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Evidence for the Involvement of Mouse Heat Shock Factor 1 in the Atypical Expression of the *HSP70.1* Heat Shock Gene during Mouse Zygotic Genome Activation

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The mouse *HSP70.1* gene, which codes for a heat shock protein (hsp70), is highly transcribed at the onset of zygotic genome activation (ZGA). This expression, which occurs in the absence of stress, is then repressed. It has been claimed that this gene does not exhibit a stress response until the blastocyst stage. The promoter of *HSP70.1* contains four heat shock element (HSE) boxes which are the binding sites of heat shock transcription factors (HSF). We have been studying the presence and localization of the mouse HSFs, mHSF1 and mHSF2, at different stages of embryo development. We show that mHSF1 is already present at the one-cell stage and concentrated in the nucleus. Moreover, by mutagenizing HSE sequences and performing competition experiments (in transgenic embryos with the *HSP70.1* promoter inserted before a reporter gene), we show that, in contrast with previous findings, HSE boxes are involved in this spontaneous activation. Therefore, we suggest that HSF1 and HSE are important in this transient expression at the two-cell stage and that the absence of typical inducibility at this early stage of development results mainly from the high level of spontaneous transcription of this gene during the ZGA.

All organisms respond to proteotoxic stress (heat shock or toxic agent exposure) by the synthesis of a group of proteins called heat shock proteins. Heat shock proteins are classified into different families on the basis of molecular mass (20, 70, and 90 kDa) and distinguished according to their inducibility: some members of heat shock families, such as heat shock cognate (hsc), are constitutively synthesized, whereas others (hsp) are expressed only following stress. Heat shock proteins interact with numerous other proteins, and their main function is the control of the accurate folding and translocation of polypeptides in the different cellular compartments (reviewed by Parsell and Lindquist [38].

More than 10 years ago, the synthesis of cognate and inducible members of the 70-kDa heat shock family was demonstrated in the two-cell mouse embryo (3). This expression coincides with the onset of zygotic genome activation, which represents a crucial event in embryonic development. It corresponds to the first step in the transition from maternal to zygotic control of development, after which inhibition of zygotic transcription leads to an immediate blockade of development. hsc protein synthesis was observed at the onset of zygotic genome activation and persisted in subsequent developmental stages (29), in agreement with the housekeeping property of this protein, which is essential for the survival of all cells (38). In contrast, two particular features concerning the expression of an inducible hsp70 gene are specific to the preimplantation period of mouse embryonic development. As mentioned above, the first feature is the high spontaneous expression, in the absence of stress, of the inducible hsp70 gene when zygotic genome transcription is initiated. The second one is that this gene does not exhibit a stress response until the blastocyst stage (33), although it is described in the literature as a paradigm to study the inducibility of gene expression. Taken together, these findings raise the question of the regulatory mechanisms controlling hsp70 gene expression during the preimplantation period of embryonic development and in particular at the two-cell stage.

Recently, we have shown that the spontaneous expression of hsp70 protein is mainly due to the transcription of the major inducible gene, *HSP70.1*, cloned by Hunt and Calderwood (22). The regulatory region of this mouse hsp70 gene, which is 85% homologous with the human sequence, includes a series of sequences known to be bound by transcriptional factors such as Sp1. In addition to these sequences identified in numerous other promoters, two specific regulatory elements called heat shock elements (HSE) are also present in the regulatory region of *HSP70.1*, each including four copies of the basal motif *n*GAA*n* arranged in tandemly inverted repeats. In eukaryotic cells induction of heat shock gene expression is dependent on the activation of a presynthesized heat shock factor (HSF), which thereafter becomes able to bind the HSE element. Depending on the species, there is one HSF (*Drosophila*) (9) or multiple HSFs (three in tomato, three in chicken, and two in human and in mouse) (36, 37, 39, 41, 42). The two factors in mouse, mHSF1 and mHSF2, which have only 38% identical residues, exhibit different states of oligomerization and DNA binding ability when they are purified from in vitro translation systems, produced in *Escherichia coli*, or extracted from established cell lines such as 3T3 (40). In the latter situation, HSF1 is a monomer when inactive and a trimer when activated, whereas HSF2 is converted from dimeric to trimeric form. These variations are most probably related to posttranslational modifications of these factors and/or their interaction with potential repressors. The intracellular localization of mHSF1 and -2 shows a diffuse pattern in cells growing in normal physiological conditions, but it is differentially modified following a stress: mHSF1 migrates to the nucleus, in agreement with its

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role in induction of hsp70 gene expression, whereas the distribution of mHSF2 remains unchanged (40).

The particular behavior of the hsp70 gene in the mouse embryo, its spontaneous expression at the two-cell stage, and the apparent absence of an inducible response until the blastocyst stage prompted us to examine whether these heat shock factors are present during the preimplantation period of development and how they are distributed in embryonic cells.

The involvement of HSE, which is preferentially bound by HSF, in the spontaneous *HSP70.1* promoter activity at the one- to two-cell stage was investigated by a competition approach (7, 18, 23, 50). We used embryos from transgenic lines of mice (8) established with a hybrid gene resulting from coupling the *HSP70.1* promoter to a sensitive reporter gene, firefly luciferase (*HSP70.1Luc*). Expression of luciferase in the early embryos of these transgenic animals resembles the transient spontaneous expression of *HSP70.1* gene at the two-cell stage. We quantitatively analyzed the level of transcriptional activity after injection of various native or mutated forms of regulatory sequences from *HSP70.1*. *HSP70.1Luc* transgenic embryos also enabled us to find a different explanation for the apparent lack of inducibility of the *HSP70.1* gene at the beginning of embryonic development.

MATERIALS AND METHODS

Transgenic mouse line. The *HSP70.1Luc* transgene (8) is a 6.6-kb linear DNA construct carrying the coding region of firefly luciferase (13) driven by the promoter of the *HSP70.1* gene (22). Several transgenic mouse lines were generated with this construct as previously described (8).

Construction and purification of promoter fragments used in competition experiments. A 470-bp fragment from the 5′ end of the *HSP70.1* gene was inserted in plasmid pBluescript (plasmid PN3 [Fig. 1A]). The promoter contains two HSEs with four imperfect inverted repeats of the basal *n*GAA*n* motif at positions −110 and −190 from the transcription initiation site and additional elements, including three SP1 elements at positions −50, −140, and −170 (Fig. 1B). This fragment has been cloned with *Xho*I ends by using oligonucleotides HC (5′-GTA CTC GAG CCC TCC CCC TCA GGA ATC CG-3′) and BL (5′-GTA CTC GAG GGC GCC GCG CTC TGC TTC TG-3′). This fragment is called "Wild." Oligonucleotide-directed mutagenesis was performed as described elsewhere (20) to produce the HSE mutations shown in Fig. 1B. These mutations were chosen in agreement with the results of Cunniff and Morgan (11): double HSE mutants were created by three substitutions (Fig. 1A), by using the oligonucleotides HSE1-2 (5′-CCG CCC TTG TCC AGG ACT CTG CAG AGG TTT GTG GGG-3′ and 5′-CCC CAC AAA CCT CTG CAG AGT CCT GGA CAA GGG CGG-3′) and HSE3-4 (5′-GGC CTT GGG GCC AGG GAT CTT GCA GCA GTT TGG CG-3′ and 5′-CGC CAA ACT GCT GCA AGA TCC CTG GCC CCA AGG CC-3′) for the mutagenesis. The mutated promoter sequences were subsequently cloned back into pBluescript at the *Xho*I site. The doubly mutated fragment was called HSE1-2.HSE3-4. The mutations in the HSE sequences and the absence of mutations in the remainder of the sequence were checked by dideoxy sequencing (Pharmacia).

DNA fragments were loaded on 0.7% agarose gels with xylene cyanol and purified with the Gene Clean II kit (Bio 101, Inc.). After purification, DNA was resuspended in water and loaded on a Quiaquick spin purification column from Qiagen (reference 28104), followed by a twofold precipitation. This DNA was then diluted to a final concentration of 125 ng/μl, which is optimal for microinjection in the embryos. Since microinjection requires a high level of purity of the DNA fragments and a good estimate of the amount of injected material, the quality and quantity of prepared fragments were checked by UV spectroscopy. As a control, we used a 450-bp fragment resulting from the digestion of pBluescript by *Pvu*II (called PvuII).

