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JCB Article

Stress-induced transcription of satellite III repeats

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Exposure of mammalian cells to stress induces the activation of heat shock transcription factor 1 (HSF1) and the subsequent transcription of heat shock genes. Activation of the heat shock response also correlates with a rapid relocalization of HSF1 within a few nuclear structures termed nuclear stress granules. These stress-induced structures, which form primarily on the 9q12 region in humans through direct binding of HSF1 to satellite III repeats, do not colocalize with transcription sites of known hsp genes. In this paper, we show that nuclear stress granules correspond to RNA polymerase II transcription factories where satellite III repeats are transcribed into large and stable RNAs that remain associated with the 9q12 region, even throughout mitosis. This work not only reveals the existence of a new major heat-induced transcript in human cells that may play a role in chromatin structure, but also provides evidence for a transcriptional activity within a locus considered so far as heterochromatic and silent.

Introduction

Exposure of cells to environmental stress conditions results in the inducible expression of a family of proteins, termed heat shock proteins (HSPs), whose function is to protect cells from stress-induced damage. Heat shock transcription factor 1 (HSF1) is responsible for the stress-induced activation of heat shock genes (Pirkkala et al., 2001). Upon stress, HSF1 redistributes from a diffuse nucleocytoplasmic population to a few nuclear foci that form through direct DNA–protein interaction on several heterochromatic loci in humans, primarily the 9q12 region (Sarge et al., 1993; Denegri et al., 2002; Jolly et al., 2002). Within this region, the DNA target of HSF1 is a chromosome 9–specific subclass of satellite III (sat III) repeats (Jolly et al., 2002). The role of nuclear stress granules is not understood. We have shown earlier that they do not correspond to the site of transcription of the three major hsp70 and hsp90 genes, thus eliminating the hypothesis that these local concentrations of HSF1 could serve as transcription factories for all heat shock genes (Jolly et al., 1997). The presence of several splicing factors within these structures further supports the assumption that they could be related to transcription and splicing events (Chiodi et al., 2000; Denegri et al., 2001). However, their possible implication in transcriptional activity is still a matter of debate, and approaches using bromo-uridine precursors to label active transcription sites have led to divergent conclusions (He et al., 1998; Chiodi et al., 2000; Jolly et al., 2002).

In this paper, we investigated the involvement of nuclear stress granules in transcriptional activity. We found that stress induces the accumulation of acetylated forms of all core histones within the 9q12, the recruitment of RNA polymerase II to the granules, and the subsequent transcription of sat III repeats essentially from one strand. All these events are HSF1 dependent. Furthermore, stress-induced sat III transcripts persist after transcription has ceased and remain associated with chromosomes throughout mitosis, suggesting a role in chromatin organization.

Results

RNA polymerase II and acetylated histones are present in nuclear stress granules

We studied the possibility that nuclear stress granules could be sites of transcription by investigating by immunofluorescence the distribution in non–heat-shocked and heat-shocked cells of the key enzymes in transcription: RNA polymerases. Results are presented in Fig. 1. In contrast to RNA pol I and III (not depicted), the intranuclear distribution of RNA pol

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Key words: HSF1; RNA polymerase II; satellite III; stress; transcription

Abbreviations used in this paper: CBP, CREB binding protein; HSF1, heat shock transcription factor 1; HSP, heat shock protein; sat III, satellite III.

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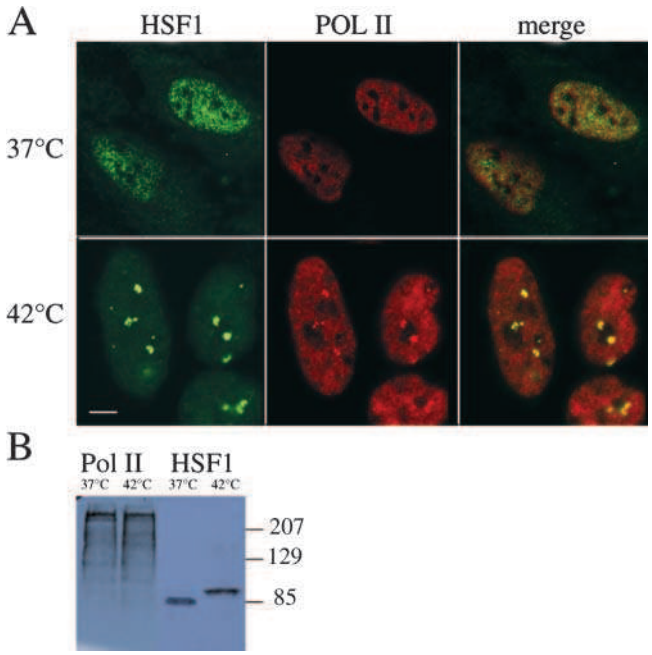


Figure 1. **RNA polymerase II is concentrated within nuclear stress granules.** (A) RNA polymerase II (red) was detected by immunofluorescence together with HSF1 (green) in non–heat-shocked and heat-shocked HeLa cells. At 37°C, HSF1 is diffusely distributed in the nucleoplasm and cytoplasm while RNA polymerase II displays a fine nuclear punctate staining. After 1 h at 42°C, HSF1 is massively recruited to nuclear stress granules, and RNA polymerase II is also found accumulated in these structures (yellow in the merged image), in addition to a remaining diffuse staining of the nucleus. Bar, 5 μm. (B) Total cell extracts prepared from non–heat-shocked (37°C) or heat-shocked (1 h at 42°C) cells were analyzed by Western blot. The blot was sequentially detected with 7C2 anti-RNA polymerase II antibody (left) and with rabbit anti-HSF1 antibody (right). No cross-reactivity of the antibodies is observed. The lowered HSF1 mobility observed in the 42°C sample is due to stress-induced posttranslational modifications of the protein (for review see Pirkkala et al., 2001).

