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Stress-induced Nuclear Bodies Are Sites of Accumulation of Pre-mRNA Processing Factors

Marco Denegri, Ilaria Chiodi, Margherita Corioni, Fabio Cobianchi, Silvano Riva, and Giuseppe Biamonti*

Istituto di Genetica Biochimica ed Evoluzionistica del Consiglio Nazionale delle Richerche, Pavia 27100, Italy

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Heterogeneous nuclear ribonucleoprotein (hnRNP) HAP (hnRNP A1 interacting protein) is a multifunctional protein with roles in RNA metabolism, transcription, and nuclear structure. After stress treatments, HAP is recruited to a small number of nuclear bodies, usually adjacent to the nucleoli, which consist of clusters of perichromatin granules and are depots of transcripts synthesized before stress. In this article we show that HAP bodies are sites of accumulation for a subset of RNA processing factors and are related to Sam68 nuclear bodies (SNBs) detectable in unstressed cells. Indeed, HAP and Sam68 are both present in SNBs and in HAP bodies, that we rename "stress-induced SNBs." The determinants required for the redistribution of HAP lie between residue 580 and 788. Different portions of this region direct the recruitment of the green fluorescent protein to stress-induced SNBs, suggesting an interaction of HAP with different components of the bodies. With the use of the 580–725 region as bait in a two-hybrid screening, we have selected SRp30c and 9G8, two members of the SR family of splicing factors. Splicing factors are differentially affected by heat shock: SRp30c and SF2/ASF are efficiently recruited to stress-induced SNBs, whereas the distribution of SC35 is not perturbed. We propose that the differential sequestration of splicing factors could affect processing of specific transcripts. Accordingly, the formation of stress-induced SNBs is accompanied by a change in the splicing pattern of the adenovirus E1A transcripts.

INTRODUCTION

In the last few years the use of light and electron microscopy imaging associated to molecular biology approaches allowed the identification of the various subdomains that compose the nucleus of a metazoan cell. Chromosomes occupy discrete territories that account for most of the nuclear volume, whereas numerous structures, among which interchromatin granules or "speckles," Cajal bodies, "gems," and promyelocytic bodies, are recognizable in the interchromatin space. However, in spite of the extensive characterization, the function of these compartments remains a matter of investigation (Lamond and Earnshaw, 1998; Matera, 1999).

In addition to these structures, several other, still poorly defined, nuclear bodies have been described that are probably implicated in important processes as different as pre-mRNA maturation, DNA replication (replication factories), and DNA repair (repair foci) (Matera, 1999). A subset of these structures, namely, the perinucleolar compartment (PNC) and the Sam68 nuclear body (SNB) are preferentially positioned in proximity to the nucleoli and are marked by specific RNA binding proteins (Huang, 2000).

SNBs are the sites of accumulation of Sam68 (Src activated during mitosis), SLM-1, and SLM-2, three members of the family of RNA binding proteins characterized by the GSG (GRP33, Sam68, GLD-1) domain, also termed signal transduction and activation of RNA domain (Chen *et al.*, 1997; Vernet and Artzt, 1997; Chen *et al.*, 1999; Venables *et al.*, 1999). The importance of SNBs is suggested by the role of Sam68 in cell cycle progression (Barlat *et al.*, 1997), RNA export (Reddy *et al.*, 2000), and splicing (Stoss *et al.*, 2001). Electron microscopy analysis demonstrates that SNBs are composed of phosphorus-rich fibers and granules, indicating the presence of abundant ribonucleoprotein complexes (Chen *et al.*, 1999). In accord with this interpretation, Sam68 interacts with a number of RNA binding proteins, including heterogeneous nuclear ribonucleoprotein (hnRNP) G, hnRNP K (Venables *et al.*, 1999), scaffold attachment factor-B (SAF-B)/HAP (Stoss *et al.*, 2001), and the splicing factor YT521 (Hartmann *et al.*, 1999). The formation and maintenance of SNBs depend on ongoing RNA polymerase II transcription, even if these nuclear bodies do not appear to concentrate newly synthesized RNAs (Chen *et al.*, 1999).

* Corresponding author. E-mail: biamonti@igbe.pv.cnr.it.

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Although the function of SNBs is still poorly understood, the role of Sam68 in RNA transport (Reddy *et al.*, 2000) suggests that these bodies could be implicated in the mRNAs trafficking through the nucleus (Chen *et al.*, 1999).

In addition to PNCs and SNBs, other perinucleolar bodies have been described that are recognized by antibodies directed against specific hnRNPs, including hnRNP L (Pinol-Roma *et al.*, 1989), hnRNP poly-pyrimidine tract binding protein (Ghetti *et al.*, 1992), hnRNP M (Datar *et al.*, 1993), and SAF-B/hnRNP A1 interacting protein (Weighardt *et al.*, 1999). However, the relationship between these bodies has not yet been investigated. hnRNP A1 interacting protein (HAP) is a novel hnRNP protein of 917 amino acids (Weighardt *et al.*, 1999) with roles in transcription, RNA maturation, and nuclear structure. Indeed, HAP was first described as SAF-B, a component of the nuclear matrix (Renz and Fackelmayer, 1996). Moreover, this protein was reported to act as a transcriptional regulator of the heat shock protein 27 gene, HET (Oesterreich *et al.*, 1997). Hereafter, for the sake of simplicity, we will only use the abbreviation HAP. Several observations support the notion of an involvement of HAP in RNA metabolism. First, HAP contains a canonical RNA binding domain and is part of the hnRNP complexes (Weighardt *et al.*, 1999). Second, in a two-hybrid assay HAP binds to hnRNP A1 (Weighardt *et al.*, 1999) and to the C-terminal domain of RNA polymerase II (Nayler *et al.*, 1998). In addition, HAP interacts, both in vitro and in vivo, with a number of splicing factors, among which are SRp30c, SF2/ASF, and htra2-beta (Nayler *et al.*, 1998), and with Sam68 and SLM-2 (Stoss *et al.*, 2001). Finally, a direct involvement of HAP in splicing is suggested by the observation that its overexpression affects splicing of the adenovirus E1A reporter gene (Nayler *et al.*, 1998).

HAP displays a punctuated distribution throughout the nucleoplasm with exclusion of nucleoli. Moreover, it is also present in a small number of nuclear bodies, some of which lie in proximity to the nucleoli (Weighardt *et al.*, 1999). Intriguingly, after stress treatments HAP is recruited to novel nuclear compartments, termed either HAP bodies (Chiodi *et al.*, 2000) or stress bodies (Jolly *et al.*, 1997), which are also sites of accumulation of heat shock factor 1 and hnRNP M (Chiodi *et al.*, 2000; Jolly *et al.*, 1997; Weighardt *et al.*, 1999). HAP bodies are relatively large structures, 1–3 μm in diameter, which consist of clusters of perichromatin granules and represent depots of RNA molecules synthesized either before or after but not during stress (Chiodi *et al.*, 2000). A careful in vivo analysis suggests that these bodies assemble on underlying nuclear structures, most likely corresponding to specific chromosomal domains. Indeed, in mitotic HeLa cells heat shock induces the recruitment of heat shock factor 1 to specific, although as yet unidentified, chromosomes (Jolly *et al.*, 1999a).

In this article we show that HAP bodies are sites of accumulation for a subset of RNA processing factors, including Sam68, SRp30c, SF2/ASF, and to a lesser extent, 9G8. Intriguingly, the same protein region that mediates the interaction with some of these SR splicing factors is also required for the recruitment of HAP to stress bodies. We propose that the differential recruitment of splicing factors to HAP bodies might be part of the posttranscriptional regulation of gene expression in heat-shocked cells.

MATERIALS AND METHODS

Cell Culture, Cell Treatments, and Transfections

HeLa cells were grown in DMEM (Invitrogen, Carlsbad, CA), 10% fetal calf serum (Invitrogen), 50 μg/ml gentamicin, and 2 mM L-glutamine. For heat-shock experiments, cells were incubated 1 h at 42°C in complete medium made 40 mM HEPES pH 7.0 and allowed to recover 1 h at 37°C before analysis.

