Induction of heat shock protein (HSP) expression by stress is initiated by binding of HSF1 to HSP gene promoters to increase their transcription. The cytoprotective functions of these HSPs are essential for cell survival, and thus it is critical that inducible HSP gene expression be executed rapidly and efficiently. Here we report an interaction between heat shock factor 1 (HSF1) and symplekin, a protein known to form a complex with the polyadenylation factors CstF and CPSF. HSF1-symplekin complexes are detected only after stress treatment, and these two proteins co-localize in punctate nuclear structures in stressed cells. HSF1 also complexes in a stress-induced manner with the 3′ processing factor CstF-64. Interfering with HSF1-symplekin interaction by overexpressing a non-DNA-binding mutant HSF1 protein significantly decreases Hsp70 mRNA polyadenylation in stressed cells, supporting the functional role for HSF1 in promoting 3′ processing of this transcript. Importantly, this was also found to result in a significant loss of Hsp70 protein induction and increased cell death in response to stress exposure. These results indicate that the HSF1-symplekin interaction functions as a mechanism for recruiting polyadenylation factors to HSP genes to enhance the efficiency/kinetics of production of mature Hsp mRNA transcripts to achieve the critical cellular need for rapid HSP expression after stress. Thus, HSF1 regulates HSP gene expression at not one but two different steps of the expression pathway, functioning both as a transcription factor and a polyadenylation stimulatory factor.

The up-regulation of heat shock protein (HSP) expression that occurs in response to cellular stress such as elevated temperature is mediated by heat shock transcription factor 1 (HSF1). In response to stress HSF1 is converted from a monomeric form that is unable to bind DNA to a trimeric form that binds with high affinity to heat shock elements in the promoters of HSP genes, particularly the HSP70 gene, to induce their transcription (1–3). The increased levels of Hsp70 and other HSP molecular chaperone proteins that result are critical for the ability of cells to survive exposure to stress conditions, and thus it is vital that the steps of HSP expression between HSF1 promoter binding and HSP mRNA translation be executed as quickly and efficiently as possible (4–7). Symplekin is a protein that has been shown to interact with the polyadenylation factors CPSF and CstF, and it has been implicated as playing an important role in polyadenylation by acting as a molecular scaffold to bring both CPSF and CstF together into the same complex (8, 9).

In this study we report the identification of an interaction between HSF1 and symplekin. The results show that HSF1 interaction with symplekin is stress-dependent and that HSF1-containing complexes in stressed cells also contain the polyadenylation factor CstF-64. Further, immunofluorescence analysis demonstrates that HSF1 and symplekin colocalize in punctate bodies in the nuclei of stressed cells. Importantly, interfering with the interaction between HSF1 and symplekin results in a decreased efficiency of polyadenylation of Hsp70 mRNA transcripts in stressed cells, loss of Hsp70 protein induction, and a significant increase in cell death upon exposure to stress. These results suggest that the HSF1-symplekin interaction plays a critical role in the cellular stress response by maximizing the kinetics and efficiency of Hsp70 mRNA polyadenylation via recruitment of polyadenylation factors to HSP genes, thereby helping to ensure that cells can produce elevated levels of the Hsp70 protein as quickly as possible.

**Experimental Procedures**

GST-symplekin and Hsp70-HSF1 Fusion Proteins and in Vitro Interaction Assays—The full-length coding region of human symplekin (amino acid residues 1–1274) was amplified from a full-length symplekin EST (expressed sequence tag) plasmid (GenBankTM AL560175) by PCR incorporating EcoRI and HindIII restriction sites and inserted into pGEX-MPB. The construct was expressed in *Escherichia coli* and purified by binding to glutathione-agarose beads (Sigma), washing, and elution. After extensive washing with buffer D (10) with 100 mM NaCl, proteins were eluted with buffer D containing 50 mM glutathione. The pQE30-HSF1β protein construct was expressed in *E. coli* and purified on nickel-nitrilotriacetic acid beads (Qiagen), washed with buffer D, and incubated with 50 ng of puriﬁed GST-symplekin at 4 °C for 1 h. Beads were collected by centrifugation, washed several times with buffer D plus 100 mM NaCl, and then subjected to Western blot analysis with mouse monoclonal anti-symplekin antibodies (Transduction Laboratories). The resulting bands were quantitated from a digital image using Kodak image processing software.

