Heat Shock Alters the Composition of Heteromeric Steroid Receptor Complexes and Enhances Receptor Activity in Vivo†

Dean P. Edwards,*,‡ Patricia A. Estes,‡ Valerie A. Fadok,‡ Betty J. Bona,‡ Sergio Oñate,‡ Steven K. Nordeen,‡ and William J. Welch§

Department of Pathology and Molecular Biology Program, University of Colorado Health Sciences Center, Denver, Colorado 80262, and Departments of Medicine and Physiology, University of California, San Francisco, San Francisco, California 94110

Received October 21, 1991; Revised Manuscript Received December 17, 1991

ABSTRACT: Under normal cellular conditions, human progesterone receptors (PR), immune-isolated from cytosols of T47D breast cancer cells, associate with two heat shock proteins (hsps), hsp 90 and hsp 70. Receptors activated by hormone binding in vivo and extracted from nuclei with 0.5 M NaCl no longer associate with hsp 90 but retain association with hsp 70. We have examined the effect of heat shock treatment of cells on hsp–receptor interactions and on receptor function. Heat shock resulted in a partial reduction in cellular levels of PR, but receptors that remained were functional for both steroid and DNA binding activities. By steady-state [^{35}S]methionine labeling prior to heat shock treatment, it was determined that heat shock did not affect the composition or maintenance of preexisting cytosolic PR·hsp 90·hsp 70 complexes. By contrast, immune isolation of PR complexes from cells pulse-labeled with [^{35}S]methionine showed that heat shock altered the composition of newly synthesized hsps associated with PR. After heat shock, both the highly inducible form of hsp 70 (72K hsp) and a 100K hsp were bound to cytosol PR, and inducible 72K hsp remained bound with the nuclear-activated PR. Neither of these hsps were associated in detectable amounts with PR under normal cellular conditions. With respect to receptor function, heat shock treatment substantially enhanced the activity of PR in vivo as determined by measuring hormone-dependent PR-mediated transcription of a target reporter gene (MMTV-CAT) that was stably transfected into T47D cells. Heat shock treatment alone, in the absence of hormone, did not stimulate MMTV-CAT expression nor did it affect transcription from a control reporter gene, pSV2-CAT, suggesting that enhanced receptor activity was due to an effect on PR-mediated processes and not to a general effect on transcription. Induction of the heat shock response by a related chemical stress (sodium arsenite) also enhanced PR activity in vivo. Interestingly, sodium arsenite produced both a greater induction of hsp 90 and hsp 70 synthesis and a greater fold enhancement of PR-mediated gene transcription than did heat shock. This suggests that enhancement of PR activity is related not only to induction of hsp synthesis but also to the severity of the stress response. The present results provide an indication that in certain cells there may exist an interrelationship between the activation pathways by which cells respond to stress and to steroid hormones. Possible mechanisms responsible for heat shock effects on PR activity are discussed.

The heat shock or stress response, that results from an abrupt elevation in temperature, is characterized by a rapid induction of synthesis of heat shock proteins (hsps). This is a highly conserved response that occurs in nearly all cells, in all organisms. In addition to elevated temperature, other agents can elicit heat shock-like responses. This includes heavy metals, ethanol, sodium arsenite, amino acid analogues, and certain viral infections (Welch et al., 1990). Heat shock proteins function to protect cells during heat shock or related stresses, but they are also important components of normal cells. Under normal cellular conditions, hsps appear to carry out a diversity of essential functions mediated primarily by their binding to other proteins. Through protein–protein interactions, hsps are thought to assist in such actions as intracellular protein trafficking and folding–unfolding of proteins and in some cases directly or indirectly influence the activity of other proteins (Lindquist & Craig, 1988; Welch, 1990; Schlesinger, 1990; Rothman, 1989, Kang et al., 1990; Gething et al., 1986; Chirico et al., 1988; Deshaies et al., 1988).

The 90-kDa heat shock protein (hsp 90) and the 70-kDa heat shock protein (hsp 70) have been shown to specifically associate with a number of interesting and physiologically important viral and cellular proteins [see Welch (1990) and references cited therein], including several members of the steroid receptor family of ligand-inducible transcriptional activators (Evans, 1988). Several different classes of steroid receptors when isolated in their inactive states have been shown to be heteromeric 8–10S complexes containing hsp 90, and in some cases hsp 70 and other proteins. The 8–10S receptor complex is unable to bind to DNA, and activation to the DNA binding state is accompanied by receptor conversion to 4S and dissociation from hsp 90 [see Pratt (1990) and references cited therein]. This and other observations have suggested that hsp 90 functions as a repressor to maintain steroid receptors in an inactive state in the absence of hormone (Mendel et al., 1986; Sanchez et al., 1987; DeMarzo et al., 1991; Dalman et al., 1990; Cadepond et al., 1991). Other studies indicate that hsp 90 may also function to maintain the unoccupied receptor in a conformation competent to bind steroid (Bresnick et al., 1989; Dalman et al., 1989). Steroid receptor association with

†This work was supported by U.S. Public Health Service Grant CA41386, by Cancer Center Core Grant P30-CA46934, by a Lucille Markey Charitable Trust (to D.P.E.), and by U.S. Public Health Service Grant GM33551 (to W.J.W.).

‡University of Colorado Health Sciences Center.

§University of California, San Francisco.

0006-2960/92/0431-2482$03.00/0

hsp 70 has been observed more recently and has been studied less extensively than hsp 90 (Estes et al., 1987; Edwards et al., 1987; Kost et al., 1989; Smith et al., 1990; Sanchez et al., 1990a; Oñate et al., 1991). Additional proteins in the range of 55–59 kDa (p55–59) have also been commonly found to associate with several different steroid receptors in their inactive 8–10S state (Tai et al., 1986; Renoir et al., 1990a,b; Oñate et al., 1991), and as recently reported, one of these may be another heat shock protein, hsp 56 (Sanchez, 1990). The functional role of receptor-associated hsp 70 and p55–59 remains much less clear than that of hsp 90. It has been suggested that hsp 90, hsp 70, and p55–59 exist as a separate higher-order complex and that all three hsps as a unit associate with receptors (Sanchez et al., 1990b).

Because steroid receptors form stable associations with several hsps under normal cellular conditions, and interactions with hsps appear to play a fundamental role in regulating receptor activity, we questioned whether heat shock would alter cellular receptor–hsp complexes and receptor function. Studies were conducted with human progesterone receptors (PR) in T47D breast cancer cells and a derivative of T47D cells stably transfected with a progesterone-responsive, PR-dependent MMTV-CAT reporter gene. Results show that induction of the stress response either by heat shock treatment or by chemical stress altered the composition of newly synthesized hsps associated with PR and dramatically enhanced receptor transcriptional activity in vivo.

Experimental Procedures

Materials. [^{3}H]R5020 (promegestone; 17,21-dimethyl-19-norpregna-4,9-dien-3,20-one; 17α-methyl-^{3}H, 87 Ci/mmol) and unlabeled R5020 were obtained from DuPont NEN products. L-Methionine [^{35}S]Translabel (1200 Ci/mmol) and [^{32}P]αdATP and [^{32}P]αdCTP (3000 Ci/mmol) were obtained from ICN. ^{35}S-Labeled protein A (30 Ci/mg) was from Amersham. Monoclonal antibodies AB-52 and B-30 (mouse IgG_1) against purified human PR were prepared as previously described (Estes et al., 1987). AB-52 recognizes both the A (94K) and B (120K) forms of human PR; B-30 recognizes B receptors only. Monoclonal antibody (mouse IgG) N27F3-4 was prepared against constitutive and inducible forms of heat shock protein 70 (hsp 70) isolated from HeLa cells (Riabowel et al., 1988). Monoclonal antibody AC-88 (mouse IgG_1) against hsp 90 was prepared as previously described (Riehl et al., 1985). All antibodies were purified from mouse ascites fluids (Estes et al., 1987).

