

## Heat Shock Induces Translocation to the Nucleus of the Unliganded Glucocorticoid Receptor\*

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There have been many reports demonstrating the specific association of several heat shock proteins with unliganded steroid hormone receptors. However, little evidence to date has been proposed to link steroid receptor action with the heat shock response in cells. In this paper, we demonstrate the effect of heat and chemical stress on glucocorticoid receptor subcellular localization in mouse L929 cells and in a stably transfected Chinese hamster ovary cell line (WCL2) which over-expresses the mouse glucocorticoid receptor. When WCL2 cells are exposed to 43 °C, there is a time-dependent decrease in glucocorticoid receptor hormone-binding capacity in the cytosolic fraction of these cells that correlates with a decrease in amount of glucocorticoid receptor protein. Analysis of both cytosolic and nuclear fractions for glucocorticoid receptor protein via quantitative Western blotting reveals that the unliganded glucocorticoid receptor of non-shocked L929 and WCL2 cells is localized primarily in the cytosolic fraction, whereas unliganded receptor of heat-shocked cells is found almost exclusively in the nuclear fraction. A similar shift to nuclear localization for unliganded glucocorticoid receptor is noted in L929 and WCL2 cells subjected to chemical shock (sodium arsenite). As treatment of these cells with glucocorticoid hormone also results in glucocorticoid receptor that is tightly bound within the nuclear fraction, it is speculated that heat and chemical stress provide a hormone-independent mechanism by which glucocorticoid receptor is transformed to the high affinity nuclear-binding state characteristic of the hormone-bound, transcriptionally active receptor.

The works of several laboratories have demonstrated that unliganded glucocorticoid receptors (GR)<sup>1</sup> reside within the cytosolic fraction of cells as complexes containing the 90-kDa heat shock protein, hsp90 (1-4). Studies within intact cells

have shown that the binding of hormone and incubation at 37 °C results in dissociation of hsp90 from the receptor complex (3, 4), and in translocation of the liganded receptor to the nucleus with simultaneous acquisition of transcription activity (5, 6). More recently, it has been reported that progesterone and glucocorticoid receptor complexes also contain the 70-kDa heat shock protein, hsp70 (7-9), as well as a newly described 56-kDa heat shock protein, hsp56 (10). This association of several heat shock proteins with steroid receptor complexes suggests a more than coincidental relationship. Yet evidence for a functional relationship between steroid receptor action and the cellular heat shock response remains lacking. The only exceptions have been one report demonstrating that glucocorticoids will increase the rate at which cells become thermotolerant to heat stress (11) and another which showed that ATP depletion of cells via dinitrophenol (DNP) treatment will cause nuclear binding of the hormone-free glucocorticoid receptor (12). As it has been known for many years that DNP can act to stimulate the cellular stress response (13), we have speculated that cellular stress could result in nuclear localization of hormone-free glucocorticoid receptors. In this paper, we report that heat shock (43 °C) and chemical stress (sodium arsenite) will cause the unliganded GR of intact L929 and WCL2 cells to become tightly bound within the nuclear fraction. The extent of stress-mediated conversion of the unliganded GR to tight nuclear binding approximates that observed in non-stressed, dexamethasone-treated cells, suggesting that heat shock may result in a hormone-independent localization of the GR to its high affinity, transcriptionally active sites within the nucleus.

### EXPERIMENTAL PROCEDURES

#### Materials

[<sup>35</sup>S]Methionine at 1133 Ci/mmol (Trans-label) and <sup>125</sup>I-conjugated goat anti-mouse IgG (11.8 μCi/μg) were obtained from ICN Radiochemicals. Autoradiography enhancer (Enlightening) and [<sup>3</sup>H] triamcinolone acetonide (42.8 Ci/mmol) were obtained from Du Pont-New England Nuclear. Sodium arsenite, acrylamide, TES, SDS, Hepes, Triton X-100, protein A-Sepharose, horseradish peroxidase-conjugated rabbit anti-goat IgG, iron-supplemented newborn calf serum, and DMEM powdered medium were obtained from Sigma. Immobilon P membranes were obtained from Millipore Corp. The BuGR2 anti-glucocorticoid receptor monoclonal antibody (hybridoma fluid) was a gift from Drs. William Hendry and Robert Harrison (University of Arkansas).

