FEBS Letters 436 (1998) 382–386 FEBS 20932

Marked differences between avian and mammalian testicular cells in the heat shock induction and polyadenylation of Hsp70 and ubiquitin transcripts

Belen Mezquita, Cristóbal Mezquita*, Jovita Mezquita

Laboratori de Genètica Molecular, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Facultat de Medicina, Universitat de Barcelona, Casanova 143, 08036 Barcelona, Spain

Received 1 September 1998

Abstract Mammalian male germ cells undergo apoptosis at the body's internal temperature of 37°C. Birds, however, are unique among homeothermic animals in developing spermatogenesis at the elevated avian internal body temperature of 40–41°C. To shed light on the mechanisms that maintain an efficient avian spermatogenesis at elevated temperatures we compared, in mouse and chicken testicular cells, the expression of genes that are essential for stress resistance: Hsp70 and ubiquitin. While the expression of Hsp70 and ubiquitin did not change upon heat shock in mouse testicular cells, both the amount and polyadenylation of Hsp70 and ubiquitin transcripts increased when male germ cells from adult chicken testis were exposed to elevated temperatures.

Key words: Apoptosis; RNA stability; Spermatogenesis; Stress resistance; Translation

1. Introduction

The homeostasis of a cell or an organism guarantees survival under stressful conditions. An essential feature of homeostasis is the rapid expression of genes whose products play a major role in protecting cells against stress. When cells or whole organisms are exposed to elevated temperatures, they respond by synthesizing a small number of highly conserved proteins, the heat shock proteins (HSP). The heat shock response was first reported as a dramatic change in gene activity induced by a brief treatment of *Drosophila hydei* larvae [1]. The heat shock proteins were also first discovered in *Drosophila* [2]. Similar findings in other eukaryotes and prokaryotes suggested that the heat shock response was an evolutionarily conserved system essential for cell survival [3–6].

Two heat shock proteins, HSP70 and ubiquitin, belong to the most highly conserved proteins in the cell. When cells are exposed to elevated temperatures the heat shock protein HSP70 is the most prominently expressed. The chaperone function of HSP70 plays a vital cytoprotective role by binding to denatured proteins and refolding them back to the native functional state [7]. Ubiquitin is also an essential component of the stress response system, specifically required for cell survival under a broad range of physiological stresses [8]. The expression of ubiquitin is greatly increased by heat stress [9,10]. Heat stress leads to a sudden increase in the level of damaged proteins that can be toxic to the cell. Covalent conjugation of ubiquitin to damaged proteins can trigger their degradation, restoring cell function.

While the heat shock response plays a major role in the protection of mammalian somatic cells from thermal injury, mammalian male germ cells undergo apoptosis at the body's internal temperature of 37°C and even slight elevations of scrotal temperature are associated with male infertility [11]. The underlying mechanisms of the inhibitory effects of elevated temperature on the process of mammalian spermatogenesis have not been elucidated.

In contrast to mammals, birds develop spermatogenesis at elevated temperature [12]. The mechanisms that maintain an efficient avian spermatogenesis at the high core body temperature of 40–41°C are unknown at present. Previously we reported a high constitutive expression of the ubiquitin gene family during avian spermatogenesis [13–16]. In addition, we detected the presence of several testis-specific transcripts of the polyubiquitin genes UbI and UbII [14,16]. One of these transcripts, from the heat-inducible chicken polyubiquitin gene UbI (t-UbI), showed a testis-specific transcription initiation site placed closer to the heat shock promoter. This transcript also undergoes alternative splicing and possesses a longer 5′ untranslated region than the somatic form [16].

To assess the differences in the heat shock response between avian and mammalian male germ cells, the main objectives of the present study were: (1) to compare the expression upon heat shock of Hsp70 and the heat-inducible polyubiquitin genes in chicken and mouse testis cells in vitro, and (2) to investigate the expression of Hsp70 and the ubiquitin gene family in chicken testis cells exposed to heat shock. Our results show that both the amount and the polyadenylation of Hsp70 and ubiquitin transcripts increased when avian male germ cells from adult chicken testis were exposed to heat shock, while no similar changes were observed in cells from mammalian testis.

