

# Transcriptional Control of Development, Protein Synthesis, and Heat-Induced Heat Shock Protein 70 Synthesis in 2-Cell Bovine Embryos<sup>1</sup>

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## ABSTRACT

Experiments were performed to evaluate the role of transcription in early development of bovine embryos. Two transcription inhibitors—5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) and actinomycin D—were used to test whether 1) the inhibitors alter the rate of early embryonic development and protein synthesis, 2) heat shock increases the steady-state amounts of mRNA for the inducible form of heat shock protein 70 (HSP70) in embryos, and 3) this latter effect is blocked by transcription inhibitors. Addition of either DRB or actinomycin D to culture medium beginning 8 h postinsemination (hpi) reduced the proportion of oocytes that had undergone cleavage by 32–34 hpi. Both transcription inhibitors also reduced the proportion of cleaved embryos that reached the 4-cell stage by 32–34 hpi. Incorporation of <sup>35</sup>S-labeled amino acids into de novo synthesized protein by bovine 2-cell embryos was lower for embryos cultured with DRB. Using reverse transcription-polymerase chain reaction, HSP70 mRNA in 2- and 4-cell embryos was increased by exposure to 42°C. Both inhibitors reduced amounts of HSP70 mRNA at 42°C. Results indicate that bovine embryos can undergo transcription in response to heat shock as early as the 2-cell stage. Moreover, the observations that transcription inhibitors reduce rates of cleavage and early development point out the importance of transcription for development from the earliest period of embryonic life.

## INTRODUCTION

Resumption of gene transcription following fertilization is a fundamental requirement for embryonic development. The timing of this event, termed activation of the embryonic genome, varies among species [1]. In cattle, it was once thought that the embryonic genome was first activated at the 8-cell stage because embryos cultured with the transcriptional inhibitor  $\alpha$ -amanitin could not develop past this stage [2]. Moreover, the embryo first develops a definitive nucleolus at this stage [3] and begins to synthesize several new proteins [4]. Subsequent experiments have confirmed that a major round of transcription occurs at the 8-cell stage. At this time, there is a burst of transcription of eukaryotic translation initiation factor eIF-1A [5], and the cell-cycle control protein cdc25 is first synthesized [6]. Nonetheless, some level of transcription probably occurs before the third cell cycle since RNA synthesis can be detected in 2- and 4-cell embryos [7–10], and expression of a microinjected gene construct was observed as early as the 1-cell stage [11]. In addition,  $\alpha$ -amanitin decreased total

protein synthesis by 2-cell embryos [12] and decreased cleavage rate [13, 14].

For successful development, an embryo must be able to adjust its physiology in response to maternal regulatory signals and to other changes in its environment. While the developmentally programmed progression of genome activation in bovine embryos has been actively studied, relatively little is known about when in development the embryonic genome can respond transcriptionally to regulatory signals or perturbations in the microenvironment such as heat, low pH, anoxia, etc. The best studied case is for heat shock protein 70 (HSP70). In cattle embryos, as in other cellular systems, there are both constitutive and inducible forms of HSP70. While heat shock has no effect on synthesis of either form of HSP70 in oocytes [15, 16], synthesis of both HSP70 molecules is increased by heat shock as early as the 2-cell stage [12, 15]. Heat-induced synthesis of HSP70 was blocked by  $\alpha$ -amanitin in 4-cell embryos but not in 2-cell embryos [12]. Thus, it is possible that, as for other cells [17, 18], heat shock exerts some posttranscriptional control of embryonic HSP70 synthesis.

The primary objectives of this study were to test whether heat shock increases the steady-state amounts of mRNA for the inducible form of HSP70 in bovine 2-cell embryos and, if so, whether this effect could be blocked by transcription inhibitors. Earlier studies on the transcriptional control of embryonic HSP70 synthesis were done with  $\alpha$ -amanitin [12], which acts by blocking binding of RNA polymerase II to the promoter [19]. However, this inhibitor may not always be effective in inhibiting transcription of HSP70 since, in *Drosophila* at least, *hsp70* gene expression is regulated at the level of transcript elongation rather than binding of RNA polymerase II to the promoter [20]. Accordingly, studies were conducted with 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB), which blocks activity of RNA polymerase II at the level of transcript elongation [21], and actinomycin D, which binds to DNA and prevents RNA elongation [22]. The design for the experiments to evaluate transcriptional regulation of HSP70 synthesis by heat shock also permitted a test of the hypothesis that transcriptional inhibitors block cleavage and overall protein synthesis and development at 32–34 h postinsemination (hpi).

