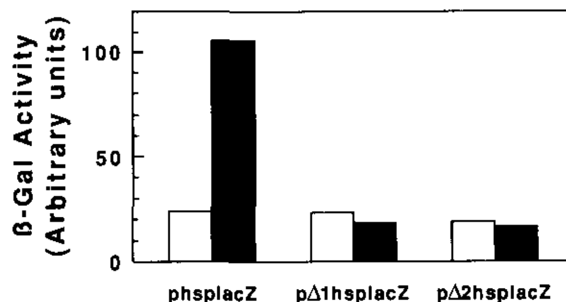

FIG. 2. Transient expression of constructs p*hsplacZ*, pΔ*1hsplacZ*, and pΔ*2hsplacZ* in transfected L-cells. L-cells were cotransfected with pCAT-CV and various *lacZ* constructs, heat-shocked, and assayed for CAT and β-galactosidase activity as described under Materials and Methods. Results are expressed as arbitrary units of β-galactosidase activity normalized for transfection efficiency, with CAT activity as the internal control. Open bars, cells were maintained under normal temperature conditions; solid bars, cells were heat-shocked prior to the assay.

Hsp68-lacZ Fusion Gene Expression in Transgenic Preimplantation Embryos

Developmental patterns of spontaneous and heat-inducible expression of *hsp68-lacZ* fusion gene were analyzed under genome integration in transgenic preimplantation embryos. We obtained three transgenic founder mice, numbered 18, 25, and 26, which had integrated 5, 12-15, and 1 transgene copies, respectively, as determined by Southern and slot-blot analyses (not shown). Among the three lines originated from the founders, lines 18 and 25 displayed Mendelian inheritance of the transgene and were therefore used to analyze *hsp68-lacZ* expression during preimplantation development.

Transgenic one-cell embryos were obtained from back-crosses between hemizygous F1 males of lines 18 and 25 and wild-type females, so that 50% of the conceptuses were expected to be hemizygous for the transgene. Embryos were cultured *in vitro* to the appropriate stage of development and then divided into two groups. One group was directly processed for cytochemical assay of reporter enzyme activity, while the other group was first subjected to heat shock and then processed for the assay. Results obtained with embryos from both transgenic lines were consistent and were therefore pooled together (Fig. 3). In agreement with the developmental pattern of *hsp68* gene expression, constitutive β-galactosidase activity was observed in approximately 50% of 2-cell and 20% of 8-cell transgenic embryos. These embryos displayed a low staining intensity (not shown), suggesting a low amount of reporter enzyme activity and in agreement with the small transgene copy number they carried. In these embryos, heat shock did not increase, but even decreased, reporter enzyme activity. At 16/32-cell and expanded blastocyst stages, on the contrary, no enzyme activity was ever detected in unstressed embryos, while approximately 30% of 16/32-cell embryos and 50% of blastocysts became X-gal-positive following heat shock.

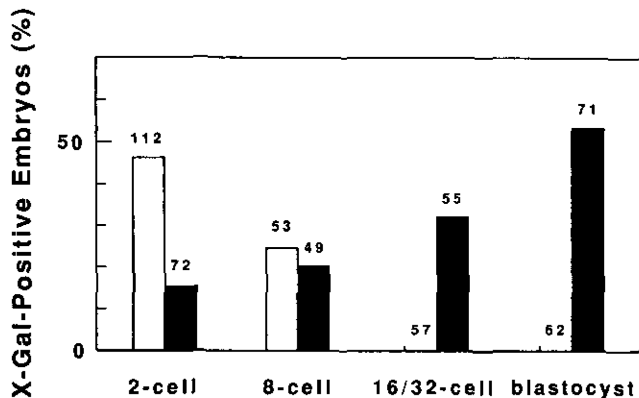

FIG. 3. Expression of the *hsp68-lacZ* fusion gene in transgenic preimplantation mouse embryos. Conceptuses were the progeny of hemizygous transgenic males with wild-type females and were expected to carry the transgene at a frequency of 50%. Histograms represent frequencies of X-gal-positive embryos at the stages indicated on the abscissa, pooled from two different transgenic lines. Embryos were assayed for β-galactosidase activity either under normal temperature conditions (open bars) or after hyperthermic treatment (solid bars), as described under Materials and Methods. Numbers of embryos assayed are indicated above bars.

