Pergamon

0960-0760(94)00138-3

J. Steroid Biochem Molec Biol. Vol. 51, No. 5/6, pp. 291–298, 1994

0960-0760/94 $7.00 + 0.00

Nanomolar Concentrations of Untransformed Glucocorticoid Receptor in Nuclei of Intact Cells

Gian Paolo Rossini* and Claudia Malaguti

Dipartimento di Scienze Biomediche, Università di Modena, Via Campi 287, 41100 Modena, Italy

The subcellular distribution of untransformed glucocorticoid–receptor complex *in vivo* has been studied by chemical crosslinking of intact cells, and using a procedure adequate for correction of experimental errors due to redistribution of components between cytosolic and nuclear fractions. We found that in HeLa S_3 cells 85.4% of total glucocorticoid–receptor complexes are located in nuclei, and 14.6% are cytosolic. If measurements were performed with MCF-7 cells, we determined that the nuclear pool of glucocorticoid–receptor complexes accounts for 75.2% of the total cellular content, whereas the remaining 24.8% are cytosolic. When the subcellular distribution of estrogen–receptor complexes was determined, instead, we found that they are almost exclusively located in nuclei of MCF-7 cells, which contain 88.9% of the total. In order to estimate the molar concentration of receptors in cytosol and nuclei of intact cells, we determined the free water content of the two compartments. The volume of solvent was found to vary in the three cell lines we have studied, and our data showed that these variations are due to the cytosolic fractions, as the free water content of nuclei is essentially the same in those cells. When the free water content and the levels of glucocorticoid–receptor complexes we have measured were used to estimate the molar concentrations of receptors, we found that these range between 0.4 and 18.9 nM in cytosols, and between 3.9 and 6.3 nM in nuclei of the three cell lines we have studied. We then concluded that the relative distribution of untransformed glucocorticoid–receptor complexes between cytosol and nuclei is cell-specific but their molar concentration in the nuclear compartment does not greatly vary among different cells.

J. Steroid Biochem. Molec. Biol., Vol. 51, No. 5/6, pp. 291–298 1994

INTRODUCTION

The cell nucleus is the compartment where steroid hormones exert their transcriptional effects through hormone–receptor complex binding to DNA [1]. The tight binding of steroid receptors to nuclear acceptor sites has been shown to occur after formation of the hormone–receptor complex at physiological temperatures in intact cells [1–4]. In the absence of the ligand and/or under low temperature [1, 4], steroid receptors are in a functional state termed untransformed, and original studies have shown that these forms are found in cytosolic extracts prepared from target tissues [2, 3], leading to the early proposal that in intact cells untransformed receptors and steroid–receptor complexes are cytosolic, and translocate into the nucleus after they are transformed by a temperature-dependent step to forms displaying high affinity for DNA [2–4].

This concept has been subjected to revision after it has been shown, by immunocytochemical [5] and enucleation [6] studies, that hormone-free estrogen receptors are mostly located at the nuclear level in intact cells. The detection of cytosolic receptors has then been interpreted as a leakage of soluble or loosely bound nuclear receptors upon cell disruption [6, 7].

These conclusions have stimulated the investigation on subcellular location of receptors for other steroid hormones, and it is now accepted that estrogen [5–8], progesterone [8–11], and androgen [12] receptors are mainly located inside nuclei. The subcellular distribution of untransformed glucocorticoid receptors has however remained controversial, as some studies show

*Correspondence to G. P. Rossini.
Received 9 Jun. 1994; accepted 8 Aug. 1994.

their presence at the nuclear level [8, 13–17], while others support a predominant cytoplasmic location [18–20].

In previous studies aimed at the characterization of the structure–function relationships of glucocorticoid–receptor complexes, we obtained data supporting the existence of untransformed forms in the nuclei of intact cells [13, 14]. This conclusion was obtained through immobilization of glucocorticoid receptors to associated components *in vivo*, upon treatment of intact cells with chemical crosslinkers. A precise assessment of cytosolic and nuclear levels of untransformed glucocorticoid–receptor complexes, however, could not be performed in those studies, due to redistribution of components between subcellular fractions upon chemical crosslinking of intact cells [13, 14].

This methodological constraint has recently been by-passed through a procedure which allows the correction of errors inherent in experimental data obtained by crosslinking, and we could establish that untransformed glucocorticoid–receptor complexes are distributed between cytosol and nuclei of intact HTC cells in a ratio which is about 2:1 [20].

In this study, we have expanded our analysis to HeLa S_3 and MCF-7 cells, and show that the relative distribution of untransformed glucocorticoid–receptor complexes between cytosol and nuclei varies, but the molar concentrations of nuclear forms is essentially the same in different cells.

EXPERIMENTAL

Materials

[2,4,6,7-^{3}H (*N*)] 17β-estradiol (104.4 Ci/mmol) and [6,7-^{3}H (*N*)]-dexamethasone (44.7 Ci/mmol) were purchased from New England Nuclear. [2-^{3}H] glycerol was purchased from Amersham. All other reagents were of analytical grade.