## MATERIALS AND METHODS

### Materials

Modified Tyrode's solutions (HEPES-Tyrode's lactate [TL], Sperm-TL, and in vitro fertilization [IVF]-TL) and Tissue Culture Medium 199 were purchased from Cell and Molecular Technologies (Lavallete, NJ). BSA (Fraction V), sodium pyruvate, and gentamicin (all from Sigma Chemical Co., St. Louis, MO) were added to these solutions to prepare HEPES-Tyrode's albumin lactate pyruvate [TALP], Sperm-TALP, and IVF-TALP as described by Parrish et al.

Accepted July 20, 1999.

Received February 26, 1999.

<sup>1</sup>Research was supported in part by USDA NRICGP #96-35205-3728. This is Journal Series No. R-06800 of the Florida Agricultural Experiment Station.

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[23]. The CR1aa medium (containing 3 mg/ml Fraction V BSA and 2.5  $\mu$ g/ml gentamicin) was prepared as described by Rosenkrans and First [24]. FSH (Super-Ov) was obtained from AgTech (Manhattan, KS), bovine steer serum was from Pel-Freez (Rogers, AK), and fetal calf serum was from Atlanta Biologicals (Norcross, GA). Frozen semen from various bulls was obtained from ABS (De Forest, WI) or Southeast Semen (Lake City, FL). Actinomycin D, DRB, and other reagents for in vitro production of embryos were from Sigma.

For radiolabeled L-<sup>35</sup>S-amino acids, TRAN <sup>35</sup>S-LABEL was purchased from ICN (Costa Mesa, CA); this is a <sup>35</sup>S *Escherichia coli* hydrolysate-labeling reagent with 70% L-[<sup>35</sup>S]methionine and  $\leq$  15% L-[<sup>35</sup>S]cysteine (specific activity = 1174–1486 Ci/mmol). Ethidium bromide and trichloroacetic acid (TCA) were from Fisher (Pittsburgh, PA). Trizol was purchased from Life Technologies (Grand Island, NY), *Taq* DNA polymerase was from Sigma, and the RETROScript kit containing reagents for reverse transcription (RT)-polymerase chain reaction (PCR) was obtained from Ambion (Austin, TX). Oligodeoxynucleotides used as PCR primers were synthesized by the DNA Synthesis Core of the Interdisciplinary Center of Biotechnology Research, University of Florida, Gainesville, or purchased from Research Genetics, Inc. (Huntsville, AL). The *Bcl*I restriction enzyme was from Promega Corporation (Madison, WI). Other reagents were from Sigma or Fisher.

#### *In Vitro* Production of Embryos

Procedures for in vitro maturation, fertilization, and culture have been described [15, 25]. Briefly, oocytes were matured for 22 h and then fertilized with Percoll-purified sperm pooled from 3 bulls. Fertilization proceeded for 8 h, and then putative zygotes were vortexed in 500  $\mu$ l of HE-PES-TALP containing 300  $\mu$ g/ml hyaluronidase to remove cumulus cells and associated spermatozoa. Putative zygotes/oocytes with no cumulus present were transferred ( $\sim$ 30/drop) to 50- $\mu$ l drops of CR1aa culture medium and cultured at 38.5°C in 5% CO<sub>2</sub>. For experiments with DRB, drops also contained either 90  $\mu$ M DRB or an equivalent volume of ethanol vehicle. For experiments with actinomycin D, drops contained either 500 ng/ml actinomycin D or an equivalent volume of water vehicle. Stage of development was recorded at 32–34 hpi, and embryos were used for subsequent RT-PCR or radiolabeling experiments.

#### *Protein Synthesis as Affected by DRB*

At 32–34 hpi, 2-cell embryos were collected from microdrops containing DRB or vehicle. Embryos were transferred in groups of 20–30 to a fresh 50- $\mu$ l microdrop of CR1aa medium prepared without essential amino acids and containing 100  $\mu$ Ci of <sup>35</sup>S amino acids. Drops were cultured at either 38.5°C for 4.33 h (i.e., 4 h 20 min) or at 42°C for 1.33 h (i.e., 80 min) and then at 38.5°C for 3 h. After culture, embryos were washed one time in CR1aa and transferred to 50  $\mu$ l solubilization buffer (5 mM K<sub>2</sub>CO<sub>3</sub> containing 9.4 M urea, 2% [v:v] Nonidet P-40, and 0.5% [w:v] dithiothreitol) and stored at –70°C until analysis. Incorporation of radiolabel into intracellular protein was determined using TCA precipitation [26].