Cell Culture, Metabolic Labeling, and Heat Shock of Cells. T47D human breast cancer cells were cultured in MEM supplemented with 5% fetal bovine serum (HyClone). Cells were plated in Falcon 175 cm^2 flasks at a density of 4×10^6 cells and grown in a humidified, CO_2 (5%) incubator at 37 °C (Estes et al., 1987; El-Ashry et al., 1989). For labeling with [^{35}S]methionine, one 175 cm^2 flask was generally used for each experimental point. For pulse-labeling studies, cells were pretreated for 30 min at 37 °C with methionine-free MEM, supplemented with 1% fetal bovine serum (Flow-Labs). Pretreatment medium was then replaced with methionine-free MEM supplemented with 25 μCi/mL [^{35}S]methionine, and cells were incubated for another 2 h at 37 °C. Flasks were then rinsed with serum-free MEM to remove excess free [^{35}S]methionine and incubated for another 1 h at 37 °C with or without 20 nM R5020. Steady-state labeling was performed by methods similar to that described (Mendel & Orti, 1988) by incubating near-confluent cell cultures for 48 h at 37 °C with 25 μCi/mL [^{35}S]methionine in 5% fetal serum and growth medium (MEM) containing a normal methionine concentration. Heat shock was performed by incubating T47D cells for 2 h and 15 min at 42 °C in a humidified, CO_2 (5%), incubator and then returning the cultures to a normal 37 °C incubator.

Receptor Preparations and Immune Isolation. All buffers used for extraction and immune isolation of PR were derived from TEG (10 mM Tris–OH, pH 7.4, 1 mM EDTA, and 10% glycerol). Harvested cells were lysed in TEDG (TEG plus 1 mM DTT) in the presence of a cocktail of protease inhibitors (Estes et al., 1987). In some experiments, sodium molybdate (20 mM) was included in the cell lysis buffer to stabilize the 8–10S cytosolic PR complex. Cell lysates were centrifuged at 105000g at 4 °C for 30 min to yield cytosol and crude nuclei in the high-speed pellet. The pellet was extracted for 60 min on ice with TEDG containing protease inhibitors and 0.5 M NaCl and then centrifuged at 105000g for 30 min to yield a salt-soluble crude nuclear extract. The crude nuclear extract was then dialyzed at 4 °C against TEDG to reduce the salt concentration prior to immune isolation. MAb–affinity matrices were prepared by chemically coupling purified AB-52 or B-30 to activated agarose (Affigel-10; Bio-Rad) at a substitution of 4.5 mg of MAb/mL of resin. One milliliter of cytosol or dialyzed nuclear extract was then incubated in suspension at 4 °C for 4 h with 100 μL of the MAb–Affigel-10 resin. MAb resins were then washed by centrifugation as described (DeMarzo et al., 1991). As controls to determine the levels of nonspecific binding, blank Affigel-10 was substituted for the receptor-specific MAb–Affigel-10.

To determine the number of steroid receptor binding sites in cytosol and nuclear extracts, binding was measured by a dextran-coated charcoal method (Estes et al., 1987; El-Ashry et al., 1989). Protein concentration in cell extracts was measured by Bradford assay (Bradford, 1976).

SDS Gel Electrophoresis, Western Immunoblotting, and Two-Dimensional Gel Analysis. Proteins bound to MAb–Affigel-10 were eluted with a 2% SDS sample buffer, boiled for 5 min, and applied directly to either 7.0% or 7.5% discontinuous SDS–polyacrylamide gels. Electrophoresis and Western immunoblotting of PR were carried out as previously described (Estes et al., 1987; El-Ashry-Stowers et al., 1988). For immunoblot of heat shock proteins, transfer to nitrocellulose was performed for 2 h at 1 A in a buffer containing 20 mM Tris–OH, pH 8.4, 150 mM glycine, and 20% methanol. Proteins metabolically labeled with [^{35}S]methionine were detected by autofluorographic enhancement. After separation of ^{35}S-labeled proteins on SDS–PAGE, gels were fixed for 30 min in 40% methanol and 10% acetic acid and then treated with Amplify (Amersham) and dried under vacuum. Dried gels were exposed at −70 °C to preflashed X-Omat film with DuPont intensifying screens. Two-dimensional gel electrophoresis, employing isoelectric focusing (70% pH 5–10 and 30% pH 3–10, Ampholines) in the first dimension followed by 12.5% SDS–PAGE in the second dimension, was performed as described (Welch, 1985). Prestained molecular weight markers (Bethesda Research Laboratories) were used for both Western immunoblots and for ^{35}S-labeled gels: myosin, 224 000; phosphorylase B, 107 500; bovine serum albumin, 71 200; ovalbumin, 46 000; carbonic anhydrase, 31 000.

Gel Mobility Shift Assay. A 32 bp double-stranded synthetic oligonucleotide, corresponding to the distal most (bp −189 to −162 from the start site of transcription) progesterone/glucocorticoid response element (PRE/GRE) of mouse mammary tumor virus (MMTV), was end-labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP by Klenow DNA polymerase fill-in. PR (50 fmol) was incubated with 0.3 ng of the

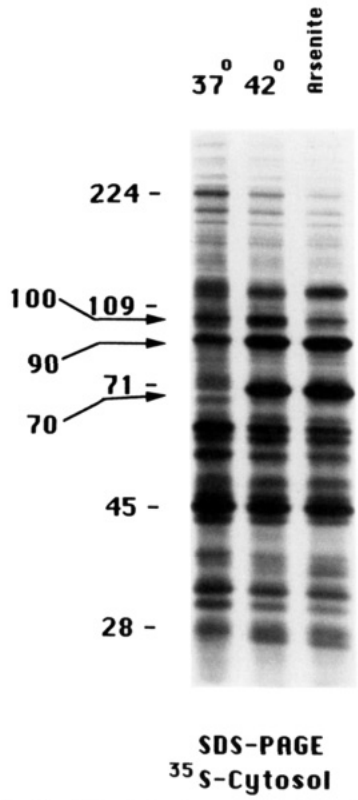

FIGURE 1: Effects of heat shock and chemical stress on protein synthesis in T47D cells. T47D cell cultures were kept at 37 °C to serve as a control, were heat-shocked by incubation at 42 °C for 2.25 h and then returned to 37 °C, or were incubated at 37 °C for 2 h with 100 μM sodium arsenite. Cells were then pulse-labeled with [^{35}S]methionine at 37 °C, cytosols prepared, and aliquots analyzed by SDS–PAGE (10%) and autoradiography.

[^{32}P]PRE oligonucleotide for 1 h at 4 °C. The DNA binding buffer conditions for gel electrophoresis have been described (El-Ashry et al., 1989). In some reactions, receptor-specific MAb, AB-52, was added at the completion of the 1-h reaction and allowed to incubate for another 30 min at 4 °C.

PR-Mediated Target Gene Transcription in Vivo. A gene construct consisting of the MMTV LTR (from −1161 bp to +102 bp), linked to the chloramphenicol acetyltransferase gene (CAT), was stably transfected into T47D cells (El-Ashry et al., 1988). The LTR contains the entire hormone responsive enhancer element (HRE) and promoter of MMTV. A cloned line was isolated (clone B-11), and induction of MMTV-CAT expression was shown previously to be both progestin- and PR-dependent (El-Ashry et al., 1988). For study of PR-mediated transcription of MMTV-CAT, clone B-11 cells were plated in six-well dishes (Falcon) at a density of 0.5×10^6 cells per well. They were allowed to attach and grow for 3 days in regular growth medium and then were incubated for 24 h with medium containing hormone-free serum (DCC-treated). Cells were then incubated with or without progestin (R5020) for the times indicated; they were harvested by scraping into TNE buffer (40 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM EDTA) and lysed by sonication in 250 μL of PBF buffer [0.25 M Tris-HCl, pH 7.4, and 0.05 mg/mL phenylmethanesulfonyl fluoride (PMSF)]. Lysates were centrifuged to pellet cell debris, and CAT enzyme activity in the supernatants was measured by a radiometric enzymatic/organic extraction method as previously described (Nordeen et al., 1987). A constitutive target gene construct, pSV2-CAT, was also stably transfected into T47D cells as previously described and used as a control non-hormone-regulated gene (Nordeen et al., 1989).

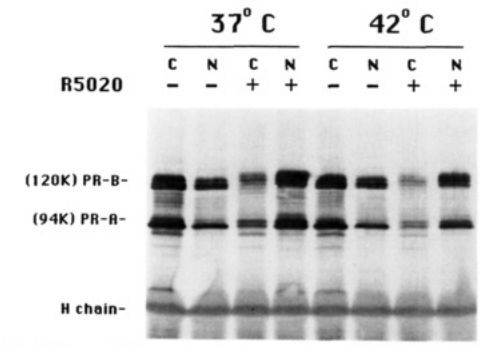

FIGURE 2: Effects of heat shock treatment on PR–protein levels and PR transformation by hormone in vivo. T47D cells were heat-shocked as in Figure 1 except that they were treated for an additional 1 h at 37 °C with or without the progestin R5020 (40 nM). Cytosol (C) and nuclear extracts (N) were prepared, and PR was immune-isolated with the receptor-specific MAb AB-52 using protein A–Sepharose as immunoabsorbent. Immobilized PR were eluted with SDS and analyzed by Western blot with the same MAb. The H-chain of the MAb also elutes from protein A–Sepharose.