2. Materials and methods

2.1. Preparation of RNA, electrophoresis, Northern hybridization and immunological detection

Total RNA was prepared with the TriPure Isolation Reagent from Boehringer Mannheim, according to the specifications of the manufacturer. Samples of total RNA (20–40 μg), obtained from prepuberal (6-week-old) and adult (25-week-old) chicken testis and adult mouse testis (10-week-old), were electrophoresed through 2 M formaldehyde, 1.2–1.6% agarose gels, in 0.02 M MOPS, 5 mM sodium acetate (pH 7) and 1 mM EDTA and transferred to positively charged nylon membranes (Nytran Plus, Schleicher and Schuell) in 10×SSC, then fixed by UV crosslinking. Blots were hybridized using ExpressHyb hybrid-

*Corresponding author. Departament de Ciències Fisiològiques I, Facultat de Medicina, Universitat de Barcelona, Casanova 143, 08036 Barcelona, Spain. Fax: (34) (93) 4035279.
E-mail: mezquita@medicina.ub.es

PII: S0014-5793(98)01172-7

ization solution (Clontech), labeled with non-radioactive digoxigenin-specific probes as described below. Hybridization conditions and the immunological chemiluminescence detection procedure were as described in [17].

2.2. DNA probes

Probes were obtained from recombinant clones containing the whole cDNA or specific segments. Labeling was done by PCR amplification using Taq DNA polymerase and a dNTP mix containing DIG-11-dUTP (Boehringer Mannheim). Primers were specific for the insert or the reverse and forward primers from the vector. Amplification conditions were as follow: 94°C 3 min; 30 cycles at 94°C 1 min; 52°C 1 min; 72°C 3 min; and final extension at 72°C, 7 min. When using specific primers the annealing temperature was changed based on the melting temperature of the primers. When the specific sequence was too short for cloning, oligonucleotide probes labeled at the 5′ end with digoxigenin were obtained from MWG-BIOTECH Synthesis Lab. Probes from recombinants having the four ubiquitin coding sequences of UbI or the three ubiquitin coding sequences of UbII hybridize unspecifically to all members of the family. We will refer to these probes as non-specific UbI or UbII probes. Specific probes have the specific exon of the testis UbI (t-UbI specific probe), the 3′ UTR sequence of UbII (UbII-specific probe), the coding sequence of the ribosomal protein T-52 (Ub-t52-specific probe) and the coding sequence of the ribosomal protein T-80 (Ub-t80-specific probe). The oligonucleotide ACTGGAAGCAGATTGACTTGCGTTGGGTACTGCGCTCAGG (labelled with digoxigenin at the 5′ end) is complementary to a sequence specific for the somatic 5′ UTR of the UbI (s-UbI-specific probe). To obtain the probe for Hsp70, the primers ATGTCTGGCAAAGGGCCGGC and TTGGGCTTGCCACCCTCG, which amplify the first 308 bp of the coding region of the gene, were used.

2.3. RNase H treatment

The method described in [18] was used: 20–30 μg of RNA was mixed with 2 μg of oligo d(T) (Pharmacia Biotech, Uppsala), denatured at 65°C for 2 min and annealed for 15 min on ice. Then, 2 units of RNase H (Pharmacia Biotech, Uppsala) were added and incubated at 37°C for 30 min. Samples were extracted with phenol-chloroform, ethanol precipitated and analyzed in agarose-formaldehyde 1.2% gels (as previously described) in parallel with control samples from the same tissues.

2.4. Heat-shock conditions

Prepuberal (6-week-old) and adult (6–12-month-old) Hubbard White Mountain chickens and adult mice (strain C57BL/6NHsd) were used in the experiments. Testes were decapsulated and the seminiferous tubules, gently dispersed with forceps, were cultured in 10 volumes of minimum essential medium (Eagle) at the appropriate temperatures. In other experiments cell suspensions were prepared from the seminiferous tubules. The tissue was finely minced with scissors, suspended in 10 volumes of minimum essential medium (Eagle) containing 0.1% trypsin, incubated at 31°C for 30 min in an orbital air incubator, and filtered through four layers of surgical gauze. Then, fetal calf serum was added to 10%. Aliquots of 1–6×10^9 cells (10 ml) were used as control and heat shock samples. Heat shock was conducted at 46°C (chicken) or 42°C (mouse) for 2 h in a water bath orbital incubator. Controls were incubated at 39°C (chicken) or 31°C (mouse) for 2 h. Cells or tissue were centrifuged at $1000\times g$ for 5 min. The pellet was extracted with TriPure (Boehringer). When actinomycin D (Sigma) was used, cells were incubated in the presence of 1 μg/ml of the inhibitor.