HeLa cells were transfected with recombinant plasmids by the calcium phosphate precipitation technique of Graham and van der Eb (1973). A total of 10^6 cells was directly plated on cover glasses. After 24 h, 2 μg of plasmid and 2 μg of high molecular mass calf thymus carrier DNA (Roche Molecular Biochemicals, Indianapolis, IN) were added. Plasmids were prepared with the QUIGEN Plasmid Midi kit (Valencia, CA).

Indirect Immunofluorescence

HeLa cells grown on coverslips were washed once with phosphate-buffered saline (PBS), fixed for 7 min in 4% formaldehyde, and subsequently permeabilized in 0.5% Triton X-100 for 7 min on ice. Primary antibodies were diluted at working concentration in PBS containing 5% skimmed milk (Difco, Detroit, MI) and then added to the coverslips. Primary antibodies used were affinity purified rabbit anti-HAP polyclonal antibody (Weighardt *et al.*, 1999), 7-1 monoclonal antibody (mAb) directed to Sam68 (Santa Cruz Biotechnology, Santa Cruz, CA), 12CA5 mAb against the HA-epitope (Roche Molecular Biochemicals), and anti-SC35 mAb (Sigma, St. Louis, MO). After 1 h at 37°C in a humid chamber, coverslips were washed three times with PBS. Secondary antibodies used were rhodamine-conjugated goat antirabbit IgG antibody, rhodamine-conjugated goat antimouse IgG antibody (Jackson Immunoresearch, West Grove, PA) and fluorescein isothiocyanate (FITC)-conjugated rabbit antimouse IgG antibody (DAKO, Carpinteria, CA). Secondary antibodies were diluted at the final concentration recommended by the supplier in PBS made 5% skimmed milk and added to coverslips. After 1 h at 37°C in a humid chamber, coverslips were washed three times with PBS, rinsed, and mounted in 90% glycerol in PBS. Confocal microscopy was performed with a Leica TCS-NT digital scanning confocal microscope equipped with a 63×/numerical aperture = 1.32 oil immersion objective. We used the 488-nm laser line for excitation of FITC (detected at 500 nm $<\lambda_{FITC}<$540 nm) and the 543-nm laser line for the rhodamine fluorescence (detected at $>$590 nm). The pinhole diameter was kept at 1 μm. Images were exported to Adobe Photoshop (Adobe Systems, Mountain View, CA).

Plasmids

Different regions of HAP were expressed in transfected HeLa cells as green fluorescent protein (GFP) fusions. Portions of the open reading frame of HAP were polymerase chain reaction (PCR)-amplified with Pwo polymerase (Roche Molecular Biochemicals) with the use of the HAP cDNA (Weighardt *et al.*, 1999) as template and suitable primers synthesized on the basis of the cDNA sequence (accession number NM-002967). Upstream and downstream primers started with an *Eco*RI and a *Sal*I site, respectively, used for cloning into the pEGFP-C1 vector (CLONTECH, Palo Alto, CA). To ensure the nuclear localization of the different GFP fusions, a double-stranded oligonucleotide encoding for the nuclear localization signal (NLS) of the simian virus 40 large T antigen (PPKKKRKV) was cloned into the *Sal*I-*Bam*HI sites of pEGFP-C1. Each plasmid was sequenced with the Seq4X4 personal sequencing system and the "Thermo Sequenase Cy5.5 Dye Terminator Cycle" sequencing kit (Amersham Pharmacia Biotech, Arlington Heights, IL). The open reading frame of HAP, either wild-type or deleted of the region encoding for amino acids 580–788, was cloned into the *Pst*I-*Eco*RI sites of the pMT2-HA plasmid that allows the expression in mammalian cells of proteins N-terminally fused to the hemagglutinin (HA)-tag. To delete the 580–788 region (cDNA sequence 1846–2735)

we used a PCR-based methodology previously described (Montecucco *et al*., 1998) and suitable primers. All primers used in this work were from MWG-Biotech (Ebersberg, Germany).

The full-length cDNAs of SRp30c and 9G8 were selected during the two-hybrid screening in yeast for proteins interacting with the region necessary for the stress-induced redistribution of HAP. The SF2/ASF cDNA was kindly provided by Dr. J. Caceres (University of Edinburgh, Edinburgh, Scotland). All the cDNAs were PCR-amplified with suitable primers and cloned into the pEGFP-C1 vector. The expression of all the fusion proteins in transfected HeLa cells was verified by Western blot analysis of total cell extracts with the use of antibodies specific for the different tagged used, GFP or HA. In all cases, the size of the transfected protein was compatible with the size expected on the basis of the cDNA sequence.

Yeast Two-Hybrid Screening

The human HeLa cDNA library, yeast strains, and cloning vectors were from CLONTECH. All library screenings and yeast manipulations were carried out according to the manufacturer. A fragment of the HAP cDNA encoding residues 580–725 was PCR-amplified with suitable primers and cloned into the *Eco*RI-*Sal*I sites of pAS2.1 that directs the expression of proteins fused to the DNA binding domain of GAL4. The *Saccharomyces cerevisiae* Y190 strain was transformed with pAS2.1-(580–725) and used as a recipient to screen a HeLa cDNA library (catalog no. HL4000AA; CLONTECH). A total of 2×10^7 transformants was plated onto 15-cm plates of leu^-, his^-, and trp^- synthetic medium containing 25 mM 3-amino-1,2,4-triazole (Sigma). Thirty-one his^+ colonies were isolated and β-galactosidase filter assay was performed by streaking the positives onto filters placed on leu^- and trp^- synthetic medium plates. Plasmids were isolated from these colonies and retrotransformed to confirm the interaction. Plasmids inserts were sequenced with the "Thermo Sequenase Cy5.5 Dye Terminator Cycle" sequencing kit (Amersham Pharmacia Biotech).

E1A Alternative Splicing

The pCMVE1A plasmid containing the E1A minigene was kindly provided by Dr. Chabot (University of Sherbrooke, Sherbrooke, Quebec) (Yang *et al*., 1994). Two micrograms of plasmid was used to transfect 5×10^5 HeLa cells by the calcium phosphate precipitation technique (Graham and van der Eb, 1973). Twenty-four hours after transfection RNAs were extracted from control transfected cell or from stressed cells, either immediately after heat shock or after increasing recovery periods at 37°C. When requested transfected cells were treated with 30 μM of cadmium sulfate for the indicated time periods. Total RNAs were extracted with the RNeasy Kit (QUIGEN) according to the extraction protocol recommended by the supplier. To avoid plasmid contamination RNA samples were digested with 100 U of RNase-free DNase I (Roche Molecular Biochemicals) for 30 min at room temperature in the presence of 50 U of RNasin (PerkinElmer Life Science Products). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described (Ghigna *et al*., 1998). Total RNA (1 μg) was retro-transcribed as recommended by the supplier with 50 U of MuLV reverse transcriptase (PerkinElmer Life Science Products) in a 20-μl reaction and the diluted to a final volume of 100 μl with H_2O. Amplification reactions (50 μl) contained 5 μl of the reverse transcription reaction, 20 pmol of primers, 2 mM $MgCl_2$, 200 μM each dNTPs, and 2.5 U of *Taq* polymerase (PerkinElmer Life Science Products, Boston, MA) in standard buffer provided by the supplier. To quantify amplification products, 1 μCi of [α-^{32}P]dCTP, 3000 Ci/mmol (Amersham Pharmacia Biotech), was added to the reactions. Primers used were E1A-569 (5′-ATTATCTGCCACGGAGGTGT-3′) and E1A-1315 (5′-GGATAGCAGGCGCCATTTTA-3′). Primers were purchased from MWG-Biotech. Amplifications were performed for 30 cycles with the following profile: 1 min at 94°C, 1 min at 56°C, and 1 min and 30 s at 72°C. No amplification was detectable if reverse transcription was omitted. An aliquot (10 μl) of each reaction was loaded onto a 5% acrylamide gel in Tris borate-EDTA buffer. Bands were revealed and quantitated with the PhosphorImager 445 SI apparatus (Molecular Dynamics, Sunnyvale, CA), with the use of the ImageQuant version 1.0 program (Molecular Dynamics).