Cell culture conditions

HeLa S_3 cells were grown in suspension culture as described previously [13]. HTC cells were grown in 5% carbon dioxide in air at 37°C, in Petri dishes, as described previously [20]. MCF-7 cells were grown in 5% carbon dioxide in air at 37°C, in Petri dishes, with a culture medium composed of Dulbecco's modified Eagle medium containing antibiotics (100 U/ml penicillin, and 100 μg/ml streptomycin), 1% nonessential amino acids, 6 ng/ml insulin, and 10% foetal calf serum. Four days before the experiment, MCF-7 cells were seeded in the culture medium lacking phenol red, and containing 10% charcoal-stripped [21] foetal calf serum.

HeLa cells were harvested by low speed centrifugation of suspensions for 8 min at 600*g*, while MCF-7 and HTC cells were harvested by treatment for 5 min at room temperature with 0.25% trypsin, 5 mM EDTA in phosphate-buffered saline buffer (PBS; 20 mM phosphate buffer, pH 7.4, 0.15 M NaCl), and were recovered by low speed centrifugation.

Formation of radioactive steroid–receptor complexes in whole cells

Cells were brought to 2°C, were recovered by low speed centrifugation, washed by suspension in PBS buffer and low speed centrifugation, and then dispersed in PBS buffer.

To study glucocorticoid receptors, cells were incubated for 2 h at 2°C with 50 nM tritiated dexamethasone, and in the presence or absence of a 200-fold molar excess of nonradioactive dexamethasone. To study estrogen receptors, cells were incubated for 2 h at 2°C with 5 nM tritiated 17β-estradiol, and in the presence or absence of a 200-fold molar excess of nonradioactive 17β-estradiol. At the end of the incubations, cells were washed once with PBS buffer, and were used for the preparation of cell extracts, after they had, or had not, been subjected to crosslinking.

Crosslinking of intact cells

Cell suspensions in PBS buffer were subjected to crosslinking by incubation for 90 min at 2°C with 0.0125% (w/v) glutaraldehyde, as described previously [14, 20]. Crosslinked cells were washed once with PBS buffer before being used for the preparation of cell extracts.

Preparation of cell extracts

Cells were disrupted by suspension in 0.8 ml of 20 mM Tris–HCl, pH 7.5 at 2°C, 1.5 mM $MgCl_2$, 10 mM NaCl (RSB buffer), containing 3% (w/v) digitonin, and incubation for 30 min at 2°C with occasional vortexing. The homogenate was centrifuged for 10 min at 1000*g* to obtain a crude nuclear precipitate and a supernatant. The nuclear precipitate was washed once by resuspension with 0.5 ml of RSB buffer and centrifugation for 10 min at 1000*g*. The precipitate was dispersed with 1 ml of RSB buffer, to yield the nuclear fraction. The supernatants of the two centrifugations were combined, and centrifuged for 1 h at 105,000*g*, to obtain the supernatant, which represented the cytosolic fraction.

Determination of glucocorticoid– and estrogen–receptor complexes in cell extracts

Cytosolic binding was determined by the radioactivity detected after extracts were treated with a dextran-coated charcoal pellet, as described previously [13].

To determine nuclear binding, nuclear suspensions were centrifuged for 10 min at 1000*g*, to obtain a supernatant and a precipitate, which was washed once with 0.4 ml of RSB buffer. The supernatants obtained in these two washes were combined, treated with a dextran-coated charcoal pellet, and used to resuspend

their own nuclear precipitate. Aliquots of these samples were then taken for determinations of radioactivity.

Specific binding was calculated by subtraction of unspecific binding (+competitor) from total binding (−competitor). The specific binding determined in cytosolic extracts was added to that measured in the nuclear fraction, to obtain the total cellular content of steroid–receptor complexes.

Determination of the free water content in whole cells and in subcellular fractions

The determination of the free water content in whole cells and subcellular fractions was based on the procedure of Horowitz and Fenichel [22].

The determination of the free water content in whole cells was performed by incubating cell suspensions in PBS buffer for 8 h at 2°C with 1 μCi of tritiated glycerol. At the end of the incubations, aliquots of the samples were centrifuged for 5 min at 800*g*. The supernatant was recovered, and the precipitate was resuspended with 150 μl of distilled water. The suspension thus obtained, and aliquots of the supernatants (20 μl) were used for determinations of radioactivity.

To determine the free water content of the nuclear and particulate fractions, cells were harvested, dispersed in 2 ml of RSB buffer, and broken with a Dounce homogenizer, after 30 min swelling at 2%C. The homogenate was then centrifuged for 10 min at 1000*g*, to obtain a nuclear precipitate and a supernatant. The nuclear precipitate was then dispersed with 1.5 ml of RSB buffer containing 0.15 M KCl, and was incubated for 30 min at 2°C, to yield the nuclear fraction. The supernatant of the previous centrifugation was brought to 0.15 M KCl, and was incubated for 30 min at 2°C, to yield the particulate fraction. The nuclear and the particulate fractions were then incubated for 2 h at 2°C with 1 μCi of tritiated glycerol. At the end of the incubations, samples containing the nuclear fractions were centrifuged for 10 min at 1000*g*, and the supernatants and the precipitates were treated as described for cell suspension. Samples containing the particulate fractions were centrifuged for 30 min at 16,000*g*, and the supernatants and the precipitates were treated as described for cell suspensions.

