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Enhancement of Glucocorticoid Receptor-Mediated Gene Expression by Constitutively Active Heat Shock Factor 1

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To further define the role of heat shock factor 1 (HSF1) in the stress potentiation of glucocorticoid receptor (GR) activity, we placed a constitutively active mutant of human HSF1 (hHSF1-E189) under the control of a doxycycline (DOX)-inducible vector. In mouse L929 cells, DOX-induced expression of hHSF1-E189 correlated with *in vivo* occupancy of the human heat shock protein 70 (hHsp70) promoter (chromatin-immunoprecipitation assay) and with increased activity under nonstress conditions at the hHsp70 promoter controlling expression of chloramphenicol acetyl transferase (CAT) (p2500-CAT). Comparison of hHSF1-E189 against stress-activated, endogenous HSF1 for DNA-binding, p2500-CAT, and Hsp70 protein expression activities showed the mutant factor to have lower, but clearly detectable, activities as compared with wild-type factor. Thus, the hHSF1-E189 mutant is capable of replicating these key functions of endogenous HSF1, albeit at reduced levels. To assess the involvement of hHSF1-E189 in GR activity, DOX-induced expression of hHSF1-E189 was performed in L929 cells expressing the minimal pGRE$_2$E1B-CAT reporter. hHSF1-E189 protein expression in these cells was maximal at 24 h of DOX and remained constant up to 72 h. hHSF1-E189 expressed under these conditions was found both in the cytosolic and nuclear compartments, in a state capable of binding DNA. More importantly, GR activity at the pGRE$_2$E1B-CAT promoter was found to increase after DOX-induced expression of hHSF1-E189. The potentiation of GR by hHSF1-E189 occurred at saturating concentrations of hormone and was dependent on at least 48 h of hHSF1-E189 up-regulation, suggesting that time was needed for an HSF1-induced factor to accumulate to a threshold level. Initial efforts to characterize how hHSF1-E189 controls GR signaling showed that it does not occur through alterations of GR protein levels or changes in GR hormone binding capacity. In summary, our observations provide the first molecular evidence for the existence of HSF1-regulated genes that serve to elevate the response of steroid receptors under stress conditions. (*Molecular Endocrinology* 18: 509–520, 2004)

THE GLUCOCORTICOID RECEPTOR (GR) is a ligand-activated transcription factor that serves as the principal target of steroids produced by the adrenal cortex (1, 2). In the absence of hormone, the GR is known to exist in a complex with several members of the heat shock protein (Hsp) family (3), proteins that are integral to the heat shock stress response found in almost all cells (4). At an organismal level, glucocorticoid hormones are known to play a variety of roles that serve to maintain homeostasis in response to stress events (5, 6). One of the best understood of these roles is the ability of glucocorticoids to protect against overactivity by immune and inflammatory pathways. In this respect, our recent finding (7, 8) that glucocorticoids can suppress the heat shock response in cells by inhibiting the actions of heat shock factor 1 (HSF1) serves to underscore the central role of GR in modulating stress responses.

In addition to suppression of HSF1 activity by GR, our laboratory and those of others have found evidence that reciprocal control of steroid receptor responses by stress can also occur (9–12). In this case, however, most studies report that heat shock and other forms of cellular stress will cause an increase in steroid receptor transcriptional activity. Key features of this response include: 1) heat shock potentiation of GR activity at all concentrations of hormone, up to and including saturating levels of hormone; 2) stress potentiation does not occur in cells devoid of GR, or containing hormone- or DNA-binding defective GR; 3) stress potentiation does not occur in response to classical GR antagonists, such as RU486; and 4) stress potentiation does not occur by cooperative binding to GR-regulated promoters by GR and any other DNA-binding transcription factor, including HSF1. Moreover, no obvious change in amount of GR protein or in hormone-induced translocation of GR to the nucleus

Abbreviations: CAT, Chloramphenicol acetyl transferase; CoA, coenzyme A; DOX, doxycycline; GFP, green fluorescent protein; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HSE, heat shock element; HSF, heat shock factor; Hsp, heat shock protein.

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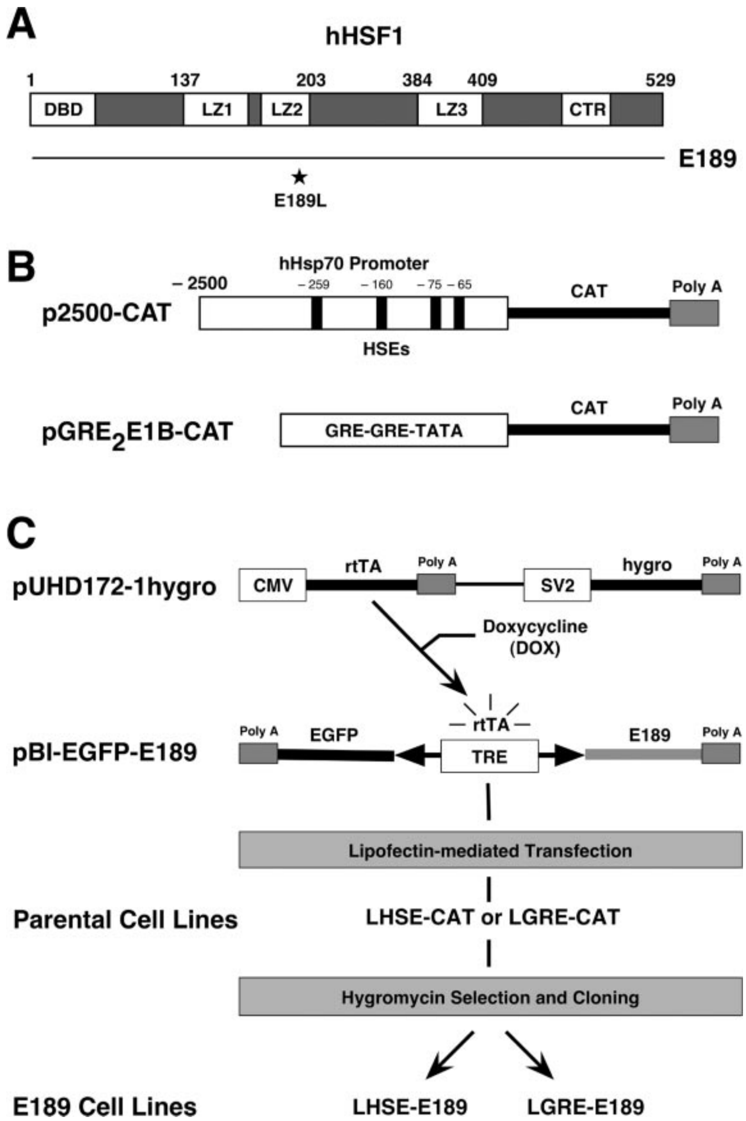

Fig. 1. Transfection of hHSF1-E189, a Constitutively Active Mutant of HSF1, into GR- and HSF1-Responsive Backgrounds

A, HSF1 is a 529-amino acid protein incorporating multiple leucine zipper regions (LZ), a DNA-binding domain (DBD), and a C-terminal transactivation domain (CTR). A point mutation at residue 189 introduced a collapse of LZ2 to produce a constitutively active form of HSF1 (E189). B, Mouse L929 cells stably selected for the p2500-CAT (Hsp70) or the minimal $pGRE_2E1B$-CAT promoters were used as parent cells for the construction of stable cell lines expressing E189. C, E189 was placed in the DOX-inducible bidirectional vector pBI-EGFP. Parent cells were cotransfected with pBI-EGFP-E189 and the transactivator vector pUHD172-1hygro, followed by hygromycin selection and cloning of GFP-positive cells to generate the LHSE-E189 and LGRE-E189 cell lines.

has been observed under the conditions of stress potentiation.

