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Expression of the *HSP 70.1* gene, a landmark of early zygotic activity in the mouse embryo, is restricted to the first burst of transcription

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SUMMARY

Activation of the mouse embryonic genome at the 2-cell stage is characterized by the synthesis of several α-amanitin-sensitive polypeptides, some of which belong to the multigenic hsp 70 family. In the present work we show that a member of this family, the *HSP 70.1* gene, is highly transcribed at the onset of zygotic genome activation. Transcription of this gene began as early as the 1-cell stage. Expression of the gene continued through the early 2-cell stage but was repressed before the completion of the second round of DNA replication. During this period we observed that the level of transcription was modulated by in vitro culture conditions. The coincidence of repression of *HSP70.1* transcription with the second round of DNA replication was not found for other transcription-dependent polypeptides synthesized at the 2-cell stage.

Key words: zygotic transcription, transgene, luciferase, *HSP 70.1*

INTRODUCTION

In the mouse embryo, activation of the zygotic genome (ZGA) has been described as a succession of two phases of transcriptional activity leading to the transition from maternal to zygotic control during the late 2-cell stage (Flach et al., 1982 ; Bolton et al., 1984). The first phase is tightly coupled to the appearance of a large complex of 70×10^3 M_r polypeptides (Flach et al., 1982; Bensaude et al., 1983; Bolton et al., 1984; Conover et al., 1991; Latham et al., 1991a,b, 1992) and is independent of the first replication (Flach et al., 1982; Bolton et al., 1984; Howlett, 1986; Schultz, 1993). It follows the acquisition of a transcriptionally permissive state that occurs during the 1-cell stage, apparently under the control of the cAMP (Latham et al., 1991a, 1992). This first phase, also termed the 'minor' activation of the zygotic genome (Vernet et al., 1992; Schultz, 1993), precedes a second phase of transcriptional activity characterized by a marked transition in polypeptide synthesis, described as the 'major' activation of the zygotic genome (Flach et al., 1982; Howlett, 1986; Vernet et al., 1992; Schultz, 1993). In contrast to the minor activation, the major activation is dependent on the first round of DNA replication (Howlett, 1986).

The description of a two-phase activation of the zygotic genome has generated the idea that this event could be regulated by two different mechanisms. Minor activation would depend on post-translational modifications of maternally derived proteins, for example, phosphorylation, (Howlett, 1986; Van Blerkom, 1981) that may regulate, either directly or indirectly, the onset of RNA polymerase activity and thus the first transcriptional burst (Latham et al., 1992; Poueymirou and Schultz, 1987, 1989; Schwartz and Schultz, 1992; Schultz, 1993). Thus, this phase depends on time elapsed from fertilization ('zygotic clock') and may correspond to a widely permissive genomic state (Martinez-Salas et al., 1989; Vernet et al., 1992). This idea is supported by the expression of a long list of genes in transient assay experiments (Ueno et al., 1987; Bonnerot et al., 1991) and by the drastic drop in the level of bulk genomic methylation (Monk et al., 1987; Kafri et al., 1992). When major activation occurs, regulatory events that govern the selectiveness of RNA polymerase II appear to operate, as evidenced by both the requirement of enhancer sequences in transient expression experiments, and the occurrence of stage-specific gene expression (Martinez-Salas et al., 1989; Wiekowski et al., 1991; Majumder et al., 1993; Rothstein et al., 1992). How the selectiveness of the RNA polymerase II is initiated remains to be elucidated.

It is striking that different profiles of gene expression are observed as early as the onset of ZGA. The transcription of the cognate heat shock gene, *hsc 70* begins with the ZGA and appears to continue beyond this stage (Bensaude et al., 1983; Manejwala et al., 1991). However, a large group of polypeptides, the TRC or 'transcription requiring complex', is only transiently expressed in the 2-cell mouse embryo, reflecting the switching on and off of RNA polymerase activity along the corresponding genes (Conover et al., 1991; Latham et al., 1991b). The gene(s) involved in the synthesis of this complex of 73, 70 and 68×10^3 M_r proteins remain unknown at present, despite some biochemical characterization (Conover et al., 1991). In contrast, a quantitatively less important group of zygotic polypeptides, also transiently expressed during the 2-cell stage were long ago identified as members of the multigenic family of inducible hsp 70 proteins (also termed hsp68, HSP72; Bensaude et al., 1983). Surprisingly, no further char-

acterization of the specific member(s) of this family (Lowe and Moran, 1986) that are expressed at the 2-cell stage has been undertaken, perhaps partly because of contradictory reports on whether or not there is constitutive expression of such genes during the onset of ZGA (Wittig et al., 1983; Hahnel et al., 1986; Schultz, 1993). Since *hsp 70* is a widely used model in the study of gene regulation, its early embryonic expression represents a unique opportunity to further investigate mechanisms of transcriptional control acting at the onset of embryonic development.

In this paper, we use both RT-PCR analysis, and transgenic lines of mice to demonstrate that *HSP70.1*, one member of the inducible heat-shock family (Hunt and Calderwood, 1990) is constitutively expressed at the first burst of ZGA. Our results show that HSP 70.1 promoter directed transcription begins at the 1-cell stage. It is highly expressed early in the 2-cell stage before rapid repression, coincident with completion of the second round of DNA replication. To our knowledge, *HSP 70.1* is the earliest gene truly identified to be expressed at the onset of ZGA and only during the first burst of transcription. These features indicate that well-defined regulatory mechanisms are involved in the control of genes expressed as soon as transcriptional competence is acquired.

MATERIALS AND METHODS

DNA constructs

The plasmid HSP70.1 *Luc* was obtained in two steps. The *Eco*RI fragment containing the promoter sequence (HSP70.1) was cloned into the *Hin*cII site of Bluescript plasmid (PBS KS+, Stratagene). This construct was then further cut by *Hin*dIII-*Bam*HI to insert the *Hin*dIII-*Bam*HI fragment corresponding to the cDNA of the firefly luciferase gene linked to the SV40 small-t intron and polyadenylation signal (DeWet et al., 1987). The different elements of the constructs described above were generous gifts from O. Bensaude. A schematic map of the construct is shown in Fig. 2A.

Microinjection of embryos and generation of transgenic mice and embryos

F_1 hybrid (C57BL6×CBA) females, superovulated by injection of 10 IU pregnant mare serum gonadotrophin (PMSG, Intervet) followed 46-48 hours later by 5 IU human chorionic gonadotrophin (hCG, Intervet, were mated with F_1 hybrid (C57BL6×CBA) males. One-cell zygotes were collected 22-24 hours post hCG and then they were injected into one pronucleus with 1-2 pl of the 7.2 kb *Sca*I linearized HSP70.1*Luc* plasmid DNA solution at a concentration of 2-3 ng/μl. Immediately after micromanipulation embryos were transferred to pseudopregnant recipients (Hogan et al., 1986).

