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Agonist-Activated Glucocorticoid Receptor Inhibits Binding of Heat Shock Factor 1 to the Heat Shock Protein 70 Promoter *in Vivo*

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We have previously shown that activation of glucocorticoid receptor (GR) signaling in stressed cells will cause inhibition of the heat shock response as mediated by heat shock transcription factor 1 (HSF1). In that work, a full-length human heat shock protein 70 (Hsp70) promoter was used to measure HSF1 transactivity, and the data suggested inhibition of HSF1 through the transactivation or transrepressive properties of GR. Here, we show that the inhibitory effect of glucocorticoid agonist (dexamethasone) upon Hsp70 promoter activity is rapid, occurring within 1 h of hormone addition. Moreover, addition of hormone during the first hour of recovery from stress was sufficient to inhibit HSF1. Thus, dexamethasone is able to rapidly reverse HSF1 transactivity, suggesting a transrepressive mode of action for GR. Yet, GR transrepression of HSF1 by analysis of putative negative glucocorticoid response elements in the Hsp70 promoter was not found. To further investigate the *in vivo* nature of this fast-acting mechanism, we used the chromatin immunoprecipitation assay with primers specific to the human Hsp70 promoter. Dexamethasone inhibited HSF1 binding at the Hsp70 promoter in response to heat or chemical shock (sodium arsenite). Moreover, dexamethasone also blocked promoter binding by a constitutively active mutant of HSF1 (hHSF1-E189) expressed under nonstress conditions. In all cases, inhibition of HSF1 recruitment to the promoter by dexamethasone was blocked by the GR antagonist RU486, a result that was consistent with promoter activity based on chloramphenicol acetyl transferase gene expression. The ability of dexamethasone to prevent HSF1 recruitment to the promoter was fast acting (occurring in as little as 15 min), and the hormone also caused release of HSF1 already bound to the promoter. Although these results suggest GR can effectively prevent HSF1 binding to Hsp promoters, fractionation and Western blot experiments showed that stress-activated HSF1 was not released from the nucleus in response to hormone. Thus, this effect of dexamethasone is either specific to the Hsp70 promoter or causes shunting of HSF1 to other high-affinity nuclear sites. These observations provide evidence of a novel mechanism for attenuation of the heat shock response by glucocorticoids: prevention or reversal of HSF1 recruitment to Hsp promoters through the rapid actions of GR. (*Molecular Endocrinology* 18: 500–508, 2004)

THE GLUCOCORTICOID RECEPTOR (GR) is a steroid-activated transcription factor (1) that is commonly thought to serve a protective function against stress. Indeed, the notion of glucocorticoids as stress hormones is an old one, with many examples in physiology serving to support this model. In comprehensive reviews on this subject by Munck and colleagues (2, 3), an evolving picture of the complex roles of glucocorticoids in stress responses has been provided. For example, these hormones can serve both a stimulatory function (such as in facilitation of cardiovascular responses to stress) and a suppressive function (as seen in the well-known ability of these hormones to moderate antiinflammatory and immune responses to stress).

Interestingly enough, the role of glucocorticoids with respect to the heat shock response, one of the most conserved and universal of stress responses, has only recently been uncovered. In work by our laboratory, it has been shown that glucocorticoid hormones are suppressive with respect to the heat shock response (4). This was demonstrated by the ability of a glucocorticoid agonist, dexamethasone (Dex), to inhibit heat shock protein (Hsp) expression in cells subjected to stress and to inhibit the transcriptional enhancement activity of heat shock transcription factor 1 (HSF1). Because this effect was not seen in GR-deficient cells, or in cells expressing certain GR mutants, it was also shown that the action of these hormones on the heat shock response required a GR-signaling mechanism.

Abbreviations: CAT, Chloramphenicol acetyl transferase; ChIP, chromatin immunoprecipitation; CoA, coenzyme A; Dex, dexamethasone; DOX, doxycycline; GR, glucocorticoid receptor; HSE, heat shock response element; HSF1, heat shock factor 1; Hsp, heat shock protein; MMTV, mouse mammary tumor virus; nGRE, negative glucocorticoid response element; SDS, sodium dodecyl sulfate.

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In this work, we extend upon these observations by showing that GR-mediated modulation of HSF1 activity is a fast-acting mechanism, occurring in as little as 15 min. We also show that GR acts on HSF1 by preventing and reversing its ability to interact with Hsp promoters within the intact cell. Such a mechanism appears to be unique with respect to GR transrepressive properties, as all other mechanisms do not appear to interfere with promoter occupancy by the affected target transcription factors.

RESULTS

Dex Inhibition of HSF1 Activity at the Hsp70 Promoter Is a Fast-Acting Effect

In earlier work, we showed that the glucocorticoid agonist, Dex, could effectively inhibit HSF1 activity at the Hsp70 promoter and that this effect was blocked by RU486 antagonist. In those studies, promoter activity was measured based on chloramphenicol acetyl transferase (CAT) gene expression. Typically, recovery of cells in the presence of Dex for a minimum of 20 h was allowed to occur before measurement of CAT enzyme levels. Thus, it was not possible to determine whether the hormone effect was occurring early or late during the heat shock response. For this reason, we also could not determine, based on the reporter gene data, whether GR-mediated repression occurred via the transrepressive or transactivation properties of the receptor. In this work, we reasoned that transrepressive actions of GR should, as a general rule, be faster acting than effects requiring GR-mediated gene expression (transactivation). To test this hypothesis, we measured the effects of Dex on expression of Hsp70 mRNA and protein during the early stages of the stress response (Fig. 1). The results show effective Dex inhibition of both Hsp70 mRNA and protein even during the first 2 h of heat shock.

