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Satellite III non-coding RNAs show distinct and stress-specific patterns of induction

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ABSTRACT

The heat shock response in human cells is associated with the transcription of satellite III repeats (SatIII) located in the 9q12 locus. Upon induction, the SatIII transcripts remain associated with the locus and recruit several transcription and splicing factors to form the nuclear stress bodies (nSBs). The nSBs are thought to modulate epigenetic changes during the heat shock response. We demonstrate here that the nSBs are induced by a variety of stressors and show stress-specific patterns of induction. While the transcription factor HSF1 is required for the induction of SatIII locus by the stressors tested, its specific role in the transcriptional process appears to be stress dependent. Our results suggest the existence of multiple transcriptional loci for the SatIII transcripts and that their activation might depend upon the type of stressors. Thus, induction of SatIII transcripts appears to be a generic response to a variety of stress conditions.

Introduction

The heat shock response is a conserved cellular response to elevated temperatures and results in the immediate induction of a group of proteins known as heat shock proteins (HSPs) [1,2]. HSPs function primarily as molecular chaperones, and thus protect cells from stress-induced damage [1,2]. The activation of HSP genes is mediated by heat shock transcription factor 1 (HSF1), which binds to the heat shock elements present in the target promoters [3]. In human cells, under the condition of heat shock, the HSF1 associates with the satellite III locus at 9q12 and activates the transcription of satellite III repeats into stable RNAs (SatIII) that eventually form the nuclear stress bodies (nSBs) [4]. The SatIII associated nSBs are known to recruit several splicing factors, serine/arginine-rich proteins (9G8, SF2/ASF, and SRp30c) and hnRNPs, besides transcription factors such as HSF1, CREB binding protein, and RNA polymerase II [5–9]. Based on these observations, the nSBs are thought to function in two distinct pathways; in chromatin remodeling and in mRNA splicing [10,11].

The SatIII transcripts are transcribed by RNA polymerase II, are polyadenylated and variable in size but all of them are known to contain numerous repeats of GGAAT motif, typical of the satellite III repeat [9,12,13]. It is curious to note that other than heat shock, treatment of cells with cadmium sulfate, azetidine, or hyperosmotic stress can also induce the SatIII locus [11,14], suggesting that the formation of nSBs is a generic response to stress in human cells. In the present study we tested this notion by exposing a human cell line to a variety of stressors and characterized the nSBs. Our results reveal stress-specific patterns for the induction of nSGs, suggesting the existence of multiple transcriptional loci for the SatIII transcripts.

Materials and methods

Antibodies and knockdown construct. The following antibodies were used in the present study; anti-ASF/SF2 (Zymed, Invitrogen), anti-HSF1 (Cell Signaling Technology, USA), and anti-γ-tubulin (Sigma–Aldrich India Pvt. Ltd.). Secondary antibodies were obtained from Jackson Immuno Research Inc. (USA). An RNAi construct (shRNAmir) for the knockdown of HSF1 was purchased from Open Biosystem, USA.

Cell culture and transfection. HeLa cells were grown in Dulbecco's modified Eagle's medium as reported [15]. For the transfection of the shRNAmir construct, the ESCORT V transfection reagent (Sigma–Aldrich India Pvt. Ltd.) was used according to manufacturer's protocol.

Treatment of cells with various stressors. For the heat shock experiments, cells were grown in monolayer up to about 80% confluence and were given a heat shock or treated with various chemicals as described in "Supplementary material". Control experiments using the resuspension solvents were performed where appropriate.

Fluorescence in situ hybridization (FISH) of SatIII transcripts. For the detection of SatIII transcripts, 5′-biotinylated probe for the

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satellite III transcripts was used as described in "Supplementary material".

Immunocytochemistry. HeLa cells were fixed and processed for immunofluorescence microscopy essentially as described earlier [15] (see "Supplementary material").

Immunoblotting analysis. Protein samples were run on a 10% SDS–PAGE, transferred onto a nitrocellulose filter and the immunoreactive proteins on the filter were visualized using a chemiluminescent detection kit as described previously [15].