Transgenic offspring were identified by slot blot hybridization or using the polymerase chain reaction (PCR) with primers specific to the firefly luciferase transgene. Copy number was determined by densitometric analysis of quantitative Southern blots. After identification of founder transgenic mice, breeding was carried out to establish the transgene in the homozygous condition.

Preimplantation transgenic embryos were obtained by mating superovulated F_1 hybrid (C57BL6×CBA) females with transgenic homozygous F_2 to F_4 hybrid (C57BL6×CBA) males. One-cell embryos were recovered at 22-24 hours post hCG. They were cultured in small drops of M16 medium (Hogan et al., 1986) under oil (light paraffin oil, BDH) and maintained in an incubator in 8% CO_2 and at 37°C until frozen prior to luciferase assay. In vivo transgenic embryos were recovered directly from females at the times indicated and were immediately frozen.

Inhibitors and enzyme

A concentrated stock solution of α-amanitin (A 2263 Sigma Chemical Co) was dissolved in phosphate-buffered saline (PBS complete, described by Hogan et al., 1986) at 1 mg/ml and stored at –20°C. Transcription was inhibited in normal embryos by incubating them from 30 hours post hCG in M16 medium supplemented with α-amanitin (0.1 mg/ml) (Kidder et al., 1985). The same medium was used to inhibit transcription in transgenic embryos with the varying times of inhibitor addition shown in Fig. 4A. Incubation in the presence of α-amanitin was carried out until the embryos were frozen for subsequent analysis.

A concentrated stock solution of aphidicolin (A0781 Sigma Chemical Co) was prepared in pure DMSO at 1 μg/μl and stored at –20°C. Replication was inhibited by incubating embryos in M16 medium supplemented with aphidicolin (2 μg/ml). Incubation with the inhibitor was initiated at 24, 30, 50 hours post hCG in order to arrest embryos in the first, second, or third round of DNA replication respectively. Embryos blocked at the 1-cell stage were frozen at 42 hours post hCG as were 2-cell control embryos, those that were blocked at the 2-cell stage were frozen at 65 hours post hCG as were 4-cell control embryos and those blocked at the 4-cell stage were frozen at 72 hours post hCG as were morula control embryos. The luciferase assay was then performed.

CuZn-superoxide dismutase (CuZn-SOD, S 2515 Sigma Chemical Co) was diluted in M16 medium to a final concentration of 6000 IU/ml. The effect of this enzyme on HSP70.1*Luc* transgene expression was tested by culturing the embryos from the 1-cell stage (24 hours post hCG) to the 2-cell stage (42 hours post hCG).

Luciferase assay

At the indicated times, individual embryos were extracted and frozen in 50 μl of reaction buffer (25 mM H_3PO_4, 10 mM $MgCl_2$, 1 mM EDTA, 1% Triton X-100, 15% glycerol, 1 mg/ml BSA, 1 mM DTT, and 0.2 mM PMSF) and stored at –80°C. The 50 μl extracts were diluted 1:1 with distilled H_2O. The assay mixture (100 μl of 1 mM firefly luciferin (SIGMA L 9504) and 20 mM ATP in the same reaction buffer) was automatically injected, and light emission was integrated for 10 seconds at 18°C in a photometer (Lumat LB 9501, Berthold). Background levels measured on reaction buffer never exceeded 150±20 RLUs (relative light units). Therefore 170 RLUs were substracted from all sample measurements before calculating the mean luciferase activity. Under our conditions, 1 RLU corresponded to 1.5 fg of purified luciferase (Sigma L9009). The stability of purified luciferase was determined by injecting a small amount (10-13 pl, volume of cytoplasmic injection estimated by Brinster et al., 1985) of a 0.1 mg/ml solution into the cytoplasm of 1-cell zygotes in order to obtain a level of luciferase activity comparable to the maximum obtained in transgenic embryos. Under these conditions, the half life of luciferase was less than 2 hours as the mean activity dropped from 3600±1254 RLUs to 116±2 RLUs, 2 to 4 hours post injection.

RT-PCR experiments

Eggs (ovulated oocytes) were produced as described above from superovulated F_1 hybrid (C57BL6×CBA) females. One-cell embryos were obtained after mating superovulated females with normal F_1 hybrid (C57BL6×CBA) males and cultured in vitro until analysis. In vivo 2-cell embryos were directly recovered from females at 42 hours post hCG. Batches of 100 eggs or embryos were frozen and stored at –80°C until assayed.

To limit loss of RNA, no purification was undertaken, and batches of eggs or embryos were simply lysed by heating them at 100°C for 1 minute in 0.4% NP-40, 1.5× MMLV-RT buffer (Gibco/BRL). To detect specifically HSP70.1 transcripts it was necessary to overcome the following difficulties: the high nucleotide sequence homology of the *hsp 70* family, and the absence of introns that increases the probability of amplifying contaminating DNA. Therefore we performed

RS-PCR (RNA Template-Specific PCR, Shuldiner et al., 1991), in which a tagging primer of poly T plus a linker sequence (5′-18xT-GCGGCCGCGGCGC-3′) was utilized to synthesize the cDNA, and the primers added in the PCR mix were a 5′ primer specific to the 3′ non coding region of the *HSP70.1* gene (5′-AGCAGCGTGCACTGT-3′) and a 3′ primer corresponding to this linker (5′-GCGCCGCGGC-CGC-3′). Egg or embryo lysates were divided into two parts and one part was supplemented with 100 ng of oligo dT primer, 0.2 mM dNTP, 1U RNasin and 100 U of MMLV reverse transcriptase (Gibco/BRL) in a final volume of 10 μl to synthesize the cDNA. After 30 minutes of incubation at 37°C, a further 100 U of reverse transcriptase was added and the reaction was terminated by a 10 minute incubation at 95°C. The same reaction was carried out on the other part of the lysate but in the absence of the MMLV enzyme to control for false positive PCR amplification of contaminating DNA. The 10 μl reaction mixture containing the synthesized cDNA was then PCR amplified in a final volume of 50 μl (1× Taq polymerase reaction buffer, 0.1 mM dNTP) in which 50 pmol of the 5′ *HSP70.1* specific primer, 50 pmol of the 3′ primer complementary to the linker coupled to the oligo dT and 0.9 U of Taq polymerase (Bioprobe) were present. Samples were denatured at 94°C for 7 minutes and then subjected to a series of amplification cycles (94°C, 30 seconds; 60°C, 30 seconds; 72°C, 1 minute) in a DNA Thermal Cycler 480 (Perkin Elmer Cetus). Aliquots of the amplified mixture corresponding to 10 eggs or embryos were run on 2% agarose gels and blotted to Hybond N+ membranes (Amersham). Hybridization was carried out overnight using a ^{32}P-labelled *HSP70.1* DNA probe and autoradiographic exposure was for 1 hour at −80°C. Quantitation analysis was performed using a PhosphorImager system (Molecular Dynamics) and the supplied software (ImageQuant, Molecular Dynamics). Signal intensity was estimated by the volume integration method, subtracting the background level surrounding the appropriate position.

