In vivo binding of active heat shock transcription factor 1 to human chromosome 9 heterochromatin during stress

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Activation of the mammalian heat shock transcription factor (HSF)1 by stress is a multistep process resulting in the transcription of heat shock genes. Coincident with these events is the rapid and reversible redistribution of HSF1 to discrete nuclear structures termed HSF1 granules, whose function is still unknown. Key features are that the number of granules correlates with cell ploidy, suggesting the existence of a chromosomal target. Here we show that in humans, HSF1 granules localize to the 9q11-q12 heterochromatic region. Within this locus, HSF1 binds through direct DNA–protein interaction with a nucleosome-containing subclass of satellite III repeats. HSF1 granule formation only requires the DNA binding competence and the trimerization of the factor. This is the first example of a transcriptional activator that accumulates transiently and reversibly on a chromosome-specific heterochromatic locus.

Introduction

Heat shock transcription factor (HSF)*1 is a key player of the cellular response leading to the expression of heat shock protein (hsp) genes under stress conditions (for review see Pirkkala et al., 2001). Upon stress, HSF1 undergoes trimerization, phosphorylation, and activation of DNA binding activity, and activates hsp gene transcription through binding to heat shock elements (HSEs) present in their promoter. A particularly distinctive feature of HSF1 resides in its dramatic redistribution during stress. Whereas the inactive factor displays a diffuse cytoplasmic and/or nuclear localization, it rapidly accumulates during stress in a few nuclear foci termed HSF1 granules whose role remains unclear (Sarge et al., 1993; Cotto et al., 1997; Jolly et al., 1997, 1999a). Although the presence of the granules correlates with the activation of the stress response (Cotto et al., 1997; Jolly et al., 1999a), the granules do not form at sites of hsp gene transcription, and they are present in heat-shocked mitotic cells lacking transcriptional activity, suggesting a role distinct from transcription regulation (Jolly et al., 1997, 1999a). Quite unexpectedly, the number of HSF1 granules correlates with cell ploidy, thus supporting the existence of a specific chromosomal target (Cotto et al., 1997; Jolly et al., 1997).

We were interested in identifying the chromosomal target of HSF1 granules. Here we show that the granules form on the pericentromeric heterochromatic region of human chromosome 9 through a direct DNA–protein interaction with a specific subfamily of satellite III repeats. HSF1 granule formation requires both the DNA binding competence and the trimerization of the protein, and does not involve stress-induced chromosome modifications.

Results and discussion

HSF1 associates in vivo with chromosome 9q11-q12 region during stress

To identify the chromosome on which HSF1 granules form, HSF1 was detected by immuno-FISH on metaphase spreads together with each human centromere. HSF1 granules were
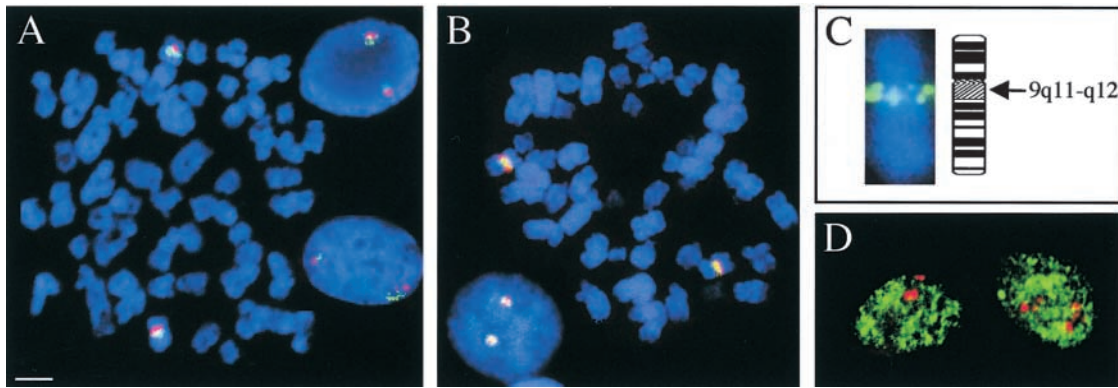
Figure 1. **Mapping of HSF1 granule chromosomal target.** HSF (green) was detected by immunofluorescence on metaphase spreads prepared from normal fibroblasts exposed for 1 h at 45°C together with either chromosome 9 centromeres (A) or pHuR98 probe (B) detected by FISH (red). DNA was counterstained with DAPI. (C) Ideogram of human chromosome 9 showing the precise location of HSF1 granules (arrow). (D) HSF1 granules (red) were codetected with RNA polymerase II transcription sites revealed by BrUTP incorporation (green) in fibroblasts exposed for 1 h at 45°C. Bar, 5 μm.