The radioactivity measured in the supernatants (dpm_S) and in the suspensions (dpm_F) was used to calculate the free water content in the fractions, after subtraction of the background.

By this procedure, we directly measured the free water content of the nuclear (Vol. N) and particulate (Vol. P) fractions, and of whole cells (Vol. C), which were normalized on the basis of the DNA content of the cellular suspensions. The free water content of the cytosolic fraction (Vol. Cy), instead, was calculated using the equation

$$\text{Vol. Cy} = \text{Vol. C} - (\text{Vol. N} + \text{Vol. P})$$

Other methods

The radioactivity was measured in a Packard liquid scintillation spectrometer, as described previously [20]. Lactate dehydrogenase activity was determined by the procedure of Wróblewski and LaDue [23]. DNA was measured by the diphenylamine test, using calf thymus DNA as the standard [24].

RESULTS

Subcellular distribution of glucocorticoid–receptor complexes after crosslinking of intact HeLa cells

The immobilization of glucocorticoid–receptor complexes in nuclear fractions induced by chemical crosslinking [13] was studied by treatments of HeLa cells with glutaraldehyde [14]. The levels of receptor complexes measured in cytosolic and nuclear fractions were then expressed as a function of the ratio between the concentrations of DNA and glutaraldehyde in the course of cell treatment with the crosslinker, as described previously [20]. By this graphical representation, a linear regression of data is obtained, and the intercepts with the ordinate yield the relative distribution of glucocorticoid–receptor complexes between cytosolic and nuclear fractions under extreme crosslinking conditions [20].

In Fig. 1 we have combined the data obtained in nine independent experiments, and it is readily apparent that a progressive decrease of the DNA: glutaraldehyde ratio in the course of crosslinking of intact cells is accompanied by accumulation of receptor complexes at the nuclear level, and a consequent loss of their cytosolic counterparts. The cytosolic and nuclear contents of glucocorticoid–receptor complexes under extreme conditions were thus found to represent 7.5 and 92.5% ($r = 0.742$, $P < 0.01$) of total cellular content, respectively.

In order to correct those data for the error introduced by artefactual redistribution of components induced by crosslinking [20], we determined the subcellular distribution of the cytosolic enzyme lactate dehydrogenase (LDH) by the same procedure. The data we obtained (Fig 2), showed that the cytosolic and nuclear levels of LDH activity under extreme crosslinking conditions represented 50.6 and 49.4% ($r = 0.764$, $P < 0.01$) of total activity, respectively.

The data on the levels of cytosolic and nuclear receptor complexes under extreme crosslinking conditions were then corrected for the error induced by cell treatment with glutaraldehyde, and using the values obtained for LDH activity, we calculated that the glucocorticoid–receptor complexes present in the two compartments represented 14.6 and 85.4% of the total cellular pool, respectively.

Therefore, according to these findings glucocorticoid–receptor complexes are prevalently distributed at the nuclear level in intact HeLa S_3 cells.

Subcellular distribution of glucocorticoid- and estrogen-receptor complexes after crosslinking of intact MCF-7 cells

The prevalent nuclear localization of glucocorticoid–receptor complexes in HeLa cells stands in striking contrast to our previous conclusion that these entities are mainly cytosolic in HTC cells [20]. We then thought it was important to expand our investigation to evaluate the subcellular distribution of glucocorticoid receptor in a third cell line, where it could be compared to that of a different steroid hormone receptor, such as the estrogen receptor, and we chose the MCF-7 cell line, which had already been used to define the subcellular distribution of estrogen receptor [5].

MCF-7 cells were thus incubated at 2°C with either dexamethasone or 17β-estradiol, were subjected to crosslinking with glutaraldehyde, and the cytosolic and nuclear levels of steroid–receptor complexes were then measured. The results we obtained in five different experiments carried out for each receptor species, including two paired determinations, are reported in Figs 3 and 4.

By linear regression of experimental data, we determined that cytosolic and nuclear levels of glucocorticoid–receptor complexes under extreme crosslinking conditions accounted for 13.8 and 86.2% ($r = 0.920$, $P < 0.001$) of the total cellular pool, respectively (Fig. 3). In the case of estrogen–receptor complexes (Fig. 4), in turn, we could determine that the cytosolic and nuclear contents, under the same conditions, represented 6.2 and 93.8% ($r = 0.913$, $P < 0.001$) of the total pool, respectively.