Although identification of the precise stage of GR signaling affected by stress has not yet been achieved, we have recently made progress by providing evidence that HSF1 activity is centrally involved in this mechanism. Through use of drugs that selectively modulate HSF1 activity under stress conditions, we have shown a corresponding modulation of GR under the same conditions (13, 14). For example, a flavonoid compound, quercetin, was used to prevent HSF1 activation in response to stress but had no effect on HSF1 after activation. Under these conditions, it was found that quercetin blocked heat shock potentiation of the GR, but only when administered before the stress event. Similarly, increasing HSF1 activity under stress conditions by treating cells with a phosphatidylinositol 3-kinase inhibitor (wortmannin) caused a concomitant increase in GR transcription enhancement activity.

Even though these pharmacological approaches provide strong evidence for the involvement of HSF1, we decided that a strictly molecular approach to this question was needed. There were several reasons for

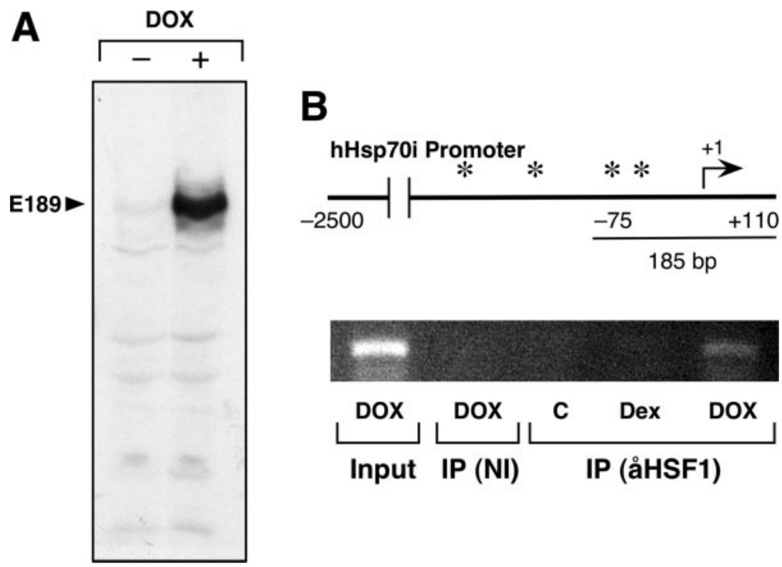

Fig. 2. DOX Induction and *in Vivo* Promoter Binding of E189 in LHSE-E189-CAT Cells

A, LHSE-E189-CAT cells were treated with DOX (10 μg/ml) for 24 h, followed by lysate preparation and analysis by Western blotting using an antibody against hHSF1. B, Chromatin immunoprecipitation assay (ChIP) was performed, as described in *Materials and Methods.* Briefly, equal amounts of cross-linked lysates were sonicated and immunoabsorbed (IP) with antibody against HSF1 (åHSF1) or nonimmune control (NI). After washing and reversal of cross-linking, PCR was performed using primers against the hHsp70i promoter (p2500-CAT), as indicated. Conditions: C, no treatment; Dex, 1 μM dexamethasone for 24 h; DOX, 10 μg/ml doxycycline for 24 h. hHsp70i, Inducible hHsp70.

this decision. First, use of drugs to inhibit HSF1 had to be performed under conditions of stress. Thus, it could not be confidently concluded that the drugs were targeting HSF1 alone, as opposed to other stress-induced signal pathways. Moreover, pharmacological approaches could not rule out the possible involvement of other members in the HSF family, such as HSF2, which are known to be expressed in the mouse (15). Lastly, if HSF1 is indeed responsible for the stress potentiation of GR, then discovery of the HSF1-regulated genes involved would be much easier using a stress-free molecular approach rather than combined conditions of stress and drug treatment. With this in mind, we report here that expression of a constitutively active mutant of HSF1 in cells can indeed up-regulate GR transcriptional enhancement activity under stress-free conditions. Thus, the mechanism by which heat shock and other forms of stress cause elevation of GR function most likely requires expression of HSF1-regulated genes during the poststress recovery period.

RESULTS

Nonstress Expression of hHSF1-E189 in Mouse L929 Cells Mimics the Function of Endogenous Stress-Activated Factor

To further define the role of HSF1 in the stress potentiation of GR, we set out to separate intrinsic HSF1 activity from all other stress-induced mechanisms. We achieved this through use of a constitutively active mutant of human HSF1 (hHSF1-E189) originally developed by Voellmy and co-workers (16). hHSF1-E189 (which we also refer to as E189) contains a single-amino acid substitution at residue 189 residing in one of three hydrophobic LZ domains (Fig. 1A). The LZ domains of HSF1 are thought to interact with heat shock protein chaperones, serving to maintain HSF1 in an inactive state. The E189 mutant, therefore, has stress-free activity because it cannot be properly chaperoned, leading to active HSF1 trimers under nonstress conditions (16, 17). As further diagrammed in Fig. 1, the cDNA for hHSF1-E189 was placed under the control of a doxycycline (DOX)-inducible vector (18) in L cells that had previously been stably transfected with a chloramphenicol acetyl transferase (CAT) reporter driven by the human (h) Hsp70 promoter (LHSE-CAT cells) or by the minimal GR-responsive glucocorticoid response element $(GRE)_2$E1B construct (LGRE-CAT cells). After selection, the stably transfected LHSE-E189 and LGRE-E189 cells were thus established.

As an initial test, LHSE-E189 cells were exposed to 10 μg/ml DOX followed by assay of hHSF1-E189 expression by Western blotting using an antibody specific to the hHSF1 (Fig. 2A). Here the results show appearance of E189 protein in response to DOX treatment. As a further test, the ability of this protein to bind the Hsp promoters *in vivo* was determined by use of the chromatin immunoprecipitation assay using primers specific to the hHsp70 promoter (Fig. 2B). The results show occupancy of the hHsp70 promoter by DOX-induced E189. To demonstrate that promoterbound E189 can indeed stimulate transcription in the absence of stress, a time course of exposure to DOX was performed in LHSE-E189 cells, followed by assay for E189 by Western blotting and CAT activity assay (Fig. 3). The results show detectable levels of hHSF1-E189 protein as early as 4 h after DOX treatment, with levels of protein appearing to plateau at about 20 h of DOX exposure. However, CAT expression from the hHsp70 promoter was not appreciably increased until 20 h of DOX and was still increasing at 40 h of treatment. As would be expected, this suggests that expression of E189-regulated genes lags behind expression of hHSF1-E189 protein itself.