II detected with the 7C2 monoclonal antibody, which recognizes both nonphosphorylated and phosphorylated forms of the enzyme (Besse et al., 1995), was altered by heat shock. Indeed, after a 1-h heat shock, three to four nuclear foci were observed in most of the cells, in addition to a persisting diffuse staining of the nucleus and cytoplasm (Fig. 1 A). The colocalization of these pol II accumulation sites with nuclear stress granules was confirmed by codetection of HSF1 (Fig. 1 A). Similar observations were obtained with the MARA3 monoclonal antibody, which recognizes a phosphoepitope in the COOH-terminal domain of RNA pol II (Patturajan et al., 1998; not depicted). The specificity of both antibodies was assessed by Western blot on total cell extracts from non–heat-shocked and heat-shocked HeLa cells, and none of these antibodies was found to cross-react with HSF1 (Fig. 1 B).

As this local concentration of RNA polymerase II does not necessarily reveal a transcriptional activity of the locus, we next looked for the presence of another marker of transcription within the granules, i.e., hyperacetylated histones. Indeed, transcription activation correlates with an increase in core histone acetylation of the transcribed region (for reviews see Turner, 2002; Fischle et al., 2003). We thus

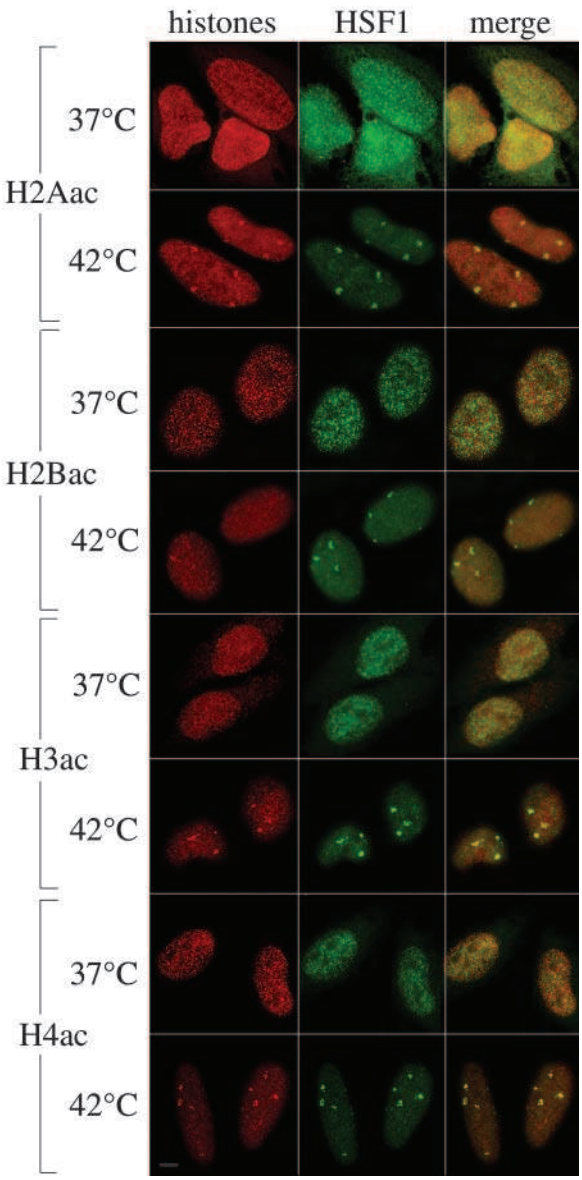


Figure 2. **Stress-induced HSF1 granules contain acetylated core histones.** HSF1 (green) and acetylated forms of each core histone (red) were codetected by immunofluorescence in HeLa cells submitted or not to a 1-h heat shock at 42°C. At 37°C, HSF1 is diffusely distributed in the nucleus and cytoplasm, and acetylated histones exhibit a punctate distribution throughout the nucleus. After heat shock, acetylated forms of the four core histones are present to various extents within stress granules. Bar, 5 μm.

performed immunofluorescence on non–heat-shocked and heat-shocked cells with a panel of specific antibodies (Turner and Fellows, 1989; White et al., 1999): H2A acetylated on K5 (H2Aac), H2B acetylated on K12 and K15 (H2Bac), H3 acetylated on K9 and K18 (H3ac), and H4 acetylated on K5, K8, K12, and/or K16 (H4ac). Results are shown in Fig. 2. At 37°C, a punctate nuclear distribution was observed with all four antibodies. In heat-shocked cells, a diffuse labeling of the nucleoplasm was still observed. In addition, most of the cells displayed nuclear accumulation sites of acetylated histones that colocalize with nuclear stress granules as shown by the codetection of HSF1 in these cells.

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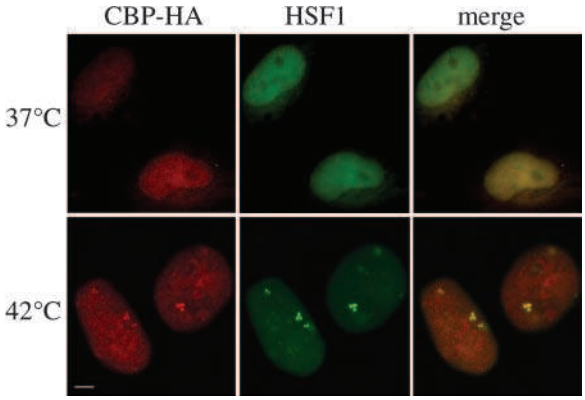


Figure 3. **CBP is recruited to nuclear stress granules during heat shock.** Transiently expressed CBP-HA (red) was codetected with HSF1 (green) in HeLa cells. In non–heat-shocked cells, CBP displays a fine punctate staining dispersed throughout the nucleoplasm. At 42°C, a fraction of the protein colocalizes with HSF1 in the granules. Bar, 5 μm.

Immunofluorescence with an antibody to the unmodified form of H2B confirmed that our observations were not merely due to a higher density of histones within stress granules (unpublished data). Interestingly, we did not find accumulation of H3 phosphorylated on serine 10 as described for transcribing heat-shock puffs on *Drosophila* polytene chromosomes (Nowak and Corces, 2000; Labrador and Corces, 2003), perhaps because human chromosome 9 sat III repeats are not structured as a canonical heat-shock promoter (unpublished data). Thus, nuclear stress granules contain acetylated core histones.