#### *Culture and RNA Preparation of Embryos*

At 32–34 hpi, 2-cell embryos were collected from microdrops containing DRB, actinomycin D, or vehicle con-

trol. Only embryos with no attached cumulus cells were used. Embryos were transferred to fresh drops of CR1aa solution (20–30 embryos/50  $\mu$ l drop) containing the same treatments (DRB, actinomycin D, or vehicle control). Drops were then exposed to 38.5°C for 4.33 h or 42°C for 1.33 h and then exposed to 38.5°C for 3 h. After heat treatment, embryos were pooled within treatment and transferred into Trizol solution (1 ml). The number of embryos extracted varied between runs from 30 to 106, but a similar number of embryos was extracted for each treatment within a given run. Embryos were extracted without carrier according to the manufacturer's instructions. After extraction, RNA was redissolved in 25  $\mu$ l diethyl pyrocarbonate (DEPC)-treated water.

#### *RT-PCR*

Primers were based on the sequence for a heat-inducible form of bovine Hsp70 [27] and were chosen to minimize hybridization with a constitutively expressed bovine *hsp70* gene [28]. The forward primer was GTCATCAACGACGGAGACAA (5' position = 436), and the reverse primer was GGTGCTGGACGACAAGGT (3' position = 973). The predicted optimum annealing temperature was 59.4°C, and predicted band size was 555 base pairs (bp).

All RT-PCR reactions were conducted in 200- $\mu$ l PCR tubes and were assembled on ice. For the RT reaction, RNA (10  $\mu$ l of final RNA pellet for embryos) was mixed with 2  $\mu$ l 10-strength RT-PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl), 2  $\mu$ l of oligo dT<sub>18</sub> (50  $\mu$ M), 4  $\mu$ l dNTP mix (2.5 mM ATP, 2.5 mM CTP, 2.5 mM TTP, and 2.5 mM GTP), and DEPC-treated water (to 20  $\mu$ l). Samples were placed in a thermocycler and incubated at 80°C for 3 min and then cooled to 4°C. Tubes were returned to the crushed ice, and 1  $\mu$ l of placental ribonuclease (RNase) inhibitor (10 U/ $\mu$ l) and 1  $\mu$ l of Moloney-Murine leukemia virus reverse transcriptase (100 U/ $\mu$ l) was added. Samples were then returned to the thermocycler and incubated at 42°C for 1 h and then at 92°C for 10 min; tubes were then cooled to 4°C. RT products were either stored at –20°C or were used immediately for the PCR step. For amplification, PCR reactions were prepared on ice and conducted in a total volume of 52.5  $\mu$ l, which contained 5  $\mu$ l cDNA product, 5  $\mu$ l 10-strength RT-PCR buffer (pH 8.3, containing 1.5 mM Mg<sup>2+</sup>), 2.5  $\mu$ l dNTP mix, 1.25  $\mu$ l (5  $\mu$ M) of each primer, 37.3  $\mu$ l H<sub>2</sub>O, and 0.2  $\mu$ l *Taq* polymerase (5 U/ $\mu$ l). Tubes were then placed in a programmable thermocycler (MJ Research, Watertown, MA) and preheated at 94°C for 4 min before 42 cycles of heating at 94°C for 1 min, 59.4°C for 1 min, and 72°C for 1 min. The PCR tubes were then heated at 72°C for 10 min and cooled to 4°C until electrophoresis. Reaction products (10–12  $\mu$ l; volume was varied slightly to adjust for differences in number of embryos extracted) were resolved on 2% (w:v) agarose gels and stained with ethidium bromide.

In one experiment, RT-PCR reaction products were digested with *Bcl*I to verify identity of the amplicons using conditions specified by the manufacturer.

#### *Statistical Analysis*

To determine effects of DRB and actinomycin on development, the percent of oocytes that cleaved and the percent of oocytes and cleaved embryos that developed to the 4-cell stage at 32–34 hpi were calculated for each microdrop. Thus, data were obtained on 9 (DRB) or 4 (actinomycin) replicates (each replicate being an IVF procedure

TABLE 1. Effect of addition of 90  $\mu$ M DRB at 8 hpi on embryonic development at 32–34 hpi.