#### Methods

**Cell Culture and Stress Treatment**—The WCL2 cells used in this study are a clone of Chinese hamster ovary cells which stably express the mouse GR cDNA (14). These cells overproduce GR relative to nontransfected cell lines and the GR expressed exhibits full steroid binding and transcription initiation activities (14). The L929 and WCL2 cells were grown in DMEM medium containing 10% iron-supplemented newborn calf serum, and all stress experiments were performed on cells that had just reached confluence. Maintenance and nonstressed cell lines were incubated in a humid 37 °C chamber containing 5% CO<sub>2</sub>. Heat shock treatment for each cell line was achieved by shifting replica flasks to a second 5% CO<sub>2</sub> incubator set at 43 °C. Typical duration of heat shock treatment was 2 h. WCL2 cells were also stressed by addition of sodium arsenite to the medium at a final concentration of 200 μM. Both the arsenite-treated and nontreated cells were incubated at 37 °C for 2 h.

**Cellular Fractionation and Immunoabsorption**—Cellular fractionation of the L929 and WCL2 cells and immune purification of

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<sup>1</sup> The abbreviations and trivial names used are: GR, glucocorticoid receptor; DNP, dinitrophenol; hsp, heat shock protein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; SDS, sodium dodecyl sulfate; triamcinolone acetonide, 9α-fluoro-11β, 16α,17α,21-tetrahydroxy-pregna-1,4-diene-3,20-dione 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-pregna-1,4-diene-3,20-dione; DMEM, Dulbecco's modified Eagle's medium.

glucocorticoid receptor were performed according to a modified procedure which generates soluble cytosolic and nuclear fractions (12). Briefly, L929 cells growing in large 175-cm<sup>2</sup> flasks were individually scraped and washed in ice-cold Hank's buffered saline solution, followed by Dounce homogenization in hypotonic buffer (10 mM Hepes, pH 7.4, at 0 °C, 1 mM EDTA, 10 mM sodium molybdate). In contrast, WCL2 cells were grown in smaller 75-cm<sup>2</sup> flasks and similarly processed. After centrifugation at 12,000 × *g* for 5 min, the cytosolic fraction was saved and the nuclear pellet was washed with hypotonic buffer one additional time and resuspended in the same buffer. Each fraction was then solubilized and denatured by addition of SDS (final concentration = 2.5%) and heating at 95 °C. After cooling and addition of Triton X-100 (final concentration = 2.5%), 20 μl of BuGR2 anti-receptor monoclonal antibody (hybridoma fluid) was added. Each sample was then adsorbed in batch to protein A-Sepharose, washed, and extracted with SDS sample buffer.

**Gel Electrophoresis and Quantitative Western Blotting**—Samples were resolved by electrophoresis in 7% polyacrylamide-SDS gels as described by Laemmli (15). The relative amounts of glucocorticoid receptor protein in the cytosolic and nuclear fractions were determined via a quantitative Western blotting technique previously described (16), which employs the BuGR2 anti-glucocorticoid receptor monoclonal antibody and both peroxidase- and <sup>125</sup>I-conjugated counter antibodies. After Western blotting and autoradiography, the peroxidase-stained receptor bands were excised and <sup>125</sup>I cpm were determined via liquid scintillation spectrophotometry. Receptor-specific cpm values were derived by subtracting the cpm value measured in a background slice of comparable area. The relative amounts of receptor protein reported in Table I were obtained by dividing the receptor cpm values of each fraction by the cpm value of control, cytosolic receptor. This normalization was done only within experiments, and thus allowed for the calculation of means and standard errors between experiments.

**[<sup>35</sup>S]Methionine Labeling and Steroid Binding**—In the experiment of Fig. 1, WCL2 cells were subjected to a time course of heat shock at 43 °C. Immediately following the stress treatment, the medium was removed and replaced with methionine-free DMEM containing 5% dialyzed calf serum and [<sup>35</sup>S]methionine at a final concentration of 9 μCi/ml. Labeling was then performed for 45 min at 37 °C (zero time control) or 43 °C (all other time points). In parallel experiments, WCL2 cells were again subjected to a time course of heat shock, but, instead of labeling with [<sup>35</sup>S]methionine, cytosols were prepared as described above. To determine specific steroid binding capacity, replicate aliquots (45 μl) of 12,000 × *g* cytosols were incubated with 50 nM [<sup>3</sup>H]triamcinolone acetonide (42.8 Ci/mmol) in the presence or absence of a 1000-fold excess of unlabeled dexamethasone as previously described (17). Prior to normalization relative to the zero time control, all hormone binding capacities were calculated as specific cpm per mg of cytosol protein. To determine the relative amounts of GR protein present at each time point, equal amounts (200 μg) of control and heat-shocked cytosols were run in a polyacrylamide gel and subjected to quantitative Western blotting as described above.

