Enhancement of glucocorticoid receptor-mediated gene expression by cellular stress: evidence for the involvement of a heat shock-initiated factor or process during recovery from stress

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Abstract Recent reports have demonstrated the ability of cellular stress to cause a large increase in the maximal levels of steroid receptor-mediated gene expression, a process we refer to as the heat shock potentiation effect (HSPE). In the present work, we have analyzed the time of appearance of the HSPE on the glucocorticoid receptor (GR) of L929 cells stably-transfected with the MMTV-CAT reporter plasmid (LMCAT2 cells). In LMCAT2 cells exposed to heat shock (43°C, 2-h) before addition of 1 µM dexamethasone, the first appearance of HSPE (CAT levels greater than hormone-alone) occurred at 8 h of recovery and continued to increase by 24 h of recovery. Treatment of LMCAT2 cells with 1 µM dexamethasone for 2 h before heat or chemical shock (sodium arsenite) resulted in the same delayed onset pattern for the HSPE. Based on a [³⁵S]methionine assay and tests of L929 cells stably transfected with the constitutive pSV2–CAT reporter, evidence is provided that the delayed appearance of the HSPE is not due to the heat shock block of general protein synthesis or to specific repression of CAT mRNA expression or translation. By using short-term incubations (4 h) with dexamethasone during the recovery period, the peaks of HSPE expression during recovery were determined to be 12–16 h for CAT enzyme activities, and 4–8 h for CAT mRNA expression. Taken together, these results provide evidence that the timing of the HSPE is not dependent on the rate of GR activation, or on the type of stress, but rather on a factor or process that is either synthesized or activated during the recovery period following stress.

INTRODUCTION

Many reports of 'cross-talk' between steroid receptor and previously unrelated signal transduction pathways have recently been published. Examples include activation of the progesterone receptor by dopamine (Power et al 1991), the involvement of protein kinase A in activation or potentiation of estrogen (ER), progesterone (PR) and glucocorticoid (GR) receptors (Denner et al 1990; Beck et al 1992; Rangarajan et al 1992; Cho and Katzenellenbogen 1993),

and the potentiation of ER by insulin-like growth factor-I (Aronica and Katzenellenbogen 1993). Yet the earliest evidence for cross-talk involving steroid receptors arose approximately 11 years ago when the presence of the 90-kDa heat shock protein, Hsp90, within untransformed steroid receptor complexes was first discovered (Catelli et al 1985; Sánchez et al 1985; Schuh et al 1985; Mendel et al 1986). This discovery quickly led to investigations into the convergence of steroid and heat shock responses. Early attempts at this question proceeded by testing the effects of steroids on levels of heat shock protein expression or other aspects of the cellular stress response. Although most of these efforts were unsuccessful, reports of the induction of low molecular weight Hsps in *Drosophila* by

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the insect steroid hormone α -ecdysterone are available (Ireland et al 1982; Berger and Woodward 1983). However, similar results in mammalian cells are few. One notable exception is a report by Fisher et al (1986) in which glucocorticoid treatment of Chinese hamster ovary cells was shown to induce a state of thermotolerance similar to that obtained in response to heat stress.

In contrast, reports describing the effects of cellular stress on steroid receptor functions have been more numerous. These studies include a report demonstrating that a short exposure of rat uterine cells to 40°C can result in a large shift of hormone-free ERs from the cytosolic to the nuclear pellet fraction (Campbell and Swanson 1989). Another study (Vedeckis et al 1989) found that heat shock treatment of a variety of mammalian cell lines results in a loss of GR steroid-binding capacity from the cytosol, presumably due to stress-induced degradation of the GR, although this assumption was not directly tested. Results from our laboratory have shown that heat shock treatment of intact cells can cause a loss of hormone-binding capacity, but that the majority of the GR in these cells is not degraded (Sánchez 1992). Instead, we observed that both heat and chemical shock treatment of these 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 (Sánchez 1992). Since then, we have shown that heat and chemical shock can result in a small but reproducible activation of GR transcription enhancement activity in the absence of hormone (Shen et al 1993). However, when cell stress is combined with hormone treatment much more dramatic increases in steroid receptor activation can be found. For example, Edwards et al (1992) showed that combined hormone and stress treatment of T47D breast cancer cells resulted in a level of PR-mediated transcription activity much higher than that seen in response to hormone alone. In our laboratory, we have found that both heat and chemical stress (sodium arsenite) can markedly increase dexamethasone (Dex)-induced GR-mediated gene transcription (Sánchez et al 1994), and we refer to this phenomenon as the heat shock potentiation effect (HSPE).