Production and manipulation of embryos. Preimplantation transgenic embryos were obtained by mating superovulated F_1 hybrid (C57BL/6 × CBA) females with transgenic homozygous F_2 to F_7 hybrid (C57BL/6 × CBA) males from line 1. One-cell embryos were recovered at 22 to 24 h post-human chorionic gonadotropin (hCG). They were cultured in small drops of M16 medium (21) under oil (light paraffin oil [BDH]) and maintained in an incubator at 37°C in 8% CO_2. Pronuclear microinjections were performed on one-cell transgenic embryos (24 to 26 h post-hCG) by the standard procedure (21). The different injected solutions are described in Results. In vivo two-cell transgenic embryos were recovered directly from females by flushing oviducts at 42 h post-hCG. For heat shock experiments, transgenic embryos at the indicated stage were incubated at 43°C for 30 min and allowed to recover at 37°C for various times after heat shock.

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 of bovine serum albumin per ml, 1 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride) 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 L9504) and 20 mM ATP in the same reaction buffer) was automatically injected, and light emission was integrated for 10 s at 18°C in a photometer (Lumat LB 9501; Berthold). Background levels measured with reaction buffer never exceeded 150 ± 20 relative light units (RLUs). Under our conditions, 1 RLU corresponded to 1.5 fg of purified luciferase (Sigma L9009).

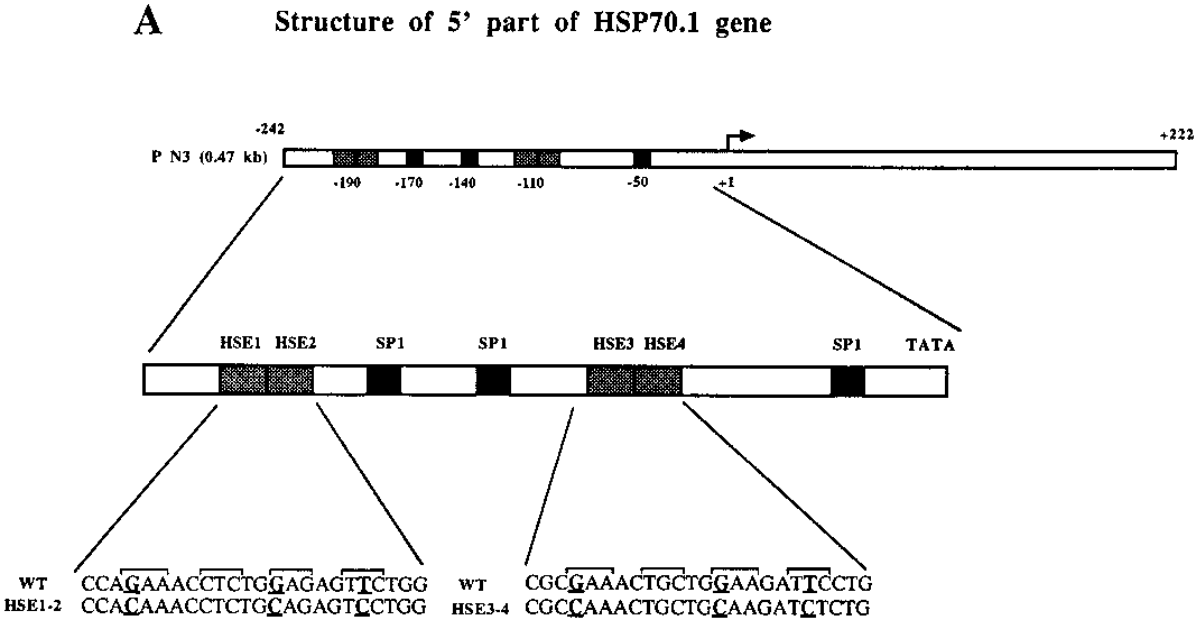
RT-PCR. Eggs (ovulated oocytes) were recovered from superovulated F_1 hybrid (C57BL/6 × CBA) females (22 to 24 h post-hCG). One-cell embryos were obtained after mating of superovulated females with normal F_1 hybrid (C57BL/6 × CBA) males and cultured in vitro until analysis (one cell, 22 to 24 h post-hCG; two cells, 44 h post-hCG; four cells, 58 to 60 h post-hCG; blastocyst, 96 h post-hCG) as described for transgenic embryos. Batches of 100 eggs or embryos were frozen and stored at −80°C until analysis. To limit loss of RNA, no purification was undertaken, and batches of oocytes or embryos were simply lysed by heating at 100°C for 1 min in 0.4% Nonidet P-40–1.5× Moloney murine leukemia virus reverse transcriptase (MMLV RT) buffer.

In the case of mHSF1, we performed RNA template-specific PCR (RS-PCR) (42), in which a tagging primer of poly(T) plus a linker sequence (5′-GCGCC GCGGCCGC-18XT-3′) was used to synthesize the cDNA, whereas the primers added in the PCR mix were a 5′ primer specific to the 3′ noncoding region of the mHSF1 cDNA (position 1382 according to Sarge et al. [39]) (5′-AGCTTCTGT CTCCACAAGAG-3′) and a 3′ primer corresponding to the linker (5′-GCGC CGCGGCCGC-3′). Oocyte or embryo lysates were divided into two fractions, one of which was supplemented with 100 ng of oligo(dT) primer, 0.2 mM deoxynucleoside triphosphate (dNTP), 1 U of RNasin, and 100 U of MMLV RT (Gibco/BRL) in a final volume of 10 μl to synthesize the cDNA. After 30 min of incubation at 37°C, an additional 100 U of RT was added, and the reaction was ended by a 10-min incubation at 95°C. The same reaction was carried out on the other fraction of the lysate but in the absence of the MMLV enzyme to look for false-positive PCR amplification of contaminating DNA. The cDNA synthesized in the 10-μl reaction mixture was then amplified by PCR in a final volume of 50 μl (1× *Taq* polymerase reaction buffer, 0.1 mM dNTP) containing 50 pmol of the 5′ mHSF1-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). Samples were denatured at 94°C for 7 min and then subjected to a series of amplification cycles (94°C for 30 s, 60°C for 30 s, and 72°C for 1 min) in a DNA thermal cycler 480 (Perkin-Elmer Cetus).

In the case of mHSF2, a region within 5′ (position 1353 according to Sarge et al. [39]; 5′-GCTACCAAGAGCAGTGTTGT-3′)- and 3′ (5′-AGGCAGTCGA GTAGCATCTG-3′; position 1874 according to Sarge et al. [39])-specific primers includes a 0.4-kb intron allowing easy discrimination between DNA and cDNA amplification. RT-PCR experiments were thus carried out as in the case of mHSF1 except for the following points. Oligo$(dT)_{12}$ was used to synthesize the cDNA strand, and 50 pmol each of 5′- and 3′-specific mHSF2 primers described above were added to the PCR mixture. Aliquots of the amplified mixture corresponding to 5 or 10 oocytes or embryos were run on 2% agarose gels and blotted to Hybond N+ membranes (Amersham). Hybridization was carried out overnight with a ^{32}P-labeled mHSF1 or mHSF2 cDNA probe, and autoradiographic exposure was for 1 h at −80°C.

Immunofluorescence-immunocytochemistry. Mouse embryos were collected at the one-cell stage and cultured as described above until they were subjected to immunofluorescence analysis. Heat-shocked embryos were incubated at 43°C for 30 min and allowed to recover at 37°C for 20 to 30 min after heat shock. Control and heat-shocked embryos were fixed at the same time in 2.5% neutral buffered paraformaldehyde for 5 min at 37°C. Fixed embryos were treated with NH_4Cl (50 mM in phosphate-buffered saline [PBS]) for 1 h. They were then permeabilized and incubated in 0.3% Triton (in PBS containing 10% fetal calf serum [FCS]) for 30 min. Incubation in the primary antibody (anti-mHSF1 or -mHSF2 specific polyclonal antibodies, kindly provided by Kevin Sarge and Rick Morimoto [40]), was carried out at 4°C overnight (dilution in PBS containing 2% FCS). The embryos were rinsed before incubation for 1 h in the second antibody, fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G (Sigma; diluted 1/600 in PBS containing 2% FCS). After a final washing, the embryos were briefly incubated in iodide propidium (5 μg/ml) and mounted in Moewiol 4-88 (Hoechst) under a coverslip. The samples were examined with a Zeiss confocal laser scanning microscope.