To corroborate these results, we refined our CAT assay so that measurement of HSF1-based Hsp70 promoter activity could be measured in as little as 3 h of total treatment time. Our initial use of this approach was performed in the LHSE-CAT cells (full-length p2500-CAT reporter) and the results are seen in Fig, 2. In this experiment, LHSE-CAT cells were pretreated with Dex for increasing amounts of time before a 2 h heat shock treatment, followed by 1 h of recovery before assay for CAT (Fig. 2A). It was found that Dex had a rapid effect on HSF1 activity. In fact, the greatest inhibition of HSF1 occurred when Dex was added immediately at the beginning of the heat shock treatment. Much to our surprise, it was also found that the inhibitory effect of Dex gradually disappeared with increasing time of pretreatment, with the inhibitory effect completely gone by 20 h of pretreatment. Although we cannot, at present, provide data to explain why the inhibitory effect should be lost with time, it is clear that the rapid effects of Dex noted are highly reproducible. Moreover, the magnitude of the inhibition (70% in Fig. 2A) is almost identical to the magnitude of inhibition seen (75%) when Dex is added during the typical 20-h recovery period (4). Thus, it appears that the fast-acting effects of Dex can completely account for the entirety of the response.

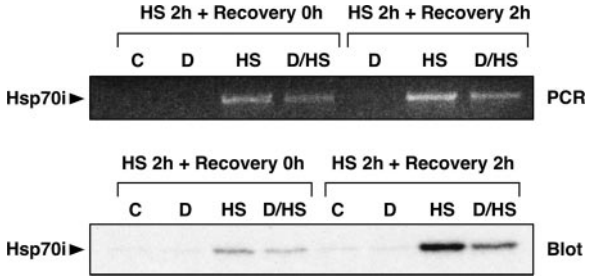

Fig. 1. Dex Rapidly Inhibits Heat Shock (HS)-Induced Expression of Hsp70 mRNA (Inducible Gene) and Protein

Replicate flasks of L929 cells were subjected to combined stress and hormone treatment, as indicated, followed by assay for Hsp70 mRNA (PCR) and protein (Blot). C, No treatment; D, Dex (1 μM) for 2 or 4 h; HS, 43 C for 2 h; D/HS, Dex (1 μM) during heat shock and recovery.

If Dex could act in such a rapid fashion, it now became possible to ask whether Dex could cause reversal of HSF1 activity by adding the hormone only during a very brief recovery period. As seen in Fig. 2B, addition of Dex during the recovery period was about as effective in repression of HSF1 activity as was addition of Dex during the heat shock period alone or during the combined heat shock and recovery. In fact, Dex exposure for as little as 1 h after heat shock was an effective treatment. Thus, we tentatively conclude that hormonal activation of GR can reverse stress-activated HSF1 activity at the Hsp70 promoter.

Analysis of Putative Inhibitory GR Response Elements in the Hsp70 Promoter

Based on the rapid effects found in Figs. 1 and 2, we reasoned that GR-mediated transrepression was the most likely mechanism by which inhibition of HSF1 activity was occurring. As the GR is well known to act as a repressor by binding to so-called negative glucocorticoid response elements (nGREs), we searched the human (h) Hsp70 promoter for binding elements with homology to known nGREs (5–8). Five such putative elements were found in the hHsp70 promoter sequence, as described by Voellmy and co-workers (9). These putative nGREs ranged from 75% to 88% homology based on half-recognition sites (Fig. 3A). To determine the potential involvement of these sites, we obtained a series of deletion constructs derived from the same intact hHsp70 promoter used in Fig. 2 and in our prior studies (4). These deletion constructs controlled expression of CAT and were stably transfected into mouse L929 cells. The results of Fig. 3B show that all of these constructs respond to hormone and heat shock in the same manner as does the intact p2500

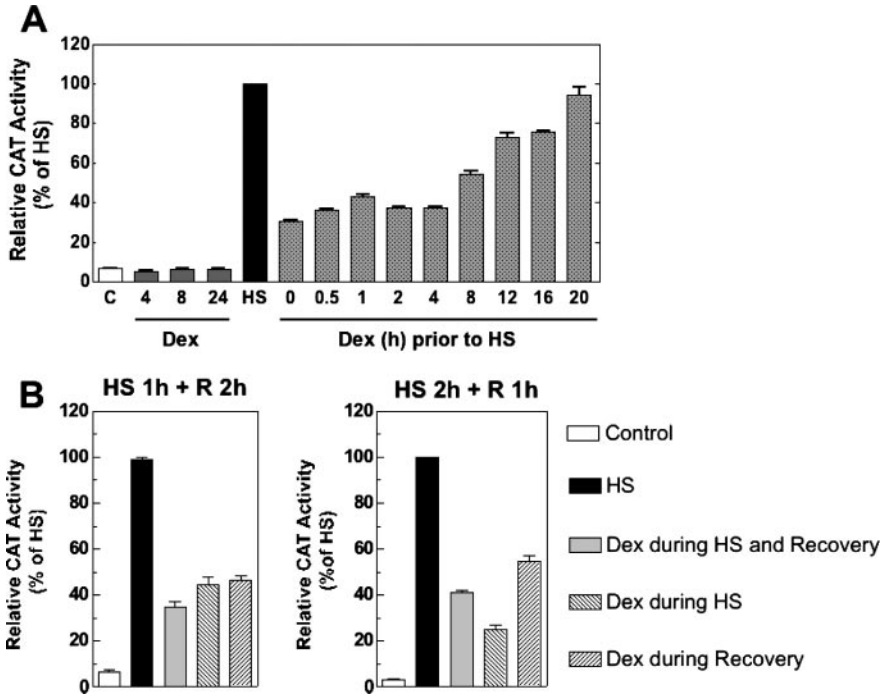

Fig. 2. Dex Causes Rapid Repression of HSF1 Transcriptional Activity at the Hsp70 Promoter