To guard against the possibility that the CAT activity observed in these cells was actually due to activation of endogenous mouse HSF1 by DOX, we treated the parental LHSE-CAT cells (no E189 vector) with this compound. DOX treatment up to 48 h had no effect on CAT expression from the hHsp70 promoter (data not shown). Because the pBI vector used for hHSF1-E189 expression also controls expression of green fluorescent protein (GFP), we also tested the possibility that GFP could be activating mHSF1, perhaps by causing recruitment of Hsp70 and other chaperones away from the inactive mouse factor. In this test (Fig. 4A), activation of E189 and mHSF1 was assayed by Western blotting using an antibody that detects both species of this factor. The results show the presence of activated mHSF1 in the nuclear fraction of heat-shocked cells and the presence of E189 in both the cytosolic and nuclear fractions. However, endogenous, activated mHSF1 has an apparent M_r larger than that of E189, and this band is not detected in cells exposed to DOX alone. Thus, it is unlikely that E189 or GFP expression leads to simultaneous activation of the endogenous factor.

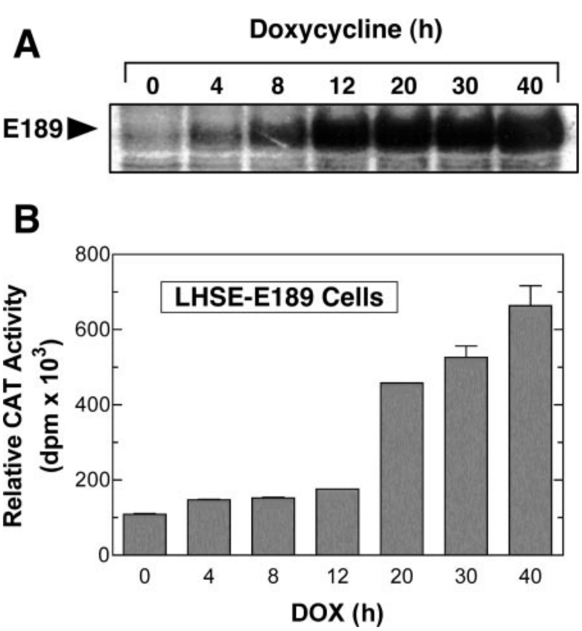

Fig. 3. Transcription Enhancement Activity of DOX-Induced E189 in LHSE-E189-CAT Cells

A, LHSE-E189-CAT cells were subjected to the indicated time course of DOX (10 μg/ml) treatment and analyzed for E189 protein expression by Western blotting with antibody against hHSF1. B, Same time course as in panel A, except that lysates were analyzed for CAT gene expression. Results represent the mean ± SEM of three independent experiments.

Although these data show that DOX-expressed E189 is active in the absence of stress at the exogenous hHsp70 promoter, we wanted to determine whether E189 could act at endogenous promoters within these cells and the extent of this activity. We chose to analyze the endogenous Hsp70 promoters by use of Western blotting with an antibody that can detect both the constitutive and inducible forms of Hsp70 (Fig. 4B). The data show that DOX treatment of LHSE-E189 cells can indeed cause increased expression of both constitutive Hsp70 and inducible Hsp70. However, the levels of induction by DOX for each of these proteins, although clearly elevated, were low compared with levels obtained in response to sodium arsenite (a potent inducer of HSF1 activity). Because of this, we reasoned that E189 activity at the exogenous hHsp70 promoter (p2500-CAT) may also be weak compared with arsenite and other stressors. The results of Fig. 5A show this to be the case, as DOX-induced CAT activity in the LHSE-E189 cells was about 50% of the activity obtained in response to heat shock and only about 15% of the activity seen in response to chemical shock with sodium arsenite. To help determine whether reduced activity by E189 was due to relative lack of DNA binding or to a deficiency of transcription activation function by this factor, we compared activation of E189 and endogenous mouse HSF1 by EMSA (Fig. 5B). Here we found that binding to DNA by DOX-expressed E189 was reduced compared with heat shock-activated mHSF1. Thus, it is likely that low E189 activity at endogenous promoters may be due to reduced promoter binding compared with that seen for stress-activated endogenous factor. Taken as a whole, however, our data clearly show that the hHSF1-E189 mutant is capable of replicating several key functions of endogenous HSF1 without the need for stress, albeit at reduced levels.

Expression of hHSF1-E189 Causes Nonstress Potentiation of GR Transcriptional Enhancement Activity

To test the effect of intrinsic HSF1 activity on GR function, we generated the LGRE-E189 cells (Fig. 1) in which DOX-regulated expression of hHSF1-E189 occurs in cells containing the pGRE$_2$E1B-CAT reporter. A time course of DOX exposure was performed in these cells to establish the kinetics of E189 expression (Fig. 6A). The results show an unusual but reproducible pattern in which E189 protein expression is not appreciably detected until 24 h of DOX exposure, with E189 levels remaining constant thereafter (up to 72 h of treatment). Because we could not directly measure

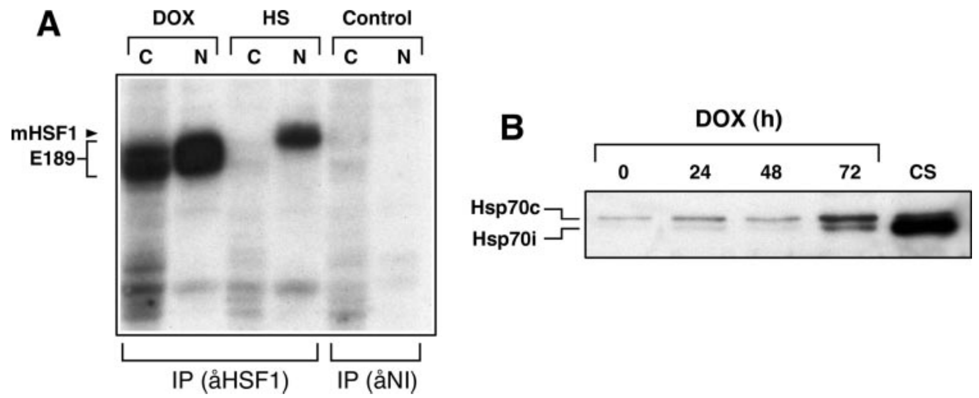

Fig. 4. DOX-Induced Expression of Hsp70 Genes by Constitutively Active E189

A, DOX treatment does not activate endogenous HSF1. LHSE-CAT cells were subjected to DOX for 72 h (DOX) or heat shock at 43 C for 2 h with no recovery (HS). After Dounce homogenization, the cells were analyzed for subcellular localization of HSF1 by immunoadsorption (IP) of cytosolic (C) and nuclear (N) fractions with nonimmune antibody (åNI) or antibody that detects both mouse and human HSF1 (åHSF1). B, DOX increases expression of inducible and constitutive Hsp70 (Hsp70i and Hsp70c, respectively). LHSE-E189-CAT cells were treated with 10 μg/ml DOX for the indicated time or were subjected to chemical shock (CS) with 200 μM sodium arsenite for 2 h and allowed to recover for 24 h. After treatment, Western blot analysis was performed using antibody against the constitutive and inducible forms of Hsp70.