RESULTS

Heat Shock Response in T47D Cells and Effect on PR Levels. T47D cells were exposed to 42 °C for 2.25 h, returned to normal 37 °C temperature, and then pulse-labeled with [^{35}S]methionine. Cells maintained at 37 °C throughout served as nonstressed controls. As shown by SDS–PAGE and autoradiography of crude cell extracts, this treatment produced a marked de novo synthesis of cellular proteins of approximately 100, 90, and 70 kDa (Figure 1). The apparent molecular weights of the induced proteins correspond with those of the major mammalian heat shock proteins, respectively; hsp 110, hsp 90, and hsp 70 (Lindquist & Craig, 1988; Welch, 1990; Schlesinger, 1990). To determine whether this heat shock response affected cellular PR levels, we examined the steroid binding capacity of cytosols prepared from heat-shocked and untreated T47D cells. Using a single saturating dose steroid binding assay, a 40% reduction (average value from four separate experiments) in progestin binding capacity was observed after heat shock treatment of cells. However, by Scatchard analysis, the sites that remained after heat shock exhibited an unaltered binding affinity for the progestin R5020. PR from untreated cells and heat-shocked cells had binding dissociation constants, respectively, of 2.4×10^{-9} and 3.2×10^{-9} M (not shown).

Human PR are produced in cells as two steroid binding proteins of different lengths, termed PR-A (94 kDa) and PR-B (120 kDa). The two forms share common hormone and DNA binding domains and differ at the amino terminus; PR-A is missing N-terminal sequences present in PR-B (Kastner et al., 1990). Western blots were performed with the PR-A- and PR-B-specific MAb AB-52 (Estes et al., 1987) to determine whether the reduction in steroid binding capacity was due to an effect on receptor activity or on lowering PR concentration. As shown in Figure 2, only a slight reduction in immunoreactive PR was observed in cytosol and nuclear extracts (both in the absence and in the presence of hormone) after heat shock treatment of cells. This was seen consistently in repeat experiments. Quantitation of Western blots by densitometry to determine the extent of reduction was not done. The slight decrease observed does indicate that the lowering in steroid binding capacity that results from heat shock treatment is due, at least in part, to a reduction in cellular PR concentration. Consistent with this, the rate of synthesis of PR was reduced after heat shock treatment (Figures 3 and 5). It should also be noted that with both control and heat shock treated cells,

the majority of PR protein was recovered in the cytosol in the absence of hormone. Addition of R5020 promoted tight binding of most PR protein to nuclei, and this occurred with both control and heat-shocked cells (Figure 2). Thus, after heat shock treatment of T47D cells, substantial amounts of receptors remained and were functional as determined by hormone binding in vitro and by their ability to bind to nuclei in response to hormone addition in vivo.

Progesterone receptors in T47D cells are phosphoproteins that exhibit basal phosphorylation in the absence of hormone and undergo an increased phosphorylation in response to hormone addition to intact cells (Sheridan et al., 1988). A distinctive feature of hormone-dependent PR phosphorylation is an increase in the apparent molecular weight of the A and B receptors on SDS–PAGE (Horwitz et al., 1985). This characteristic upshift of PR on SDS–PAGE was detected in both control and heat-treated cells in response to R5020 addition. Thus, at this level of resolution, hormone-dependent phosphorylation of PR also appeared to be unimpaired in T47D cells after induction of the heat shock response (Figure 2).

Heat Shock Altered the Composition of Newly Synthesized hsps Associated with Cytosolic PR. Since the majority of cellular PR remained intact and functional with respect to hormone binding after heat shock, we next examined whether heat shock might affect PR association with hsp 90 and hsp 70. Untreated and heat shock treated T47D cells were pulse-labeled with [^{35}S]methionine as in Figure 1. Cytosols were then prepared in low ionic strength buffer that also contained sodium molybdate to stabilize the heteromeric 8–10S PR complex, and receptors were immune-isolated and analyzed by SDS–PAGE and autoradiography. In the unactivated state, PR-A and PR-B each form separate associations with hsp 90 and hsp 70 (Kost et al., 1989). Therefore, we chose to analyze only PR-B in this experiment, by use of the PR-B-specific MAb B-30 (Estes et al., 1987). Figure 3A shows that the cytosolic PR complex isolated from normal cells contained a radiolabeled protein of 120 kDa that corresponds to PR-B, and three other associated proteins of ≈90, 70, and 55 kDa. The 90- and 70-kDa proteins have been previously identified immunologically as hsp 90 and hsp 70, respectively (Oñate et al., 1991). The 55-kDa component is likely to be the same receptor-associated 56–59-kDa protein identified by others and recently shown to be hsp 56 (Sanchez, 1990). The 55-kDa protein associated with cytosol human PR has the same behavior on two-dimensional electrophoresis as hsp 56 (Figure 3B) (Sanchez et al., 1990b) and undergoes increased synthesis in response to heat shock treatment (Figure 3A). Neither PR-B nor the other three proteins were bound to any appreciable extent to control resins (Figure 3A, right panel), indicating that hsp 90, hsp 70, and the 55-kDa protein were coisolated through specific association with PR. We do see trace amounts of hsp 90 and hsp 70 binding to control antibodies, but this is substantially less than that bound with receptor-specific antibodies. The cytosolic PR complex isolated from heat-shocked cells contained the same three associated proteins, except the extent of labeling of hsp 90 and hsp 70 was dramatically increased. The 55-kDa protein also exhibited an increased rate of synthesis, but less so than hsp 90 and hsp 70. It should also be noted that the total amount of radiolabeled PR-B was slightly decreased compared with control cells, indicating that the rate of PR synthesis was reduced somewhat in response to heat shock. After heat shock, an additional labeled protein of ≈100 kDa was typically observed to associate with PR in an immune-specific manner that was

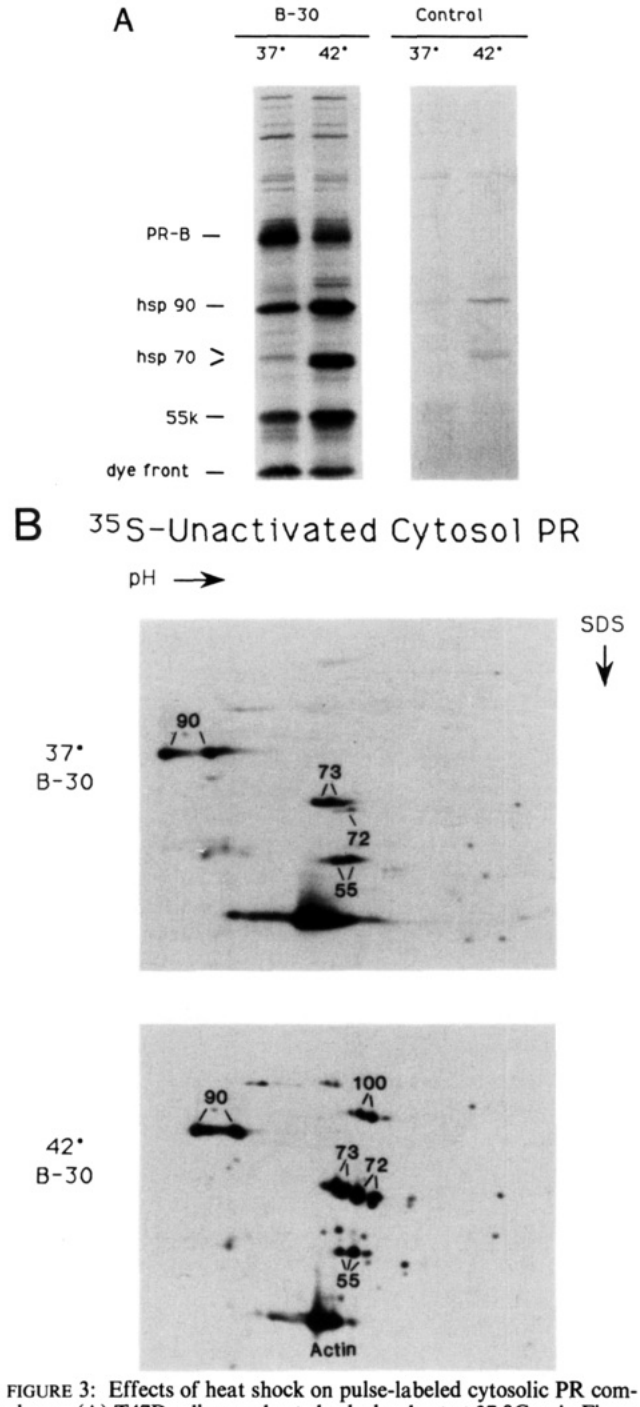

FIGURE 3: Effects of heat shock on pulse-labeled cytosolic PR complexes. (A) T47D cells were heat-shocked or kept at 37 °C as in Figure 1 and then pulse-labeled with [^{35}S]methionine. Cytosols were prepared in low ionic strength buffer with sodium molybdate, and PR complexes were immune-isolated with the PR-B-specific MAb B-30 coupled to Affigel-10. Samples were immune-isolated in parallel with a control Affigel-10. Immobilized PR complexes were eluted from Affigel-10 with SDS and analyzed by SDS–PAGE (7%) and autoradiography. (B) Two-dimensional gel electrophoresis of pulse-labeled hsps associated with cytosol PR from control and heat shock treated cells. Cytosol PR prepared from pulse-labeled cells were immune-isolated as in (A), and then immobilized PR complexes were eluted with IEF sample buffer and submitted to two-dimensional gel electrophoresis as outlined under Experimental Procedures.