A, Dex inhibits HSF1 when added during or just before the stress event. Replicate flasks of L929 cells stably transfected with the p2500-CAT reporter (LHSE cells) were subjected to a time course of treatment with Dex (1 μM), as indicated, followed by heat shock (HS) (43 C for 2 h) and 1 h of recovery. Cells were harvested at the end of recovery and assayed for CAT activity. The results represent means ± SEM of three independent experiments. B, Dex inhibits HSF1 when added during a short 1-h recovery period. Replicate flasks of LHSE cells were subjected to various temporal combinations of HS (43 C), recovery, and Dex (1 μM), as indicated. After recovery, the cells were assayed for CAT activity. The results represent means ± SEM of six independent experiments. Control, No treatment; HS, 43 C for 1 or 2 h; R, recovery for 1 or 2 h; Dex during HS and recovery, Dex (1 μM) during HS and recovery in the continued presence of Dex; Dex during HS, Dex (1 μM) during HS, followed by recovery in absence of Dex; Dex during recovery, HS followed by Dex (1 μM) treatment during recovery.

promoter: inhibition of HSF1 activity by Dex and blockade of this effect by RU486. Because inhibition of HSF1 was seen with the shortest of these deletions (p146-CAT), which contains a putative nGRE at position −110, it can be argued that this site may be essential for this effect. Based on the results that follow, however, we do not believe that this site serves this purpose.

Rapid Effects of Glucocorticoid Agonist to Prevent and Reverse Binding of HSF1 to the Hsp70 Promoter

The results of Fig. 2 provide evidence that Dex can prevent and reverse HSF1 transcriptional activity under stress conditions. In our previous study (4), we used EMSAs to show that Dex did not prevent binding of HSF1 to DNA oligonucleotides containing consensus heat shock response elements (HSEs). Taken together, this would suggest that GR-mediated signaling acts to suppress the transactivation properties of HSF1 while it is still localized to its high-affinity promoter sites. Recently, the chromatin immunoprecipitation assay (ChIP) has been shown to be a more precise and, arguably, more physiologically relevant assay that can measure actual promoter occupancy under intact cell conditions (10–15). We, therefore, have used this assay to measure binding of HSF1 to the hHsp70 promoter in our intact LHSE-CAT cells. In our system, primers were designed to span a proximal portion of the hHsp70 promoter containing two active HSE sites, as well as the start site of transcription (Fig. 4A). After cross-linking and fragmentation of the chromatin, immunoprecipitation was performed with an antibody against HSF1 that recognizes the endogenous mouse form of this protein. Our results show occupancy of this promoter by heat shock-activated HSF1. Similar ChIP assays performed on the parental L929 cells showed no detectable PCR product (data not shown). Moreover, ChIP assays using primers to the endogenous mouse actin promoter showed no PCR product when the antibody against HSF1 was used for immunoprecipitation (Fig. 4B). Thus, the results of Fig. 4A are specific to the stably transfected p2500-CAT reporter used in this and prior studies to measure HSF1 activity.

To test the effect of GR-mediated signaling on HSF1 occupancy of the Hsp70 promoter, we repeated the ChIP assay of Fig. 4 but used a 4-h pretreatment with