found to localize in all heat-shocked cells to chromosome 9, in a region below the centromere (Fig. 1 A). No signal was observed on chromosomes prepared from nonheat-shocked cells (unpublished data). The same experiments were then repeated with a probe specific of the 9q11-q12 region where the granules seemed to localize. This region, also known as secondary constriction or 9qh region, is a large block of heterochromatin composed primarily of satellite III repeats (Jones et al., 1973). Codetection of HSF1 and the pHuR98 probe corresponding to a chromosome 9 subclass of satellite III (Moyzis et al., 1987) showed the colocalization of the two signals (Fig. 1 B, yellow), thus demonstrating that the granules localize to the 9q11-q12 region (Fig. 1 C).

Because HSF1 granules localize to a region of heterochromatin that is supposedly silent, we investigated further the involvement of HSF1 granules in transcriptional activity. We found that HSF1 granules were spatially distinct from RNA polymerase II transcription sites labeled by BrUTP incorporation (Fig. 1 D, absence of yellow).

HSF1 granule formation requires both the DNA binding and trimerization domains

The domains of HSF1 required for targeting to the granules were identified by the construction of HSF1 deletion mutants fused to the green fluorescent protein (GFP) and transiently expressed in HeLa cells. Results are shown in Fig. 2. The full-length HSF1 only formed granules after heat shock (an average of three granules was observed because HeLa cells contain three 9qh regions). The mutants lacking either the DNA binding domain (ΔDBD) or the trimerization domain (ΔTRIM) displayed a diffuse nuclear distribution excluding nucleoli both at 37°C and 42°C. Likewise, mutants that retained either the DBD or the TRIM domain exhibited a diffuse nuclear and cytoplasmic staining both at control and heat shock temperature. In contrast, the DBD + TRIM construct which retained both the DNA binding and trimerization domains formed granules in all cells, both at 37°C and 42°C, whereas the complementary construct ΔDBD–ΔTRIM always displayed a diffuse nuclear and cytoplasmic distribution. The nuclear structures observed for

the DBD + TRIM mutant corresponded to HSF1 granules as confirmed by the subsequent FISH detection of chromosome 9 centromeres in these cells (unpublished data). Moreover, the endogenous HSF1 visualized with a monoclonal antibody that recognizes an epitope in the LZ4 domain displayed a diffuse nuclear distribution in these cells at 37°C, thus confirming that the constitutive granules observed for the DBD + TRIM mutant did not result from the induction of a stress response (unpublished data).

Altogether, these results demonstrate that the targeting of HSF1 to 9qh requires both the DNA binding activity and the trimerization of the protein, but not its transactivation capacity, confirming that only the active HSF1 can be targeted to the granules. Moreover, the constitutive appearance of HSF1 granules with the DBD + TRIM mutant reveals that granule formation does not require a stress-induced nuclear modification.

In vitro reconstitution of HSF1 granules on the 9qh shows a direct DNA–protein interaction