## RESULTS AND DISCUSSION

When WCL2 cells are subjected to a time course of heat shock at 43 °C, there is a rapid decline in the overall level of protein synthesis within the first half hour (Fig. 1, *inset*). This is followed by increases in the rates of synthesis of the major heat shock proteins: hsp110, hsp100, hsp90, hsp70, and hsp25. The synthesis rates for all of these heat shock proteins continue to increase even after 4 h of treatment. In contrast, glucocorticoid receptor steroid binding capacity is rapidly lost in response to heat shock (Fig. 1), such that only 13% of the initial steroid binding activity remains after 4 h. The loss of steroid binding correlates with a heat shock-induced decrease in the amount of glucocorticoid receptor protein present in the cytosolic fraction of these cells (Fig. 1). This loss of glucocorticoid receptor protein from the cytosols of heat-shocked cells could be due to: 1) depression in the rate of receptor translation combined with an increased rate of receptor protein degradation, as has been noted for the majority of proteins in heat-shocked cells (18); or 2) movement of the receptor from the cytosolic compartment to the nucleus.

To test the latter hypothesis, the intracellular locations of

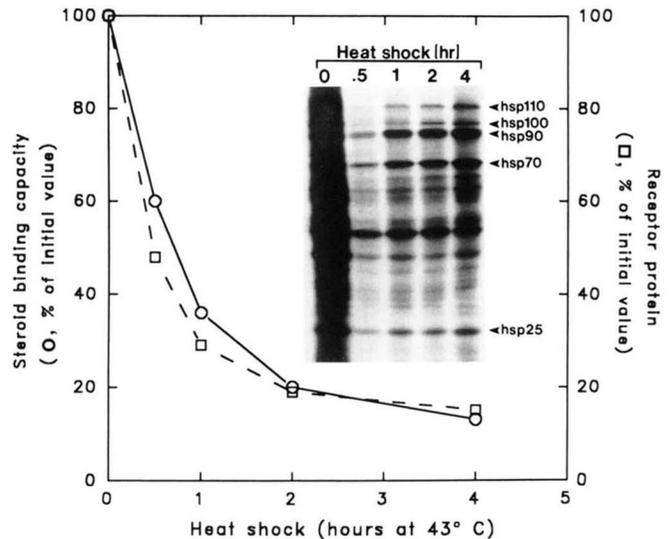


FIG. 1. Time course of heat shock-induced decreases in glucocorticoid-binding capacity and glucocorticoid receptor protein in WCL2 cell cytosols. WCL2 cells, maintained in culture as previously described (9), were incubated in a humid 5% CO<sub>2</sub> atmosphere set at 43 °C. At intervals of 0, 0.5, 1, 2, and 4 h, individual flasks were removed and either subjected to *in vivo* pulse-labeling with [<sup>35</sup>S]methionine (*inset*) or subjected to fractionation, followed by measurements within the cytosolic fraction of glucocorticoid hormone binding capacity and glucocorticoid receptor protein as described under "Experimental Procedures." All data points within the graphs represent the mean values obtained from two separate experiments. The major induced heat shock proteins (*hsp*) are indicated by arrows in an autoradiogram of a polyacrylamide gel (*inset*) in which equal amounts (100 μg) of <sup>35</sup>S-labeled cytosols were loaded.