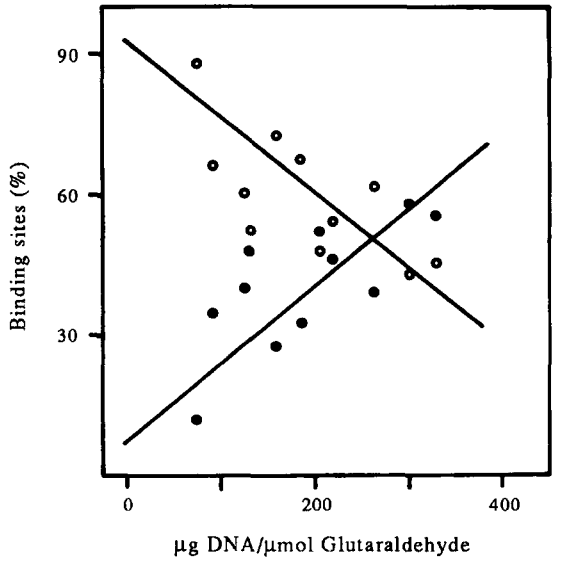

Fig. 1. Linear regression of glucocorticoid binding in cytosolic and nuclear fractions from crosslinked HeLa cells. HeLa cells were incubated for 2 h at 2°C with 50 nM tritiated dexamethasone and in the presence, or absence, of a 200-fold molar excess of nonradioactive competitor. At the end of the incubations, cells were recovered, dispersed in PBS buffer, and diluted with different volumes of PBS buffer. Cells were then subjected to crosslinking, as described in the Experimental, before being processed to determine specific glucocorticoid binding in cytosolic (●) and nuclear (○) fractions, which has been expressed as the percentage of total cellular binding. The total content of receptor complexes in crosslinked cells was 93.0 ± 4.0% of that measured in paired control cells. Data have been obtained in nine separate experiments, and the specific binding has been plotted as a function of the ratio between the DNA and the glutaraldehyde contained in 1 ml of sample during crosslinking.

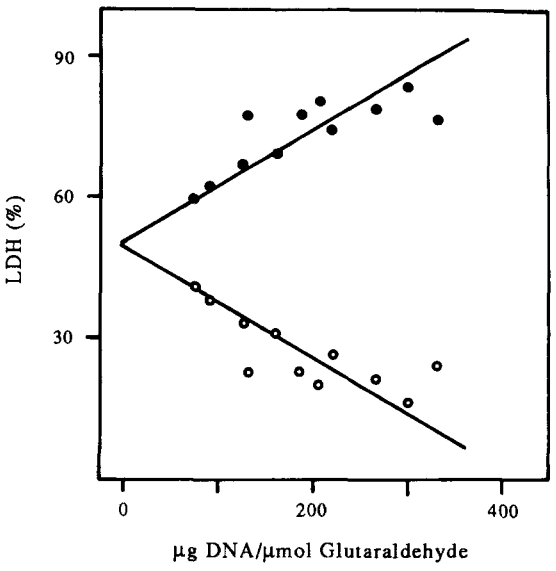

Fig. 2. Linear regression of LDH activity in cytosolic and nuclear fractions from crosslinked HeLa cells. HeLa cells were recovered, dispersed in PBS buffer, and diluted with different volumes of PBS buffer. Cells were then subjected to crosslinking, as described in the Experimental, before being processed to measure LDH activity in cytosolic (●) and nuclear (○) fractions, which has been expressed as the percentage of total cellular activity. The total activity of crosslinked cells was 96.1 ± 7.4% of that measured in paired control cells. The data were obtained in seven separate experiments, and have been plotted as a function of the ratio between the DNA and the glutaraldehyde contained in 1 ml of sample during crosslinking.

In order to correct these data, we also determined LDH activity in cytosolic and nuclear fractions obtained in six independent experiments. The values we obtained (Fig. 5) showed that the cytosolic and nuclear levels of LDH activity under extreme crosslinking conditions corresponded to 55.8 and 44.2% ($r = 0.721$, $P < 0.01$) of total activity, respectively.

The data on the subcellular distribution of glucocorticoid- and estrogen–receptor complexes in MCF-7 cells could then be corrected, and we calculated that cytosolic and nuclear levels of the former were 24.8 and 75.2%, whereas in the case of the latter they corresponded to 11.1 and 88.9% of the total cellular receptor pool, respectively.

These findings, therefore, confirmed that estrogen receptors are almost entirely confined inside nuclei [5, 6] even when they are in a form displaying low affinity for chromatin acceptor sites, and showed that

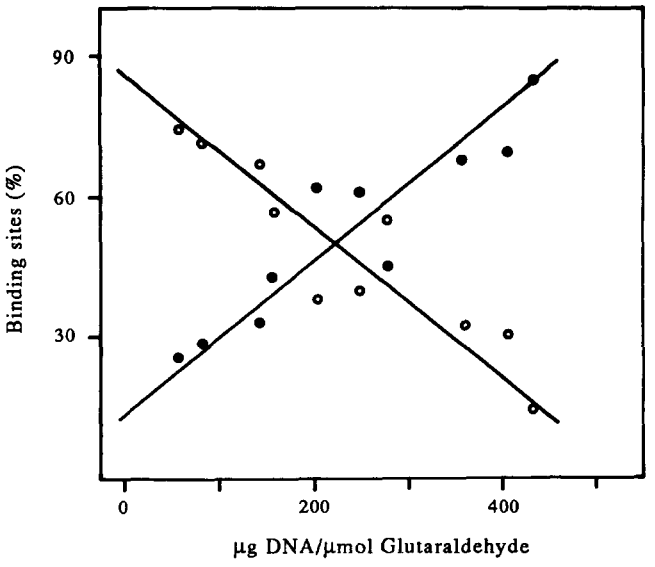

Fig. 3. Linear regression of glucocorticoid binding in cytosolic and nuclear fractions from crosslinked MCF-7-cells. The experimental conditions were as described in the legend to Fig. 1. The data on specific glucocorticoid binding in cytosolic (●) and nuclear (○) fractions were obtained in five separate experiments. The total content of receptor complexes in crosslinked cells was 97.4 ± 2.5% of that measured in paired control cells.

