CMLS, Cell. mol. life sci. 53 (1997) 168–178
1420-682X/97/020168-11 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Multi-author Review

Developmental control of heat shock and chaperone gene expression*

Hsp70 genes and heat shock factors during preimplantation phase of mouse development

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Abstract. Heat shock genes are found in all organisms, and synthesis of heat shock proteins is induced by various stressors in nearly all the cells forming these organisms. However, a particular situation is noticed for *hsp70* genes in mouse embryos at the beginning of their development. First, spontaneous expression of *hsp70* is observed at the onset of zygotic genome activity. Second, inducible expression is delayed until morula or early blastocyst stages. A better understanding of both these points depends on a more careful analysis of *hsp70* expression in relation to their major regulators, the heat shock factors. In this review, we will see how the development of the preimplantation embryo highlights the complexity of heat shock gene regulation involving *trans-cis* interactions and the cellular and nuclear environment.

Key words. Hsp70; mouse heat shock factor (mHSF); zygotic gene activation; 2-D gel electrophoresis; transient expression; transgene; mouse embryo.

Introduction

Heat shock genes are among the most conserved genes throughout the plant and animal kingdoms and several common features concerning the expression, regulation and function of these genes have been described in numerous organisms. In the past few years, several reviews have analysed the developmental control of these genes in cells and embryos [1–3]. This contribution will focus exclusively on the early mouse embryo and will take into account specific parameters of embryonic development in this mammalian species.

In contrast to frog (*Xenopus laevis*) or fish (zebra fish), the mouse embryo is small, divides very slowly, and cannot be kept under the eyes of the observer from fertilization until full completion of embryonic development because of its implantation in the maternal uterus. Before implantation, there is a 3.5 to 4 day period called preimplantation during which embryos developing from the one cell (zygote) to the blastocyst (about 100 cells) stage are migrating freely in the oviduct to the uterus [4]. Distinctive features of heat shock gene expression are directly linked to major events occurring during the preimplanation phase in mammals: 1) spontaneous *hsp70* expression and the onset of zygotic genome activity (ZGA) which is defined by the appearance of the first zygotic transcripts (reviewed by Schultz in [5]), 2) inducible *hsp70* expression and the formation of the blastocyst which marks the differentiation of two types of embryonic cells forming the inner cell mass and the trophectoderm layer (fig. 1). Since their first description [6], several aspects of expression and control of heat shock genes have been examined in detail using various experimental approaches: analysis of protein synthesis by two-dimensional gel electrophoresis, transcript detection by reverse transcription-polymerase reaction (RT-PCR), gel shift analysis of DNA binding complexes and investigation of transcriptional activity with hybrid genes. It should be pointed out that hybrid genes carrying the coding region of a reporter gene driven by an appropriate 5′-regulatory region offer the opportunity to circumvent the limited amount of material available to study gene expression in the mouse embryo. In particular, in our group we have taken advantage of this reporter approach using the easily quantifiable and labile firefly luciferase which is very useful in following the kinetics of preimplantation gene expression [7]. It allows us to draw a relatively precise profile of gene activity during early development. Afterwards, we will analyse how such a defined *hsp70* expression might be subject to nonspecific and specific regulatory pathways, pointing out involvement of heat shock factors.

* This Multi-author Review consists of two parts: Part I. Plants and nonmammals (published in the previous issue of CMLS, Vol. 53, 1). Part II. Mammals (this issue).
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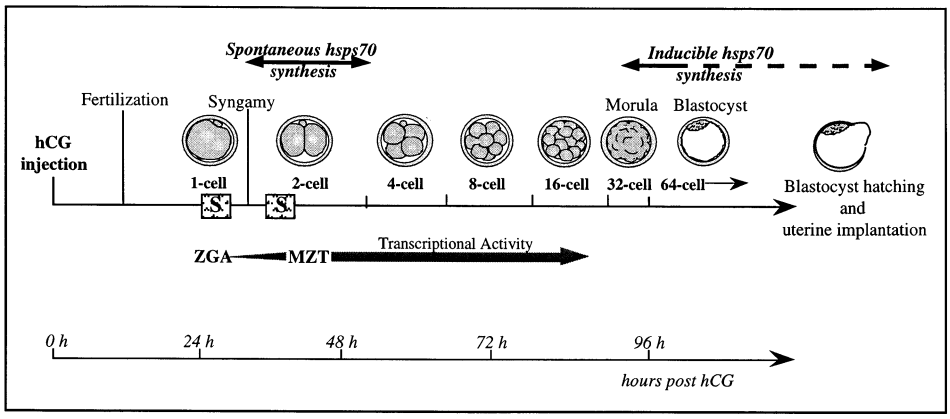

Figure 1. Schematic representation of mouse embryonic preimplantation phase. Timing indicated is given in hours post hCG (human Chorionic Gonadotrophin) injection as mouse embryos are usually obtained from superovulated females. Zygotes or one cell embryos are formed following the fusion of a spermatozoon with an ovulated oocyte. Both maternal and paternal genomes remain separated until they are intermingled during syngamy. Zygotic genome activity occurs at the end of the single cell stage. The two cell stage is long (about 18 hours) and is characterized by spontaneous hsp70 synthesis. Beyond this stage, zygotic transcription is required to sustain further development (MZT, maternal to zygotic control transition). By the eight cell-morula stage, hsp70 synthesis becomes inducible and heat shock response is fully established at the blastocyst stage. Between day 3 and day 4, embryos enter the uterus and bind the mucosa following hatching from zona pellucida.