Results

SatIII transcription is induced by a wide range of stressors

Previous studies have shown that apart from the heat shock nSBs can be induced by a few other stress conditions [11,14]. We were therefore interested in checking the inducibility of nSBs by a wide range of stressors. Because SatIII transcription is regulated by HSF1, and because HSF1 also regulate the expression of HSP70, we looked into the literature for chemicals that are known to increase Hsp70 levels and tested them for the SatIII induction. These include 8-hydroxyquinoline (8HQ), zinc sulfate ($ZnSO_4$), and ibuprofen [16,17]. HeLa cells were treated with 8HQ or $ZnSO_4$ for 6 h at 37 °C or with ibuprofen for 1 h at 37 °C and were then immediately processed for fluorescence in situ hybridization (FISH) analysis with SatIII repeats as probe for the detection of nSBs. All the three chemicals were able to induce the nSBs in a significant proportion of cells (Fig. 1A). Similarly, treatment of HeLa cells with MG132, lactacystin (reversible and non-reversible proteasomal inhibitors, respectively), and puromycin (a protein biosynthesis inhibitor) also led to the induction of nSBs (Fig. 1A). MG132, lactacystin, and puromycin are known to increase the cellular levels of unfolded/abnormal proteins. Thus, the SatIII locus appears to be induced by abnormal/unfolded protein stress.

Autophagosome–lysosomal blockers do not induce SatIII transcripts

Since the proteasome blockade led to the induction of nSBs, we wanted to examine whether impairment in other proteolytic pathways, such as lysosome and autophagosome, would also result in the formation of nSBs. For this HeLa cells were treated with 3-methyl adenine (3MA; a specific inhibitor of autophagy), bafilomycin A1 (an ATPase inhibitor that interferes with the autophagosome–lysosome fusion), or ammonium chloride (disrupts trafficking and lysosomal processing of proteins), as reported in one of our recent studies [15], and processed for FISH for the detection of nSBs. However, none of these chemicals induced nSBs (data not shown). Thus, inhibition of general protein clearance pathway does not seem to induce nSBs.

Qualitative difference in the nSBs induced by various stressors

Two of the most striking features that we observed with regard to the SatIII positive nSBs induced by various stressors are the following: (i) with the exception of ibuprofen, the induction of nSBs was not always 100%. As shown in Fig. 1B, almost 100% of the cells that were treated with ibuprofen exhibited nSBs while for puromycin it was around 35%. Thus, there could be a "threshold" level with regard to the inducibility of SatIII positive nSBs by a given chemical. The concentration of chemicals used in the present study was based on previous reports, and also based on our pilot experiments wherein the highest concentration (or the duration of treatment) which did not result in significant cell death (>20%) was arrived at. (ii) The second observation was the number of nSBs induced in each cell by a given chemical. Consistent with the previous report [14], in HeLa cells, the heat shock treatment lead to the formation of 5–8 nSBs (per nucleus) in a majority of cells while for the ibuprofen treatment the number never exceeded 5 per cell (see Fig. 1C). On the other hand, 8HQ treatment resulted in the formation of 8-to-12 nSBs in a great majority of cells (Fig. 1C). Overall there was a distinct and reproducible pattern with regard to the number of cells showing nSBs and the number of nSBs seen in each cell for each of the six stressors tested in the present study.

The SatIII RNA induced by various stressors recruit known components of nSBs

The heat shock induced SatIII transcripts are essential for the formation of nSBs and the transcripts are known to recruit several RNA binding proteins involved in pre-mRNA processing activities [10]. Such proteins include the splicing factor ASF/SF2 which directly binds to SatIII transcripts [7]. We therefore wanted to test whether the SatIII transcripts induced by various inducers recruit the known components of nSBs. For this, HeLa cells were treated with MG132, 8HQ, or puromycin, and processed for FISH followed by immunostaining for the endogenous SF2/ASF protein – one of the established components of the nSBs [7]. As a control set, heat shocked cells were used. As shown in Fig. 2, the SatIII RNA induced by all three chemicals colocalized with the SF2/ASF protein, and the localization pattern was similar to that of heat shocked cells.