In the latter study, we provided evidence that the mechanism of the HSPE may involve, amongst other possibilities, the induction in heat shocked cells of a factor that mediates the interaction between the DNA-bound GR and the polymerase II (Pol II) transcription complex. In the present study, we have analyzed the time of appearance during recovery of the HSPE on the GR. In cells stably transfected with a GR-inducible chloramphenicol acetyltransferase (CAT) reporter gene, we find that there is a delay in the onset of the HSPE on the GR relative to hormone treatment alone. In addition, short-term exposure to hormone in cells recovering from stress

revealed the presence of well-defined peaks of heat shock potentiation activity on the GR, as analyzed by both CAT protein and mRNA. These results and others suggest that the HSPE on GR-controlled gene expression is mediated by a heat shock-inducible factor that is either synthesized or activated during the recovery period.

MATERIALS AND METHODS

Materials

[³H]Acetate (10.3 μ Ci/mmol) and [³⁵S]methionine ('Trans-label'; 1133 Ci/mmol) were obtained from ICN Radiochemicals. [α -³²P]dCTP (6000 μ Ci/nmol) and Hybond N+ charged nylon membranes were obtained from Amersham Corporation. Sodium arsenite, dexamethasone, G418 (Geneticin) antibiotic, acetyl CoA synthetase, acetyl Co-enzyme A, tris, Hepes, EDTA, protein A-Sepharose, DMEM powdered medium, trichloroacetic acid, cycloheximide and BSA were from Sigma Chemical Company. Iron-supplemented newborn calf serum and dialyzed fetal bovine serum were from Hyclone. Tri Reagent for the isolation of mRNA was obtained from Molecular Research Center, Inc. The Prime-It II random primer labeling system was obtained from Promega, Inc.

The pMMTV-CAT plasmid DNA was obtained from Mark Danielsen and Gordon Ringold. The pMMTV-CAT plasmid contains the complete mouse mammary tumor virus-long terminal repeat promoter (MMTV-LTR) upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (Danielsen et al 1986). Hormonally driven expression of CAT by this reporter is principally controlled by glucocorticoid response elements (GREs) known to reside within the LTR region (Cato et al 1986). The pCAT-control plasmid was obtained from Promega, Inc. In this plasmid, high-level, constitutive expression of the CAT reporter gene is controlled by the SV40 early promoter.

Cell culture and stress treatment

The LMCAT2 cells were established as previously described (Sánchez et al 1994). Briefly, mouse L929 cells were co-transfected with the pSV2neo and pMMTV-CAT plasmids, followed by selection for a stably transfected, cloned cell line using G418 (Geneticin) antibiotic. The LSVCAT cell line was similarly derived by co-transfecting L929 cells with the pSV2neo and pCAT-Control plasmids. The LMCAT cells and LSVCAT cells were grown in an atmosphere of 5% CO₂ at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 0.2 mg/ml G418 and 10% iron-supplemented newborn calf serum. 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 are obtained with subconfluent cultures. Heat shock treatment was achieved by shifting replicate flasks to a second 5% CO₂ incubator set at 43°C. Typical duration of heat shock treatment was 1–2 h. Cells were also stressed by addition of 200 µM sodium arsenite to the medium. Both the arsenite-treated and non-treated cells were incubated at 37°C for 2 h and were then washed with DMEM medium and allowed to recover. In the experiments of Figure 2B, Dex was added to both the stress and recovery media.