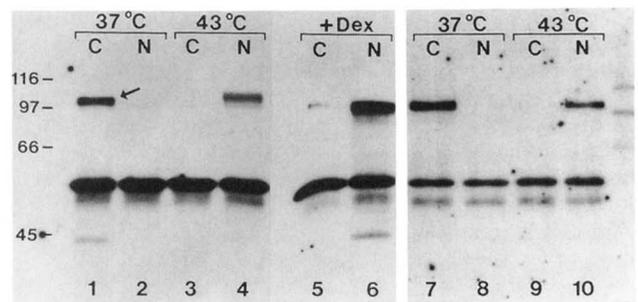


FIG. 2. Heat shock treatment of L929 and WCL2 cells induces nuclear binding of unliganded glucocorticoid receptors. L929 cells (lanes 1–4) and WCL2 cells (lanes 7–10) were incubated at 37 or 43 °C for 2 h. In a separate experiment, L929 cells were incubated with 1 μM dexamethasone (lanes 5 and 6) for 2 h at 37 °C. Cytosolic (C) and nuclear (N) fractions of these fractions were prepared, and glucocorticoid receptors (arrow) from each of these fractions were immunopurified and Western-blotted with BuGR2 anti-receptor antibody (29) serving as both purification and probe antibody. The figure represents autoradiograms made from each Western blot in which <sup>125</sup>I-conjugated counter antibody was employed. The sizes (*M<sub>r</sub>* × 10<sup>-3</sup>) of molecular mass standards are shown. The immune reactive bands seen in each lane and migrating between the 66- and 45-kDa standards correspond to the heavy chains of the BuGR2 antibody used during immune purification.

glucocorticoid receptors in heat-shocked L929 and WCL2 cells were determined, utilizing a cell-rupturing procedure which generates soluble cytosolic and nuclear fractions (12). Glucocorticoid receptors present in each of these fractions were immune-purified with BuGR2 anti-glucocorticoid receptor monoclonal antibody and analyzed by quantitative Western blotting (16). Autoradiograms of the Western blots are seen in Fig. 2, while quantitation of the amounts of receptor

TABLE I

Quantitation of receptor protein in the cytosolic and nuclear fractions of heat- and chemically stressed L929 and WCL2 cells

The Western blot experiments of Figs. 2 and 3, as well as those of several replicate experiments, employed both  $^{125}\text{I}$ - and peroxidase-conjugated counter antibodies as signals for the reaction of the BuGR2 anti-receptor antibody with receptor protein. The peroxidase-stained receptor bands of each blot were excised and subjected to liquid scintillation spectroscopy. Receptor specific cpm values were obtained by subtracting the cpm of a background slice (no discernible protein band) of equal surface area from the cpm of each receptor slice. Dex, dexamethasone.

Cell line	Treatment	Relative amount of receptor protein by $^{125}\text{I}$ Western blotting <sup>a</sup>	
		Cytosol	Nuclear pellet
L929	37 °C	1.00	0.26 ± 0.10
L929	43 °C	0.16 ± 0.06	0.95 ± 0.16
L929	-Arsenite	1.00	0.06
L929	200 μM	0.11 ± 0.02	0.64 ± 0.21
L929	-Dex	1.00	0.07
L929	+Dex	0.16 ± 0.02	1.02 ± 0.25
WCL2	37 °C	1.00	0.15 ± 0.04
WCL2	43 °C	0.11 ± 0.02	0.72 ± 0.21
WCL2	-Arsenite	1.00	0.06 ± 0.02
WCL2	200 μM	0.13 ± 0.01	0.85 ± 0.26
WCL2	-Dex	1.00	0.12 ± 0.06
WCL2	+Dex	0.53 ± 0.16	0.61 ± 0.20

<sup>a</sup> The amount of receptor-specific radioactivity measured in each fraction was normalized relative to receptor-specific radioactivity found in the cytosol fraction of control cells (37 °C, -arsenite, or -Dex). Values expressed are the means ± standard errors of three to seven separate determinations.

protein in each fraction is presented in Table I. When L929 cells were maintained at 37 °C (Fig. 2), the majority of the receptor (see Table I) was found in the cytosolic fraction (compare lanes 1 and 2). In contrast, heat shock treatment (2 h at 43 °C) resulted in receptor that is primarily localized within the nuclear fraction (lanes 3 and 4). The amount of receptor bound to the nuclear pellet in response to heat shock (95%) closely approximates the percentage of receptor (102%) moved into the nucleus in response to dexamethasone hormone (lanes 5 and 6). Similar results were obtained with WCL2 cells subjected to heat shock (Fig. 2). Glucocorticoid receptors of WCL2 cells maintained at 37 °C (lanes 7 and 8) were found predominantly within the cytosolic fraction, while receptors of cells grown at 43 °C (lanes 9 and 10) showed a marked shift to the nuclear fraction (72%). It was interesting to note that incubation of WCL2 cells with hormone resulted in only 61% recovery of the GR within the nuclear fraction (Table I). This percentage was highly consistent and was unaffected by culture conditions, hormone concentration (0.1, 1, and 10 μM) or time of incubation with hormone (data not shown). The authors who developed this cell line and other lines expressing variable amounts of GR have demonstrated that saturation of hormone-inducible transcription activity does not occur with increasing levels of expressed receptor (14). However, it should be noted that the assay they employed for transcription activity involved transfection with the pMMTVCAT reporter plasmid. By design, this procedure introduces additional high affinity acceptor sites into these cells. Thus, the possibility still remains that the "normal" Chinese hamster ovary cell genome contains a relatively limited number of nuclear acceptor sites which can become saturated when nontransfected WCL2 cells are subjected to hormone treatment. In any case, the data so far presented indicate that heat shock can cause a hormone-independent