Transient Expression of phsplacZ, pβactinlacZ, and pΔ1hsplacZ in Preimplantation Embryos

Construct phsplacZ. Spontaneous *hsp68* gene promoter activity in preimplantation mouse embryos was studied in detail by determining the developmental pattern of transient p*hsplacZ* expression. Circular or linear p*hsplacZ* was injected into one-, two-, and four-cell embryos, and construct expression was then assayed cytochemically at increasing postinjection (pi) times. When circular p*hsplacZ* (Fig. 4A) was injected at the one-cell stage, X-gal-positive embryos appeared with increasing frequency 3–5 hr after the first embryonic cleavage, i.e., according to the timing of zygotic transcriptional activation. Most of these embryos displayed a high staining intensity (not shown), suggesting a high rate of construct expression and in agreement with the large construct copy number (approximately 5×10^2/nucleus) they carried. Similar X-gal staining frequency and intensity appeared concomitantly in the small fraction of embryos that spontaneously failed to cleave following injection, in agreement with our previous observations (Bevilacqua and Mangia, 1993). When circular p*hsplacZ* was injected at the mid two-cell stage, X-gal-positive embryos appeared very rapidly after the injection, having a staining intensity similar to that observed with previous injection protocol (not shown). When the injection was performed at the four-cell stage, X-gal-positive embryos appeared soon after the injection, but with frequency and staining intensity (not shown) significantly lower than that observed on embryos injected at previous stages. With all injection protocols, frequencies of X-gal-positive embryos slightly decreased during subsequent embryo development to blastocyst, suggesting a slow β-galactosidase degradation rate in the absence of new enzyme synthesis. Results obtained with linear p*hsplacZ* (Fig. 4B) were similar to those observed with the circular construct.

Amounts of reporter enzyme activity produced by p*hsplacZ* were measured by biochemistry in single embryos subjected to different injection protocols. Results obtained in experiments in which the expression of p*hsplacZ* was compared to that of other constructs are reported in Table 1. In agreement with cytochemical observations, two-cell embryos appeared to synthesize β-galactosidase at a high rate and irrespective of the stage of injection, while four-cell embryos produced a lower amount of reporter enzyme activity. Similar results were obtained with injections of linear p*hsplacZ* (not shown).

Construct pβactinlacZ. To evaluate the possibility that

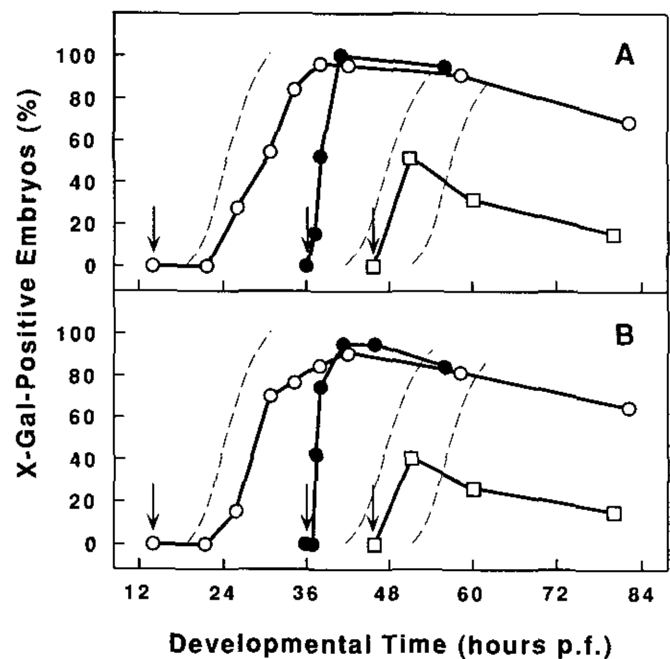

FIG. 4. Expression of construct p*hsplacZ* during mouse preimplantation development. Dashed curves indicate frequencies of injected embryos undergoing cleavage (from left to right: first, second, and third cleavage), as described under Materials and Methods. Circular (A) or linear (B) p*hsplacZ* was injected into the male pronucleus of one-cell embryos (○) or the nucleus of a single blastomere of two-cell (●) or four-cell (□) embryos. Arrows indicate the time of injection: one-cell embryos, 14 hr pf; two-cell embryos, 36 hr pf; four-cell embryos, 46 hr pf. Symbols indicate percentages of embryos progressed to the appropriate stage of development that were positively stained with X-gal at the times indicated on the abscissa. Each value was obtained with 20-60 embryos, pooled from at least three independent experiments.