RESULTS

HAP and Sam68 Colocalize in Nuclear Bodies Both before and after Heat Shock

We have previously shown that in exponentially growing HeLa cells HAP displays a punctated distribution throughout the nucleoplasm with exclusion of nucleoli (Weighardt *et al*., 1999). Moreover, brighter dots, preferentially positioned in proximity to the nucleoli, are clearly detectable in most cells (Chiodi *et al*., 2000). This pattern closely resembles the distribution of Sam68, a protein recently proven to interact with HAP (Stoss *et al*., 2001).

To investigate the relationship between the distribution of these two proteins, HeLa cells, costained with the 7-1 mAb directed to Sam68 and with the anti-HAP polyclonal antibody (Weighardt *et al*., 1999) were analyzed by confocal laser microscopy. As exemplified in Figure 1, top, in untreated cells both proteins showed a punctated distribution in the nucleoplasm, with some sites of preferential accumulation in the perinucleolar regions. Merged images proved that HAP colocalized with Sam68 in SNBs, with poor overlapping in other nuclear compartments.

We have described that after stress treatments, such as heat shock, HAP is recruited to a small number of large nuclear bodies, a subset of which lies in the perinucleolar region as SNBs (Weighardt *et al*., 1999; Chiodi *et al*., 2000). We asked whether heat shock could cause redistribution of Sam68 as well. HeLa cells were, therefore, heat shocked 1 h at 42°C and after 1 h of recovery at 37°C were costained with anti-Sam68 and anti-HAP antibodies. As shown in Figure 1, bottom, Sam68 was massively recruited to the HAP bodies. A time course experiment demonstrated that the two proteins were recruited with identical kinetics (Denegri, unpublished results).

Thus, this analysis adds Sam68 to the list of proteins present in HAP bodies, which already includes heat shock factor 1 (Weighardt *et al*., 1999) and hnRNP M (Chiodi *et al*., 2000). The similar subnuclear distribution of HAP bodies and SNBs suggests a tight relationship between these nuclear compartments. This possibility is supported also by the fact that both correspond to sites of accumulation of RNA molecules synthesized in other nuclear regions (Chen *et al*., 1999; Chiodi *et al*., 2000). On the basis of these similarities, we propose to rename HAP bodies as stress-induced SNBs.

RE Region Governs Subnuclear Distribution of HAP

As a first step to understand the mechanisms underlying the formation of stress-induced SNBs, we searched for the protein motif that directs the recruitment of HAP.

HAP is a large protein of 917 amino acids composed of a few recognizable domains (Figure 2): 1) the first part of the protein (residues 1–319) is characterized by a high content in acidic residues. A putative DNA binding motif, called SAF-A/B Acinus and PIAS domain (residues 31–65), is located within this region. The SAF-A/B Acinus and PIAS domain is shared with other nuclear proteins and is thought to be

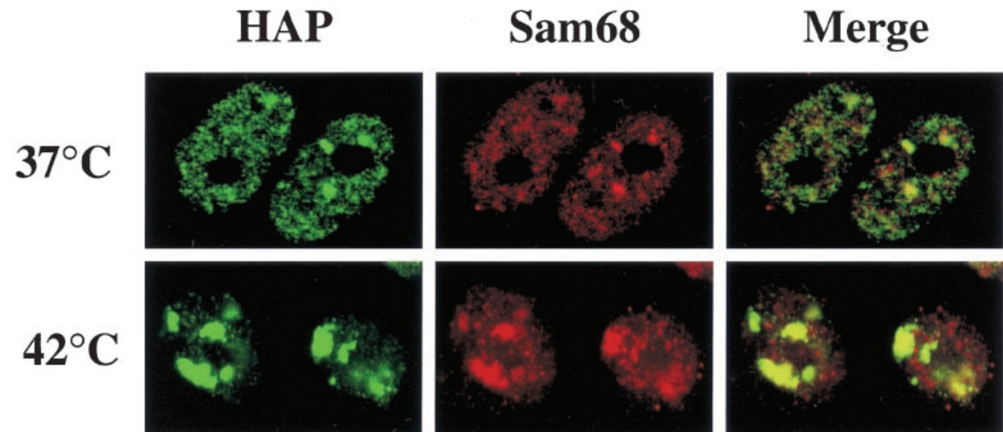


Figure 1. HAP and Sam68 colocalize in nuclear bodies both in unstressed and in heat-shocked cells. HeLa cells, either unstressed (37°C) or heat-shocked 1 h at 42°C and allowed to recover 1 h at 37°C (42°C), were costained with the anti-HAP polyclonal antibody and with the 7-1 mAb to Sam68. The distribution of HAP was revealed with an FITC-conjugated goat antirabbit antibody, whereas Sam68 was revealed with a rhodamine-conjugated goat antimouse antibody. Antigen–antibody complexes were visualized by confocal laser scanning microscopy. Colocalization of the two antigens is revealed by yellow in the merged images.

involved in chromosomal organization (Aravind and Koonin, 2000). 2) A canonical RNA binding domain occupies the central part of the protein (residues 398–482) and is followed by 3) an extended region (residues 483–621) rich in serine-lysine (SK) dipeptides and hydrophilic residues. 4) A region rich in arginine-glutammic acid (RE) dipeptides spans from position 622 to 788. Sequence analysis shows that a portion of this region (residues 638–699) has a very high probability (0.96) to exist in a coiled-coil conformation. 5) Finally, the C-terminal part of the protein (residues 789–917) is rich in glycine (24%) and arginine (16.3%) and shows some similarities with the RGG motif described in hnRNP U and A1 (Kiledjian and Dreyfuss, 1992; Weighardt *et al.*, 1996).

We initially studied the subnuclear distribution of four GFP fusions that together covered the entire HAP protein, namely, GFP-[1–407], GFP-[306–509], GFP-[474–788], and GFP-[773–917]. A nuclear localization signal (NLS) was cloned at the C terminus of the fusion proteins to ensure their nuclear accumulation. Of the four fusions, only GFP-[474–788] displayed at 37°C a punctated distribution in the nucleoplasm, with exclusion of nucleoli, similar to the endogenous protein (Figure 2). The same fusion was the only one that after heat shock was recruited to nuclear bodies indistinguishable in size, number, and distribution from stress-induced SNBs. Immunostaining of the transfected cells with the anti-HAP antibodies proved that GFP-[474–788] was indeed present in stress-induced SNBs (Figure 2).

To narrow down the region that mediated the redistribution of HAP after heat shock, we generated two further constructs, GFP-[474–652] and GFP-[580–788], containing, respectively, the SK and RE regions. The results in Figure 2 demonstrated that only GFP-[580–788] was recruited to stress-induced SNBs. Notably, the distribution of GFP-[580–788] in unstressed cells was indistinguishable from that of the endogenous protein (Figure 2), suggesting a major role of the RE region in determining the subnuclear localization of HAP. Concerning GFP-[474–652], this fusion, similarly to the GFP-NLS reporter protein, was mostly confined to the nucleoli of unstressed cells (Figure 2). After heat shock GFP-[474–652] was present also in a small number of nuclear sites distinct from stress-induced SNBs (Figure 2) and recognized by the anti-SC35 mAb (Denegri, unpublished results).

To understand whether the 580–788 region was not only sufficient but also necessary for the recruitment, we studied the subnuclear distribution of the deletion mutant HAP-Δ[580–788] in heat-shocked HeLa cells. The wild-type and the mutated proteins, both N-terminally tagged with the HA epitope, were expressed in transiently transfected HeLa cells together with GFP-[580–788] that, on the basis of the analysis in Figure 2, was chosen as a marker of stress-induced SNBs. Immunofluorescence analysis showed that, as expected, the wild-type protein colocalized with GFP-[580–788] in stress-induced SNBs. In contrast, HAP-Δ[580–788] remained homogeneously distributed throughout the nucleoplasm (Figure 3). Together these results demonstrate that the 580–788 region is both necessary and sufficient for the recruitment of HAP to stress-induced bodies.

