Potentiation of Glucocorticoid Receptor-Mediated Gene Expression by Heat and Chemical Shock

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We have examined the effects of heat shock on glucocorticoid receptor (GR)-mediated gene transcription in an L929 cell line derivative (LMCAT2) stably transfected with the mouse mammary tumor virus-chloramphenicol acetyltransferase (MMTV-CAT) reporter plasmid. Exposure of the LMCAT2 cells to heat or chemical shock resulted in a large increase in dexamethasone (Dex)-induced expression of CAT enzyme activity. This potentiation of hormone-induced MMTV-CAT expression was dependent on the magnitude of the stress event and on the Dex concentration, with maximal increases observed for 1 μM Dex after 2 h at 43 C or 2 h at 200 μM sodium arsenite. Heat shock potentiation of MMTV-CAT expression was not seen in an L929 cell derivative devoid of GR or in LMCAT2 cells treated with RU486 antagonist, suggesting that this effect of stress on CAT gene expression was mediated by the GR. Using a quantitative Western blot procedure, the amount of GR protein in the nucleus of cells subjected to combined heat shock and Dex treatment was no greater than the amount of nuclear GR in cells treated with hormone alone, indicating that the stress potentiation effect was not the result of increased nuclear translocation or retention by the GR. In addition, equally strong potentiations of MMTV-CAT expression were observed for cells subjected to heat shock either before or after Dex-mediated translocation of the GR to the nucleus. Thus, the major effect of stress on GR transcription enhancement activity appears to occur after the GR is bound to its high affinity nuclear acceptor sites. We have used a series of MMTV-CAT reporter constructs containing varying portions of the long terminal repeat regulatory region to show that a putative heat shock transcription factor-binding sequence at position −437 of the long terminal repeat is not required for this effect of heat shock on MMTV-CAT expression. A stress-induced increase in hormone-mediated CAT gene expression was observed for a minimal CAT reporter controlled by two synthetic glucocorticoid response elements and a TATA box sequence. Thus, it is unlikely that any DNA-binding transcription factor, other than GR, is required for this effect of stress on transcription by the hormone-bound GR. Based on these results, a model of heat shock enhancement of GR-mediated gene expression is developed in which stress acts on the DNA-bound GR, on a putative heat shock-activated adaptor, or on components of the RNA-polymerase-II complex. (Molecular Endocrinology 8: 408–421, 1994)

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INTRODUCTION

The glucocorticoid hormone receptor (GR) is a member of steroid/thyroid receptor family and as such serves to control differential gene expression by acting as an enhancer of specific gene transcription (1, 2). Although many of the details of GR activation are not known, it is generally accepted that the primary event in this signal pathway is the binding of a glucocorticoid agonist to the GR. This event is rapidly followed by a series of steps that include 1) dissociation of the cytoplasmically localized heteromeric receptor complex, 2) translocation of the hormone-bound receptor to the nucleus, 3) high affinity binding of the GR to glucocorticoid response elements (GREs) present in the regulatory regions of target genes, and 4) the interaction of GRE-bound receptors with elements of the transcription machinery, resulting in initiation of transcription.

Among these steps, the best understood may be the hormone-mediated dissociation of the untransformed steroid-receptor complex (3). Analysis of this complex in both intact cells and cell lysates has revealed the presence of a variety of cellular heat shock proteins (hsps). One of these, the 90-kilodalton (kDa) hsp

(hsp90), is not only a component of the GR complex (4–6), it is also found in untransformed progesterone-, estrogen-, and androgen-receptor complexes (7, 8). In addition to hsp90, two other heat shock proteins, hsp70 and hsp56, have been shown to be components of steroid-receptor complexes. In the case of hsp70, there is evidence that both the untransformed and transformed progesterone receptors (PR) contain this hsp (9, 10); under certain circumstances, hsp70 is also found associated with the GR (11). Hsp56 is a newly described low abundance 56- to 59-kDa heat shock protein (12) that was originally discovered as a component of untransformed steroid receptors by Tai and co-workers (13). Although very little is known about the functions of hsp56 in the heat shock response or steroid receptor action, it is interesting to note that this protein is also a member of the immunophilin class of proteins and is capable of binding the immunosuppressive macrolides FK506 and rapamycin (14–16). In support of these observations is the recent report that treatment of intact cells with FK506 and rapamycin results in the potentiation of GR-mediated gene expression (17). Taken together, these findings point to steroid receptors as a potential locus for the convergence of at least three previously distinct signal pathways: steroid hormones, immunophilins, and the heat shock response.

The cellular heat shock response is a process of adaptation to environmental stress in which a variety of stress-induced proteins play integral roles (18, 19). Although the heat shock response occurs after thermal injury, a variety of agents, such as amino acid analogs and metabolic poisons, are also known to increase the expression of hsps. Almost all of these agents will result in the accumulation of abnormally folded proteins (20), and the presence of misfolded proteins may be the initial trigger for the induction of hsps (21). Consistent with this model is the fact that many of the best-studied hsps function to insure proper polypeptide folding, often in the assembly or disassembly of protein complexes (22, 23). The association of steroid receptors with several hsps has led to the speculation that one possible role of steroid receptor-associated hsps may be to insure the proper conformation and consequent activity of untransformed steroid receptors. Several lines of *in vitro* evidence have been reported to support this idea. First, *in vitro* translations of the glucocorticoid (24) and estrogen (25) receptors were found to result in the association of hsp90 with these receptors during the terminal stages of translation. Second, hormone-mediated dissociation of hsp90 from the GR is irreversible in cytosol preparations, and this dissociation can result in some GR molecules that become nonfunctional with respect to DNA binding due to misfolding (26). Third, successful reconstitutions of the progesterone (27) and glucocorticoid (28) receptors with both hsp90 and hsp70 have been achieved using a rabbit reticulocyte lysate system. In each case, reconstitution of the receptor-hsp complex requires receptor that is hormone free, and in the case of the GR, reconstitution results in a simultaneous loss of receptor DNA-binding function and recovery of steroid-binding activity (28).

Although the above studies provide fairly comprehensive *in vitro* evidence for the nature of the receptor-hsp interaction, they do so only under nonstress conditions. On the other hand, *in vivo* studies on the effects of cellular stress on steroid receptor action are limited. These studies include a report demonstrating the effects of homogenization temperature on compartmentalization of estrogen receptors (29). Although the latter study was not designed to examine the interaction of the cellular heat shock response on steroid function, it did provide evidence that even a short exposure of rat uterine cells to 40 C can result in a large shift of hormone-free estrogen receptors from the cytosolic to the nuclear pellet fraction. In contrast, a later study has reported that heat shock treatment of a variety of mammalian cell lines results in a loss of GR steroid-binding capacity, presumably due to stress-induced degradation of the GR (30). Results from our laboratory have shown that heat shock treatment of intact L929 cells can cause a loss of hormone-binding capacity, but that the majority of the GR in these cells is not degraded (31). Instead, we observed that both heat and chemical shock treatment of L929 cells will cause unliganded GRs to become tightly bound within the nucleus, suggesting the possibility that cellular stress could play a role in controlling GR-mediated gene expression (31). In support of this speculation is a report by Edwards and co-workers (32), in which the researchers demonstrate enhancement of hormone-dependent PR-mediated transcription activity in cells subjected to heat or chemical shock. Their study employed human T47D breast cancer cells stably transfected with a mouse mammary tumor virus-chloramphenicol acetyltransferase (MMTV-CAT) reporter plasmid. As the 5′-regulatory region of this reporter construct is large (~1200 base-pairs) and contains putative binding sites for a variety of transcription factors, including a consensus binding site for the heat shock transcription factor (HSF), it was speculated that one possible explanation for the observed enhancement by heat shock on PR-mediated transcription was due to cooperativity between hormone-bound PR and HSF (32).