TABLE 1
β-GALACTOSIDASE ACTIVITY PRODUCED BY CLEAVING MOUSE EMBRYOS AFTER MICROINJECTION OF CIRCULAR DNA CONSTRUCTS

	Protocol of injection and assay[a]			
Construct	A (1- to 2-cell)	B (2- to 2-cell)	C (2- to 4-cell)	D (4- to 4-cell)
p*hsplacZ*	8.94 ± 0.79[b]	8.76 ± 0.77*	n.d.[c]	3.50 ± 0.70
pΔ*1hsplacZ*	9.49 ± 0.92**	8.33 ± 0.82*,**	n.d.	2.77 ± 0.65**
pΔ*2hsplacZ*	8.65 ± 0.82**	7.95 ± 0.76*,**	n.d.	0.32 ± 0.08***
p*βactinlacZ*	0.87 ± 0.24	0.81 ± 0.15*	5.56 ± 1.10****	4.59 ± 0.55

[a] Experimental protocols were as follows: A, injection at one-cell stage and assay at early/mid two-cell stage (20 hr pi); B, injection at mid two-cell stage and assay at late two-cell stage (5 hr pi); C, injection at mid two-cell stage and assay at mid four-cell stage (20 hr pi); D, injection at early/mid four-cell stage and assay at mid/late four-cell stage (5 hr pi).

[b] Values represent pmoles of 4-MU produced by single embryos in biochemical assays of β-galactosidase activity, as described under Materials and Methods. Each value represents the mean ± SEM of 25-35 individual determinations, pooled from three independent experiments. Difference between constructs using the same protocol, pΔ*1hsplacZ* or pΔ*2hsplacZ* vs p*hsplacZ*: $**P > 0.6$; $***P < 0.005$. Difference between protocols using the same construct, protocols B or C vs protocol D: $*P < 0.005$; $****P > 0.5$. Calculated by ANOVA.

[c] Not determined.

the low level of p*hsplacZ* expression observed with injections at the four-cell stage depended on a lower injection efficiency in these embryos, we measured β-galactosidase activity produced in early cleaving embryos following injection of p*βactinlacZ*. This construct was selected for this purpose because its expression increases from the two- to the four-cell stage (Bevilacqua and Mangia, 1993) according to transcriptional activation of endogenous *β-actin* genes (Taylor and Pikó, 1990; Rothstein *et al.*, 1992; Temeles *et al.*, 1994), thus providing an expression pattern opposite to that of p*hsplacZ*. Circular/linear p*βactinlacZ* was injected into two- and four-cell embryos, and reporter enzyme activity was then determined by both cytochemistry (Fig. 5) and biochemistry (Table 1) at the two- and four-cell stages. In these experiments, p*βactinlacZ* appeared to be expressed by four-cell embryos at a level significantly higher than that observed at the two-cell stage, regardless of both the injection protocol used and injected DNA topology. These results showed that in our hands, injections at the two- and four-cell stages had similar efficiency.

Construct pΔ1hsplacZ. We next investigated whether spontaneous p*hsplacZ* expression at two- and four-cell stages was dependent on the presence of the two canonical HSEs of its promoter region. To this purpose, either circular or linear pΔ*1hsplacZ* was injected into one-, two-, and four-cell embryos according to the protocols described above, and patterns of construct transient expression were then determined by cytochemistry during further embryo development (Fig. 6). In parallel experiments, embryo β-galactosidase activity was measured by biochemistry (Table 1). Results obtained with pΔ*1hsplacZ* in two- and four cell-embryos were virtually identical to those observed with p*hsplacZ*, in terms of both X-gal staining frequencies and reporter enzyme activity.

Effect of Heat Shock on Expression of Constructs phsplacZ and pΔ1hsplacZ during Preimplantation Development

The dependence of construct heat inducibility on HSEs was analyzed by injecting circular/linear p*hsplacZ* or pΔ*1hsplacZ* at either the 1- or the 4-cell stage and then allowing embryos to develop to the mid 2-cell stage or the 8/16-cell and 16/32-cell stages, respectively, under normal temperature conditions. At these times, embryos were randomly distributed into two experimental groups. The first group was subjected to heat shock and subsequent recovery at 37°C, while the second group was constantly maintained at 37°C. Construct expression in embryos of both groups was eventually assayed by either cytochemistry or biochemistry. Heat shock did not increase, but even decreased, expression of circular p*hsplacZ* and pΔ*1hsplacZ* at the 2- and 8/16-cell stages (Figs. 7A, 7B, 7C, and 7D), in agreement with results obtained with heat-shocked transgenic embryos (see Fig. 3). At the 16/32-cell stage, however, heat shock appeared to stimulate p*hsplacZ* activity about fourfold, in terms of both embryo staining frequency and enzyme activity, while it had no apparent effect on activity of pΔ*1hsplacZ* (Fig. 7E and 7F). Results obtained with linear constructs were similar to those observed with the circular ones (not shown).