3. Results

3.1. Heat shock increases the amount of Hsp70 and polyubiquitin UbI transcripts in adult chicken testis cells but not in cells from adult mouse testis

An attractive possibility to explain the striking differences in thermotolerance of mammalian and avian spermatogenesis could be the existence of a differential expression of heat shock genes, such as Hsp70 and polyubiquitin. We first examined this possibility. Cells from adult chicken testis were exposed to 46°C for 2 h. Control cells were incubated at the internal body temperature of 40°C for the same length of time. Total RNA was prepared from heat-shocked cells and control cells and analyzed by Northern blot hybridization with probes from chicken Hsp70 and the coding sequence of chicken polyubiquitin UbI. Both transcripts were more abundant in heat-shocked cells than in control cells (Fig. 1). The increase of the polyubiquitin transcript is much less than the increase of Hsp70 induced under the same circumstances. The length of transcripts of Hsp70 and polyubiquitin UbI also increased upon heat shock, giving rise to broader bands in the Northern blots (Fig. 1). The same results were obtained using cell suspensions or seminiferous tubules.

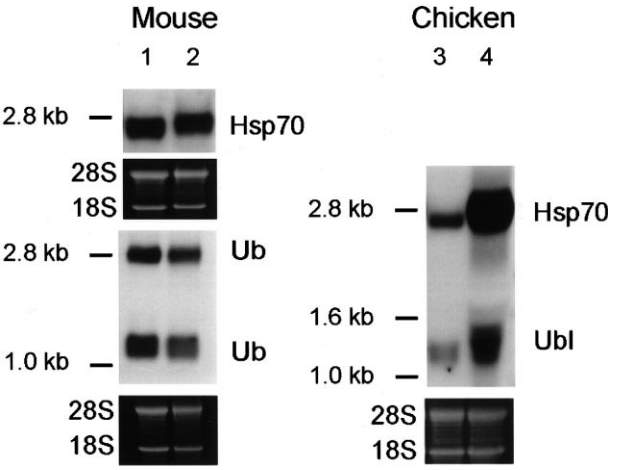

Fig. 1. Northern blotting analysis of Hsp70 and polyubiquitin mRNAs from mouse and chicken testicular cells incubated at physiological temperatures (lanes 1, 3) or exposed to heat shock (lanes 2, 4). Cells from mouse and chicken adult testis were incubated at the temperatures indicated in Section 2. Panels with ribosomal RNAs show the equalization of RNA samples used for analysis. The polyubiquitin UbI probe used recognizes the somatic and testicular transcripts of UbI.

To test whether a similar response was observed in heat-shocked cells from adult mouse testis, seminiferous tubules

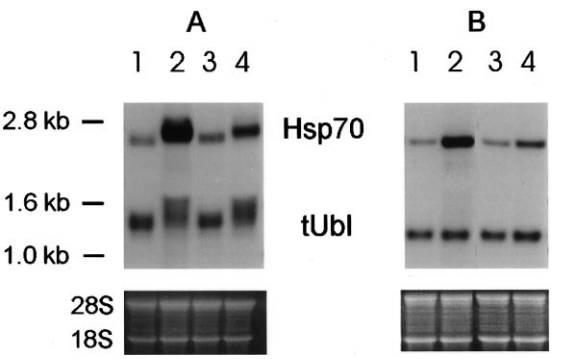

Fig. 2. Effect of heat shock on polyadenylation of Hsp70 and polyubiquitin t-UbI transcripts. A: Northern blotting analysis of Hsp70 and polyubiquitin t-UbI mRNAs from chicken testicular cells incubated at physiological temperatures (lanes 1, 3) or exposed to heat shock (lanes 2, 4). Actinomycin D (1 μg/ml) was used to inhibit transcription (lanes 3, 4). B: Same as A, except that mRNAs were treated with RNase H to remove poly(A) tails. Panels with ribosomal RNAs show the equalization of RNA samples used for analysis. The UbI probe used recognizes the testis-specific UbI transcript (t-UbI).