Heat shock factor 1 is essential for the induction of SatIII transcripts by various stressors

The heat shock factor 1 (HSF1) is the critical player that triggers heat shock response [3]. In unstressed cells HSF1 is present in the cytoplasm as an inactive protein. Upon heat shock, HSF1 relocates to nucleus and activates the transcription of heat shock genes, including the SatIII locus [9]. The nuclear translocation of HSF1 is associated with the hyperphosphorylation of its key residues [18,19]. We therefore tested whether the induction of nSBs by various stressors is mediated by HSF1 or independent of it. For this, we transiently transfected HeLa cells with a knockdown construct (shRNA) for HSF1 or an empty vector as control and treated the cells with heat shock, MG132, 8HQ, or puromycin, and visualized the nSBs by FISH using probes specific to the SatIII repeat followed by immunostaining for the endogenous HSF1. As shown in Fig. 3A, a great majority of cells that were transfected with the HSF1 RNAi construct lacked nSBs, suggesting that the heat shock, MG132, 8HQ, or the puromycin treatment induce nSBs through the activation of HSF1. Moreover, the nSBs induced by MG132 and 8HQ also colocalized with HSF1 in the empty vector transfected cells (Fig. 3A), corroborating earlier observations that HSF1 is recruited to the SatIII locus for the transcription of SatIII RNA [9]. However, it is intriguing to note that the nSBs induced by puromycin did not colocalize with HSF1 after 12 h of treatment, although HSF1 is translocated to the nucleus and was found to be essential for the induction of nSBs by puromycin (Fig. 3A). Since the activation of HSF1 is known to be associated with its hyperphosphorylation, we have also examined the phosphorylation status of HSF1 subsequent to treating the cells with MG132, 8HQ, or puromycin. As controls, cells that were treated with heat shock and untreated cells were used. Equal quantity of proteins from the control set and the treated set was electrophoresed and immunoblotted with an anti-HSF1 antibody. As shown in Fig. 3B, and as also reported earlier for the heat shock response [18], a slower mobility hyperphosphorylated form of HSF1 was observed, although to different extent, in samples that were treated with MG132, 8HQ, or puromycin. Taken together, our observations suggest that HSF1 is a critical player for the induction of nSBs by all the stressors tested.