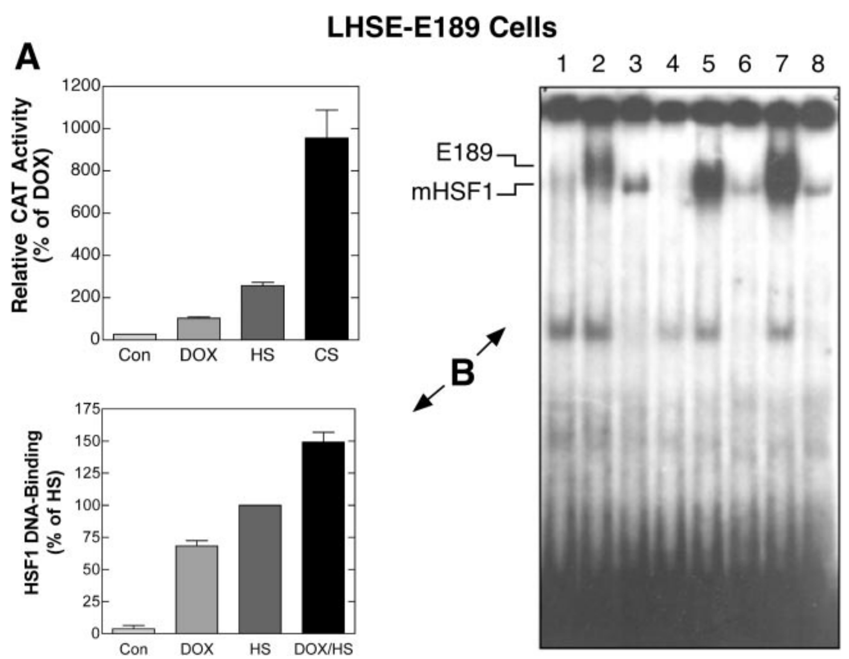

Fig. 5. Comparison of Transcription Activity and DNA-Binding Properties of E189 and Endogenous mHSF1

A, Promoter activities by E189 and mHSF1 in LHSE-E189-CAT cells were measured in response to no treatment (Con), 10 μg/ml DOX for 24 h (DOX), heat shock at 43 C for 2 h (HS), or chemical shock using 200 μM sodium arsenite for 2 h (CS). Stressed cells were allowed to grow for an additional 24 h under normal conditions before harvesting. The results represent the mean $\pm$ SEM of three to six independent experiments. B, DNA-binding activities of E189 and mHSF1 in LHSE-E189-CAT cells were measured by EMSA and subsequent quantitation by densitometric scanning. Cells were subjected to the following conditions. Lane 1, No treatment (Con); lane 2, 10 μg/ml DOX for 24 h (DOX); lane 3, lysates of DOX-treated cells incubated with antibody against HSF1; lane 4, lysates of DOX-treated cells incubated with unlabeled oligonucleotide; lane 5, heat shock at 43 C for 2 h with no recovery (HS); lane 6, lysates of HS-treated cells incubated with antibody against HSF1; lane 7, DOX treatment for 24 h followed by HS (DOX/HS); lane 8, lysates of DOX/HS-treated cells incubated with antibody against HSF1. Results represent the mean $\pm$ SEM of nine independent experiments.

E189 transcriptional activity in these cells, we tested the ability of E189 to localize to the nucleus (Fig. 6B) and to bind DNA (Fig. 6C). As in the LHSE-E189 cells (Figs. 4 and 5), E189 in the LGRE-E189 cells was found both in the cytosolic and nuclear compartments and was capable of binding DNA.

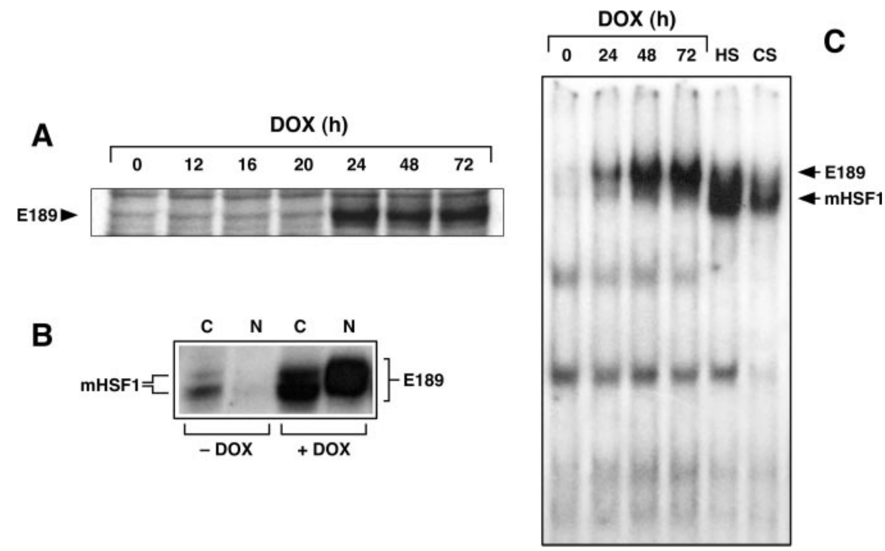

Fig. 6. DOX-Induced E189 Nuclear Expression and DNA Binding in LGRE-E189 Cells

A, LGRE-E189 cells were subjected to a time course of 10 μg/ml DOX followed by Western blot analysis of E189 protein. Results are representative of four independent experiments. B, LGRE-E189 cells were treated with DOX for 72 h (DOX) or vehicle control, followed by Dounce homogenization and analysis for subcellular localization of HSF1. Aliquots of cytosolic (C) and nuclear (N) fractions were immunoabsorbed with antibody against mouse and human HSF1 followed by Western blotting. C, LGRE-E189 cells were treated with 10 μg/ml DOX for 0, 24, 48, and 72 h or were subjected to heat shock (HS) or chemical shock (CS). Cells were harvested immediately after the stress or DOX treatment and analyzed by EMSA. Results are representative of four independent experiments.

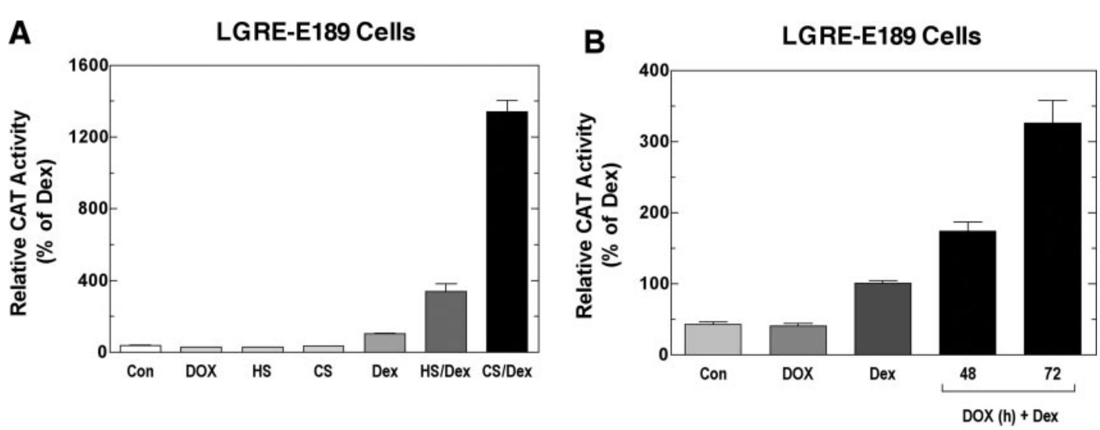

Fig. 7. DOX-Induced E189 Expression Increases GR Transcription Activity

A, LGRE-E189 cells were subjected to no treatment (Con), 10 μg/ml DOX for 72 h (DOX), heat shock for 2 h followed by 24 h of recovery without hormone (HS), chemical shock for 2 h followed by 24 h of recovery without hormone (CS), 1 μM dexamethasone for 24 h (Dex), heat shock for 2 h followed by 1 μM dexamethasone for 24 h (HS/Dex), or chemical shock for 2 h followed by 1 μM dexamethasone for 24 h (CS/Dex). B, LGRE-E189 cells were subjected to no treatment (Con), 10 μg/ml DOX for 72 h (DOX), 1 μM dexamethasone for 24 h (Dex), or 10 μg/ml DOX for 48 and 72 h with 1 μM dexamethasone present during the last 24 h of treatment (DOX + Dex). Each panel shows relative CAT activity from the GR-responsive promoter. Results represent the mean ± SEM of three (panel A) and 12–18 (panel B) independent experiments.