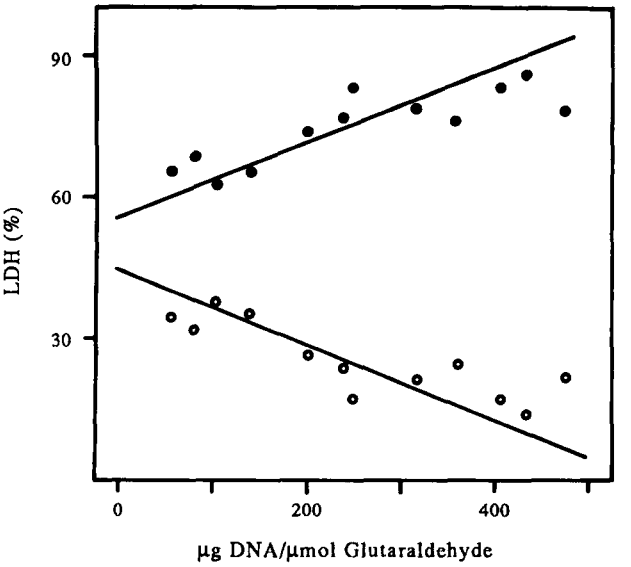

Fig. 5. Linear regression of LDH activity in cytosolic and nuclear fractions from crosslinked MCF-7 cells. The experimental conditions were as described in the legend to Fig. 2. The data on LDH activity in cytosolic (●) and nuclear (○) fractions were obtained in six separate experiments. The total activity of crosslinked cells was 92.5 ± 5.3% of that measured in paired control cells.

glucocorticoid receptors are mainly nuclear in MCF-7 cells, but their distribution does not quantitatively coincide with that of estrogen receptor.

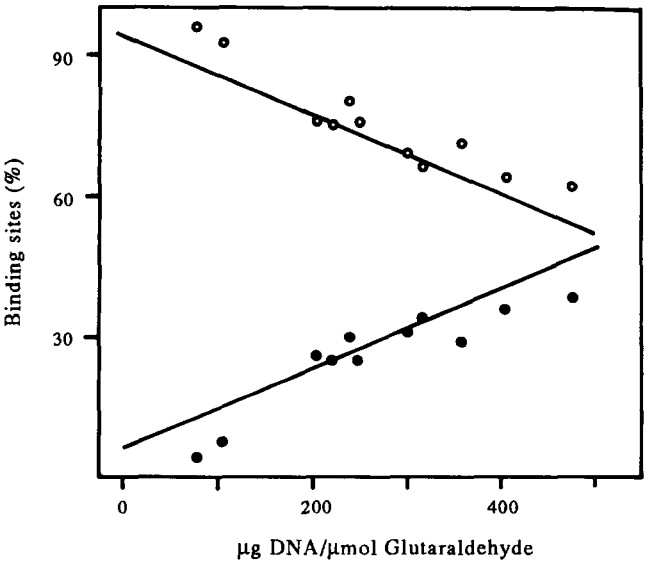

Fig. 4. Linear regression of 17β-estradiol binding in cytosolic and nuclear fractions from crosslinked MCF-7 cells. MCF-7 cells were incubated for 2 h at 2°C with 5 nM tritiated 17β-estradiol and in the presence, or in the absence, of a 200-fold molar excess of nonradioactive estradiol. The experimental conditions were as described in the legend to Fig. 1. The data on specific 17β-estradiol binding in cytosolic (●) and nuclear (○) fractions were obtained in five separate experiments. The total content of receptor complexes in crosslinked cells was 94.9 ± 7.7% of that measured in paired control cells.

If the data we obtained in the case of HTC [20], HeLa S_3 (Fig. 1), and MCF-7 (Fig. 3) cells are taken together, it can be concluded that the subcellular distribution of glucocorticoid–receptor complexes varies among cell lines. While this conclusion indicates that the relative distribution of glucocorticoid receptors between cytosol and nuclei is cell-specific, our data cannot provide information regarding the mechanism responsible for such a phenomenon, whose evaluation required measurements of the actual molar concentrations of receptors in the two subcellular compartments in intact cells.

Determination of glucocorticoid receptor concentrations in cytosols and nuclei of intact cells

The measurements of receptor concentrations in cytosols and nuclei can be achieved if the free water content of the two subcellular compartments in intact cells can be preliminarily determined. We approached this issue by a radioisotopic procedure, using tritiated glycerol, which has been shown to have access to the free water content of intact cells [22]. While this procedure allows a direct estimate of free water contents in whole cells and organelles, any measurements of cytosolic water must be indirect. We than directly measured the free water content of intact cells, nuclei, and other particulate fractions, and calculated that of cytosols by a subtractive method, as described the Experimental section.