Different Portions of 580–788 Region Are Able to Direct Recruitment to Stress-induced SNBs

To map more precisely the targeting signal, we generated N-terminal and C-terminal–deleted versions of the 580–788 region, as depicted in the scheme of Figure 4. These protein fragments were expressed in transiently transfected HeLa cells as GFP fusions and their distribution was determined by confocal laser microscopy and compared with that of the endogenous HAP, stained by the anti-HAP polyclonal antibody. As detailed below, the analysis of all these mutants revealed a complex pattern of subnuclear distributions, which most likely reflected the ability of the 580–788 region to interact with proteins preferentially located in different nuclear districts such as nucleoli, speckles and stress-induced SNBs.

The four C-terminal mutants clearly pointed to a role of the coiled-coil domain (residues 638–699) in the subnuclear distribution of HAP (Figure 4). All the constructs containing the entire coiled-coil were, in fact, efficiently recruited to stress-induced SNBs. In contrast GFP-[580–659], which comprised only the first 20 residue of this domain, was not detectable in these bodies and was mostly found in the nucleoli of both unstressed and heat-shocked cells. Notably, the three fusions targeted to stress-induced SNBs were also consistently present in nuclear speckles stained by the anti-SC35 mAb (Denegri, unpublished results), suggesting an

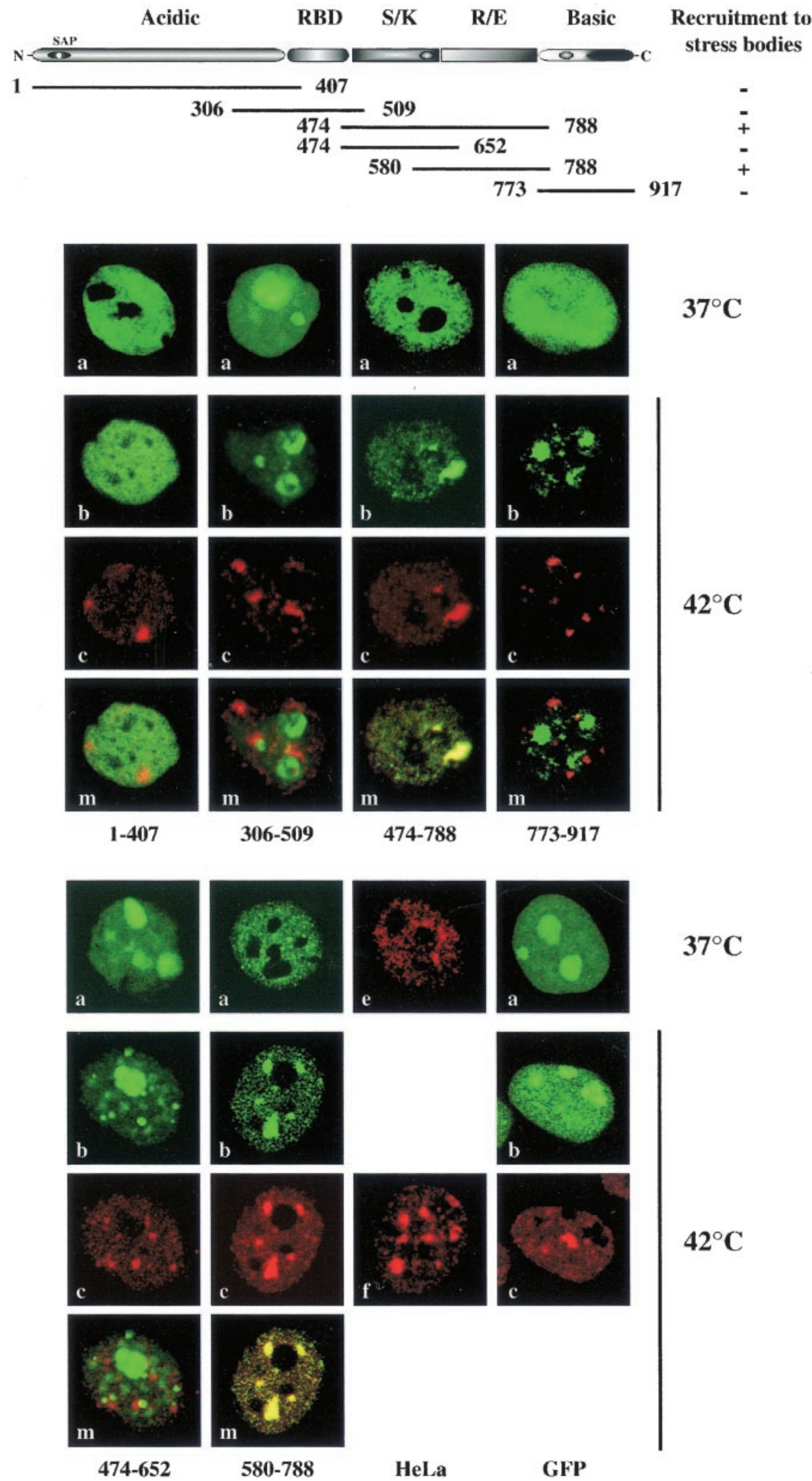


Figure 2. Identification of the region of HAP able to direct the GFP reporter protein to stress-induced SNBs. A schematic representation of the HAP structure, as detailed in the text, is shown in the top part of the figure. Regions expressed in transfected HeLa cells as GFP fusions are also indicated. Numbers refer to the first and to the last residue of each region. The ability of the different GFP fusions to associate with stress-induced SNBs was scored as + (targeting proficient) or − (targeting deficient) on the basis of the results shown in the figure. The distribution of the different GFP fusions in either unstressed (a) or heat-shocked (b) HeLa cells was determined by visualizing the autofluorescent signal of the GFP protein in confocal laser microscopy. Heat-shocked cells were also stained with the anti-HAP polyclonal antibody and the distribution of the antigen–antibody complex was revealed by indirect immunofluorescence with a rhodamine-conjugated sheep antirabbit antibody (c). Images of the same cells were merged (m) to reveal colocalization of the endogenous and of the transfected protein, resulting in yellow. The distribution of the GFP reporter protein, fused to the NLS of the simian virus 40 large T-Ag, in unstressed and heat-shocked cells is also shown. (e and f) Distribution of the endogenous HAP protein in nontransfected unstressed and heat-shocked cells, respectively.

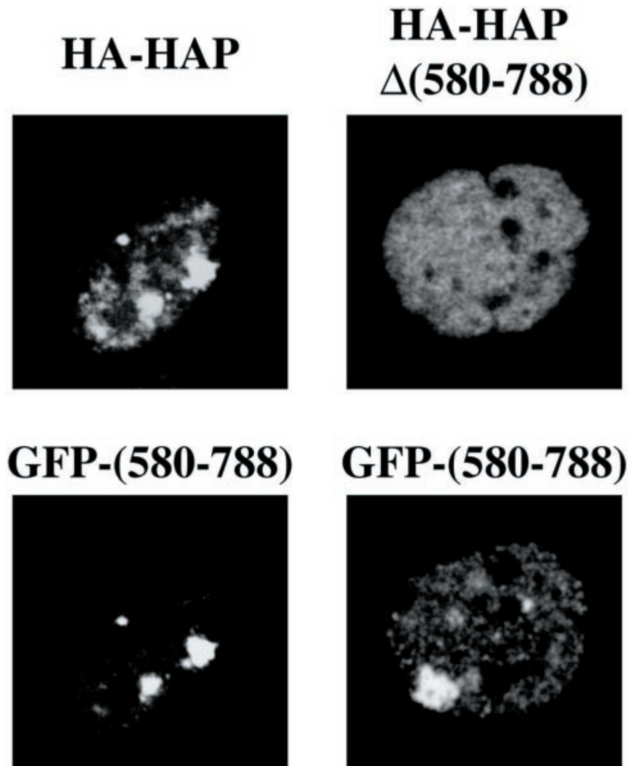


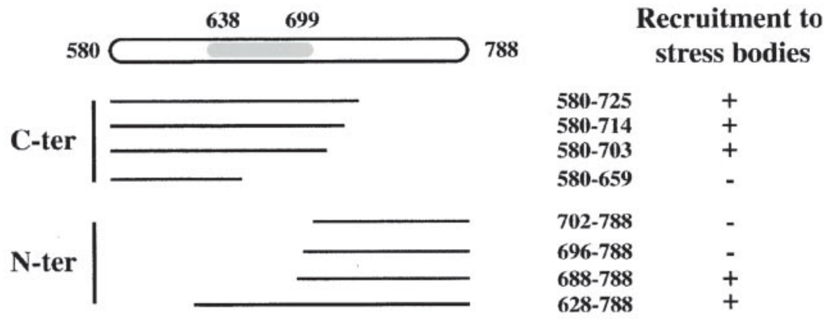
Figure 3. The 580–788 region is necessary for the stress-induced redistribution of HAP. HeLa cells were transfected with plasmids that direct the expression either of the wild-type HAP or of a deletion mutant lacking the 580–788 region (HAP-Δ[580–788]), both fused to the HA epitope. Cells were cotransfected with a plasmid that directs the expression of GFP-[580–788], a marker of stress-induced SNBs. Transfected cells were heat shocked 1 h at 42°C and then allowed to recover 1 h at 37°C. Cells were then fixed with formaldehyde and stained with the 12CA5 mAb against the HA-epitope. The distribution of the GFP fusion was determined by visualizing the autofluorescent signal of GFP, whereas the HA-tagged protein was revealed with a rhodamine-conjugated goat antimouse IgG antibody. Confocal laser images of the same cells are shown.