Treatment	Replicates (n)			Development at 32–34 hpi		
	Oocytes	Drops	Days	Oocytes cleaved (%)	Oocytes to 4-cell stage (%)	Cleaved embryos to 4-cell stage (%)
Control	668	33	9	58.5 $\pm$ 2.5	18.8 $\pm$ 1.7	31.8 $\pm$ 3.3
DRB	786	36	9	37.7 $\pm$ 2.3	9.0 $\pm$ 1.6	20.9 $\pm$ 3.1
<i>P</i>				0.001	0.004	0.043

performed on a specific day), using several microdrops of embryos per treatment for each replicate. Data were analyzed by least-squares ANOVA using the General Linear Models (GLM) procedure of the Statistical Analysis System (SAS) [29]. Data were subjected to arc sine transformation before analysis. Each experiment was analyzed with effects of treatment, replicate, and treatment  $\times$  replicate in the model. All main effects were considered as fixed.

Data for TCA-precipitable radioactivity were determined for a group of embryos from one microdrop and then mathematically expressed as cpm per embryo. The experiment was replicated 3–4 times, and treatment effects were determined by least-squares ANOVA using the PROC GLM procedure of SAS [29] with effects of DRB, temperature, replicate, and their interactions.

## RESULTS

### Cleavage and Development

Addition of either DRB (Table 1) or actinomycin D (Table 2) to culture medium beginning 8 hpi significantly reduced the proportion of oocytes that had undergone cleavage by 32–34 hpi. The proportion of cleaved embryos that reached the 4-cell stage by 32–34 hpi was reduced ( $P = 0.04$ ) by DRB (Table 1) and actinomycin D (Table 2). Thus, both transcription inhibitors delayed the rate of embryonic development among embryos that did undergo cleavage.

### Protein Synthesis

Incorporation of  $^{35}$ S-labeled amino acids into de novo synthesized protein by bovine 2-cell embryos was lower ( $P < 0.01$ ) for those cultured at 42°C for 1.33 h followed by 38.5°C for 3 h than for embryos cultured at 38.5°C for 4.33 h (Fig. 1). Regardless of temperature treatment, exposure of embryos to 90  $\mu$ M DRB reduced ( $P < 0.02$ ) de novo synthesis of protein as measured by incorporation of  $^{35}$ S-labeled amino acids into TCA-precipitable protein.

### RT-PCR for HSP70

RT-PCR of RNA extracted from 2-cell embryos and 4-cell embryos resulted in synthesis of a 555-bp amplicon corresponding to the predicted size for HSP70 mRNA (Fig. 2). The amplicon was absent in 2-cell embryos cultured at 38.5°C but was present in 2-cell embryos exposed to 42°C for 1.33 h followed by 38.5°C for 3 h (Figs. 2–3). For 4-

cell embryos, a faint PCR product was observed for embryos at 38.5°C and a more intense band for embryos exposed to 42°C (Fig. 2). The PCR product from 2-cell embryos was not present when reverse transcriptase was excluded from the reaction (Fig. 3). The HSP70 amplicon was cleaved by *Bcl*I into a smaller product that corresponded to the 407-bp product predicted for HSP70 (Fig. 3).

In one replicate, 2-cell embryos were cultured at 42°C in the presence or absence of DRB: the amount of PCR product was nearly undetectable in the DRB-treated embryos but was abundant in control embryos (Fig. 4). When 26 (not shown) or 44 2-cell embryos per group (Fig. 4) were subjected to RNA extraction, actinomycin D completely blocked the heat-induced appearance of PCR product (Fig. 4). When a larger number of 2-cell embryos were extracted ( $n = 105$ ), heat shock (42°C) caused a slight increase in the amount of HSP70 amplicon in actinomycin-treated embryos (Fig. 4). This increase appeared less than that observed in embryos without actinomycin D. There was also a slight amount of HSP70 mRNA detectable at 38.5°C in the absence of actinomycin D (Fig. 4).

## DISCUSSION

The present findings indicate that heat shock-induced synthesis of HSP70 in bovine 2-cell embryos depends, at least in part, upon new transcription. Moreover, present results confirm the notion that, in addition to mediating stress responses, transcription is generally important for development of early bovine embryos since addition of transcription inhibitors reduced cleavage rate, slowed development, and inhibited overall protein synthesis. Thus, rather than viewing the early development as a process dependent solely on proteins synthesized by preformed maternal transcripts, it is apparent that gene activation is important for development from the earliest period of embryonic development.