The values we obtained for the free water content of whole HTC, HeLa S_3 and MCF-7 cells, and of their subcellular fractions are reported in Table 1, and show

Table 1. Free water content of cellular fractions and whole cells

Cell line	Free water content (μl/10^6 cells)			
	Whole cells	Nuclei	Particulate	Cytosol
HeLa S_3	9.91 ± 1.85	3.27 ± 0.87	0.79 ± 0.21	5.85
MCF-7	6.36 ± 1.11	3.98 ± 0.77	0.51 ± 0.14	1.87
HTC	6.13 ± 1.17	3.38 ± 0.45	0.34 ± 0.08	2.41

The estimation of the free water content of cellular fractions and whole cells was carried out as described in the Experimental Data of whole cells, nuclei, and particulate fractions have been expressed as means ± SD of values obtained in 4–9 separate determinations. The free water content of cytosols has been calculated as described in the Experimental.

that the volume of solvent varied in the three cell lines, but it was relatively constant in the case of their nuclei. It can also be noticed that the free water contents of the particulate fractions account for less than 10% of the total. Consequently, major differences in the free water content among the three cell lines involve their cytosols. Thus, if the relative water content of subcellular fractions in the three cell lines is considered, it is readily apparent that the volume of solvent in nuclei is twice as much as that of cytosol in MCF-7 cells, whereas in HeLa cells it is about half as much (Table 1).

The free water content and the levels of steroid–receptor complexes of cytosolic and nuclear fractions we have determined in the three cell lines, were then used to calculate the molar concentration of receptors in cytosol and nuclei of intact cells (Table 2). When the data we obtained for any cell line are considered, it is readily apparent that glucocorticoid–receptor complexes are more concentrated in either cytosol or nuclei,

Table 2. Receptor concentrations in cytosolic and nuclear compartments in intact cells

	Cell line			
			MCF-7	
	HTC GR	HeLa S_3 GR	GR	ER
Cytosol				
fmol/10^6 cells	45.6	2.2	8.3	6.7
$[R_C]$ (10^{-9} M)	18.9	0.4	4.4	3.7
Nuclei				
fmol/10^6 cells	19.0	12.7	25.1	53.8
$[R_N]$ (10^{-9} M)	5.6	3.9	6.3	13.5
K	0.3	9.7	1.4	3.6

The total receptor content in whole cells was measured as described in the Experimental, and the mean values obtained from 7–11 separate determinations were used to calculate the cytosolic and nuclear levels, based on their relative proportion under extreme crosslinking conditions, and after correction for artefactual subcellular redistribution caused by glutaraldehyde. The molar concentrations of receptors in cytosolic (R_C) and nuclear (R_N) compartments were calculated according to the mean values determined for the free water content of these fractions. The distribution constant (K) was calculated as the R_N/R_C ratio.

depending on the cell line. When the molar concentrations in the two compartments are considered, instead, it can be observed that they range between 0.4 and 18.9 nM in cytosols, whereas they are maintained within 3.9 and 6.3 nM in nuclei. Thus, in the three cell lines we have studied, the molar concentrations of glucocorticoid–receptor complexes showed a 50-fold variation in cytosols, but was almost the same in nuclei, leading to distribution constants ranging between 0.3 and 9.7.

The data we obtained in the case of estrogen receptor, in turn, showed that its molar concentration in nuclei remains higher than in cytosol of MCF-7 cells, and the distribution constant we calculated was 3.6.

DISCUSSION

In the present study we have investigated the concentrations of glucocorticoid–receptor complexes in cytosol and nuclei by chemical crosslinking of intact cells. The experimental conditions we chose involved the incubation of intact cells with hormone at 2°C, which allowed formation of steroid–receptor complexes and their maintenance in an untransformed functional state(s), as judged by three criteria, i.e. the low affinity for nuclear acceptor sites, which resulted in recovery of most receptor complexes in cytosolic extracts of non-crosslinked cells (Figs 1, 3 and 4 and Refs [13, 14]); the ionic properties of receptors, as assessed by anion exchange chromatography [13, 14]; and the DNA-binding properties of steroid–receptor complexes, as determined by DNA–cellulose chromatography [13]. Furthermore, we have already shown that chemical crosslinking of intact cells at 2°C does not cause by itself the transformation of glucocorticoid–receptor complexes [13, 14]. The data we have obtained in the present study then refer to the subcellular distribution of glucocorticoid–receptor complexes before they acquire high affinity for DNA.

By a procedure we established in a previous study [20], we could measure the relative proportion of LDH activity and steroid–receptor complexes in cytosolic and nuclear samples prepared after crosslinking of intact cells. The essentially identical redistribution of LDH activity found in the three cell lines we studied under extreme crosslinking conditions (Figs 2 and 5 and Ref. [20]) indicates that the values determined by our procedure are not affected by the variability among cell types, provided that crosslinking has been brought to completion, i.e. when the DNA:glutaraldehyde ratio is zero. These findings then provide additional support to our conclusion that the redistribution of LDH activity under extreme crosslinking conditions can be used as a correction factor to define the distribution of steroid–receptor complexes between cytosolic and nuclear fractions in intact cells [20]. This conclusion is also confirmed by our estimate on subcellular distribution of estrogen–receptor complexes after

crosslinking of MCF-7 cells, which coincides with that already established by different experimental approaches [5, 6].

In the case of glucocorticoid–receptor complexes, we found that their partitioning between cytosolic and nuclear fractions varies among the different cell lines, being mostly cytosolic in HTC cells [20], and nuclear in HeLa and MCF-7 cells (Figs 1 and 3). Thus, according to our estimates, the relative distribution of untransformed glucocorticoid–receptor complexes in cytosol and nuclei of intact cells is cell-specific.