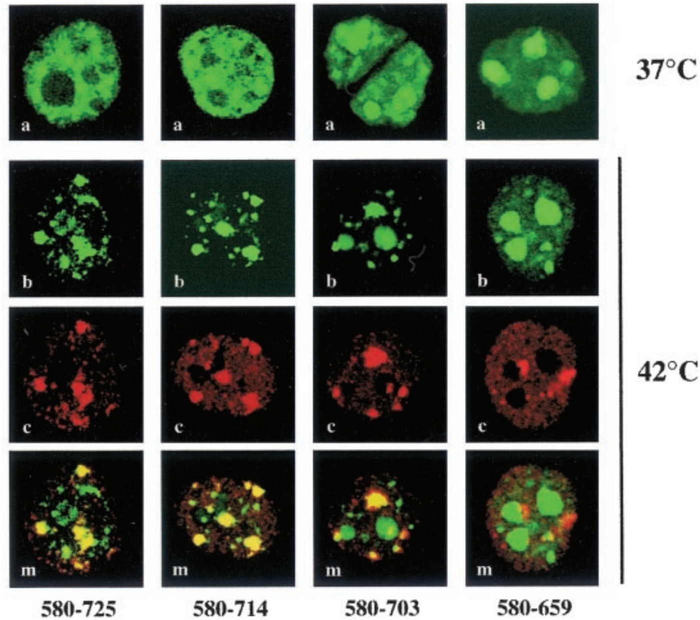
interaction with proteins, such as SR factors, normally present in these compartments. The importance of the coiled-coil region in directing the subnuclear distribution of HAP was confirmed by the fact that N-terminal mutants lacking this domain failed to produce the recruitment of the GFP reporter protein (Figure 4). However, a good recruitment was observed with GFP-[688–788] that contained only the last 11 residues of the coiled-coil. In conclusion, the results in Figure 4 identify two protein regions, i.e., 580–703 and 688–788, able to target GFP to stress-induced SNBs. The fact that the last 11 residues of the coiled-coil domain are present in both regions suggests a role of this sequence in the redistribution of HAP triggered by heat shock.

To investigate more in detail this aspect, we studied the distribution of three additional constructs bearing internal deletions in the 580–788 region: 1) GFP-[580–788 Δ638–699], which lacked the entire coiled-coil; 2) GFP-[580–788 Δ699–725], in which a short sequence immediately downstream of the coiled-coil was deleted; and 3) GFP-[580–788 Δ638–725], in which both sequences were removed (Figure 5). Confocal laser microscopy analysis of unstressed cells showed that the two fusions without the coiled-coil (constructs 1 and 3) were mostly found in the nucleoli. The remaining GFP-fusion displayed a punctated distribution in the nucleoplasm similar to the endogenous protein (Figure 5). The analysis of the same three constructs in heat-shocked cells, unexpectedly, proved that neither the coiled-coil nor the immediately downstream sequence was, individually, necessary for the association with stress-induced SNBs (Figure 5), arguing against the possibility that this event could be mediated by a single short motif, such as the last 11 amino acids of the coiled-coil. On the contrary, these results and those in Figure 4 suggested that different portions of the 580–788 region could cooperate to direct the redistribution of HAP after stress treatments. In support of this hypothesis we found that the simultaneous deletion of the entire coiled-coil and of the immediately downstream sequence in GFP-[580–788 Δ 638–725] completely abolished the recruitment (Figure 5). Notably, a fraction of GFP-[580–788 Δ638–725] colocalized with SC35 in speckles (Denegri, unpublished results), suggesting an interaction with proteins normally present in these nuclear compartments. The GFP-[638–725] construct proved that the 638–725 region was not only necessary but also sufficient to direct the association of the GFP reporter protein to stress-induced SNBs (Figure 5). The fact that short deletions from either end, as in GFP-[638–703] and GFP-[653–725], completely prevented the recruitment (Figure 5) identified the 638–725 region as a targeting signal.

Splicing Factors Interacting with HAP Are Recruited to Stress-induced SNBs

We used a region spanning from position 580–725 as bait to screen a human cDNA library by the "two-hybrid" approach in yeast (see MATERIALS AND METHODS). From the screening of 2×10^7 transformants, we obtained 31 clones most of which (24 clones) corresponded to the complete open reading frame (ORF) of the splicing factor SRp30c. In addition, we selected two clones containing the entire ORF for 9G8, another member of the family of SR splicing factors, and a few clones for as yet uncharacterized proteins. The interaction, both in vitro and in vivo, between HAP and SR splicing factors, particularly SRp30c, is not a novelty (Nayler *et al.*, 1998). However, it is intriguing that these splicing factors can bind to the region necessary for the recruitment of HAP to stress-induced SNBs as if the interaction with SRp30c and association of HAP with the bodies after heat shock were intrinsically connected. To test this hypothesis, we investigated whether SRp30c and 9G8 were recruited to stress-induced SNBs in response to heat shock. Both splicing factors were expressed as GFP fusions in transiently transfected HeLa cells and their association with stress-induced SNBs was verified by confocal laser microscopy on cells stained with the anti-HAP antibody. As exemplified in Figure 6, SRp30c was recruited to the bodies as efficiently as HAP, suggesting that the two factors not only physically interacted with each other (Nayler *et al.*, 1998) but also participated to a common metabolic pathway that entailed their redistribution after stress treatments. In regard to 9G8, the recruitment to stress-induced SNBs, albeit clearly detectable, occurred only in a fraction (~30%) of the trans-





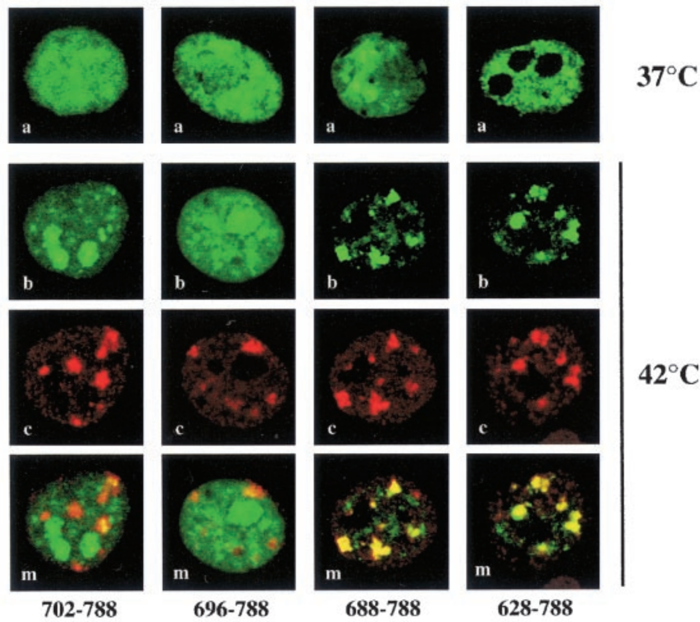
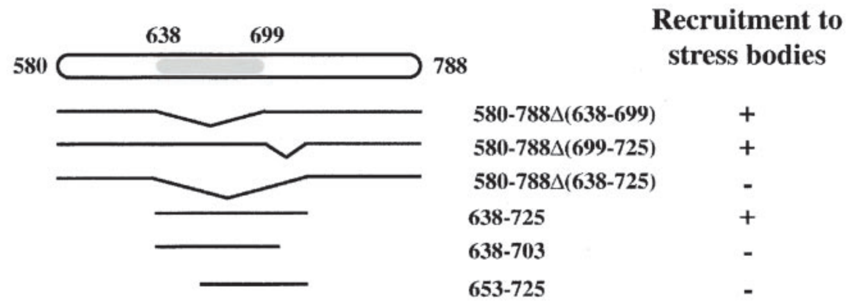