not detectable from control cells, and the hsp 70 component was resolved as a doublet band (Figure 3A).

It has been shown in most mammalian cells that there is a constitutive form of hsp 70 synthesized in the absence of stress (referred to here as 73K hsp) and related forms of hsp 70 that are highly inducible by stress and often referred to as

72K hsp (Welch, 1990). To further examine the composition of hsp 70 associated with PR after heat shock, cytosolic PR complexes from pulse-labeled cells were immune-isolated and analyzed by two-dimensional gel electrophoresis. By standard two-dimensional electrophoresis, PR does not enter equilibrium isoelectric focusing gels. Only receptor-associated proteins are resolved. As shown in Figure 3B, constitutive 73K hsp is the primary form of hsp 70 associated with cytosolic PR from normal 37 °C cells, and this resolved into two isoelectric species. After heat shock, two additional charged species of the inducible 72K hsp became associated with cytosolic PR, and rates of synthesis of receptor-associated 73K hsps were also dramatically increased. hsp 90 associated with cytosol PR resolved as the same two charged isoforms from both control and heat-shocked cells (Figure 3B), and both forms exhibited an increase in labeling. On two-dimensional gel electrophoresis, ^{35}S-labeled spots at 100 kDa were also found associated with cytosolic PR after heat shock which were undetectable from control cells. This is sometimes evident by single-dimension SDS–PAGE (Figure 3A), but more so by two-dimensional analysis (Figure 3B). It should be noted that the 100-kDa protein exhibits isoelectric points similar to that of hsp 110 (Welch et al., 1990).

An additional protein that resolved on two-dimensional gels with the position expected of actin was also found to associate with cytosol PR in an immune-specific manner. This protein did not exhibit an increased rate of synthesis, and it runs in the dye front of our 7.0% single-dimensional SDS gels. Recent studies have suggested that the 8S glucocorticoid receptor (GR) is able to bind to both endogenous and exogenous actin filaments through the hsp 90 component of the receptor complex. GR in the 4S state, dissociated from hsp 90, did not bind actin (Miyata & Yahara, 1991). Thus, we conclude from this analysis that heat shock treatment affected newly assembled PR complexes by increasing the complexity of hsps associated with PR, particularly so for hsp 70 isoforms, and by increasing the rate of synthesis of associated hsps.

To examine whether heat shock might also affect the maintenance of preexisting PR·hsp 90·hsp 70 complexes, T47D cells were labeled to steady-state with [^{35}S]methionine (48 h) and afterward were submitted to heat shock. Cells were then returned to normal 37 °C for 1 h and harvested. Under these conditions, the composition of cytosolic PR complexes immune-isolated from heat-shocked cells appeared to be unchanged from that of normal cells. Also, PR complexes isolated from both groups of cells exhibited the same relative amounts of steady-state-labeled PR and associated components; hsp 90, hsp 70, and the 55-kDa protein (Figure 4). These results indicate that heat shock does not affect the composition or stoichiometry of preexisting receptor–hsp complexes.

The Inducible Form of hsp 70 (72K) Associates with Activated Nuclear PR. PR appears to bind differently with hsp 90 than it does with hsp 70, since receptors dissociate from hsp 90 upon hormone and salt activation, while association with hsp 70 is retained under these conditions. The continued association of activated PR with hsp 70 suggests that this component of the receptor complex serves a function different from that of hsp 90 (Kost et al., 1989; Smith et al., 1990; Oñate et al., 1991). We were therefore curious to see whether the inducible 72K hsp present in the cytosolic PR complex after heat shock was also associated with the hormone-activated receptor.

Control 37 °C cells or cells subjected to heat shock were pulse-labeled with [^{35}S]methionine as in Figure 3, except that

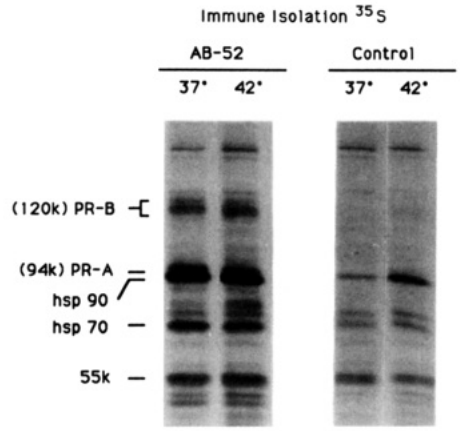

FIGURE 4: Effect of heat shock treatment on the preexisting steady-state-labeled cytosol PR complex. T47D cell cultures were incubated for 48 h at 37 °C with [^{35}S]methionine to label PR to steady-state. Cells were then washed, replaced with regular growth medium (containing normal methionine content), and heat-shocked at 42 °C. Cells incubated continuously at 37 °C served as controls. Cytosols were then prepared in the presence of molybdate, and PR complexes were immune-isolated with the receptor MAb AB-52 (PR-A and PR-B specific) coupled to Affigel-10. Immobilized PR were analyzed by SDS–PAGE (7%) and autoradiography.

afterward cells were treated for an additional 1 h at 37 °C with R5020 in order to activate receptors in vivo. In vivo activated PR were then extracted from nuclei with 0.5 M NaCl and immune-isolated with AB-52–Affigel-10 resins (PR-A- and PR-B-specific MAb). As shown by SDS–PAGE in Figure 5A, activated nuclear PR from control 37 °C cells were recovered with one other radiolabeled component, hsp 70. No hsp 90 or p55 was recovered with activated nuclear PR. It should be noted that human hsp 70 contains half the number of methionines (Hunt & Morimoto, 1985) as human PR and is thus expected to label less efficiently. After heat shock, hsp 70 associated with activated nuclear PR exhibited a dramatic increase in the rate of synthesis and was now composed of both 72K hsp and 73K hsp (Figure 5A). As with cytosolic PR, the amount of newly synthesized activated nuclear PR isolated from heat-shocked cells was reduced compared with normal 37 °C cells. Results from Figure 2 show this is not due to a reduced ability of PR after heat shock to respond to hormone in vivo and transform to the tight nuclear binding state. As shown by two-dimensional gel electrophoresis, hsp 70 recovered with activated nuclear PR from control cells resolved as a single 73-kDa spot, while activated nuclear PR from heat-shocked cells was recovered with multiple isoforms of hsp 70 (Figure 5B) including two charged species of 72K hsp. Thus, the inducible 72K hsp that binds to cytosolic PR after heat shock retains association with nuclear PR after activation by hormone.