In the present study, we have directly addressed this question and others by measuring the effects of heat and chemical shock on GR-mediated gene expression using CAT reporter constructs controlled by a variety of 5′-regulatory regions. The various promoters of CAT gene expression used include a series of truncation mutants of the MMTV-long terminal repeat (LTR) region as well as a series of synthetic minimal promoters containing GREs linked to the TATA box. Our results indicate that CAT gene expression is markedly increased when cells are sequentially exposed to heat or chemical stress and glucocorticoid hormone [dexamethasone (Dex)]. This potentiation effect on GR-mediated gene expression is dependent on both the magnitude of the stress event and the Dex concentration. However, the potentiation effect is not dependent on

the presence of heat shock elements (HSEs) or other transcription factor recognition sequences, as this effect is still observed for the GRE/TATA minimal promoter. For this reason and others, we discuss the possibilities that this effect of cellular stress on GR-mediated gene expression is the result of an intrinsic modification of the GR or of a more efficacious interaction between the GR and components of the transcription initiation complex.

RESULTS

Heat and Chemical Shock Potentiate Dex-Induced MMTV-CAT Expression

To test the effect of cellular stress on GR-mediated gene transcription, we stably transfected mouse L929 cells with the pMMTV-CAT reporter plasmid. This plasmid contains the CAT gene linked to the MMTV-LTR sequence containing several GREs. After transfection, a cloned cell line (LMCAT2) was established by selection with G418 (Geneticin) antibiotic. In the experiments shown in Fig. 1, LMCAT2 cells were first subjected to cellular stress, followed by the addition of 1 μM Dex or vehicle, and the cells were then cultured for an additional 24 h. From the results obtained, it is clear that both heat and chemical shock (sodium arsenite) will cause a large increase in Dex-induced CAT gene expression in a manner that is dependent on the magnitude of the stress event. This stress-mediated potentiation of GR transcription enhancement activity was maximal after a 2-h treatment at 43 C or a 2-h treatment with 200 μM arsenite. Using these maximal stress conditions, a Dex concentration dependency experiment was then performed. As shown in Fig. 2, the addition of Dex resulted a concentration-dependent increase in MMTV CAT expression for the control, heat-shocked, and chemically shocked cells. However, both heat and chemical shock resulted in levels of CAT gene expression that were considerably higher than those in nonstressed cells under saturating concentrations of Dex (10^{-6} M).

From the data shown in Figs. 1 and 2, it can be seen that chemical shock consistently resulted in a greater potentiation effect than heat shock. This observation is similar to that reported by Edwards *et al.* (32), in which arsenite treatment of T47D cells resulted in a potentiation of PR-mediated CAT gene expression that was approximately 4 times greater than that observed in response to heat shock. Although it is not clear why chemical shock should result in a larger potentiation effect than heat shock, it is clear that chemical shock employing sodium arsenite will result in a cellular heat shock response (induction of hsps) qualitatively similar to that seen after thermal stress (33).

The results shown in Fig. 1 also indicate that stress-mediated potentiation of CAT expression does not occur in the absence of hormone. This observation was somewhat unexpected because we had previously shown that cellular stress results in nuclear translocation of the hormone-free GR in mouse L929 cells (31). Moreover, we subsequently demonstrated a stress effect on MMTV-CAT expression under hormone-free conditions in Chinese hamster ovary cells (WCL2), which stably overexpress the mouse GR (34). In the latter report, the level of Dex-independent stress-induced GR-mediated CAT gene expression was a small (2.3%), but reproducible, fraction of the very high CAT activity seen in Dex-treated WCL2 cells. Thus, we concluded that heat shock could result in a partial activation of the unliganded GR. For the cell lines used in the present study, observation of a hormone-independent stress potentiation effect has been variable. In the experiments shown in Fig. 1, no hormone-independent increase in CAT was observed, but in the experiments in Fig. 3B, a small increase (2.4-fold) in CAT was measured in response to heat shock alone. One possible explanation for this inconsistency is that a similarly small fraction of hormone-free activation can occur in the LMCAT2 cells, but that this increase happens to fall near the limits of detection for the particular CAT assay we have employed. For this reason, the use

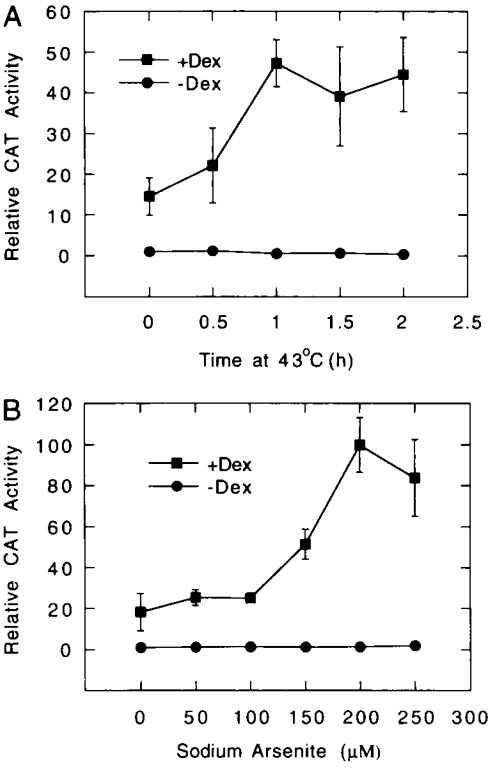

Fig. 1. Heat and Chemical Shock Treatments Increase Dex-Induced Transcription Enhancement Activity *in Vivo*

LMCAT2 cells were subjected to either a time course of heat shock at 43 C (A) or 2 h of chemical shock using increasing concentrations of sodium arsenite (B). Immediately after the stress events, Dex (1 μM final) or vehicle was added, and the cells were allowed to grow under nonstress conditions for an additional 24 h. Lysates were then prepared and analyzed for relative CAT enzyme activity (fold induction relative to basal, no treatment, controls). The results shown represent the mean ± SEM of three independent experiments.

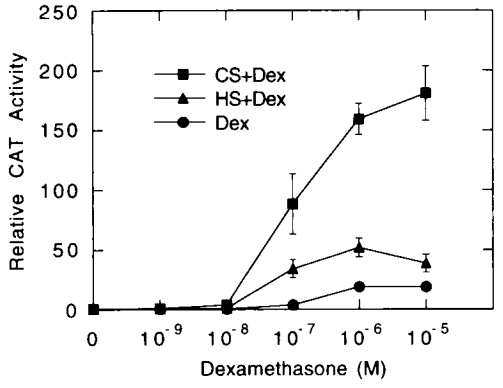

Fig. 2. Effects of Heat and Chemical Shock on Dex Dose-Dependent MMTV-CAT Gene Expression

LMCAT2 cells were subjected to no treatment, 200 μM sodium arsenite for 2 h (CS), or 43 C for 2 h (HS). After stress treatments, Dex was added at the indicated concentrations, and the cells were cultured for an additional 24 h under nonstress conditions. Lysates were then prepared and analyzed for relative CAT enzyme activity. The results shown represent the mean ± SEM of three independent experiments.

of more sensitive CAT assays (35) should help to resolve this issue.