Figure 4. Mapping the targeting signal within the 580–788 region by means of N-terminal or C-terminal deletion mutants. A schematic representation of the 580–788 region is shown in the top part of the figure. The gray oval indicates the coiled-coil domain. Regions expressed in transfected HeLa cells as GFP fusions are also indicated. Numbers refer to the first and to the last residue of each considered region. The ability of the different GFP fusions to associate with stress-induced SNBs was scored as + (targeting proficient) or − (targeting deficient) on the basis of the results shown in the bottom part of the figure. The distribution of the different GFP fusions in transiently transfected HeLa cells, either unstressed (a) or heat-shocked (b), was determined by visualizing the autofluorescent signal of GFP by confocal laser microscopy. Heat-shocked cells were also stained with the anti-HAP polyclonal antibody and the protein localization was revealed by indirect immunofluorescence with a rhodamine-conjugated goat antirabbit antibody (c). Images of the same cells were merged (m) to reveal colocalization of the endogenous and of the transfected protein, resulting in yellow.



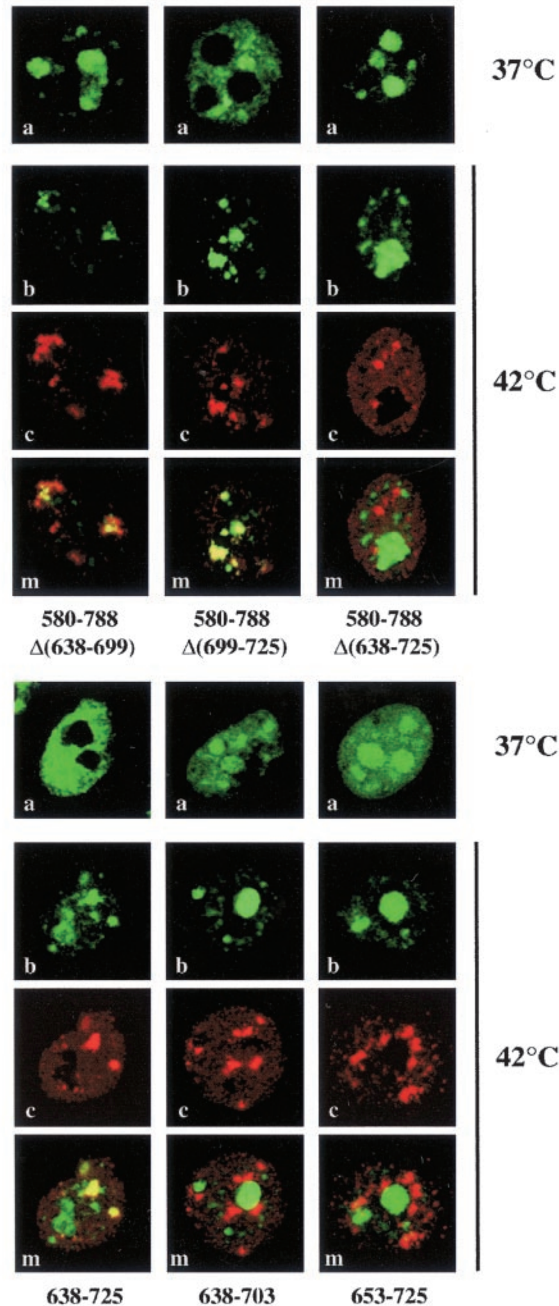


Figure 5. Identification of the minimal sequence able to direct the recruitment to stress-induced SNBs. A schematic representation of the 580–788 region is shown in the top part of the figure. The gray oval indicates the coiled-coil domain. Regions expressed in transfected HeLa cells as GFP fusions are also indicated. Numbers refer to the first and to the last residue of each considered region. The ability of the different GFP fusions to associate with stress-induced SNBs was scored as + (targeting proficient) or − (targeting deficient) on the basis of the results shown in the bottom part of the figure. The distribution of the different GFP fusions in transiently transfected HeLa cells, either unstressed (a) or heat-shocked (b), was determined by visualizing the autofluorescent signal of GFP in confocal laser microscopy. Heat-shocked cells were also stained with the anti-HAP polyclonal antibody and the protein localization was revealed by indirect immunofluorescence with a rhodamine-conjugated goat antirabbit antibody (c). Images of the same cells were merged (m) to reveal colocalization of the endogenous and of the transfected protein, resulting in yellow.

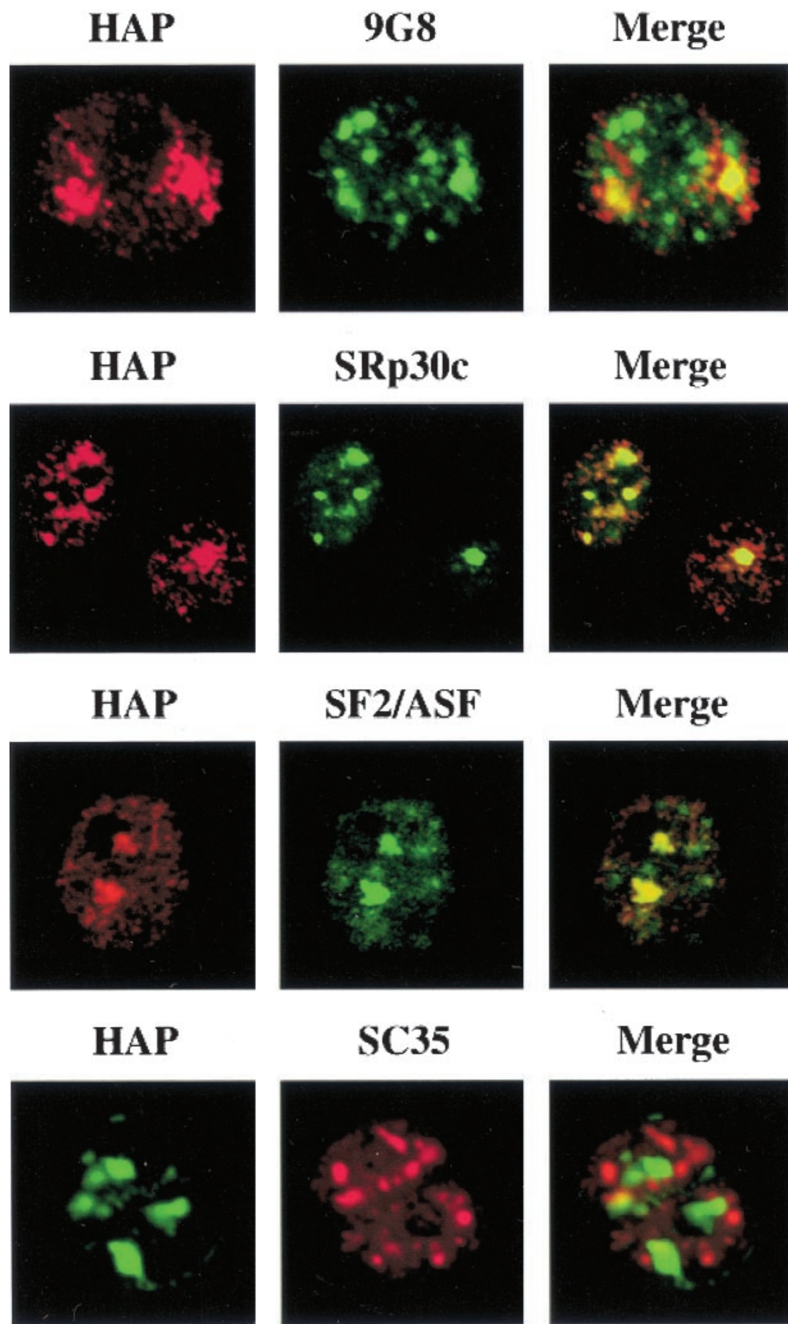


Figure 6. Splicing factors SRp30c, 9G8, and SF2/ASF are recruited to stress-induced SNBs. HeLa cells were transfected with a fusion between GFP and the entire ORF of SRp30c, 9G8, or SF2/ASF. The distribution of the different GFP fusions in heat-shocked HeLa cells was determined by visualizing the autofluorescent signal of GFP in confocal laser microscopy. The same cells were stained with the anti-HAP polyclonal antibody and the protein distribution was revealed with a rhodamine-conjugated goat antirabbit antibody. As a control, nontransfected cells were heat-shocked and costained with the anti-SC35 mAb and the anti-HAP polyclonal antibody. Protein distribution was revealed with an FITC-conjugated goat antirabbit antibody and a rhodamine-conjugated goat antimouse antibody. Colocalization resulted in the appearance of yellow in the merged images.