Whole-cell extract and DNA-protein complex analysis by gel electrophoresis. Mouse embryos were collected at the one-cell stage and cultured as described above until the two-cell stage (42 to 44 h post-hCG). Some groups of two-cell embryos were subjected to heat shock treatments (43°C for 30 min or 45°C for 15 min), after which they were immediately rinsed and frozen together with the other groups continuously cultured at 37°C. Embryos were stored at −80°C until further use. The detailed methods of extraction and DNA-protein complex analysis were essentially those described by Mezger et al. (31). Supershift experiments with antibodies against HSF1 (a1) or against HSF2 (a2) were performed by the method of Mezger et al. (32). F9 cells were cultured in vitro and heat shocked (45°C for 15 min) or not, and extracts were prepared as described by Mezger et al. (31, 32). The amount of F9 extract loaded for each lane contains 20 μg of protein. The radioactivity present in the retarded complexes was quan-



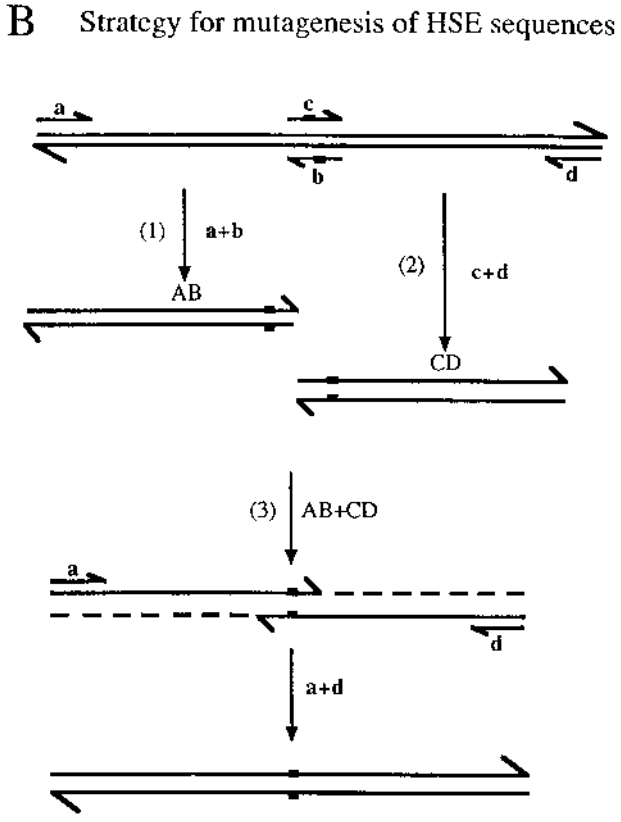


FIG. 1. (A) Structure of the 5′ region of the mouse *HSP70.1* gene. Regions containing sequences known to regulate transcription are boxed and identified. WT, wild type. (B) Diagram outlining the strategy used for site-directed mutagenesis. DNA and synthetic oligonucleotides are represented by lines with arrows indicating the 5′-to-3′ orientation. The sites of mutagenesis are indicated by the small black boxes. PCR products are indicated by pairs of uppercase letters, and oligonucleotides are indicated by lowercase letters. In the first step (1 and 2) two smaller fragments containing the extremities and the area of mutagenesis are amplified by PCR. Oligonucleotides a and d correspond to the extremities of the 470-bp *Xho*I-*Xho*I fragment used for competition. Oligonucleotides c and d contain the mutations in the HSE1-2 or HSE3-4 boxes. The product of step 3 represents the proposed intermediate where the denatured fragments annealed by the overlapping sequences are extended 3′ by DNA polymerase (dashed line) to form a mutated 470-bp fragment. By adding primers a and d, the mutated fragment is further amplified by PCR.

tified by PhosphorImager (Molecular Dynamics) with ImageQuant version 3.3 software following overnight exposure with a Phosphor Screen (Molecular Dynamics).

Statistical analysis. Transgenic embryos harboring three copies of the *HSP70.1Luc* construct were injected at the one-cell stage with different DNA fragments. Their luciferase activities measured at the two-cell stage were compared with that of controls isolated from the same initial batch of transgenic embryos for each replicate experiment. Mean values were compared by *t* tests with the correction of Chocram and Cox for unequal variances, as proposed by the SAS user guide (version 6.03; SAS Institute, Inc., Cary, N.C.) and after their transformation into $\log(1 + x)$ for adjustment to a normal distribution (12).

RESULTS

Presence of mHSF1 and mHSF2 RNAs in mouse embryos. RT-PCR experiments were used to detect the presence of mHSF1 and mHSF2 poly(A)$^+$ transcripts at the two-cell stage, when spontaneous expression of *HSP70.1* is observed; at the four- to eight-cell stage, when there is neither spontaneous nor inducible expression of this gene; and finally at the blastocyst stage, when the embryo becomes truly able to respond to a stress. As we have found that the expression of the *HSP70.1* promoter begins in fact at the end of the one-cell stage (8), we also looked for the presence of mHSF1 and mHSF2 poly(A)$^+$ RNAs in the matured oocyte to see whether these factors should be considered maternal factors.

Specific primers were chosen in the 3′ region of these genes beyond the very homologous region of oligomerization and DNA binding. Since we found only a very short intron included in the region flanked by the 5′- and 3′-specific primers of mHSF1, leading to confusion between cDNA and DNA amplification, we decided to use the RS RT-PCR technique (44) (see Materials and Methods for experimental details). Two fragments 0.4 and 0.6 kb in length were amplified following this RT-PCR procedure, while both were absent in the control reaction (heart cells) without RT (Fig. 2). The mHSF2 RNA amplification was performed according to the classical RT-PCR protocol. The amplification reaction specific to mHSF2 also gave two fragments, 1 and 0.6 kb in length. As the longer one (not shown here) did not disappear in the control reaction

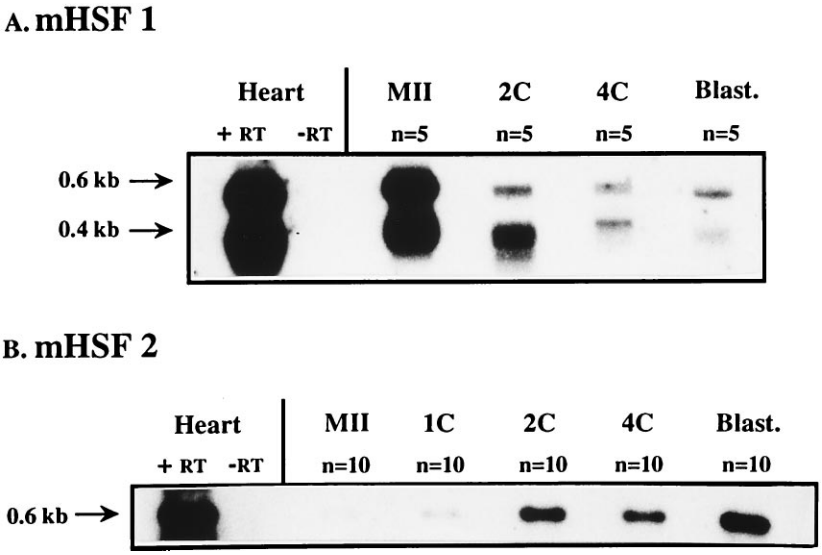


FIG. 2. Analysis of mHSF1 (A) and mHSF2 (B) mRNAs in mouse tissue (heart); mature oocytes at the metaphase II stage (MII); and preimplantation embryos at the one-cell (1C), two-cell (2C), four-cell (4C), and blastocyst (Blast.) stages. RT-PCR experiments were performed as described in Materials and Methods. Lanes +RT and −RT, reactions performed in the presence or absence of MMLV-RT. For mHSF1 analysis, each lane represents the signal obtained from 5 oocytes or embryos; for mHSF2 analysis, the signal corresponds to 10 oocytes or embryos. Molecular sizes of the different amplicons are indicated.