Heat Shock Enhances PR-Mediated Target Gene Transcription in Vivo without Altering PR–DNA Binding Activity. To assess PR activity in vivo, a cloned derivative of T47D cells (B-11), stably transfected with an MMTV-CAT construct, was used to examine the effects of heat shock on PR-mediated target gene expression in vivo. B-11 cells were heat shocked, returned to 37 °C, and then incubated for another 24 h at 37 °C without and with R5020. B-11 cells maintained at 37 °C throughout served as nonstressed controls. As shown in Figure 6A, R5020 stimulated target gene expression in a dose-dependent manner in both control and heat shock treated cells. We were surprised to observe that maximal target gene induction achieved with saturating concentrations of hormone was higher by 3-fold in heat shock treated cells. The higher level of induction was not due to an activation of MMTV-CAT

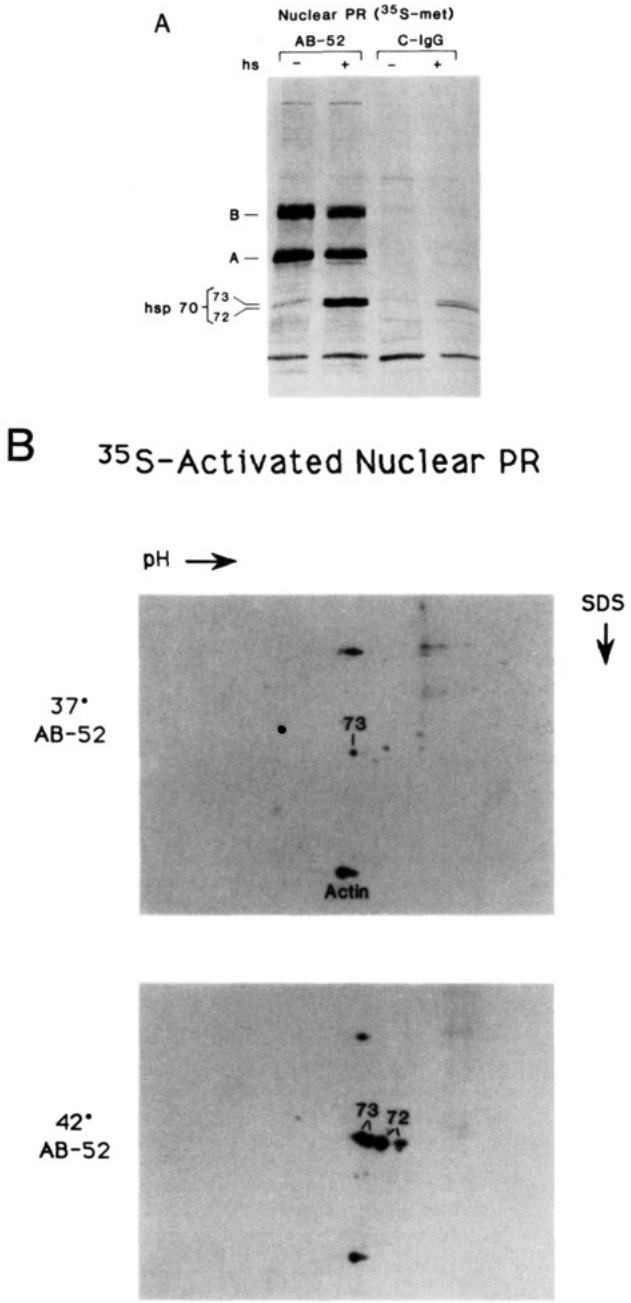

FIGURE 5: Effect of heat shock on activated nuclear PR. (A) T47D cell cultures were heat-shocked and pulse-labeled as in Figure 3 except that at the end of pulse-labeling, PR were activated in vivo by addition of the progestin R5020 (40 nM) for 1 h at 37 °C. Nuclear extracts were then prepared, and PR complexes were immune-isolated with AB-52 MAb coupled to Affigel-10. The immobilized receptors were eluted and analyzed by SDS–PAGE (7%) and autoradiography. (B) Two-dimensional gel electrophoresis of nuclear-activated PR immune-isolated from pulse-labeled cells with AB-52–Affigel-10.

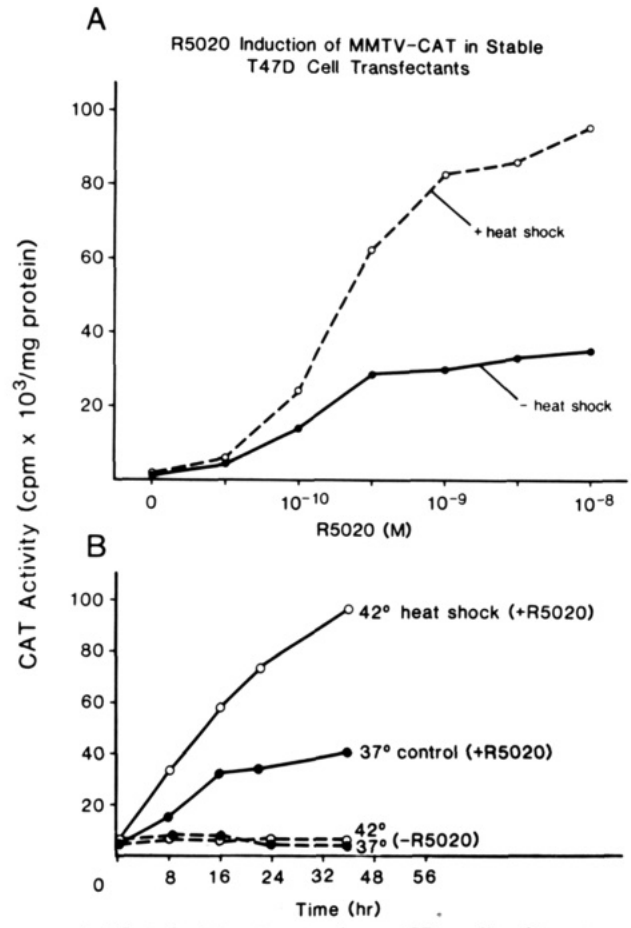

FIGURE 6: Heat shock treatment enhances PR-mediated target gene transcription in vivo. (A) T47D cells (clone B-11) stably transfected with an MMTV-CAT construct were heat-shocked, returned to normal 37 °C, and then incubated for 24 h with the concentrations of R5020 indicated. B-11 cells incubated continuously at 37 °C served as controls. Cells were then lysed, and CAT enzyme activity was measured. (B) Heat-shocked or 37 °C control cells were incubated with a single concentration of R5020. At various times after hormone addition, cells were then harvested and lysed, and CAT enzyme activity was measured. CAT enzyme activity was expressed as the cpm of [^{3}H]acetyl-CoA converted to acetyl chloramphenicol per hour per microgram of protein. Values are averages from duplicate determinations. The results are representative of five replicate experiments.

expression in the absence of hormone, since the basal level of expression that occurs without hormone remained low and was not measurably different in normal or heat shock treated cells. In Figure 6B, PR-mediated induction of the MMTV-CAT gene was analyzed at different times after addition of hormone. Hormonal induction of CAT was observed to be greater in heat shock treated cells at all time points measured, indicating that the effects of heat shock were not due to an alteration in the kinetics of target gene induction.

In Figure 7, R5020 was added to B-11 cells at various times after heat shock treatment. In each case, cells were incubated with a single dose of R5020 (20 nM), and the total time of exposure to hormone was the same (24 h) before harvest of cells for CAT enzyme assay. Maximal enhancement of PR-mediated target gene expression that resulted from heat shock treatment was obtained when R5020 was added immediately after cells were shifted from 42 to 37 °C. This effect diminished progressively with time over a period of several hours. When R5020 addition was delayed by 8 h after heat shock, the magnitude of hormone-dependent MMTV-CAT stimulation had nearly returned to that obtained with control 37 °C cells (Figure 7). The gel inset in Figure 7 shows results of immunoprecipitation of ^{35}S-labeled hsp 70 with an hsp 70 specific MAb (N27), from cells pulse-labeled at the different times indicated after heat shock. Induced synthesis of hsp 70 was also transient. Maximal induction occurred immediately following heat shock, and by 8 h after the return of cells to 37 °C, hsp 70 synthesis rates had returned to near control levels. Thus, we observed a temporal relationship between enhanced hormonal induction of MMTV-CAT and maximal induction of hsp 70 synthesis. This temporal relationship was not observed for hsp 90. Elevated synthesis of hsp 90 continued for 24 h after return of cells to normal temperature (not shown). We also examined the effect of heat shock on a constitutive reporter gene construct, pSV2-CAT, that was stably transfected into T47D cells (Nordeen et al., 1989).