fected cells. Moreover, even in cells displaying GFP-9G8 in stress-induced SNBs, the association with the bodies was incomplete and a significant fraction of the protein persisted in the typical speckled pattern (Figure 6). We asked whether SF2/ASF, another SR protein shown to interact with HAP (Nayler *et al.*, 1998) and highly similar to SRp30c, was also directed to stress-induced SNBs in response to heat shock. SF2/ASF was, therefore, expressed in HeLa cells as a GFP fusion and its distribution in heat-shocked cells was determined by confocal laser microscopy. As shown in Figure 6, SF2/ASF, similarly to SRp30c, moved from speckles to stress bodies. However, the recruitment to stress-induced SNBs does not seem to be a general feature of splicing factors of the SR family. Indeed, costaining with anti-SC35 and anti-HAP antibodies showed that in heat-shocked cells, in agreement with previous data (Jolly *et al.*, 1999b; Chiodi *et al.*, 2000), SC35 did not colocalize with HAP (Figure 6) and with SF2/ASF and SRp30c. Together, these results show that a subset of SR proteins is recruited to stress-induced SNBs and suggest the intriguing possibility that these nuclear compartments may play a role in the fate of transcripts in heat-shocked cells.

Stress Treatments Affect Alternative Splicing Program of E1A Transcripts

The results in the previous section indicate that the subnuclear distribution of SR factors is differentially affected by heat shock. Indeed, although SF2/ASF and SRp30c are massively recruited to stress-induced SNBs, only a fraction of 9G8 and no SC35 is present in these structures. Thus, heat shock appears to drastically perturb the relative abundance of SR factors in the nucleoplasm simply by altering their subnuclear distribution. We expect that the alternative splicing program of specific genes is a likely target of this redistribution. We selected the adenovirus E1A gene as a model to verify this hypothesis. Alternative splicing of the E1A pre-mRNA generates three major isoforms (13S, 12S, and 9S) and two minor isoforms (11S and 10S) (Figure 7A) (Caceres *et al.*, 1994). Several proteins, among which are hnRNP A1, SF2/ASF, 9G8, and SC35, have been shown to modulate the production of these isoforms by altering 5′ splice-site selection (Gattoni *et al.*, 1991; Caceres *et al.*, 1994). We have shown that the recruitment of HAP, and other RNA processing factors, to stress-induced SNBs peaks at 3 h of recovery from heat shock, and an additional 3 h is required before the distribution of the protein becomes again comparable with that observed in unstressed cells (Weighardt *et al.*, 1999). We tested whether altered levels of the major E1A transcript isoforms accompanied the formation and the dispersal of stress-induced SNBs. After transfection with the E1A minigene, HeLa cells were heat shocked 1 h at 42°C and then allowed to recover for increasing time intervals (0–6 h) at 37°C. Total RNAs were extracted from untreated and from stressed cells and analyzed by RT-PCR to detect the different E1A transcript isoforms (Figure 7B). Quantitation of the amplification bands (see MATERIALS AND METHODS; Figure 7C) showed that heat shock induced an increase in the relative abundance of 13S molecules and a concomitant decrease of the 12S and 9S transcripts. This effect was even more evident after 1 and 2 h of recovery at 37°C (Figure 7, B and C). At longer times (6 h of recovery) the splicing pattern of E1A transcripts was similar to that observed in unstressed cells. Thus, these results are in agreement with the hypothesis that the formation of stress-induced SNBs is accompanied by a transient alteration of the splicing program of the E1A reporter gene. To confirm this conclusion we studied alternative splicing of E1A transcripts in cells treated with cadmium sulfate, another inducer of stress bodies. We have previously shown (Chiodi *et al.*, 2000) that stress-induced SNBs are detectable in ~30% of HeLa cells grown for 3 h the presence of 30 μM cadmium sulfate, and in almost all the cells 2 h later. Therefore, we determine the relative level of the 13S, 12S, and 9S isoforms in cells grown for 3 and 5 h in the presence of cadmium sulfate. As shown in Figure 7, B and C, cadmium produced a change in the splicing pattern of the E1a transcripts comparable with that triggered by heat shock.

The fact that two different treatments triggering the formation of stress bodies have similar effects on the alternative splicing of the E1A gene strongly suggests a link between the recruitment of RNA processing factors and alteration of splicing programs.

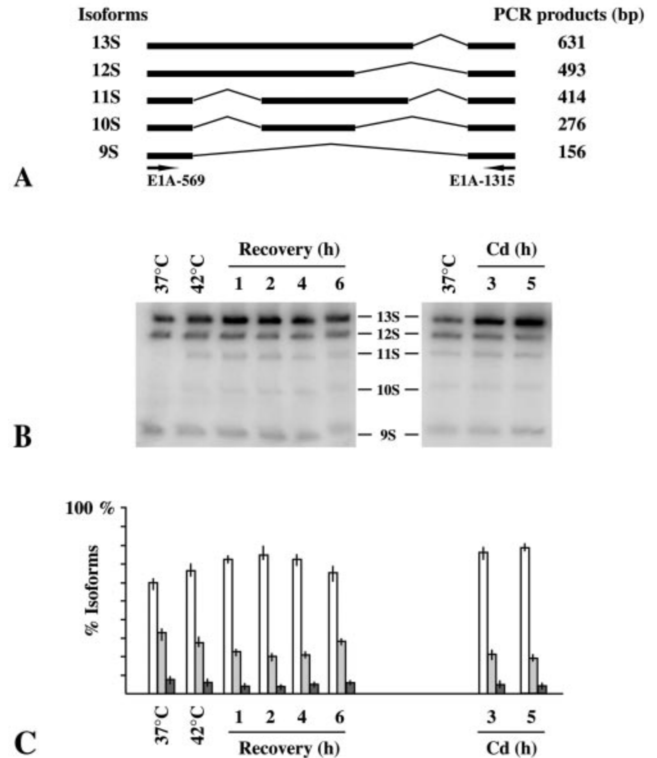


Figure 7. Heat shock and cadmium sulfate change the alternative splicing pattern of the adenovirus E1A minigene. (A) Schematic representation of the alternative spliced isoforms of E1A pre-mRNA. The major isoforms 9S, 12S, and 13S are generated by alternative selection of the 5′ splice sites. The minor isoforms 10S and 11S involve the usage of additional internal 3′ splice sites. Sizes, in base pairs, of the corresponding RT-PCR products obtained with the use of the E1A-569 and E1A-1315 primers are shown. (B) In vivo alternative splicing of E1A pre-mRNA is affected by heat shock and by cadmium sulfate. The pCMVE1A plasmid containing the E1A minigene was transfected in HeLa cells. Twenty-four hours after transfection total RNAs were extracted from unstressed cells (37°C) or from cells heat-shocked for 1 h at 42°C. RNAs were also prepared from cells allowed to recover 1, 2, 4, or 6 h at 37°C after heat shock. RNAs were also extracted from cells treated for 3 or 5 h with 30 μM of cadmium sulfate, another induced of HAP bodies. RNA were analyzed by RT-PCR with E1A-569 and E1A-1315 primers. RT-PCR products were resolved on 5% polyacrylamide gel and detected by autoradiography. (C) ImageQuant PhosphorImager quantification of the major E1A isoforms: white bars, light gray bars, and dark gray bars, represent 13S, 12S, and 9S molecules, respectively. Percentages of the three isoforms are the average of at least three independent experiments. Error bars are shown.