To confirm that the presence of acetylated histones within the granules is a stress-induced event, we investigated the possibility that a histone acetyltransferase (HAT) is also recruited to the granules upon stress. Therefore, we analyzed the distribution of transiently expressed fusion proteins encoding either GCN5, Tip60, or CREB binding protein (CBP) in HeLa cells (Col et al., 2001; Legube et al., 2002). GCN5 and Tip60 both displayed a punctate nuclear staining that was unaffected by stress (unpublished data). In contrast, a fraction of the overexpressed CBP protein was detected at 42°C in a few granular structures that coincided with HSF1-containing granules, in addition to the persisting punctate nucleoplasmic staining (Fig. 3). CBP is thus able to relocate to the granules during stress, likely accounting for the stress-induced accumulation of acetylated histones within these structures.

Stress induces the transcription of sat III repeats

Based on these observations, we sought to determine if transcription occurs within the granules. Nuclear stress granules form primarily on the 9q12 juxtacentromeric region in humans, with putative secondary sites on chromosomes 12 and 15 and perhaps other chromosomes (Denegri et al., 2002; unpublished data). As the only characterized target sequence for HSF1 within the granules is a sat III repeat of the 9q12 region characterized by the clone pHuR98 (Jolly et al., 2002), we investigated the possibility that these repeats could be inducibly transcribed during stress. We first per-

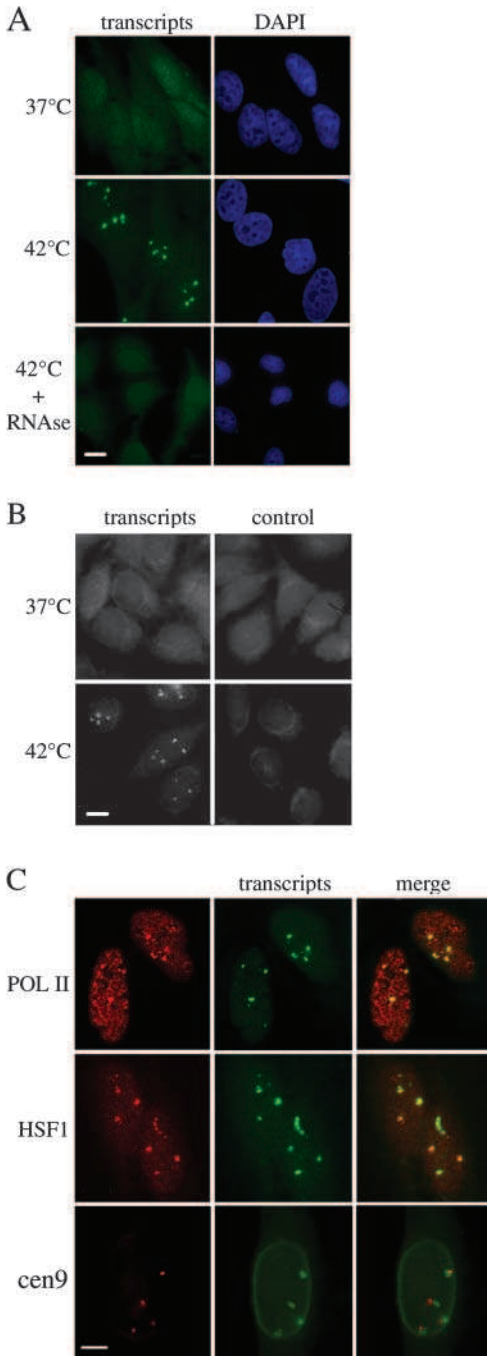


Figure 4. **Stress induces the transcription of chromosome 9 sat III repeats.** (A) RNA FISH was performed on HeLa cells with clone pHuR98, which is specific for sat III repeats, as a probe. At 37°C, a faint nuclear and cytoplasmic staining is observed. In cells exposed to a 1-h heat shock at 42°C, three to four bright nuclear foci are detected in each nucleus, in addition to a persisting diffuse staining of the nucleus and cytoplasm. These foci are absent in heat-shocked cells treated with 100 μg/ml RNase A for 15 min before hybridization. (B) Comparison of the RNA FISH signals obtained with the pHuR98 probe and when the probe was omitted (control). Both at 37°C and 42°C, the intensity of the diffuse nuclear and cytoplasmic staining is comparable to the background level (control). (C) Codetection of pHuR98 transcripts by RNA FISH (green) with RNA polymerase II or HSF1 detected by immunofluorescence, or with chromosome 9 centromeres revealed by DNA FISH (red). Transcript foci coincide perfectly with HSF1 and RNA polymerase II within nuclear stress granules, and each transcript focus is found in the vicinity of a chromosome 9 centromere. Bars, 5 μm.