CAT assay

Measurement of CAT enzyme activity was performed according to the method of Nordeen et al 1987) with minor modifications. 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 an enzymatic reaction mixture containing acetyl CoA synthetase, [³H]sodium acetate, coenzyme A (CoA) and ATP. Radioactively labeled acetyl coenzyme A was first generated enzymatically 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 the entire organic phase was dried and counted.

Measurement of protein synthesis

In the experiment shown in Figure 3, the medium was removed and replaced with methionine-free medium containing 10% dialyzed fetal bovine serum and [³⁵S]methionine at a final concentration of 50 µCi/ml. The cells were labeled at 37 °C for 45 min. All subsequent steps were carried out on ice. The cells were washed and resuspended in 10 pellet volumes of 10 mM Hepes (pH 7.4), 1 mM EDTA, 0.5 M NaCl. The cells were ruptured by Dounce homogenization and centrifuged at 8000 × *g* for 5 min. The supernatant (whole cell extract) was stored at –20 °C or used immediately. Aliquots (10 µg) of whole cell extracts, to which were added 500 µg of BSA as carrier, were TCA precipitated (20%) and counted.

RNA Northern blotting and quantitation

Total mRNA was isolated from the LMCAT cells by using the Tri Reagent method of the Molecular Research Center, Inc. The RNA samples were then fractionated on formaldehyde-containing 1.2% agarose gels (20 µg RNA

per lane) and transferred to charged Hybond N+ nylon membranes by vacuum blotting. CAT cDNA (1 kb) was excised from the pMAMneo-CAT plasmid by digestion with *Sal* I, and was labeled with [α -³²P]dCTP using the Prime-It II random primer labeling system of Promega, Inc. After transfer, the membranes were incubated in pre-hybridization buffer (5X SSC, 5X Denhardt's solution, 0.5% SDS, 1 µg/ml sheared salmon sperm DNA) in a rotating oven for 2 h at 68°C, then overnight in the same buffer containing [³²P]CAT cDNA (100 × 10⁶ cpm/µg DNA) at 68°C. Following hybridization, the membranes were washed once in 1X SSC/0.1% SDS for 20 min at room temperature, then three times for 20 min each in 0.2X SSC/0.1% SDS at 68°C. The membranes were exposed to Kodak XAR-5 X-ray film with an intensifying screen for ≈2 h at –80°C and the relative amounts of CAT mRNA were calculated by densitometry scanning using the Bio-Rad Molecular Analyst software.

RESULTS

Delayed onset of heat and chemical shock potentiation of Dex-induced MMTV-CAT expression during the stress recovery period

In the experiments shown in Figure 1 we have performed a time-course for the appearance of the HSPE on GR-mediated CAT gene expression in a mouse L929 cell line (LMCAT2 cells) stably transfected with the pMMTV-CAT reporter. In these experiments, flasks of LMCAT2 cells were either left untreated or were subjected to heat shock at 43°C for 1 h. Both sets of cells were then incubated with 1 µM Dex [a concentration of steroid previously documented to yield maximum expression of CAT in these cells (Sánchez et al 1994)] and the levels of CAT enzyme activity were measured at precise intervals during recovery. Under these circumstances it can be seen that the HSPE is not detectable until 8 h of recovery and is very strong at 24 h. At time intervals before 8 h, the levels of CAT gene expression in response to the combined heat shock and hormone treatment were actually lower than those seen in response to hormone treatment alone. These data suggest that the ability of heat shock to potentiate the transcription enhancement activity of the GR requires a finite amount of time during the recovery period.