Radiolabelling of embryos and two-dimensional gel electrophoresis

Non-transgenic embryos obtained from superovulated F_1(C57BL6×CBA) females were cultured until the indicated stage, when groups of 20-30 embryos were radiolabelled by incubating them in microdrops of medium containing 1.5 mCi/ml [^{35}S]methionine for 3 hours at 37°C. At the end of the radiolabelling period, they were rinsed in a 0.9% NaCl solution supplemented with 0.4% polyvinylpyrrolidone and stored with a minimum of medium in Eppendorf tubes at −20°C.

Between 105 and 185×10^3 c.p.m. were applied to each gel. Non-equilibrium pH gradient electrophoresis (NEPHGE) was performed according to a modification of the protocol described by O'Farrell et al. (1977) (Richoux et al., 1991; Vernet et al., 1993). The resulting gels were fixed, dried and exposed to Hyperfilm-TM (Amersham) for 5-15 days.

Signal intensity was estimated by the volume integration method, subtracting the background level surrounding the appropriate position. To take into account the level of [^{35}S]methionine incorporation in embryos, the volume (in pixels) measured for each polypeptide was divided by the number of c.p.m. loaded on the gel. This was the resulting value we used to compare the patterns of protein synthesis obtained with the different types of embryos.

RESULTS

Transcriptional activity of the HSP70.1 promoter during preimplantation development

RT-PCR experiments were carried out to demonstrate that the regulatory region of the *HSP70.1* gene is activated at the beginning of development, in particular, at the 2-cell stage. We first examined the transcriptional activity of the endogenous gene by examining the presence of HSP 70.1 mRNA in embryos at different preimplantation stages and in eggs (Fig. 1A,B). Twenty-five amplification cycles were sufficient to detect HSP 70.1 cDNA corresponding to the transcripts from the equivalent of 10 eggs or embryos. To verify that the different signals observed between stages were not due to amplification artefacts, egg, 2-cell embryo, and blastocyst extracts were each subjected to 20, 25, and 30 cycles of amplification (Fig. 1C). The signal given by 2-cell embryos was several orders of magnitude higher than those obtained from other stages and this result was reproduced in each replicate. A faint signal was obtained with embryos at the 4-cell (Fig. 1A), 8-cell, morula (data not shown) and blastocyst stages (Fig. 1B), and a strong signal was produced by blastocysts (Fig. 1B) that had been heat shocked.

To confirm that the marked burst of transcriptional activity observed at the 2-cell stage was dependent on zygotic transcription, embryos were cultured in the presence of α-amanitin from the 1-cell stage (30 hours post hCG) under conditions that have been shown to inhibit zygotic transcription (Kidder et al., 1985). Under those conditions, 2-cell embryos displayed a signal comparable to that observed in eggs (Fig. 1A,C). To assess whether *HSP70.1* transcriptional activity was an artefact of in vitro culture, in vivo 2-cell embryos were collected directly from females. These embryos gave a 5 times higher signal than that found in eggs (Fig. 1A,C) though this was 15 times lower than the signal generated by embryos cultured in vitro from the 1-cell stage. These results show that although in vitro culture modulates the level of *HSP70.1* expression, the gene is transcribed in vivo.

From these data, we conclude that the *HSP70.1* gene exhibited a profile of expression similar to that described for the hsp 70 family of proteins as determined by two-dimensional electrophoretic analysis (Bensaude et al., 1983; Morange et al., 1984). This strongly suggested that *HSP70.1* was one of the genes expressed in vivo at the onset of zygotic genome activity.

Profile of HSP 70.1 *Luc* expression in transgenic embryos during the preimplantation period

We then established three lines of transgenic mice from C57BL6×CBA F_1 embryos using the 7.2 kb *Sca*I linearised Bluescript plasmid HSP70.1 *Luc* (Fig. 2A). These lines, which contained 2 to 3 copies of the transgene per haploid genome, were bred to homozygocity. They all presented a profile of expression (Table 1) that clearly reproduced that of the hsp 70 family during mouse preimplantation development, that is, a marked constitutive expression at the 2-cell stage and inducible expression from the blastocyst stage onwards. Quantitative measurements of luciferase activity in (C57BL6×CBA) F_1 eggs fertilized by F_2-F_4 transgenic males showed that mean constitutive expression was at least 9 to 27 times higher at the 2-cell stage than at the 4-cell stage (lines nos. 1 and 30) and either nonexistent or very low at the blastocyst stage. A dramatic increase was obtained at the blastocyst stage following heat shock (857, 1293 and 60 fold for lines nos. 1, 14 and 30, respectively). Since the main characteristics of HSP70.1*Luc* expression were similar in each of the 3 lines, only results obtained with line no. 1 are further presented.

