Temporary loss of glucocorticoid receptor-mediated regulation of gene expression in heat-shocked cells

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Abstract The effect of heat shock on the transcriptional activity of glucocorticoid receptor was assessed using HeLa cells stably transfected with the chloramphenicol acetyltransferase (CAT) gene the transcription of which is controlled by two glucocorticoid-responsive elements placed directly upstream of a core promoter. Heat shock inactivated the high-affinity glucocorticoid binding capacity of the cells and nullified the rate of accumulation of CAT mRNA in the presence of hormone. Hormonal responsiveness was restored on return to normal temperature concomitantly with recovery of high-affinity glucocorticoid binding capacity. Heat inactivation of the receptor was coincident with loss of its solubility and apparently unrelated to receptor degradation.

Key words: Glucocorticoid receptor; Heat shock; Gene expression

1. Introduction

The glucocorticoid receptor is a ligand-activated transcription factor capable of interacting with the glucocorticoid-responsive elements (GREs) of specific target genes as well as with components of the transcription machinery, thus affecting the rate of transcription initiation by RNA polymerase II ([1–3] and references therein). Previous studies have demonstrated that the ligand-free receptor is recovered in the cytosol as a non-DNA binding, 350-kDa complex comprising hsp90 (heat shock protein with M_r 90,000 Da) [4] and that the same [5] or a similar heterocomplex [6] is also present in the cell (reviewed in [4]). Hormone binding to the cytosol receptor was found to promote dissociation of the complex and receptor binding to the GRE [1,4]; the hsp90-stripped receptor thus produced was reported to possess significantly lower affinity for the hormone [7]. It is thought that in the cell, hormone-mediated dissociation of the 350-kDa complex allows receptor dimerization, translocation to the nucleus (in cells that exhibit cytoplasmic localization of the receptor) and receptor regulation of gene expression [8].

Heat-shock treatment of cells was shown to potentiate the expression of several hsp's, apparently as a means of preventing aggregation and assisting renaturation of proteins partially unfolded as a result of thermal injury [9–11]. Loss of solubility due to thermal denaturation was found to be reversible [10,12,13] and resolubilization was reported to be dependent on hsp's, namely hsp70 [10] and, in particular, hsp110 [14]. Those of the hsp's that are highly expressed in unshocked cells are thought to be involved in house-keeping processes such as stabilization of partially folded intermediates, assembly and dissociation of protein oligomers and protein transport. Hsp90, in particular, is thought to maintain proteins in the inactive, unassembled state at the expense of ATP (reviewed in [11]). With respect to steroid receptors, hsp90 was shown to 'shape' a high-affinity, hormone binding state, competent for transcriptional regulation [15]. It has been proposed that, in the cell, unliganded steroid receptors maintain competence by consecutive cycles of binding to and release from hsp90 and that hormone binding to the competent receptor produces a transcriptionally active form not binding to hsp90 [16].

Several studies have focused on the effect of heat-shock treatment of cells on steroid receptor structure and function [17–22]. It has been shown that heating cells results in rapid loss of the glucocorticoid binding capacity of cells and cytosol [18–20], apparently due to receptor deficiency for high-affinity hormone binding [19] and possibly, even loss of receptor protein [20]. Since the missing receptor protein was not fully regained in the crude nuclear fraction of the heat-shocked cells [20] and recovery of initial binding capacity and amount of receptor protein in the cytosol upon return of the heat-shock cells to 37°C was incomplete [19,20,22], speculation was raised that heat shock promotes receptor degradation [18,20]. Interestingly, however, in heat-shocked cells recovering at normal temperature as compared to unshocked cells, glucocorticoid and progesterone receptors apparently were capable of mediating a higher fold enhancement by the respective hormone of the expression of chloramphenicol acetyltransferase (CAT) activity in L929 and T47D cell line derivatives stably transfected with a hormone-regulated CAT reporter gene [21,22]. Since basal expression of the reporter in the absence of hormone as well as constitutive reporter expression driven by the SV-40 promoter were unaffected by heat shock, it was concluded that heat potentiation of reporter expression was a receptor-mediated event, probably involving stimulation of receptor transcriptional activity or activation of a transcriptional intermediary factor (coactivator or adapter) synergizing with the receptor [21,22]. In contrast to these observations, Wolffe et al. [17] reported a transient debilitation of estrogen regulation of vitellogenin gene transcription paralleled by inactivation of hormone binding capacity of estrogen receptor as well as a decay of pre-existing vitellogenin mRNA, following heat shock of *Xenopus* hepatocytes.

In this report, we investigated whether the glucocorticoid receptor of heat-shocked cells is transcriptionally active. Using a HeLa cell derivative stably transfected with a CAT reporter gene transcribed at a core promoter (i.e. a TATA sequence and a start site) under the regulated control of two adjacent copies of a synthetic GRE placed directly upstream of the TATA box, we show that heat shock transiently nullified the rate of synthesis of CAT mRNA in the presence of hormone. The ligand-free

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receptor was found to be tightly bound in a non-ligand binding state to cytoplasm-free nuclei and to a Triton-X-100-insoluble cytoskeleton fraction from heat-treated cells, suggesting that hormone binding and the ensuing activation of transcriptional regulatory functions of the receptor are debilitated by heat shock. In heat-shocked cells recovering at normal temperature, recovery of glucocorticoid responsiveness coincided with restoration of glucocorticoid binding capacity, further suggesting that heat-induced loss of hormonal responsiveness is due to receptor inactivation. In addition, since the amount of receptor protein in the cytosol was fully regained in heat-shocked cells recovering in the presence of cycloheximide, the previously postulated heat-induced degradation of glucocorticoid receptor appears unlikely.