In the experiment in Figure 1, the LMCAT2 cells were subjected to heat shock before addition of hormone. As previously documented (Sánchez 1992), heat shock treatment of these cells results in nuclear translocation of the GR, with simultaneous loss of hormone-binding function. Because of this, we have reasoned that the delay in the onset of the HSPE on the GR observed in Figure 1 may be due to a mechanism in which the GR must either recycle to the cytoplasm or be synthesized *de novo* during the

Fig. 1 Time-course of Dex-induced CAT expression in LMCAT2 cells recovering from heat shock. LMCAT2 cells were subjected to heat shock (43°C, 1-h), or were left untreated. Immediately after treatment, 1 μ M Dex was added and the cells were allowed to culture under non-stress conditions for the indicated time intervals. Lysates were prepared and analyzed for relative CAT enzyme activity (fold induction relative to no-treatment, zero-time controls). The results shown represent the means $\pm$ SEM of three independent experiments. Inset: data for the 0-, 1-, 2- and 4-h time points only. Large panel: data for the complete time-course.

recovery period before the hormone-dependent, heat shock potentiation can take place. Indeed, we have already reported evidence in support of this notion, as GR capable of binding hormone can be recovered in the cytosolic fraction of heat shocked cells within 4 h of recovery (Sánchez et al 1994). As a test of this hypothesis, we performed the experiments seen in Figure 2, in which LMCAT2 cells were exposed to 1 μ M Dex for 2 h before heat or chemical shock treatment, and the cells were grown in the continued presence of Dex during the recovery period. Under these conditions, it is known that the majority of the GR translocates to the nucleus in response to the hormonal signal (Sánchez 1992; Sánchez et al 1994). Thus, the need for GR recycling or de novo synthesis before appearance of the HSPE should be eliminated, as the GR is already bound with hormone and residing in the promoter regions of regulated genes. To our surprise, addition of hormone before the stress event still resulted in a delayed pattern of onset for the potentiation effect by both heat (Fig. 2A) and chemical shock (Fig. 2B).

There are two conclusions that can be drawn from the above results. First, it is unlikely that GR recycling or de novo synthesis is the reason for this delay. Second, the onset of the potentiation effect appears to be highly consistent, appearing at 8 h of recovery regardless of

Fig. 2 Recovery time-course of Dex-induced CAT expression in LMCAT2 cells exposed to hormone prior to heat or chemical shock. LMCAT2 cells were exposed to 1 μ M Dex for 2 h followed by heat shock at 43°C for 2 h (panel A), or chemical shock employing 200 μ M arsenite for 2 h (panel B), or no stress treatment (panels A and B). After shock treatment, all cells were cultured under non-stress conditions for the indicated time intervals. Lysates were prepared and analyzed for relative CAT enzyme activity (fold induction relative to no-treatment, zero-time controls). The results shown represent the mean $\pm$ SEM of three independent experiments.

whether Dex was added before or after stress (compare Figs 1 and 2), or regardless of the type of stress (compare Figs 2A and 2B). Thus, the onset of the stress potentiation effect appears to be dependent on an event initiated by stress and occurring in the stressed cells at a fixed time in the recovery period.

Delayed onset of stress potentiation of Dex-induced MMTV-CAT expression is not due to depressed general protein synthesis or depressed synthesis of CAT enzyme

We have previously speculated that the above mentioned 'event' could take the form of a heat shock-inducible factor that is either activated or synthesized in

Fig. 3 Relative protein synthesis in LMCAT2 cells recovering from heat or chemical shock. LMCAT2 cells were subjected to heat shock (43°C, 1-h), or chemical shock (200 μ M arsenite, 2-h), or were left untreated. Immediately after shock treatment, 1 μ M Dex was added and the cells were allowed to culture under non-stress conditions for the indicated time intervals. At each time point, [35 S]methionine incorporation into protein was measured as described under Methods. CPM values obtained are expressed as percentages of untreated control cells. The results shown represent the mean $\pm$ SEM of three independent experiments.