Earlier observations that the 2-cell embryo can undergo increased HSP70 synthesis in response to heat [12, 15] illustrated the idea that the early embryo has some capacity for changing its physiology in response to stress in an effort to stabilize cellular function. HSP70 is most likely important for protecting embryonic cells from the adverse effects of heat shock because injection of HSP70 mRNA into mouse oocytes increased their resistance to heat shock [30] and HSP70 is important for survival following heat shock for other cells [31]. Present results indicate that the increase

TABLE 2. Effect of addition of 500 ng/ml actinomycin D at 8 hpi on embryonic development at 32–34 hpi.

Treatment	Replicates (n)			Development at 32–34 hpi		
	Oocytes	Drops	Days	Oocytes that cleaved (%)	Oocytes to 4-cell stage (%)	Cleaved embryos to 4-cell stage (%)
Control	481	19	4	62.1 $\pm$ 2.1	19.7 $\pm$ 1.6	31.0 $\pm$ 3.1
Actinomycin D	437	19	4	31.6 $\pm$ 2.1	6.6 $\pm$ 1.6	21.2 $\pm$ 3.1
<i>P</i>				0.001	0.001	0.036

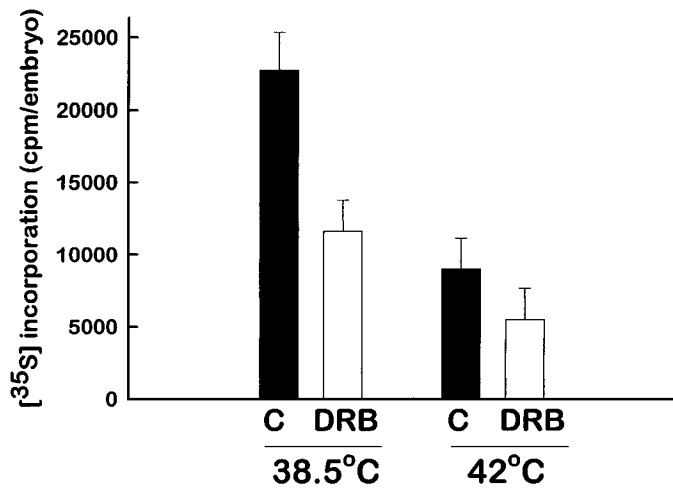


FIG. 1. Effect of heat shock and DRB on incorporation of <sup>35</sup>S-labeled amino acids into de novo synthesized protein by bovine 2-cell embryos. Embryos were exposed to either 38.5°C for 4.33 h or 42°C for 1.33 h followed by 38.5°C for 3 h. Data represent least-squares means of 3–4 pools of embryos/treatment. Incorporation was affected by temperature ( $P < 0.01$ ) and DRB treatment ( $P < 0.02$ ).

in HSP70 synthesis caused by heat shock is the result of new transcription. Heat shock caused an increase in steady-state amounts of HSP70 mRNA in 2-cell embryos, and this increase was reduced if embryos were cultured with DRB or actinomycin D. When large numbers of embryos were extracted for RT-PCR (105 instead of the 30–40 used in most procedures), a slight increase in HSP70 mRNA was seen in the presence of actinomycin D. This increase could reflect an increase in mRNA stability caused by heat shock, similar to that reported for chicken reticulocytes and human HeLa and 293 cells [32, 33], or simply reflect incomplete inhibition of transcription.

Heat shock caused a more severe reduction of subsequent development when applied to the 2-cell stage than when applied at the 4- to 8-cell or morula stage [16, 34]. Thus, the 2-cell bovine embryo appears to be more sensitive to heat shock than embryos at later stages even though gene expression of products such as HSP70 that increase resistance to heat shock can be induced at the 2-cell stage. Perhaps, the amounts of stress proteins synthesized are lower for 2-cell embryos than at other stages. Alternatively, stage-dependent differences in systems other than stress proteins may be responsible for determining the degree to which embryos are sensitive to heat shock. One action of heat shock on mouse embryos, for example, is to cause a reduction in the cellular antioxidant glutathione [35], and it may be that 2-cell embryos are less able to ameliorate

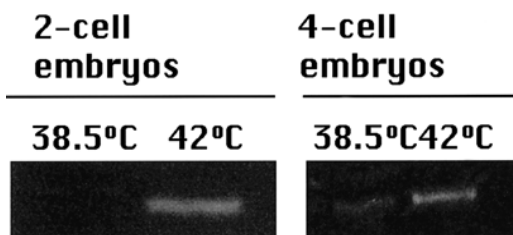


FIG. 2. Induction of HSP70 mRNA in 2- and 4-cell bovine embryos by heat shock. Embryos were exposed to either 38.5°C for 4.33 h or 42°C for 1.33 h followed by 38.5°C for 3 h. RNA was extracted from pools of 40 embryos and subjected to RT-PCR using primers specific for the inducible form of HSP70.