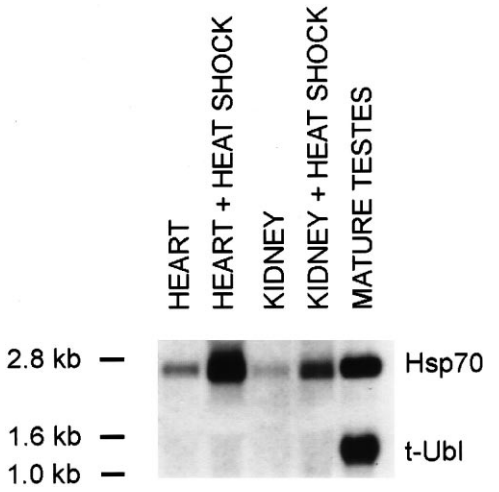

Fig. 3. Northern blotting analysis of Hsp70 and polyubiquitin t-UbI transcripts in chick embryo heart and kidney at physiological temperatures or after heat shock. Chick embryos (17-day-old) were incubated at 38°C (control) or exposed to 44°C for 3 h. RNA from adult testis was extracted from the tissue without previous exposure to heat shock. The UbI probe used recognizes the testis-specific UbI transcript (t-UbI).

were exposed to 42°C for 2 h. Controls were incubated at 31°C, the physiological temperature of scrotal mouse testis. Total RNA from heat-shocked and control tissues were analyzed by Northern blot hybridization with probes from chicken Hsp70 and the coding region of chicken polyubiquitin UbI. The chicken Hsp70 probe hybridizes with a band of 2.7 kb that is expressed in non-stressed mouse testicular cells [19]. The chicken polyubiquitin UbI probe hybridizes to two bands that are the major mammalian ubiquitin transcripts induced by heat shock in somatic cells [10]. When testis cells from adult mouse testis were exposed to heat shock, no differences were observed in the amount and length of the Hsp70 and polyubiquitin UbI transcripts in relation to control cells (Fig. 1). These results show a remarkable difference in the response to heat shock between avian and mammalian spermatogenesis.

3.2. Heat shock increases polyadenylation of Hsp70 and polyubiquitin UbI transcripts in adult chicken testis

We next examined whether the increase in length of Hsp70 and polyubiquitin UbI transcripts upon heat shock was a consequence of polyadenylation. To test this possibility we treated Hsp70 and polyubiquitin UbI mRNAs with RNase H, which removes poly(A) tails. After RNase treatment, the broad bands induced by heat shock were replaced by narrow bands with the same electrophoretic mobility as control samples (Fig. 2). This observation indicates that heat shock induces polyadenylation of Hsp70 and polyubiquitin UbI transcripts in cells from adult chicken testis. To see whether the heat shock-induced expression of Hsp70 and polyubiquitin UbI was regulated transcriptionally, we examined the effects of a transcriptional inhibitor (actinomycin D) on the induction of the transcripts. As shown in Fig. 2, actinomycin D prevented almost completely the induction of Hsp70 and showed no effect on the expression of polyubiquitin UbI. These results suggest that the heat-induced expression of Hsp70 in adult testis is regulated at the transcriptional level, while the changes in the polyubiquitin UbI transcript are independent of transcription.