2. Materials and methods

2.1. Constructs

pGEM4, pWLneo and pTRI-GAPDH (human glyceraldehyde-phosphate dehydrogenase antisense template) were obtained from Promega (Madison, WI), Stratagene (La Jolla, CA) and Ambion (Austin, TX), respectively. ptkCAT [23], 2GRE-37Tk [24] and pRGAPD.13 [25] were kindly provided by Drs. M. Beato, G. Schütz and P. Fort, respectively. Plasmid ptkCAT5.1 was constructed by cloning the *Pvu*II/*Hin*dIII fragment (containing the tk promoter and the CAT coding region up to nucleotide 142) from plasmid ptkCAT into the *Hin*cI/*Hin*dIII site of plasmid pGEM4.

2.2. Cells and cell protein labelling; CAT and whole-cell, hormone binding assays

HeLa cells, cultured to approx. 50% confluence in Dulbecco's modified Eagle's medium (DMEM; Seromed/Biochrom KG) supplemented with 10% steroid-stripped fetal calf serum, were stably transfected with 19.5 μg of 2GRE-37Tk and 0.5 μg of pWLneo per 100-mm dish, using the calcium phosphate coprecipitation method [26]. 20 h after transfection the cells were washed twice with phosphate-buffered saline, fed with fresh medium and 44 h later refed with medium containing 0.5 mg/ml geneticin. Colonies were isolated 2 weeks later and tested for CAT activity in the presence or absence of 1 μM triamcinolone acetonide for 24 h. CAT enzyme activity was determined according to Gorman et al. [27] using equal amounts of cell extract protein to determine percent conversion of the substrate to the acetylated form; non-acetylated and acetylated ^{14}C-labelled chloramphenicol was extracted and counted in a liquid scintillation counter. Clone 1β5 that was selected for this study, exhibited a 8.5-fold enhancement of CAT enzyme activity in the presence of the hormone. [^{35}S]Methionine labelling of cellular proteins synthesized in culture was carried out as described by Beckmann et al. [28].

The high-affinity hormone binding capacity of cells was determined according to Reese and Katzenellenbogen [29] using 10 nM [^{3}H]triamcinolone acetonide in the presence or absence of 100-fold excess of radioinert hormone.

2.3. Heat-shock treatment of cells

Cells were heat shocked having first reached confluence in 100-mm dishes. Before heating, HEPES, pH 7.0, was added to the culture medium to a final concentration of 25 mM, the dishes were sealed with parafilm and immersed in a water bath at 44°C for 1 h. Heat-shocked cells were either used immediately or allowed to recover at 37°C in fresh medium for appropriate periods of time either in the presence or absence of cycloheximide (10 μg/ml). Cells were harvested by trypsinization, washed twice, pelleted and either directly homogenized (preparation of nuclei) or immediately frozen in liquid N_2 (preparation of cytosol and RNA). It was found that 88.2 ± 3.5% (n = 5) of the cells remained viable 24 h after heat shock at 45°C for as long as 5 h, as judged by the Trypan blue exclusion assay; 95.5 ± 6.5% (n = 3) of unshocked cells were viable to start with.

2.4. Preparation, SDS-PAGE and quantitative immunoblotting of cellular fractions

Preparation of cytosol and isolation of cytoplasm-free nuclei were carried out as previously described [6,30]. For crude cytosol and nuclear fractions, frozen cell pellets were rapidly thawed in 2 vols. of 20 mM Tris, pH 7.5, 80 mM NaCl, 20 mM sodium molybdate, 5 mM EGTA, 8 mM $MgCl_2$, 10% (by vol.) glycerol, 0.05% (by vol.) Triton X-100, 1 mM leupepetin, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and 10 mg/ml trypsin inhibitor and fractionated by centrifugation at 12,000 × g for 5 min. Triton-X-100-insoluble cytoskeleton fraction was prepared as described by Horvath et al. [31].

SDS-PAGE was performed as already described [6]. PAGE marker proteins were as follows (M_r): myosin 205,000; β-galactosidase 116,000; phosphorylase b 97,400; bovine serum albumin 66,000; and ovalbumin 45,000.

Receptor immunoblotting using rabbit antiserum EP to the human glucocorticoid receptor peptide G499-N597 and ^{125}I-labelled protein A has been described [6]. The EP antiserum was found to possess a weak, hsp70 immunoblotting (non-immunoadsorbing) reaction (Fig. 1), probably the result of a humoral immune response to microbial infection [32]. Following autoradiography, the nitrocellulose membranes were developed with peroxidase-conjugated second antibody, the stained receptor bands were cut out and radioactivity was measured in a γ-counter; receptor-specific cpm values were corrected for values of background slices and compared to those of a calibration curve obtained with known amounts of immunoabsorbed HeLa receptor, as determined from the amount of specifically bound [^{3}H]triamcinolone acetonide.

2.5. Isolation of RNA, Northern-blot analysis and ribonuclease protection assay

Heat-shocked and unshocked cells were incubated at 37°C for the indicated periods of time and for one more hour in the presence or absence of 1 μM triamcinolone acetonide. Total RNA was extracted from frozen cells using RNAzol B (Tel-Test, Friendswood, TX) as recommended by the manufacturer and was quantified at 260 nm.

Northern-blot analysis using cDNA clone pRGAPD.13 was performed as previously described [30]. After autoradiography, the mRNA bands were cut out and the radioactivity was measured in a liquid scintillation counter.