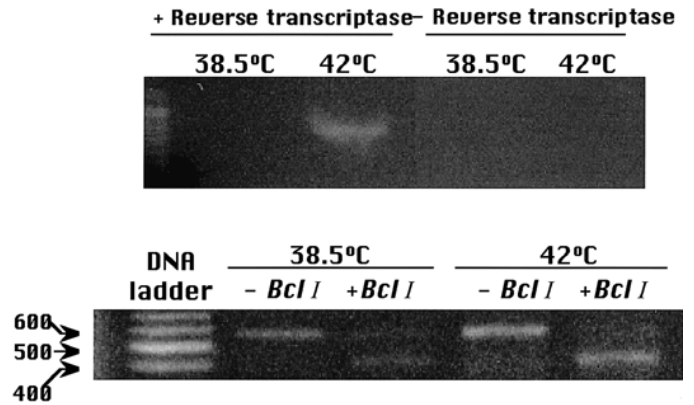


FIG. 3. Validation of RT-PCR for HSP70 in bovine 2-cell embryos. RNA was extracted from groups of 45 (top panel) or 105 (bottom panel) embryos exposed to either 38.5°C for 4.33 h or 42°C for 1.33 h followed by 38.5°C for 3 h. The top panel illustrates that 1) heat shock increased the amount of HSP70 amplicon and 2) that the amplicon was absent when reverse transcriptase was not included (lanes on right) in the RT-PCR protocol. The bottom panel shows that restriction digestion of HSP70 RT-PCR products produced a product of the predicted size (407 bp).

effects of free radicals induced by heat shock. It is also possible that 2-cell embryos are not really more sensitive to heat shock than more advanced embryos but that the choice of developmental endpoints obscures differences in the ability of embryos of different developmental stages to survive heat shock. A recent report in the mouse [36] indicated that 2-cell embryos appeared to be more susceptible to heat shock than 4-cell and morula-stage embryos when the endpoint used was development to the blastocyst stage; however, the reduction in development to the hatched blastocyst stage was similar for all embryos.

It is clear from the present results that, rather than relying simply on mRNA formed during oocyte growth, the bovine embryo depends upon new transcription for early developmental processes leading to cleavage and continued growth. In fact, both DRB and actinomycin D reduced the numbers of inseminated oocytes that underwent cleavage and reduced the numbers of cleaved embryos that had developed to the 4-cell stage by 32–34 hpi. Moreover, DRB inhibited de novo protein synthesis by 2-cell embryos. While it is possible that effects of DRB and actinomycin

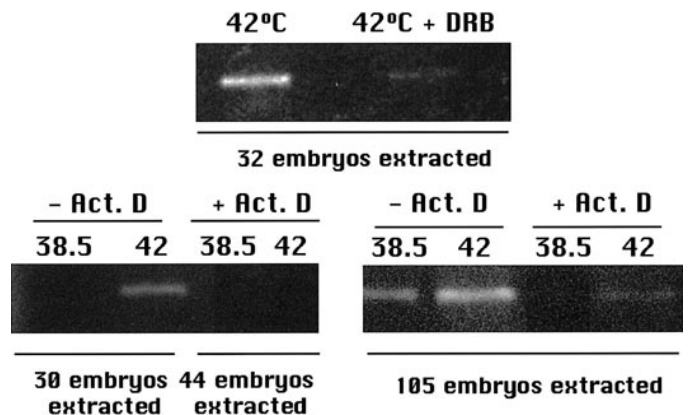


FIG. 4. Inhibition of heat shock-induced changes in HSP70 mRNA in embryos cultured with transcription inhibitors DRB and actinomycin D (Act. D). Embryos were exposed to either 38.5°C for 4.33 h or 42°C for 1.33 h followed by 38.5°C for 3 h. RNA was extracted from pools of embryos and subjected to RT-PCR using primers specific for the inducible form of HSP70.