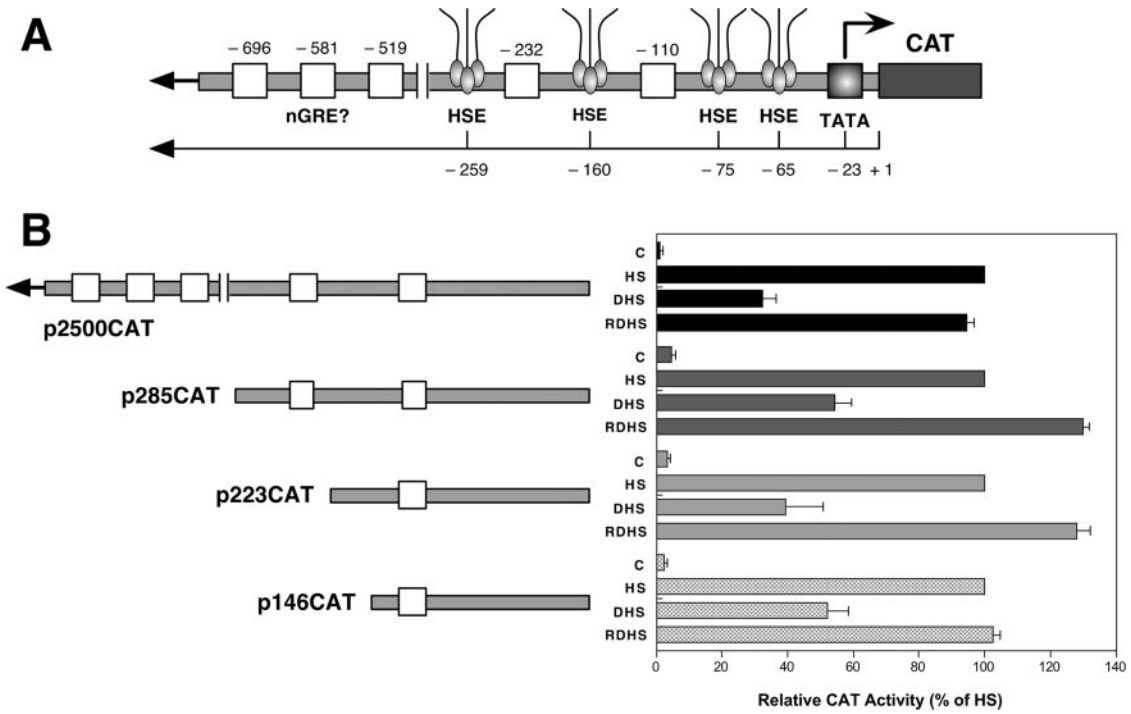

Fig. 3. Contribution of Putative nGRE Sequences within the hHsp70 Promoter to the Inhibitory Effect of Dex on HSF1

A, Representation of the hHsp70 promoter indicating the relative positions of established HSEs and putative nGRE sequences (□). B, Effect of Dex on HSF1 transactivity using various truncation constructs of the Hsp70 promoter. Replicate flasks of L929 cells stably expressing various deletion constructs of Hsp70 promoter linked to CAT reporter gene were subjected to a combination of stress and hormone treatments. Following heat shock (HS), all cells were allowed to recover for 1 h before assay for CAT. The results represent means ± SEM of six independent experiments. C, No treatment; HS, 43 C for 2 h followed by 1 h recovery; DHS, Dex (1 μM) during 2 h of HS and 1 h of recovery; RDHS, RU486 (1 μM) and Dex (1 μM) for 2 h during HS and 1 h of recovery.

hormone. The results show heat shock-induced occupancy of the promoter by HSF1 (Fig. 5A), as expected. To our surprise, Dex pretreatment resulted in loss of promoter binding by HSF1. To corroborate these results, similar experiments were performed using chemical shock (sodium arsenite) as the form of stress. Here too, promoter occupancy by HSF1 was blocked by Dex (Fig. 5B). As a further test, we performed the same ChIP assay on L929 cells that contain stably integrated p2500-CAT reporter and a constitutively active form of human HSF1 (hHSF1-E189). In these cells (LHSE-E189 cells), expression of hHSF1-E189 is under the control of a doxycycline (DOX)-inducible vector. We have previously shown DOX-induced hHSF1-E189 activity in these cells under nonstress conditions and that this activity can be blocked by Dex (4). The results demonstrate DOX-induced occupancy of the Hsp70 promoter and that this occupancy can be blocked by Dex (Fig. 5C). It should be noted that Dex inhibition of promoter occupancy by HSF1 was effectively reversed by RU486 under all of the above conditions. This observation was an important test of the relevancy of the ChIP assay to our system, as all of our data to date using various CAT reporters (Fig. 3 and Ref. 4) show that Dex inhibition of HSF1 transactivity is antagonized by RU486.

In the above ChIP experiments, Dex was added 4 h before heat shock and remained in the medium for the 2 h of stress treatment. According to the results of Fig. 2, this condition of Dex treatment should result in effective inhibition of HSF1 transactivity at the p2500-CAT promoter. However, the results of Fig. 2 also showed that treatment with Dex for only 1 h during recovery was enough to effectively inhibit this activity. We therefore measured the effects of Dex on the kinetics of HSF1 promoter occupancy (Fig. 6). It can be seen that the Dex effect on promoter binding by HSF1 is also fast acting. Indeed, onset of inhibition can be detected in as little as 15 min of Dex exposure. Moreover, the results of Fig. 6B show that 15 min of Dex treatment can also cause release of heat shock-activated HSF1 from the promoter.

Based on the above, we have concluded that agonist-activated GR can both prevent and reverse binding of HSF1 to the Hsp70 promoter. To explain the role of GR in this response, we reasoned that it might occur by displacement of HSF1 through binding of GR to the Hsp70 promoter. However, ChIP assays against the Hsp70 promoter using an antibody against GR

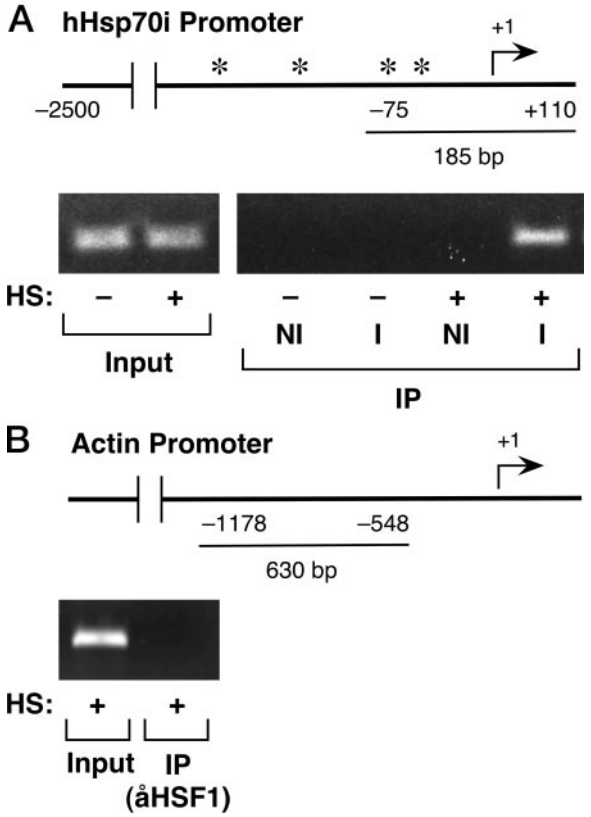

Fig. 4. *In Vivo* Occupancy of the hHsp70 Promoter by Heat Shock (HS)-Activated HSF1