Characteristics of the 70 kDa heat shock family in mouse

The mouse genome includes several heat shock genes classified into different families on the basis of molecular weight and distinguished according to their inducibility: some members of the heat shock families, such as heat shock cognate (hsc), are constitutively synthesized whereas others (hsp) are only expressed following a stress. A previous review has described in detail the different mammalian heat shock protein families [8] and this paper will deal mainly with the *hsp70* gene family including two inducible (*hsp70.1*, *hsp70.3*) and three cognate (*hsp70.2*, *hsc70t*, *hsc70* genes). Since members of this family have been variously named by different authors, we have summarized the nomenclature of the different genes to avoid any confusion in the following text (table 1).

The regulatory region of heat shock genes is characterized by short sequences named heat shock elements, HSE, which are arranged as contiguous arrays of several units of 5 base pairs (bp): 5′ nGAAn3′. These elements are mainly responsible for heat-induced transcription [9] and are recognized by heat shock factors (HSF), either mHSF1 in response to elevated temperature or mHSF2 after activation by some process of differentiation, e.g. spermatogenesis [10–12]. The promoter region of both inducible *hsp70.1* and *hsp70.3* genes contains two double overlapping HSEs but also a rich array of sequence elements which have been shown to regulate transcription: a TATA-like element, an inverted CCAAT box, and at least two Spl boxes [13, 14]. It should be noticed that *hsp70.1* and *hsp70.3* have only 22 divergent nucleotides in the proximal 500 bp, including all these important regulatory elements. The promoter region of the noninducible testis-specific *hsp70.2* gene shows the same type of regulatory elements but they are differently organized. It includes a TATA-like element, two inverted CAAT boxes, and three Sp1 boxes, and contains a number of potential HSE motifs, targets for mHSF2 rather than mHSF1 [12, 15]. So far there is no complete description of the regulatory regions of the remaining *hsc* genes, *hsc70t* and *hsc70*, in mouse but following a possible homology with the structure of the human *HSC70* promoter, these promoters might also contain a TATA-like element, Sp1 boxes and potential imperfect HSE motifs [16].

Developmental profile of spontaneous expression

Triggering of heat shock gene expression at the onset of zygotic genome activity

The onset of zygotic genome activity (ZGA) is tightly coupled to the synthesis of a large group of 70×10^3 Mr polypeptides including heat shock proteins, since this synthesis is suppressed when transcription is blocked by α-amanitin, an inhibitor of RNA polymerase II [6, 17–19]. Two-dimensional gel electrophoresis is required to resolve this group of 70 kDa polypeptides into its different subgroups: a major complex called TRC or 'transcription requiring complex' which itself contains several components [19] and both hsps70, hscs70 spots closely located to the TRC ones [6, 7] (fig. 2). Such a pattern of protein synthesis has been found in embryos from three different mouse strains as well as in parthenogenotes and androgenotes [20]. This last result interestingly demonstrates that hsps70 and hscs70 expression is the result of transcriptional activity beginning in both maternal and paternal counterparts of the embryonic genome.

To identify further which members of the *hsp70* genes family are expressed at this stage, heat shock transcripts

Table 1.

*Hsp*70 family	Promoter proximal region	Other designations	DNAc[a]	Chromosomal localization[b]
Inducible genes				
HSP70.1	-250 -30 *	*hsp70A2*	MHS 214	17
HSP70.3	-250 -30 *	*hsp70A1* HSP70B1	MHS 243/ MHS 213	17
Cognate genes				
Hsp70.2	-250 -30 *		–	12
Hsp70t	–	–	–	17
Hsc70-74	–	–	–	–

*: TATA box SPI AP2 CAAT double HSE imperfect HSE

[a]Lowe D. G. and Moran L. A. (1986) Molecular cloning and analysis of DNA complementary to three mouse Mr = 68,000 heat shock proteins mRNAS*. J. Biol. Chem. **261:** 2102–2112.
[b]Hunt C. R., Gasser D. L., Chaplin D. D., Pierce J. C. and Kozak C. A. (1993) Chromosomal localization of five murine HSP70 gene family members: Hsp70-1, Hsp70-2, Hsp70-3, Hsc70t and Grp78. Genomics **16:** 193–198.

can be specifically analysed by the reverse transcription-polymerase chain reaction (RT-PCR). Such experiments have demonstrated that the major inducible heat shock gene, called *hsp70.1*, and the cognate *hsc70*, are both highly transcribed at the two cell stage [7, 21]. So far one cannot conclude that *hsp70.1* and *hsc70* are the only 70 kDa hsp genes expressed in mouse embryos as there are no available RT-PCR data for the second inducible gene, *hsp70.3*, and the two testis-specific genes, *hsp70.2* and *hsc70t*. Nevertheless it is now clear that early embryonic hsp expression detected by 2D gel electrophoresis is not due to a specific gene only transcribed at this particular developmental stage as suggested by Burel et al. (1992) [8]. Futhermore, elevated spontaneous transcriptional ability of both *hsp70.1*, and *hsp70.3* promoters has been shown at the two cell stage using the reporter approach (*HSP70.1Luc-hsp70.1* with firefly luciferase [7, 22] or Vargula luciferase [23], p*hsplacZ*-hsp70.3 with *E. coli* β-galactosidase [24, 25]. In fact, analysis of *HSP70.1Luc* expression demonstrates that the hsp promoter is activated as soon as mouse embryos become transcriptionally competent at the end of the one cell stage [7, 26] (fig. 3).