shift of glucocorticoid receptor protein from the cytosolic to the nuclear fractions, regardless of whether the receptor is present endogenously (L929 cells) or as a result of gene transfer (WCL2 cells). In the case of the WCL2 cells, this shift in fractions may not mean that the GR shifts from the cytoplasm to the nucleus within the intact cell, because, in contrast to the GR of L929 cells, recent immunofluorescence studies of WCL2 cells suggest that the GR is already present within the nucleus even in the absence of hormone (9, 19). Instead, the shift of the WCL2 GR from the cytosolic to the nuclear fraction in response to heat shock or to hormone probably means that the GR is converted from a form with low affinity for nuclear sites to one of high affinity for nuclear sites, resulting in GR that is retained within the nuclear fraction upon cell rupture.

Although heat shock is the most commonly used form of stress in experimental systems, many others, such as chemical poisoning and heavy metal toxicity, will also cause induction of heat shock proteins and the stress response. In order to test if glucocorticoid receptor nuclear binding could also be induced by chemical shock, WCL2 cells were exposed to sodium arsenite (Fig. 3, Table I), which is known to produce a cellular stress response similar to that induced by thermal shock (20). As expected, glucocorticoid receptor protein in untreated cells (lanes 1 and 2) was largely confined to the cytosolic fraction, while treatment with 200 μM sodium arsenite (lanes 3 and 4) resulted in a shift of receptor to the nuclear fraction (85%). Similar results were obtained when L929 cells were treated with 200 μM sodium arsenite (see Table I). These observations suggest that glucocorticoid receptor nuclear binding may be inducible by any condition which can elicit the cellular stress response. With this in mind, it is possible that the previously reported ability of DNP to cause nuclear binding of the unliganded glucocorticoid receptor (12) may actually be due to stimulation of the stress response by DNP. Although it is thought that DNP acts to generate the nuclear-bound unliganded receptor via depletion of ATP stores, it is possible that ATP depletion is secondary to a direct effect of DNP on the stress response or that ATP depletion itself can elicit the same response. Given that DNP was the first agent other than heat to demonstrate a heat shock response in cells (13), this explanation seems plausible. Further evidence to

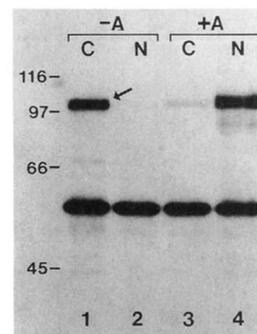


FIG. 3. Intracellular localization of unliganded glucocorticoid receptors in sodium arsenite-treated WCL2 cells. WCL2 cells were left untreated (-A, lanes 1 and 2), or were treated with 200 μM sodium arsenite (+A, lanes 3 and 4) for 2 h at 37 °C. The relative amounts of glucocorticoid receptor protein (arrow) in the cytosolic (C) and nuclear (N) fractions were resolved by immune purification, gel electrophoresis, and Western blotting as described under "Experimental Procedures." The figure represents an autoradiogram made from the Western blot in which  $^{125}\text{I}$ -conjugated counter antibody was employed. The molecular mass standards ( $M_r \times 10^{-3}$ ) are indicated. The immune reactive bands above the 45-kDa standard correspond to the heavy chains of the BuGR2 antibody.

support a common mechanism for nuclear localization of unliganded receptors in DNP-treated and heat-stressed cells can be seen in Table II. The data show that receptors localized to the nuclei of heat-shocked L929 and WCL2 cells have lost the ability to bind hormone, as is the case for the so-called "null receptor" which is found in the nuclei of ATP-depleted cells (12).