Cell Culture and Heat Shock Conditions—HeLa cells were cultured at 37°C with 5% CO2 in Dulbecco’s modiﬁed Eagle’s medium with 10% fetal calf serum and 50 μg/ml gentamycin. Human erythroleukemia K562 and K562 HSF1-PD (K562 cells stably transfected with HSF1 point mutant, which lacks DNA binding ability) were the kind gifts of Stuart Calderwood (11). They were cultured at 37 °C with 5% CO2 in RPMI 1640 containing 10% fetal calf serum and 50 μg/ml gentamycin.

Immunoprecipitation and Immunoblotting Analysis—Nuclei were prepared from HeLa cells (~10^6) by a hypotonic lysis protocol (10).
HeLa cell nuclear extracts were immunoprecipitated as described previously (12) using 3 μl of anti-HSF1 polyclonal antibody or 10 μl of anti-IgG control antibody (Sigma) bound to 20 μl of protein G-Sepharose beads and then analyzed by Western blot using anti-symplekin mouse monoclonal antibody (Transduction Laboratories) or anti-CstF64 mouse monoclonal antibody (generously provided by Yoshio Takagaki, University of Virginia) (13). The resulting bands were quantitated from a digital image using Kodak image processing software. Western blot analysis of Hsp70 levels was performed using antibody SPA-812 (StressGen) specific for the stress-inducible Hsp70i protein or anti-CstF-64 mouse monoclonal antibodies.

Fig. 1. Interaction between HSF1 and symplekin. A, yeast pJ694A containing pGBD-HSF1 bait and pVP16-symplekin(1–124) target construct identified in a library screen or HSF1 bait and empty pVP16 plasmid were streaked on -TL, -HTL, and -ATL plates. B, location of an HSF1-interacting sequence in the N-terminal region of symplekin (amino acids 1–124) in the context of mouse symplekin (GenBank X95835 AHA98652). Arrowsheads indicate the position of putative nuclear localization signals (NLS). C, purified GST-symplekin expressed in and purified from E. coli was incubated with nickel-nitrotriacetic acid beads containing bound purified His6-HSF1 or beads alone (Control) for 60 min at 4 °C. After washing, bound symplekin was detected by SDS-PAGE and Western blot using anti-symplekin antibodies (Transduction Laboratories).

Yeast Two-hybrid Interaction Assays—Yeast strain pJ694A was transfected with pGBD-HSF1 bait and used to screen a mouse embryo yeast two-hybrid library. The interaction between the pGBD-HSF1 and pVP16-symplekin(1–124) was verified by streaking the yeast containing these constructs or pGBD-HSF1 bait and empty pVP16 plasmid (as negative control) on -TL, -HTL, and -ATL plates (12).

Immunofluorescence Analysis—Control and heat-treated (42 °C for times indicated) HeLa cells grown on coverslips were fixed using 2% paraformaldehyde in phosphate-buffered saline + 2% bovine serum albumin at room temperature and subjected to immunofluorescence as described previously (14) using 1:100 dilutions of HSF1 polyclonal antibody and symplekin mouse monoclonal antibody (Transduction Laboratories).

RNase Protection Assay—To generate a probe for the human Hsp70 coding region, a 192-bp PCR product yielding a 173-nucleotide RNA transcript of which 131 nucleotides are homologous to Hsp70 sequence was amplified by sense primer 1 (5’-tgctctctcacatgtagcatcttgac-3’, antisense primer 2 (5’-gttgtctctcacatgtagcatcttgac-3’), lowercase letters indicate a nonhomologous sequence) and antisense primer 2 (5’-TTATACGACTCATACTAGGagctcgtagAGCTATCTACTTCTAAGTTG-3’; a 19-bp T7 promoter is underlined, and lowercase letters indicate a 12-bp nonhomologous sequence). Sense primer 3 (5’-agctgtgtgtgtgtgtggtgg-3’; a 30-bp nonhomologous sequences) and antisense primer 4 (5’-TTATACGACTCATACTAGGagctcgtagACGCTATCTACTTCTAAGTTG-3’) and antisense primer 4 (5’-TTATACGACTCATACTAGGagctcgtagACGCTATCTACTTCTAAGTTG-3’; a 19-bp T7 promoter is underlined, and lowercase letters indicate a 12-nucleotide nonhomologous sequence) were used for amplification of human Hsp70 3’-untranslated region (a 427-bp PCR product yielding a 409-nucleotide RNA transcript, of which 280 nucleotides are homologous to the Hsp70 sequence surrounding the site of poly(A) addition). Antisense riboprobes were labeled with biotin-16-UTP using T7 RNA polymerase (MAXScript kit, Ambion) and resuspended in water. Total RNA was isolated from heat-treated K562 and K562 HSF-1-PD cells using TRIZOL reagent (Invitrogen). RNase protection assays were performed using the Ambion protocol with minor modification. The 1 ng of labeled probe was co-precipitated with 5 μg of total DNA and then resuspended in 10 μl of hybridization buffer. Following a 16-h incubation at 56 °C, samples were digested at 37 °C for 30 min with 2.5 units/ml RNase A and 100 units/ml RNase T1 (Ambion), ethanol-precipitated, separated on an 8% urea-6% polyacrylamide gel, and then transferred to nylon membrane, UV-cross-linked, and visualized with a SuperSignal kit (Pierce). The resulting bands were quantitated from a digital image using Kodak image processing software.