Stress Potentiation of MMTV-CAT Expression Is GR Mediated

To address the possibility that the observed increases in CAT gene expression in stressed LMCAT2 cells is the result of some nonspecific increase in generalized gene transcription, we measured the effects of stress on L929 cells stably transfected with the constitutive pSV2CAT reporter. In these cells, CAT gene expression was found to be unchanged after either heat or chemical shock treatment and varying times of recovery (data not shown). However, as the pSV2CAT construct typically results in high level expression of CAT, the possibility still remained that a small stress-induced increase in SV2-controlled CAT expression would not be detected. Accordingly, we have addressed this question more directly by measuring the effects of stress on MMTV-CAT expression either in the absence of GR or in the presence of GR bound with the glucocorticoid antagonist RU486. In the experiment shown in Fig. 3A, a subclone of L929 cells (E82.A3 cells) that does not contain GR protein (36) was stably transfected with the MMTV-CAT reporter. The resultant cell line, LGR⁻CAT, showed no increases in CAT gene expression in response to Dex, heat shock, or combined Dex and heat shock treatments. This lack of response could not be attributed to a faulty or nonexistent MMTV-CAT reporter because transient transfection of the LGR⁻CAT cells with the pSV2wrec plasmid, which constitutively expresses the wild-type mouse GR, resulted in a Dex-induced increase in CAT expression (Fig. 3A). In the experiment shown in Fig. 3B, LMCAT2 cells were subjected to various treatments with glucocorticoid agonists (Dex), antagonists (RU486), and heat shock. As expected, RU486 by itself had no effect on CAT gene

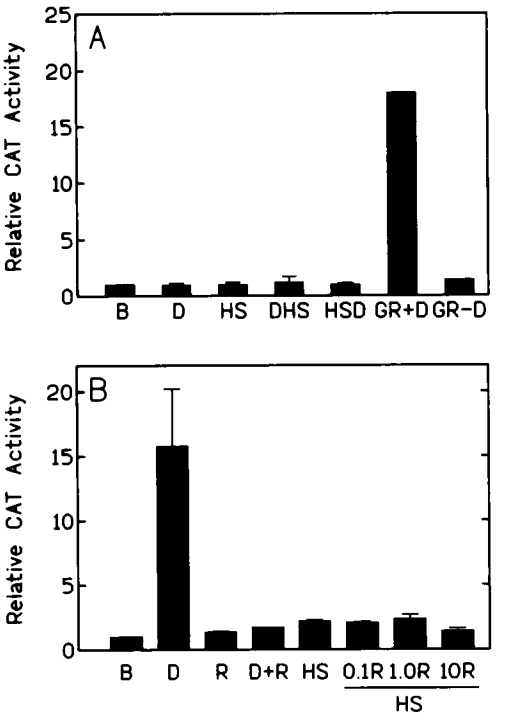

Fig. 3. Heat Shock Potentiation of Dex-Induced Transcription Enhancement Activity Requires GR and Does not Occur in the Presence of RU486 Antagonist

LGR⁻CAT cells (A) and LMCAT2 cells (B) were subjected to a variety of hormone and heat shock conditions, as indicated. The LGR⁻CAT cells are a subclone of L929 cells deficient in GR (GR-D) and stably transfected with the MMTV-CAT reporter. After 24 h of culture under nonstress conditions, relative CAT enzyme activities were measured, as described in *Materials and Methods*. The results depicted (except for the GR-deficient condition of A) represent the mean ± SEM of three independent experiments. Conditions in A: B, no treatment; D, 1 μM Dex; HS, heat shock (43 C, 2 h) alone; DHS, 1 μM Dex followed by heat shock; HSD, heat shock followed by Dex; GR+D, LGR⁻CAT cells transiently transfected with a GR-expressing plasmid (pSV2wrec) and subjected to 1 μM Dex; and GR-D, LGR⁻CAT cells transiently transfected with pSV2wrec and not treated with Dex. Conditions in B: B, no treatment; D, 1 μM Dex; R, 1 μM RU486 alone; D+R, 1 μM Dex plus 1 μM RU486; HS, heat shock (43 C, 2 h) alone; 0.1R/HS, 1.0R/HS and 10R/HS, 0.1, 1.0, and 10 μM RU486, respectively, followed by heat shock.

expression and acted as a competitive inhibitor of Dex-induced CAT expression. More importantly, no heat shock potentiation effect was observed for RU486 concentrations ranging from 0.1–10 μM. Taken together, the results shown in Fig. 3 provide evidence that heat shock potentiation of MMTV-CAT expression in the LMCAT2 cells is a GR-mediated process and not the result of a generalized effect of stress on MMTV-CAT gene expression.

Stress Potentiation Is not the Result of Increased Nuclear Retention of the GR and Occurs Subsequent to Hormone-Mediated GR Transformation

In an earlier report, we had shown that heat shock treatment of L929 cells in the absence of Dex results

in nuclear translocation of the GR (31). Under these conditions, about 95% of the GR was recovered in the nuclear pellet after a 2-h heat shock treatment at 43 C, whereas about 64% of the GR was nuclear after chemical shock treatment with 200 μM sodium arsenite. Given these results, we reasoned that the effects of cellular stress on GR-mediated gene expression reported here could be the result of an increased retention of GR within the nuclear compartment after combined exposure to stress and hormone. Accordingly, we performed the experiment shown in Fig. 4, in which the amounts of GR protein in the cytosolic and nuclear fractions were measured using a quantitative Western blotting procedure previously described (37). In this experiment, the subcellular distributions of GR were determined immediately after a short 2-h exposure to hormone and after 24 h of recovery following combined heat shock and hormone treatment. From the results obtained, it is clear that the amount of GR retained within the nuclear compartment after combined heat shock and Dex exposure is no greater than the amount of GR recovered in the nuclear pellet after a 24-h exposure to Dex alone. In fact, results from this and replicate experiments suggest that 24-h nuclear GR levels are actually reduced in cells subjected to both heat shock and Dex compared to those in the Dex only controls. As the latter conditions are identical to the conditions under which heat shock potentiation of GR-mediated MMTV-CAT expression was observed (Figs. 1, 2, 8, and 9), we have concluded that this potentiation phenomenon cannot be the result of an increase in GR retention within the nuclear compartment. Indeed, the data argue that enhancement of GR-mediated transcription activity by cellular stress occurs in spite of a reduction in the amount of nuclear GR.

Although the results shown in Fig. 4 eliminated increased nuclear retention as an explanation for heat shock potentiation, the possibility still remained that heat shock could be affecting the process by which GR is transformed to the nuclear-bound, transcriptionally active state (transformation), perhaps resulting in GR with altered conformation or covalent modifications. To address this issue, we reasoned that if heat shock potentiation of GR-mediated gene expression occurred at the level of GR transformation, then subjecting GR to normal hormone-mediated transformation before heat shock treatment should eliminate the heat shock potentiation effect. (Up to this point, all of the data on GR-mediated MMTV-CAT expression have been for cells subjected to stress before the addition of hormone.) With this in mind, we first treated LMCAT2 cells with 1 μM Dex for 2 h, a condition that results in near-complete translocation of the GR to the nucleus (see Fig. 4), before exposure of these cells to heat shock (43 C, 2 h). After recovery at 37 C for 24 h, CAT activities were measured, and the results are shown in

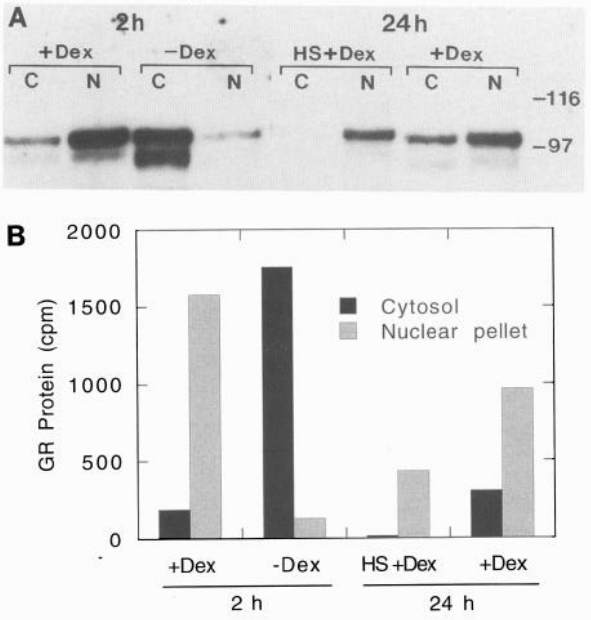

Fig. 4. Heat Shock Potentiation of GR Transcription Enhancement Is not the Result of Increased Nuclear Retention of the GR

Replicate flasks of L929 cells were subjected to the following conditions: 1 μM for 2 h (+Dex/2h), no treatment for 2 h (−Dex/2 h), 43 C for 2 h followed 1 μM Dex for 24 h at 37 C (HS+Dex/24 h), and 1 μM Dex for 24 h (+Dex/24 h). Cytosolic (C) and nuclear (N) extracts were then prepared as described in *Materials and Methods*. GR protein was immune purified with BuGR2 anti-GR monoclonal antibody and protein-A-Sepharose. Quantitative Western blot analysis of GR protein was performed using both peroxidase- and ^{125}I-conjugated counterantibodies. A, Autoradiogram from the Western blot. The bands just below the 97-kDa marker are proteolytic fragments of the intact GR, which migrates as a 100-kDa band. Both the intact GR and the fragments were subjected to quantitation. B, Quantitation of GR after excision of peroxidase-labeled GR bands and liquid scintillation spectroscopy.