A, Use of ChIP assay to demonstrate binding of HSF1 to the Hsp70 promoter (inducible gene). L929 cells stably transfected with the p2500-CAT reporter were subjected to the indicated treatments and assayed for ChIP using an antibody against HSF1, as described in *Materials and Methods*. The results shown are representative of three independent experiments. *Asterisks* (*) designate the relative positions of HSEs. HS, 43 C, 2 h; Input, chromatin extract before immunoprecipitation; IP (åHSF1), immunoprecipitation of chromatin using antibody against HSF1 (I) or nonimmune serum (NI). B, ChIP using primers to the actin promoter to demonstrate specificity of HSF1 antibody to HSF1 on the Hsp70 promoter. ChIP assay was performed in LHSE cells as described above, except with primers against the endogenous mouse actin promoter. HS, 43 C, 2 h; IP (åHSF1), IP using antibody against HSF1.

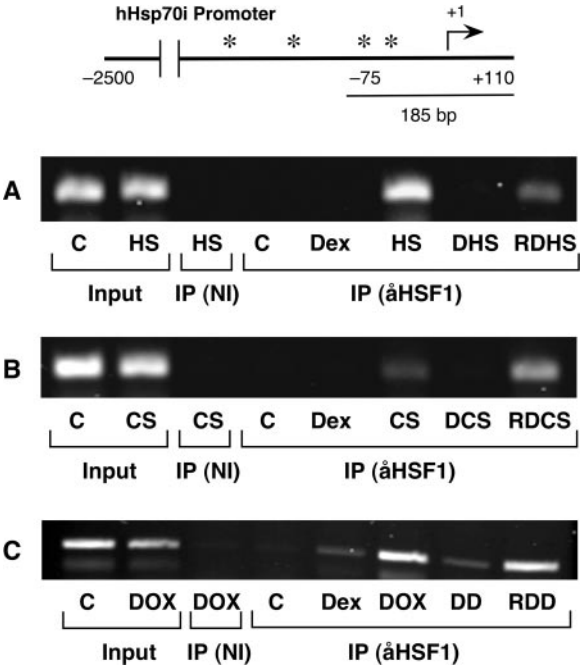

Fig. 5. Glucocorticoid Agonist Inhibits Hsp70 Promoter Occupancy of Stress-Activated Mouse HSF1 and Constitutively Active hHSF1

Replicate flasks of LHSE cells (panels A and B) or cells expressing the constitutively active hHSF1-E189 mutant (panel C) were subjected to various combinations of agonist (Dex), antagonist (RU486), and stress, as indicated. The cells were then assayed by ChIP for HSF1 occupancy of the hHsp70 promoter, as described in *Materials and Methods*. The results are representative of six to nine independent experiments. Input, Chromatin before Immunoprecipitation; IP (NI), immunoprecipitation using nonimmune IgG; IP (åHSF1), immunoprecipitation using HSF1 antibody. Panel A: C, no treatment; D, Dex (1 μM) for 2 h; HS, 43 C for 2 h; DHS, Dex (1 μM) for 2 h during HS; RDHS: Dex (1 μM) and RU486 (1 mM) for 2 h during HS. Panel B: Same as panel A, except that chemical shock (CS, 200 μM sodium arsenite for 2 h) was used as the form of stress. Panel C: Same as panel A, except that DOX was used to induce hHSF1-E189 expression in the absence of stress. DOX, 10 μg/ml DOX for 20 h; DD, Dex (1 μM) for 2 h after 20 h of DOX treatment.

have not shown GR occupancy of the Hsp70 promoter, even under conditions (Dex plus heat shock) that cause inhibition of HSF1 binding (data not shown). Thus, direct displacement of HSF1 by GR is not a likely mechanism by which this inhibition takes place.

Because GR signaling caused loss of HSF1 from the Hsp70 promoter, we speculated that this would result in either loss of HSF1 binding to all high-affinity sites within the nucleus or that the result was more or less specific to the Hsp70 promoter, leaving HSF1 free to bind other nuclear sites. To test this model, we assayed for HSF1 cellular localization by fractionation and Western blotting (Fig. 7). It can be seen that Dex treatment of heat-shocked cells does not cause HSF1 to be released into the cytosolic fraction, suggesting that HSF1 is still bound to high-affinity nuclear sites under conditions where binding to the Hsp70 promoter is blocked. Thus, HSF1 may still be active at other promoters, even in the presence of glucocorticoid agonist.