As we have previously shown (14), the response to hormone in LGRE-CAT cells is relatively low due to the intrinsic limitations of the minimal $pGRE_2E1B$-CAT reporter. However, in these same cells the response at this promoter can be dramatically increased when heat shock or arsenite treatment is combined with hormone. It can be seen in Fig. 7A that the LGRE-E189 cells show a similar pattern of responses to hormone and stress conditions, with arsenite typically giving a much higher potentiation of GR activity than heat shock. It should also be noted that no increase in promoter activity is seen in response to heat shock or chemical shock alone (no hormone). In Fig. 7B, we measured GR activity in the same cells under conditions of E189 up-regulation (DOX). An increase in GR activity at the $pGRE_2E1B$-CAT reporter was seen in

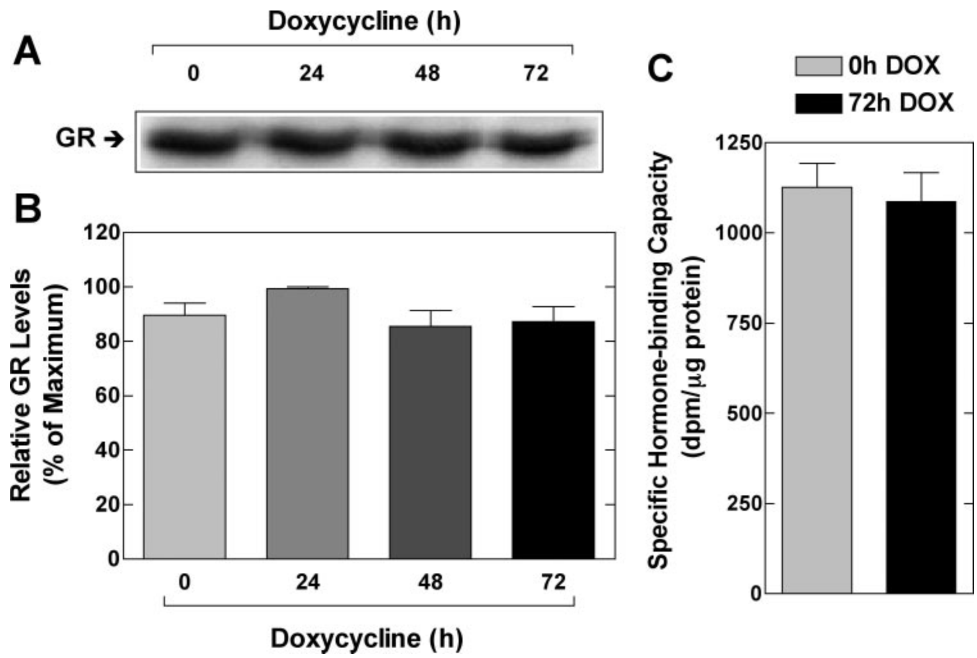

Fig. 8. DOX Induction of E189 Expression Has No Effect on GR Protein Levels or Hormone Binding Capacity

A, LGRE-E189 cells were subjected to a time course of 10 μg/ml DOX, as indicated, followed by Western blot analysis of GR and quantitation by densitometric scanning. Results are the mean SEM of four independent experiments. B, LGRE-E189 cells were subjected to 0 or 72 h of 10 μg/ml DOX, followed by measurement of specific hormone-binding capacity using [^{3}H]dexamethasone. Results are the mean ± SEM of six independent experiments.

response to DOX treatment for 48 h, and the response was even greater at 72 h of treatment. DOX alone had no effect on this activity. Moreover, DOX had no effect on the GR response in the parental LGRE-CAT cells containing no E189 vector (data not shown). Thus, it appears that intrinsic HSF1 activity can indeed control ligand-induced GR responses in these cells. It should be noted that the magnitude of the effect seen at 72 of DOX (Fig. 7B) is starting to approach the level of response seen after heat shock treatment in these same cells (Fig. 7A).

Because HSF1 is known to be the major regulator of Hsp70 and Hsp90 levels in cells (4) and because these Hsps are known to associate with unliganded GR heterocomplexes (3), we reasoned that E189 could be causing potentiation of the GR by altering GR heterocomplexes in a way that leads either to more GR or to GR with increased hormone-binding capacity. Interestingly enough, both of these possible effects of E189 up-regulation were not observed (see Fig. 9). Thus, it is likely that HSF1 is targeting a site of action downstream of the hormone-free GR heterocomplex.

DISCUSSION

We have shown that the E189 mutant under nonstress conditions can effectively replicate most of the key properties of HSF1, including the ability to activate Hsp gene expression at both heterologous (p2500-CAT) and endogenous (Hsp70) genes. In so doing, we have been able to reconcile a long-standing issue with respect to the mechanism by which heat shock and other forms of stress cause enhancement of GR activity, *i.e.* whether HSF1 signaling itself (as opposed to other stress-activated events) was the principal mechanism responsible for GR up-regulation. Although in prior publications we have shown evidence for involvement of HSF1 in the GR potentiation (13, 14), the approaches taken in those studies involved the use of pharmacological agents applied to cells experiencing stress. Thus, a complete separation of all possible stress-activated signal mechanisms from the HSF1 pathway was not possible until the present study. It is now clear that intrinsic HSF1 activity can indeed lead to a potentiation of GR transactivation.

It is still not clear, however, whether the stress effect on GR can be completely explained by HSF1 activity alone. Although the results of Fig. 7 show that DOX up-regulation of E189 yields a level of GR potentiation approaching that seen in response to heat shock, there are simply too many unknown variables in this comparison to allow us to make this claim. Moreover, E189 potentiation of GR is nowhere near as potent as that seen in response to chemical shock (Fig. 7), suggesting that this form of stress, at least, may act on GR signaling by additional mechanisms. An obvious way to resolve this issue would be to assess GR activity after stress in HSF1-deficient cells. However, HSF1 knockout mice, although viable under normal condi-