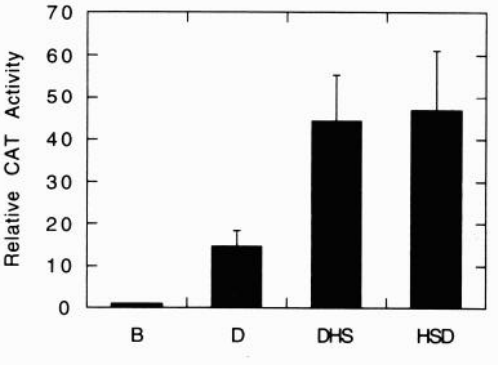

Fig. 5. Heat Shock Potentiation of GR Transcription Enhancement Activity Can Occur after Hormone-Mediated Transformation of the GR

LMCAT2 cells were subjected to no treatment (B), 1 μM Dex (D), 1 μM Dex for 2 h followed by heat shock at 43 C for 2 h (DHS), or 2 h of heat shock at 43 C followed by 1 μM Dex (HSD). After treatment, all cells were cultured under nonstress conditions for an additional 24 h. Relative CAT enzyme activities were then measured as described in *Materials and Methods*. The results depicted represent the mean ± SEM of five independent experiments.

Fig. 5. As before, heat shock followed by Dex treatment resulted in a marked potentiation of GR-mediated MMTV-CAT expression. However, Dex treatment followed by heat shock also yielded a similar level of potentiation. We performed similar experiments using sodium arsenite and found that chemical shock potentiation can also occur after incubation with Dex (data not shown). Thus, by these criteria, the heat shock potentiation effect appears to be acting at a stage subsequent to GR transformation. For this conclusion to be valid, however, we had to first address an apparent contradiction. This contradiction was that heat shock alone, as already stated, could result in translocation to the nucleus of the hormone-free GR, and that this nuclear-localized GR was not capable of binding hormone (31). How, then, could the addition of hormone after heat shock result in an increase in GR-mediated MMTV-CAT expression? We have attempted to resolve this question by assaying for GR protein and steroid-binding capacity in L929 cells recovering from heat shock in the absence of hormone (Fig. 6). The results of these experiments show that both cytosolic GR protein and glucocorticoid-binding capacity return to approximately 70% of the initial values within 8 h. Moreover, as much as 30% of the GR protein and 20% of the steroid-binding capacity are recovered within 2 h. Although these data do not discriminate between *de novo* synthesis of GR and recycling of GR from the nucleus, they do provide evidence that GR capable of binding hormone can return to near-normal levels during the 24-h incubation period we have used before measurement of CAT activities. This reappearance of hormone-binding GR may explain the potentiation effects observed on CAT gene expression in cells exposed to Dex after heat shock (Figs. 1, 2, and 5).

In the experiments shown in Fig. 7, we further explored this possibility by measuring the rate of onset of CAT gene expression in LMCAT2 cells exposed to Dex after stress treatment. In this experiment, relative CAT activities were expressed as a percentage of maximum values to allow comparison of the rates of onset between a condition with relatively low absolute levels of induction (Dex alone) and conditions with much higher levels of induction. In cells exposed to Dex, but not stressed, the rate of onset of CAT expression was relatively fast, as about 20% of the maximal CAT activity was observed after 4 h and about 50% was observed after 8 h. In contrast, both heat and chemical shock resulted in a delayed pattern of CAT expression. In cells recovering from chemical shock, Dex-mediated induction of CAT was only 2% of maximum at 4 h and 14% at 8 h. In cells recovering from heat shock, Dex-induced CAT remained at basal levels after 8 h and did not significantly increase until 24 h. One interpretation of these results is that delayed onset of CAT expression in stressed cells is the result of a generalized stress-induced reduction in protein synthesis. Indeed, stress-induced decreases in mRNA transcription, processing, and translation have been widely reported. However, few reports exist demonstrating levels of protein synthesis in cells recovering from stress events. In one such report, rat embryo fibroblast cells (REF-52 cells) were subjected to either heat shock at 42.5 C or chemical shock using sodium arsenite, and the levels of overall protein synthesis were examined during recovery (33). It was found that protein synthesis had returned to normal within 7–8 h of recovery after heat shock and within 4–5 h of recovery after chemical shock. As these time frames for recovery of protein synthesis in the REF-52 cells correspond to time points when maximal CAT activity has not yet been achieved in stressed LMCAT2 cells (Fig. 7), we speculate that reduced levels of protein synthesis in response to stress cannot wholly account for the delay. However, we cannot rule out the possibility that these two cell lines (REF-52 and L929) differ markedly in their rates of recovery of protein synthesis after stress, or that the rate of recovery for any specific protein (*e.g.* GR) can vary markedly from the overall cellular rate of recovery.

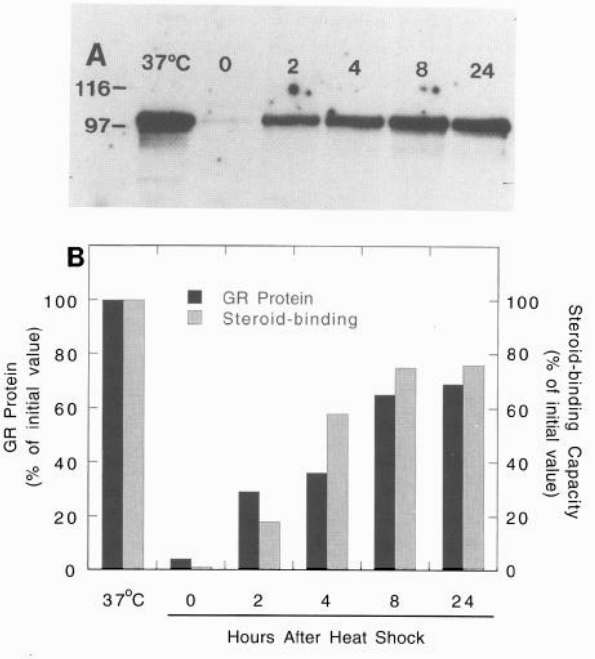

Fig. 6. Quantitation of GR Protein and Glucocorticoid-Binding Capacity in the Cytosolic Fractions of L929 Cells Recovering from Heat Shock

Cytosolic fractions of mouse L929 cells were prepared from control cells (37 C) and cells recovering from heat shock (43 C, 2 h) for the indicated time intervals (0, 2, 4, 8, and 24 h). The relative amounts of GR protein in each fraction were then determined by immunoadsorption of GR to protein-A-Sepharose using the BuGR2 anti-GR monoclonal antibody followed by Western blotting (A), using BuGR2antibody as probe and both peroxidase- and ^{125}I-conjugated counter antibodies. Quantitation of relative GR protein (B) was achieved by excision of the peroxidase-stained GR protein bands, followed by liquid scintillation spectroscopy and normalization relative to the 37 C control (initial value). The relative steroid-binding capacity in each cytosolic fraction (B) was measured using [^{3}H]triamcinolone acetonide (TA), as described in *Materials and Methods*. Specifically bound [^{3}H]TA values were normalized to protein content and to the 37 C control (initial value).