To address whether HSF1 interacts directly with DNA within the 9qh, we designed an in vitro assay to reconstitute the granules on human chromosomes prepared by standard cytogenetic procedures, which are known to result in the loss of most chromosome-associated proteins (Ronne et al., 1979). Immunofluorescence detection of HSF1 on such preparations indeed confirmed that HSF1 was not detectable on chromosomes 9 (unpublished data). We attempted to reconstitute the granules on these HSF1-depleted chromosomes by the addition of recombinant human HSF1, and the subsequent detection of the protein by immunofluorescence. The quality and purity of HSF1 were checked on silver-stained acrylamide gel and by Western blot (unpublished data). In addition, previously published data showed that recombinant HSF1 exhibits the properties of native active HSF1 trimers that appear during stress (Kroeger et al., 1993). 50 ng of HSF1 and 5 min of incubation with the protein were sufficient to observe, in all nuclei and chromosome spreads, two bright fluorescent signals on one pair of chromosomes which were subsequently confirmed by FISH
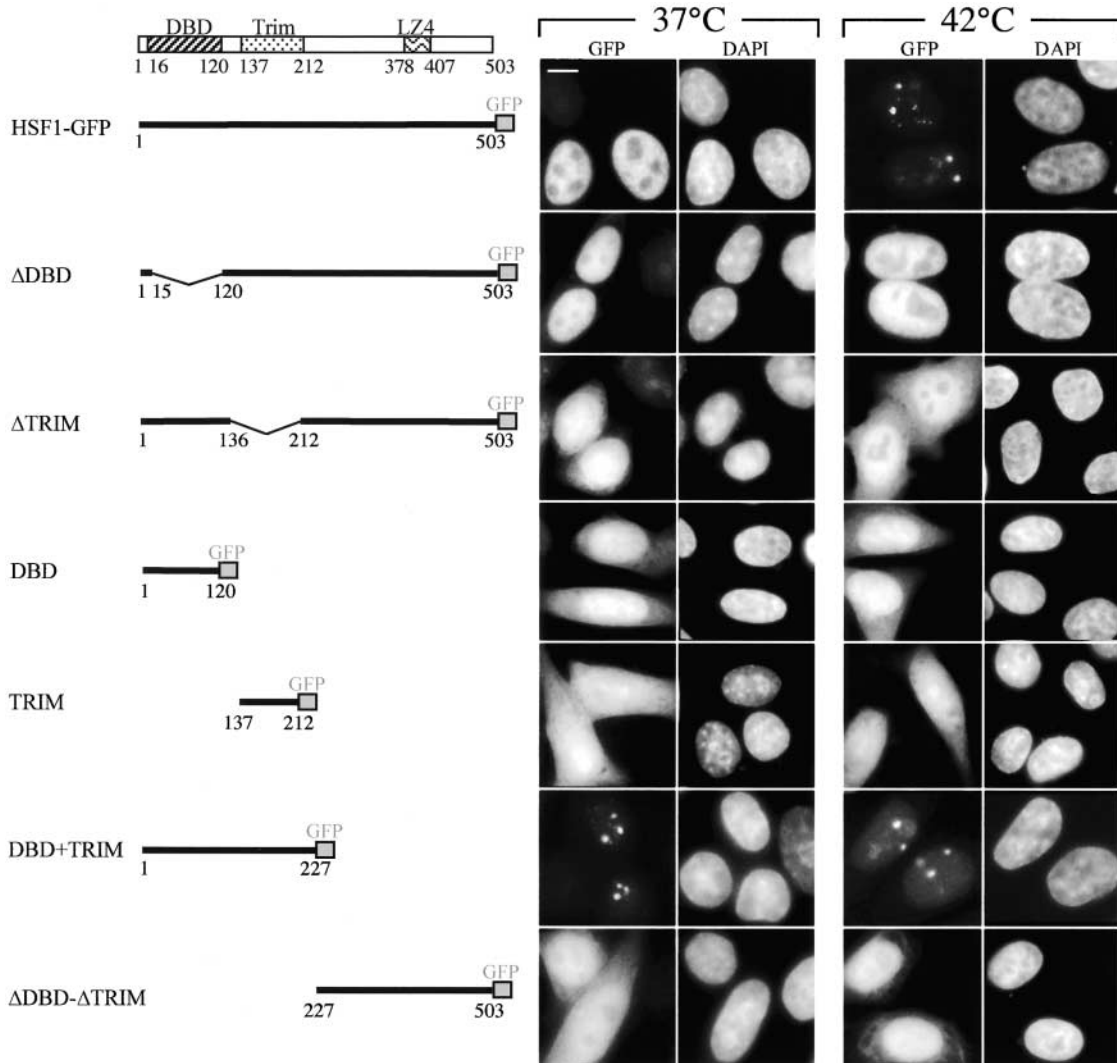
Figure 2. **Mapping of the HSF1 protein domains required for the targeting to the granules.** Images of the distribution at 37°C or after 30 min at 42°C of the different HSF1–GFP mutants transiently expressed in HeLa cells are shown with the corresponding DAPI image. Bar, 5 μm.

to be chromosomes 9 (Fig. 3 A). The same experiment performed with a nonrelated DNA binding factor, HBP1 (Lesage et al., 1994), yielded no detectable signal, confirming the specificity of the signal observed with HSF1 (unpublished data). Thus, the key information for HSF1 granules to form is contained within the 9qh. The association of