DISCUSSION

In this work, we have provided evidence for a fast-acting mechanism by which glucocorticoid agonists cause inhibition of HSF1 activity under stress conditions. According to our evidence, this inhibition occurs

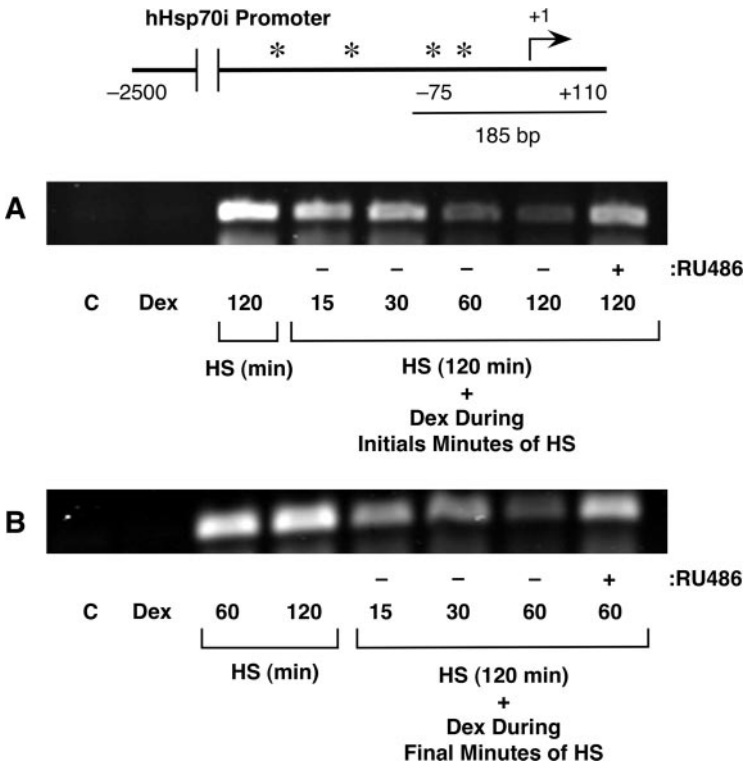

Fig. 6. Dex Inhibition of Hsp70 Promoter Occupancy by Stress-Activated HSF1 Is a Rapid Event

A, Dex during the early stages of heat shock (HS) prevents binding of HSF1 to the Hsp70 promoter (inducible gene). Replicate flasks of LHSE cells were subjected to various hormone and stress treatments, as indicated, followed by measurement of HSF1 binding to the Hsp70 promoter by ChIP assay. C, No treatment; Dex: Dex (1 μM) for 2 h; HS, 43 C for 120 min; HS+Dex during initial minutes of HS, Dex (1 μM) was added to each of the flasks at the beginning of HS. At indicated time intervals during HS, Dex was washed away, and preheated (43 C) media were added to the flasks. Flasks were subjected to continued HS until cross-linking. B, Dex causes rapid release of promoter-bound HSF1. Replicate flasks of LHSE cells were treated as above, except that Dex was added to the final minutes of HS, as indicated. Results of panels A and B are representative of three independent experiments.

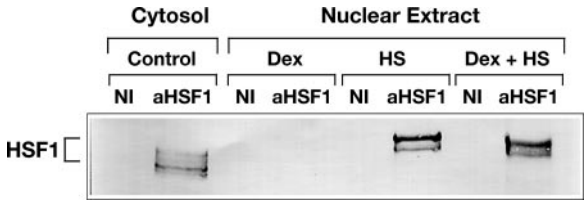

Fig. 7. Dex-Induced Release of HSF1 from the Hsp70 Promoter Does Not Cause Release of HSF1 from the Nuclear Fraction

Replicate flasks of LHSE cells were subjected to the indicated conditions and separated into cytosolic and nuclear fractions, as described in *Materials and Methods*. Fractions were then immunoadsorbed with monoclonal antibody against HSF1 (aHSF1), or with nonimmune rat IgG (NI), followed by SDS-PAGE and Western blotting using antibody against HSF1 as probe. Control, No treatment; Dex**,** Dex (1 μM) for 2 h; HS, 43 C for for 2 h; Dex + HS, 1 μM Dex during HS.

primarily by prevention or reversal of HSF1 recruitment to the Hsp70 promoter. Although the only evidence provided in this work that this response is mediated by GR is antagonism by RU486, our prior study showed a requirement for GR protein, as the response was not seen in GR-deficient cells or in cells expressing DNA-binding- or hormone-binding-incompetent receptor (4). Thus, we conclude that this fast-acting mechanism is mediated by GR.

The above conclusion is largely based on results obtained with the ChIP assay (Figs. 5 and 6). In an earlier study, however, measurement of HSF1 activity by EMSA did not show loss of this activity in response to GR activation (4). Although it is not yet possible to determine which assay is correct, it is likely (for the reasons that follow) that the ChIP assay provides the more relevant data. First and foremost, it should be noted that a major failing of EMSA is lack of chromatin context. There are a myriad of well-documented chromatin parameters that undoubtedly influence promoter binding by HSF1. Thus, signaling events by GR that control chromatin organization, histone acetylation, or corecruitment of additional transcription factors, *etc.*, could lead to displacement of HSF1, even though intrinsic binding affinity of HSF1 for DNA is unaffected. In EMSA, only intrinsic ability to bind DNA

is measured. Moreover, it is possible that HSF1 DNA binding affinity is altered, for example, by a weak interaction with GR that does not survive nuclear extraction before EMSA. Indeed, one conclusion we can make, based on both the ChIP and EMSA results, is that GR signaling is probably not inducing a covalent modification of HSF1 (*e.g*. phosphorylation) that leads to reduced DNA-binding function, because such a change would persist in the EMSA. Instead, it is more likely that a protein-protein interaction is responsible for the GR effect we see.