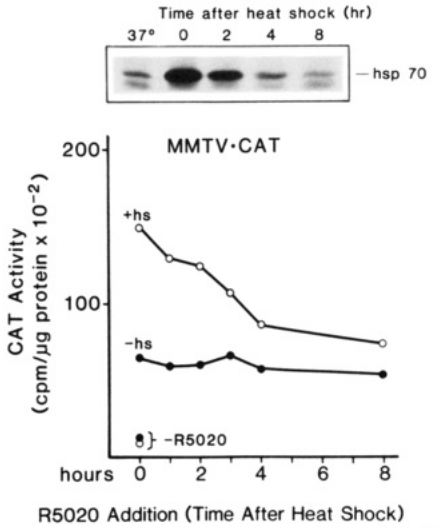

FIGURE 7: Effect of heat shock treatment on PR activity is transient. B-11 cells incubated continuously at 37 °C (−hs) or heat-shocked for 2 h at 42 °C (+hs) and then returned to 37 °C were incubated without or with R5020 (40 nM). Either R5020 was added immediately following heat shock, or addition was delayed for the times indicated. In each case, exposure of cells to R5020 was for the same length of time (24 h). Cells were then harvested, and CAT enzyme activity was measured and expressed as in Figure 6. A singe time point was taken for 37 °C cells (−hs) and heat shock cells (+hs) without R5020 addition. Inset: Immune isolation of hsp 70 (MAb, N27) from cells that were pulse-labeled with [^{35}S]methionine (2 h) at the various times indicated after heat shock. The inset shows the region of the SDS gel containing ^{35}S-labeled hsp 70.

Constitutive CAT expression drived by the SV-40 promoter was found to be similar in both normal and heat shock treated cells and at all times points examined after heat shock (not shown). This is a non-hormone-responsive reporter construct, and hormone also had no effect on pSV2-CAT expression either in normal cells or after heat shock (not shown). Results with pSV2-CAT indicate that enhanced PR activity is not due to a general effect of heat shock on transcription or on stability of the CAT enzyme but may be due to an effect on PR-mediated processes.

To determine whether enhanced PR-mediated transcription might to due to a modification of receptor–DNA binding activity, PR from control and heat shock treated cells were assayed by electrophoretic mobility shift for binding to a [^{32}P]oligonucleotide containing a progesterone response element (PRE) derived from the MMTV promoter. As shown in Figure 8, PR from control and heat shock treated cells did not bind to the [^{32}P]PRE–oligonucleotide in the absence of hormone. With both groups of cells, binding required addition of hormone, and approximately the same amount of PR–DNA binding was obtained after hormone addition when equal numbers of receptors were added to the DNA binding reaction. It should also be noted that the electrophoretic mobilities of PR–DNA complexes were identical with receptors isolated from control and heat shock treated cells. Thus, heat shock did not activate PR–DNA binding in the absence of hormone or produce an obvious effect on the DNA binding activity of PR–hormone complexes that could account for the observed enhancement of receptor activity in vivo.

Chemical Stress Induces hsp Synthesis and Also Enhances PR Activity in Vivo. When T47D cells were subjected to a chemical stress by incubation for 2 h at 37 °C with 100 μM sodium arsenite, this resulted in an increased rate of synthesis of hsp 70 and hsp 90, as detected by SDS–PAGE of cytosols prepared from cells pulse-labeled with [^{35}S]methionine (Figure 1). Induction of hsp 70 and hsp 90 synthesis was in fact

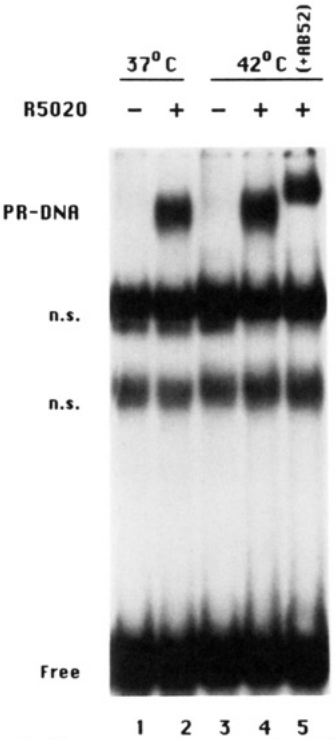

FIGURE 8: Heat shock treatment does not alter PR–DNA binding activity as measured by gel mobility shift assay. Control T47D cells (37 °C) and cells subjected to heat shock treatment were incubated for 1 h at 37 °C with or without R5020. Whole cell extracts were prepared and incubated with a [^{32}P]PRE oligonucleotide. PR–DNA complexes were separated from free DNA by nondenaturing gel electrophoresis.

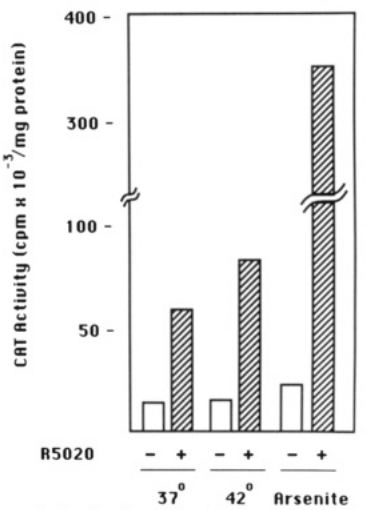

FIGURE 9: Effect of chemical stress on PR-mediated gene transcription in vivo. B-11 cells stably transfected with MMTV-CAT were left untreated at 37 °C, were heat-shocked for 2.25 h at 42 °C, or were incubated with 100 μM sodium arsenite for 2 h at 37 °C. All cells were then incubated at 37 °C for 24 h without or with R5020 (40 nM). Cells were lysed, and CAT enzyme activity was measured and expressed as in Figure 6.

greater than that obtained by heat shock treatment (Figure 1). When untreated and sodium arsenite treated cells were pulse-labeled with [^{35}S]methionine and cytosol PR complexes immune-isolated with PR-specific MAbs, results similar to those in Figure 3 were obtained. As with heat shock, sodium arsenite treatment resulted in an increased rate of synthesis of hsp 90 and hsp 70 associated with cytosolic PR and binding of the inducible 72K hsp to PR (not shown). The effect of chemical stress on PR-mediated target gene transcription in vivo is shown in Figure 9. Hormone-dependent PR-mediated

transcription was enhanced as a result of sodium arsenite treatment, and the enhancement was even greater than that which occurs after heat shock. Sodium arsenite also reproducibly stimulated a small but measurable increase in MMTV-CAT expression in the absence of hormone. This effect in the absence of hormone was not seen with the heat shock conditions used in this study. The results with sodium arsenite indicate that enhancement of PR activity is not just a peculiarity of heat shock treatment but appears to be related to induction of hsp synthesis. It is also of interest that the severity of the stress response, as gauged by the extent of hsp 70 synthesis, was related to the fold enhancement of PR-mediated gene transcription.

Discussion

Steroid receptors have been studied almost exclusively under normal cellular conditions. There have been few reports on the effect of heat shock on steroid receptors. In two earlier studies, heat shock treatment was shown to dramatically inhibit steroid responsiveness. Heat shock of primary cultures of *Xenopus laevis* hepatocytes resulted in a transient inhibition of estrogen stimulation of transcription of the vitellogenin gene (Wolffe et al., 1984). This was paralleled by a total loss of estrogen receptor–hormone binding activity. Whether loss of hormone binding activity resulted from inactivation of receptors or from receptor degradation was not determined. In the water mold *Achlya ambisexualis*, heat shock and treatment with sodium arsenite each resulted in a severe inhibition of responsiveness to the fungal steroid antheridiol (Riehl, 1988). A detailed analysis of the *Achlya* steroid receptor showed a sizable reduction in the concentration of receptors in response to heat shock, but the receptors that remained exhibited no alteration in steroid binding affinity. Curiously, sodium arsenite did not lower the concentration of steroid receptors. Short-term exposure (2 h) of mammalian cell cultures to 42 °C was also reported to result in a 90–95% reduction of glucocorticoid–receptor binding activity and GR protein levels (Vedeckis et al., 1989). In contrast to these earlier studies, we observed an enhancement of steroid responsiveness as a result of both heat shock and treatment with sodium arsenite. We also did not observe a reduction in cellular receptor concentrations to the extent reported in other studies. We can only speculate that this may be due to differences in cell lines, species, or possibly classes of steroid receptors. There are data to indicate that the stability of hsp 90 association with different receptor classes can vary considerably. For example, the 8S dioxin receptor was reported to be more stable in vitro under the same conditions as the 8S glucocorticoid receptor–hsp 90 complex (Nemoto et al., 1990). We have found that GR interaction with hsp 90 is more salt-resistant than is PR interaction with hsp 90[1] and at least two members of the nuclear receptor family, thyroid and retinoic acid receptors, do not appear to associate with hsp 90 at all (Dalman et al., 1990, 1991). Steroid receptors under certain conditions may also exhibit different behavior with respect to their ability to associate with hsp 70 (Sanchez et al., 1990a,b). It is possible that, depending on the nature or stability of preexisting receptor–hsp complexes, classes of steroid receptors will be affected differently by heat shock treatment and related stresses.

