Unoccupied binding sites for oestrogen in nuclei of a breast tumour cell line (MCF-7)

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1. A method to measure both occupied and unoccupied oestrogen receptors directly in the crude nuclear fraction of the MCF-7 cells was developed. The receptors had high affinity for oestradiol (K_d approx. 0.7 nM) and binding specificity characteristics of oestrogen receptors. 2. A substantial amount of the unoccupied receptors were found in the crude nuclear fraction. 3. Several experiments excluded the possibility that the unoccupied nuclear receptor might be a cytoplasmic contaminant. (a) Multiple extractions with Tris buffer released about 75% of the total receptor content, leaving the rest unextractable in the crude nuclear fraction. (b) Nuclei purified by centrifugation through 1.8 M-sucrose and treatment with 0.7% Triton X-100, or by centrifugation through 50% glycerol with 0.1% Triton X-100 contained similar amounts of unoccupied receptors to that found in the crude nuclear fraction. (c) In cells cultured during 5 days after preconfluency a 3-fold increase in the amount of unoccupied cytoplasmic receptors occurred, whereas the amount of unoccupied nuclear receptors did not change significantly and conversely in cells exposed to increasing concentrations of oestradiol the unoccupied cytoplasmic receptor was continuously depleted but no considerable change in the unoccupied nuclear receptor was found.

The mechanism of action of oestradiol on its target cell assumed the presence of an oestradiol occupied receptor in the nucleus. This ER_n originated from cytoplasmic receptor, which translocated to the nucleus after binding to oestradiol (Jensen *et al.*, 1968; Gorski *et al.*, 1968; Jensen & De Sombre, 1973; Gorski & Gannon, 1976).

More recently the presence of an unoccupied oestrogen receptor in the nucleus has been demonstrated in the MCF-7 cell line, derived from pleural effusion of a breast-cancer patient (Zava *et al.*, 1977; Zava & McGuire, 1977; Geier *et al.*, 1979a), and in human cancerous breast tumours (Garola & McGuire, 1977a,b; Panko & McLeod, 1978; Geier *et al.*, 1979a,b). It was assumed that these tissues contain a defective oestrogen mechanism of action that differs from that found in normal

target tissue, i.e. translocation of the unoccupied cytoplasmic receptor to the nucleus.

Recently it was demonstrated that a substantial number of the nuclear receptors in normal human endometrium (Geier *et al.*, 1980; Levy *et al.*, 1980) and myometrium (Giannopoulos *et al.*, 1980), during the menstrual cycle, are unoccupied. The presence of unoccupied receptors was demonstrated in purified uterine nuclei from ovariectomized and adrenalectomized pigs (Jungblut *et al.*, 1978) and in the uterine nuclear fraction from untreated immature rats (Carlson & Gorski, 1980). This finding may indicate that the unoccupied receptor is a necessary product in the normal mechanism of oestradiol action in its target tissue.

Edwards *et al.* (1980) found that the later passages of the MCF-7 cells in their possession did not contain the high concentrations of R_n reported originally (Zava *et al.*, 1977; Zava & McGuire, 1977). They claim that the R_n measured in the crude nuclear fraction previously was a contamination of the preparation with cytoplasmic receptor.

The purpose of the present study was to examine

Abbreviations used: R_c , unoccupied cytoplasmic oestrogen receptor; ER_c , occupied cytoplasmic oestrogen receptor; R_n , unoccupied nuclear oestrogen receptor; ER_n , occupied nuclear oestrogen receptor.

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critically the existence of R_n in the MCF-7 cells in our possession. Both R_n and ER_n were measured and characterized directly in the nuclear fraction.

Materials and methods

Cell culture

MCF-7 cells were generously provided by Dr. Charles M. McGrath (Michigan Cancer Foundation) and grown as previously described (Geier *et al.*, 1979a). Preconfluent or confluent cells in the Falcon flasks were washed with cold phosphate-buffered saline without Ca^{2+} and Mg^{2+} and were stored for 1–5 days at $-90^{\circ}C$ until assayed.

Preparation of nuclear and cytoplasmic fractions

The frozen cells were thawed by adding 3 ml of TED buffer [10 mM-Tris/HCl/1.5 mM EDTA/0.5 mM-dithiothreitol (pH 7.4)] per flask and harvested by scraping the Falcon flask with a rubber policeman. The cells were homogenized in a Dounce homogenizer (Kontes Co., Vineland, NJ, U.S.A.) with the B pestle (60–70 strokes). The homogenate was centrifuged at 800 g for 10 min to obtain the nuclear pellet. This pellet was washed twice by resuspending in TED buffer (3 ml of buffer/flask) and centrifuging at 800 g for 10 min. The washed pellet was slightly rehomogenized (10 strokes) in TED buffer (1.5 ml of buffer/flask) and considered as the nuclear fraction. The low-speed supernatant was centrifuged at 110 000 g for 30 min at $1^{\circ}C$ to obtain the cytosol fraction.

Preparation of purified nuclei

Frozen cells were thawed and harvested in Tris/sucrose buffer (3 mM- $MgCl_2$ /0.33 M-sucrose in 10 mM-Tris/HCl buffer, pH 7.4). The cells were homogenized as described above and centrifuged at 800 g for 10 min. The supernatant was removed and the nuclear pellet was further purified by two procedures.

(1) The pellet was resuspended in Tris/sucrose buffer containing 1.8 M-sucrose and centrifuged at 40 000 g for 60 min, thus eliminating cytoplasmic contamination (Chauveau *et al.*, 1956). The pellet so obtained was resuspended in Tris/sucrose buffer containing 0.7% (v/v) Triton X-100, to eliminate remaining cytoplasmic contamination, and centrifuged at 800 g for 10 min. The pellet was then washed twice with Tris/sucrose buffer to eliminate the Triton.