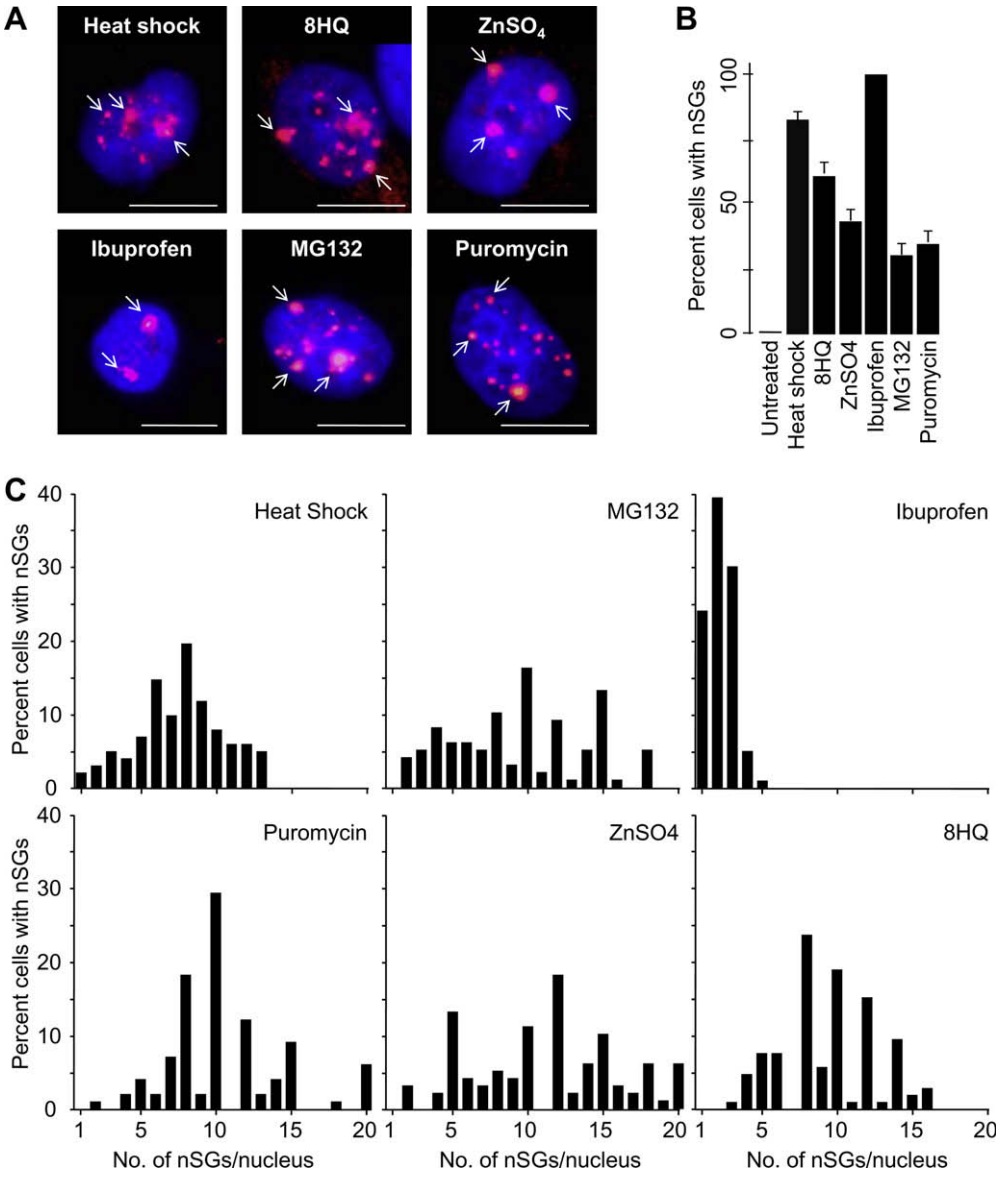

Fig. 1. Novel inducers of nSBs: (A) representative images of the DAPI stained nuclei of HeLa cells treated with stressors as indicated and subjected to FISH for detecting SatIII positive nSBs (indicated by arrows). Scale bar, 10 μm. (B) Bar diagram showing the proportion of cells exhibiting SatIII positive nSBs under various treatments, as indicated. The value of each bar represents the mean average of three independent transfections, with a minimum of 200 cells scored in each set. Error bars indicate standard deviation of the mean. (C) Bar diagram showing variation in the number of SatIII positive nSBs observed per cell under various treatments as indicated. For each treatment, a minimum of 200 nSB positive cells were scored.

CREB binding protein colocalizes with the puromycin induced nSBs even in the absence of HSF1

Under heat shock, in addition to the HSF1, transcription factor CREB binding protein (CBP) is known to translocate to the nSBs [9]. While the specific function of the CBP in nSBs is not known it could be suggested that CBP is involved in the transcription of SatIII locus because HSF1 and CBP are not seen in the nSBs during the recovery phase after heat shock – a stage when the SatIII locus are no longer transcribed [9]. Since HSF1 did not colocalize with nSBs induced by puromycin, but it was required for the induction, we wanted to check whether or not these nSBs represent transcribing loci. For this we treated HeLa cells with puromycin for various time periods (2, 4, 6, or 8 h) and processed for FISH followed by immunostaining for the endogenous HSF1 or CBP. As shown in Fig. 4, HSF1 granules were found to colocalize with the nSBs only in cells that were treated with puromycin for 2 h. On the other hand, CBP colocalized with the nSBs in cell that were treated for 2, 4, 6, or 8 h (Fig. 4). Intriguingly, the FISH signal was brightest in the cells that were treated with puromycin for 8 h, suggesting an increase in the amount of target RNA in the SatIII loci. Taken together our results suggest that while HSF1 may be required for the induction of SatIII locus by puromycin, its transcriptional status may be maintained by CBP.

Discussion

One of the suggested functions of SatIII transcripts is its role in regulating RNA processing when the cells are under heat shock [10]. Sequestration of factors like ASF/SF2 onto the nSBs is likely to inhibit RNA processing in stressed cells and may also modulate the alternative splicing pattern of some as yet unknown target transcripts as part of the stress response process [10]. We show here that nSBs are induced by chemical stressors, non-steroidal