No evidence for stress-induced degradation fragments was noted on the blots or autoradiograms of Figs. 2 and 3. Yet, it is apparent from the results seen in Table I that both heat and chemical shock can result in a moderate loss of glucocorticoid receptor as a result of stress-induced proteolysis. For example, the amount of receptor protein recovered in the nuclear fraction of L929 cells treated with arsenite was approximately 64% of the receptor originally present in the unstressed cytosol. As the amount of missing receptor (36%) could not be accounted for by the amount found in the cytosols of arsenite-treated cells (11%), it is likely that at least a small portion (25%?) of the GR is degraded within the stressed cell. Similar numbers and conclusions can be drawn from the results of WCL2 cells subjected to heat shock. But in the case of L929 cells subjected to heat shock and WCL2 cells treated with arsenite, most of the receptor protein can be found in the nuclear fraction (95 and 85%, respectively), and that which is not can be accounted for in the cytosols of these cells. Although these numbers will certainly vary with other cell lines and treatments, it is safe to say that the majority of glucocorticoid receptor protein in the two cell lines employed in this study will escape stress-induced proteolysis and eventually become localized to the nucleus. Indeed, it is interesting to speculate that the very act of sequestration into the nucleus is what protects the receptor from stress-induced degradation.

Unliganded estrogen and progesterone receptors are known to be located in the nucleus of cells (21–24). But it is clear that most hormone-free glucocorticoid receptors are found in the cytoplasm (25–27). As already stated, one exception to this is the glucocorticoid receptor of the WCL2 cells used in this study, which is located in the nucleus in the absence of hormone when intact cells are examined by indirect immunofluorescence (9). However, like estrogen and progesterone receptors (21–24) and like the GR of L929 cells, unliganded WCL2 receptors are recovered in the cytosolic fraction upon cell rupture (9) (Figs. 2 and 3), indicating that the hormone-free receptors in these cells are loosely bound within the nuclear compartment. A recent analysis of WCL2 cells by

confocal microscopy has shown that GR does not appear to undergo a redistribution within the nucleus upon incubation of these cells with glucocorticoid hormone (19), suggesting that the unliganded GR of WCL2 cells is already present at its final nuclear destination and that the binding of hormone simply serves to increase the affinity of the GR for this site. With this in mind, it will eventually be interesting to determine if the GR of WCL2 cells is similarly not redistributed within the nucleus in response to heat shock. In the case of L929 cells, the results reported here suggest that heat shock may also cause the unliganded GR to actually translocate from the cytoplasm to the nucleus. As we and others have shown that dissociation of the GR-hsp90 complex *in vitro* results in acquisition of DNA-binding function by the unliganded GR (28), it is possible that heat shock-mediated translocation of the unliganded GR and its conversion to high affinity nuclear binding are the direct result of dissociation of the GR-hsp90 complex within the intact cell.

Although it is not currently known where the heat-shocked glucocorticoid receptor is bound within the nucleus, it is possible that a hormone-independent unmasking of the DNA-binding domain has occurred. If so, the high affinity nuclear binding noted in this study may result from binding of the unliganded glucocorticoid receptor to its specific chromosomal sites, suggesting the possibility that heat shock-mediated nuclear binding may ultimately result in hormone-independent induction of glucocorticoid receptor transcription activity.

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TABLE II

*Steroid binding capacities of cytosolic and nuclear glucocorticoid receptors from normal and heat shock treated L929 and WCL2 cells*

Specific steroid binding capacities of receptors present in cytosols and nuclear pellets derived from control and stressed cells were determined by incubating replicate aliquots of cytosol or replicate suspensions of nuclear pellet with 50 nM [<sup>3</sup>H]triamcinolone acetonide in the presence and absence of a 1000-fold excess of nonradioactive dexamethasone. Receptor specific cpm values in each entire fraction were then calculated.

Cell line	Temperature	Fraction	Specific binding capacity
	°C		cpm/fraction
L929	37	Cytosol	107,810
L929	37	Nuclear pellet	40,240
L929	43	Cytosol	10,830
L929	43	Nuclear pellet	4,760
WCL2	37	Cytosol	381,810
WCL2	37	Nuclear pellet	25,930
WCL2	43	Cytosol	64,330
WCL2	43	Nuclear pellet	0