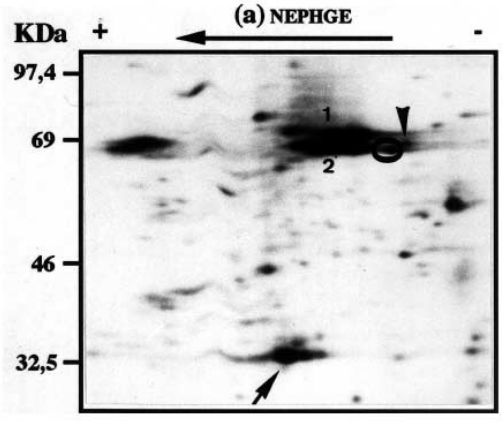

Figure 2. Fluorograms of [^{35}S] methionine-radiolabelled proteins synthesized by two cell stage mouse embryos. Embryos are cultured from the single cell stage (22 h post hCG) to the two cell stage (42–44 h post hCG), when they are radiolabelled for 3 hours. Numbers 1 and 2 designate two main visible portions of transcription requiring complex (TRC). Arrowhead indicates hsc70 and arrow shows some maternal polypeptides dependent on fertilization (35 kDa). Spot corresponding to inducible hsp70 proteins is encircled.

Repression of heat shock gene expression during S phase of the two cell stage

After the onset of zygotic genome activity, there is a clear difference between hscs 70 and hsps 70 expression. Cognate 70 kDa heat shock proteins are continuously synthesized during the preimplantation period and beyond [27, 28]. In contrast, expression of inducible 70 kDa heat shock proteins is repressed and becomes barely detectable as early as the four cell stage [7, 27]. The more sensitive RT-PCR approach has shown that *hsp70.1* transcripts do not completely disappear but remain present at a very low level at all embryonic stages and even in all adult tissues studied [7, 22]. Like endogenous inducible *hsp70*, *HSP70.1Luc* transgenes have exhibited a drastic decrease in spontaneous transcriptional activity at the four cell stage [7, 22] but the p*hsplacZ* transgenes have not [25]. In fact more than 20% of these *LacZ* transgenic embryos are still X-gal positive at the eight cell stage, most probably because of the higher stability of β-galactosidase. Therefore *HSP70.1Luc* transgenic embryos provided us with a convenient tool to study the timing of repression of spontaneous *hsp70* transcriptional activity in mouse em-

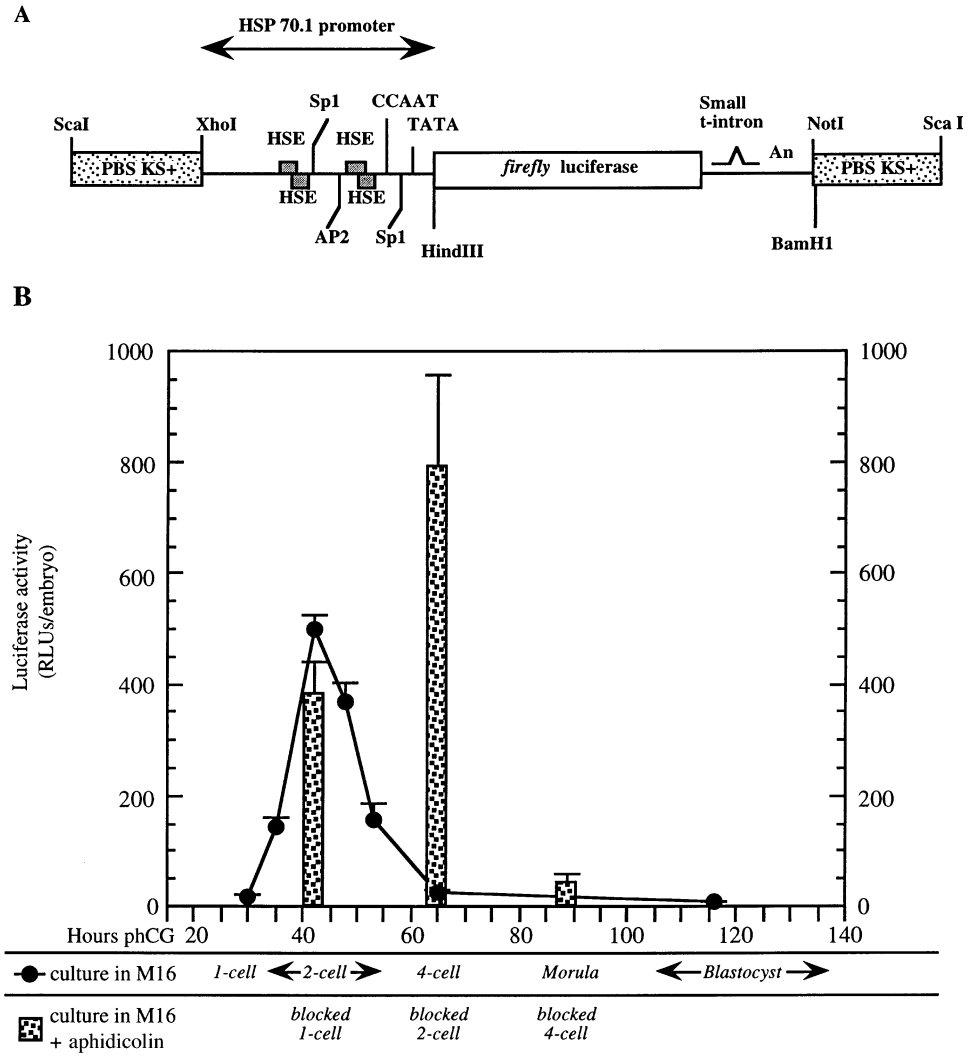

Figure 3. Spontaneous expression of *HSP70.1Luc* transgene. (*A*) Structure of the *HSP70.1Luc* transgene. The transgene corresponds to the whole construct linearized by ScaI including the plasmic sequence (Bluescript KS+) and *hsp70.1* promoter placed upstream of firefly luciferase cDNA [7]. (*B*) Profile of transgene expression measured in hemizygous embryos cultured from the single cell stage in M16 medium (–●–); transgene expression in hemizygous embryos blocked by aphidicolin at various stages (▦).