Measurement of Cell Viability—K562 and K562 HSF-1-PD cells were pretreated at 43 °C for 30 min, returned to 37 °C for 4 h to develop...
thermotolerance, exposed to a stringent 45 °C heat treatment for 20 min, and then placed at 37 °C for 40 h, after which they were subjected to a trypan blue exclusion assay to determine the number of viable cells. A parallel aliquot of cells kept at 37 °C for the duration of this experiment (45 h) was also counted by trypan blue exclusion assay as a control. The percent viability was calculated and expressed according to the following equation: viability (%) = [(number of viable cells with heat stress)/(number of viable cells under nonstress conditions (kept at 37 °C))] × 100%.

RESULTS

To advance the understanding of the critical role of HSF1 in mediating stress-induced transcription of HSP genes, we undertook a yeast two-hybrid screen to identify HSF1-interacting proteins. One of the partners identified was symplekin, a protein previously found to associate with the CPSF and CstF factors involved in mRNA polyadenylation (Fig. 1A) (8, 9). The clone identified in our screen represented a region at the N terminus of symplekin comprising amino acids 1–124 (Fig. 1B).

As an independent test of the interaction between HSF1 and symplekin, and to determine whether the interaction is direct, we performed an in vitro pull-down assay. The results, shown in Fig. 1C, indicate that His₆-HSF1 specifically bound purified recombinant symplekin. Quantitation of the data revealed that 62% of the input symplekin was bound, indicating the efficiency of interaction of these two proteins.

To test for interaction between endogenous HSF1 and symplekin we performed immunoprecipitation experiments. The results indicate the existence of a specific interaction between the endogenous proteins and demonstrate that this complex is only found in cells that have been exposed to heat shock treatment (Fig. 2A). To test whether HSF1 also associates with other components of the polyadenylation machinery such as the CstF complex, we performed an immunoprecipitation analysis of HSF1 interaction with the CstF-64 protein. The results indicate that HSF1 is indeed found in a complex with CstF-64 and that, as with symplekin, this association is observed only after cells have been exposed to stress conditions (Fig. 2B). We quantitated these data and determined that in the symplekin immunoprecipitation 0.74% of the symplekin protein in the
input was recovered, and in the CstF-64 immunoprecipitation 0.80% of the CstF-64 protein in the input was recovered. This is consistent with our expectations that only a fraction of the total cellular symplekin and CstF-64 would likely be associated with HSF1. We also noted that, because our results would represent only the complexes that survived the antibody binding incubations and washes of the immunoprecipitation protocol, these are likely to be conservative estimates of the amounts of HSF1-symplekin and HSF1-CstF-64 complexed in the cell.

Previous studies demonstrated that stress causes some of the HSF1 protein, which is predominantly nuclear, to concentrate in punctate nuclear bodies (10, 15–16). To test whether symplekin was also found in these bodies we performed a co-immunofluorescence analysis of these two proteins in nonstressed and heat-treated HeLa cells. The results of this experiment, shown in Fig. 3, reveal that a portion of the symplekin protein colocalizes with HSF1 in these foci in a stress-dependent manner, suggesting that these bodies may represent a site where HSF1 associates with symplekin, perhaps promoted by the concentration of these two partners in the bodies.