HSF1 with chromosome 9 could be disrupted by treatment of the chromosomes with various nucleases, including DNAse I, micrococcal nuclease, and the restriction endonuclease Alu I prior to addition of HSF1 (unpublished data). In contrast, pretreatment of the chromosomes with 100 μg/ml proteinase K or 0.01% pepsin/0.01 N HCl did not pre-
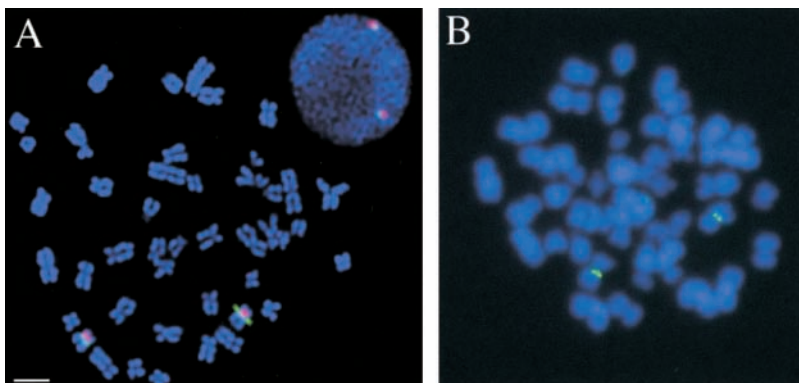

Figure 3. **In vitro reconstitution of HSF1 granules on metaphase chromosome spreads.** (A) Cytogenetic preparations of chromosomes were incubated with recombinant human HSF1 protein, processed for immunofluorescence for HSF1 (green), and subsequently processed for chromosome 9 centromeres detection by FISH (red). (B) The same experiment was performed on chromosome spreads treated with 100 μg/ml proteinase K for 15 min at 37°C prior to incubation with HSF1 (green). DNA was counterstained with DAPI. Bar, 5 μm.
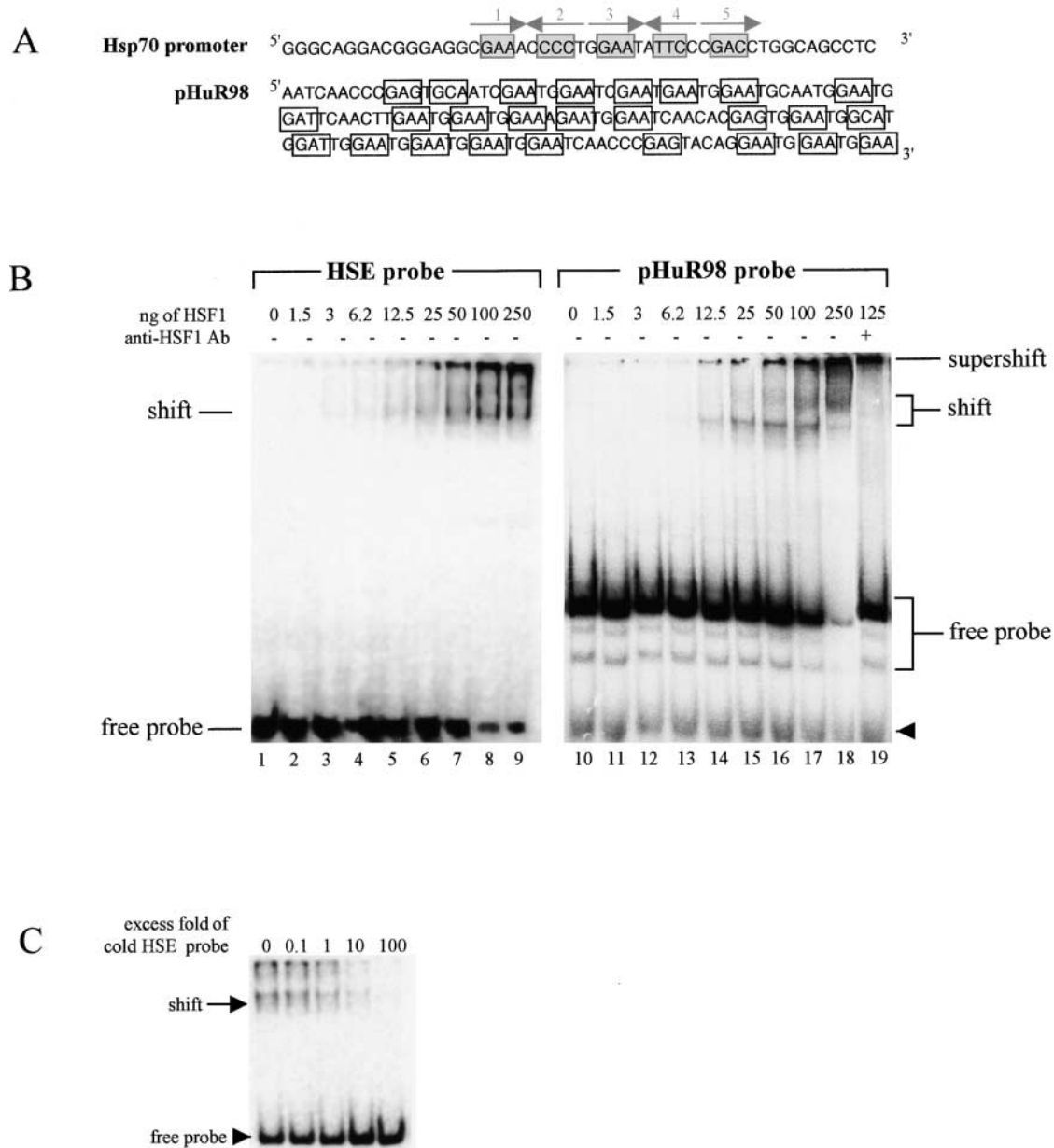
Figure 4.   In vitro binding of HSF1 to chromosome 9 satellite III repeats and to HSP70 heat shock element. (A) Sequence comparison of the proximal HSE of HSP70 promoter (nt-130 to -81) and pHuR98. The plain grey boxes indicate the individual HSF1 binding sites (Kroeger et al., 1993). The empty grey boxes indicate the possible HSF1 binding sites. (B) EMSA was performed by incubating a labeled HSE (lanes 1–9) or pHuR98 probe (lanes 10–19) with various amounts of recombinant HSF1. For supershift assay, 1 μl of polyclonal anti-HSF1 antibody was added to the reaction (lane 19). The arrowhead points to a high mobility form of pHuR98 free probe which is not shifted by HSF1. (C) HSF1 (12.5 ng) was incubated with various excesses of unlabeled HSE competitor probe prior to the addition of a fixed amount of labeled pHuR98 probe.