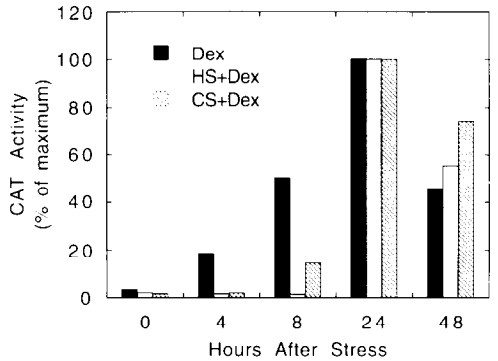

Fig. 7. Dex-Induced MMTV-CAT Expression in LMCAT2 Cells Recovering from Heat and Chemical Shock

LMCAT2 cells were subjected to heat shock (HS; 43 C, 2 h) or chemical shock (CS; 200 μM arsenite, 2 h) or were left untreated. Immediately after treatment, 1 μM Dex was added to all three conditions, and the cells were allowed to grow under nonstress conditions for the indicated time intervals (0, 4, 8, 24, and 48 h). Lysates were prepared and relative CAT activities were measured and expressed as a percentage of the maximum for each condition.

An alternative interpretation of these results is that reappearance of cytosolic GR is required (in cells subjected to stress before hormone treatment) before that GR can bind hormone and take part in stress-induced potentiation of CAT gene expression. Under this scenario, normal hormone-mediated transformation of the GR presumably occurs (once it reappears in the cytoplasm), and it is the interaction of the hormone-bound GR with heat shock-altered nuclear acceptor sites or transcription machinery that results in stress-induced potentiation of GR-mediated transcription enhancement.

Potentiation of GR-Mediated Gene Expression Requires Only GRE Minimal Promoters

In a recent report, Edwards *et al.* (32) demonstrated that heat shock could enhance PR-mediated target gene transcription. This observation was made in T47D cells stably transfected with an MMTV-CAT reporter plasmid. As the construct employed by the authors was shown to contain a consensus HSF-binding site (HSE) within the MMTV LTR, it was proposed that one possible explanation for the heat shock potentiation effect was through an interaction between factors (HSF?) binding this site and hormone-bound PR. A search of the LTR promoter region has revealed the presence of a HSE, which must be arrayed in an inverted repeat pattern of head to head (nGAAnnTTCn) or tail to tail (nTTCnnGAAn) configurations to stably bind HSF (73), at a position 437 basepairs up-stream of the transcription start site. To determine the role of this putative HSE in heat shock potentiation of GR-mediated transcription enhancement, we stably transfected L929 cells with a series of MMTV-CAT reporter constructs in which varying portions of the LTR region were deleted. These constructs include the pMMTV-CAT reporter containing the intact LTR promoter region; the pΔMTV-CAT reporter, in which three of the four GREs were deleted; the p631-CAT reporter, in which all sequences up-stream of position −631 were deleted; and the p236-CAT reporter, in which all sequences up-stream of position 236 were deleted. Cell lines containing these constructs were then subjected to chemical shock in the presence or absence of Dex (Fig. 8). From the results obtained, it is clear that a large chemical shock potentiation effect can be achieved with all of these reporters except the pΔMTV-CAT. Thus, the large potentiation effects observed require not only combined chemical shock and Dex treatment, but also functional GRE sites. More importantly, the results obtained demonstrate that the putative HSE site at −437 is not involved in stress potentiation of GR action, and that no DNA sequence up-stream of −236 is involved.

Having obtained the results shown in Fig. 8, the possibility still remained that the stress potentiation effect was the result of cooperativity between the hormone-bound GR and a putative heat shock-activated, DNA-binding factor. According to this model, then, one or more cognate recognition sequences for these heat shock-activated factors must exist on the MMTV-LTR between −236 and the transcription start site. However, it seemed just as likely that the heat shock potentiation effect was not the result of cooperativity between an unknown DNA-binding factor(s) and the GR, but, rather, was due to a mechanism requiring GR as the only transcriptional enhancer. With this in mind, we performed the experiments shown in Fig. 9, in which cells stably transfected with a variety of minimal CAT reporters were subjected to chemical shock potentiation conditions. The minimal promoters employed included the $pGRE_2NF1E1B$-CAT construct, in which two tandem synthetic GREs were linked to an nuclear factor-1-binding sequence (NF1) site and a TATA box immediately up-stream of a CAT reporter gene; the pNF1E1B-CAT construct, which contained the NF1 and TATA sequences up-stream of CAT; the $pGRE_2E1B$ CAT construct, containing the GRE sites and TATA up-stream of CAT; and the pE1B-CAT construct, in which CAT expression is controlled only by the TATA sequence. The results shown in Fig. 9 demonstrate that potentiation of GR-mediated CAT expression only occurs with the minimal promoters containing GRE sites: $pGRE_2NF1E1B$-CAT and $pGRE_2E1B$-CAT. Thus, GRE sites are both necessary and sufficient for stress-mediated enhancement of GR transcription activity.

DISCUSSION

Until recently, differential gene expression by steroid hormone receptors was viewed as an independent signal transduction pathway principally controlled by steroidal agonists. Evidence is now accumulating that steroid receptor action may also be controlled by effector molecules of previously unrelated signal pathways.

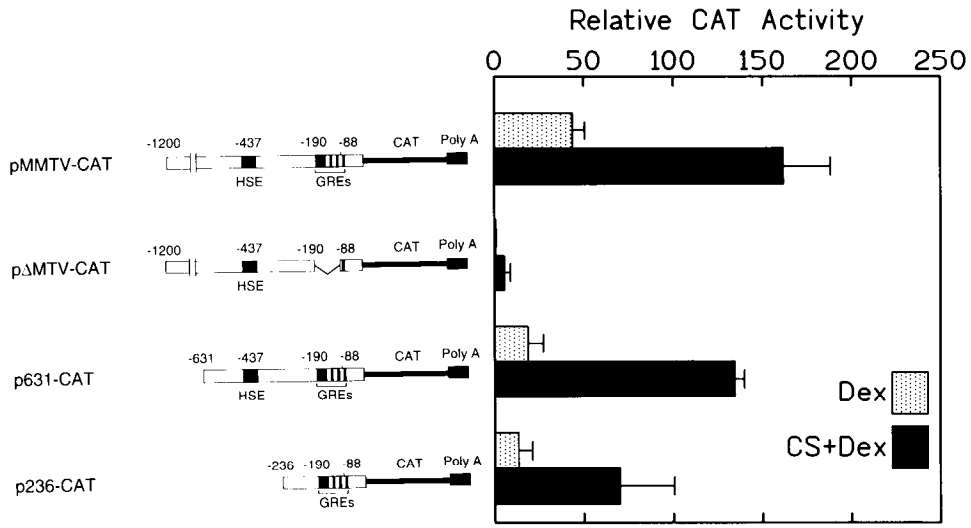

Fig. 8. A Consensus HSF-Binding Sequence (HSE) at Position −437 of the MMTV-LTR Is not Required for Stress Potentiation of GR-Mediated MMTV-CAT Expression

L929 cells were stably transfected with a variety of MMTV-CAT reporter plasmids, as indicated. Relative CAT inductions in each cell line were then measured in response to treatment with 1 μM Dex alone (Dex) or to chemical shock (200 μM arsenite) followed by 1 μM Dex (CS+Dex). Fold CAT inductions were calculated relative to basal (no treatment) control values for each cell line. The results shown represent the mean ± SEM of three or four independent experiments.