Transient Expression of Construct pΔ2hsplacZ

Relevance of distal *hsp68* promoter sequences for spontaneous expression of p*hsplacZ* at the two- and

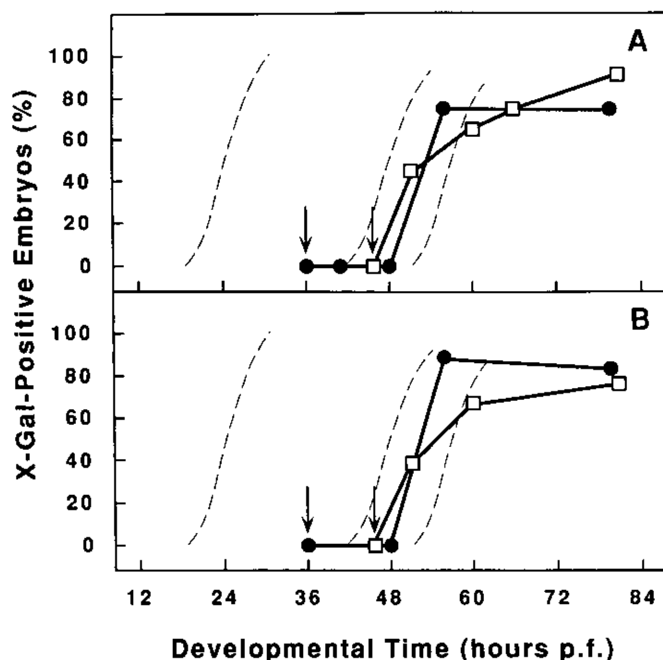

FIG. 5. Expression of construct p*βactinlacZ* during mouse preimplantation development. Dashed curves indicate frequencies of injected embryos undergoing cleavage (from left to right: first, second, and third cleavage), as described under Materials and Methods. Circular (A) or linear (B) p*βactinlacZ* was injected into the nucleus of a single blastomere of two-cell (●) or four-cell (□) embryos. Arrows indicate the time of injection: two-cell embryos, 36 hr pf; four-cell embryos, 46 hr pf. Symbols indicate percentages of embryos progressed to the appropriate stage of development that were positively stained with X-gal at the times indicated on the abscissa. Each value was obtained with 20-60 embryos, pooled from at least three independent experiments.

four-cell stages was then investigated by using pΔ*2hsplacZ*. In matched experiments, embryos were subjected to injection of circular/linear p*hsplacZ*, pΔ*1hsplacZ*, or pΔ*2hsplacZ*, according to the following protocols: (a) injection at the one-cell stage and β-galactosidase assay at the early/mid two-cell stage (20 hr pi); (b) injection at the mid two-cell stage and assay at the mid/late two-cell stage (5 hr pi); and (c) injection at the early/mid four-cell stage and assay at the mid/late four cell-stage (5 hr pi). Levels of β-galactosidase activity produced by different constructs were compared by both cytochemistry (Fig. 8) and biochemistry (Table 1).

When embryos received circular p*hsplacZ*, pΔ*1hsplacZ*, or pΔ*2hsplacZ* at the one- or two-cell stage and were then assayed at the early/mid or mid/late two-cell stage (protocols a and b), the three constructs appeared to be expressed at similar levels, in terms of both staining frequency and reporter enzyme activity. However, when injections and assays were performed at the four-cell stage (protocol c), p*hsplacZ* and pΔ*1hsplacZ* were both expressed, although at a level lower than that of the two-cell stage, while no enzyme activity was ever detected with pΔ*2hsplacZ*. Results obtained with linear constructs were similar to those obtained with the circular ones (not shown).

DISCUSSION

The present study confirms our previous observations that DNA constructs driven by *hsp68* promoter sequences are spontaneously expressed at the two-cell stage in the mouse, independently of the stage of injection (Bevilacqua and Mangia, 1993), and thus represent suitable probes for studying the onset of zygotic transcription. We have now compared developmental patterns of *hsp68-lacZ* expression under genome-integrated and episomic conditions and studied the effect exerted on transient activity of this fusion gene by deletion of different promoter regions.