without RT, it thus corresponds to the amplification of the genomic DNA revealing the presence of a 0.4-kb intron in this part of the gene. The results given by these two slightly different strategies to synthesize and amplify the cDNA from both factors are presented in Fig. 2. A rough examination of these results shows that there are obvious differences between the signals given by the two factors at the different embryonic stages studied. At the oocyte stage, a very strong signal was obtained with primers specific to mHSF1, while the signal was barely detectable with primers specific to mHSF2. To avoid any confusion with amplification of transcripts from extraneous granulosa cells picked with oocytes, we repeated this experiment at the one-cell stage, and the result was similar to that obtained at the oocyte stage. At subsequent stages of development, the signal given by mHSF1 amplification seemed to decrease while that given by mHSF2 became easily detectable. Our data can be interpreted only qualitatively insofar as we have not attempted to perform an exact quantification of the mHSF1 and mHSF2 transcripts. However, they clearly indicate that mHSF1 transcripts are abundant in the matured oocyte and thus represent maternal factors present when zygotic genome transcription starts, while both factors are present at the end of the preimplantation period. The difference in the levels of signals obtained with the two factors should reflect their different levels.

Intracellular localization of mHSF1 and mHSF2 in preimplantation embryos. Since mHSF1 and mHSF2 transcripts were present in preimplantation embryos, we performed immunofluorescence experiments to see whether the corresponding polypeptides were also present and how they were distributed in the embryonic cells. Intracellular localization of HSF has been previously reported in different organisms. For instance, in *Drosophila* cells it is exclusively nuclear. In mouse 3T3 cells, mHSF1 and mHSF2 are distributed equally in the cytoplasm and nucleus in the absence of stress while mHSF1 migrates from the cytoplasm to the nucleus in heat-shocked cells (40). In our immunofluorescence analysis we used the same mHSF1 and mHSF2 polyclonal antisera as Sarge and collaborators (40). The results are presented in Fig. 3.

In two-cell mouse embryos, mHSF1 appeared almost exclusively localized in the nucleus with a clear exclusion from the nucleoli (Fig. 3A). A 10-fold increase in the concentration of the polyclonal antiserum used to detect the presence of mHSF1 (diluted 1/300 instead of 1/3,000) gave only a weak increase in cytoplasmic staining (data not shown). Heat shock of embryos for 30 min at 43°C before fixation and staining also resulted in a similar marked nuclear localization (Fig. 3B), while no labeling was obtained when the first antibody was omitted (Fig. 3C). Similar patterns were observed for in vitro and in vivo two-cell embryos (data not shown), indicating that in vitro manipulation of the embryos, which has previously been shown by us to influence the level of *HSP70.1* expression (8), did not artifactually induce the nuclear localization of mHSF1. In contrast to the strong labeling by mHSF1 antiserum, only a faint cytoplasmic signal excluding the nucleus was obtained with mHSF2 antiserum (Fig. 3D), even after a 10-fold increase in the concentration used (1/300) (data not shown). As in the case of mHSF1, this pattern of labeling was similar when two-cell embryos were directly collected in vivo (data not shown) instead of being obtained in vitro from the one-cell stage. Heat shock did not lead to any significant change in subcellular localization (Fig. 3E).

At later stages and up to the blastocyst stage, mHSF1 still exhibited nuclear labeling but also became clearly detectable in the cytoplasm (Fig. 3F). Staining by the mHSF2 antiserum became more intense in both the cytoplasm and the nucleus (Fig. 3H). With both mHSF1 and mHSF2 antisera, the intensity of nuclear staining was variable in the different nuclei of the same embryo. As for two-cell embryos, no modifications of mHSF1 and mHSF2 immunofluorescent staining were observed following heat shock treatment at either the morula (data not shown) or the blastocyst stage (Fig. 3G and I). This result is unexpected in the case of the blastocyst stage, as a consistent correlation is found between the nuclear relocalization of mHSF1 and its role in heat-induced transcription of hsp genes, which first appears at this developmental stage.

DNA binding properties of mHSF1 at the two-cell stage. From RT-PCR experiments and immunofluorescence analysis,

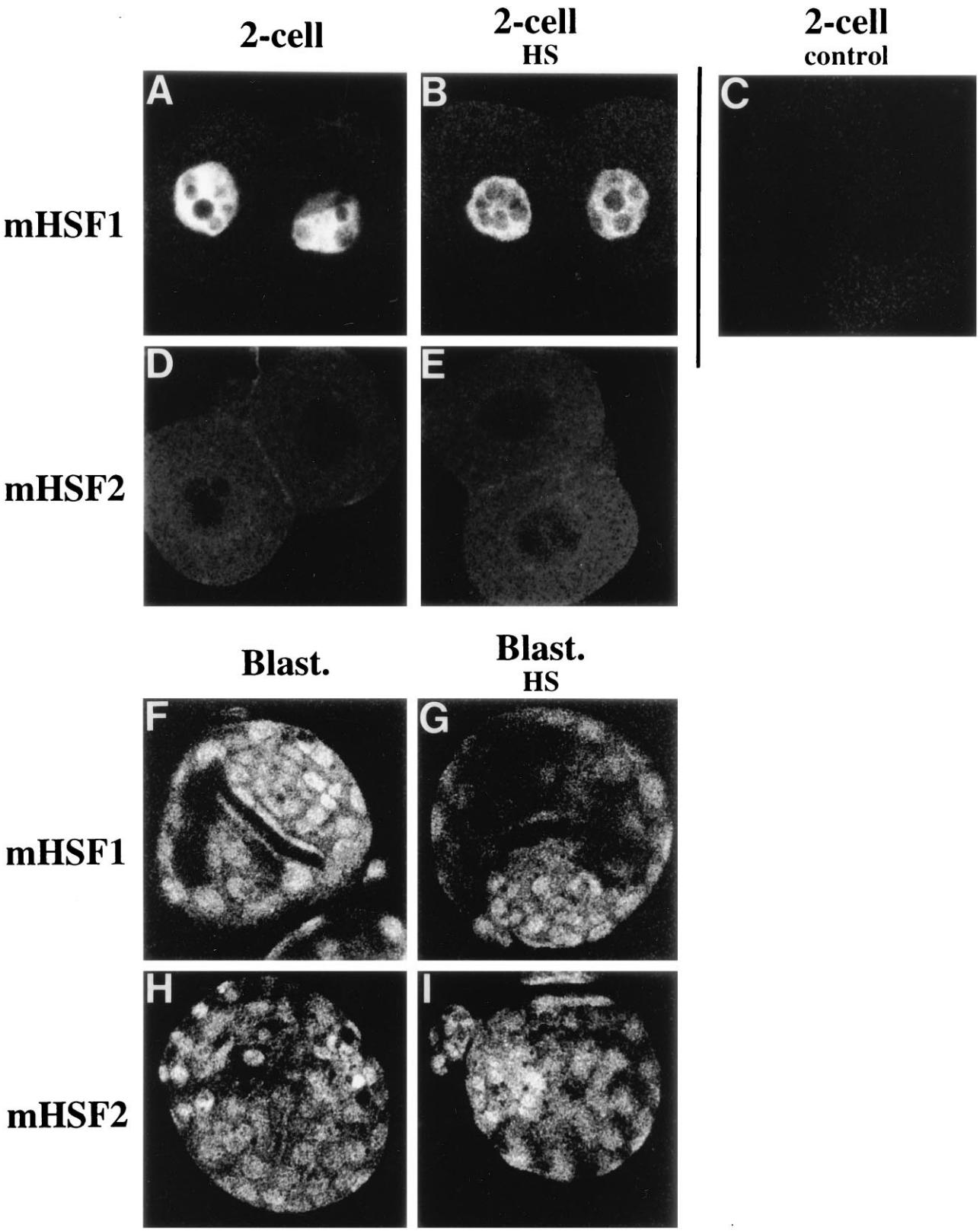


FIG. 3. Immunolocalization of mHSF1 and mHSF2 in preimplantation mouse embryos. Embryos collected at the one-cell stage and cultured to the two-cell or blastocyst (Blast.) stage were heat shocked (HS) (43°C for 30 min) or not and then subjected to immunofluorescence analysis using mHSF1 and mHSF2 polyclonal antibodies (see Materials and Methods).