The stress-induced nature of the interaction of HSF1 with symplekin and CstF-64 suggests that it could be important for assisting HSF1 function in up-regulating expression of HSP genes. Specifically, in light of the known functions of symplekin/CstF-64, we postulated that this interaction could serve as a mechanism for enhancing the efficiency of polyadenylation of Hsp70 mRNA transcripts by recruiting symplekin-CstF to HSP gene promoters and the associated polymerase II complex, similar to the described function of TFIID in recruiting CPSF to polymerase II on promoters (17). To test this hypothesis we examined the polyadenylation efficiency of Hsp70 mRNA transcripts in heat-treated cells, either in K562 cells or K562 cells that stably express a mutant HSF1 protein that is unable to bind DNA because of a point mutation in its DNA-binding domain (K562 HSF1-PD cell line) (11). The reasoning was that the mutant HSF1 protein would bind to symplekin, thereby reducing the amount of symplekin available for association with the endogenous wild-type HSF1 and decreasing the efficiency of Hsp70 mRNA polyadenylation. Parental K562 cells and the K562 HSF1-PD cells were subjected to heat shock treatment at 42 °C for 30 or 60 min, after which total RNA isolated from these cells was analyzed by RNase protection assay using two different probes, one hybridizing to the 3′ cleavage site and the other to an internal region of the transcript for normalization purposes (positions shown in Fig. 4A).

The results demonstrate that the cells harboring the mutant HSF1 protein exhibit a significantly lower efficiency of 3′ end formation than the parental K562 cells (Fig. 4B). The total levels of Hsp70 mRNA transcripts (detected by internal probe) are not appreciably affected by the presence of the HSF1 mutant protein, suggesting that it does not affect Hsp70 gene transcription to any significant extent. Because this mutant protein is unable to bind the Hsp70 promoter, it presumably is unable to interfere with the assembly of transcription complexes on this promoter. Quantitation of the RNase protection results reveal approximate 60 and 70% decreases in Hsp70 mRNA 3′ end processing in the K562 HSF1-PD cells for the 30- and 60-min heat shock treatment samples, respectively (Fig. 4C).

This loss of Hsp70 mRNA polyadenylation is correlated with a significant decrease in induction of Hsp70 protein in response to heat stress (Fig. 5, A–C). Similar results were obtained for both K562 (Fig. 5, A and C) and CHO versions (Fig. 5B) of HSF1-PD cell lines, using both a direct Hsp70 Western blot approach (Fig. 5, A and B) and 35S in vivo pulse labeling/im munoprecipitation measurements of Hsp70 levels (Fig. 5C).

Importantly, the reduced Hsp70 protein induction observed in HSF1-PD cells is associated with a significant increase in the levels of cell death following stress exposure (Fig. 5D), indicating the critical importance of HSF1-enhanced polyadenylation for the ability of cells to survive stress treatment.

**DISCUSSION**

A fascinating development in the study of gene expression has been the finding that transcription and mRNA processing
events such as capping, splicing, and polyadenylation are not independent events and can be coupled (18–22). For example, interaction between the C-terminal domain of RNA polymerase II and CPSF/CstF polyadenylation factors allows coupling to occur between transcription and polyadenylation events (23–27). TFIIID interaction with CPSF represents a mechanism for loading CPSF onto the C-terminal domain, but whether a similar mechanism existed for recruitment of the CstF complex to promoters is not known (17). In addition, and more relevant to our present study, because these mechanisms involve components involved in transcription of all class II genes, RNA polymerase II and TFIIID, it was also not known whether related mechanisms exist that could couple transcription to 3' processing in a gene- or gene family-specific manner. Such a mechanism would make particular biological sense in the case of genes whose expression needs to be rapidly induced in response to some signal.

Our results indicate that this mechanism does exist, at least in the case of stress-induced transcription of HSP genes, and that it is mediated by interaction between HSF1 and the symplekin protein. It will be fascinating to explore whether symplekin interacts with other transcription factors responsible for regulating genes in which products are required to be rapidly inducible. Indeed, symplekin makes an ideal partner for such interactions because it associates with both CstF and CPSF complexes (8, 9), and thus interacting with symplekin could allow a transcription factor to efficiently recruit both of these components critical for 3' processing to promoters it binds.

Finally, these data reveal that HSF1 positively regulates stress-induced HSP gene expression at not one but two distinct steps in the gene expression pathway. Thus, in addition to its well-characterized function in stimulating the transcription of HSP genes, our results now show that HSF1 also acts to enhance polyadenylation of the resulting HSP mRNA transcripts via its interaction with the symplekin-3' processing complex. The existence of this mechanism demonstrates even further the critical role played by HSF1 in the cellular stress response and underscores the vital importance of stress-induced HSP gene expression for cell function and survival.