bryos further. This was performed by collecting embryos from a superovulated F1(C57BL/6 × CBA) female mated with a homozygous transgenic male. Three different types of embryos were assayed for luciferase activity: normal developing embryos which were either nonsynchronized or synchronized by pick-off, and embryos which were synchronized by pick-off and treated at various time with α-amanitin. This enabled us to determine that transcriptional activity ceases about 4–6 hours after the first cleavage which corresponds to S phase at the two cell stage. Furthermore, when replication is inhibited by aphidicolin at the two cell stage, blocked transgenic embryos continue to express the hybrid gene at a high level. This observation strongly suggests that some events occur during this second S phase which are able to repress the spontaneous expression of inducible *hsp* [7] (fig. 3). This repression is apparently not restricted to the heat shock regulatory pathway since, among the 38 polypeptides which are similarly transiently expressed during the two cell stage [29], one identified as the protein translation factor eIF-4C seems to be similarly repressed by S phase during the cell stage [30].

Characteristics of spontaneous *hsp70* gene transcription at the two cell stage

Transcriptional efficiency of *hsp70* genes

As their name suggests, inducible *hsp70* genes are only expressed by cultured cells in response to a thermal stress [13] and it is worth underlining that in these circumstances, general gene transcription and protein synthesis slow down. In contrast, in mouse two cell embryos, inducible *hsps70* are spontaneously and highly expressed at normal temperatures when zygotic tran-

scription is just starting. This raises the question of the transcriptional efficiency of *hsp70* genes in comparison to the other genes activated at this embryonic stage.

The reporter approach used in transient assay is a convenient way to test directly the transciptional efficiency of *hsp70* genes. Supercoiled plasmid is injected at the one cell stage and expression is assayed about 15 hours later at the two cell stage. In these conditions, the highest level of luciferase activity is observed with the *hsp70.1* promoter followed by the *hsp70.2* and *hsp70.3* promoters. In contrast, the *Droso 87CI* promoter give a significantly lower level of luciferase activity [31]. This may reflect the absence of Sp1 motifs which have been proved to be important for transciptional activity in preimplantation embryos [32] (see below). Unfortunately there are only a few data available to compare these *hsp70* promoters with some other types of regulatory regions. Numerous studies by De Pamphilis and colleagues have focussed on the interplay between promoter and enhancer using the polyomavirus (PyV) early gene enhancer and promoter as a model (reviewed in [33] by Nothias et al.). In order to create the most powerful enhancer capable of stimulating PyV promoter activity, they produced several mutated PyV enhancers including a duplicated symmetrical sequence which has a strong homology to the HSE motif [34]. However, even if it is associated with one of the most efficient enhancers, PyV promoter have still exhibited an 8-fold lower activity than the *hsp70.1* promoter (our unpublished data, fig. 4). Therefore it could be concluded that the organization of regulatory motifs in the *hsp70.1* promoter has very efficiently fulfilled all the requirements for transcriptional activity starting in mouse embryos.

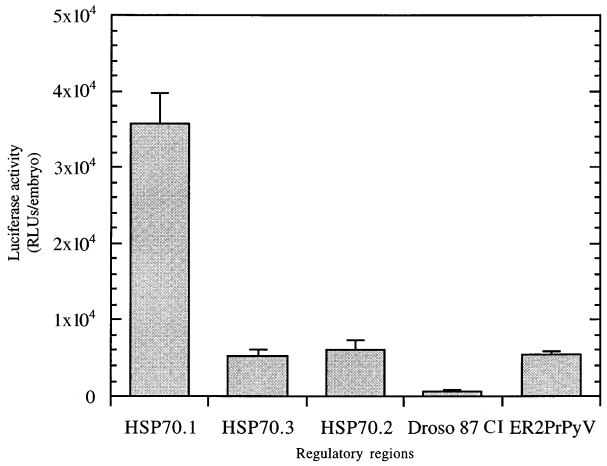

Figure 4. Comparison of promoter strength by transient expression assay. Plasmids used in these experiments include one of the following regulatory regions (*hsp70.1* [13], *hsp70.2* [15], *hsp70.3* [47], *Droso87*CI [31] and *ER2PrPyV* [34] coupled to luciferase reporter gene. Zygotes (22–24 h post hCG) are microinjected with 50ng/µl DNA constructs and then cultured until two cell stage (42–44 h post hCG) when luciferase assay is performed on single embryo. Experiments were repeated at least twice and mean luciferase activity calculated from a minimum of 20 embryos. Errors bars denote the standard error of the mean.

An estimate of the transcriptional efficiency of the endogenous *hsp70* genes may be roughly obtained by comparing the amount of *hsp70.1* and *hsc70* transcripts in two cell embryos untreated and treated with α-amanitin [7, 21]. This relative level is 18-fold higher for the inducible *hsp70.1* than for the cognate *hsp70* gene, but this assessment has to include the possibility of a different turnover of both types of transcripts since postranscriptional regulation of the *hsp70* gene is well established [21, 35]. In fact such postranscriptional regulation in addition to translational control could explain the fact that inducible hsps70 and hscs70 products represent about 0.1% of the total protein synthesis in two cell embryos. This percentage is quite low when it is compared with the two following data: first, TRC at the same embryonic stage are equivalent to 4% of the total protein synthesis [19] and second, stress-induced synthesis of hsp70 proteins can reach 20% of translational activity [36].