it is clear that mHSF1 and also, at a minor level, mHSF2 products are present at the two-cell stage. Since we previously demonstrated by gel mobility shift assays that an inducible but not constitutive DNA-binding activity was present at the two-cell stage (31), we wanted to determine whether mHSF1, known in somatic cells to be responsible for this inducible DNA-binding activity (39), was effectively involved at this precise period of mouse embryonic development. To this end we incubated extracts from 100 two-cell stage embryos, heat shocked (43°C for 30 min) or not, with antibodies targeted against mHSF1 or mHSF2. Antibodies were used at a final dilution of 1/3,000. In these experimental conditions anti-mHSF2 antibodies did not alter the HSE complex, while anti-mHSF1 antibodies induce a weak decrease of this complex (Fig. 4A). In order to ascertain the role played by mHSF1 in the formation of this HSE complex, we decided to examine the patterns obtained with modified heat shock treatment (45°C for 15 min) and different antibody dilutions.

In a preliminary experiment, we looked for the dilution of antibodies (final dilution, 1/200 or 1/1,000) inducing a specific supershift of the mHSF1-DNA complex using untreated or heat-shocked embryonal carcinoma (F9) cell extracts. Such extracts were demonstrated in previous experiments to give rise to HSF-DNA complexes containing only mHSF2 or mHSF1 (35). At a final dilution of 1/200, anti-mHSF1 markedly reduced the HSE complex obtained with extracts from heat-shocked cells, but it also decreased the intensity of the HSE complex observed with extracts from untreated cells which are known to contain only mHSF2 (Fig. 4B and C; compare lanes 2 and 5 to lanes 1 and 4). Therefore, at this dilution, the anti-mHSF1 antibodies were no longer specific. In contrast, the 1/1,000-diluted anti-mHSF1 antibodies decreased the HSE complex from heat-shocked cells (Fig. 4B, lane 6) without altering the HSE complex seen with extracts from control cells (Fig. 4B, lane 3). For this dilution (1/1,000) with heat-shocked two-cell embryos, a marked reduction in the intensity of the HSE complex was observed together with an increase of the signal located in the corresponding well (Fig. 4B and C, lanes 9).

Involvement of HSE sequences in *HSP70.1* promoter activity. To study the transcriptional control of hsp gene expression during development, we have established transgenic lines of mice bearing the promoter of the major inducible hsp gene, *HSP70.1*, coupled to the firefly luciferase gene as a reporter (13, 22). By using transgenic homozygous males crossed with normal superovulated females, large batches of transgenic embryos were easily produced, allowing us to draw a precise profile of expression of the transgene from the one-cell stage to the blastocyst stage. In summary (8), *HSP70.1Luc* is constitutively expressed starting from the end of the one-cell stage, and expression reaches a maximum during the first part part of the two-cell stage and then progressively decreases to background levels. This profile closely mimics that of the endogenous gene obtained from the analysis of bidimensional gel electrophoresis of groups of embryos. However, the main advantage of our transgenic model is that it allows monitoring of quantitative variations in luciferase activities from individual embryos.

This prompted us to investigate the nature of the regulatory elements involved in spontaneous *HSP70.1* promoter activity at the two-cell stage by means of competition experiments. DNA fragments that include normal or mutated promoter sequences are microinjected and tested for their capability to trap transactivating factors responsible for *HSP70.1* promoter activation, looking at the level of reporter gene expression (6, 28). For these experiments, we chose to microinject large DNA fragments from the gene promoter, specifically mutated or not

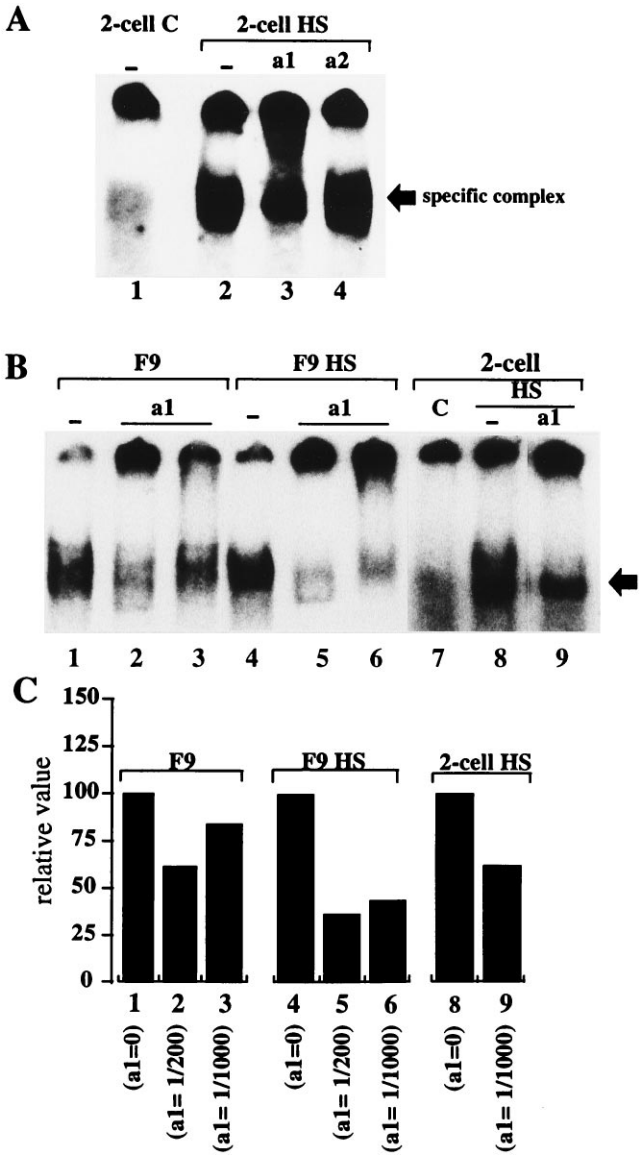


FIG. 4. Effects of polyclonal antisera against mHSF1 and mHSF2 on the migration of DNA-protein complexes with whole-cell extracts. (A) Antiserum raised against mHSF1 (a1) or mHSF2 (a2) at a final dilution of 1/3,000 was incubated with extracts from groups of 100 heat-shocked (HS) (43°C for 30 min) two-cell embryos prior to the gel shift assay. No antisera were added to extracts from control (C) or 100 HS (43°C for 30 min) two-cell embryos (lanes 1 and 2). (B) Antiserum raised against mHSF1 was incubated with extracts from control, untreated (lanes 2 and 3) or HS (45°C for 15 min) (lanes 5 and 6) F9 cells at a dilution of either 1/200 (lanes 2 and 5) or 1/1,000 (lanes 3 and 6). No antiserum was added to extracts from control or HS (45°C for 15 min) F9 cells (lanes 1 and 4). Antiserum raised against mHSF1 was incubated with extracts from 100 HS (45°C for 15 min) two-cell embryos at a dilution of 1/1,000 (lane 9). No antiserum was added to extracts from control or HS (45°C for 15 min) two-cell embryos (lanes 7 and 8). (C) Phosphorimager quantification of the results shown in panel B. The signal intensities are reported as percentages of the value obtained in the absence of antibodies (a1=0).

within the HSE sequences. This strategy is actually more valuable than the direct microinjection of HSE sequences for two reasons: first, large DNA fragments are less vulnerable to degradation, and second, this strategy can demonstrate the involvement of a factor, in our case HSF, even if this factor requires other factors to bind to its target sequences.