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formed FISH to detect transcripts with the clone pHuR98 as a probe (Grady et al., 1992). Results are presented in Fig. 4. Both at 37°C and 42°C, a diffuse nucleoplasmic and cytoplasmic staining was observed (Fig. 4 A). In addition, at 42°C, three to four bright nuclear foci were also visible in most cells. These granular foci corresponded to transcripts as confirmed by their absence in cells treated with RNase A before hybridization (Fig. 4 A). The intensity of the nuclear and cytoplasmic diffuse staining is comparable to the background level both at 37°C and 42°C, thus demonstrating that sat III transcripts are essentially concentrated in the three to four nuclear foci (Fig. 4 B). Similarly RNA FISH experiments performed with probes corresponding to either chromosome 9 classical satellites (D9Z1), chromosome 9 centromeric repeats, or chromosome 16 sat II repeats (pHuR195) did not reveal any signal (not depicted). Codetection of sat III transcripts with HSF1 or RNA pol II showed a colocalization of these transcript foci with nuclear stress granules (Fig. 4 C). Finally, to confirm that the presence of three to four transcript foci was reflecting the number of copies of the 9q12 locus in HeLa cells, we also performed codetection of sat III transcripts with chromosome 9 centromeric repeats revealed by DNA FISH. As shown in Fig. 4 C, each transcript focus was found in the vicinity of a centromere, confirming that each RNA FISH signal corresponded to sat III transcripts emerging from one chromosome 9. Altogether, these observations show that heat shock specifically induces the transcription of sat III repeats from the three to four 9q12 loci present in HeLa cells. Interestingly, the 9q12 region has been described as heterochromatic based on its enrichment in methylcytosine-rich DNA (Miller et al., 1974). To better determine the chromatin flavour of the 9q12, we investigated the distribution of one of the best characterized markers of heterochromatin: the heterochromatin protein HP1β (Eissenberg and Elgin, 2000). Cells were transiently transfected with an HP1-GFP construct, and nuclear stress granules were revealed either by a mutant HSF1 (DBD+TRIM), which forms constitutive granules at 37°C (Jolly et al., 2002), or by the endogenous HSF1 at 42°C. As shown in Fig. 5, stress granules were always juxtaposed to an HP1β-rich focus, which corresponded to the close centromere of chromosome 9, but never enriched in HP1β protein, demonstrating that the juxtacentromeric 9q12 region does not display conventional heterochromatic features.

HSF1 is the key determinant in sat III transcription

The question of whether HSF1 is the key determinant in the induced transcription of sat III repeats was first addressed by using the DBD+TRIM deletion mutant of HSF1, which only contains the DNA-binding and trimerization domains and forms granules constitutively (Jolly et al., 2002). Interestingly, this mutant acts as a dominant negative, by preventing the endogenous HSF1 from accumulating into the granules upon heat shock (Fig. 6 A). We found that acetylated histone H4, CBP-HA, RNA polymerase II, and sat III transcripts were never present in the granules formed by the DBD+TRIM mutant, both at 37°C and at 42°C (Fig. 6, B–E). As a confirmation to this, we also prevented the targeting of HSF1 to the granules by overexpressing HSP70,

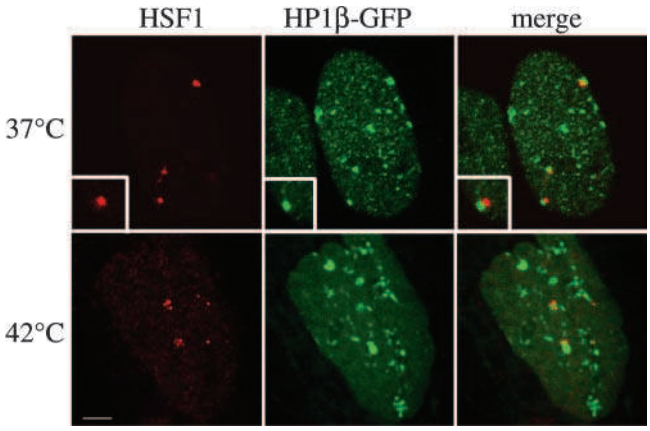


Figure 5. **The heterochromatin protein HP1β is not concentrated in the 9q12 locus.** HP1β-GFP construct (green) was transiently expressed in HeLa cells and codetected with either the DBD-TRIM mutant at 37°C or the endogenous HSF1 at 42°C (red). Projections of confocal series are shown. In both cases, nuclear stress granules are juxtaposed to, but never coincident with, HP1β-enriched foci (10 nuclei analyzed for each case). Insert shows a magnification of one stress granule. Bar, 5 μm.

which is a negative regulator of HSF1 activity (Shi et al., 1998). As expected, upon heat shock, HSP70 overexpression prevented the accumulation of HSF1, acetylated histones, and CBP-HA in the granules, and the subsequent transcription of sat III repeats (Fig. 6 F).

Stress-induced sat III transcripts are large and stable RNAs that remain associated with the 9q12

We were next interested in the characterization of sat III transcripts. To estimate the induction level induced by heat exposure, and to determine from which strand the transcripts are generated, we performed reverse transcription on total RNA extracts from non–heat-shocked and heat-shocked HeLa cells with the following primers: antisense pHuR98, sense pHuR98, or antisense hsp70 (corresponding to the major heat-induced gene). The reaction samples were then spotted onto nitrocellulose membrane and quantified using the PhosphorImager® system (normalized to the 37°C value for each primer). Results are presented in Fig. 7 A. We found that both sense (gray) and antisense (black) sat III transcripts as well as hsp70 transcripts were present at very low levels in the 37°C samples as compared with input. After a 1-h heat shock at 42°C, we observed a twofold decrease in the amount of antisense sat III transcripts and a ninefold increase in the amount of sense sat III transcripts as compared with the 37°C samples. Likewise, a ninefold increase in hsp70 transcripts was observed at 42°C. A run-on assay was also performed with nuclei prepared from stressed and unstressed cells (Fig. 7 B). This experiment showed that stress induces a 11.9-fold and a 13.4-fold induction in sat III and hsp70 transcription, respectively. Sat III repeats are thus inducibly transcribed during stress, essentially from one strand.

We also performed a Northern blot analysis of these RNA extracts with pHuR98 as a probe to determine the size of sat III transcripts. Hsp70 transcripts were used as a control. Ethidium bromide staining of the gel before transfer confirmed that equal amounts of extracts were loaded in each