The spontaneous expression of an inducible member of the *hsp70* gene family in two-cell mouse embryos, originally analyzed by two-dimensional gel electropho-

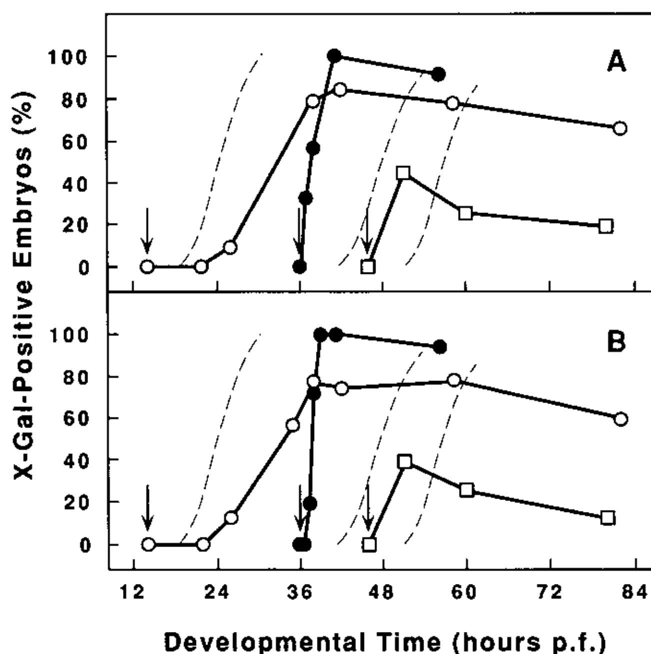

FIG. 6. Expression of construct pΔ*1hsplacZ* during mouse preimplantation development. Dashed curves indicate frequencies of injected embryos undergoing cleavage (from left to right: first, second, and third cleavage), as described under Materials and Methods. Circular (A) or linear (B) pΔ*1hsplacZ* was injected into the male pronucleus of one-cell embryos (○) or the nucleus of a single blastomere of two-cell (●) or four-cell (□) embryos. Arrows indicate the time of injection: one-cell embryos, 14 hr pf; two-cell embryos, 36 hr pf; four-cell embryos, 46 hr pf. Symbols indicate percentages of embryos progressed to the appropriate stage of development that were positively stained with X-gal at the times indicated on the abscissa. Each value was obtained with 20-60 embryos, pooled from at least three independent experiments.

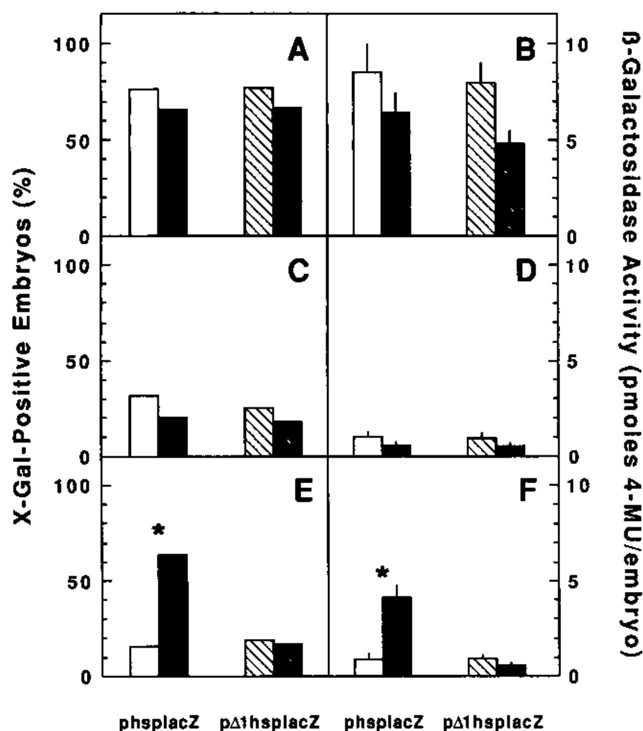

FIG. 7. Effect of heat shock on expression of circular p*hsplacZ* and pΔ*1hsplacZ* in preimplantation embryos. (A and B) Embryos were injected at the 1-cell stage and assayed at the 2-cell stage. (C and D) Embryos were injected at the 4-cell stage and assayed at the 8-cell stage. (E and F) Embryos were injected at the 4-cell stage and assayed at the 16/32-cell stage. (A, C, and E) Histograms represent X-gal staining frequencies, while those in B, D, and F represent mean values of β-galactosidase activity obtained in biochemical assays on single embryos. Injected embryos, cultured to the appropriate stage of development, were individually assayed for β-galactosidase activity by cytochemistry (A, C, and E) or biochemistry (B, D, and F), either under normal temperature conditions (□ and ▧) or after hyperthermic treatment (■ and ▧). Asterisks above bars indicate significant difference ($P < 0.005$) between control and heat-shocked embryos. P was calculated by χ^2 (A, C, and E) and ANOVA (B, D, and F).

resis in terms of synthesized protein (Bensaude *et al.*, 1983), was recently demonstrated at the transcriptional level by reverse transcriptase-polymerase chain reaction (RT-PCR) and by analyzing the activity of *hsp70.1* promoter coupled to a reporter luciferase gene in transgenic embryos (Christians *et al.*, 1995). *Hsp70.1* gene (Hunt and Calderwood, 1990) shares a high degree of identity with *hsp70A1* (Lowe and Moran, 1986; Perry and Moran, 1987), the promoter of which was used in our study. In fact these genes have very similar coding regions and identical proximal promoter sequences, but significantly diverge in promoter sequences upstream from the distal HSE.