Taking into account all these observations, it is obvious that even if *hsp70* promoters have a powerful transcriptional efficiency, hsp70 proteins are not major products of the newly activated zygotic genome.

Modulation of *hsp70* spontaneous transcription

In mouse embryos, switch on-off of inducible heat shock genes is precisely programmed and timed. Nevertheless, we report here several observations indicating that the level of *hsp70* expression can be modulated by both the cytoplasm and embryonic environment.

It has been demonstrated that inducible *hsp70* genes are recruited by the earliest active transcriptional apparatus appearing in mouse embryos. Most of the components of this apparatus which have been stored in the oocyte cytoplasm remain unknown and are globally designated as maternal factors (see below about mHSF). Transcriptional efficiency of zygotic genome is directly linked to the abundancy and composition of these maternal factors which can vary among the different strains of mice. This has been shown in particular by modulation of *hsp70* spontaneous expression: hsp70 synthesis measured at the two cell stage is 2-fold higher in embryos produced by C3H females than those having a BALB/c mother [37]. Once again we do not know if this effect involves a heat shock-specific regulatory mechanism since some other genes and transgenes have shown similar variable expression [38].

The embryo environment itself may influence *hsp70* expression observed at the beginning of development. Preimplantation embryos which normally develop in the oviduct can also grow in vitro in a defined culture medium from the single cell to the blastocyst stage without hampering their further normal development

after transfer into foster mothers. Using various approaches, we have quantified expression of both endogenous and exogenous *hsp* genes in in vitro and in vivo two cell embryos: *hsp70* expression is 5- to 15-fold higher in cultured embryos [7]. It is striking that *hsp70* transcription increases with in vitro culture conditions whereas expression of several other genes decreases [39]. Such cultured embryos have to cope with handling, variations of temperature and a completely different cellular environment. All these parameters might be considered as a source of stress in addition to oxidative stress phenomena directly associated with in vitro culture [40] and might specifically induce *hsp* gene expression by the heat shock regulatory pathway. Nevertheless there is no experimental evidence to support this hypothesis, e.g. DNA binding of heat shock factor.

Evidence of a progressive acquisition of the inducible response during the preimplantation stage

The main characteristic of *hsp70* genes is their ability to respond to a stress with a sharp and rapid increase in their transcriptional activity. This stress inducibility is observed in nearly all cells except some mouse embryonic cell lines and preimplantation embryos [27, 41, 42], and it is also the case for early embryos from various organisms such as *Xenopus*, sea urchin [2].

In mouse, heat inducibility disappears in fully grown oocytes some time before their maturation (progress in meiosis I) whereas they are still fully transcriptionally active [43]. After fertilization, the heat shock response remains largely absent until the blastocyst stage as observed by several groups from protein synthesis analysis [27, 41, 44, 45]. Nevertheless, in some circumstances, others were able to detect an induced hsp70 synthesis after the eight cell stage [46]. In fact, it is not very easy to compare these different studies as different parameters were used to heat shock preimplantation embryos and these heat shocked embryos were not always allowed to recover at 37 °C before their labelling to check neosynthesized proteins.

Considering the different heat shock transgenic lines established so far, all of them (*HSP70.1Luc*, p*hsplacZ*) have displayed a strong, rapid heat shock response at the blastocyst stage [7, 22, 25, 47] as previously described for the *hsp70* endogenous gene. In fact, p*hsplacZ* seems to be already inducible at the eight cell stage according Bevilacqua et al. [25], though this recent result is inconsistent with the previous study of Kothary et al. [47] who could not show any X-gal positive staining at this stage. It could be simply a question of level of expression, which was not investigated in these two studies. Therefore we have reexamined the timing of this response in *HSP70.1Luc* embryos at various stages of development. HSP70.1Luc expression is modified by heat shock at all stages of preimplantation

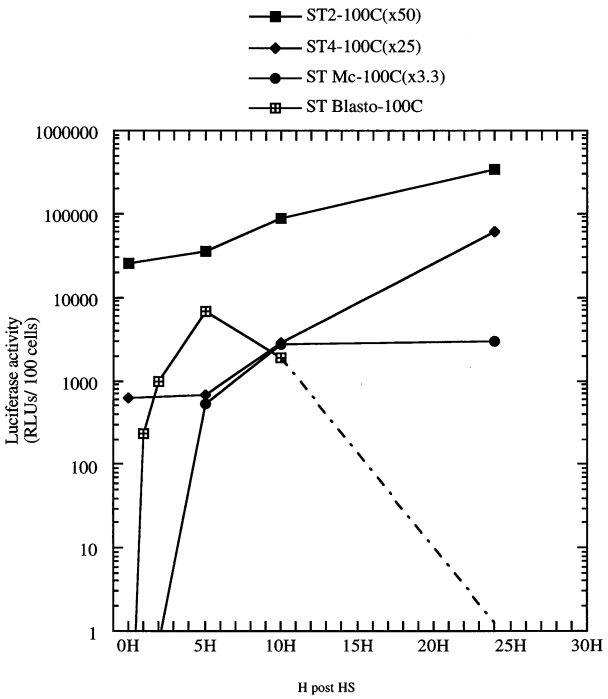

Figure 5. Effect of heat shock on *HSP70.1Luc* transgene activity in preimplantation mouse embryos. Transgenic embryos obtained from normal females mated with homozygous *HSP70.1Luc* male were collected at the single cell stage and cultured until treatment. They were subjected to heat shock (43 °C, 30 minutes) and frozen for the luciferase assay after indicated recovery times at 37 °C. Results are presented as mean luciferase activity calculated for 100 embryonic cells at each stage.