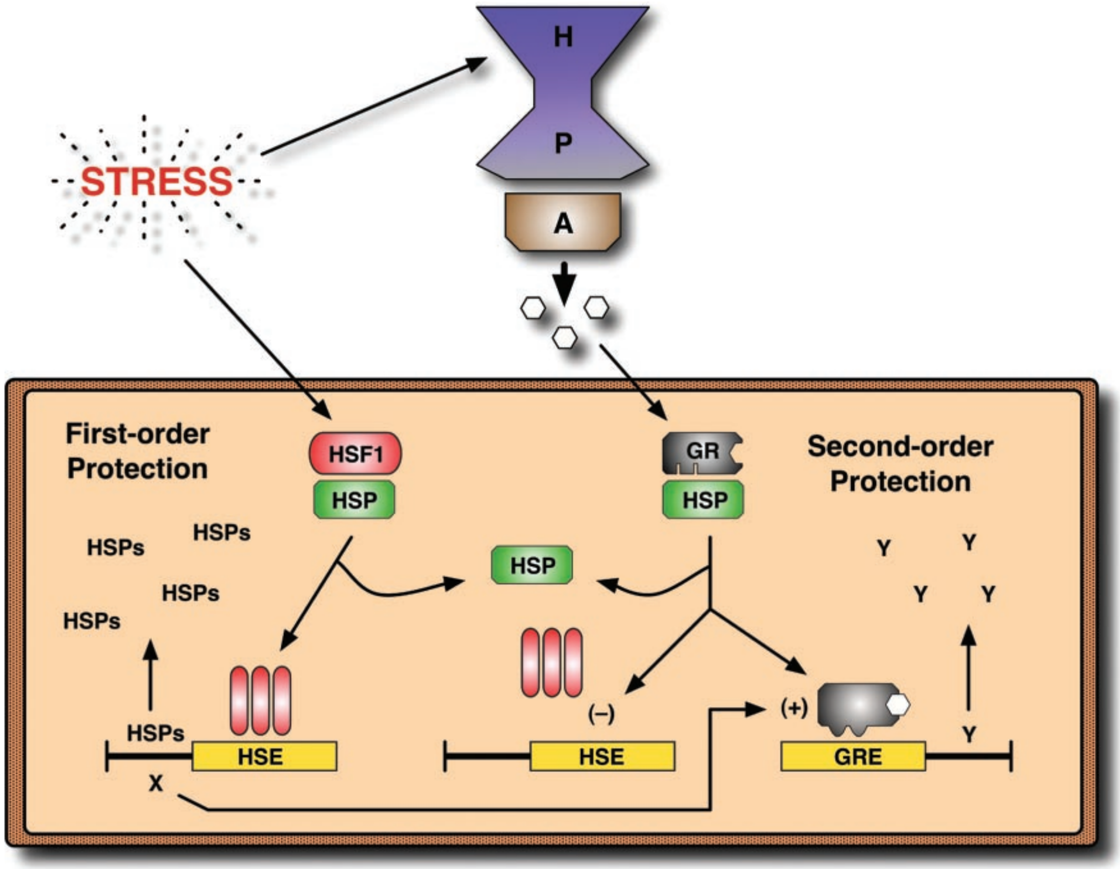

Fig. 9. Model for Reciprocal Regulation of GR and HSF1 Signaling

In this work and in our companion paper (8), we have provided evidence for a complex functional relationship between GR and HSF1 with the following overall properties. In stressed cells, activation of GR signaling causes rapid inactivation of HSF1 by blocking its actions at the promoter of Hsp70 and probably other Hsp genes. Meanwhile, the stress event also leads to a stimulation of GR transcription enhancement activity that requires, at least in part, functional HSF1. As detailed in the *Discussion*, we believe that the best model by which to understand these seemingly opposing processes is one in which full recovery of cells after a stress event occurs through precise, ordered activities on the part of HSF1 and the GR. According to this model, the initial stress event is likely to occur under conditions of low glucocorticoid hormone concentration. Under these conditions, HSF1 action would be unimpeded and would provide "first-order protection," principally through up-regulation of molecular chaperones (Hsps). At some later point, the stress event is likely to lead to elevated secretion of glucocorticoids as controlled by the hypothalamus-pituitary-adrenal (HPA) axis. Glucocorticoid action at the stressed cell at this stage of recovery is likely to be 2-fold: rapid inactivation of HSF1 (to prevent overstimulation) and production of gene products (Y) that likely serve to complete the reestablishment of cellular homeostasis. We call the latter effect "second-order protection," and it should be noted that one or more gene products (X) produced by HSF1 earlier in the stress response are likely to serve as facilitators of this GR activity.

tions, show high levels of lethality in response to certain stressors, such as endotoxin challenge (19), suggesting that viability under stress conditions may be problematic. In contrast, primary fibroblasts derived from HSF1 −/− animals (20) or cell lines transfected with dominant-negative HSF1 (21) are able to survive moderate stress events, principally because basal levels of constitutive Hsp70 are unchanged. For this reason, follow-up experiments using these or similar cells are under consideration.

Because HSF1 is a transcription factor best known for its regulation of Hsp90 and Hsp70 expression, our results with E189 would suggest HSF1-induced gene expression as the most likely mechanism causing stress potentiation of GR. Aside from this fact, other aspects of our data support this conclusion. First and foremost is that fact that an apparent delay exists between expression of E189 protein, activation of its DNA-binding activity, and the potentiation effect on the GR. In Fig. 6A, DOX-induced expression of E189 is not detectable until 24 h and remains steady thru 48 and 72 h of DOX exposure. This atypical kinetic pattern is highly reproducible in the LGRE-E189 cells (data not shown), in contrast to the more typical incremental increase seen in the LHSE-E189 cells (Fig. 3). Although maximal expression of E189 protein occurs at about 24 h, maximal DNA-binding activity by E189 is not seen until 48 h (Fig. 6C), suggesting that additional posttranslational steps are required for activation of E189. (Although we do not know what they may be, a reasonable assumption is that the trimerization step needed for activation of HSF1 is involved.) In contrast, potentiation of GR-mediated CAT activity by E189 is relatively weak at 48 h of DOX but becomes clearly established at 72 h, suggesting that additional time post-DNA-binding is needed for HSF1 to exerts its effects on the receptor. One caveat, however, that must be considered is that the perceived delay between DNA binding by E189 and GR CAT activity could be due to a slow rate of expression for CAT enzyme. However, we have measured stress potentiation of GR in similar cells in as little as 4 h of exposure to hormone (22). Thus, response by this reporter construct can be rapid when activated by receptor under stress conditions. Lastly, the kinetics of expression for endogenous Hsp70i (Fig. 4B) also support this model, as detectable amounts of this protein (albeit in the LHSE-E189 cells) are not seen until 72 h of exposure to DOX. In summary, these observations are consistent with a model in which potentiation of GR cannot occur until E189 rather inefficiently causes up-regulation of endogenous products. We have also considered a more remote possibility, *i.e*. a direct or indirect protein-protein interaction between GR and HSF1. Unfortunately, efforts to show this interaction by coimmunoprecipitation experiments have been inconclusive (our unpublished results).

If an HSF1-induced product is responsible for the stress potentiation of GR, it would be logical to predict that one of the major known heat shock proteins would serve this function. Because Hsp70 and Hsp90 are known to regulate assembly of GR heterocomplexes (23) and the ability of receptor to bind hormone (24), we reasoned that HSF1-induced changes to GR cytoplasmic heterocomplexes could explain increased activity by the receptor. However, this mechanism now seems less likely, as E189 potentiation of GR transcription activity occurred without any changes to GR expression levels or overall hormone-binding function (Fig. 8). Yet, it remains a possibility that GR complexes are altered in a way that can still affect transactivation without changing hormone-binding capacity. One such mechanism is through up-regulation of immunophilins, such as FK506-binding protein-52 and cyclophilin-40, both of which show increased expression after stress (25, 26). In the case of FK506-binding protein-52, this protein appears to play a role in the targeting of GR to the nucleus after the hormone-binding event (27, 28). Of course, it is also possible that an HSF1-regulated gene will control GR at any number of other steps in the GR signal pathway, including the transactivation stage. Our demonstration here that E189 activity under nonstress conditions can essentially replicate the stress potentiation of GR will now make it easier to identify this HSF1-induced product(s), *e.g*. through use of genomic or proteomic approaches.