stressed cells and that mediates the potentiation of GR-mediated gene expression (Sánchez et al 1994). Before this alternative could be seriously considered we had to first address the possibility that the delay in appearance of the HSPE on the GR was simply due to the generalized depression of protein synthesis that is known to occur in heat shocked cells. To test this question, we did the experiment seen in Figure 3, in which the relative levels of general protein synthesis were measured in LMCAT2 recovering from stress and exposed to hormone. As expected, both heat and chemical shock resulted in large decreases in the levels of [35 S]methionine incorporation into protein as compared to unstressed control cells. When these cells were allowed to recover, the levels of protein synthesis gradually rose over time, but only reaching $\approx$ 40% (heat shock) and $\approx$ 60% (chemical shock) of the unstressed level by 24 h of recovery. Although these data appear to support the hypothesis that the heat shock block of protein synthesis and its gradual recovery are responsible for the delayed onset of the HSPE, aspects of the data are also inconsistent with this conclusion. As already shown, the levels of GR-mediated CAT gene expression increased dramatically in the 4- to 8-h recovery period: from $\approx$ 40-fold to $\approx$ 200-fold in heat shocked cells (Fig. 2A), and from $\approx$ 25-fold to $\approx$ 175-fold in chemically shocked cells (Fig. 2B). If the heat shock block and its release were indeed responsible for the pattern of CAT activity seen in stressed cells (Figs 1 and 2), then we would expect to find a similar prominent lifting of the protein synthesis block, especially during the 4- to 8-h

Fig. 4 Time-course of CAT expression in LSV2CAT cells recovering from heat or chemical shock. LSV2CAT cells (L929 cells stably transfected with pSV2CAT) were subjected to heat shock at 43°C for 1 h (HS), or chemical shock employing 200 μ M arsenite for 2 h (CS), or were left untreated (Control). Immediately after treatment, the cells were allowed to culture under non-stress conditions for the indicated time intervals. Lysates were then prepared and analyzed for relative CAT enzyme activity (fold induction relative to no-treatment, zero-time controls). The results shown represent the mean $\pm$ SEM of three independent experiments.

transition. When the changes in levels of protein synthesis between 4 and 8 h of recovery are compared (Fig. 3), it is seen that these levels only increase from $\approx$ 25% to $\approx$ 35% in heat shocked cells, and barely at all (from $\approx$ 42% to $\approx$ 45%) in chemically shocked cells. Thus, the large increase in CAT gene expression observed at 8 h of recovery from stress does not appear to correlate in magnitude with the corresponding increase in general protein synthesis.

The experiments shown in Figure 3 tested the effects of stress on general protein synthesis. In order to test the effect of stress on specific synthesis of CAT protein, we measured CAT enzyme levels during recovery from stress in L929 cells (LSV2CAT) stably transfected with a plasmid (pSV2-CAT) in which expression of CAT is under the constitutive control of the SV40 promoter (Fig. 4). The results show that both heat and chemical shock have little effect on the levels of CAT gene expression, either immediately after the stress event or during the recovery period. Although it is not clear why CAT protein synthesis should be unaffected by the heat shock block of general protein synthesis, these data do support the notion that the pattern of delayed onset for the HSPE on GR-mediated expression of CAT is not the result of a heat shock-induced decrease in CAT mRNA translation followed by rebound synthesis during recovery.

Determination of peak stress potentiation activity

Taken together, the experiments in Figures 1–4 are consistent with a model of the HSPE in which a heat shock-inducible factor is either synthesized or activated during

Fig. 5 Determination of peak heat shock potentiation activity by 'pulse' incubation with Dex of LMCAT2 cells recovering from heat or chemical shock. LMCAT2 cells were subjected to heat shock at 43°C for 2 h (HS), or chemical shock employing 200 μ M arsenite for 2 h (CS), or were not stressed (Dex). Immediately after treatment, the cells were allowed to culture under non-stress conditions and 1 μ M Dex was added for 4 h intervals beginning at 0, 4, 8, 12, 20, and 40 h of recovery. Each series of samples also included non-hormone-treated controls (C). Lysates were prepared and analyzed for relative CAT enzyme activity (fold induction relative to no-treatment, zero-time controls). The results shown represent the mean $\pm$ SEM of three independent experiments.