Since the majority of cellular PR was retained after heat shock treatment of T47D cells, this enabled us to examine the effect of heat shock on both the maintenance and composition of PR·hsp 90·hsp 70 complexes. On the basis of analysis of steady-state-labeled PR, it appeared that heat shock treatment did not affect PR·hsp 90·hsp 70 complexes that were preexisting under normal cellular conditions. Thus, the maintenance and stability of preformed PR complexes may account for the retention of the majority of cellular PR in a biologically active form. When newly synthesized hsp PR complexes were analyzed by pulse-labeling after heat shock, the stress-inducible forms of hsp 70 (72 hsp) and a 100-kDa stress protein became associated with PR that were not associated in detectable amounts prior to heat shock. This indicates that the protein composition of newly assembled PR complexes was altered by heat shock. One difficulty with these pulse-labeling experiments is that they cannot distinguish between increased association of newly synthesized hsps with newly synthesized PR or with preexisting unlabeled PR, since both receptor forms will be immune-isolated with receptor-specific MAbs. However, that no obvious change in the stoichiometry of prelabeled PR complexes was observed as a result of heat shock argues against exchange of inducible hsp with unlabeled preexisting hsps bound to receptors. In addition, heat shock and chemical stress also resulted in a dramatic increase in the rates of synthesis of associated hsp 70 and hsp 90 relative to that of PR. Whether this represents a change in the relative mass of the protein components in newly synthesized PR complexes or a change in specific activity is not known. Estimating changes in the stoichiometry of protein components in the newly synthesized PR complex will require measuring the specific activity of both free hsps and hsps associated with PR. It would be interesting to compare the fate (half-life) and function of newly synthesized PR–hsp complexes and preexisting PR complexes after stress.

The heat shock response activates expression of genes that encode hsps, while expression of most other genes are inhibited (Lindquist & Craig, 1988; Welch, 1990; Schlesinger, 1990; Rothman, 1989). For the most part, normal biochemical pathways that have been examined have been found to be compromised as a result of induction of the stress response (Welch, 1990). Although we did observe a reduction in synthesis of PR after heat shock, consistent with inhibition of PR gene expression and thus a classical heat shock response, we were quite surprised to find that progesterone receptor activity with respect to its ability to mediate induction of target gene transcription in vivo was considerably enhanced after heat shock. Moreover, heat shock treatment appeared to selectively affect receptor transcriptional activity, since steroid binding and DNA binding activities of PR were not stimulated. The fact that heat shock treatment did not stimulate a measurable increase in MMTV-CAT in the absence of hormone and did not affect expression from a constitutive reporter gene (pSV2-CAT) also suggested that heat shock treatment was affecting PR-mediated processes and not just general transcription. This, however, does not demand that PR itself was directly modified. Heat shock treatment, for example, could affect another transcription factor intimately involved in PR-mediated gene transcription of target genes.

A related chemical stress produced by incubating cells with sodium arsenite also resulted in enhancement of receptor activity in vivo, indicating that the effect of heat shock treatment on receptor was related to induction of the stress response and not merely to an effect of cell warming on steroid solubility or to stimulation of other general cellular processes. Interestingly, Riehl (1988) showed that heat shock and sodium arsenite treatment of *Achlya ambisexualis* did not affect cellular uptake of steroid hormone. Sodium arsenite in the

[1] C. A. Beck and D. P. Edwards, unpublished results.

present study produced a more severe stress response, as gauged by the degree of induction of hsp 70 synthesis, and also stimulated receptor activity to a greater extent than did heat shock. This raises the interesting possibility that enhancement of receptor activity may be related to the relative fold stimulation of hsp synthesis.

The mechanism(s) responsible for enhanced receptor activity as a result of induction of the stress response is (are) not known. It is unlikely for a number of reasons that we are observing a posttranscriptional effect on the CAT reporter mRNA or on CAT enzyme activity. First, most non-hsps mRNAs are inhibited by heat shock, not enhanced (Lindquist & Craig, 1988), and at least one study has reported that two reporter enzymes, luciferase and β-galactosidase, expressed from non-hsp promoters in mouse cells were in fact inactivated by heat shock treatment (Nguyen et al., 1989). Second, the same CAT reporter gene driven from a constitutive SV-40 promoter was not stimulated by heat shock. Furthermore, in a separate study, glucocorticoid receptor mediated gene transcription has also been shown to be enhanced by heat shock using a glucocorticoid-responsive MMTV promoter linked to a different reporter enzyme, luciferase (Moyer and Nordeen, unpublished results).

We propose two possible mechanisms that are not necessarily mutually exclusive. One is the possibility that the MMTV promoter may contain a heat shock response element (HSE). This is a cis-acting binding site for the heat shock transcription factor (HSTF), a trans-acting factor that controls expression of hsp genes (Wu et al., 1990). The MMTV-CAT reporter gene was constructed with the entire MMTV-LTR (El-Ashry et al., 1989), and a search of MMTV revealed the presence of a reverse orientation consensus HSE (-TTC--GAA-) (Lis et al., 1990). This putative HSE is located 288 bp upstream of the distal-most GRE/PRE. Several other sites with a single bp mismatch are also present in the vicinity of the GRE/PRE. Since heat shock treatment alone did not induce a measurable stimulation of MMTV-CAT expression and sodium arsenite had only minimal effects without hormone, these sites are not functional, or poor at best, in the absence of hormone. They may be functional in the presence of hormone, possibly through cooperation with PR.

Another possibility is that heat shock and related stresses may stimulate phosphorylation pathways that affect PR or PR-mediated processes. In support of this idea, we have demonstrated in a separate study that cellular modulators of protein kinases and phosphatases in T47D cells enhance PR-mediated target gene transcription in a manner very reminiscent of the present results with heat shock and sodium arsenite (Beck et al., 1992). Additionally, heat shock and related stresses are known to stimulate phosphorylation of certain specific protein substrates [see Legagneuz et al. (1990) and references cited therein] including the heat shock transcription factor (Mosser et al., 1990; Larson et al., 1988; Hensold et al., 1990; Price & Calderwood, 1991). Also, two specific protein kinases have been shown to be stimulated by heat shock (Legagneux et al., 1990), and a rapid increase in intracellular cAMP levels occurs shortly after stress (Kiang et al., 1991).

Several recent studies have demonstrated "cross talk" between steroid-responsive pathways and other signal transduction pathways that were not previously thought to be linked. Examples include the reciprocal transcriptional interference that occurs between AP-1 activity and glucocorticoid receptor (Yang et al., 1990; Schule et al., 1990) and AP-1 repression of estrogen receptor activity (Doucas et al., 1991). It has also been shown that several steroid receptors and an orphan receptor can be activated in a ligand-independent manner by dopamine receptor coupled phosphorylation pathways (Power et al., 1991a,b). The results of the present study showing a stimulatory effect of stress on steroid responsiveness may represent another previously unrecognized coordinate regulation between two different pathways. The data in Figure 7 showing that enhanced steroid responsiveness is a short-lived effect that correlates with the time of maximal induction of hsp 70 synthesis provides a particularly strong indication for an interrelatedness between stress-responsive and steroid-responsive pathways. Stress proteins have clearly been shown to be involved in cellular protection after stress. It is possible that in certain cells or tissues common pathways have evolved for the cell to respond both to environmental stress and to stimulation by steroid hormones. The two pathways working in concert to reestablish cellular homeostasis after stress may offer a physiological advantage to the cell. These are potentially interesting new questions that will require further investigation.

ACKNOWLEDGMENTS

We thank Drs. David O. Toft (Mayo Clinic) and Nancy L. Weigel (Baylor College of Medicine) for critical reading of the manuscript and other helpful discussions. We also thank Nancy Hart and Clairene Mraz for their assistance with the manuscript preparation.

Registry No. $AsHO_2$·Na, 7784-46-5; progesterone, 57-83-0.

REFERENCES

Beck, C. A., Weigel, N. L., & Edwards, D. P. (1992) *Mol. Endocrinol.* (in press).

Beckmann, R. P., Mizzen, L. A., & Welch, W. J. (1990) *Science 248*, 850–854.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Bresnick, E. H., Dalman, F. C., Sanchez, E. R., & Pratt, W. B. (1989) *J. Biol. Chem. 264*, 4992–4997.

Cadepond, F., Schweizer-Groyer, G., Segard-Maurel, I., Jibard, N., Hollenberg, S. M., Giguere, V., Evans, R. M., & Baulieu, E.-E. (1991) *J. Biol. Chem. 266*, 5834–5841.

Dalman, F. C., Bresnick, E. H., Patel, P. D., Perdew, G. H., Watson, S. J., & Pratt, W. B. (1989) *J. Biol. Chem. 264*, 19815–19821.