We have used three different probes to analyze the expression induced by heat shock of the polyubiquitin UbI transcript. One of the probes, from the 5′ untranslated region of the transcript, recognizes a testis-specific form, t-UbI (testis-specific UbI probe) (Fig. 2). This form has been previously described in our laboratory [16]. The testis-specific transcript results from an alternative initiation placed closer to the heat shock promoter of the gene, and an alternative splicing of the 5′ intron. Polyubiquitin t-UbI is not expressed in unstressed or stressed somatic tissues and it is constitutively expressed in adult testis (Fig. 3). The testis specific form was polyadenylated upon heat shock (Fig. 2). A second probe of UbI, obtained from the coding region (non-specific UbI probe), recognizes both the somatic and the testis-specific form and showed also a widened band in Northern blots upon heat shock (Figs. 1 and 4). Finally, a third probe specific for the somatic form (s-UbI-specific probe) displayed a band in Northern blots from adult chicken testis that underwent polyadenylation during heat shock (results not shown). Therefore, all the forms of polyubiquitin UbI were polyadenylated in adult chicken testis during heat shock.

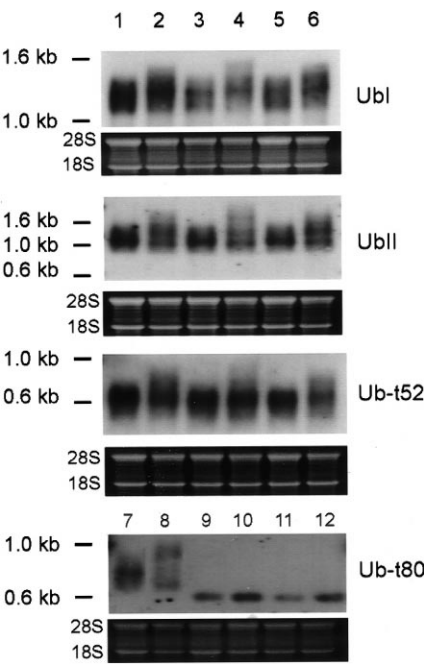

Fig. 4. Northern blotting analysis of ubiquitin transcripts from chicken adult testis cells incubated at physiological temperatures (lanes 1, 3, 5, 7) or exposed to heat shock (lanes 2, 4, 6, 8). Lane 3 shows the effect of 4 h incubation, and lane 4 the result of 2 h of heat shock followed by 2 h of recovery at 40°C. Lanes 5 and 6 show the effect of actinomycin D (1μ g/ml), an inhibitor of transcription. Lanes 9 and 10 show the same samples as lanes 7 and 8, but mRNAs were treated with RNase H to remove poly(A) tails. Samples in lanes 11 and 12 contained the same samples as lanes 7 and 8, but cells were incubated in the presence of actynomycin D and the RNAs were treated with RNase H to remove poly(A) tails. The UbI probe used recognizes the somatic and the testis-specific UbI transcript. The rest of the probes are specific for the different transcripts. Panels with ribosomal RNAs show the equalization of RNA samples used for analysis.

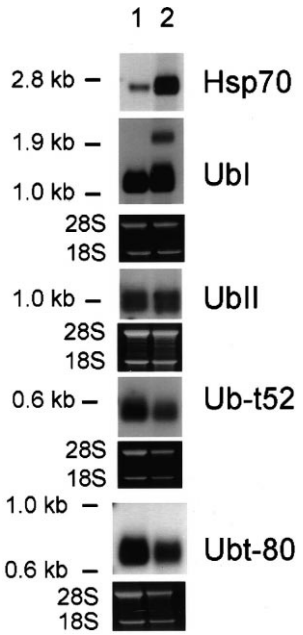

Fig. 5. Northern blotting analysis of Hsp70 and ubiquitin transcripts obtained from prepuberal chicken testis cells at physiological temperatures (lane 1) or after heat shock (lane 2). The seminiferous tubules prepared from prepuberal chicken testis were incubated at the chicken body internal temperature of 40°C for 2 h or exposed to 46°C for the same length of time. Panels with ribosomal RNAs show the equalization of RNA samples used for analysis. The polyubiquitin UbI probe used recognizes the somatic and testicular transcripts of UbI.