vent the association of HSF1 with 9qh, despite the alteration of chromosome structure as attested by the puffy morphology of chromosomes observed after DAPI counterstaining (Fig. 3 B). Thus, these data demonstrate that HSF1 interacts directly with chromatin within the granules. In addition, HSF1 granules can be reconstituted on non-heat- and heat-shocked chromosomes with the same efficiency, supporting the proposal that granule formation does not require a stress-induced chromosomal modification. Likewise, micrococcal nuclease digestion experiments revealed that like other heterochromatic regions, chromo-

some 9 satellite III repeats are packaged into highly regular nucleosome arrays and that this organization is not altered by stress (unpublished data).

HSF1 binds to chromosome 9 satellite III repeats in vitro

Next, we investigated the possibility that pHuR98 repeats were the DNA target of HSF1 granules by electrophoretic mobility shift assay (EMSA) using either a consensus HSE or pHuR98 as a probe. Recombinant HSF1 was used in these experiments because extracts from control cells contain unidentified proteins that bind pHuR98 in vitro

(Grady et al., 1992). With the HSE probe, an HSF1–HSE complex was detected with amounts of HSF1 >3 ng (Fig. 4 B, lanes 3–9). The intensity of the band corresponding to this complex increased with the amount of HSF1, whereas the intensity of the band corresponding to the free probe decreased at the highest HSF1 concentrations (Fig. 4 B, lanes 8 and 9). For pHuR98, DNA–protein complexes were detected only at HSF1 levels >12.5 ng (Fig. 4 B, lanes 14–18). Several complexes were detected at the highest concentrations of HSF1, perhaps representing the binding of multiple trimers to the probe (Fig. 4 B, lanes 17 and 18). The specificity of these complexes as HSF1–pHuR98 was confirmed by supershift assay (Fig. 4 B, lane 19). In addition, no shift was observed with a related satellite II of the 16qh (Moyzis et al., 1987) or with the α-satellite of chromosome 16 centromere (unpublished data). As with HSE, the signal corresponding to the free pHuR98 probe declined with high amounts of HSF1 (lanes 17 and 18); however, HSF1 only shifted slower-migrating forms (Fig. 4 B, free probe) that may correspond to species of the probe forming secondary structures.