development, but the increase in luciferase activity exhibits different profiles. First, maximal levels attained are higher at the two cell and four cell stages than in morula and blastocyst (when estimated for 100 embryonic cells). Second, the speed with which this maximal level was reached is higher in blastocysts than in the other stages. The blastocyst is the only embryonic stage where a rapid, high and transient elevation of *HSP70.1* transcription rate is observed (fig. 5). Thus both parameters (maximal level of expression, rate of expression increase) used to describe a heat shock response are different at each embryonic stage, and their evolution indicates the progressive acquisition of heat inducibility during the preimplantation period. One could imagine that *hsp70* genes are not as heat inducible at the two cell stage as at the blastocyst stage because they are already highly spontaneously activated. In other words, this suggests that, for example, *hsp70.1* has reached its maximal rate of transcription which cannot increase further. Though this hypothesis is conceivable, it does not account for the absence of inducibility at the subsequent stage (four cell) when this spontaneous transcription is repressed. Furthermore, this hypothesis does not explain the lack of inducibility observed in early embryos from other mammalian species which do not display any spontaneous expression of inducible *hsp* during the beginning of their embryonic development – e.g. rabbit

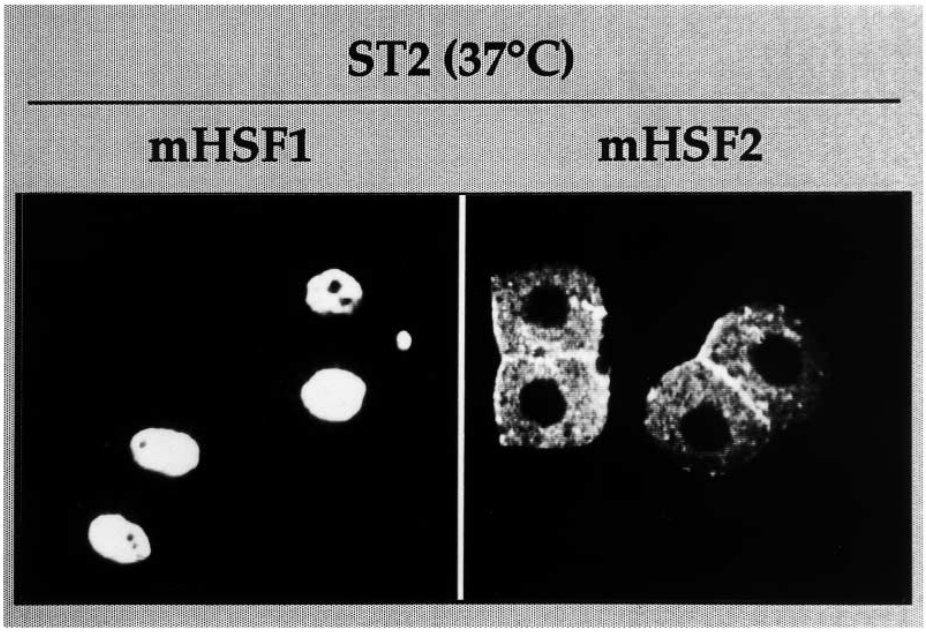

Figure 6. Immunolocalization of mHSF2 and mHSF at the two cell stage. Embryos collected at the one cell stage and cultured normally to the two cell stage were submitted to immunofluorescence analysis using mHSF1 or mHSF2 polyclonal antibodies (kindly provided by K. Sarge and R. Morimoto). Samples were examined with a Zeiss confocal laser scanning microscope.

([2], our unpublished observations). Finally, this spontaneous expression is unable to make mouse two cell embryos thermoresistant as they are irreversibily blocked when they are subjected to heat shock, which is similar to the situation in other mammalian embryos which do not produce hsp70 during the preimplantation period (our unpublished observations).

Different status of mHSF1 and mHSF2 during preimplantation phase

The regulatory region of inducible *hsp* genes is characterized by a nGAAn motif arranged in tandemly inverted repeats called heat shock elements (HSE). Specific transcriptional factors, heat shock factors (HSF) bind these HSE sequences when the cell is subjected to various stressful treatments including heat shock. In mouse, two different genes coding for mHSF1 and mHSF2 have been cloned by Sarge et al. [10]. Even if both these factors are able to recognize the same consensus sequence, they display a limited degree of homology and some difference in terms of cellular amount and localization and in conditions of binding activity (table 2).

During the preimplantation period, several changes can be noticed in both mHSF1 and mHSF2. When specific transcripts are analysed by RT-PCR, *mHSF1* is found to be very abundant in oocyte and zygote in contrast to *mHSF2* which is then barely detectable. The *mHSF2* transcripts appear later at the two cell stage and slightly increase until the end of the preimplantation period, whereas *mHS1* transcripts become comparatively less abundant [47a]. Both *mHSF1* and *mHSF2* transcripts found in mouse oocytes or embryos remain to be further characterized in terms of the different transcript isoforms previously described in various tissues by Goodson et al. [48] and Fiorenza et al. [49]. Immunocytochemistry using specific antibodies for mHSF1 and mHSF2 suggests that protein and transcript abundancies are in good correlation for each mHSF. More interestingly, it shows that the cellular localizations of mHSF1 and mHSF2 markedly differ. In two cell mouse embryos, mHSF1 appears almost concentrated in the nuclear compartment to the exclusion of the nucleoli (fig. 6); at later stages, mHSF1 still exhibits a nuclear labelling but also becomes detectable in the cytoplasm. In contrast, mHSF2 seems to be preferentially localized to the cytoplasm at the beginning of development; when mHSF2 is more abundant by the blastocyst stage, it is found in nucleus as well as in cytoplasm. It should be noted that in the blastocyst, nucleo-cytoplasmic partition of these factors is variable between cells but it is not significantly modified by heat shock treatment. This result is quite unexpected, since a consistent correlation was described between nuclear relocalization of mHSF1 and its role in heat shock response which is clearly observed when embryos reach the blastocyst stage (our unpublished observations).