Ribonuclease protection assays were performed using uniformly [α-^{32}P]GTP-labelled antisense probes transcribed from *Hin*dIII-restricted ptkCAT5.1 and *Dde*I-restricted pTRI-GAPDH templates, respectively. 40 μg aliquots of total RNA were hybridized for 16 h at 45°C with 150,000 cpm of each probe in 20 μl of hybridization buffer (80% formamide, 80 mM NaCl, 8 mM PIPES, pH 6.7, and 0.2 mM EDTA). Following hybridization, samples were chilled, diluted with 300 μl of 300 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 46 μg/ml ribonuclease A and 2 μg/ml ribonuclease T_1 and digested for 30 min at 37°C. The RNase-treated hybridization reactions were supplemented with 0.5% SDS and 300 μg/ml proteinase K, incubated at 37°C for 15 min, phenol-extracted, ethanol-precipitated in the presence of carrier tRNA, and the samples were denatured and analyzed on a 5% acrylamide/7 M urea sequencing gel [33]. After autoradiography, protected CAT and GAPDH-specific mRNA fragments were quantified using a personal laser scanning densitometer (Molecural Dynamics) and expressed in arbitrary densitometric units; values were normalized with respect to those of the respective GAPDH mRNA fragments and expressed relative to the normalized CAT mRNA of hormone-treated, unshocked cells. Plasmid pAT153, restricted with *Hin*fI and end-labelled with [γ-^{32}P]ATP, was used as size marker.

3. Results

In HeLa 1β5 cells growing at 37 °C in the presence of fetal calf serum and Phenol red, part of the ligand-free glucocorticoid receptor was conspicuously nuclear, as judged by indirect immunofluorescence of paraformaldehyde-fixed cells using immunopurified anti-receptor antibodies (data not shown). The immunoblot of Fig. 1 shows, however, that the nuclear receptor leaked to the cytoplasm upon cell fractionation. Furthermore, the receptor was absent from nuclei, isolated by a procedure that uses Triton X-100 to reduce cytoplasmic contamination to the minimum (lanes C; filled arrow), and from Triton X-100-

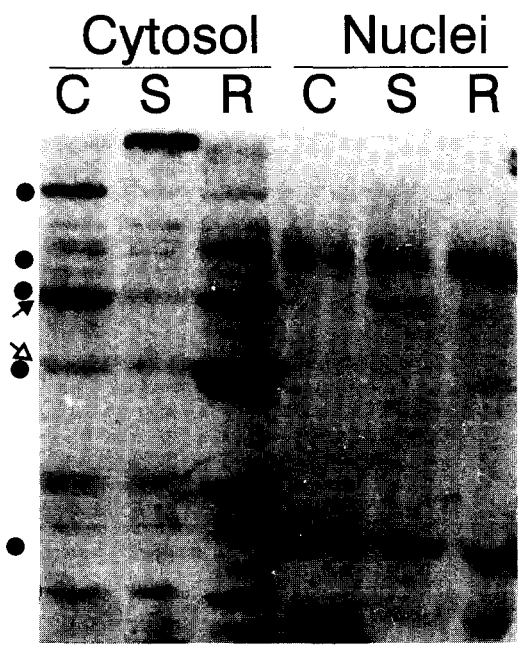

Fig. 1. Presence of ligand-free glucocorticoid receptor in the nucleus following heat-shock treatment of HeLa cells. Cytosol and cytoplasm-free nuclei from 10^6 control cells (C), cells heat-shocked at 44°C for 1 h (S) and cells recovering from the shock at 37°C for 24 h (R), were analysed by SDS-PAGE and immunoblotting using ^{125}I-labelled protein A, followed by autoradiography. The positions of the receptor (filled arrow) and hsp70 (open arrow) are indicated. Dots point to the positions of the marker proteins described in section 2.

insoluble cytoskeletal fraction. Following heat shock, receptor was lost from the cytosol and approximately 45% of the amount of receptor protein initially present in the cytosol of unshocked cells was found to reside in the nucleus (lanes S) whereas the rest was associated with the cytoskeleton (not shown). Both these fractions were insoluble in non-ionic detergents. Receptor was fully regained in the cytosol of cells recovering from shock at 37°C for 24 h (lanes R), at which time the amount of receptor protein was 120% of that present in the cytosol of unshocked cells. This amount is in full agreement with the whole-cell, hormone binding data in Table 1 showing that the glucocorticoid binding capacity of 24-h recovering cells was 130% that of unshocked cells. The loss of receptor solubility suggests that thermal denaturation of the receptor exposes an interactive domain leading to receptor aggregation. This is corroborated by the data in Table 1 showing that few high-affinity glucocorticoid binding sites remained active after heat shock. However, the hormone binding capacity was fully regained within 6 h of recovery from the shock and was kept somewhat above control values as long as 24 h after the shock. Fig. 1 shows, in addition, that hsp70 (open arrow) was strongly induced by the treatment. Furthermore, it shows that although heat shock moderately increased the nuclear fraction of insoluble hsp70, it had no effect on the solubility of a few other proteins non-specifically reacting with antiserum EP.