D represent some toxic or transcriptional-independent action of these molecules, such an explanation is unlikely because both molecules, acting to inhibit transcription through different mechanisms, exerted similar actions. Moreover, another transcriptional inhibitor,  $\alpha$ -amanitin, reduced cleavage rate of bovine embryos [13, 14] and de novo protein synthesis by 2-cell bovine embryos [12]. Cordycepin, which inhibits posttranscriptional adenylation of nuclear RNA, also reduced cleavage rate at 48 hpi [14]. Also consistent with the idea that early development is regulated in part by transcriptionally controlled events are the observations that RNA synthesis occurs in 2- and 4-cell embryos [7–10] and that a microinjected gene construct was expressed at the 1-cell stage [11]. Now that it is known that embryonic gene expression can be modified in response to environmental stresses, in what is probably an attempt of the embryo to stabilize cellular function, it will be instructive to determine whether the pattern of embryonic gene expression can also be altered by other signals such as regulatory molecules of maternal or embryonic origin that might act to coordinate embryonic development with progressive changes in the maternal reproductive tract during pregnancy.

#### ACKNOWLEDGMENTS

The authors thank Rocio Rivera and Mohammed Khaled for technical assistance; Tommy Bryan, owner of Center Packing Co. (Center Hill, FL) and his employees for assistance in obtaining ovaries; and William Farmerie and DNA Synthesis Core Laboratory of the University of Florida Interdisciplinary Center for Biotechnology Research for invaluable assistance in designing PCR primers.