If HSF1 can indeed stimulate GR activity, what may be the cellular or physiological significance of this event? Although we do not yet have an answer to this question, one possible explanation is that GR activity under stress serves to promote cell survival and that heat shock signaling stimulates this activity. In this sense, GR may serve a similar function to HSF1, whose role in protecting against stress-induced cellular lethality is well documented (29). Although studies showing a protective role of glucocorticoids are numerous [see reviews by Munck and colleagues (5, 6)], an interesting example is the ability of glucocorticoid agonists (in the absence of stress) to induce a state of thermotolerance similar to that seen when cells are subjected to a conditioning, sublethal heat stress (30, 31). In our laboratory, we have observed that combined stress and glucocorticoid treatment leads to a rate of cell survival that is dramatically higher than that seen after stress treatment alone (our unpublished observations). Thus, by the measure of cellular viability, a synergistic relationship between the heat shock response and GR activity does appear to occur.

However, the simplicity of the above model must be tempered by our concurrent observation that glucocorticoid agonists appear to have an inhibitory effect on the heat shock response itself, principally by inhibiting the ability of HSF1 to act as a transcription factor (8). How, therefore, can these seemingly contradictory phenomena be reconciled? One explanation is that HSF1 potentiation of GR is simply a mechanism by which to ensure its own down-regulation (potentiated negative feedback). Yet, the rapid nature of GR actions on HSF1 makes this mechanism unlikely (8). Moreover, the feedback model does not explain how HSF1 can cause potentiation of GR when it is being simultaneously inhibited. A potential, albeit quantitative, solution to this problem is that glucocorticoid inhibition of HSF1 activity is not 100% effective. Typically, about 30% of HSF1 activity remains when glucocorticoid treatment occurs before the stress event, even at a concentration of 1 μM dexamethasone (7). Thus, under the most stringent conditions, enough HSF1 activity may remain to cause the actions on GR documented in this work. In most experiments of this kind, however, we typically add the hormone after the stress event, as this appears to yield a higher potentiation effect on the receptor, although a rigorous investigation of this has proven difficult to do in a way that maintains both equal exposure time to hormone and equal recovery time after stress.

The above issues aside, we believe that a more relevant model involves intertwined actions of HSF1 and GR that are not simultaneous (Fig. 9). In most experiments designed to inhibit HSF1, we have added hormone to cells at or before the time of stress. It is likely that such treatment is an artificial condition that most cells do not experience in a physiological context. Instead, the stress event is likely to occur first in an environment of relatively low glucocorticoid hormone concentration. In this case, the heat shock response in affected tissues would proceed uninhibited until the stress event triggers a rise in glucocorticoid secretion as controlled by the hypothalamus-pituitary-adrenal axis, a result that has indeed been observed in rats exposed to restraint stress (32). Elevated hormone levels would then lead secondarily to a rapid attenuation of the heat shock response, presumably to prevent overstimulation by this response, or to provide an alternative mechanism of cell survival, or both. Yet at this point in the course of events, HSF1-controlled genes responsible for potentiation of GR activity would have already been expressed, producing an elevated response to hormone that most likely serves to restore normal cellular functioning through gene products that cannot be produced by the heat shock pathway itself. One way to look at this model is that the heat shock response is the cell's initial survival mechanism that, in addition to producing protective heat shock proteins, also serves to prime optimal response for a later-acting survival mechanism mediated by the GR, a mechanism that involves rapid moderation of the heat shock response itself and increased production of gene products that likely serve to reestablish cellular homeostasis.

Although many aspects of the above model remain to be confirmed, we do know that the peak time for either heat or chemical shock potentiation of GR occurs approximately 16 h into the recovery period (22), kinetics that are consistent with a temporal pattern in which the protective role of hormone follows the initial stress event. Lastly, we believe that our elucidation of this complex relationship between GR and HSF1 has important implications for the treatment of disorders arising from pathophysiological stress, especially if novel GR-regulated genes can be identified with primary roles in the restoration of cellular homeostasis.

MATERIALS AND METHODS

Materials

[^{3}H]Dexamethasone (NET467; 42.8 Ci/mmol), [^{3}H]acetate (10.3 μCi/mmol), and ^{125}I-labeled conjugates of goat antimouse IgG (NET159; 11.8 μCi/μg) and goat antirabbit IgG (NET155; 9.0 μCi/μg) were obtained from New England Nuclear (Boston, MA). DOX, ATP, dimethylsulfoxide, sodium arsenite, dexamethasone, G418 (Geneticin) antibiotic, hygromycin, acetyl-coenzyme A (CoA) synthetase, acetyl CoA, Tris, HEPES, EDTA, protein A-Sepharose, protein G-Sepharose, and DMEM-powdered medium were from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase conjugates of goat antimouse and goat antirabbit IgG were from Calbiochem (La Jolla, CA). Iron-supplemented newborn calf serum was from Hyclone Laboratories, Inc. (Logan, UT). Imobilon polyvinylidine difluoride membranes were obtained from Millipore Corp. (Bedford, MA). GenePorter transfection reagent was obtained from Gene Therapy Systems, Inc. FiGR mouse monoclonal antibody against GR (33) was a gift from Jack Bodwell (Dartmouth Medical School, Hanover, NH) and was expressed and affinity purified by Biocon (Rockville, MD). The Stressgen (Victoria, British Columbia, Canada) SPA-812 antibody was used to detect the inducible and constitutive forms of Hsp70. To analyze HSF1, several antibodies were employed. Neomarker's HSF1-AB4 antibody (Fremont, CA) was used to detect both mouse and hHSF1, whereas the PA3–017 (Affinity BioReagents, Inc., Golden, CO) and the SPA-901 (Stressgen) antibodies showed selectivity for hHSF1. Rat monoclonal antibody against hHSF1 (HSF1-AB4) was purchased from Neomarkers. The PA3–017 antibody against mouse HSF1 was from Affinity BioReagents, whereas the SPA-901 antibody recognizing mouse and human HSF1 was from Stressgen. Technical grade rat IgG and mouse IgG2a were bought from Sigma.

In the p2500-CAT reporter used in this study, expression of CAT is controlled by the hHsp70 promoter. This promoter contains consensus heat shock elements (HSEs) that are activated by binding of HSF1 (34). The pGRE$_2$E1B-CAT minimal reporter is composed of two synthetic GREs derived from the tyrosine aminotransferase promoter linked to the adenovirus E1B TATA sequence (35). The pBI-EGFP vector was obtained from CLONTECH Laboratories, Inc. (Palo Alto, CA). In this vector, expression is controlled by a tetracycline-response element and two minimal cytomegalovirus promoters arranged in opposite orientations. Expression of enhanced GFP was used to isolate positive cell colonies. The pUHD172–1hygro vector (18), expressing the reverse tet transactivator and hygromycin resistance genes, was obtained from Hermann Bujard (Universitat Heidelberg, Heidelberg, Germany). The cDNA for the E189 mutant of hHSF1 (16) was the generous gift of Richard Voellmy.