Taking these data together, it appears that mHSF1 is typically a maternal factor stored in oocytes before ovulation, and therefore present and available in the embryonic nucleus when zygotic genome transcription starts by producing heat shock proteins. Nevertheless, involvement of mHSF1 in early spontaneous transcrip-

Table 2.

	mHSF1			mHSF2		
	mRNA	immunolocalization	DNA binding	mRNA	immunolocalization	DNA binding
M II oocyte	+ (abundant)	ND	+	−	−	−
2-cell	+	+ n > c	+	+	−/+ c > n	−
4-cell	+	+ n/c	−	+	+ n/c	−
blastocyst	+	+ n/c	+	+	+ n/c	+

ND: not determined, +: present, −: undetectable, n: nuclear, c: cytoplasmic.

tion of *hsp* genes remains to be further demonstrated (see below). The second factor, mHSF2, is likely to be an embryonic product dependent on the onset of zygotic transcription. So there is a complex regulatory-pathway controlling first expression of these transcriptional factors and then the spontaneous and inducible expression of their target genes, the *hsp70* genes.

Interaction between HSFs (heat shock factors) and HSE (heat shock element) during preimplantation phase

DNA binding activity

It is well established that HSF specifically binds HSE found in the *hsp* promoter and that this binding is a key step in the regulation of heat shock gene transcription. Gel shift assay and footprinting are both appropriate techniques to assess heat shock element-binding activities (HSE-BA) in cells directly. So far the only study dealing with HSE-BA in preimplantation embryos was made by Mezger et al. [50, 55]. Using a double-stranded consensus oligonucleotide known to be an efficient site for HSF binding, they showed that oocyte and embryo extracts from all stages except the four cell stage contain an inducible HSE-BA and that embryos from the eight cell stage and beyond also display a constitutive HSE-BA. Both inducible and constitutive HSE-BA appear as equal migrating complexes which are are supershifted by specific aantisera for mHSF1 and mHSF2 respectively [51]. It is striking that mHSF2 binding is visible while preimplantation embryos become able to respond to heat shock. There is some indication of cooperation between mHSF1 and mHSF2 to elicit the heat shock response which would be consistent with these data, but this remains to be clearly demonstrated. In addition, Kim et al. [52] have showed in HeLa cells that another constitutive HSE-binding factor, called CHBF, is able to bind the murine *hsp70.1* promoter, limiting its heat inducibility. This CHBF can be easily identified because it produces a faster migrating complex than HSF1. As no such complex has been detected Mezger et al. [50, 51], this factor seems to be absent in the preimplantation embryo.

Transcriptional stimulating activity

Taking into account the absence of any constitutive binding activity involving mHSF1 or mHSF2 at the two cell stage, it might be considered rather unlikely that HSEs have an important part in the regulation of spontaneous *hsp* transcription at this stage. However it could also be suggested that experimental conditions might have not allowed the detection of such a DNA-binding activity at the two cell stage. The two following observations support this hypothesis. The transience of *hsp70* spontaneous transcription can be associated with a very brief DNA binding of heat shock factor which would have already disappeared by the time two embryos have been analysed [50]. The high nuclear concentration of mHSF1 in two cell embryos can facilitate a spontaneous DNA binding in situ, while relative dilution in the total embryonic extract makes this binding unstable in vitro. In any case, these speculative explanations have strengthened the need for experiments addressing this issue directly.

The role played by HSE has been more precisely investigated by Bevilacqua and collaborators [25] using the reporter approach with the *hsp70.3* promoter (p*hsplacZ*). Three different plasmids were injected into mouse zygotes: the native p*hsplacZ* plasmid and two constructs derived form the native plasmid by deleting first, a fragment containing two tetrameric HSEs and one Sp1 binding site (p∂1 p*hsplacZ*) and second, a larger fragment from nucleotide −90 (p∂2p*hsplacZ*). All these constructs are constitutively expressed at the two cell stage but also in growing dictyate oocytes and in transfected granulosa cells. Therefore regulatory motifs located in the first 90 nucleotides (1 CAAT box, 1 Sp1, 1 TATA box) seem to be sufficient for this constitutive expression. These results are consistent with previous observations made with the human promoter in somatic cells [53].

In another attempt to verify the involvement of HSF-HSE interaction in this spontaneous expression of inducible *hsp70*, we have designed experiments to create a competition between microinjected fragments containing the *hsp70.1* proximal regulatory region and the already integrated *HSP70.1Luc* transgene. If effective

this competitive will titrate transcriptional factors which normally bind this proximal region, leading to a significantly reduced *HSP70.1Luc* transcription and a lowered luciferase activity. Comparison of *HSP70.1Luc* activity following injection of competitor fragments with native or mutated HSE motifs demonstrates that HSE binding plays a significant part in the regulation of *hsp* expression at the two cell stage (our unpublished observations). As they indicated that HSE sites are not required for hsp expression, previous experiments by Bevilacqua et al. [24, 25] might appear in contradiction with our findings. However, their results were based on a transient assay with nonintegrated constructs so, that they could not take into account the role played by promoter interactions with transcriptional factors inside nuclear chromatin. In any case, further investigations are required to define precisely the relative importance of HSE binding in comparison with the other binding sites described in the *hsp* promoter (e.g. Sp1), and the final clarification might require knock-out and transgenic analysis of heat shock factor.