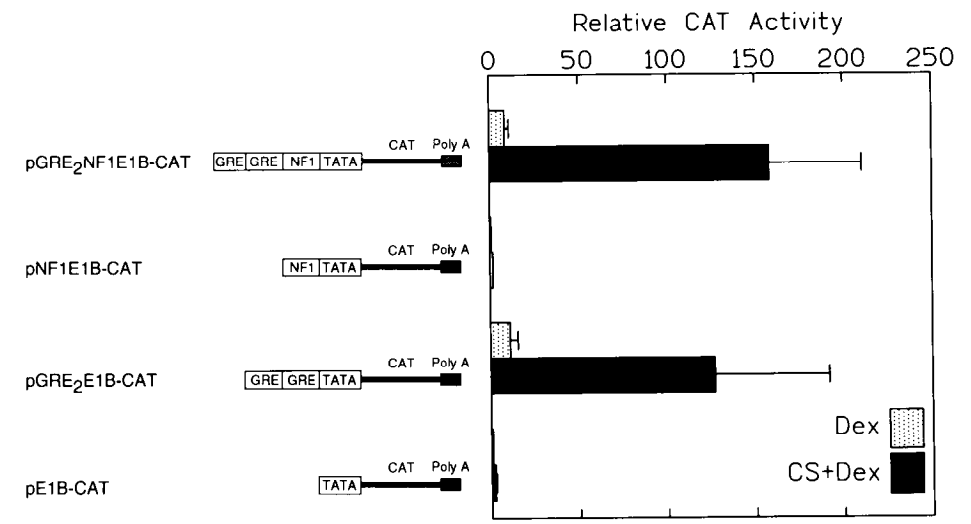

Fig. 9. Stress Potentiation of GR-Mediated CAT Gene Expression Occurs with Synthetic Promoters Containing Only GREs and a TATA Box

L929 cells were stably transfected with the indicated synthetic minimal promoters. Relative CAT inductions in each cell line were then measured in response to treatment with 1 μM Dex alone (Dex), or to chemical shock (200 μM arsenite) followed by 1 μM Dex (CS+Dex). Fold CAT inductions were calculated relative to basal (no treatment) control values for each cell line. The results shown represent the mean ± SEM of four independent experiments.

For example, several laboratories have demonstrated that treatment of intact cells with 8-bromo-cAMP, an activator of protein kinase-A (PKA), can either activate or potentiate transcription enhancement activity by the progesterone (38, 39), estrogen (40), and GRs (41). In support of these findings and of the involvement of phosphorylation mechanisms are the observations that an inhibitor of PKA (H8) can block hormone-mediated activation of PR (38, 39); that okadaic acid, a protein phosphatase inhibitor, can also potentiate hormone-mediated activation of progesterone (38, 39) and glucocorticoid (42) receptors; and that PKA itself can potentiate GR transcription enhancement (41).

The results of this study provide evidence that heat shock treatment of intact cells can dramatically increase the ability of the hormone-bound GR to act as an enhancer of specific gene transcription. The observations supporting this conclusion were made by stably

transfecting the GR-expressing L929 cell line with the pMMTV-CAT reporter construct and assessing the effect of cellular stress on Dex-induced CAT gene expression. Using this approach, we have developed the model of heat shock effects on GR action presented in Fig. 10. According to this model, heat or chemical shock will increase GR-mediated gene expression at both low and saturating concentrations of hormone, resulting in a significant increase in the degree of transcriptional enhancement activity. In light of the data presented in this work, there are several possible mechanisms, not mutually exclusive, to account for this effect of stress on GR transcription activity. As depicted in Fig. 10, one effect of stress may be to induce an intrinsic hyperactivation of the steroid-bound receptor. Such a hyperactivation may be the result of a covalent modification on the receptor, such as phosphorylation. As stated above, there is evidence from several laboratories that phosphorylation mechanisms may be involved in steroid receptor actions. For example, it has been shown that PR and GR transcription enhancement activities can be modulated by PKA itself (41), by activators of PKA (38, 39), or by inhibitors of protein phosphatases (42). Yet, direct studies on the effects of these treatments on steroid receptor phosphorylation have been few and largely negative. Moyer *et al.* (43) were not able to detect changes in GR phosphorylation under conditions where GR-mediated transactivation was potentiated by activators of PKA, protein kinase-C, and protein phosphatase inhibitors. Similarly, Mason and Housley (44) used site-directed mutagenesis techniques to show that phosphorylation sites in the mouse GR are not required for hormone-mediated activation of GR transactivation at the MMTV-LTR promoter. Although we have not examined stress-induced changes in GR phosphorylation in this work, we have provided evidence that any modification that occurs must take place after the GR is transformed to its high affinity, nuclear-bound state. The major observation in support of this conclusion is that the heat shock potentiation effect is observed even after hormone-mediated transformation and translocation of the GR has occurred (see Fig. 5).

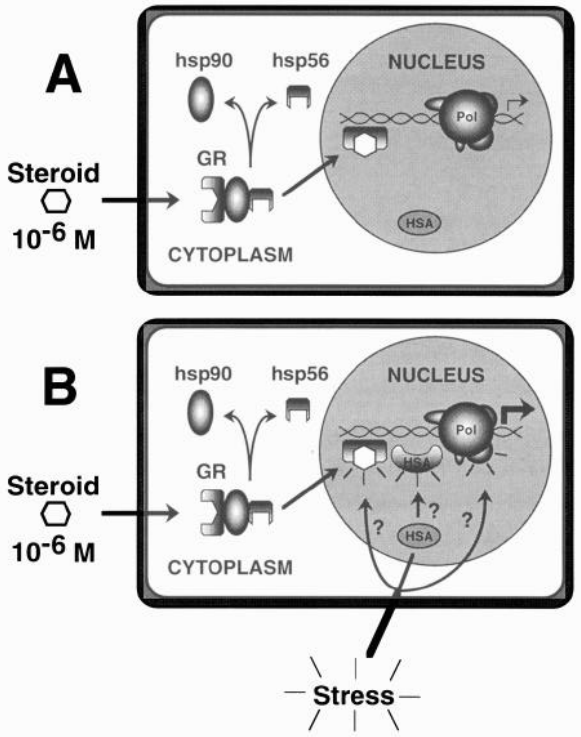

Fig. 10. Model of Heat Shock-Induced Potentiation of GR Transcription Enhancement Activity

In the nonstressed cell (A), exposure to saturating concentrations of Dex results in full steroid occupancy of the GR followed by dissociation of the GR complex, nuclear translocation, and binding by the hormone-bound GR to 5′-regulatory regions of target genes. Enhancement of target gene transcription by the GR then occurs by a poorly understood process that involves either a direct or indirect interaction between the hormone-bound GR and the RNA-polymerase-II complex (Pol). In cells subjected to stress after the addition of Dex (B), the efficiency of communication between the hormone-bound GR and Pol is altered, resulting in a higher level of transcription activity than that seen under hormone-alone conditions. It is postulated that this stress-induced increase in GR-mediated transcription enhancement is the result of changes in the DNA-bound GR, changes in components of the Pol complex, or activation of a putative HSA that serves a coactivation function.