Transfection of Cell Lines

The LHSE-CAT and LGRE-CAT cell lines were established as previously described (10, 13). Briefly, mouse L929 cells were cotransfected with pSV2neo and a 2-fold excess of p2500-CAT (to yield LHSE-CAT cells) or pGRE$_2$E1B-CAT (to yield LGRE-CAT cells) using GenePorter as carrier. This was followed by selection for stably transfected, cloned cell lines using G418 (Geneticin) antibiotic at 0.4 mg/ml. Once established, each cell line was grown in an atmosphere of 5% CO_2 at 37 C in DMEM containing 0.2 mg/ml G418 and 10% iron-supplemented newborn calf serum. The tetracycline-inducible LHSE-E189 and LGRE-E189 cells were made by stably transfecting LHSE-CAT or LGRE-CAT cells with the pUHD172–1hygro plasmid and a 7-fold excess of pBI-E189 plasmid, followed by selection and cloning using 0.4 μg/ml hygromycin. The pBI-E189 construct was made by excising the cDNA for the constitutively active hHSF1-E189 mutant from the pGEM-E189 vector originally developed by Voellmy and co-workers (36). This cDNA was then inserted into the multiple cloning site of the pBI-EGFP vector (CLONTECH).

Stress Treatment of Cell Lines

For all experiments, the newborn calf serum was stripped of endogenous steroids by extraction with dextran-coated charcoal. Most stress experiments were performed on cells that were at or near confluence; although similar results were obtained with subconfluent cultures. Heat shock treatment was achieved by shifting replicate flasks to a second 5% CO_2 incubator set at 43 C. Duration of heat shock treatment was 2 h, or as indicated. Cells were also subjected to chemical shock by addition of 200 μM sodium arsenite to the medium. In the chemical shock experiments, the arsenite-treated and nontreated cells were incubated at 37 C for 2 h and were then washed with DMEM and allowed to recover, or were harvested immediately after stress.

Chromatin Immunoprecipitation Assay

To detect binding of HSF1 to the hHsp70 promoter *in vivo*, chromatin immunoprecipitation assay was performed according to the method of Nissen and Yamamoto (37) with some modifications. Briefly, replicate flasks of LHSE-E189 cells were treated as described in the legend to Fig. 2, followed by cross-linking with formaldehyde and preparation of nuclear extracts. After sonication, crude fragments of protein-linked chromatin were further subjected to immunoprecipitation using an antibody specific to hHSF1 or an equivalent amount of nonimmune serum as control, followed by immobilization on protein G sepharose. The samples were washed, and then digested with proteinase K, and cross-links were reversed by heating. DNA was extracted and purified and subjected to 25 cycles of PCR. The 20-bp forward primer 5′-GGA AGG TGC GGG AAG GTT CG-3′ was designed to bind at −75 of upper strand of the hHsp70 promoter used in the p2500-CAT reporter. The backward primer 5′-TTC TTG TCG GAT GCT GGA-3′ was chosen to bind at +110 of the lower strand. The size of product obtained was 185 bp. PCR products were run on 2% agarose gels containing ethidium bromide and photographed.

Fractionation, Immune Purification, and Western Blotting

In the experiments of Figs. 4A and 6B, cells were fractionated into cytosolic and nuclear portions by Dounce A homogenization in hypotonic buffer, followed by centrifugation at 1000 × *g*. The cytosolic fractions were saved and the nuclear pellets were washed two times by resuspension and pelleting in hypotonic buffer. Hypotonic buffer containing 0.5 M NaCl was added to the pellet fractions and incubated on ice with occasional vortexing for 1 h. After salt extraction, the nuclear pellets were centrifuged at 14,000 × *g* and the supernatants were saved. Cytosolic and nuclear fractions were adsorbed in batch to protein A-Sepharose, using the HSF1-AB4 antibody recognizing both the human and mouse forms of HSF1. Sepharose pellets were washed with TEG buffer (10 mM TES, 1 mM EDTA, 10% glycerol, 50 mM NaCl, 10 mM sodium molybdate; pH 7.6) and eluted with 2× sodium dodecyl sulfate sample buffer.

In the experiments of Figs. 2A, 3A, 4B, 6A, and 8A, whole-cell extracts were prepared by freezing of cells at −80 C and resuspension in WCE buffer (20 mM HEPES, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.5 mM dithiothreitol; pH 7.9) followed by centrifugation at 100,000 × *g* for 10 min.

All samples were resolved by electrophoresis in 10% polyacrylamide sodium dodecyl sulfate gels, followed by transfer to Imobilon polyvinylidine difluoride membranes. The relative amounts of hHSF1-E189, endogenous mouse HSF1, or GR were determined via a Western blotting technique previously described (38), employing primary antibody and both peroxidase- and ^{125}I-conjugated counter antibodies. After color development, the blots were exposed to Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY) with an intensifying screen at −80 C. Relative amounts of proteins were determined by densitometric scanning of films using a Molecular Dynamics (Sunnyvale, CA) scanner and software.

CAT Assay

Measurement of CAT enzyme activity was performed according to the method of Nordeen *et al.* (39) with minor modifications. In this assay, a reaction mixture containing acetyl CoA synthetase, [^{3}H]sodium acetate, CoA, and ATP is briefly preincubated to enzymatically generate labeled acetyl CoA from CoA and labeled acetate. Acetylation of chloramphenicol was then initiated by addition of cell lysate containing CAT enzyme. The reaction was stopped by extraction with cold benzene, and 75% of the organic phase was counted. Cell lysates were prepared by sequential freezing and thawing in 0.25 M Tris, 5 mM EDTA (pH 7.5) and centrifugation at 14,000 × *g*. Aliquots of lysate containing equal protein content were added to the enzymatic reaction mixtures. As the HSE- and GRE-containing promoters employed in this study have distinct basal and inducible activities, all data are represented as percent of control, maximum, or the equivalent. In this way, the relative effects of each treatment can be readily seen.

EMSA

EMSA assays for HSF1 were performed according to the protocol of Mosser *et al.* (40), with minor modifications. Briefly, cells were harvested, centrifuged, and rapidly frozen at −80 C. The frozen pellets were resuspended in WCE buffer and centrifuged at 100,000 × *g* for 10 min. The supernatants were either stored at −80 C or used immediately. EMSA was performed by mixing 10 μg of whole cell extract with 0.1 ng (50,000 cpm) of ^{32}P-labeled HSE oligonucleotide (5′-GAT CTC GGC TGG AAT ATT CCC GAC CTG GCA GCC GA-3′) and 1.0 μg poly (dI-dC) in 10 mM Tris (pH 7.8), 50 mM NaCl, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, in a final volume 25 μl. For competition experiments, the binding reactions contained 0.1 ng of the ^{32}P-labeled HSE and a 100-fold molar excess of unlabeled HSE. Reactions were incubated at 25 C for 30 min and loaded onto 4% polyacrylamide gels in 0.5× Tris-borate-EDTA. The gels were run at room temperature for 1.5 h at 150 V and were exposed to Kodak XAR-5 film with an intensifying screen at −80 C. The relative amounts of probe-bound HSF1 were measured by densitometric scanning of the film using the Bio-Rad Molecular Analyst system (Bio-Rad Laboratories, Inc., Hercules, CA).

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