Since no receptor fragments were present in detectable amounts in the cytosol and nuclei of heat-shocked cells, the previously postulated degradation of the receptor in heat-shocked cells appears unlikely. This is corroborated by the experiment of Fig. 2A which shows an SDS-PAGE and immunoblotting analysis of proteins in low speed cytosol and nuclear fractions of heat-shocked cells recovering in the presence of cycloheximide for 0, 2, 4, 6 and 10 h after the shock. Cycloheximide reduced the rate of synthesis of total protein, as well as the receptor, in unshocked and recovering heat-shocked cells to 6.5 ± 0.9% (n = 4) of that in its absence, as determined by TCA precipitation and immunoadsorption, respectively, of [^{35}S]methionine-labelled proteins (not shown). To account for protein loss to the insoluble fraction following heat shock [27], SDS-PAGE samples deriving from the same number of cells were analysed. The compromise, by cycloheximide alone, of the amount of receptor protein in the cytosol of unshocked cells is also shown for comparison. In unshocked cells, receptor solubility was unaffected by cycloheximide (data not shown). As expected, heat shock reduced receptor solubility (filled arrowhead) but not that of two non-specifically immunoreacting proteins featuring below the receptor band (open arrowhead). Evidently, receptor resolubilization was complete after 10 h of recovery in the presence of cycloheximide. Quantitative immunoblotting analysis and expression of receptor protein levels in the cytosol of recovering heat-shocked cells relative to the levels of unshocked cells treated with cycloheximide for the same time period shows that receptor solubility was fully regained 10 h after return of the heated cells to 37°C (Fig. 2B; dashed line). That control levels of soluble receptor protein were recovered in the absence of protein synthesis implies that heat shock had no effect on the rate of receptor degradation. Fig. 2B further shows that regain of receptor solubility preceded in time the recovery of high-affinity hormone binding capacity of whole cells (full line), as expressed relative to the capacity of unshocked cycloheximide treated cells. In fact, the binding capacity of 10-h recovering cells was 66.8% of that of unshocked cells, implying that a fair fraction of soluble receptors, incompetent for high-affinity hormone binding, existed in these cells. That this fraction became significant in heat-shocked cells recovering for 6 h or longer while resolubilization apparently proceeded at the same pace suggests that reconstitution of the 350-kDa complex becomes rate-limiting during late recovery. Significantly, recovery of glucocorticoid binding to the receptor was much slower in the presence of cycloheximide than in its absence (Table 1), suggesting that inhibition of de novo hsp synthesis may have a causative effect. Inhibition of heat-induction of hsp70, in particular, is expected to hold up reconstitution of the 350-kDa complex [16] and consequently, acquisition of competence for high-affinity hormone binding to the receptor [15].

Table 1
Glucocorticoid binding sites in unshocked and heat-shocked (44°C for 1 h) HeLa cells at various times after treatment

Unshocked	Hours after heat shock				
	0	2	4	6	24
100	14 ± 5 (6)	27 ± 7 (2)	69 ± 7 (2)	112 ± 6 (2)	130 ± 17 (7)

Incubation of cells with [^{3}H]TA in the presence and absence of excess radioinert TA was carried out as described in section 2. The amount of [^{3}H]TA specifically bound to 10^6 heat-shocked cells was expressed as a percent of that bound by the same number of unshocked cells, present in each separate determination. Values are mean ± S.D. of determinations from independent experiments. The number of experiments is shown in parentheses. Unshocked cells were found to express 39,000 ± 6,000 (n = 18) high-affinity, glucocorticoid binding sites per cell. TA, triamcinolone acetonide.