A second possible mechanism to account for the heat shock potentiation is that the hormone-bound GR in heat-shocked cells is interacting in a cooperative manner with a putative, HSF(s), a speculation that has also been advanced by Edwards *et al.* (32). Initially, we considered the HSF, which is responsible for the transcription of heat shock protein genes during stress (39), as the most likely candidate for this factor. This speculation was particularly appealing because an examination of the MMTV LTR DNA sequence (GenBank) revealed the presence of a consensus HSE at −437 and a total of 14 possible HSEs when mismatch is allowed to occur at one base; with four of these near-perfect HSEs clustered between −265 and −132. However, the results we have reported here do not support a role for HSF or HSEs in heat shock potentiation, as cellular stress alone had no effect on MMTV-CAT expression (Fig. 1), and deletion of the HSE at −437 did not eliminate the potentiation effect (Fig. 8). The results shown in Fig. 8 did leave open the possibility that DNA-binding transcription factors other than HSF could be involved in heat shock potentiation. This idea seemed plausible, because DNA-binding sites for a variety of factors (*e.g.* NF1) had been shown to cause dramatic increases in the rates of GR-mediated CAT gene transcription (45). In addition, Oshima and Simons (46) reported the presence of a 21-basepair glucocorticoid modulatory element in the regulatory region of the tyrosine aminotransferase (TAT) gene that increases agonist-mediated expression of TAT. Yet, our observations with minimal promoters containing GRE sites and a TATA box (Fig. 9) suggest that, with the possible exception of the TATA sequence, the heat

stress effect is not the result of an interaction between GRE sites and other *cis*-acting elements.

Stress potentiation of GR-mediated transcription could also result from cooperative binding by GR to tandem GRE sites. As previously reported (47), this would involve the binding of GR dimer to the first GRE site, which would then facilitate binding by another GR dimer to the second GRE site. Although stress potentiation of GR transcription enhancement was observed with the $pGRE_2E1B$-CAT reporter, which contains two tandemly linked GREs, the observed stress potentiation occurred at high concentrations of Dex (1 μM). At this concentration, all of the steroid-binding sites on the GR should be occupied, and consequently, all GRE sites would be bound with GR. Consistent with this is our observation that 1 μM Dex results in near-complete translocation of the GR to the nucleus (see Fig. 4). Thus, our data suggest that heat shock potentiation does not result from cooperative binding by GR dimers to multiple GRE sites.

An alternative mechanism to explain stress-mediated potentiation of GR transcription enhancement is that this process is facilitated by molecules that interact with the DNA-bound GR and the transcription initiation complex. A mechanism such as this has been proposed by several laboratories to explain synergistic activation of transcription in a variety of procaryotic and eucaryotic systems (48–52), and the effector molecules involved have been referred to as adaptors (48, 51) or coactivators (52). Recently, evidence for the adaptor model of synergistic transcription enhancement has been reported for steroid receptors. For example, Bastian and Nordeen (53) provided indirect evidence that stimulation of GR-mediated CAT gene expression by Dex is limited by a constitutively present coactivator, and that unmasking of latent RU486 agonist activity by activators of PKA is mediated by a PKA-inducible coactivator (54). A related proposal has been put forward by Cho and Katzenellenbogen (55) to explain synergistic enhancement of estrogen receptor-mediated transcription by activators of PKA. In this case, PKA activators serve to stabilize or facilitate interaction between the estrogen receptor and components of the polymerase-II transcription complex. In large part, these conclusions by the laboratories of Nordeen and Katzenellenbogen have been based on results using minimal promoters containing the appropriate hormone response elements. In this paper, we show that heat shock potentiation of GR-mediated gene expression can also be observed with minimal promoters containing synthetic GRE sequences. For this reason, we have incorporated some of these ideas into the model shown in Fig. 10. In addition to possible intrinsic changes in the GR itself, we propose that the effect of cellular stress on GR-mediated transcription activity may be due to the presence of a heat shock-activated adaptor (HSA) or to some change in a preexisting component of the polymerase-II transcription initiation complex. With regard to the latter, one possible target may be the TATA-binding protein of the basal transcription factor TFIID (56). In the case of heat shock adaptors, it should be emphasized that the putative HSA may not be different from the adaptor(s) proposed to be activated by the PKA pathway. Indeed, one of the major attractions of the adaptor/coactivator model is that a common factor could be the mechanism by which diverse signal pathways cause synergistic enhancement of transcription from target genes (57).

We have also considered the roles that hsps could play in controlling the transactivation by GR in heat-shocked cells. It has been well documented that hsp90 is required for establishing and maintaining the high affinity hormone-binding state of the GR and other steroid receptors, and that hsp70 is involved in the assembly of steroid receptor/hsp90 complexes (3). Yet a third steroid receptor-associated hsp has been described, hsp56, whose receptor-related function remains unknown (12). These hsps have been shown to comprise the core of a hsp complex found in the cytosolic fraction of cells (58, 59). In heat-shocked cells, the hsp90-hsp70-hsp56 complex remains stable and changes only to the extent that both the inducible and constitutive forms of hsp70 are now present (12). This observation has led us to speculate that the appearance of the inducible form of hsp70, in either the hsp core complex or the GR complex, may be related to the stress-induced increase in GR transcription activity we report here. A similar speculation was made by Edwards *et al.* (32) when it was found that chemical shock (sodium arsenite) resulted in both a higher level of hsp70 synthesis and a higher level of PR transcription enhancement than did heat shock, suggesting a correlation between the relative fold stimulation of hsp synthesis and the stress potentiation effect. However, the exact nature of this correlation between levels of hsp induction and GR transactivation function remains far from resolved. One possible explanation is derived from the facts that hsps appear to function in the cellular adaptation to stress by binding to misfolded proteins (20, 21), and that hsp90 may be essential for maintaining the cytoplasmically localized, hormone-binding state of the GR (3). Given these observations, it is possible that hsp90 is recruited off the GR in the heat-shocked cell due to the sudden appearance of large numbers of substrates, and that this then leads to loss of GR steroid-binding capacity and premature binding to DNA. Although this mechanism provides a logical explanation for our observation that cellular stress causes the unliganded GR to translocate to the nucleus (31), it does not supply a satisfactory explanation for the effects of stress on Dex-induced GR transcription enhancement, because as we have shown, the stress potentiation effect requires the presence of GR capable of binding hormone. A more plausible mechanism may be derived from the fact that both hsp90 and hsp70 are known to accumulate in the nucleus of heat-shocked cells (60, 61). Thus, under heat shock conditions, increased amounts of nuclear hsp90 and hsp70 may serve a stabilizing or stimulatory role on GR-mediated transcription activity. Although no evidence exists at present for

an effect by hsp90 or hsp70 on transcription factor functions, evidence does exist that hsp70 remains complexed to glucocorticoid (11) and progesterone (9, 10) receptors even after these receptors are hormonally transformed to the DNA-binding state, prompting speculation that hsp70 may be involved in the nuclear functions of steroid receptors (3, 11). With this in mind, it will be interesting to determine if hsp70 or another hsp serves the function of the putative HSA adaptor proposed in our model (Fig. 10).

The results presented in this work provide evidence that the steroid hormone and heat shock signal transduction pathways may converge to control GR-mediated expression of the MMTV-CAT reporter gene. One interesting possibility that arises from this observation is that GR-mediated gene expression may be required for cellular adaptation to stress. This idea seems plausible for a variety of reasons. For example, it is clear that hsps, among their varied functions, provide protection to the cell after a stress event. In addition, it has been know for many years that conditions of organismal stress, such as trauma, infection, and fever, lead to a rapid increase in glucocorticoid secretion by the adrenal cortex (see Ref. 62 for review). This observation has led to a model in which glucocorticoids can provide an organism with increased resistance to stress (62). The results presented herein may provide a molecular explanation for this phenomenon, in that certain GR-induced gene products may be required for cell and organ survival after a stress event. With this in mind, it will interesting to determine whether heat shock activation and potentiation of GR-mediated transcription also occur for endogenous genes known to be positively regulated by glucocorticoid hormones.