Observations on transgenic embryos show that the *hsp68-lacZ* fusion gene used in the present study contains all regulatory sequences needed for transcriptional activation during preimplantation development, suggesting that the lack of expression previously observed at the two-cell stage with a similar *hsp68-lacZ* transgene (Kothary *et al.*, 1989) was likely dependent on the prokaryotic translation initiation signal carried by that construct. Present results agree with those of Christians *et al.* (1995), showing that *hsp68* gene(s): (i) is among the earliest genes spontaneously activated with the onset of zygotic genome expression; (ii) is then transcribed through the two-cell stage; and (iii) is eventually down-regulated around the second embryonic cleavage. As for expression of *hsp68*-directed transgene at the late one-cell stage recently reported for a small fraction of transgenic embryos (Christians *et al.*, 1995), this possibility was not directly evaluated in the present study. It remains to be elucidated whether such expression before the first embryonic cleavage, if confirmed by further study, reflects an early transcriptional leakage of randomly integrated transgenes or whether it actually represents a feature of zygotic genes and is restricted to the paternal or maternal pronucleus (Wiekowski *et al.*, 1993).

Transient *hsp68* promoter activity at the 2-cell stage was further confirmed by present microinjection experiments, in which episomic p*hsplacZ*: (i) was recruited by the embryo's first transcriptional wave with a time schedule similar to that of TRC expression (Conover *et al.*, 1991) when injected at the 1-cell stage; (ii) was immediately expressed when injected at the 2-cell stage; and (iii) was still transcribed by 4-cell embryos, although at a level lower than that observed at the previous stage. As for stress inducibility of *hsp68-lacZ* fusion gene, it was first observed at late preimplantation stages in both transgenic and injected embryos, in agreement with previous observations (Morange *et al.*, 1984; Hahnel *et al.*, 1986, Christians *et al.*, 1995). The decrease in β-galactosidase activity consistently displayed by 2- to 8-cell embryos after heat shock was likely dependent on loss of enzyme activity caused by the protein-denaturing effect of heat shock (Rothman, 1989) in the absence of heat shock response. The 16/32-cell stage was the earliest one at which heat inducibility of *hsp68-lacZ* was first observed during development, suggesting that this, and not the blastocyst (Morange *et al.*, 1984; Hahnel *et al.*, 1986; Kothary *et al.*, 1989), is the preimplantation stage at which endogenous *hsp68*, and likely other heat shock genes, become heat-inducible. Since at this stage mouse embryo blastomeres differentiate into inner and outer cell compartments representing the first embryonic primordia (Pedersen, 1986), acquisition of heat shock gene inducibility may be considered among the markers of incipient blastomere differentiation (Johnson and Maro, 1986).

In microinjection experiments, we have addressed

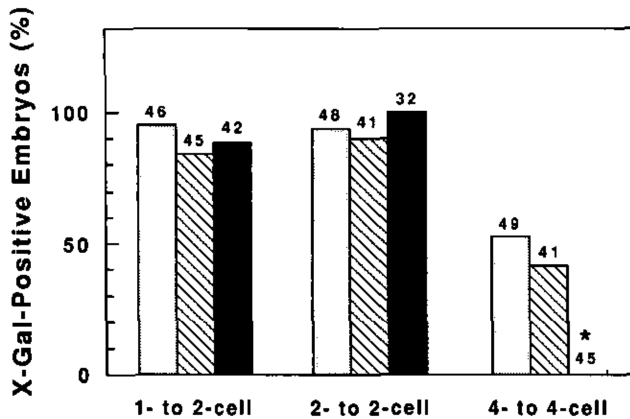

FIG. 8. Cytochemical comparison of spontaneous activities of circular p*hsplacZ*, pΔ*1hsplacZ*, and pΔ*2hsplacZ* in two- and four-cell embryos. Histograms represent fractions of X-gal positive embryos that had received p*hsplacZ* (stippled), pΔ*1hsplacZ* (striped), and pΔ*2hsplacZ* (solid). Experimental protocols: 1- to 2-cell, embryos were injected at the one-cell stage and assayed at the early to mid two-cell stage; 2- to 2-cell, embryos were injected and assayed at the two-cell stage; 4- to 4-cell, embryos were injected and assayed at the four-cell stage. Numbers above bars indicate total embryos assayed, pooled from three independent experiments. An asterisk indicates significant difference ($P < 0.005$) with embryos injected with p*hsplacZ* (calculated by χ^2).