As alluded to above, one simple and attractive hypothesis to explain the inhibitory effect of GR on HSF1 is a direct binding event by these two transcription factors. However, our recent efforts to show a GR-HSF1 interaction using coimmunoprecipitation approaches have been inconclusive: only a fraction of the experiments have shown positive results (our unpublished observations). Another hypothesis is that GR can displace HSF1 from the promoter by competing for binding sites or through allosteric interactions caused by GR occupancy of nearby promoter sites. We have tested this mechanism in two ways. In the first approach, we screened for the presence of GR binding sites in the hHsp70 promoter, using consensus nGRE sequences as a guide. Five such sites were found (Fig. 3), and analysis of deletion constructs of the hHsp70 promoter showed that at least four of them did not contribute to GR inhibition of HSF1 activity. Although we cannot rule out potential contribution by the last site located at position −110, it should be noted that this site was not a perfect match of the published sequence (88% homology). Moreover, we have shown that Dex inhibition of HSF1 leads to decreased expression of Hsp90 and Hsp110, in addition to Hsp70 (4). Yet, the Hsp90 and Hsp110 promoters do not contain a putative nGRE element homologous to the element identified at −110 (our unpublished observation). In the second approach, GR occupancy of the hHsp70 promoter was tested by the ChIP assay. But, here too, the results have been negative (data not shown). Thus, we conclude that GR inhibition of HSF1 probably does not occur via direct binding by GR to the Hsp70 promoter.

Because GR complexes contain Hsp90 (16), and under some circumstances (17), and because both of these Hsps are chaperones that serve to maintain the inactive, cytosolic form of HSF1 (18, 19), we have considered that GR-mediated inhibition of HSF1 may occur by release of Hsps from the receptor heterocomplex. But results from an earlier effort did not support the hypothesis, as RU486 causes dissociation of this complex without resulting in repression of HSF1 (4). Unfortunately, these observations taken together have not provided much insight into how the GR can achieve this dramatic effect on HSF1. However, a still-active model in the laboratory is the possibility of a relatively weak interaction between GR and HSF1, and efforts are underway to determine the proper conditions under which to uncover this interaction.

With respect to HSF1, however, we now appear to have a mechanistic explanation that is highly consistent with the totality of data obtained to date in this system. Dex inhibition of promoter binding by HSF1 is, of course, in complete agreement with the inhibitory effect seen on promoter-driven expression of CAT. More importantly, antagonism of the Dex effect on reporter gene expression by RU486 is corroborated by the ability of RU486 to prevent the Dex effect on HSF1 promoter occupancy. Lastly, inhibition of constitutive promoter occupancy by HSF1-E189 under nonstress conditions is also in complete agreement with the effect of Dex on promoter activity. This last condition is important, as it suggests that this particular inhibitory ability of GR is not dependent on a heat shock (stress)-induced signaling event.

MATERIALS AND METHODS

Materials

[^{3}H]acetate (10.3 μCi/mmol) and [^{125}I]conjugated goat antimouse IgG (11.8 μCi/μg) were obtained from ICN Radiochemicals (Cleveland, OH). ATP, dimethylsulfoxide, sodium arsenite, Dex, G418 (Geneticin) antibiotic, hygromycin, acetyl coenzyme A (CoA) synthetase, acetyl CoA, Tris, HEPES, EDTA, protein A-Sepharose, DMEM powdered medium, and horseradish peroxidase-conjugated goat antimouse IgG were from Sigma Chemical Co. (St. Louis, MO). The steroidal antagonist RU486 was obtained from Roussel-Uclaf. Iron-supplemented newborn calf serum was from Hyclone Laboratories, Inc. (Logan, UT). Imobilon polyvinylidine difluoride membranes were obtained from Millipore Corp. (Bedford, MA). The FiGR monoclonal antibody against GR (20) was a gift from Jack Bodwell (Dartmouth Medical School, Hanover, NH) and was expressed and affinity purified by Biocon, Inc. (Rockville, MD). HSF1 rat monoclonal antibody HSF1-AB4 (cocktail) was obtained from Neomarkers (Fremont, CA). 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 containing consensus HSEs known to be activated by the binding of HSF1 (21). The p2500-CAT reporter and its deletion constructs (9) were supplied by Richard Voellmy (University of Miami, Miami, FL). The pMMTV-CAT plasmid contains the complete mouse mammary tumor virus (MMTV)-long terminal repeat promoter upstream of CAT. Hormonally driven expression of CAT by this reporter is controlled by GREs residing within the long terminal repeat region (22). The pBI-EGFP vector was obtained from CLONTECH (Palo Alto, CA). In this vector, tetracycline-induced expression is controlled by a tetracycline response element and two minimal cytomegalovirus promoters in opposite orientations. The pUHD172–1hygro vector (23) 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 (24) was the generous gift of Richard Voellmy.

Transfection of Cell Lines

The LHSE (p2500-CAT) and LMCAT2 (pMMTV-CAT) cell lines were established as previously described (25, 26). Briefly, mouse L929 cells were cotransfected with pSV2neo and a 2-fold excess of p2500-CAT or pMMTV-CAT using Gene-

Porter 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. L929 cells stably expressing the p285-CAT, p223-CAT, and p146-CAT deletion constructs of p2500 were generated in similar fashion. The tetracycline-inducible LHSF1-E189 cells were made by stably transfecting LHSE cells with the pUHD172–1hygro plasmid and a 7-fold excess of pBI-E189 plasmid, as previously described (4).

Stress and Hormone 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.

CAT Assay

Measurement of CAT enzyme activity was performed according to the method of Nordeen *et al.* (27) 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 adding 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 $\times$ *g*. Aliquots of lysate containing equal protein content were added to the enzymatic reaction mixtures. As the HSE-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 inhibitory effect of each treatment can be readily seen.