the stress recovery period. Assuming that such a factor exists and that it eventually disappears or loses activity during recovery (the cells eventually return to normal with respect to Dex-induced CAT expression), then we would expect to see a defined peak of activity for the HSPE during recovery. Although no such peak of activity

is evident in the experiments shown in Figures 1 and 2, this result was not unexpected as the cells in these experiments were continually exposed to hormone during the recovery intervals. Thus, the CAT activities measured under these conditions are simply the result of accumulating enzyme over increasing intervals of Dex exposure. In order to determine the peak time of HSPE activity, we have performed the experiments seen in Figure 5. In these experiments, LMCAT2 cells were subjected to heat or chemical shock followed by a time-course of recovery in which the cells were incubated with hormone for short 4-h intervals. As expected, unstressed control cells exhibited a uniform response to hormone over the entire recovery period. Also as expected, both heat and chemical shock resulted in potentiation of Dex-induced CAT expression, but interestingly both forms of stress resulted in peaks of HSPE activity during the 12- to 16-h interval of recovery.

There are several conclusions that can be drawn from these results. First, the observed decline in HSPE activity at 20–24 h from the peak at 12–16 h is further evidence that the heat shock block of general protein synthesis and its rebound are not responsible for the pattern of HSPE activity in these cells, as the levels of general protein synthesis continue to rise at 24 h of recovery (Fig. 3), while the HSPE effect is declining (Fig. 5). Second, the fact that heat and chemical shock can generate similar peaks of activity suggests that both forms of stress are acting by a common mechanism. Third, the presence of defined peaks of HSPE activity may serve to identify the temporal conditions under which to attempt purification of the factor(s) involved in this process.

Fig. 6 Time-course of CAT mRNA expression in LMCAT2 cells 'pulse' incubated with Dex during recovery from heat or chemical shock. LMCAT2 cells were subjected to heat shock at 43°C for 2 h (HS), or chemical shock employing 200 μ M arsenite for 2 h (CS), or were not stressed (Dex). Immediately after treatment, the cells were allowed to culture under non-stress conditions and 1 μ M Dex was added for 4 h intervals beginning at 0, 4, 8, 12, 20, and 40 h of recovery. Each series of samples also included non-hormone-treated controls (C). Total mRNA was isolated at each time point and analyzed for CAT mRNA by Northern blotting. Panel A: autoradiogram of typical Northern blot. Panel B: quantitation of CAT mRNA (fold induction relative to no-treatment, zero-time controls) as derived by densitometric scanning of the autoradiograms. The results shown represent the means of two independent experiments.

Fig. 7 Effect of cycloheximide on the heat and chemical shock potentiation of GR-mediated CAT mRNA expression in LMCAT2 cells. Replicate flasks of LMCAT2 cells were subjected to the following conditions: no treatment (C), heat shock at 43°C for 2 h (HS), or chemical shock employing 200 μ M arsenite for 2 h (CS). Immediately after treatment, the cells were allowed to culture under non-stress conditions and 1 μ M Dex (D) was added for 4 h in the presence or absence of 50 μ M cycloheximide (CHX). Total mRNA was isolated and analyzed for CAT mRNA by Northern blotting. Panel A: autoradiogram of typical Northern blot. Panel B: quantitation of CAT mRNA (fold induction relative to no-treatment controls) as derived by densitometric scanning of the autoradiograms. The results shown represent the means of two independent experiments.