We first examined the effect of increased concentrations of

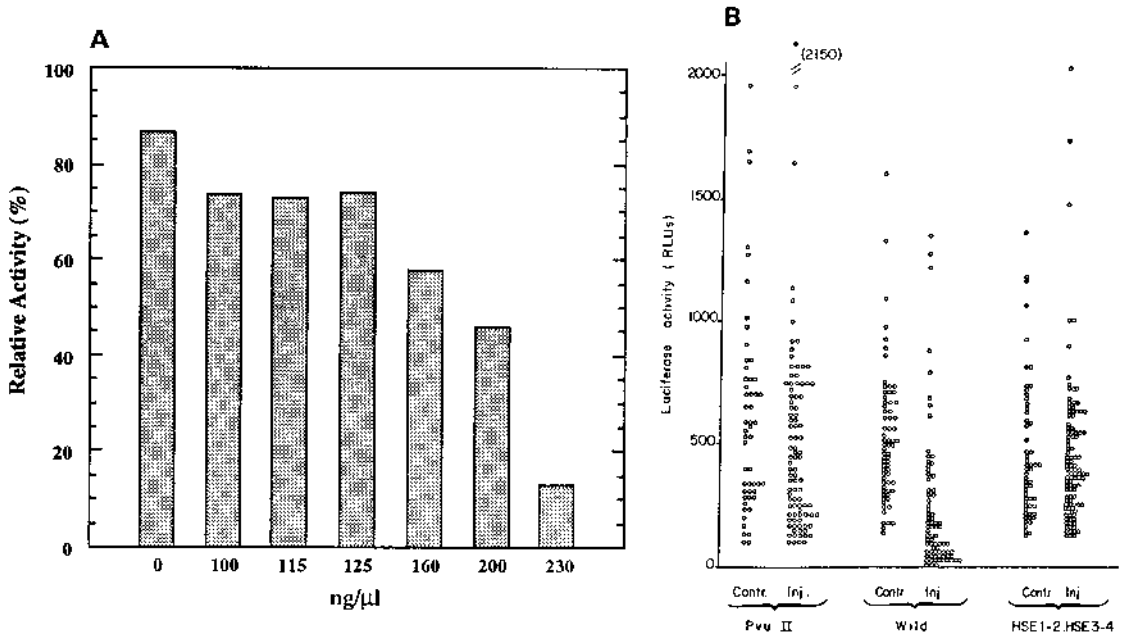


FIG. 5. (A) Effect of increasing concentration of a microinjected DNA control fragment (PvuII [450-bp pBluescript fragment]) on *HSP70.1Luc* transgene expression. Transgenic embryos were microinjected at the one-cell stage, cultured to the two-cell stage, and then assayed for luciferase activity. A minimum of 20 embryos were analyzed for each indicated concentration (0 ng, saline only). Results are presented as percent mean luciferase activities of injected embryos relative to that of noninjected embryos from the same initial batch. (B) Competition assay. Luciferase activities of single embryos injected (Inj.) or not (Contr.) with DNA solutions at a concentration of 120 ± 5 ng/µl. PvuII, 450-bp pBluescript fragment; Wild, 470-bp *HSP70.1* promoter fragment; HSE1-2.3-4, 470-bp mutated *HSP70.1* promoter fragment. Each fragment was injected in three different experiments with at least 20 embryos.

an unrelated control DNA fragment on the level of luciferase activity in transgenic embryos. For this, we injected a 450-bp PvuII pBluescript fragment at the one-cell stage and measured luciferase activities at the two-cell stage. The results show that mean luciferase activities relative to that of noninjected control embryos from the same batches remained higher than 70% for concentrations up to 125 ng/µl but then declined rapidly for more concentrated solutions, leading to a residual relative activity of only about 10% following injection of a DNA solution of 230 ng/ml (Fig. 5A). We chose a window of DNA concentrations of 120 ± 5 ng/µl, compatible with a limited nonspecific inhibition of two-cell-stage luciferase activity.

We then injected 470-bp fragments corresponding to the *HSP70.1* promoter and bearing either no (wild type) or one mutated site in each of the four (HSE1-2.HSE3-4) HSE sequences of the promoter. Wide variability of luciferase activity, at least partly reflecting differences in the amounts of DNA injected, was observed between embryos (Fig. 5B). However, when the mean luciferase activity obtained in each duplicate experiment was compared with that of noninjected controls, a significant reduction was observed only following injection of the wild-type fragment (Table 1). With the mutated fragment, as with control DNA, no significant reduction of luciferase activity was observed. From these data we conclude that only the injected wild-type sequences were able to trap the transactivating factors which bind to the endogenous promoter and that mutation in the HSE sequences altered this competition.

This suggests that the main factor or factors required for luciferase activity under the control of the *HSP70.1* promoter bind to the HSE sequences but are unable to bind to the mutated HSE sequences. This result, in contradiction with the previous observations in the literature (5), demonstrates the importance of HSE sequences in *HSP70.1* promoter activity. It is reasonable to assume that it is mHSF1 which is involved in this spontaneous expression, since we demonstrated previously that mHSF1 is much more abundant than mHSF2 at these first steps of mouse development.

Inducibility of the hybrid gene *HSP70.1Luc* during the preimplantation period. mHSF1 is present throughout the entire preimplantation period, and it exhibits inducible HSE DNA-binding activity except at the four- to eight-cell stage (this study; 31). In contrast, no inducible hsp70 protein synthesis was found in several studies (17, 33, 34, 48). In order to solve this paradox, we used the *HSP70.1Luc* transgenic mice to estimate heat shock gene transcriptional activity more precisely than in the previous experiments.

The effects of heat shock at the two-cell stage were first

TABLE 1. Effects of DNA fragments microinjected at the one-cell stage on the activity of the *HSP70.1* transgene at the two-cell stage

Injected fragment and group of embryos (no. injected[a])	Luciferase activity (mean ± SE)	t^b	P
*Pvu*II			
Injected (93)	5.42 ± 0.12		
Control (52)	5.57 ± 0.11	0.85	0.39
Wild			
Injected (78)	4.97 ± 0.15		
Control (69)	5.67 ± 0.85	3.73	0.003[c]
HSE1-2.HSE3-4			
Injected (107)	5.40 ± 0.10		
Control (57)	5.29 ± 1.24	0.55	0.58

[a] Three replicates each.
[b] Determined as indicated in Materials and Methods.
[c] Significant difference ($P < 0.01$).

examined. As in vitro manipulations of the embryos are known to possibly affect their transcriptional efficiency (8), we measured luciferase activity at various times following heat shock treatment (time zero) performed at the two-cell stage both on embryos collected at the one-cell stage and cultured until the two-cell stage and on in vivo embryos collected directly at the two-cell stage. The results expressed as mean luciferase activity relative to those for non-heat-treated in vitro and in vivo embryos are presented in Fig. 6A. Five hours after heat shock, relative luciferase activities were increased 2-fold for in vitro embryos and 22-fold for in vivo embryos. At 24 h posttreatment, the respective increases were 150- and 300-fold. That these increases in luciferase activities were the result of embryonic transcription was further demonstrated by allowing heat-shocked in vitro embryos to recover in α-amanitin, an inhibitor of RNA polymerase II. In these conditions there was no increase in luciferase activity even after 10 h of recovery at 37°C (data not shown). From these results it is clear that transcription of the *HSP70.1* hybrid gene is affected by heat shock treatment at the two-cell stage and that the conditions in which the two-cell embryos are produced (in vitro or in vivo) only modulate this effect. With this result in hand, we quantified the heat shock response of embryos at later preimplantation stages using in vitro embryos. To allow comparison between stages, *HSP70.1Luc* transgene expression was calculated for the equivalent of 100 cells from each stage. The results (Fig. 6B) show that, at all stages of preimplantation development, embryos respond to heat shock, but the increase in luciferase activity exhibits different profiles in two different respects: maximal levels attained are lower between the four-cell stage and the morula stage than at the two-cell and blastocyst stages, and, as development proceeds, these maximal levels are reached more rapidly, resulting (but only from the blastocyst stage) in a heat shock response quite similar to that displayed by differentiated cells. These data clearly demonstrate that preimplantation mouse embryos do respond to heat shock treatment, but in an atypical manner and differently at different embryonic stages.