The expression profile from the end of the 1-cell stage (30

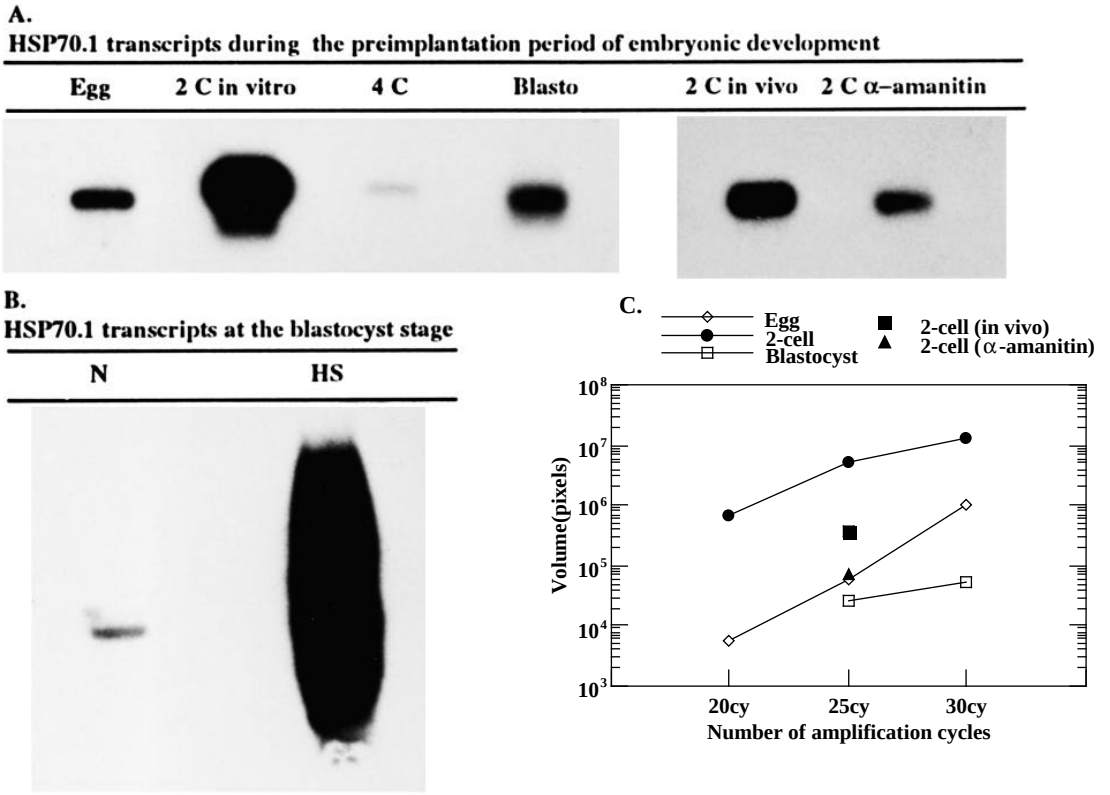

Fig. 1. Preimplantation profile of HSP70.1 mRNA transcripts. Preimplantation mouse embryos or eggs (ovulated oocytes) were obtained in groups of 100 from superovulated females and cultured in vitro until the indicated stages, or obtained directly from females at 42 hours post hCG. RT-PCR experiments were performed as described in the Materials and Methods. (A) Constitutive expression of HSP70.1: ovulated oocytes (Eggs, 30 hours post hCG); in vitro 2-cell stage (42 hours post hCG); 4-cell stage (65 hours post hCG); blastocyst stage (Blasto, 96 hours post hCG); in vivo 2-cell stage (42 hours post hCG); α-amanitin 2-cell stage (42 hours post hCG). Each lane represents the signal obtained from 10 eggs or embryos. (B) Blastocyst stage: constitutive expression (N); heat induced expression (HS: 43°C, 30 minutes) after a 5 hour recovery period (37°C). Each lane represents the signal obtained from 8 embryos. (C) Signal volume obtained following hybridization of RT-PCR products (equivalent to 10 eggs or embryos) as determined by phosphoimaging analysis. Data are plotted logarithmically for PCR amplification of 20, 25, and 30 cycles following reverse transcription of transcripts contained in eggs, 2-cell embryos and blastocysts.

hours post hCG) to the blastocyst stage (112 hours post hCG) is presented in Fig. 2B. At the 1-cell stage about 10% of the embryos with fully developed pronuclei exhibited luciferase activity (responders) above the background level (128±32 RLUs, *n*=8). When we proceed to a pick-off experiment where luciferase assays were carried out on embryos that were at the 2-cell stage for less than 1 hour (in order to estimate luciferase activity already present at the 1-cell stage), we found that these few responders cleaved according to the normal timing (32-37 hours in our culture conditions) (data not shown). This indicated that these embryos were not abnormal ones in which cleavage could have been uncoupled from the cell cycle. Taken

Table 1. Expression of HSP70.1*Luc* transgene

Line (no.)		Number of copies	Constitutive expression 1-cell	2-cell	4-cell	Blastocyst	Inducible expression Blastocyst
1	homoz	3	15.6±6.4 (65)	501±23 (389)	25±78 (203)	8±1 (79)	6855±313 (218)
14	homoz	2	9±4.9 (78)	325±28 (102)	36±10 (20)	2.4±0.05 (21)	3104±317 (86)
30	homoz	3	40.3±23.2 (40)	721±42 (139)	26±10 (37)	91.8±15 (93)	5502±317 (86)

Transgenic embryos were cultured from the 1-cell stage (24 hours post hCG) and assayed at the 1-cell stage (30 hours post hCG), the 2-cell stage (42 hours post hCG), the 4-cell stage (65 hours post hCG) and the blastocyst stage (112 hours post hCG) to measure the constitutive expression. The inducible expression was determined from blastocysts that were exposed to heat shock (43°C, 30 minutes) and then allowed to recover at 37°C for 5 hours.

Values are: mean luciferase activity (RLUs ± s.e.m.); numbers in parentheses, number of embryos measured.

together these data clearly demonstrated that a zygotic transcriptional activity is already turned on at the 1-cell stage.

The proportion of responders was high (up to 90-100%) during the 2-cell stage, dropped back to 25% at the 4-cell stage and to less than 10% at the blastocyst stage. The profile of HSP70.1*Luc* increased from the end of the 1-cell stage (30 hours post hCG) to the 2-cell stage (41-44 hours post hCG) where the maximal level of activity was attained (502 RLUs). Thereafter, there was a continuous and rapid decrease leading up to the 4-cell stage.

Similar expression profiles were observed with in vivo collected embryos, though the level of luciferase activity was reduced about 5-fold for each point analysed. In other words, some parameters linked to our conditions of in vitro culture seemed to induce the HSP70.1 transcriptional activity. One of these parameters could be the oxidative stress phenomenon associated with in vitro culture (Nasr-Esfahani et al., 1990) because it is known to modify the embryonic transcriptional activity (Vernet et al., 1993) and to affect the expression of *hsp 70* genes (Donati et al., 1990). To test this hypothesis, we used an antioxidant, CuZn-superoxide dismutase (CuZn-SOD), and compared the hybrid gene expression in the presence and absence of the enzyme (Table 2). The level of luciferase activity of transgenic 2-cell embryos developed in vitro was significantly reduced in the presence of CuZn-SOD ($P \leq 0.05$) but still remained higher than in 2-cell embryos developed in vivo.

Relationship between time post cleavage and the pattern of HSP 70.1*Luc* expression

The above results showed that increased luciferase activity was limited to the early 2-cell stage and followed by a rapid drop suggesting the establishment of a repressive mechanism. In order to examine this aspect more precisely we measured luciferase activity on individual embryos at 42 hours post hCG following an hourly pick-off after completion of the first mitosis. Since cleavage occurred primarily over a period of 5 hours (from 32 to 37 hours post hCG, when 100% of the embryos were at the 2-cell stage), luciferase assays were performed from the 6 th to the 10 th hour of the second cell cycle. The mean level of activity calculated from the pooled data (x=583±32 RLUs, n=332) was not significantly different from that obtained with asynchronous embryos (x=501±23 RLUs, n=389, Table1) demonstrating that under our experimental conditions, repetitive pick-off of a given batch of embryos did not alter their expression.