MATERIALS AND METHODS

Materials

The pMMTV-CAT and pSV2Wrec plasmid DNAs were obtained from Mark Danielsen and Gordon Ringold. The pMMTV-CAT plasmid contains the complete MMTV-LTR promoter upstream of the CAT reporter gene (63). Hormonally driven expression of CAT by this reporter is principally controlled by GREs known to reside within the LTR region (64). The pSV2Wrec plasmid contains the cDNA for the mouse wild-type GR gene under the control of the simian virus-40 promoter (63). The pΔMTV-CAT plasmid (65) was obtained from Vincent Giguire. This plasmid DNA contains a modified MMTV-LTR sequence up-stream of the CAT reporter gene in which bases 190 to −88 have been deleted, thus eliminating three of the four GREs present in the MMTV-LTR (64). The pSV2neo plasmid (66), in which aminoglycoside phosphotransferase (*neo*) is constitutively expressed from the simian virus-40 early promoter, was obtained from Margaret Hirst. The p236-CAT and p631-CAT constructs were obtained from Miguel Beato. In these reporters, expression of CAT is controlled by MMTV-LTR regions in which all sequences up-stream of −236 (p236-CAT) or −631 (p631-CAT) have been deleted. Several minimal CAT reporter plasmids were used in this study. The $pGRE_2NF1E1B$-CAT construct contains two tandem synthetic GREs linked to a synthetic NF1 site and a TATA box immediately up-stream of a CAT reporter gene. The pNF1E1B-CAT construct contains the NF1 and TATA sequences up-stream of CAT. The $pGRE_2E1B$-CAT construct contains two GRE sites and TATA up-stream of CAT, whereas in the pE1B-CAT construct, CAT expression is controlled only by the adenovirus-E1B TATA sequence. These constructs were developed by Victoria Allgood and John Cidlowski by introducing synthetic GREs derived from the TAT promoter, as well as a synthetic NF1-binding site, into the pE1B-CAT plasmid (67).

Lipofectin was obtained from BRL (Gaithersburg, MD). [^{14}C] Chloramphenicol (51.4 mCi/mmol) and ^{125}I-conjugated goat antimouse immunoglobulin G (11.8 μCi/μg) were obtained from ICN Radiochemicals (Irvine, CA). [^{3}H]Triamcinolone acetonide (42.8 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA). Silica gel-60 TLC plates were obtained from EM Separations, Inc. (Gibbstown, NJ). Immobilon P membranes were obtained from Millipore Corp. (Bedford, MA). Sodium arsenite, Dex, G418 (Geneticin) antibiotic, acetyl coenzyme-A, Tris, EDTA, protein-A-Sepharose, horseradish peroxidase-conjugated rabbit antigoat immunoglobulin G, Dulbecco's Modified Eagle's powdered medium (DMEM), and iron-supplemented newborn calf serum were obtained from Sigma Chemical Co. (St. Louis, MO). The BuGR2 anti-GR monoclonal antibody (68) was a gift from Drs. William Hendry and Robert Harrison (University of Arkansas, Little Rock).

Cell Culture and Stress Treatment

The mouse L929 cell line and its MMTV-CAT-transfected derivatives were grown in an atmosphere of 5% CO_2 at 37 C in DMEM containing 10% iron-supplemented newborn calf serum. The E82.A3 cell line is a subclone of L929 selected for glucocorticoid resistance and does not contain GR mRNA or GR protein (36). The E82.A3 cell line and its MMTV-CAT transfected derivative (LGR^-CAT) were grown in DMEM containing 5% iron-supplemented newborn calf serum. For all experiments, the newborn calf serum was first stripped of all 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. The typical duration of heat shock treatment was 2 h. Cells were also stressed by the addition of sodium arsenite to the medium at the indicated final concentrations (usually 200 μM). Both the arsenite-treated and nontreated cells were incubated at 37 C for 2 h and then washed with DMEM medium and allowed to recover.

Stably Transfected CAT Reporter Cell Lines

Establishment of the LMCAT cell line was achieved by cotransfecting one flask (75 cm^2) of L929 cells at approximately 50% confluence with 10 μg pMMTV-CAT DNA and 5 μg pSV2neo DNA, using 100 μg lipofectin reagent (69) as carrier. After an overnight incubation with DNA and lipid, the transfected cells were allowed to grow for 48 h. The cells were then subcultured (1:2) in DMEM medium containing 0.4 mg/ml active G418 (Geneticin) antibiotic and incubated at 37 C, with frequent replacement of the selection medium, until colony growth was noted. The pooled cell culture thus derived was designated LMCAT. Cloned cell lines were isolated, with 70% of these demonstrating strong Dex induction of CAT gene expression. One of these, LMCAT2, was selected for the studies reported here, although similar results have been obtained with a sister clone (LMCAT1) and the pooled parental cell line (LMCAT). Establishment of the other CAT reporter cell lines used in this study was achieved by cotransfecting 10 μg of the appropriate CAT plasmid DNA with 5 μg pSV2neo DNA. All subsequent steps, including isolation of clonal derivatives, were performed as described above.

CAT Assay

Measurement of CAT enzyme activity was performed essentially according to the method of Gorman *et al.* (70). Briefly, cell lysates were prepared by sequential freezing and thawing in 0.25 M Tris-5 mM EDTA (pH 7.5) and centrifugation at 8000 × *g*. Aliquots of lysate containing equal protein content were added to the enzymatic reaction mixture, and the reaction was stopped by extraction with ethyl acetate. The acetylated forms of [^{14}C]chloramphenicol were separated by TLC. Quantitation was achieved by separately excising the spots corresponding to substrate and product and performing liquid scintillation spectroscopy. The counts per min values obtained were then normalized to the basal (no treatment) controls to yield relative (fold induction) CAT activities. Percent substrate conversion values ranged from 1–20% for all experiments reported.

Cellular Fractionation and Immunoadsorption

In the experiment shown in Fig. 4, individual 75-cm^2 flasks of L929 cells subjected to various Dex and heat shock conditions were fractionated into cytosolic and nuclear portions by Dounce A homogenization in hypotonic buffer (10 mM HEPES and 1 mM EDTA, pH 7.4). After centrifugation at 1000 × *g* for 5 min, the cytosolic fractions were saved, and the nuclear pellets were washed twice by resuspension and pelleting in hypotonic buffer containing 250 mM sucrose. Hypotonic buffer was then added to both the pellet and cytosolic fractions to a final volume of 0.5 ml. Each fraction was made 0.5 M for NaCl by the addition of 0.5 ml of a 1-M stock solution and incubated on ice with occasional vortexing for 1 h. After salt extraction, the nuclear pellets were centrifuged at 8000 × *g*, and the supernatants were saved. BuGR2 anti-GR monoclonal antibody (20 μl) was added to both the cytosolic and nuclear extract fractions, and each sample was then adsorbed in batch to protein-A-Sepharose, washed, and eluted with 2 × sodium dodecyl sulfate (SDS) sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% mercaptoethanol). In the experiment shown in Fig. 6, cytosolic fractions only were prepared. These cytosols were subjected to GR immunoadsorption as described above, except that the step involving the addition of NaCl was eliminated.

Gel Electrophoresis and Quantitative Western Blotting

Samples were resolved by electrophoresis in 7% polyacrylamide-sodium dodecyl sulfate gels, as described by Laemmli (71). The relative amounts of GR protein in the cytosolic and nuclear fractions were determined via a quantitative Western blotting technique, previously described (37), which employs the BuGR2 anti-GR monoclonal antibody and both peroxidase- and ^{125}I-conjugated counterantibodies. After Western blotting and autoradiography, the peroxidase-stained receptor bands were excised, and ^{125}I counts per min were determined via liquid scintillation spectrophotometry. Receptor-specific counts per min were derived by subtracting the counts per min measured in a background slice of comparable area.

Steroid Binding Assay

To determine the specific steroid-binding capacity, replicate aliquots (45 μl) of 12,000 × *g* cytosols were incubated with 50 nM [^{3}H]triamcinolone acetonide (42.8 Ci/mmol) in the presence or absence of a 1000-fold excess of unlabeled Dex, as previously described (72). All hormone-binding capacities were calculated as specific counts per min/mg cytosol protein.

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