#### REFERENCES

- de Sousa PA, Caveney A, Westhuisin ME, Watson AJ. Temporal patterns of embryonic gene expression and their dependence upon genetic factors. *Theriogenology* 1998; 49:115–128.
- Barnes FL, First NL. Embryonic transcription in *in vitro* cultured bovine embryos. *Mol Reprod Dev* 1991; 29:117–123.
- Kopecny V, Flechon JE, Camous S, Fulka J. Nucleogenesis and the onset of transcription in the eight-cell bovine embryo: fine ultrastructural autoradiographic study. *Mol Reprod Dev* 1989; 1:79–90.
- Frei PG, Schultz GA, Church RB. Qualitative and quantitative changes in protein synthesis occur at the 8–16 cell stage of embryogenesis in the cow. *J Reprod Fertil* 1989; 86:637–641.
- de Sousa PA, Watson AJ, Schultz RM. Transient expression of a translation initiation factor is conservatively associated with embryonic gene activation in murine and bovine embryos. *Biol Reprod* 1998; 59:969–977.
- Jones JM, First NL. Expression of the cell-cycle control protein cdc25 in cleavage stage bovine embryos. *Zygote* 1995; 3:133–139.
- Plante L, Plante C, Shepherd DL, King WA. Cleavage and  $^3\text{H}$ -uridine incorporation in bovine embryos of high *in vitro* developmental potential. *Mol Reprod Dev* 1994; 39:375–383.
- Viuff D, Avery B, Greve T, King WA, Hyttel P. Transcriptional activity in *in vitro* produced bovine two- and four-cell embryos. *Mol Reprod Dev* 1996; 43:171–179.
- Hyttel P, Viuff D, Avery B, Laurincik J, Greve T. Transcription and cell cycle-dependent development of intranuclear bodies and granules in two-cell bovine embryos. *J Reprod Fertil* 1996; 108:263–270.
- Memili E, Dominko T, First NL. Onset of transcription in bovine oocytes and preimplantation embryos. *Mol Reprod Dev* 1998; 51:36–41.
- Saeki K, Matsumoto K, Kaneko T, Hosoi Y, Kato H, Iritani A. Onset of RNA synthesis in early bovine embryos detected by reverse transcription-polymerase chain reaction following introduction of exogenous gene into their pronuclei. *Theriogenology* 1999; 51:192 (abstract).
- Edwards JL, Ealy AD, Monterroso VH, Hansen PJ. Ontogeny of temperature-regulated heat shock protein 70 synthesis in preimplantation bovine embryos. *Mol Reprod Dev* 1997; 48:25–33.
- Memili E, First NL. Developmental changes in RNA polymerase II in bovine oocytes, early embryos, and effect of  $\alpha$ -amanitin on embryo development. *Mol Reprod Dev* 1998; 51:381–389.
- Park E-H, Chian R-C, Chung H-M, Lim J-G, Ko J-J, Cha K-Y. The stage of embryonic genome activation in bovine embryos following *in vitro* fertilization. *Theriogenology* 1999; 51:188 (abstract).
- Edwards JL, Hansen PJ. Elevated temperature increases heat shock protein 70 synthesis in bovine two-cell embryos and compromises function of maturing oocytes. *Biol Reprod* 1996; 55:340–346.
- Edwards JL, Hansen PJ. Differential responses of bovine oocytes and preimplantation embryos to heat shock. *Mol Reprod Dev* 1997; 46:138–145.
- Banerji SS, Theodorakis NG, Morimoto RI. Heat shock induced translational control of HSP70 and globin synthesis in chicken reticulocytes. *Mol Cell Biol* 1984; 4:2437–2448.
- Colbert RA, Young DA. Detection of mRNAs coding for translationally-regulated heat-shock proteins in non-heat-shocked thymic lymphocytes. *J Biol Chem* 1987; 262:9939–9941.
- Lindell TJ, Weinberg F, Morris PW. Specific inhibition of nuclear RNA polymerase II by  $\alpha$ -amanitin. *Science* 1970; 257:967–961.
- Gilmour DS, Lis JT. RNA polymerase II interacts with the promoter region of the noninduced hsp70 gene in *Drosophila melanogaster* cells. *Mol Cell Biol* 1986; 6:229–239.
- Yamaguchi Y, Wada T, Handa H. Interplay between positive and negative elongation factors: drawing a new view of DRB. *Genes Cells* 1998; 3:9–15.
- Sobell HM. Actinomycin and DNA transcription. *Proc Natl Acad Sci USA* 1985; 82:5328–5331.
- Parrish JJ, Susko-Parrish JL, Critser ES, Eyestone WH, First NL. Bovine *in vitro* fertilization with frozen-thawed semen. *Theriogenology* 1986; 25:591–600.
- Rosenkrans CF, Zeng GQ, McNamara GT, Schoff PK, First NL. Development of bovine embryos *in vitro* as affected by energy substrates. *Biol Reprod* 1993; 49:459–462.
- Paula-Lopes FF, de Moraes AAS, Edwards JL, Justice JE, Hansen PJ. Regulation of preimplantation development of bovine embryos by interleukin-1 $\beta$ . *Biol Reprod* 1998; 59:1406–1412.
- Mans RJ, Novelli D. Measurement of the incorporation of radioactive amino acids into proteins by a filter-paper disk method. *Arch Biochem Biophys* 1961; 94:48–53.
- Gutierrez JA, Guerriero V. Chemical modifications of a recombinant bovine stress-inducible 70 kDa heat-shock protein (Hsp70) mimics Hsp70 isoforms from tissues. *Biochem J* 1995; 305:197–203.
- deLuca-Flaherty C, McKay DB. Nucleotide sequence of the cDNA of a bovine 70 kilodalton heat shock cognate protein. *Nucleic Acids Res* 1990; 18:5569.
- SAS. Statistical Analysis System: A User's Guide. Version 6, 4th edition. Cary, NC: Statistical Analysis System Institute, Inc.; 1989.
- Hendrey JJ, Kola I. Thermolability of mouse oocytes is due to the lack of expression and/or inducibility of Hsp70. *Mol Reprod Dev* 1991; 25:1–8.
- Johnson RN, Kucey BL. Competitive inhibition of *hsp70* gene expression causes thermosensitivity. *Science* 1988; 242:1551–1554.
- Theodorakis NG, Morimoto RI. Posttranscriptional regulation of hsp70 expression in human cells: effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. *Mol Cell Biol* 1987; 7:4357–4368.
- Theodorakis NG, Banerji SS, Morimoto RI. HSP70 mRNA translation in chicken reticulocytes is regulated at the level of elongation. *J Biol Chem* 1988; 263:14579–14585.
- Ealy AD, Howell JL, Monterroso VH, Aréchiga CF, Hansen PJ. Developmental changes in sensitivity of bovine embryos to heat shock and use of antioxidants as thermoprotectants. *J Anim Sci* 1995; 73:1401–1407.
- Aréchiga CF, Ealy AD, Hansen PJ. Evidence that glutathione is involved in thermotolerance of preimplantation mouse embryos. *Biol Reprod* 1995; 52:1296–1301.
- Aréchiga CF, Hansen PJ. Response of preimplantation murine embryos to heat shock as modified by developmental stage and glutathione. *In Vitro Dev Cell Biol Anim* 1998; 34:655–659.