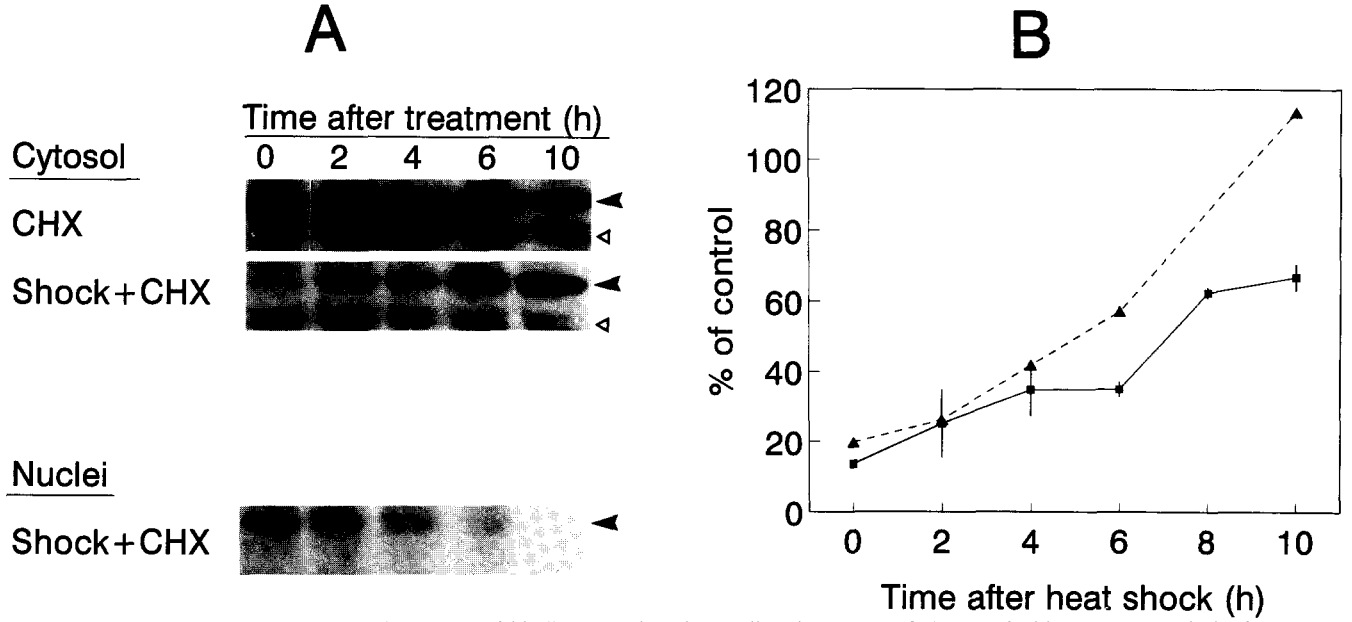

Fig. 2. Time–course of reconstitution of glucocorticoid-binding capacity of the cell and recovery of glucocorticoid receptor protein in the cytosol on return of heat-shocked HeLa cells to 37°C. (A) Low speed cytosol and nuclear fractions were prepared from 10^6 control and heat-shocked cells (44°C for 1 h) recovering from shock for 0, 2, 4, 6 and 10 h in the presence of CHX and were analyzed by SDS-PAGE and immunoblotting using ^{125}I-labelled protein A, followed by autoradiography. The positions of the receptor (filled arrowhead) and two non-specifically immunoreacting proteins (open arrowhead) are indicated. (B) Glucocorticoid binding capacity of heat-shocked cells recovering in the presence of CHX for the indicated times (■; mean ± S.D. (error bars) of determinations from two independent experiments), and amount of receptor protein in the cytosol of heat-shocked cells of Fig. 2A (▲), both normalized with respect to CHX-treated, unshocked cells and expressed relative to unshocked cells maintained in the absence of CHX (cycloheximide).

Since ligand binding to the receptor is a prerequisite for transcriptional activation, the inference from the above data is that heat shock is likely to inhibit receptor-mediated enhancement of gene expression, as previously suggested for estrogen receptor [17]. The experiment of Fig. 3 shows, however, a 3-fold-higher CAT enzyme expression in the presence of hormone in 10-h recovering heat-shocked cells (SH) and even a 4-fold-higher expression in cells exposed to the hormone 30 min before the shock (HS), as compared to unshocked hormone-treated cells (H). CAT expression in the absence of hormone was only 1.3-fold-higher in recovering heat-shocked cells (SB) as compared to unshocked cells (B). Since CAT gene transcription in HeLa 1β5 cells initiates at a core promoter regulated by two synthetic GREs placed directly upstream of the TATA box, the inference is that heat potentiation of CAT expression is receptor-mediated. In this light, our findings show that the previously observed potentiation of glucocorticoid receptor-mediated CAT enzyme accumulation during 24 h of recovery from heat shock [22], holds even for a 10-h recovery during which hormone binding to the receptor is debilitated for quite sometime (Table 1).

To find out whether heat potentiation of hormone-induced CAT expression was the result of stimulation of receptor transcriptional activity, the rate of CAT gene transcription was estimated from the amount of CAT mRNA accumulated within 1 h of hormone treatment, as determined by the RNase protection assay depicted in Fig. 4A. The autoradiogram of Fig. 4B shows that the correctly initiated CAT mRNA, a read-through CAT transcript initiating upstream of the TATA box and GAPDH mRNA protected fragments of 216, 255 and 134 nt from the respective probes. Transcription initiation from sites upstream of the TATA box have also been observed in transient transfection assays with synthetic [23,24] as well as authentic templates [34]. As was the case with transient assays, we observed glucocorticoid regulation of expression of the correctly initiated as well as the read-through transcript. As was not expected from the data of Fig. 3, however, Fig. 4B shows that heat shock completely inhibited hormonal induction of the synthesis of both CAT mRNAs whereas it had no effect on the level of GAPDH mRNA. The Northern blot analysis of Fig. 4C shows indeed that the steady-state level of GAPDH mRNA

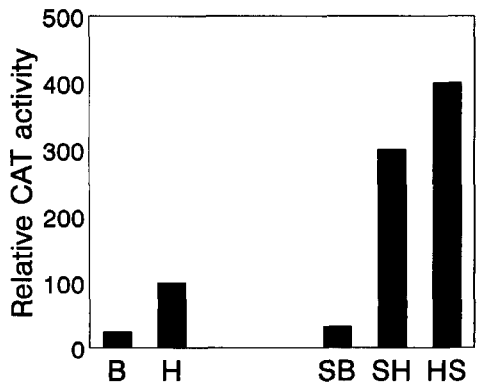

Fig. 3. Potentiation of glucocorticoid-induced CAT enzyme activity following heat-shock treatment of HeLa cells. The CAT activity of unshocked (B) and heat-shocked (44°C for 1 h) cells (SB) in the absence of TA, and that of cells exposed to TA either 30 min before (HS) or immediately after heat shock treatment (SH) was determined 10 h after treatment and expressed relative to the activity of unshocked cells exposed to TA for 10 h (H). TA, triamcinolone acetonide; CAT, chloramphenicol acetyltransferase.