We next showed that pHuR98 and HSE sequences can compete for HSF1 binding in vitro (Fig. 4 C). The intensity of pHuR98 shifted band decreased progressively with increasing amounts of cold HSE to become barely visible with a 100-fold excess of cold probe (Fig. 4 C, lanes 3–5). Thus, HSF1 can bind specifically to chromosome 9 satellite III repeats through a direct DNA–protein interaction, which is most likely mediated by the DNA binding domain.

Taken together, our data show that the key determinant for the accumulation of active HSF1 into granules is contained within the 9qh satellite III repeats. Interestingly, repeats of HSEs to which HSF binds during stress have been found in the telomeres of *Chironomus thummi* (Martinez et al., 2001), suggesting that the stress-induced redistribution of HSF on heterochromatin may be a conserved phenomenon. In this context, it is worth noting that pHuR98 sequences are conserved among higher eukaryotes (Grady et al., 1992), and that HSF1 granules also form in monkey cells (unpublished data). In humans, the specificity of association between HSF1 and the 9qh may rely on the presence of NGAAN elements in these repeats, although their organization, which differs from that of a canonical HSE, argues against this hypothesis (Perisic et al., 1989). Alternatively, the active factor may recognize secondary structures formed by pHuR98 repeats, as suggested by their unusual thermal stability in vitro (Grady et al., 1992) and supported by our EMSA experiments. In addition, several observations underscore the unique structural features of the 9qh, in particular a decondensation of the chromatin within this region (Hungerford, 1971; Bobrow et al., 1972; Mitchell et al., 1986; Heslop-Harrison et al., 1989). Thus, it is conceivable that the 9qh region adopts a specific conformation that allows HSF1 to bind and organize as granules.

Heterochromatin has been implicated in several functions, such as gene regulation or chromosome segregation (Marshall et al., 1997; Renauld and Gasser, 1997). The present work also suggests a possible role for heterochromatin in the stress response. Interestingly, patterns of heterochromatic lo-

calization have been reported for several transcriptional regulators (Raff et al., 1994; Brown et al., 1997; Wang et al., 1997; Platero et al., 1998; Saurin et al., 1998; McDowell et al., 1999; Ryan et al., 1999; Tang and Lane 1999). What could be the functional significance of the stress-induced targeting of HSF1 to human heterochromatin? The possibility that chromosome 9 satellite III repeats form a repressive microenvironment involved in the negative regulation of hsp genes is unlikely, as inactive hsp genes never colocalize with the 9qh region, either in unstressed cells or during attenuation of the stress response (Jolly et al., 1997; unpublished data). Another hypothesis is that HSF1 granules serve as sites of storage and/or buffering of the active factor to ensure the coordinate activation and down-regulation of all target genes located at distant sites both during activation and attenuation of the heat shock response. Alternatively, HSF1 granules may coordinate some yet undescribed aspects of the stress response, perhaps via other proteins targeted the granules (Weighardt et al., 1999; Denegri et al., 2001). Finally, one can imagine that HSF1 granules serve in protecting a hypersensitive region of the genome from stress. Indeed, large heterochromatic blocks like the 9qh are known to be fragile regions presenting a high incidence of rearrangements in normal and tumor cells (Bartlett et al., 1998; Lamszus et al., 1999), as well as mitotic/meiotic abnormalities (Boue et al., 1985).

Materials and methods

Antibodies and probes
The probes specific for each human centromere were obtained from Dr. M. Rocchi (Università degli Studi di Bari, Bari, Italy) (Archidiacono et al., 1995). Clone pHuR98 (D9Z3) is a 158-bp probe specific for human 9qh satellite III repeats (Moyzis et al., 1987). For FISH, probes were labeled by random priming with biotin-14-dATP (GIBCO BRL). The 10H8 rat monoclonal antibody (Cotto et al., 1997) and a rabbit polyclonal antibody (Sarge et al., 1993) were used to detect HSF1 in immunofluorescence and gel shift assays.

Cloning of HSF1 deletion mutants
All HSF1 mutants were generated by PCR using the GFP–HSF1 construct (Cotto et al., 1997) as a template. PCR products were cloned into pEGFP-N1 or pEGFP-N2 vector (CLONTECH Laboratories, Inc.). All constructs were verified by sequencing across the coding region.