If the peaks of HSPE activity observed in Figure 5 are indeed the result of a factor that potentiates the transcription enhancement function of the GR, then the peak of its effects on CAT gene transcription should precede that observed for CAT enzyme activity. Moreover, although the results in Figure 4 suggest that the potentiation effect of stress on GR-mediated CAT gene expression is not the result of a post-transcriptional event, such as mRNA stabilization, direct evidence that the HSPE can actually result in increased levels of CAT mRNA has yet to be reported. For these reasons, we performed the experiments seen in Figure 6, in which CAT mRNA levels were measured in cells recovering from stress and pulse incubated with hormone at the same 4 h intervals employed in the experiments shown in Figure 5. The results show that both heat and chemical shock can indeed potentiate CAT mRNA levels. Although both stress treatments resulted in peaks of HSPE activity that were less well-defined than those observed for CAT enzyme levels, it is clear that CAT mRNA levels are increased in response to stress at a faster rate; within 4 h, CAT mRNA levels were $\approx$ 70% of maximal.

The results shown in Figure 6 demonstrated that the HSPE could be measured at the level of CAT mRNA expression. We reasoned that this observation would now make it possible to test whether the HSPE required the synthesis of a factor during the recovery period by incubating the recovering cells with cycloheximide. In the experiments shown in Figure 7, it can be seen that cycloheximide did not prevent the HSPE as measured by CAT mRNA levels. Indeed, cycloheximide increased the HSPE on CAT mRNA for both the heat and chemical

shock conditions. Although this could be seen as evidence against the synthesis of a stress-mediating factor, this conclusion would be premature, as cycloheximide also caused an increase in CAT mRNA for cells treated with hormone only. In fact, the level of this increase was about the same as either HSPE condition. Thus, cycloheximide appears to be acting as a form of chemical stress, negating its usefulness under these conditions.

DISCUSSION

In a previous report (Sánchez et al 1994) we proposed a model in which three possible mechanisms could account for the ability of heat and chemical stress to potentiate the transcription enhancement function of the hormone-bound GR. According to this model, the HSPE could result either from an intrinsic hyper-activation of the GR, from changes in the Pol II complex, or from the induction of a non-DNA-binding co-activator that mediates the interaction between the GRE-bound GR and the Pol II complex. This model was based, in part, on the observations that the HSPE required agonist-activated GR; that it was not the result of increased nuclear translocation of the GR; and that it could be achieved with promoters containing only synthetic GRE elements and a TATA box. In that report, we also observed that the onset of the HSPE during the recovery period from stress was somewhat delayed with respect to the onset of unstressed, hormone-driven CAT gene expression. As those experiments were done by subjecting the cells to stress before addition of hormone, we provided evidence that the delay may be due to the need for reactivation of

GR hormone-binding function following its inactivation during stress. In the present study we provide evidence that this delayed onset of the HSPE is not the result of a GR inactivation/reactivation cycle, as the same pattern and timing of delay is observed in cells that were incubated with hormone before the stress event (Fig. 2).

The above result caused us to question if the delayed pattern of HSPE could simply be the result of a depressed and slowly recovering protein synthesis machinery. We found that the levels of general protein synthesis do indeed fall in response to stress and rise during the recovery period (Fig. 3). However, there are several reasons the effects of heat shock on protein synthesis cannot adequately account for the pattern of HSPE we observe in our cells. First, general protein synthesis rose very slowly (if at all) during the same time points (4–8 h of recovery) when CAT gene expression was rising dramatically (Figs 1 and 2). Second, general protein synthesis continued to rise (indeed it had not yet reached normal levels) at 24 h of recovery, a time at which the HSPE was already beginning to fall (Fig. 5). Third, although heat shock could reduce general protein synthesis, it did not reduce synthesis of CAT (as controlled by the constitutive SV2 promoter), suggesting that translation of CAT mRNA itself was not subject to the inhibitory effect of stress (Fig. 4).