ChIP Assay and PCR

ChIP assays were performed according to the method of Nissen and Yamamoto (12), with modifications. Briefly, cells subjected to various treatments were fixed with 37% formaldehyde, followed by quenching with 125 mM glycine. After harvest and washing with cold PBS, cells were resuspended in buffer A (50 mM HEPES, pH 8.0; 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10% glycerol, 0.5% Nonidet P40, 0.25% Triton X-100, protease inhibitors) and crude nuclei were collected by centrifugation at 600 $\times$ *g* at 4 C. Nuclei were washed in buffer B (10 mM Tris-HCl, pH 8; 1 mM EDTA; 0.5 mM EGTA; 200 mM NaCl; protease inhibitors) followed by addition of 1$\times$ RIPA buffer [10 mM Tris-HCl, pH 8; 1 mM EDTA; 0.5 mM EGTA; 140 mM NaCl; 1% Triton X-100; 0.1% deoxycholate; 0.1% sodium dodecyl sulfate (SDS); protease inhibitors[and sonication to yield chromatin fragments ranging from 0.2 to 1.2 kb. Samples were centrifuged at 16,000 $\times$ *g* at 4 C and supernatants were collected as chromatin extracts.

Immunoprecipitation was achieved by addition of HSF1-AB4, FiGR, rat IgG, or mouse IgG to the chromatin extracts and incubation at 4 C for 2 h. Samples were transferred to tubes containing 50 μl of a 20% protein G-Sepharose slurry in 1$\times$ RIPA buffer containing 100 μg/ml sonicated salmon sperm DNA. After nutation at 4 C, samples were sequentially washed with 1$\times$ RIPA buffer, 1$\times$ RIPA buffer containing 100 μg/ml salmon sperm DNA, 1$\times$ RIPA buffer containing 500 mM NaCl plus 100 μg/ml salmon sperm DNA, LiCl buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA; 0.5 mM EGTA; 250 mM LiCl; 1% Triton X-100; 1% deoxycholate; protease inhibitors) and finally with 1$\times$ RIPA buffer. Digestion buffer (50 mM Tris, pH 8; 1 mm EDTA; 100 mM NaCl; 0.5% SDS; 100 μg/ml proteinase K) was added to each sample followed by heating for 3 h at 55 C and 6 h at 65 C to reverse cross-links. DNA was extracted with phenol-chloroform, ethanol precipitated, and resuspended in Tris-EDTA buffer.

PCRs, 50 μl each, were programmed for 25 cycles with 2 μl of DNA sample. Primer oligonucleotides (Fisher Scientific, Pittsburgh, PA), *Taq* DNA polymerase and buffer (QIAGEN, Chatsworth, CA), 10 mM dNTPs (Invitrogen, San Diego, CA) were added to the template to set up PCR. Titrations were performed to ensure a linear range of amplification. One fifth of each PCR was electrophoresed on 2% agarose gels in 0.5% Tris-borate-EDTA. PCR fragments were observed and gels were scanned with a Typhoon phospho imager adjusted to detect ethidium bromide staining.

PCR primer sets for the ChIP were as follows. For the hHsp70 promoter, the −75/+105 region was amplified with the primer pairs 5′-GGAAGGTGCGGGAAGGTTCG-3′ (forward) and 5′-TTCTTGTCGGATGCTGGA-3′ (backward). A 185-bp product is formed in the PCR. For the endogenous mouse actin promoter, the −548/−1178 region was amplified with 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ (forward) and 5′CTAGAAGCATTTGCGGTGGACGATGGAGGGG-3′ (backward) primers to yield a 630-bp product.

PCR primers sets used in Fig. 1 for detection of endogenous mouse Hsp70 (inducible gene) were as follows: 5′-TGGAGATCATCGCCAACGACC-3′ (forward) and 5′-TCCTCCACGAAGTGGCTCACC-3′ (backward).

Immune Purification and Western Blotting

In the experiment of Fig. 1, L929 cells were treated as described and lysed by Dounce A homogenization in HEPES buffer (10 mM HEPES; 3 mM EDTA, pH 7.4) to yield cytosolic fractions. Equal protein aliquots were then Western blotted (as described below) using the SPA810 (Stressgen Biotech Corp., Victoria, British Columbia, Canada) antibody specific to the inducible form of Hsp70.

In the experiment of Fig. 7, cells were fractionated into cytosolic and nuclear portions by Dounce A homogenization in HEPES buffer, followed by centrifugation at 1000 $\times$ *g*. The cytosolic fractions were saved and the nuclear pellets were washed two times by resuspension and pelleting in HEPES buffer. HEPES 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 $\times$ *g* and the supernatants saved. HSF1-AB4 was added to the cytosolic and nuclear fractions, and each sample was adsorbed in batch to protein A-Sepharose, followed by washing with TEG buffer (10 mM TES; 1 mM EDTA; 10% glycerol; 50 mM NaCl, pH 7.6) and elution with 2$\times$ SDS sample buffer. All samples were resolved by electrophoresis in 7% polyacrylamide SDS gels, followed by transfer to Imobilon polyvinylidine difluoride membranes and probing with HSF1-AB4 antibody. Detection of HSF1 was achieved via a combination of peroxidase- and ^{125}I-conjugated counter antibodies, as previously described (28). After color development, the blots were exposed to Kodak XAR-5 film with an intensifying screen at −70 C.

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