Development evolution of chromatin structure and HSP expression

The nuclear environment created by chromatin structure and its evolution is deeply involved in the regulatory control of gene expression [54]. The beginning of embryogenesis is a unique period of extensive chromatin remodelling. In zygotes, pronuclei are built up from hypercondensed chromatin provided either by the Meta II oocytes or the spermatozoa [55, 56]. Then at syngamy, maternal and paternal genomes are mixed in an embryonic nucleus which will undergo a complex series of modifications leading to its maturation by the eight cell stage [57–59]. Therefore, it is now necessary to find out how the complex pattern of *hsp70* spontaneous and inducible expression could be controlled by chromatin remodelling.

Since this suggests regulatory steps mediated by chromatin organization, it is interesting to compare the expression given by hybrid genes composed of the *hsp70* promoter and a reporter gene when integrated into the embryonic genome or not. For instance, it was shown by various approaches, including *HSP70.1Luc* transgenic lines, that spontaneous inducible hsp expression observed at the two cell stage has disappeared by the four cell stage. However, when an *HSP70.1Luc* plasmid is directly introduced by microinjection into normal four cell embryos, a high level of luciferase activity is detected. This clearly indicates that transcriptional factors are not limiting at this stage and that another mechanism responsible for this repression could be the chromatin remodelling that occurs during this period. Moreover, the correlation found between this repression and the second S phase reinforces this point of view, since replication can be coupled to changes in chromatin structure [7, 58].

Recently, several studies have focussed on histone acetylation which is an indication of transcriptionally active chromatin [58, 59]. When embryonic chromatin is maintained hyperacetylated by inhibition of histone deacetylases, *HSP70.1Luc* transgene not only remains active but it is actually stimulated at the four cell stage. It is interesting to note that a peculiar distribution of an isoform of acetylated histone H4 (H4.Ac5-12) occurs within the period of spontaneous transcription of *HSP70.1* and does not exist any more at the four cell stage [59].

Unfortunately, there are no such observations which could be linked with the establishment of a typical heat shock response by the eight cell to blastocyst stages. Wittig et al. [44] had already suggested that 'studies concerning the nucleosome location on *hsp* genes should give insight as to how genes are activated during embryonic development'. This would be a very informative investigation since several studies in *Drosophila* or *Xenopus* have provided strong evidence that heat shock-regulated transcription depends on the interaction between HSF and nucleosome assembly [60, 61].

All the features described above convincingly suggest the regulatory role of chromatin. However this must be reexamined in the light of the following observations. First, we have derived more than ten different *HSP70.1Luc* transgenic lines, and though position effect phenomena are known to affect transgene expression, nearly all of these lines have displayed the endogenous pattern of *hsp* expression perfectly [7, 22, 23]. Second, addition of scaffold attachment regions (SARs) flanking the transgene did not modify this pattern [22, 58]. Therefore it could be suggested that regulatory motifs included in the *hsp70.1* promoter are the major elements by which the main aspects of *hsp70.1* transcription are controlled through interactions with transcriptional factor, repressor or nucleosome.

Conclusion

Heat shock genes are often considered as a paradigm for the study of gene regulation. Especially in the case of the preimplantation mouse embryo, this is one of the rare opportunities where specific transcriptional factors and target genes are known, cloned and available to investigation as described in this review.

Schematically, three different levels of *hsp70* expression can be described in early mouse embryos. First, a low level or basal transcription leads to the permanent small amount of *hsp70.1* transcripts we have observed by RT-PCR. Such basal activity is well-described for the corresponding human *HSP* gene [53]. Second, the onset of zygotic genome activity introduces a transient peak of *hsp70.1* transcription which represents a 5 to 88 times increase in *hsp70.1* transcripts from oocytes to in vivo or

in vitro two cell stage embryos respectively. The third level is observed after a heat shock and is several hundred times higher than the basal one. One hypothesis could be that the involvement of mHSF1 increases from the first to the third level of *hsp70* expression; mHSF1 would be unnecessary for the basal expression, required for the spontaneous expression and essential for the inducible activity of the *hsp70* gene. According to observations made in several species [2, 62] mouse embryos seem to be the only mammalian embryos to exhibit this particular pattern of spontaneous hsp70 expression. In contrast, the establishment of a canonical rapid heat shock response as the embryos develop to the blastocyst stage can be taken as a general feature. Therefore, acquisition of heat inducibility may be considered among the markers of blastomere differentiation and can be studied in this way. Finally, while our knowledge of inducible *hsp* expression and regulation is increasing, the function of these inducible genes, especially in early mouse embryos, remains to be investigated.

Acknowledgments. Work referred to from the laboratories of the authors has been supported by grants from the Ministere de la Recherche et de la Technologie, and Rhône-Merieux (Rhône-Merieux-INRA-MRT, n°90T0968) and from the Association pour la Recherche sur le Cancer (grant n°6505). We wish to thank E. Campion for the numerous two-dimensional gel electrophoreses with mice and rabbit embryo proteins. We are grateful to Dr. P. Adenot for confocal analysis. During the preparation of this manuscript, E.C. was supported by a postdoctoral grant from MRC (UK, Prof. M. Monk). We acknowledge Prof. M. Monk for having permitted studies on heat shock proteins in her laboratory.

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