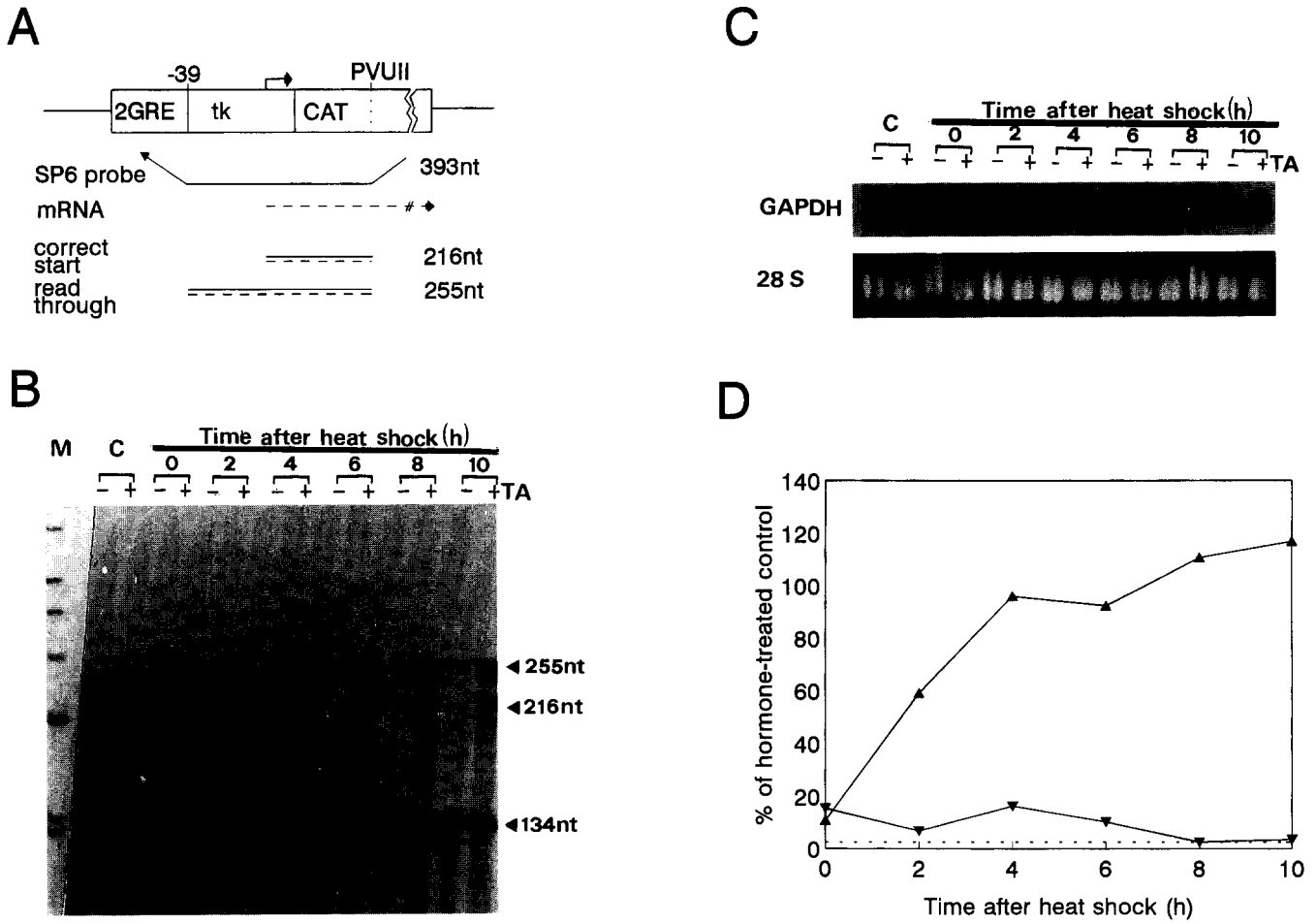

Fig. 4. Time–course of reconstitution of glucocorticoid receptor-mediated CAT mRNA expression on return of heat-shocked HeLa cells to 37°C. (A) In plasmid 2GRE-37Tk, expression of CAT mRNA is controlled by two glucocorticoid responsive elements (GREs) and the thymidine kinase (tk) promoter. The 393-nt long antisense CAT probe from plasmid ptkCAT5.1 protects from RNases the correctly initiated (216-nt long) and a read-through (255-nt long) CAT mRNA. (B) Autoradiogram of electrophoresed, RNase-protected CAT mRNAs and a 134-nt long GAPDH mRNA protected by the antisense GAPDH probe. Total RNA (40 μg), from unshocked (lane C) and heat-shocked (44°C for 1 h) cells recovering for 0, 2, 4, 6, 8 and 10 h in the absence (−) or presence (+) of TA for 1 h more, was used for the RNase protection assay. The ^{32}P-labelled *Hin*fI-restricted pAT153 fragments of 1630, 517, 396, 298, 221, 220, 154 and 145 nt, used as size marker, are shown (lane M). (C) Autoradiogram of Northern blot analysis of total RNA (20 μg) from the unshocked and heat-shocked cells of Fig. 4B, using a GAPDH cDNA probe. The part of the ethidium bromide-stained gel showing 28 S ribosomal RNA is also presented. (D) Basal (▼) and TA-induced (▲) CAT mRNA levels in the autoradiogram of Fig. 4B, as determined by laser densitometry, were normalized with respect to the respective GAPDH mRNA levels and expressed relative to the normalized CAT mRNA level of TA-induced unshocked cells. The dotted line indicates the normalized CAT mRNA level of non-induced unshocked cells. CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde-phosphate dehydrogenase; TA, triamcinolone acetonide.