three major questions: (1) the effect of injected DNA topology on construct transient expression; (2) the need for HSEs in *hsp68* spontaneous activation at the two-cell stage and/or heat inducibility at late preimplantation stages; and (3) the role played by other proximal promoter elements and/or enhancer(s) in controlling *hsp68* activity at the two-/four-cell stages. The first question was of interest in light of different transcriptional activities we previously observed in mouse oocytes and preimplantation embryos with injection of circular or linear constructs (Bevilacqua *et al.*, 1992). In mouse oocytes and somatic cells, circular DNA is assembled into chromatin and is strongly expressed, while linear DNA is not assembled into chromatin and is poorly expressed (Chalifour *et al.*, 1986; Bevilacqua *et al.*, 1992; Bevilacqua and Mangia, 1993). On the contrary, early mouse embryos appear to activate injected DNA molecules regardless of their circular/linear topology (Ueno *et al.*, 1987; Bevilacqua and Mangia, 1993). Present results fully confirm previous observations by further showing that in early mouse embryos, injected DNA topology does not affect construct expression. A simple explanation for this finding is that early mouse embryos rapidly join linear DNA into free-ended concatemers (Burdon and Wall, 1992), which in turn may behave like circular molecules in terms of transcriptional regulation (Weber and Shaffner, 1985). A second possibility, not exclusive of the first one, is that in contrast to oocytes, early mouse embryos efficiently transcribe relaxed plasmid DNA. In fact, it was previously reported that one- and two-cell mouse embryos assemble injected circular DNA into chromatin very slowly with respect to oocytes and that the embryo's ability to express injected constructs inversely correlates with the fraction of superhelical DNA molecules (Chalifour *et al.*, 1986; Martínez-Salas *et al.*, 1989).

The role played by HSEs and other genetic elements of proximal/distal promoter sequences in *cis*-regulating *hsp68-lacZ* activity during preimplantation development was investigated by deletion analysis of *hsp68* promoter regions typically observed in members of eukaryotic *hsp70* genes (Hunt and Morimoto, 1985; Perry and Moran, 1987; Wu *et al.*, 1986; Amin *et al.*, 1987; Morgan *et al.*, 1987; Hunt and Calderwood, 1990) (see Fig. 1): (1) a proximal 90-bp-long promoter region, including transcription start site, a TATA box, a CCAAT element, and an SP1 binding site; (2) an intermediate 210-bp-long region, including two functional tetrameric HSEs at positions −105 and −190, and an SP1 binding site; and (3) a distal 345-bp-long region, containing mismatched dimeric HSEs, a CCAAT element, and other sequences still uncharacterized in terms of putative genetic elements.

The recent observation that heat shock factor (HSF) DNA-binding activity is present in mouse preimplantation embryos (Mezger *et al.*, 1994) made of particular interest the question of whether HSE(s) is needed for *hsp68* spontaneous activation at the two-cell stage. Present results obtained with pΔ*1hsplacZ* show that activation/inactivation of injected *hsp68* promoter during early cleavage is independent of HSEs, suggesting that HSF(s) is unlikely to play a canonical role, if any, in transcriptional regulation of endogenous *hsp68* at these stages. This conclusion is in agreement with the lack of HSF DNA-binding activity in one- and two-cell mouse embryos under normal temperature conditions (Mezger *et al.*, 1994). As for the HSF DNA-binding activity observed at these stages after heat shock (Mezger *et al.*, 1994), it remains to be elucidated whether it is actually generated by stress in intact cells and/or whether it plays any function at the *hsp68* promoter level. In contrast to the spontaneous expression, *hsp68* heat inducibility appeared to depend strictly on the presence of the two canonical HSEs in both L-cells and late preimplantation embryos, as expected by the central role of these elements in stress-induced activation of heat shock genes (Sorger, 1991) and in agreement with previous observations on mouse growing oocytes and granulosa cells (Bevilacqua and Mangia, 1993).