Dalman, F. C., Koenig, R. J., Perdew, G. H., Massa, E., & Pratt, W. B. (1990) *J. Biol. Chem. 265*, 3615–3618.

Dalman, F. C., Stinzenbecker, L. J., Levin, A. A., Lucas, D. A., Perdew, G. H., Petkovitch, M., Chambon, P., Grippo, J. F., & Pratt, W. B. (1991) *Biochemistry 30*, 5605–5608.

DeMarzo, A. M., Beck, C. A., Oñate, S. A., & Edwards, D. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 72–76.

Doucas, V., Spyrou, G., & Yan, V. M. (1991) *EMBO J. 10*, 2237–2245.

Edwards, D. P., Estes, P. A., El-Ashry, D., Suba, E., & Lawler-Heavner, J. (1987) in *Immunological Approaches to the Diagnosis and Therapy of Breast Cancer*, pp 157–168, Plenum Publishing Corp., New York.

Edwards, D. P., DeMarzo, A. M., Oñate, S. A., Beck, C. A., Estes, P. A., & Nordeen, S. K. (1991) *Steroids 56*, 271–278.

El-Ashry, D., Oñate, S. A., Nordeen, S. K., & Edwards, D. P. (1989) *Mol. Endocrinol. 3*, 1545–1558.

Elashry-Stowers, D., Zava, D. T., Speers, W. C., & Edwards, D. P. (1988) *Cancer Res. 48*, 6462–6474.

Estes, P. A., Suba, R. J., Lawler-Heavner, J., Elashry-Stowers, D., Wei, L. L., Toft, D. O., Sullivan, W. P., Horwitz, K. B., & Edwards, D. P. (1987) *Biochemistry 26*, 6250–6262.

Evans, R. M. (1988) *Science 240*, 889–895.
Gething, M. J., McCannon, K., & Sambrook, J. (1986) *Cell 46*, 939–950.
Hensold, J. O., Hunt, C. R., Calderwood, S. K., Housman, D. E., & Kingston, R. E. (1990) *Mol. Cell. Biol. 10*, 1600–1608.
Horwitz, K. B., Francis, M. D., & Wei, L. L. (1985) *DNA 4*, 451–460.
Hunt, C., & Morimoto, R. I. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6455–6459.
Kang, P. J., Ostermann, J., Shilling, J., Neupert, W., Craig, E. A., & Pfanner, N. (1990) *Nature 348*, 137–143.
Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., & Chambon, P. (1990) *EMBO J. 9*, 1603–1614.
Kiang, J. G., Wu, Y. Y., & Lin, M. C. (1991) *Biochem. J. 276*, 683–689.
Kost, S. L., Smith, D. F., Sullivan, W. P., Welch, W. J., & Toft, D. O. (1989) *Mol. Cell. Biol. 9*, 3829–3838.
Larson, J. S., Schuetz, T. J., & Kingston, R. E. (1988) *Nature 335*, 372–375.
Legagneux, V., Morange, M., & Bensaude, O. (1990) *Eur. J. Biochem. 193*, 121–126.
Lindquist, S., & Craig, E. A. (1988) *Annu. Rev. Genet. 22*, 631–677.
Lis, J. T., Xia, H., & Perisic, O. (1990) in *Stress Proteins in Biology and Medicine* (Morimoto, R., Ed.) pp 411–428, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mendel, D. B., & Orti, E. (1988) *J. Biol. Chem. 263*, 6695–6702.
Mendel, D. B., Bodwell, J. E., Gametchu, B., Harrison, R. W., & Munck, A. (1986) *J. Biol. Chem. 261*, 3758–3763.
Miyata, Y., & Yahara, I. (1991) *J. Biol. Chem. 266*, 8779–8783.
Mosser, D. D., Kotzbauer, P. T., Sarge, K. D., & Morimoto, R. I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3748–3752.
Nemoto, T., Mason, G. G. F., Wilhelmisson, A., Cuthill, S., Happgoal, J., Gustafsson, J.-A., & Poellinger, L. (1990) *J. Biol. Chem. 263*, 2269–2277.
Nguyen, V. T., Morange, M., & Bensuade, O. (1989) *J. Biol. Chem. 264*, 10487–10492.
Nordeen, S. K., Green, P. P., III, & Fowlkes, D. M. (1987) *DNA 6*, 173–178.
Nordeen, S. K., Kühnel, B., Lawler-Heavner, J., Barber, D. A., & Edwards, D. P. (1989) *Mol. Endocrinol. 3*, 1270–1278.
Oñate, S. A., Estes, P. A., Welch, W. J., Nordeen, S. K., & Edwards, D. P. (1991) *Mol. Endocrinol. 5*, 1993–2004.
Ostermann, J., Horwich, A. L., Neupert, W., & Ulrich Hartl, F. (1989) *Nature 341*, 125–130.
Power, R., Lydon, J. P., Coneelly, O. M., & O'Malley, B. W. (1991a) *Science 252*, 1546–1548.
Power, R. F., Mani, S. K., Codina, J., Coneelly, O. M., & O'Malley, B. W. (1991b) *Science 254*, 1636–1639.
Pratt, E. B. (1990) *Mol. Cell. Endocrinol. 74*, C69–C76.
Price, B. D., & Calderwood, S. K. (1991) *Mol. Cell. Biol. 11*, 3365–3368.
Renoir, M. J., Radanyi, C., Faber, L. E., & Baulieu, E. E. (1990a) *J. Biol. Chem. 265*, 10740–10745.
Renoir, M. J., Radanyi, C., Jung-Testas, I., Faber, L. E., & Baulieu, E. E. (1990b) *J. Biol. Chem. 265*, 14402–14406.
Riabowol, K. T., Mizzen, L. A., & Welch, W. J. (1988) *Science 242*, 433–436.
Riehl, R. M. (1988) *Exp. Cell Res. 179*, 462–476.
Riehl, R. M., Sullivan, W. P., Vronman, B. T., Bauer, W. J., Pearson, G., & Toft, D. O. (1985) *Biochemistry 24*, 6586–6591.
Rothman, J. E. (1989) *Cell 59*, 591–601.
Sanchez, E. R. (1990) *J. Biol. Chem. 265*, 22067–22070.
Sanchez, E. R., Meshinchi, S., Tienrugaroj, W., Schlesinger, M. J., & Toft, D. O. (1987) *J. Biol. Chem. 262*, 6986–6991.
Sanchez, E. R., Hirst, M., Scherrer, L. C., Tang, H. I., Welsh, M. J., Harmon, J. M., Simmons, S. S., Ringold, G. M., & Pratt, W. B. (1990a) *J. Biol. Chem. 265*, 20123–20130.
Sanchez, E. R., Faber, L. E., Henzel, W. J., & Pratt, W. B. (1990b) *Biochemistry 29*, 5145–5152.
Schlesinger, M. J. (1990) *J. Biol. Chem. 265*, 12111–12114.
Schüle, R., Rangarajan, P., Kliewer, S., Ransone, L. J., Bolado, J., Yang, N., Verma, I. M., & Evans, R. M. (1990) *Cell 62*, 1217–1226.
Sheridan, P. L., Krett, N. L., Gordon, J. A., & Horwitz, K. B. (1988) *Mol. Endocrinol. 2*, 1329–1342.
Smith, D. F., Faber, L. E., & Toft, D. O. (1990) *J. Biol. Chem. 265*, 3996–4003.
Tai, P. K., Maeda, Y., Nakao, K., Wakin, N. J., Duhring, J. L., & Faber, L. E. (1986) *Biochemistry 25*, 5269–5275.
Vedeckis, W. V., Ali, M., & Allen, H. R. (1989) *Cancer Res. 49*, 2295s–2302s.
Welch, W. J. (1985) *J. Biol. Chem. 260*, 3058–3065.
Welch, W. J. (1990) in *Stress Proteins in Biology & Medicine* (Morimoto, R., & Georgopoulos, C., Eds.) pp 238–278, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Wolffe, A. P., Perlman, A. J., & Tata, J. R. (1984) *EMBO J. 3*, 2763–2770.
Wu, C., Zimarino, V., Tsai, C., Walker, B., & Wilson, S. (1990) in *Stress Proteins in Biology & Medicine* (Morimoto, R., Tissières, A., & Georgopoulos, C., Eds.) pp 429–442, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Yang-Yen, H.-F., Chambard, J.-C., Sun, Y.-L., Smead, T., Schmidt, T. J., Drovin, J., & Karin, M. (1990) *Cell 62*, 1205–1215.