3.3. *Heat shock also increases polyadenylation of other ubiquitin transcripts in adult chicken testis cells*

Two polyubiquitin and two monoubiquitin genes are abundantly expressed in adult chicken testis [13–15] giving rise to high levels of ubiquitin in chicken testicular cells [20]. We wanted to know whether all the ubiquitin transcripts were modified by heat in a similar way as the heat-inducible polyubiquitin UbI. Northern blot analyses of polyubiquitin UbI (non-specific probe), polyubiquitin UbII (specific probe) and monoubiquitin transcripts Ub-t52 and Ub-t80 (specific probes) showed that during heat shock the length of all transcripts increases (Fig. 4). This pattern is independent of transcription because it did not change when samples were incubated with actinomycin D (Fig. 4). The pattern persists 2 h after cell recovery at 40°C. After RNase treatment, the broad bands generated by heat shock are replaced by narrow bands of the same size as in control samples, as shown for the monoubiquitin transcript Ub-t80 (Fig. 4). This result indicates that the increase in size of the transcripts during heat shock was due to polyadenylation.

3.4. *Polyadenylation of ubiquitin transcripts upon heat shock is characteristic of adult chicken testis but it is not apparent in prepuberal testis*

Finally we addressed the question of whether the heat shock response characteristic of chicken adult testis, enriched in meiotic and postmeiotic cells, was also present in prepuberal testis enriched in Sertoli cells and spermatogonia. Heat shock did not induce polyadenylation of ubiquitin transcripts when seminiferous tubules from chicken prepuberal testis were incubated at 46°C for 2 h (Fig. 5). A band of lower electrophoretic mobility than UbI appeared upon heat shock in the Northern blots (Fig. 5). This extra band has been previously reported in heat-stressed chicken fibroblasts [9].

4. Discussion

This study reveals substantial differences in the expression of Hsp70 and ubiquitin between mammalian and avian testicular cells exposed to heat shock. Cells from adult chicken testis respond to heat shock with a marked increase in transcription of Hsp70 and polyadenylation of Hsp70 and ubiquitin transcripts. In striking contrast to this observation, no major changes in the amount and polyadenylation of Hsp70 and polyubiquitin transcripts were observed in heat-shocked cells from adult mouse testis. Conflicting results have been previously reported on the expression of HSP70 proteins in mouse male germ cells exposed to heat shock [19,21–23]. Our results demonstrate the constitutive expression of a unique-sized Hsp70 mRNA of 2.7 kb in adult mouse testis, and the lack of induction under heat shock of this transcript, in accordance with previous reports [19,24]. In addition, the two heat-inducible polyubiquitin transcripts expressed upon heat shock in somatic mammalian cells [10] are constitutively expressed in adult mouse testis and their expression did not change during heat shock. The absence of induction and polyadenylation of Hsp70 and polyubiquitin transcripts in heat-shocked mouse testicular cells may contribute to the known lack of thermotolerance of mammalian testicular cells, due to the essential role that HSP70 proteins and ubiquitin play restoring damaged proteins.

In contrast to the lack of response of mammalian testis, the expression and polyadenylation of Hsp70 and ubiquitin transcripts induced by heat shock may contribute to the development of thermotolerance during avian spermatogenesis. We have reported previously the induction and polyadenylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts in adult chicken testis exposed to heat shock [25]. GAPDH is a heat shock protein in several systems [4,26] and it has been proposed that it may induce thermotolerance in *Xenopus laevis* embryos [27].

Our analysis has not yet approached the mechanism by which the increased polyadenylation of Hsp70 and ubiquitin transcripts may contribute to the thermotolerance of avian spermatogenesis. One possibility is through stabilization of the transcripts. It is known that heat-induced stress results in the degradation of many mRNAs, while others such as Hsp70 and ubiquitin may be protected. Polyadenylation plays a key role in regulating Hsp70 expression [28] and could be potentially important to control the expression of other transcripts expressed during heat shock. One observation reports an enhanced polyadenylation in response to thermal stress after infection of human B cells with Epstein-Barr virus [29]. In addition to a possible increase in the stability of the transcripts, polyadenylation enhances the efficiency of translation and the efficiency of mRNA export from the nucleus [30,31]. These effects may contribute to ensure the synthesis of essential proteins which achieve thermotolerance during avian spermatogenesis.

Acknowledgements: We thank Virginia Mas for her collaboration as a summer research student and Montserrat Pau for technical assistance.

Supported by Grants from Marató TV3 37/95, and CICYT SAF97-0062 and SAF98-0020.