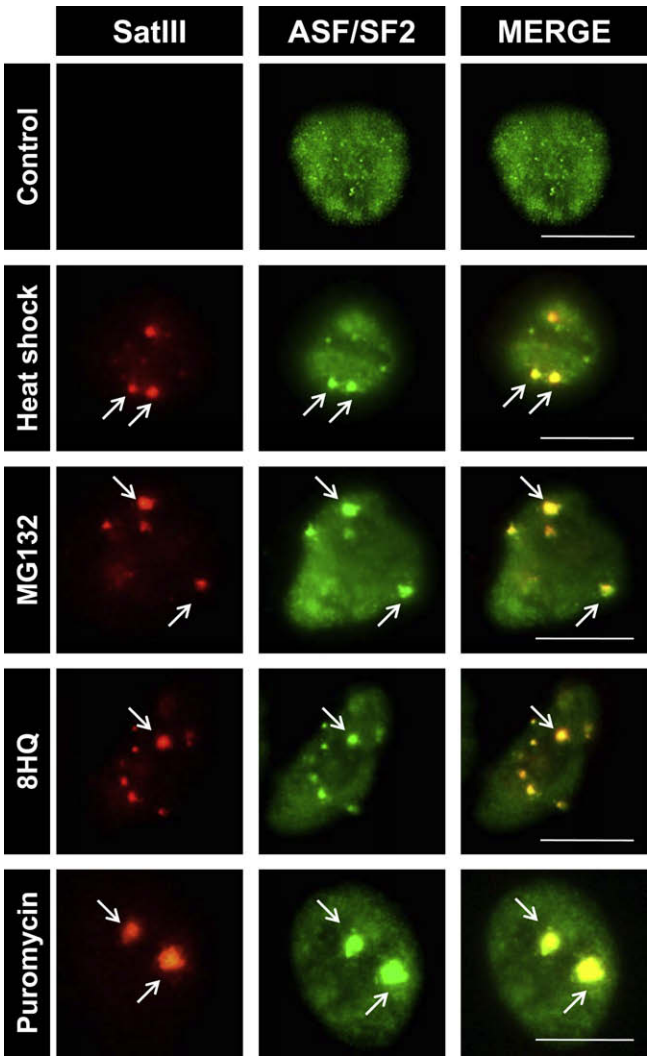

Fig. 2. The SatIII transcripts induced by various stressors are associated with ASF/SF2 protein. HeLa cells treated with various stressors, as indicated, were processed for FISH with SatIII repeat specific biotinylated probe (SatIII) followed by immunostaining for the ASF/SF2 protein. Untreated cells are identified as "control". Arrows identify SatIII positive nSBs that also co-stained for the ASF/SF2 protein. Scale bar, 10 μm.

anti-inflammatory drug, and inhibitors of proteasome, and the nSBs in all these conditions recruited the ASF/SF2 protein. Our present study, along with a recent report of Valgardsdottir et al. [11], which appeared when this work was in progress, demonstrates that the induction of nSBs could possibly represent a generic response to stress – not limited to heat shock, and that the induction of SatIII transcripts are involved in the stress response pathway. It is intriguing to note that treatment of cells with blockers for the autophagosome, lysosome, or the fusion of these two failed to induce nSBs while proteasomal blockade led to the formation of nSBs. One possible explanation could be that the functions of nSBs are limited to the stress response pathways that are mediated by HSF1, and where HSP70 is induced. This notion is partly supported by the observation that majority stressors that induced nSBs are also known to induce HSP70 [17] and that HSF1 was essential for the stressors tested in the present study to induce nSBs. However, a recent study demonstrates that, under hyperosmotic stress, induction of SatIII is facilitated by tonicity enhancer-binding protein (TonEBP), a transcription factor, which is also recruited to nSBs [11]. It is worth noting here that while HSF1