During the first 10 hours post-cleavage (Fig. 3), luciferase activity consistently increased, reaching a level of 1200 RLUs. As the 2-cell stage lasted about 30 hours, this suggested that HSP70.1 transcription was restricted to the first third of this stage. Because of the sensitivity and short half-life of the reporter gene product (2 hours, see Materials and Methods), we considered that the variations of luciferase activity corresponded to the variation of transcriptional activity. We thus decided to determine the length of this period of HSP70.1 transcriptional activity at the 2-cell stage by adopting the following procedure (Fig. 4A): groups of embryos were transferred into culture medium containing α-amanitin at different times from

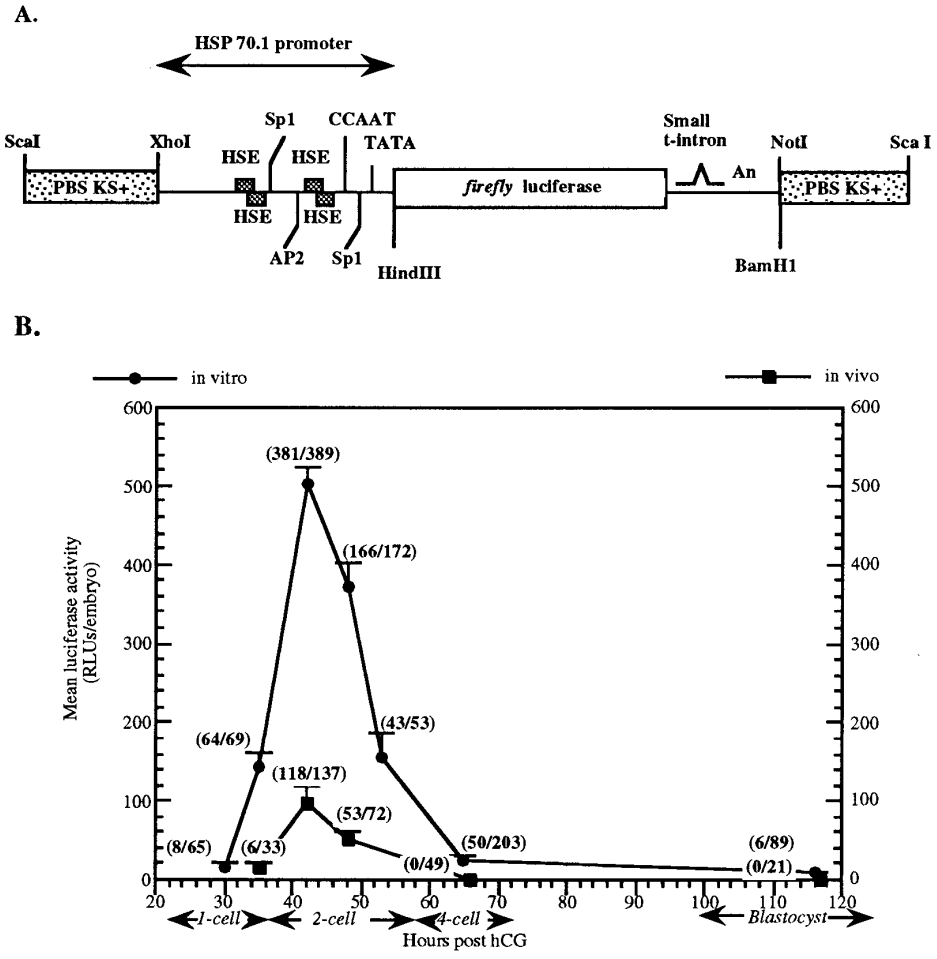

Fig. 2. Constitutive expression of the HSP70.1*Luc* transgene. (A) Structure of the HSP70.1*Luc* construct. The different segments of the hybrid gene were as follows: plasmid sequences from the Bluescript (PBS KS+, Stratagene) vector (stippled boxes); the HSP70.1 promoter containing 4 tandemly arranged heat shock elements (HSE) bound by specific heat shock factors, an inverted CCAAT box, TATA box, 2 Sp1 binding sites, and one AP2 binding site (Hunt and Calderwood, 1990); the cDNA for firefly luciferase; small-t intron, and SV40 polyadenylation signal (DeWet et al., 1987). (B) Heterozygous transgenic embryos were cultured from the 1-cell stage (22-24 hours post hCG) until luciferase assay at the indicated times, or collected directly from females at the corresponding time. The corresponding developmental stages are noted. For each point, the number of responders and the total number of embryos that were assayed for luciferase activity are shown in brackets. The level of expression is given in relative light units (RLUs) as mean luciferase activity after deduction of background. Error bars denote the standard error of the mean.

Table 2. Effects of developmental conditions on HSP70.1*Luc* expression in 2-cell transgenic embryos (42 hours post hCG)

	Expression of HSP70.*1Luc* transgene	
Embryos developed	Mean luciferase activity ± s.e.m. (total number of embryos assayed)	Percentage expression
in vitro		
M16 (control)	576±57	100
M16 + CuZn-SOD	440±39.3*	76.4
(600 IU/ml)	(66)	
in vivo	130±15.6**	17.4
	(63)	

t test: **P*≤0.05; ***P*≤0.001.

cleavage, and luciferase activities were calculated relative to those found in non α-amanitin treated (controls) embryos. The following time groups were considered: 2 hours before cleavage (when the pronuclei were no longer visible, group designated −2 hours) or every 2 hours up to 8 hours after cleavage (groups designated as 0, +2, +4, +6 and +8 hours post division). To take into account variability in the timing of cleavage among embryos, luciferase assays were performed at 42, 48 and 53 hours post hCG on groups of early, mid, and late cleaving embryos. Since no differences were noticed between the relative activities of these groups of embryos data were subsequently pooled.