at 1.3 kb was unaffected by heat shock and that this was the case also with 28 S ribosomal RNA. Return of the heat-shocked cells to 37 °C resulted in rapid reconstitution of receptor-mediated responsiveness, already observed in 2-h recovering cells (Fig. 4B). Laser densitometry of the autoradiogram of Fig. 4B and expression of the correctly initiated CAT mRNA levels of recovering cells, as normalized with respect to the respective GAPDH mRNA levels, relative to the normalized level of the hormone-treated, unshocked cells reveals that hormonal responsiveness was restored in 4-h recovering cells (Fig. 4D). Reconstitution of glucocorticoid responsiveness correlates well with the recovery of high-affinity glucocorticoid binding to whole cells (Table 1), suggesting that the loss of receptor competence for transcriptional activation is due to dissociation of the 350-kDa complex rather than to inactivation of a general transcription factor(s) or a transcriptional intermediary factor cooperating with the receptor. Furthermore, the restored rate of CAT mRNA accumulation only marginally exceeded that of unshocked cells, in agreement with data (Table 1) showing that a somewhat higher than control amount of glucocorticoid receptor, competent for high-affinity hormone binding, is present in recovering heat-shocked cells. In this light, the previously postulated hyperactivation of glucocorticoid receptor by heat shock [22] appears unlikely. It is also noteworthy that in the absence of hormone, correctly initiated CAT mRNA levels were somewhat higher in heat-shocked (full line) as compared to unshocked cells (dotted line) and apparently this was the case also with the read-through transcript (Fig. 4B). In this light, the 1.3-fold higher CAT activity in recovering heat-shocked cells (SB) as compared to unshocked cells (B) (Fig. 3) is probably due to higher CAT enzyme expression rather than to heat-induced increase in CAT enzyme activity or stability. Similarly,

heat-shock treatment of T47D and HeLa cell derivatives stably expressing CAT and β-galactosidase, respectively, apparently had no effect on the stability or activity of these enzymes [12,21].

4. Discussion

Given that heat-shock proteins associated with steroid hormone receptors play a fundamental role in receptor structure and function, the link between the signal transduction pathways by which cells respond to thermal stress and steroid hormones is of special interest. This and other studies [21,22] have shown that heat-shock treatment of cells, stably transfected with a CAT reporter gene, the transcription of which is regulated by progestin and glucocorticoid receptors, has a strong stimulatory effect on CAT enzyme expression in the presence of hormone. These findings have raised speculations as to what extent the transcriptional activity of these receptors is thermally stimulated. The present study gives the first evidence that severe heat-shock treatment of HeLa 1β5 cells at 44°C for 1 h is adequate to completely, yet reversibly, abolish the glucocorticoid receptor-mediated enhancement of CAT mRNA expression. That thermal injury of the receptor is directly responsible for the heat-induced loss of hormonal responsiveness was inferred from data (Table 1 and Fig. 4D) showing that recovery of glucocorticoid responsiveness correlated well with reactivation of high-affinity hormone binding to the receptor. Normal responsiveness was re-established in heat-shocked cells recovering for 4 h or longer. In the light of this and findings that CAT mRNA translation is unlikely to resume in heat-shocked HeLa cells before a recovery period has elapsed [35], it appears likely that CAT mRNA accumulation higher than that of unshocked cells, probably due to impaired mRNA turnover, could account for the potentiation of CAT enzyme expression in heat-shocked cells. That heat shock might decrease the rate of CAT mRNA degradation is also suggested by the fact that cells exposed to the hormone 30 min before heat shock exhibited significantly higher CAT enzyme expression as compared to cells exposed to hormone after the shock (Fig. 3), and that a transient increase in basal CAT mRNA levels was observed following heat shock (Fig. 4D). Significantly, it has been shown that pre-existing mRNAs remain translationally repressed but stable during heat shock and are efficiently translated during incubation at normal temperature [36]. In contrast to this finding, a decline was observed in the half-life of pre-existing vitellogenin mRNA during heat shock of primary *Xenopus* hepatocytes [17]. In view of this controversy it is reasonable to speculate that in contrast to vitellogenin mRNA, that is highly stabilized by an estrogen-induced proteinaceus factor [37] which possibly gets destabilized during heat shock, normally turning over mRNAs probably are stabilized for as long as factors involved in their degradation have not recovered from thermal injury.

As regards receptor structure, heat-induced loss of receptor solubility appears to correlate with thermal denaturation of the receptor, as suggested by the loss of its hormone-binding activity and as already shown for the interferon-induced p68 kinase of HeLa cells [13]. That receptor aggregation in the nucleus and the cytoskeletal fraction of heat-shocked cells was the concomitant of loss of the glucocorticoid binding activity suggests that heat-induced dissociation of the 350-kDa complex and, in particular, dissociation of hsp90 from the receptor, in addition to compromising high-affinity hormone binding to the receptor, probably exposes an interactive domain leading to aggregation of the ligand-free receptor. Similarly, in ATP-depleted cells, reconstitution of the 350-kDa complex is inhibited and the receptor is kept in inactive form, insoluble to non-ionic detergents ([4,16]; and references therein). Hsp70 is thought to bind to exposed interactive domains and prevent damaged proteins from aggregating [11]. Appreciable amounts of protein rendered insoluble by thermal denaturation co-localizes with hsp70 and hsp110 in the perinuclear aggregate of collapsed filaments as well as in the nucleus [13,14,28]. Both proteins are thought to play a role in assisting dissolution of protein aggregates (hsp110 in the nucleus and hsp70 predominantly in the cytoplasm) while simultaneously maintaining unfolded proteins soluble and potentially in a state competent for refolding [11,14]. Significantly, immunoadsorbed glucocorticoid and progesterone receptors stripped of hsp90 by high salt treatment, bind stoichiometric amounts of hsp70 (D.J. Mitsiou and N.M. Alexis, unpublished results) and are competent for hsp70-assisted proper folding in vitro upon incubation with reticulocyte lysate ([4,16]; and references therein). Resolubilized receptor that is unable to refold to the 350-kDa complex, as observed in heat-shocked cells recovering in the presence of cycloheximide (Fig. 2), might remain bound to hsp70 until it eventually gets degraded.