Acknowledgments—We are very grateful to Stuart Calderwood for generously providing K562 and CHO versions of the HSF1-PD cell lines, Yoshi Takagaki for the generous gift of anti-CstF-64 antibodies, and to Yiling Hong, Eric Lubert, Roland Hilgarth, Chad Wilkerson, Hollie Skaggs, and Lynea Murphy for helpful discussions.

REFERENCES
1. Christians, E. S., Yan L. J., and Benjamin, I. J. (2002) Crit. Care Med. 30, (suppl.) S43–S50
2. Pirkkalala, L., Nykanen, P., and Sistonen, L. (2001)FASEB J. 15, 1118–1131
3. Morano, K. A., and Thiele, D. J. (1999)Gene Expr. 7, 271–282
4. Naylor, D. J., and Hartl, F. U. (2002) Biochem. Soc. Symp. 68, 45–68
5. Kregel, K. C. (2002)J. Appl. Physiol. 92, 2177–2186
6. Jaattela, M. (1999)Ann. Med. 31, 261–271
7. Kaufman, R. J. (1999) Biochem. Biophys. Acts 1423, R13–R27
8. Takagaki, Y., and Manley, J. L. (2000) Mol. Cell. Biol. 20, 1515–1525
9. Hofmann, I., Schnitzler, M., Kaufmann, I., and Franke, W. W. (2002) Mol. Biol. Cell 13, 1665–1676
10. Sarge, K. D., Murphy, S. P., and Morimoto, R. I. (1993) Mol. Cell. Biol. 13, 1392–1407
11. Chen, C., Xie, Y., Stevenson, M. A., Auron, P. E., and Calderwood, S. K. (1997)J. Biol. Chem. 272, 26803–26806
12. Hong, Y., and Sarge, K. D. (1999) J. Biol. Chem. 274, 12967–12970
13. Takagaki, Y., Manley, J. L., MacDonald, C. C., Wilusz, J., and Shenk, T. A. (1990) Genes Dev. 4, 2112–2120
14. Goodson, M. L., Hong, Y., Rogers, R., Matunis, M. J., Park-Sarge, O. K., and Sarge, K. D. (2001)J. Biol. Chem. 276, 18513–18518
15. Cotto, J., Fox, S., and Morimoto, R. I. (1997) J. Cell Sci. 110, 2925–2934
16. Holmberg, C. I., Illman, S. A., Kallio, M., Mikhailov, A., and Sistonen, L. (2000) Cell Stress Chaperones 5, 219–228
17. Dantzig, J. C., Murthy, K. G., Manley, J. L., and Tora, L. (1997) Nature 389, 399–402
18. Minvielle-Sebastia, L., and Keller, W. (1999) Curr. Opin. Cell Biol. 11, 352–357
19. Hirose, Y., and Manley, J. L. (2000) Genes Dev. 14, 1415–1429
20. Orphanides, G., and Reinberg, D. (2002) Cell 108, 439–451
21. Proudfoot, N. J., Furger, A., and Dye, M. J. (2002) Cell 108, 501–512
22. Maniatis, T., and Reed, R. (2002) Nature 416, 499–506
23. McCracken, S., Fong, N., Yankulov, K., Ballantyne, S., Pan, G., Greenblatt, J., Patterson, S. D., Wickens, M., and Bentley, D. L. (1997) Nature 385, 357–361
24. Hirose, Y., and Manley, J. L. (1998) Nature 395, 93–96
25. Fong, N., and Bentley, D. L. (2001)Genes Dev. 15, 1783–1785
26. Ryan, K., Murthy, K. G., Kaneko, S., and Manley, J. L. (2002) Mol. Cell. Biol. 22, 1684–1692
27. Lacalatiosi, D. D., Geiger, G., Minet, M., Schroeder, S., Cilli, K., McNeil, J. B., and Bentley, D. L. (2002) Mol. Cell 9, 1101–1111
HSF1 Modulation of Hsp70 mRNA Polyadenylation via Interaction with Symplekin
Hongyan Xing, Christopher N. Mayhew, Katherine E. Cullen, Ok-Kyong Park-Sarge and
Kevin D. Sarge

J. Biol. Chem. 2004, 279:10551-10555.
doi: 10.1074/jbc.M311719200 originally published online January 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311719200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 26 references, 11 of which can be accessed free at
http://www.jbc.org/content/279/11/10551.full.html#ref-list-1