Taken together then, the results of this work are consistent with a model in which the HSPE is controlled by a heat shock-inducible factor that is either activated or synthesized in the recovering cell and that mediates the potentiation of GR-mediated gene expression. If this heat shock-inducible factor exists, it is probably not activated in response to stress, as this type of regulation should have resulted in a faster onset of the HSPE in cells that were treated with hormone before the stress event (Fig. 2) as opposed to after (Fig. 2). Treatment with hormone before stress is a condition in which the 'activated' factor would not have to wait for reappearance of hormone-competent receptors. Of course, it is still possible that a slow mechanism may be operating by which a pre-existing factor is activated in these cells, but examples of such mechanisms are not known to these authors. Instead, most forms of activation, such as phosphorylation, are mechanisms that act in the order of seconds rather than hours.

If the putative factor is not activated, then it must be synthesized. Our observation that the HSPE can be measured at the level of GR-controlled CAT mRNA transcription (Fig. 6) allowed us to test the synthesis hypothesis by asking if cycloheximide could block the potentiation of CAT mRNA expression (Fig. 7). To our surprise, addition of cycloheximide to LMCAT2 cells at the start of the recovery period did not cause an attenuation of the HSPE effect on CAT mRNA. Even more surprising was

the fact that cycloheximide caused a potentiation of Dex-induced CAT mRNA expression on its own, as well as increasing the potentiation effect of either heat or chemical shock on CAT mRNA. Thus, cycloheximide appears to be acting as a form of chemical shock with respect to its effects on GR-mediated gene expression. This conclusion is in good agreement with a variety of reports demonstrating that protein synthesis inhibitors (cycloheximide, puromycin and anisomycin) can either increase c-fos mRNA expression, just like heat and chemical stressors (Gubits and Fairhurst 1988), can cause a super-induction of c-fos mRNA in combination with heat shock (Andrews et al 1987), or can actually induce the heat shock response, as measured by increased synthesis of the major heat shock proteins (Hightower 1980). As the initial event in the induction of the cellular heat shock response is widely accepted to be the accumulation of misfolded proteins (Anathan et al 1986), it is likely that chain-terminating compounds, which result in the accumulation of abnormal peptides, may induce the heat shock response by a similar mechanism. Thus, the inability of cycloheximide to block the heat shock potentiation of Dex-induced CAT mRNA expression may not mean that the putative heat shock factor involved in this process is not synthesized in response to stress. The same mechanism that allows heat shock proteins and, presumably, the putative HSPE factor to be synthesized in cells with otherwise depressed protein synthesis levels may be operating in cycloheximide-treated cells, which we now know can induce a similar heat shock protein response.

In a similar work by Edwards et al (1992), the authors also performed a time-course of hormone addition during the recovery period for cells exposed to heat shock. In this particular case, the cells were PR-expressing T47D cells stably transfected with the MMTV-CAT reporter and the hormone used was a synthetic progestin. The authors found that heat shock could potentiate PR-mediated CAT gene expression but that the effect gradually diminished from an initial high. It was concluded that the HSPE effect in these cells was transient, with the maximal effect occurring at the end of the heat shock period. However, the authors did not perform short-term incubations with hormone during the recovery time-course. Instead, hormone was added to the cells for 24 h beginning at 0, 1, 2, 3, 4 or 8 h of recovery. Under these conditions it is possible that a peak of potentiation activity could still be occurring during the recovery period, say at 12 h, and that the gradual decline in CAT activity simply represents a gradual degradation of enzyme over the long recovery times employed (24–32 h). Thus, it is still possible that the temporal pattern of HSPE expression reported here may be a widespread response of cells, and further tests of other cell lines will be required to resolve this issue. If the original conclusions of Edwards

and co-workers are correct, this would suggest that the timing of any putative HSPE factor involvement in steroid receptor transcription enhancement activity can vary from cell line to cell line. In the case of T47D cells, the putative HSPE factor may be synthesized or activated during the actual stress period, much in the way heat shock proteins are synthesized during this period. In either case, the data shown here, although indirect in nature, present compelling evidence for the existence of a heat shock-induced pathway by which the actions of steroid receptors and perhaps other trans-acting factors can be controlled.

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