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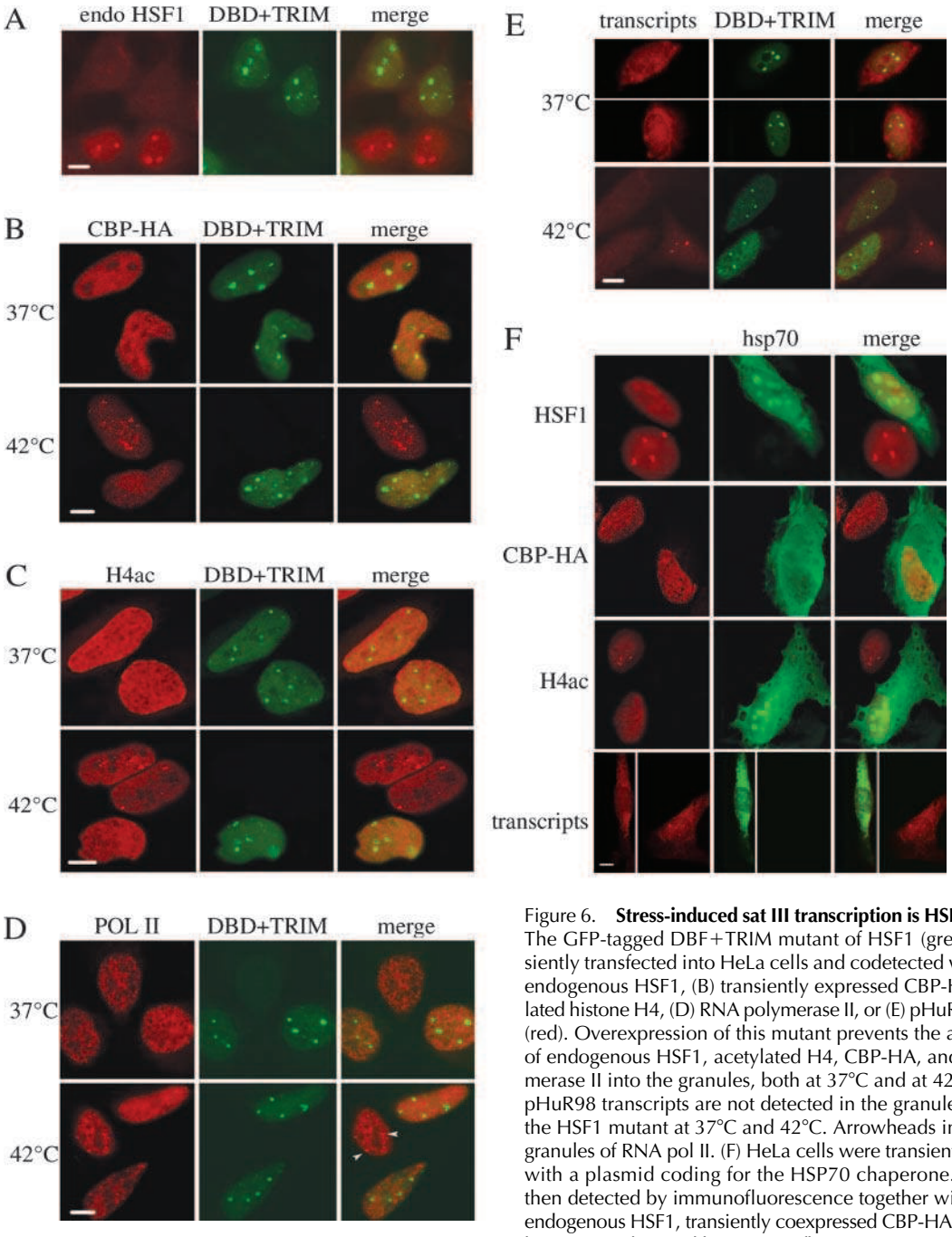


Figure 6. **Stress-induced sat III transcription is HSF1 dependent.** The GFP-tagged DBF+TRIM mutant of HSF1 (green) was transiently transfected into HeLa cells and codetected with either (A) endogenous HSF1, (B) transiently expressed CBP-HA, (C) acetylated histone H4, (D) RNA polymerase II, or (E) pHuR98 transcripts (red). Overexpression of this mutant prevents the accumulation of endogenous HSF1, acetylated H4, CBP-HA, and RNA polymerase II into the granules, both at 37°C and at 42°C. Likewise, pHuR98 transcripts are not detected in the granules formed by the HSF1 mutant at 37°C and 42°C. Arrowheads in D point to granules of RNA pol II. (F) HeLa cells were transiently transfected with a plasmid coding for the HSP70 chaperone. HSP70 was then detected by immunofluorescence together with either the endogenous HSF1, transiently coexpressed CBP-HA, or acetylated histone H4 detected by immunofluorescence, or with pHuR98 transcripts revealed by RNA FISH. Only images of cells exposed for 1 h at 42°C are shown. In HSP70-overexpressing cells, the endogenous HSF1, CBP-HA, and acetylated histone H4 are not recruited to nuclear stress granules, and pHuR98 transcript foci are not present. The white lines in E and F indicate that the cells were taken from different fields. Bars, 10 μm.

lane (Fig. 7 C). While a faint signal was observed in the 37°C extracts both for sat III and hsp70 transcripts, strong signals were present for both transcripts in the 42°C samples. The signal corresponding to hsp70 transcripts was found at the expected size (2.3 kb), whereas the signal corresponding to sat III transcripts was close to the wells (Fig. 7 C), demonstrating that these transcripts are very large. No signal corresponding to small RNAs was detected with this probe, even when the gel was not washed with NaOH before transfer (not depicted). The same experiment was also performed with sense and antisense oligonucleotides and confirmed that heat-induced transcription of sat III repeats only occurs in the sense direction (Fig. 7 C).

The question of the intracellular distribution of sat III transcripts was then addressed. Indeed, our RNA FISH data show that in contrast to hsp70 transcripts (not depicted), the fluorescent signal for sat III RNAs is essentially localized at the site of transcription with no diffuse signal in the nucleus and/or cytoplasm (Fig. 4 B). These observations strongly suggest that sat III transcripts are not exported but rather remain associated with the 9q12 even after transcription is completed. A further support for this assumption