Cell culture and transient transfection
Human normal primary fibroblasts obtained from a skin biopsy performed on a female donor were grown in RPMI medium supplemented by 10% fetal calf serum. HeLa cells were grown in DME supplemented with 5% fetal bovine serum. Transient transfections were performed using ExGen 500 (Euromedex).

Metaphase chromosome preparation
Cytogenetic preparation of chromosomes. Metaphase spreads were prepared from blood lymphocytes according to standard cytogenetic techniques.

Preparation using formaldehyde fixation. Fibroblasts were treated with 0.01 μg/ml colcemid for 8 h. Mitotic cells collected by mechanical shock were submitted to hypotonic treatment in 75 mM KCl for 30 min at 37°C, spread onto glass slides by cytospin centrifugation for 1 min at 1,200 rpm, fixed in 4% formaldehyde/PBS for 10 min, and processed for immunofluorescence.

Immunofluorescence, FISH, and microscopy
Detection of HSF1 by immunofluorescence was performed as described (Jolly et al., 1999a, 1999b). Anti-HSF1 antibodies (1:300 dilution) were detected with FITC-conjugated secondary antibodies (Sigma-Aldrich). DNA was counterstained with 250 ng/ml DAPI in an anti-fading solution (90% glycerol, 2.3% diaza-bicyclo-octane). Cells transfected with

GFP-tagged HSF1 mutants were fixed in formaldehyde and directly counterstained. Images were acquired on a Zeiss axiophot microscope equipped with a cooled charge-coupled device camera (C4880 Hamamatsu), using the 63×, 1.25 NA oil immersion objective and an intermediate magnification of 1.25×. The subsequent detection of DNA sequences by FISH was performed as described (Jolly et al., 1999b). Probes were detected using avidin-TRITC (Sigma-Aldrich). The cells which were previously pictured for HSF1 were photographed again to acquire the FISH signal.

In vivo labeling of transcription sites using BrUTP and combined immunofluorescence

BrUTP incorporation was performed as described previously (Jolly et al., 1999b). After the in vivo transcription reaction, cells were fixed in 4% formaldehyde/PBS and transcription sites were detected with a mouse anti-BrdU antibody (Sigma-Aldrich) and anti–mouse-FITC (Sigma-Aldrich). Subsequent detection of HSF1 was performed as described above.

In vitro reconstitution of HSF1 granules on metaphase chromosomes

Chromosome spreads prepared according to standard cytogenetic techniques were incubated for 1 h at 37°C in a blocking solution (10% fetal bovine serum, 0.3% Triton, PBS). Slides were then incubated at 37°C for various times (5 min to 4 h) with different amounts (10 ng to 2 μg) of human recombinant HSF1 (StressGen) or GST-HBP1 protein, provided by Dr. S. Khochbin (Institut A. Bonniot, La Tronche, France) (Lesage et al., 1994) resuspended in Hepes 50 mM, pH 7.4, EDTA 0.1 mM, NaCl 0.2 M. Slides were subsequently processed for immunofluorescence. GST-HBP1 protein was detected using a goat anti-GST antibody (1:200 dilution) and an anti–goat-FITC antibody (Sigma-Aldrich). In some experiments, chromosome spreads were treated, prior to the incubation with HSF1, with either 100 μg/ml proteinase K in Tris-HCl 20 mM, pH 7.2, CaCl₂ 2 mM or 0.01% pepsin/0.01 N HCl for 15 min at 37°C, or for 8 h at 37°C with 100 units of micrococcal nuclease, Alu I, or DNAse I.

Electrophoretic mobility shift assay

The following primer and its complementary primer were used as a HSE probe: 5′-TCGGCTGGAATATTCCCGACCTGGCAGCCGA-3′. 100,000 cpm of ³²P end-labeled pHuR98 probe (0.5 ng) or double-strand HSE (1.4 ng) were incubated for 20 min at 25°C with various amounts (0.75 ng to 250 ng) of recombinant HSF1 protein, and samples were run on a 4% polyacrylamide gel. For supershift assay, 1 μl of rabbit polyclonal anti-HSF1 antibody was added to the reaction. For competition experiments, the reaction was first carried out for 20 min with 12.5 ng of HSF1 in the presence of various amounts of cold HSE probe (0.1–100-fold molar excess). 1 nM of labeled pHuR98 was then added and the reaction was run again for 20 min.

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