As regards the inter-relationship of cell responses to heat shock and steroid hormones, it is noteworthy that the rate of recovery of glucocorticoid binding to the receptor was much slower in the presence of cycloheximide. Compromise of the heat-shock response by cycloheximide is likely to limit the amount of hsp110 and hsp70 that is available to bind thermally damaged proteins, thus preventing these proteins from commencing proper folding. Significantly, it has been shown that the extent of heat-shock response of the cell, as assessed from the amount of heat-induced hsp70, was related to the extent of heat potentiation of responsiveness of CAT enzyme expression to progestin in T47D cells stably transfected with a progestin-regulated CAT reporter gene [21]. In the above light, heat-induced de novo synthesis of hsp70 and hsp110 is likely to accelerate receptor recovery and restoration of hormonal responsiveness which, if assisted by stabilization of CAT mRNA following heat shock, could lead to a higher fold enhancement by glucocorticoids of CAT enzyme expression in heat-shocked as compared to unshocked cells.

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References

[1] Beato, M. (1989) Cell 56, 335–344.
[2] McEwan, I.J., Wright, A.P.H., Dahlman-Wright, K., Carlstedt-Duke, J. and Gustafsson, J.-Å. (1993) Mol. Cell. Biol. 13, 399–407.
[3] Brou, C., Wu, J., Ali, S., Scheer, E., Lang, C., Davidson, I., Chambon, P. and Tora, L. (1993) Nucleic Acids Res. 21, 5–12.
[4] Pratt, W.B. (1993) J. Biol. Chem. 268, 21455–21458.
[5] Rexin, M., Busch, W., Segnitz, B. and Gehring, U. (1988b) FEBS Lett. 241, 234–238.
[6] Alexis, M.N., Mavridou, I. and Mitsiou, D.J. (1992) Eur. J. Biochem. 204, 75–84.
[7] Nemoto, T., Ohara-Nemoto, Y., Denis, M. and Gustafsson, J.-Ä. (1990) Biochemistry 29, 1880–1886.

[8] Picard, D. and Yamamoto, K.R. (1987) EMBO J. 6, 3333–3340.
[9] Lewis, M.J. and Pelham, H.R.B. (1985) EMBO J. 4, 3137–3143.
[10] Skowyra, D., Georgopoulos, C. and Zylicz, M. (1990) Cell 62, 939–944.
[11] Becker, J. and Craig, E.A. (1994) Eur. J. Biochem. 219, 11–23.
[12] Pinto, M., Morange, M. and Bensaude, O. (1991) J. Biol. Chem. 266, 13941–13946.
[13] Dubois, M. F., Hovanessian, A.G. and Bensaude, O. (1991) J. Biol. Chem. 266, 9707–9711.
[14] Parsell, D.A., Kowal, A.S., Singer, M.A. and Lindquist, S. (1994) Nature 372, 475–478.
[15] Bohen, S.P. and Yamamoto, K.R. (1993) Proc. Natl. Acad. Sci. USA 90, 11424–11428.
[16] Smith, D.F. (1993) Mol. Endocrinol. 7, 1418–1429.
[17] Wolffe, A.P., Perlman, A.J. and Tata, J.R. (1984) EMBO J. 3, 2763–2770.
[18] Vedeckis, W.V., Ali, M. and Allen, H.R. (1989) Cancer Res. 49, 2295s–2302s.
[19] Anderson, R.L., Kraft, P.E., Bensaude, O. and Hahn, G.M. (1991) Exp. Cell Res. 197, 100–106.
[20] Sanchez, E.R. (1992) J. Biol. Chem. 267, 17–20.
[21] Edwards, D.P., Estes, P.A., Fadok, V.A., Bona, B.J., Oñate, S., Nordeen, S.K. and Welch, W.J. (1992) Biochemistry 31, 2482–2491.
[22] Sanchez, E.R., Hu, J.-L., Zhong, S., Shen, P., Greene, M.J. and Housley, P.R. (1994) Mol. Endocrinol. 8, 408–421.
[23] Cato, A.C.B., Miksicek, R., Sch_tz, G., Arnemann, J. and Beato, M. (1986) EMBO J. 5, 2237–2240.
[24] Strähle, U., Schmid, W. and Schütz, G. (1988) EMBO J. 7, 3389–3395.
[25] Fort, P., Marty, L., Piechaczyk, M., El Sabrouty, S.E., Dani, C., Jeanteur, P. and Blanchard, J.M. (1985) Nucleic Acids Res. 13, 1431–1442.
[26] Graham, F.L. and van der Eb, A.J. (1973) Virology 52, 456–467.
[27] Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982) Mol. Cell. Biol. 2, 1044–1051.
[28] Beckmann, R.P., Lovett, M. and Welch, W.J. (1992) J. Cell Biol. 117, 1137–1150.
[29] Reese, J.C. and Katzenellenbogen, B.S. (1991) Nucleic Acids Res. 19, 6595–6602.
[30] Baki, L. and Alexis, M.N. (1994) Eur. J. Biochem. 225, 797–803.
[31] Horvath, A.R., Muszbek, L. and Kellie, S. (1992) EMBO J. 11, 855–861.
[32] Mollenhauer, J. and Schulmeister, A. (1992) Experientia 48, 644–649.
[33] Maxam, A. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560–564.
[34] Jantzen, H.-M., Strähle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R. and Schütz, G. (1987) Cell 49, 29–38.
[35] Mizzen, L.A. and Welch, W.J. (1988) J. Cell Biol. 106, 1105–1116.
[36] Storti, R.V., Scott, M.P., Rich, A. and Pardue, M.L. (1980) Cell 22, 825–834.
[37] Shapiro, D.J. and Dodson, R.E. (1994) Mol. Cell. Biol. 14, 3130–3138.