When embryos were exposed 2 hours before their first cleavage, their luciferase activity was reduced to less than 15% of controls (Fig. 4B). Increasing the time between first cleavage and exposure to the drug resulted in a progressive increase in the luciferase activity measured in the α-amanitin-

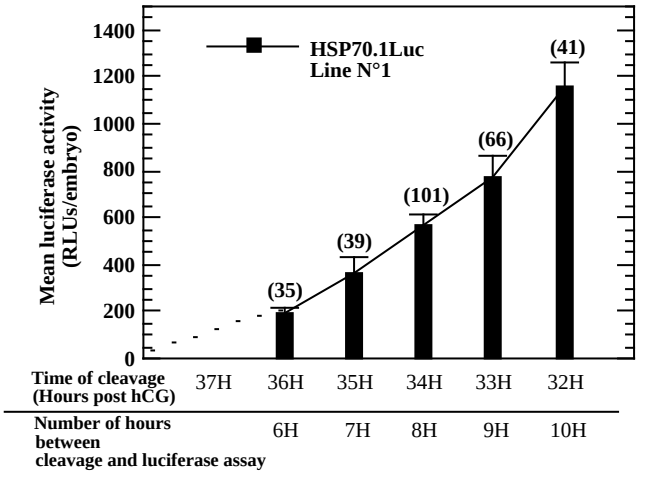

Fig. 3. HSP70.1*Luc* expression versus time post first cleavage. Transgenic 1-cell zygotes were produced, recovered and cultured as described in the Materials and Methods. They were examined briefly at 1-hour intervals from 31 hours post hCG to 37-38 hours post hCG. Two-cell embryos formed within the previous hour were picked-off and cultured separately until luciferase assay (42 hours post hCG). The mean level of luciferase activity was determined relative to time post cleavage from three replicate experiments. The number of embryos assayed at each interval is indicated above each bar. Error bars denote the standard error of the mean

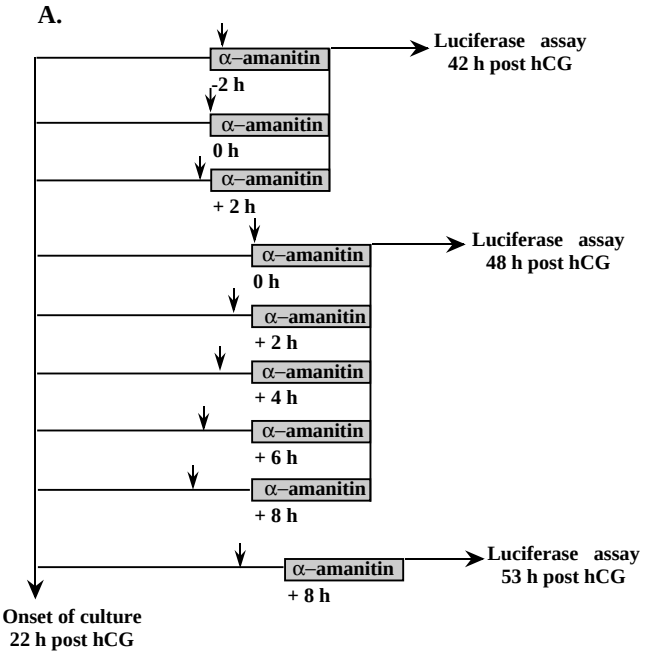

Fig. 4. Effect of timed transcriptional inhibition on transgene expression. (A) Schema of the experimental procedure. Transgenic embryos were collected, cultured in M16 medium and synchronized by pick-off. The period of culture in normal medium is indicated by a thick line. The time where cleavage and thus pick-off procedure have occurred is shown as a vertical arrow. At various intervals post division, embryos were transferred to M16 supplemented with 100 µg/ml α-amantin, cultured for 8 hours (stippled bars) and then assayed for luciferase activity. The earliest exposure of embryos to α-amanitin was at 31 hours post hCG when they were still at the 1-cell stage. Control embryos were maintained in normal medium and assayed according to the same schedule. (B) Mean luciferase activity was calculated for treated and control embryos from pooled data of five replicate experiments. Transgene expression in treated embryos was plotted relative to that of control embryos as a function of the time interval between first cleavage and exposure to α-amanitin. The number of embryos assayed at each interval is noted in parantheses.

treated group. When embryos began exposure to α-amanitin at 8 hours post cleavage, they exhibited the same level of luciferase activity as the control ones. From these data we concluded that under our culture conditions HSP70.1 tran-

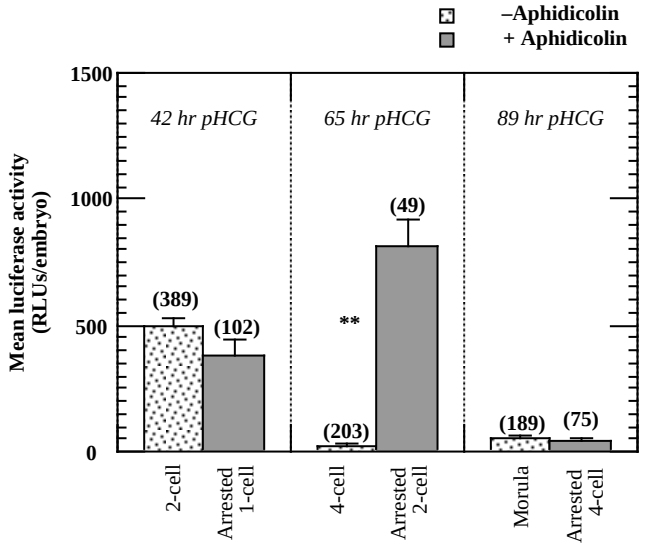

Fig. 5. Constitutive expression of the HSP70.1*Luc* transgene in embryos blocked in S phase. Transgenic embryos were collected at 22-24 hours post hCG and cultured in the presence or absence of aphidicolin until luciferase assay. One-cell blocked embryos were cultured in aphidicolin (0.2 μg/ml) from 22 to 42 hours post hCG, two-cell blocked embryos were incubated in aphidicolin from 30 to 65 hours post hCG, and 4-cell blocked embryos were treated with aphidicolin from 53 to 89 hours post hCG. Control embryos, cultured without aphidicolin were harvested at 42, 65, and 89 hours post hCG.

scriptional activity was restricted to the first 4-6 hours post cleavage.

Effect of S phase arrest on the transcriptional activity of HSP 70.1

When pronuclear embryos were blocked at the first S phase by aphidicolin, (Fig. 5) the level of luciferase activity measured 42 hours post hCG was not markedly modified. When blocked at the second S phase (following exposure to the drug starting 30 hours post hCG), luciferase activity was increased 16-fold compared with controls that had progressed to the 4-cell stage at the time of measurement (65 hours post hCG). This was not observed at the third S phase, in which 4-cell embryos were blocked following exposure to the drug beginning at 53 hours post hCG. The significant effect obtained at the second S phase was due to the action of aphidicolin and not to the solvent used for the addition of the drug to the medium (0.4% DMSO), since no stimulation was observed when embryos were exposed to the same concentration of DMSO alone (data not shown). Thus, in the absence of passage through the second S phase, constitutive expression of HSP70.1*Luc* was not repressed as it was when 2-cell stage embryos develop normally.