Cis-regulation of spontaneous *hsp68* promoter activity during early cleavage was further investigated by using pΔ*2hsplacZ*, a construct driven by minimal promoter se-

quences. Expression of this construct at the two-cell stage was similar to that of p*hsplacZ*, showing that distal promoter sequences are fully dispensable for spontaneous *hsp68* activity at this stage. This finding is reminiscent of human *hsp70* basal expression, which is regulated at the proximal promoter level by SP1 transcription factor (Morgan, 1989) and CCAAT element binding factor(s) (CTF) (Williams *et al.*, 1989; Lum *et al.*, 1992; Agoff *et al.*, 1993) and is induced by serum (Wu and Morimoto, 1985) and adenovirus E1a (Wu *et al.*, 1986). In early mouse embryos, SP1 activity was recently reported to increase two- to sixfold from the one- to the two-cell stage (Majumder *et al.*, 1993, Worrad *et al.*, 1994), thus making this factor a putative *trans*-activator of *hsp68* expression in early two-cell embryos. Actual relevance of SP1, CTF, and other factors present in mouse preimplantation embryos, including E1a-like (Dooley *et al.*, 1989) and AP-1-like (Schwartz and Schultz, 1992) activities for zygotic gene activation deserves further investigation.

Expression of episomic p*hsplacZ* at the four-cell stage was of particular interest, in light of the finding that endogenous *hsp68* is not transcribed at this stage (Christians *et al.*, 1995). Lack of endogenous *hsp68* expression has also been observed in our laboratory by RT-PCR, using oligonucleotide primers specific to sequences common to both *hsp70A1* and *hsp70.1* genes (M. T. Fiorenza, unpublished results). In present microinjection experiments, the *hsp68* promoter activity observed at the four-cell stage was significantly lower than that of two-cell embryos, as conclusively shown by microinjection of p*βactinlacZ* that ruled out the possibility of injection artefacts. Injected construct activity at this stage appeared to be regulated by mechanisms substantially different from those acting before the second embryonic cleavage, being strictly dependent on the presence of distal promoter sequences, excluding HSEs. Distal *hsp68* promoter region may carry functional enhancer(s), as shown by the finding that insertion of a retinoic acid response element at its distal end conferred a pattern of spatial and temporal expression specific to the RARβ gene on p*hsplacZ* in transgenic mouse postimplantation embryos (Rossant *et al.*, 1991). As for the human *hsp70*, the distal promoter region was found to be necessary for species-specific basal activity in transfected cell lines (Greene *et al.*, 1987).

The role of enhancers in regulating zygotic genome expression in the mouse has been thoroughly studied by microinjection experiments (Martínez-Salas *et al.*, 1989; Wiekowski *et al.*, 1991, 1993; Majumder *et al.*, 1993; Mélin *et al.*, 1993). It was shown that enhancerless constructs are efficiently transcribed by mouse embryos blocked at the one-cell stage either spontaneously or by treatment with aphidicolin, while the presence of an enhancer(s) is required for transcription of injected DNA at the two-cell stage (Martínez-Salas *et al.*, 1989; Majumder *et al.*, 1993). However, the need for enhancers inversely correlates with the strength of basal promoter, being weak when the enhancer is linked to a strong promoter and strong when it is linked to a weak promoter (Majumder *et al.*, 1993). Levels of activity observed with present *hsp68-lacZ* constructs are in agreement with these findings, strongly favoring the conclusion that, although a complementary role of HSE/HSF(s) cannot be ruled out, activation of injected *hsp68* promoter at this stage is mainly, if not all, regulated at the proximal promoter level.

Present experiments have also shown that expression of episomic *hsp68-lacZ* fusion gene is down-regulated at the four-cell stage. While mechanisms responsible for such down-regulation are still unknown, the lack of pΔ*2hsplacZ* expression in four-cell embryos suggests that such mechanisms also act at the proximal promoter level. Several possibilities can be hypothesized, including inactivation/sequestration of basal transcription factor(s), appearance of a repressor(s), and/or inhibition by a higher order chromatin structure. As for actual down-regulation of endogenous *hsp68* expression at the late two-cell stage (Christians *et al.*, 1995), it will require further study to be elucidated. In any case, however, enhancer(s) that stimulates expression of episomic *hsp68* at the four-cell stage appears not to be functional in the case of endogenous *hsp68*.

Results obtained in the present study allow us to propose a testable model for transcriptional regulation of *hsp68* during early mouse embryogenesis, involving activation regulated at the proximal promoter level at the onset of zygotic genome expression and down-regulation at the late two-cell stage (Christians *et al.*, 1995; M. T. Fiorenza, unpublished results). While HSEs do not appear to play an essential role in spontaneous *hsp68* activation during cleavage, they mediate promoter stress inducibility at late preimplantation stages.

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