This conclusion could explain the conflicting data reported in literature regarding the intracellular localization of glucocorticoid-free receptors [8, 13–20], on the basis of the fact that the binding of hormone, under our experimental conditions, did not promote the conversion of receptors to forms displaying high affinity for DNA, as discussed above. Indeed, we have performed our measurements using untransformed glucocorticoid–receptor complexes, as previous investigations by others [25] and by us [13] have shown that the steroid binding capacity of hormone-free receptors is impaired by crosslinking, preventing precise determinations of the content of functional glucocorticoid receptors in subcellular fractions.

The experiments carried out to establish the molar concentration of glucocorticoid–receptor complexes in cytosol and nuclei of intact cells were devised to gain insight into the mechanistic bases underlying the cell-specific distribution we detected. In particular, we considered it was important to establish whether the different partitioning of receptor we found in the subcellular compartments solely depends on the volume of their free water content, so that it could be explained by the existence of a simple equilibrium between cytosol and nuclei, as proposed by others [7].

The data we obtained (Table 2) clearly show that the concentration of cytosolic receptors differs from that in nuclei in the three cell lines we studied, indicating that a simple equilibrium between the two compartments does not occur in intact cells. Thus, the dynamic equilibrium of the distribution of receptors between cytosolic and nuclear fractions [7] would explain the behaviour of soluble forms in cell-free systems, such as those generated by cell homogenization, when destruction of complex units leads to alteration of the physicochemical parameters existing inside intact cells [6].

The importance of the conditions existing in whole cells in determining the subcellular distribution of steroid hormone receptors is strongly suggested by the finding that both the amount and the molar concentration of untransformed glucocorticoid–receptor complexes in nuclei are essentially the same in the three cell lines we studied, and that the nuclear concentrations of different receptor species do not coincide in the same cell, as in the case of glucocorticoid and estrogen receptors in MCF-7 cells (Table 2). Interestingly, the concentration of nuclear estrogen receptors we measured is similar to the theoretical estimate made by others [26].

The molecular mechanism responsible for the maintenance of a constant concentration of nuclear untransformed glucocorticoid–receptor complexes, in spite of variable concentrations of cytosolic pools, is presently unknown. The simplest explanation of this phenomenon, however, is that limited amounts of specific nuclear components loosely interacting with receptor proteins play a role of docking sites for untransformed steroid receptors, maintaining the proper concentration of these *trans*-acting transcription factors in the subcellular compartment where they perform their function.

While we do not presently know whether a relationship might occur between postulated nuclear sites and those proposed to exist in the cytosolic compartment [27, 28], the finding that the concentration of untransformed glucocorticoid–receptor complexes in nuclei can be lower than that in cytosol, as in the case of HTC cells (Table 2), would suggest that the levels of nuclear sites might be the limiting factor in determining the concentration of receptors in this subcellular compartment. Furthermore, if different receptor species can be present in nuclei of the same cell, as in the case of glucocorticoid and estrogen receptors in MCF-7 cells (Table 2), and of glucocorticoid, estrogen and progesterone receptors in GH_3 cells [8], the possibility should be considered that the subcellular distribution of receptors might be controlled by several parameters, such as the cytosolic and nuclear concentrations of one or more docking components and/or the different affinities of each receptor species for any single component.

If our interpretation is correct, the transformation of steroid–receptor complexes to forms displaying high affinity for DNA would involve changes in the topological arrangement(s) of receptor complexes, leading to shifts from docking sites to steroid responsive elements on DNA and/or establishment of different interactions and contacts with components of the transcription machinery.

Acknowledgements—This work was supported by a grant from the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (Quota 60%). We thank Fabrizio Zucchi for his excellent technical assistance.

REFERENCES

1. Beato M.: Gene regulation by steroid hormones. *Cell* **56** (1989) 335–344.
2. Gorski J., Toft D. O., Shymala G., Smith D. and Notides A. C.: Hormone receptors: Studies on the interaction of estrogen with the uterus. *Recent Prog. Horm. Res.* **24** (1968) 45–80.
3. Jensen E. V., Suzuki T., Kawashima T., Stumpf W. E., Jungblut P. W. and DeSombre E. R.: A two-step mechanism for the interaction of estradiol with rat uterus. *Proc. Natn. Acad. Sci. (U.S.A.)* **59** (1968) 632–638.
4. Schmidt T. J. and Litwack G.: Activation of the glucocorticoid-receptor complex. *Physiol. Rev.* **62** (1982) 1131–1192.