Two dimensional gel electrophoresis of 2-cell blocked and control embryos (Fig. 6A,C) confirmed that blocking the second round of replication also affected endogenous *hsp 68-70* gene(s) expression, which remained elevated in 2-cell blocked but not in control 4-cell embryos (Fig. 6D). Relative quantification of the different zygotic products (hsc 70, hsp 70, TRC) synthesized at the 2-cell stage (Fig. 6B) indicated that the link between repression of constitutive HSP70.1 expression and the second round of replication is not applicable to the TRC complex nor to the hsc 70 polypeptides, the expression of which were only marginally, or not at all, influenced by aphidicolin incubation.

DISCUSSION

Identification of a specific inducible hsp during preimplantation mouse development

Since the work of Flach et al. (1982) and Bensaude et al. (1983), numerous studies have considered the synthesis of the different 70×10^3 M_r polypeptides as markers for the onset of zygotic genome activity (Howlett, 1986; Howlett et al., 1987; Wiekowski et al., 1991; Vernet et al., 1992). Various designations have been used to describe this synthesis such as putative hsp, hsp-like or TRC for 'transcription requiring complex' (Schultz, 1986; Howlett, 1986; Poueymirou and Schultz, 1987; Latham et al., 1991a,b, 1992). Moreover, contradictory data were obtained concerning the constitutive synthesis of an inducible hsp 70 protein since Hahnel et al. (1986), using in vivo 2-cell stage embryos, found neither mRNAs on northern blots nor a 68×10^3 M_r protein band on one-dimensional gels, and Kothary et al. (1989) were unable to detect *lac*Z expression in transgenic (HSP68-*lacZ*) 2-cell embryos.

Our data clearly demonstrate that the onset of zygotic genome activity is marked by the expression of the *HSP70.1* gene, the major inducible heat shock gene (Hunt and Calderwood, 1990). This is supported by RT-PCR detection of mRNA transcripts from the *HSP70.1* endogene that show a transient 6-fold α-amanitin-dependent increase over a basal activity at the 2-cell stage, and a high inducible transcription after a heat treatment at the blastocyst stage. This pattern is similar to that obtained for polypeptides identified as hsp proteins from peptide mapping analysis (Bensaude et al., 1983; Morange et al., 1984). Further evidence was obtained with the promoter sequence included in the HSP70.1*Luc* hybrid gene used in transgenic lines. This promoter contained the minimal regulatory sequences required to direct preimplantation expression since luciferase activity was constitutively high at the 2-cell stage and strongly inducible at the blastocyst stage. As three different transgenic lines have displayed comparable expression profiles, the site of integration does not appear to be of major significance in our observations.

In contrast to the data of Hahnel et al. (1986), our results show that the *HSP 70.1* gene is actually expressed during normal embryonic development and thus does not correspond to an artefact linked to in vitro manipulation as hypothesized by Johnson and Nasr-Esfahani (1994). The level of expression obtained after in vitro culture from the 1-cell stage was, however, about 5 times higher than that observed from in vivo embryos, revealing that our conditions of in vitro culture exert some inductive effect on the HSP70.1 promoter. Recently, in vitro culture conditions were also shown to affect the transcriptional activity of hybrid transgenes during early development (Kothary et al., 1992; Vernet et al., 1993). Involvement of oxidative stress was clearly shown in the case of an HIV*lacZ* transgene since the addition of a reducing agent (superoxide dismutase, CuZn-SOD) to the culture medium completely abolished its expression (Vernet et al., 1993). In contrast, these authors were unable to establish any correlation between such