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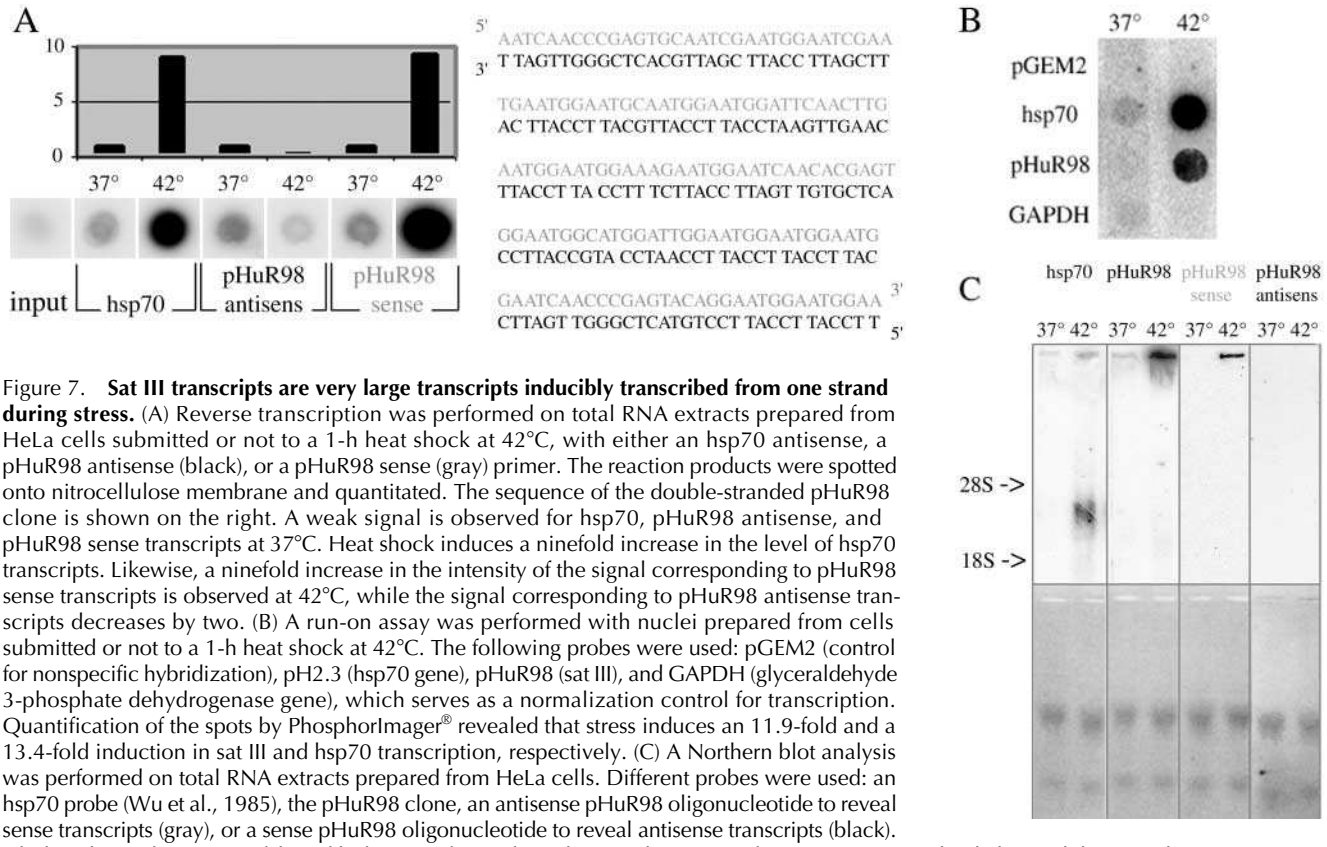


Figure 7. **Sat III transcripts are very large transcripts inducibly transcribed from one strand during stress.** (A) Reverse transcription was performed on total RNA extracts prepared from HeLa cells submitted or not to a 1-h heat shock at 42°C, with either an hsp70 antisense, a pHuR98 antisense (black), or a pHuR98 sense (gray) primer. The reaction products were spotted onto nitrocellulose membrane and quantitated. The sequence of the double-stranded pHuR98 clone is shown on the right. A weak signal is observed for hsp70, pHuR98 antisense, and pHuR98 sense transcripts at 37°C. Heat shock induces a ninefold increase in the level of hsp70 transcripts. Likewise, a ninefold increase in the intensity of the signal corresponding to pHuR98 sense transcripts is observed at 42°C, while the signal corresponding to pHuR98 antisense transcripts decreases by two. (B) A run-on assay was performed with nuclei prepared from cells submitted or not to a 1-h heat shock at 42°C. The following probes were used: pGEM2 (control for nonspecific hybridization), pH2.3 (hsp70 gene), pHuR98 (sat III), and GAPDH (glyceraldehyde 3-phosphate dehydrogenase gene), which serves as a normalization control for transcription. Quantification of the spots by PhosphorImager® revealed that stress induces an 11.9-fold and a 13.4-fold induction in sat III and hsp70 transcription, respectively. (C) A Northern blot analysis was performed on total RNA extracts prepared from HeLa cells. Different probes were used: an hsp70 probe (Wu et al., 1985), the pHuR98 clone, an antisense pHuR98 oligonucleotide to reveal sense transcripts (gray), or a sense pHuR98 oligonucleotide to reveal antisense transcripts (black). Ethidium bromide staining of the gel before transfer confirms that equal amounts of RNA extracts were loaded in each lane. For hsp70 transcripts, a very weak signal is observed for the 37°C sample, while a signal at the expected size (2.3 kb) is observed in the 42°C sample (the positions of the 28S and 18S rRNAs are indicated). For pHuR98 transcripts, a faint signal is present with all three probes at 37°C, while in the 42°C sample, a very slowly migrating band is revealed, both with the pHuR98 probe and the antisense oligonucleotide (gray). No signal is observed with the sense oligonucleotide (black).

comes from the observation that sat III transcript foci were still visible in the majority of the cells after 3 h of recovery following heat shock, whereas the other actors of transcription (HSF1, CBP, acetylated histones, and RNA polymerase II) were no longer present in the granules at that time (Fig. 8 A). To confirm this proposal, we looked for the presence of sat III transcripts associated with transcriptionally inactive mitotic chromosomes. As heat shock can arrest or delay cell cycle progression (for review see Kühl and Rensing, 2000), cells were allowed to recover for 3–6 h after heat shock. As shown in Fig. 8 B, we observed the presence of mitotic cells with transcripts associated with the 9q12. Neither HSF1 nor RNA polymerase II was found within the same loci when codetected (unpublished data), thus confirming that these transcripts do not correspond to nascent RNAs but to transcripts produced before entry into mitosis and which remained associated with chromosome 9 during cell cycle progression.