5. King W. J. and Greene G. L.: Monoclonal antibodies localize oestrogen receptor in the nuclei of target cells. *Nature* **307** (1984) 745–747.
6. Welshons W. V., Lieberman M. E. and Gorski J.: Nuclear localization of unoccupied oestrogen receptors. *Nature* **307** (1984) 747–749.
7. Sheridan P. J., Buchanam J. M., Anselmo V. C. and Martin P. M.: Equilibrium: the intracellular distribution of steroid receptors. *Nature* **282** (1979) 579–582.
8. Welshons W. V., Krummel B. M. and Gorski J.: Nuclear localization of unoccupied receptors for glucocorticoids, estrogens, and progesterone in GH_3 cells. *Endocrinology* **117** (1985) 2140–2147.
9. Press M. F. and Greene G. L.: Localization of progesterone receptor with monoclonal antibodies to the human progestin receptor. *Endocrinology* **122** (1988) 1165–1175.
10. Guiochon-Mantel A., Lescop P., Christin-Maitre S., Loosfelt H., Perrot-Applanat M. and Milgrom E.: Nucleocytoplasmic shuttling of the progesterone receptor. *EMBO Jl* **10** (1991) 3851–3859.
11. Tuohimaa P., Pekki A., Blåuer M., Joensuu T., Vılja P. and Ylikomi T. Nuclear progesterone receptor is mainly heat shock protein 90-free *in vivo*. *Proc. Natn. Acad. Sci. (U.S.A.)* **90** (1993) 5848–5852.
12. Husmann D. A., Wilson C. M., McPhaul M. J., Tilley W. D. and Wilson J. D.: Antipeptide antibodies to two distinct regions of the androgen receptor localize the receptor protein to the nuclei of target cells in the rat and human prostate. *Endocrinology* **126** (1990) 2359–2368.
13. Rossini G. P.: Particulate untransformed glucocorticoid-receptor complexes from HeLa cells crosslinked *in vivo*. *Biochim. Biophys Acta* **1011** (1989) 183–191.
14. Rossini G. P. and Masci G.: Chemical crosslinking: a useful tool for evaluations of steroid receptor structures and their functional states in intact cells. *J. Steroid Biochem.* **34** (1989) 363–367.
15. Sanchez E. R., Hirst M., Scherrer L. C., Tang H.-Y., Welsh M. J., Harmon J. M., Simons S. S. Jr, Ringold G. M. and Pratt W. B.: Hormone-free mouse glucocorticoid receptors overexpressed in chinese hamster ovary cells are localized to the nucleus and are associated with both hsp70 and hsp90. *J. Biol. Chem.* **265** (1990) 20123–20130.
16. Martins V. R., Pratt W. B., Terracıo L., Hirst M. A., Ringold G. M. and Housley P. R.: Demonstration by confocal microscopy that unliganded overexpressed glucocorticoid receptors are distributed ın a nonrandom manner throughout all planes of the nucleus. *Molec. Endocr.* **5** (1991) 217–225.
17. Brink M., Humbel B. M., De Kloet E. R. and Van Driel R. The unliganded glucocorticoid receptor is localized in the nucleus, not in the cytoplasm. *Endocrinology* **130** (1992) 3575–3581.
18. Fuxe K., Cintra A., Agnati L. F., Härfstrand A., Wikström A.-C., Okret S., Zoli M., Miller L. S., Greene G. L. and Gustafsson J.-Å.: Studies on the cellular localization and distrıbution of glucocorticoid receptor and estrogen receptor immunoreactivity in the central nervous system of the rat and their relationship to monoaminergic and peptidergic neurons of the brain. *J. Steroid Biochem.* **27** (1987) 159–170.
19. Wikström A.-C., Bakke O., Okret S., Brőnnegård M. and Gustafsson J.-Å.: Intracellular localization of glucocorticoid receptor: evidence for cytoplasmıc and nuclear localization. *Endocrinology* **120** (1987) 1232–1242.
20. Rossini G. P. and Malaguti C.: The subcellular distribution of glucocortıcoid-receptor complexes as studied by chemical crosslinking of intact HTC cells. *J. Steroid Biochem. Molec. Biol.* **48** (1994) 517–521.
21. Horwitz K. B. and McGuire W. L.: Nuclear estrogen receptors. *J. Biol. Chem.* **255** (1980) 9699–9705.
22. Horowitz S. B. and Fenichel I. R.: Analysıs of glycerol-^{3}H transport ın the frog oocyte by extractive and radioautographic techniques. *J. Gen. Physiol.* **51** (1968) 703–730.
23. Wróblewski F. and LaDue J. S.: Serum glutamic pyruvic transaminase in cardiac and hepatic disease. *Proc. Soc Exp. Bıol. Med.* **91** (1956) 569–571.
24. Burton K.: A study on the conditions and the mechanısm of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62** (1956) 315–323.
25 Rexin M., Busch W. and Gehring U.: Chemical cross-linking of heteromeric glucocorticoıd receptors *Biochemistry* **27** (1988) 5593–5601.
26. Murdoch F. E. and Gorski J.: The role of lıgand ın estrogen receptor regulation of gene expressıon. *Molec. Cell. Endocr.* **78** (1991) C103–C108.
27. Pratt W. B., Sanchez E. R., Bresnıck E. H., Meshinchi S., Scherrer L. C., Dalman F. C. and Welsh M. J.: Interaction of the glucocorticoıd receptor with the Mr 90,000 heat shock protein: an evolving model of ligand-mediated receptor transformatıon and translocation. *Cancer Res.* **49** (Suppl.) (1989) 2222s–2229s
28. Akner G., Sundqvist K. G., Denis M., Wikstrőm A-C. and Gustafsson J.-Å.: Immunocytochemical localization of glucocorticoid receptor ın human gengival fibroblasts and evidence for a colocalızation of glucocorticoid receptor with cytoplasmıc microtubules. *Eur. J. Cell Biol.* **53** (1991) 390–401.