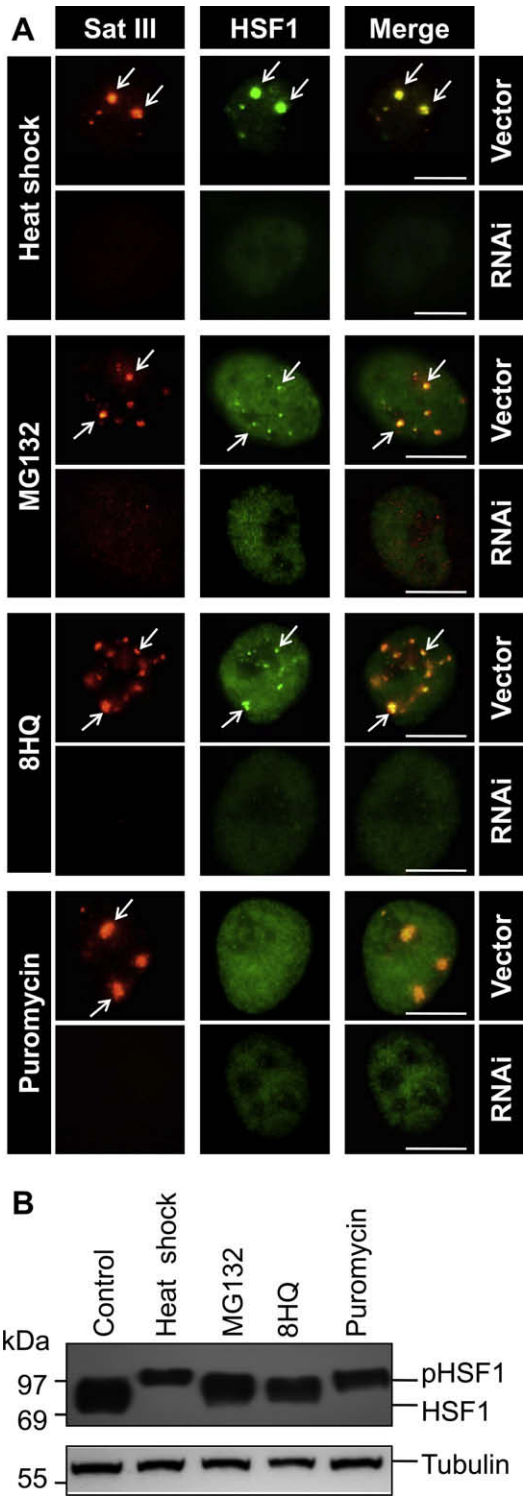

Fig. 3. HSF1 is essential for the induction of SatIII transcription by various stressors. (A) HeLa cells were transiently transfected with the shRNA construct for the knockdown of HSF1 (RNAi) or with an empty vector (vector), treated with various stressors, and processed for FISH with SatIII repeat specific biotinylated probe (SatIII) and immunostaining for the HSF1 protein. Note that the SatIII positive nSBs that colocalize with HSF1 protein (identified by an arrows) in control cells ("vector") are absent in the cells that were transfected with shRNA for the HSF1. Scale bar, 10 μm. (B) Immunoblot analysis of cell lysates showing the phosphorylation status of HSF1 after various treatments, as indicated. The anti-hsf1 immunoreactive band that showed a shift because of the hyperphosphorylation is identified as "pHSF1". The same blot was immunoreacted with anti-γ-tubulin to serve as loading control.