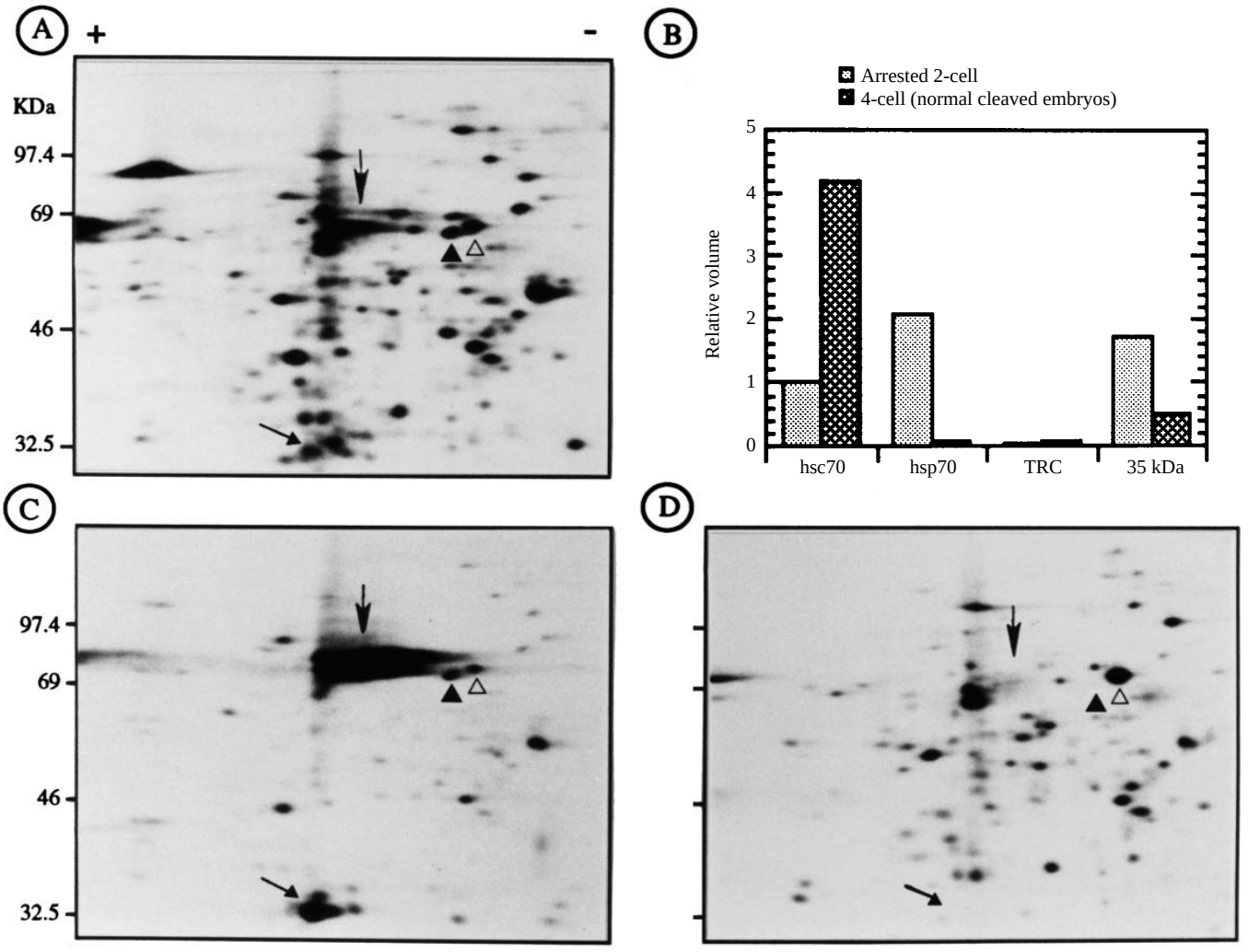

Fig. 6. Polypeptide expression in C57BL6×CBA embryos. (A) Two-cell embryos blocked in S phase (65 hours post hCG) by incubation in aphidicolin from 30 hours post hCG. (B) Quantification of the protein synthesis level by phosphoimaging. Data are shown as the volume (pixels) measured in 2-cell blocked embryos or 4-cell embryos relative to those measured in control embryos (see Materials and Methods). **(**C) Control 2-cell embryos (42 hours post hCG) (D) Control 4-cell embryos (65 hours post hCG). Large arrow, TRC complex; small arrow, 35×10^3 M_r complex; open arrowhead, hsc 70; solid arrowhead, hsp 70.

an oxidative stress and the hsp 70 synthesis at the onset of zygotic genome activation. Nevertheless their experimental approach (2-D gel electrophoresis) did not allow them to estimate a quantitative effect of such a stress. Using similar culture conditions but a quantitative reporter gene approach, we obtained a significant but not so drastic reduction of HSP70.1*Luc* expression at the 2-cell stage. Thus our data show that, during a short period of culture, the use of CuZn-SOD can only modulate the expression of this gene. Since the ability of CuZn-SOD to remove intracellular superoxides has not been demonstrated (Johnson and Nasr-Esfahani, 1994) it could be considered that the protective effect of CuZn-SOD against oxidative stress was only partial. Therefore our results suggest that other mechanisms (e.g., damage induced by the presence of free transition metals in culture medium) must be involved in order to explain the stimulation of HSP70.1*Luc* hybrid gene expression in embryos developed in vitro (Johnson and Nasr-Esfahani, 1994 and references therein).

Constitutive expression of HSP 70.1 reveals the onset of zygotic genome activity at the 1-cell stage

Our work demonstrates that transcriptional activity of the HSP70.1 *Luc* hybrid gene begins during G2 of the first cell cycle. In all transgenic lines, luciferase activity was detected in pronuclear embryos (30 hours post hCG) obtained by mating homozygous transgenic males with normal F_1 (C57BL6×CBA) females. Thus, the male pronucleus exhibits very early transcriptional capacity, following remodelling of the highly condensed chromatin of the spermatozoa. Early zygotic expression was first suspected by Clegg and Pikò (1982, 1983). It has been evidenced also from transient reporter gene expression (Vernet et al., 1992; Ram and Schultz, 1993). However, these experiments are based on the injection of constructs directly into male pronuclei and thus the measured activity resulted mainly from the transcription of non-integrated sequences (Brinster et al., 1985). Furthermore, the relatively large quantities of injected DNA delayed cleavage and

resulted in lower rates of blastocyst formation. Recently acquisition of a transcriptional state during the 1-cell stage has been demonstrated by nuclear transplantation and analysis of 'TRC' expression (Latham et al., 1992) but this approach involved extensive manipulations of the embryos. In comparison, the HSP70.1*Luc* transgenic embryos represent a particularly well adapted tool for analysis of gene regulation during the earliest phases of embryonic transcription.

Transcription of HSP 70.1 is repressed prior to completion of the second round of replication

In agreement with qualitative analysis of hsp 68-70 protein synthesis (Flach et al., 1982; Bolton et al., 1984), our results in the presence and absence of α-amanitin show that active zygotic synthesis of HSP70.1 *Luc* is restricted to the first 4-6 hours of the second cell cycle. This corresponds to the transition to S phase (Bolton et al., 1984) and suggests that the second round of replication, or some concurrent event(s), represses HSP70.1 expression. This repressive event would mark the end of the first burst of zygotic transcription. This conclusion is substantiated by the correlation we observed between aphidicolin induced arrest in S phase and maintenance of HSP70.1 expression. There is some evidence in the literature concerning modulation of *hsp 70* gene expression in relation to S phase during cell growth. Kao et al. (1985) and Milarski and Morimoto (1986) have reported increased activity of the human *hsp 70* gene during, or at the end of S phase. However it is apparent from our results that blockading in S phase *per se* did not sustain high constitutive expression of the HSP70.1*Luc* hybrid gene and that time elapsed from fertilization ('zygotic clock') was not sufficient to define repression of this gene.

When embryos are arrested in their second S phase, the appearance of embryo-encoded late 2-cell polypeptides and the disappearance of maternally coded early 2-cell polypeptides are roughly maintained (Flach et al., 1982; Bolton et al., 1984). Reduced HSP70.1 expression at the 4-cell stage was probably not due to the disappearance of maternally transcribed transcription factors as microinjection of the HSP70.1*Luc* plasmid at the 2-cell stage resulted in transient expression at the 4-cell stage (data not shown). Alternative hypotheses to explain the repression of HSP 70.1 expression during the 2-cell stage would involve repressor trans-acting factors or the establishment of a repressive chromatin structure. Our results of transient expression at the 4-cell stage cannot distinguish among these possibilities, as microinjection of the regulatory region of *HSP 70.1* gene might either saturate the repressor or provide a template that escapes genomic chromatin structure.

The first burst of transcription: a regulated step in zygotic genome activation

Our results suggest that the first burst of transcription could be described as a period beginning at the 1-cell stage with the acquisition of transcriptional competence and ending with the second round of DNA replication. The narrow window of intense expression exhibited by *HSP 70.1* reveals the presence of regulatory mechanisms during this early phase of the onset of zygotic genome activity. The second round of DNA replication marks the end of this phase and the beginning of the second major phase of zygotic genome activity (Rothstein et al., 1992; Flach et al., 1982; Bolton et al., 1984). Whether other members of the group of 38 polypeptides shown to be transiently expressed during the 2-cell stage follow the same pattern of regulation is at present unknown (Latham et al., 1991b). Our preliminary data from 2-D gel analysis indicate that this is not the case, suggesting that differential regulative events controlling zygotic transcriptional activity are already operative in the mouse embryo at the 2-cell stage.

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