References

[1] Ritossa, F. (1962) Experientia 18, 571–573.
[2] Tissieres, A., Mitchell, H.K. and Tracy, U.M. (1974) J. Mol. Biol. 84, 389–398.
[3] Lindquist, S. (1986) Annu. Rev. Biochem. 55, 1151–1191.
[4] Lindquist, S. and Craig, E.A. (1988) Annu. Rev. Genet. 22, 631–677.
[5] Parsell, D.A. and Lindquist, S. (1993) Annu. Rev. Genet. 27, 437–496.
[6] Nover, L. and Scharf, K.D. (1997) Cell. Mol. Life Sci. 53, 80–103.
[7] Morimoto, R.I., Tissieres, A. and Georgopoulos, C. (1994) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[8] Finley, D., Ozkaynak, E. and Varshavsky, A. (1987) Cell 48, 1035–1046.
[9] Bond, U. and Schlesinger, M.J. (1985) Mol. Cell. Biol. 5, 949–956.
[10] Fornace Jr., A.J., Alamo Jr., I., Hollander, M.C. and Lamoreaux, E. (1989) Nucleic Acids Res. 17, 1215–1230.
[11] Zorgniotti, A.W. and Sealfon, A.I. (1988) J. Reprod. Fertil. 82, 563–566.
[12] Beaupre, C.E., Tressler, C.J., Beaupre, S.J., Morgan, J.L., Bottje, W.G. and Kirby, J.D. (1997) Biol. Reprod. 56, 1570–1575.
[13] Mezquita, J. and Mezquita, C. (1991) FEBS Lett. 279, 69–72.
[14] Mezquita, J., Lopez-Ibor, B., Pau, M. and Mezquita, C. (1993) FEBS Lett. 319, 244–248.
[15] Mezquita, J., Pau, M. and Mezquita, C. (1997) Gene 195, 313–319.
[16] Mezquita, J., Pau, M. and Mezquita, C. (1997) Mol. Reprod. Dev. 46, 471–475.
[17] Engler-Blum, G., Meier, M., Frank, J. and Muller, G.A. (1993) Anal. Biochem. 210, 235–244.
[18] Kleene, K.C., Distel, R.J. and Hecht, N.B. (1984) Dev. Biol. 105, 71–79.
[19] Zakeri, Z.F. and Wolgemuth, D.J. (1987) Mol. Cell. Biol. 7, 1791–1796.
[20] Agell, N. and Mezquita, C. (1988) Biochem. J. 250, 883–889.
[21] Allen, R.L., O'Brien, D.A., Jones, C.C., Rockett, D.L. and Eddy, E.M. (1988) Mol. Cell. Biol. 8, 3260–3266.
[22] Allen, R.L., O'Brien, D.A. and Eddy, E.M. (1988) Mol. Cell. Biol. 8, 828–832.
[23] Sarge, K.D. and Cullen, K.E. (1997) Cell. Mol. Life Sci. 53, 191–197.
[24] Zakeri, Z.F., Welch, W.J. and Wolgemuth, D.J. (1990) J. Cell Biol. 111, 1785–1792.
[25] Mezquita, J., Pau, M. and Mezquita, C. (1998) J. Cell. Biochem. (in press).
[26] Laxalt, A.M., Cassia, R.O., Sanllorenti, P.M., Madrid, E.A., Andreu, A.B., Daleo, G.R., Conde, R.D. and Lamattina, L. (1996) Plant Mol. Biol. 30, 961–972.
[27] Nickells, R.W. and Browder, L.W. (1988) J. Cell. Biol. 107, 1901–1909.
[28] Dellavalle, R.P., Petersen, R. and Lindquist, S. (1994) Mol. Cell. Biol. 14, 3646–3659.
[29] Kumar, A., Kurl, R.N., Kryworuchko, M., Diaz-Mitoma, F. and Sharma, S. (1995) Leukocyte Res. 19, 831–840.
[30] Sachs, A.B., Sarnow, P. and Hentze, M.W. (1997) Cell 89, 831–838.
[31] Huang, Y. and Carmichael, G.C. (1996) Mol. Cell. Biol. 16, 1534–1542.