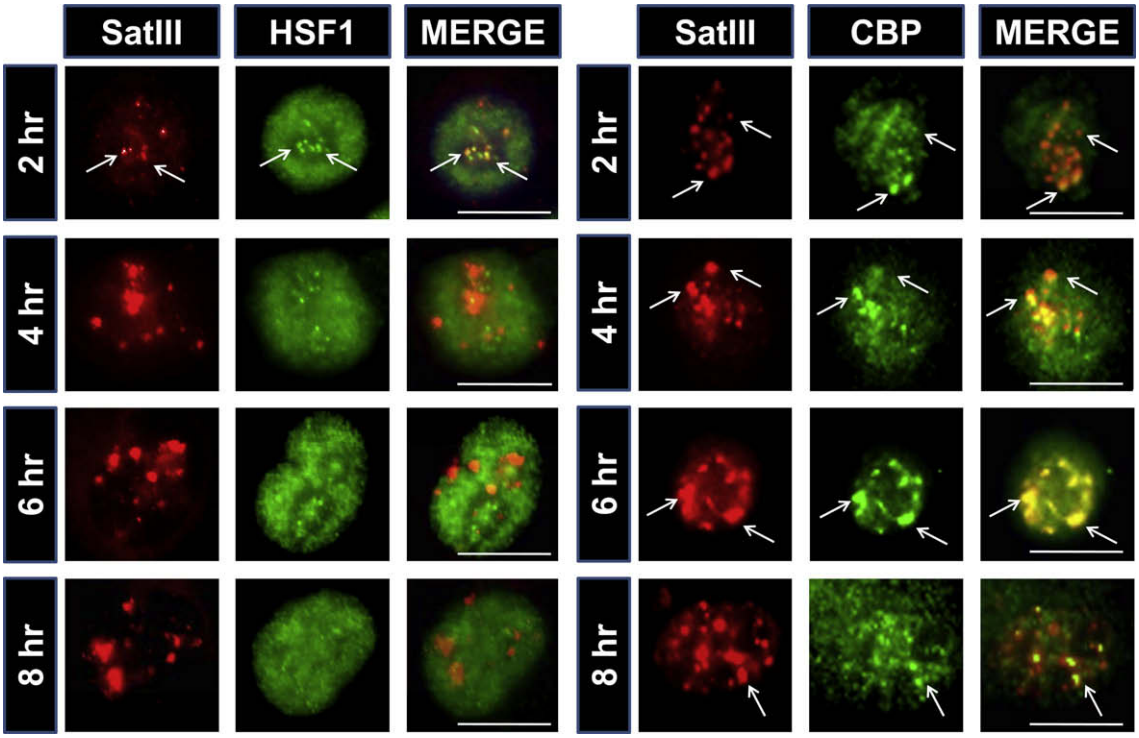

Fig. 4. CREB binding protein (CBP) is recruited to the puromycin induced nSBs. HeLa cells treated with puromycin for 2, 4, 6, or 8 h (indicated on the left) were processed for FISH with SatIII repeat specific biotinylated probe (SatIII) followed by immunostaining for the endogenous HSF1 or the CBP protein. Arrows identify SatIII positive nSBs that also co-stained for the HSF1 or the CBP. Scale bar, 10 μm.

was required for the induction of SatIII by puromycin, its recruitment to the nSBs was limited to the first 2 h of the treatment. CBP on the other hand was found to colocalize with the nSBs at least up to 8 h of the treatment. Under the condition of heat shock, however, HSF1 and CBP are known to dislodge together from the nSBs during the recovery period [9]. In this regard it is of interest to note that CBP is a transcription factor and CBP interacts with HSF1 [20]. Since HSF1 is known to function as a complex with other transcription factors under the condition of heat shock [21], it is tempting to speculate that HSF1, together with CBP, might transiently bind to the SatIII locus upon exposure to puromycin and induce the transcription. However, the transcriptional status of the locus might be maintained by the CBP and this may not require the presence of HSF1. Thus, it is tempting to suggest the existence of multiple transcription factors for the SatIII locus and their involvement might depend upon the type of stressors. This conclusion calls for further studies to identify additional regulatory factors that govern the expression of SatIII transcripts and the specific functions of nSBs in stress response pathway.

In the present study, we found the number of nSBs to vary with the kind of stressors used although the number of nSBs observed in the heat shocked cells was consistent with earlier reports [14,22]. It has been demonstrated that SatIII transcripts remain associated mainly with the 9q12 locus of synthesis, and recruit associated proteins to form nSBs [8–10]. The cell-to-cell variation observed for the number of nSBs was suggested to be because of the ploidy level seen in the HeLa cells [14,22]. Considering the fact that the same stock of cells was used for various stressors in the present study and yet the number of nSBs observed showed stress-specific patterns, it is tempting to suggest that depending on the stressor, multiple and distinct set of chromosomal loci could be induced to transcribe the SatIII repeats. In addition to the 9q12 locus, chromosomes 12, 15, and 21 are known to transcribe SatIII repeats [23,24], and our in situ hybridization analysis on the metaphase chromosomal spreads did suggest multiple loci for the SatIII repeats (S.S. and S.G., unpublished observations). Thus, the possible occurrence of stress-, and locus-specific induction of SatIII transcripts needs to be studied. Nonetheless, our observation that the nSBs induced by various stressors are associated with ASF/SF2 protein suggests a similar role for these functional complexes in the cellular response processes that are triggered by various stressors. Our results also suggest the existence of multiple transcriptional loci for the SatIII transcripts, that their activation might depend upon the type of stressors and that the specific role of transcription factors could be dependent on the stressor. The specific role of SatIII transcripts in the stress response pathway is being investigated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.02.137.

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