DISCUSSION

In this article, we have investigated in further detail the nature of the nuclear bodies to which HAP is recruited in response to stress treatments. We have reported the existence of a close relationship between stress-induced HAP bodies and SNBs detectable in a subset of transformed cell lines (Chen *et al.*, 1999). Moreover, we have shown that, contrary to what was previously thought (Jolly *et al.*, 1999b; Chiodi *et al.*, 2000), HAP bodies are also sites of accumula-

tion of a subset of splicing factors, suggesting a role of these structures in RNA metabolism.

Relationship between SNBs and HAP Bodies

In unstressed cells, HAP and Sam68 display a punctated distribution in the whole nucleus with exclusion of nucleoli. In spite of this widespread nuclear distribution, however, colocalization of these two factors mainly occurs in a small number of relatively large bodies (Figure 1), often associated to the nucleoli, which are known as SNBs. Intriguingly, after heat shock, Sam68 and HAP are both massively recruited to stress-induced nuclear structures that we previously termed HAP bodies. We have found that the association of these two factors with stress bodies is temporally coincident, suggesting the existence of a common underlying mechanism. Several observations support the idea of a close relationship between SNBs and HAP bodies. First, these structures occupy similar positions in the nucleus, being frequently associated to the nucleoli. Second, both SNBs and HAP bodies contain RNA molecules. We have reported that transcripts synthesized either before or after but not during heat shock (Chiodi *et al.*, 2000) are present in HAP bodies, which, therefore, most likely function as depots for RNA molecules whose synthesis is not triggered by stress treatments. Consistently with their depot nature, HAP bodies do not contain RNA polymerase II and do not represent sites of transcription (Jolly *et al.*, 1997, 1999b; Chiodi *et al.*, 2000). Indeed, they originate from the continuous recruitment of highly packed forms of ribonucleoprotein complexes, namely, the perichromatin granules (Chiodi *et al.*, 2000) from the surrounding nucleoplasm. Similarly, SNBs are not sites of transcription and contain RNA molecules most likely synthesized in other nuclear districts (Chen *et al.*, 1999). In accord with the idea that RNA is an important component of SNBs and HAP bodies, the formation and stability of both structures require RNA synthesis and are strongly affected by inhibitors such as actinomycin D and 5,6-dichlorobenzimidazole riboside (DRB) (Chen *et al.*, 1999; Weighardt *et al.*, 1999). On the basis of these considerations, we propose that HAP bodies originate from preexisting SNBs and, therefore, we tentatively rename them as stress-induced SNBs.

It has been suggested (Chen *et al.*, 1999) that transcripts bound by Sam68 would pass through SNBs along their path toward the nuclear envelope. This possibility is consistent with the fact that the nucleoplasmic pool of Sam68 exists in a dynamic equilibrium with the protein present in SNBs (Chen *et al.*, 1999). We speculate that heat shock could alter this equilibrium and, by slowing down or even blocking the exit of ribonucleoprotein complexes from SNBs, it would induce the appearance of the stress-induced SNBs. Thus, the formation of these large bodies would result from a stress-sensitive step in the movement of ribonucleoprotein complexes in the cell nucleus.

Specific Splicing Factors Accumulate in Stress-induced SNBs: Functional Implications

As a first step to investigate the composition of stress-induced SNBs, we sought to identify the protein region that mediates the recruitment of HAP to these nuclear structures. We have found that a portion of HAP spanning from residue 580 to 788 is both sufficient to direct the GFP reporter protein to the bodies and necessary for the correct redistribution of the protein upon stress. This region is characterized by the presence of an extended sequence (residues 638–699) rich in arginine-glutamic acid (RE) dipeptides almost regularly interspersed with hydrophobic amino acids, usually leucine or methionine, which has a high p (0.96 on average) to exist in a coiled-coil conformation. Our analysis indicates that different portions of the 580–788 region contribute to the recruitment to stress-induced SNBs. Indeed, two partially overlapping sequences, spanning, respectively, from residue 638 to 725 and from residue 688 to788, are equally able to target the GFP reporter protein to the bodies (Figure 4). It is conceivable that these regions act by mediating the interaction of HAP with other components of stress-induced SNBs assembled in higher order multiprotein complexes. In accord with this possibility, we have previously shown, through a detailed electron microscopy analysis, that, after heat shock, HAP associates to highly packed forms of ribonucleoprotein complexes, i.e., the perichromatin granules, that compose the bodies (Chiodi *et al.*, 2000). It is known that numerous proteins interact with HAP both in vitro and in vivo. Among them the C-terminal domain of RNA polymerase II, CLK2, hnRNP A1, the YT521 protein, Sam68, and the splicing factors SRp30c, htra2-beta1, and SF2/ASF (Nayler *et al.*, 1998; Chiodi *et al.*, 2000). A weaker binding was detected also with U2AF35 and SRp55, whereas no interaction was observed with the other members of the SR protein family, including SRp40 and SRp75 (Nayler *et al.*, 1998). We have found that the 580–725 sequence, which comprises one of the targeting signals identified in this article, mediates the interaction, in the two-hybrid assay, with SRp30c and 9G8, two members of the SR protein family. The interaction with SRp30c appears to be tightly connected to the recruitment of HAP. Indeed, in heat-shocked cells SRp30c is sequestered in stress-induced SNBs, suggesting the possibility that this factor acts as the recruiter that directs HAP to the bodies. It is possible that the association of HAP with the ribonucleoprotein complexes present in stress-induced SNBs is not mediated by RNA, but rather by the interaction with some protein factors among which is SRp30c. This hypothesis is supported by the observation that the HAP-Δ[580–788] mutant, which contains an intact RNA binding domain, fails to be recruited to stress-induced SNBs.

A major result of our analysis is the demonstration that stress-induced bodies are sites of accumulation of SR splicing factors, a conclusion missed by previous studies (Jolly *et al.*, 1999b; Chiodi *et al.*, 2000). Intriguingly, heat shock affects to a different extent the distribution of different SR splicing factors: whereas SRp30c and SF2/ASF efficiently accumulate in stress-induced bodies, 9G8 is detectable both in stress-induced SNBs and in speckles and, finally, the distribution of SC35 is not affected by heat shock. This finding allows a classification of SR factors not only on the basis their the activity in splicing but also on their subnuclear distribution and on the participation to higher order protein–RNA assemblies occurring in different growth conditions. In this regard it is worth noticing that SC35 was recently reported to associate to genes transcribed under stress conditions regardless of the presence of introns (Jolly *et al.*, 1999b),

increasing the possibility that this splicing factor could have other functions at the sites of transcription in addition to intron excision.

Finally, the results reported in this article support of the idea that stress treatments can affect posttranscriptional control of gene expression by controlling the subcellular distribution of specific RNA processing factors. Another stress treatment, i.e., osmotic shock, has been recently shown to trigger the cytoplasmic accumulation of hnRNP A1, without any effect on the distribution of SF2/ASF, through the activation of the p38 mitogen-activated protein kinase pathway (van der Houven van Oordt *et al.*, 2000). In this manner, osmotic stress perturbs the alternative splicing of the adenoviral E1A gene. Our results identify a novel mechanism through which stress treatments could affect splicing. Contrary to osmotic stress, in fact, heat shock does not alter the distribution of hnRNP A1 (Weighardt *et al.*, 1999) but induces the recruitment of SF2/ASF and other splicing factors to stress-induced SNBs. Although the signal transduction pathway involved is as yet unknown, the fact that stress-induced SNB formation is insensitive to treatment with the p38 MAP kinase inhibitor SB203580 suggests that this kinase is not involved (our unpublished results). Interestingly, and similarly to what was observed in cells subjected to osmotic stress, the alternative splicing pattern of the E1A reporter minigene is significantly affected in heat-shocked cells.

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