DISCUSSION

Since 1983, it has been known that spontaneous expression of the *HSP70* genes, both *hsp* and *hsc70*, occurs in the two-cell mouse embryo at the same time as the onset of zygotic genome activation (3). As HSP genes are considered to be a model for the study of transcriptional regulation, analysis of this expression is of particular interest for two main reasons. From the embryological point of view, a better understanding of the developmental process requires determination of the mechanisms involved in the regulation of gene expression at the time of the zygotic genome activation. Concerning heat shock gene regulation, it remains to be explained how inducible hsp70 genes can be spontaneously expressed without a well-defined heat shock response. Since the initial two-dimensional gel electrophoresis analyses from Bensaude et al. (3), numerous studies using different approaches have focused on the latter question (4, 10, 17, 25, 29, 33, 34, 48). Recent studies (4, 5, 8, 46) have revealed spontaneous transient expression of *HSP70 lacZ* or *Luc* hybrid genes at the two-cell stage, demonstrating that the sequences driving the spontaneous expression of *HSP70* at the two-cell stage are localized within the *HSP70* regulatory region included in these constructs. Furthermore, using this approach, Bevilacqua et al. (5) have suggested that HSE which are bound by HSF are not involved in this spontaneous expression, since they obtained the same results with a mutant form in which the two proximal HSE sequences of the *HSP70A1* promoter were deleted.

Precise identification and cloning of mHSF1 and mHSF2 (39) prompted us to study their expression and activity in the early mouse embryo. Our RT-PCR data show that transcripts are present from the two-cell stage to the blastocyst stage. mHSF1 is already abundant in oocytes and might thus be considered a maternal factor, while mHSF2 is undetectable in oocytes and increases during the preimplantation period. This result is in agreement with the Northern analysis performed by Sarge et al. (39) which demonstrated high expression of mHSF1 in the ovary. Two different forms of mHSF1, a and b, and mHSF2 have been described (14–16). As the oligonucleotides we used in RT-PCR experiments are located downstream, we cannot compare our results with those previously published. Nevertheless, our RT-PCR experiments suggest that additional splicing sites are present in this downstream 3′ region in both mHSF1 and mHSF2. Thus, HSF transcripts are subjected to numerous posttranscriptional modifications which could modulate production and/or activity of the different forms of mHSF1 and -2. It would therefore be interesting to further characterize the types of transcripts produced during the preimplantation period of development, either those previously described or specific embryonic ones.

The results of the immunofluorescence analysis are well correlated with those of the RT-PCR experiments. At the start of preimplantation development, mHSF1 is more abundant than mHSF2. At later stages (morula-blastocyst) mHSF1 and mHSF2 intensities become comparable. Concerning the embryonic localization of these factors, we have observed with mHSF1 a quite exclusive nuclear staining in nonstressed two-cell stage embryos. The migration of mHSF1 from cytoplasm to nucleus was described by Sarge et al. (40) as a first mark of the activation of the factor before its binding to HSE and the activation of its transcriptional capacity. Our results might reflect the spontaneous activation of this factor at the two-cell stage or the atypical localization of this factor when it is very abundant (as shown in 3T3 cells overexpressing mHSF1 [40]; see below).

The demonstration of the presence of both mHSFs raises the question of their activity and their role in heat shock gene expression during the preimplantation period of embryonic development. Mezger et al. (31) have detected an inducible DNA-binding activity in extracts of heat-shocked mouse embryos from the one-cell to the blastocyst stage (except at the four- to eight-cell stage), while a constitutive DNA-binding activity has been found only in embryonic extracts from the blastocyst stage. At the latter stage, the respective correlation between mHSF1 and mHSF2 and the inducible and constitutive binding capacities has been established by supershifting the complexes with polyclonal antibodies (32). In the present study similar observations were made concerning the inducible binding activity present in embryonic extracts from the two-cell stage. As could be expected in the presence of such a large amount of nuclear mHSF1, this factor is involved in the inducible DNA-binding activity present at the two-cell stage and detected in vitro by gel shift assays. In our experimental conditions, however, the HSE complex obtained with embryonic extracts from the two-cell stage is not completely supershifted by mHSF1 polyclonal antibodies. Such an observation leaves open the possibility that another mHSF can also be activated at this stage (e.g., a putative murine equivalent of the avian HSF3 [37] or another, yet uncharacterized HSF).

Competition experiments demonstrate that HSE sequences play a role in the spontaneous expression of the *HSP70.1* promoter at the two-cell stage. This observation appears to be

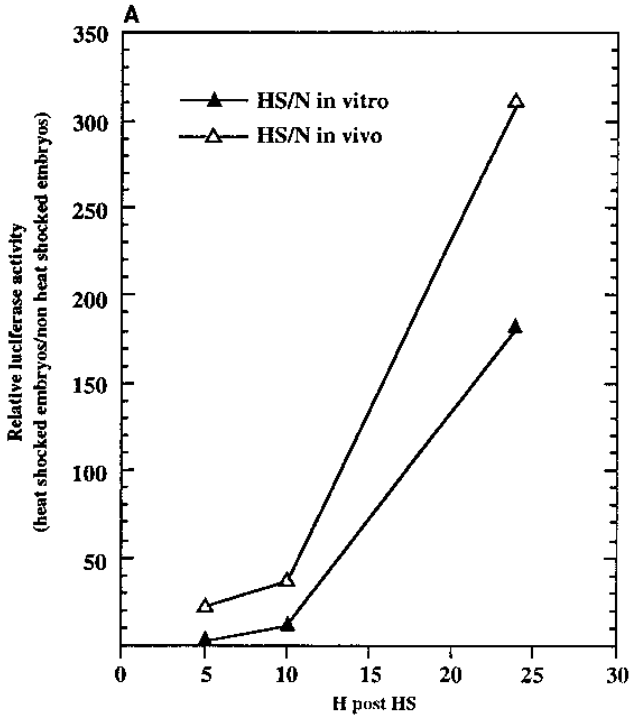
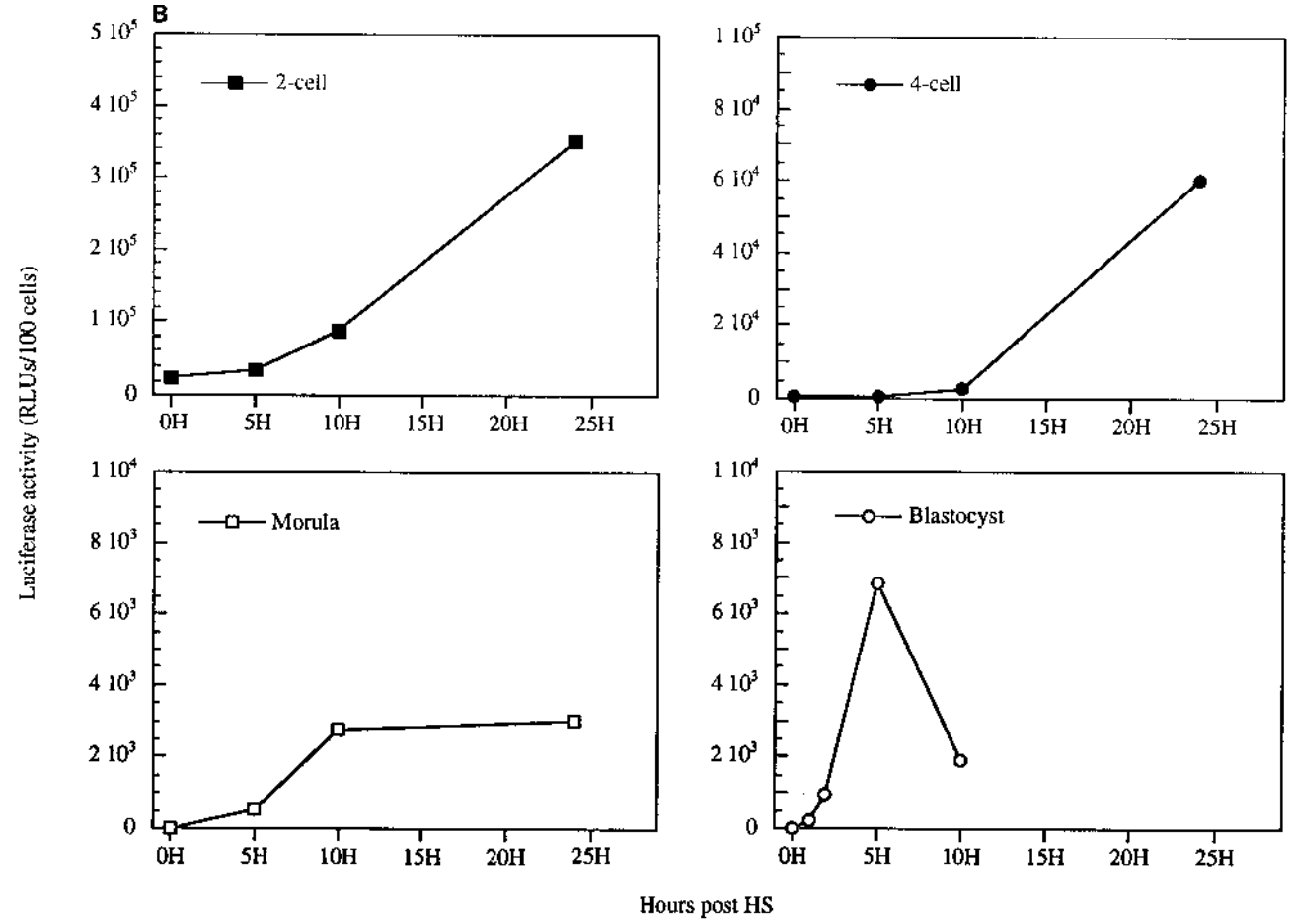