The stability of sat III transcripts was further investigated by the use of the transcription inhibitor actinomycin D. We found that a treatment of 20–60 min with 100 μg/ml actinomycin D just after the 1-h heat shock did not significantly affect the number of cells containing sat III transcript foci, with 80% of the cells still displaying foci versus 81% in untreated cells (unpublished data). As a comparison, hsp70 transcript foci were no longer detected after 15 min of actinomycin D (Jolly et al., 1998). Altogether, our findings show that sat III transcripts are very large and stable RNAs that, at least in part, remain associated for a certain time with the 9q12 locus after synthesis, even throughout mitosis.

Discussion

In this paper, we describe the role of the HSF1-containing nuclear stress granules in the stress-induced transcription of chromosome 9 sat III repeats. This transcription is HSF1 dependent and driven by RNA polymerase II. The transcripts generated are very large, stable, and most likely noncoding as they remain associated with the 9q12 locus from which they originate. These observations are particularly interesting with regard to the growing evidence that repeated sequences of the eukaryotic genome are transcribed. In the past few years, a large number of reports have led to the emerging view that RNA can play a crucial role in the negative regulation of gene expression and in chromatin structure (for reviews see Stevenson and Jarvis, 2003; Wutz, 2003). The organization of silent domains, such as pericentric heterochromatin or the inactivated X chromosome, is dependent on noncoding RNA molecules (Maison et al., 2002; for reviews see Avner and Heard, 2001; Cohen and Lee, 2002). For example, small interfering RNAs bind to complementary RNA sequences and target them for destruction, thereby regulating gene expression. As to chromatin structure, the role of RNAs in

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A	1h	1h+rec
HSF1	+	-
CBP-HA	+	-
acetylated histones	+	-
POL II	+	-
sat III transcripts	+	+

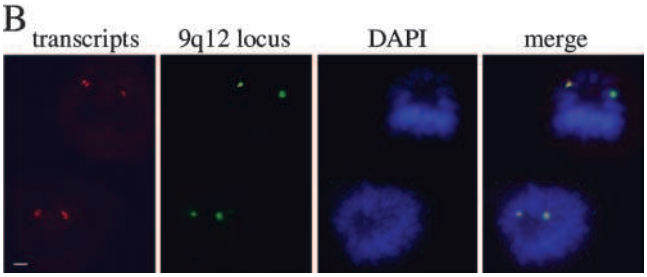


Figure 8. **Stress-induced sat III transcripts are stable transcripts that remain associated with the 9q12 locus.** (A) Table showing the composition of nuclear stress granules after 1 h of heat shock at 42°C or for 3 h at 37°C after a 1-h heat shock at 42°C (200 nuclei analyzed in each case). While all components are detected in the granules after 1 h of heat shock (HSF1, overexpressed CBP-HA, acetylated histones, RNA polymerase II, sat III transcripts), only sat III transcripts are still detected in a large majority of cells after recovery. (B) Sat III transcripts (green) were detected by RNA FISH along with the 9q12 locus, revealed by DNA FISH with a probe specific for chromosome 9 classical satellites (D9Z1) in HeLa cells allowed to recover for 3 h at 37°C after a 1-h heat shock. Two mitotic cells with sat III transcripts (red) associated to the 9q12 locus (green) are shown. Only two spots are visible in each cell because the two others are not in the same focal plan. Bar, 5 μm.

heterochromatin formation and/or maintenance, for example, is now widely recognized, although most of these RNAs have not been identified yet. In yeast, for example, double-stranded RNAs generated from centromeric repeats have been proposed to play a role in heterochromatin formation via the production of small heterochromatic RNAs (Reinhardt and Bartel, 2002; for review see Jenuwein, 2002). In mouse, a bidirectional transcription of major (gamma) satellite repeats has been reported, suggesting that this regulatory pathway of heterochromatin formation may also exist in mammalian cells (Rudert et al., 1995; for review see Jenuwein, 2002).

Notably, there are a few examples of heat-induced transcription of repeated sequences. In mouse, for example, heat shock induces the unidirectional transcription by RNA polymerase III of B2 repetitive elements into small polyadenylated transcripts that are exported to the cytoplasm (Fornace and Mitchell, 1986). Heat shock also induces a transient increase in the RNA polymerase III–transcribed Alu and SINE RNAs in human, mouse, rabbit, and silkworm cells (Fornace and Mitchell, 1986; Liu et al., 1995; Kimura et al., 1999). This stress-induced transcription of repeated sequences can also occur in physiological stress conditions in living animals, suggesting that it is functionally relevant (Li et al., 1999; Kimura et al., 2001). Conceivably, the function of these transcripts may only be revealed under certain physiological or pathological conditions, explaining the lack of functional data available so far. Interestingly, overexpressed Alu RNA can stimulate the expression of a reporter gene under certain circumstances, providing the first evidence for a functional role of stress-induced repeated transcripts (Chu et al., 1998). Our observation that sat III transcripts remain associated with the 9q12 locus, as does Xist RNA with the inactive X chromosome (for review see Avner and Heard, 2001), supports a role for sat III transcripts in chromatin structure. Interestingly, Grady et al. (1992) show that the transcribed strand of the sat III sequence, and most likely the corresponding transcript, has an unusually high thermal stability and could form unusual structures by nonconventional base pairing, perhaps explaining how these transcripts anchor to chromatin. One hypothesis as to the role of sat III transcripts is that they serve to protect a fragile region of the genome from stress-induced damage. Indeed, the 9q12 is a fragile region often rearranged in certain pathologies such as cancer or the triple A syndrome (Bartlett et al., 1998; Lamszus et al., 1999; Reshmi-Skarja et al., 2003). Another hypothesis based on the fact that several splicing factors are also present in nuclear stress granules is that these structures play a role in the titration of key components of transcription and splicing activities, thereby providing a powerful way to control both functions during stress (Chiodi et al., 2000; Denegri et al., 2001). Whatever the hypothesis, our work reveals not only a transcriptional activity within a locus considered so far as heterochromatic and silent, but also the existence of a new major heat-induced transcript in human cells that may play a role in chromatin structure.