FIG. 6. (A) Comparison of heat shock effects on the *HSP70.1Luc* transgene in in vivo and in vitro two-cell embryos. Transgenic two-cell embryos were cultured at the one-cell stage (in vitro) or directly obtained from females (in vivo). Groups of both categories of embryos were subjected to heat shock (43°C for 30 min) and then cultured at 37°C for 5, 10, or 24 h before luciferase assay. The ratios of *HSP70.1Luc* activity in heat-shocked embryos (HS) to that in non-heat-shocked embryos (N) were calculated for each recovery time at 37°C. Experiments were performed in duplicate, and 30 to 100 embryos were analyzed. (B) Analysis of *HSP70.1Luc* activity following HS performed at various preimplantation stages. Transgenic embryos were cultured at the one-cell stage until HS treatment (two-cell, four-cell, morula, or blastocyst stage). They were subjected to HS (43°C for 30 min) and frozen for the luciferase assay after the indicated recovery periods at 37°C. Results are presented as mean luciferase activity calculated for 100 embryonic cells at each stage.



in disagreement with the results recently reported by Bevilacqua et al. (5), who conclude that HSE is not required for this expression. Nevertheless, it should be noticed that the experimental systems are different: we used transgenic mice with copies of the transgene stably integrated in the genome, whereas these authors followed the transient expression of large amounts of microinjected constructs. These discrepancies might be explained by differences in the chromatin state of the *HSP70.1* promoter, which has been demonstrated in different systems to be essential for the correct regulation of this heat shock gene (2, 30), in particular during early embryogenesis (26, 27, 47). As shown by Landsberger and Wolffe (27), HSF is involved in hsp70 gene expression in *Xenopus* oocytes. However, this role is revealed only when the hsp70 gene is located in correctly assembled chromatin. In fact, chromatin structure is extensively modified during mouse development from the two-cell stage (47, 49).

HSE sequences are also able to bind factors different from HSFs (24). Therefore, competition alone does not demonstrate the involvement of HSF. However, the absence of inhibition of a microinjected DNA fragment mutated in the HSE sequence at positions which specifically alter HSF binding is a very strong argument in favor of the role of HSF in hsp70 expression at the two-cell stage. Taking into account the competition results, we have to explain the fact that our gel shift assays did not reveal any HSF-like DNA-binding activity in one- and two-cell mouse embryos under normal temperature conditions (31). These two results are not in disagreement if we consider that binding of HSF requires prior binding of another transcription factor; in this case it will not be possible to detect binding of HSF with oligonucleotides containing only the HSE sequences. On the other hand, we also cannot rule out the possibility that constitutive HSE binding activity exists only during a short period corresponding to the G_1 phase of the two-cell stage, to which *HSP70.1* expression is restricted (8). We could have failed to detect this binding because the two-cell embryos used in HSE binding assays were collected later, during the long G_2 phase. Since it has been demonstrated (40) that, when overexpressed, mHSF1 becomes active and migrates to the nucleus, another attractive hypothesis would be that the high level of this factor at the two-cell stage leads to its concentration in the nucleus which is responsible for its spontaneous DNA-binding activity. This concentration-dependent activation of mHSF1 would not be revealed by gel shift assays performed with diluted whole-cell extracts (43). As shown by Sarge et al. (40), this activation in DNA-binding activity would be parallel to an increase in transcriptional efficiency.

In order to study the relationship between inducible DNA-binding activity and inducible transcription of heat shock genes, we have used *HSP70.1Luc* transgenic mice and analyzed transgene expression at the two-cell, four-cell, morula, and blastocyst stages during the 24-h following heat shock. From this study, it appears that at the two-cell stage thermal stress leads to a relatively moderate increase in activity of the transgene which persists for 24 h after heat shock treatment. At the morula stage, the profile of response becomes quite different, as there is a somewhat more rapid increase in luciferase activity, but its level is lower at 24 h after heat shock than that observed at the four-cell stage; this could be considered the first preliminary step in the establishment of the heat shock response. The high and very rapid response observed at the blastocyst stage reflects the acquisition of the full capacity to induce heat shock gene expression following stress as described previously (19, 33, 48).

How can this difference between the heat shock response at the two-cell stage and blastocyst stage be explained? It seems that the basic elements of the heat shock response are already present in the two-cell embryo, as mHSF1 is present and able to bind to the HSE after heat shock during this period. Moreover, it is known that the two-cell embryo, but not blastocysts, is characterized by a spontaneous *HSP70.1* transcriptional activity. On the basis of *HSP70.1Luc* expression estimated for 100 embryonic cells from each stage, it appears that spontaneous *HSP70.1* transcriptional activity at the two-cell stage is high, comparable to the heat-induced activity observed at the other stages. Thus, the absence of a further increase in *HSP70.1* transcriptional activity following heat shock treatment may simply be due to the fact that the promoter is already transcribed at its maximal rate. This explanation is strengthened by our results with in vivo two-cell embryos: spontaneous expression is lower in in vivo than in in vitro two-cell embryos, while heat shock treatment induces a larger increase of *HSP70.1* activity in the in vivo two-cell embryos, which then exhibit the same maximal level of *HSP70.1* transcription.

These results taken together with all the previously reported experiments suggest a model capable of explaining both spontaneous and inducible *HSP70.1* expression during the preimplantation period. At the two-cell stage, spontaneous expression would involve the action of mHSF1, which is very abundant in the nuclei of these embryos; this does not preclude the possibility that mHSF1 cooperates with other transcription factors. What remains unknown is whether the exclusive nuclear localization of mHSF1 at the two-cell stage results only from the abundance of this factor or requires a mechanism specific to the early embryo. As development proceeds and nuclei form, a new chromatin organization is built up in addition to mHSF1 dilution, resulting in the disappearance of the spontaneous activation of the heat shock genes as well as in a momentary loss of inducible DNA-binding activity. The latter could be explained by an autoregulation by heat shock proteins synthesized at the two-cell stage which remain stable for 48 h (1). Inducibility is restored only at the blastocyst stage, probably in parallel with the synthesis of new mHSF1 by transcription of the zygotic genome. Cooperation between mHSF1 and mHSF2 at this stage of development remains possible (45).

Altogether, our results provide a precise description of the transcriptional factors mHSFs in relation with the activity of their target gene (*HSP70.1*), bringing convincing support to an effective role of mHSF1 through these early stages of development.

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