Materials and methods

Cell culture, transient transfection, and drug treatments

HeLa cells were grown in DME supplemented with 10% fetal bovine serum. Transient transfections were performed using Polyfect lipid solution (QIAGEN). Transcription inhibition was achieved by treating the cells for 20 min at 37°C with 100 μg/ml actinomycin D.

Plasmids and antibodies

Clone pHuR98 specific for sat III repeats was obtained from R. Moyzis (University of California, Irvine, CA) (Grady et al., 1992). Human CBP-HA expression plasmid was provided by A. Harel-Bellan (Institut A. Lwoff, Villejuif, France). pCMV-hsp70 construct (Michels et al., 1997) and pH2.3 clone corresponding to human hsp70 sequence (Wu et al., 1985) were provided by R.I. Morimoto (Northwestern University, Evanston, IL). The HP1β-GFP construct was provided by P.-Y. Perche (CreaCell™, INSERM U309, Grenoble, France). The DNA probes specific for chromosome 9 classical satellites (D9Z1) and chromosome 9 centromeres (Rocchi et al., 1991) were provided, respectively, by M.G. Mattei (Faculté de Médecine, Marseille, France) and M. Rocchi (Università di Bari, Bari, Italy). The rat monoclonal and rabbit polyclonal anti-HSF1 antibodies (Sarge et al., 1993; Cotto et al., 1997) and the mouse anti-HSP70 antibody were purchased from Stressgen. The MARA3 (Patturajan et al., 1998) and the 7C2 (Besse et al., 1995) mouse monoclonal antibodies against RNA polymerase II were obtained, respectively, from B.M. Sefton (Salk Institute, La Jolla, CA) and M. Vigneron (IGBMC, Strasbourg, France). The antibodies working dilution for immunofluorescence were, respectively, 1:100 (polyclonal anti-acetylated H2A, H2B, and H3 and anti-HSP70) (Turner and Fellows, 1989; White et al., 1999), 1:200 (polyclonal anti-acetylated H4 and MARA3), 1:300 (anti-HSF1 antibodies), and 1:400 (7C2). The rat monoclonal anti-HA antibody (Santa Cruz Biotechnology, Inc.) was used at 1:50 in immunofluorescence experiments.

Cell extracts and Western blot analysis

Whole cell extracts were prepared according to Dignam et al. (1983). The Western blot analysis of extracts was performed on 8% acrylamide gels for HSF1 and RNA polymerase II detection. The working dilutions for the rabbit anti-HSF1 and the 7C2 mouse anti-RNA polymerase II antibodies were, respectively, 1:5,000 and 1:10,000.

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Immunofluorescence, RNA FISH, DNA FISH, and microscopy

Immunofluorescence was performed on formaldehyde-fixed cells as described previously (Jolly et al., 2002), except for endogenous CBP, which was detected on cells fixed for 5 min in 1% formaldehyde and subsequently for 2 min in acetone at −20°C. Secondary antibodies conjugated to either Alexa 546 or Alexa 488 (Molecular Probes) were used. DNA was counterstained with 250 ng/ml DAPI. RNA FISH alone and in combination with immunofluorescence or DNA FISH was performed as described previously (Jolly et al., 1997). In control experiments, cells were treated for 15 min at 37°C with 100 μg/ml RNase A. Images were acquired on a Zeiss axiophot microscope equipped with a cooled charge-coupled device camera (C4880; Hamamatsu), using the 63x, 1.25 NA oil immersion objective and an intermediate magnification of 1.25x. HP1β-transfected cells were analyzed by confocal microscopy using a Zeiss LSM 510 microscope. Confocal sections were then projected on the same focal plane using the Zeiss LSM image browser.

RNA extraction, Northern blot, and reverse transcription

Total RNAs were extracted with Tri-reagent® (Sigma-Aldrich) following the manufacturer's instructions. For Northern blot analysis, 10 μg of RNA was loaded on a 1% agarose denaturing gel. Before transfer, the gel was stained with ethidium bromide to check the loading, treated or not with 75 mM NaOH for 10 min, and washed in 0.5 M Tris/1.5 M NaCl, pH 7.0. The RNAs were then transferred onto Hybond N membrane (Amersham Biosciences) and hybridized with pH2.3 or pHuR98 probe labeled with α-[^{32}P]dCTP, or with either a sense (nt 1–92) or an antisense (nt 92–1) oligonucleotide to pHuR98 end labeled with γ-[^{32}P]ATP. Reverse transcription was performed on 3 μg of total RNA in the presence of α-[^{32}P]dCTP as previously described (Buisson et al., 1999). The sequences of the primers used for sat III transcripts were 5′AATCAACCCGAGTGCAATC-GAATGGAATCG 3′ (sense) and 5′ TCCATTCCATTCCTGTACTCGG 3′ (antisense). The primers used for hsp70 transcripts were the same as previously described (Wang et al., 1999). After the reaction, the nonincorporated α-[^{32}P]dCTP was eliminated by spinning on Sephadex G50 columns. The samples were spotted on Hybond N membrane, and the signals were quantified with the PhosphorImager® system (Molecular Dynamics).

Run-on assay

Nuclear run-on transcription experiments were performed on nuclei isolated from non–heat-shocked or heat-shocked cells in the presence of 50 μCi of α-[^{32}P]UTP as described in Mathew et al. (2000). After precipitation, labeled RNAs were hybridized to the following DNA probes: pGEM2 (control for nonspecific hybridization), pHuR98, pH2.3 (hsp70 gene), and the rat GAPDH gene (Fort et al., 1985) as a normalization control for transcription. The intensity of the radioactive signals was quantitated using the PhosphorImager® system (Molecular Dynamics).

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