(2) The pellet was resuspended in Tris/glycerol buffer [10 mM-Tris/HCl (pH 7.4)/10 mM-NaCl/1.5 mM- $MgCl_2$ /50% (v/v) glycerol/0.1% (v/v) Triton X-100] and centrifuged at 3000 g at $0^{\circ}C$ for 10 min (Schibler & Weber, 1974). The pellet was then washed twice with Tris/sucrose buffer.

The purity of the nuclei was assessed by light and scanning electron microscopy. Receptor assay was

carried out on purified nuclei resuspended in TED buffer (1.5 ml/flask).

Nuclear and cytoplasmic oestrogen-receptor assays

Single-point assay. Triplicate portions (200 μ l) of nuclear or cytosol fraction were incubated with 7.5 nM- 17β -[2,4,6,7- 3H]oestradiol (sp. radioactivity 104 Ci/mmol; New England Nuclear Corp., Boston, MA, U.S.A.) in the presence or absence of 750 nM-diethylstilboestrol. Specific oestradiol-binding sites were calculated by subtraction of the non-specific binding measured in the presence of diethylstilboestrol from total [3H]oestradiol bound in the absence of diethylstilboestrol. Unoccupied nuclear and cytoplasmic receptors were determined by incubation at $0^{\circ}C$ for 4 h. Total nuclear and cytoplasmic receptors were determined by incubation at $30^{\circ}C$ for 3 h. Occupied receptors were calculated as the difference between total and unoccupied binding sites. Separation of bound and free oestradiol in cytosol was accomplished by adsorption with 20 μ l of charcoal/dextran mixture (5% Norit A and 0.5% dextran grade C in 10 mM-Tris/HCl, pH 8.0) at $0^{\circ}C$ for 15 min and centrifugation at 5000 g for 7 min. The supernatant was collected and the radioactivity was counted in 5 ml of scintillation fluid that contained 7.0 g of 2,5-diphenyloxazole, 0.3 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 100 g of naphthalene in 1000 ml of dioxan. At the end of the incubation of the nuclear fraction 1 ml of cold TED buffer was added to each of the incubation tubes, which were subsequently centrifuged at 1600 g for 10 min. Then the nuclei were washed twice with 1 ml of TED buffer. The bound [3H]oestradiol was extracted with 1 ml of ethanol at $37^{\circ}C$ for 30 min. The extract was evaporated to dryness under air and the radioactivity was counted as described above.

Scatchard analysis. Nuclear and cytoplasmic fractions were incubated in duplicate with 0.1–7.5 nM- 3H]oestradiol. Unoccupied receptors were measured at $0^{\circ}C$ for 4 h and occupied receptors at $30^{\circ}C$ for 3 h. Parallel samples contained [3H]oestradiol plus diethylstilboestrol (750 nM). The non-specific binding for both nuclear and cytoplasmic receptors ranged from 5 to 10%. The data were analysed by using the Scatchard plot (Scatchard, 1949). Each plot was constructed by seven to eight points obtained from five pooled flasks.

DNA, used for calculation of the numbers of cytoplasmic and nuclear receptor sites per mg of DNA, was determined in parallel cultured flasks by the diphenylamine method of Burton (1956).

Scanning electron microscopy

Samples for examination by scanning electron microscopy were fixed in cold 1% glutaraldehyde in a 0.1 M-cacodylate buffer, pH 7.4. The purified nuclei

were allowed to be bound to poly- α -lysine-coated glass coverslips, then dehydrated through graded alcohols and Freons, followed by critical-point drying with liquid CO_2 . After coating with gold, the nuclei were examined in a JEOL-35 scanning electron microscope.

Results

Binding of oestradiol

It is usually accepted that unoccupied receptors are measured at a low temperature, whereas oestradiol-occupied receptors are determined by exchange at an elevated temperature. Fig. 1 illustrates the effect of incubation time and temperature on the binding of [^3H]oestradiol to nuclear and cytosol fraction, prepared from confluent MCF-7 cells. Two binding components could be demonstrated in both the nuclear and cytosol fraction. One component, which bound [^3H]oestradiol at 0°C , was saturated after 2 h of incubation and remained constant for 6 h, was considered to be the unoccupied receptor. The other component bound [^3H]oestradiol at 30°C , was saturated after 2 h of incubation in the nuclear fraction and after 0.5 h in the cytosol. This component remained stable up to 6 h of incubation and was considered to be the occupied receptor. To demonstrate that at 0°C [^3H]oestradiol bound only to unoccupied receptors and that oestradiol occupied receptors can be exchanged by [^3H]oestradiol at 30°C , we exposed confluent cells for 1 h to a medium fortified with

Fig. 1. Binding of (^3H)oestradiol to the nuclear fraction (N) and cytosol (C) as a function of time and temperature

Nuclear fraction and cytosol prepared from confluent cells were incubated with 7.5 nM [^3H]oestradiol at 0°C (O) and at 30°C (●). Non-specific binding, measured with 750 nM diethylstilboestrol, was subtracted. Each point on the curves represents the mean of three determinations from 10 pooled flasks (experiment repeated three times).

Fig. 2. Effect of time and temperature on the exchange of [^3H]oestradiol into oestradiol-occupied nuclear (N) and cytoplasmic (C) receptors

Nuclear fraction and cytosol prepared from confluent cells exposed for 1 h to a medium fortified with 10 nM oestradiol were incubated with 7.5 nM [^3H]oestradiol at 0°C (O) and at 30°C (●). Non-specific binding, measured with 750 nM diethylstilboestrol, was subtracted. Each point on the curves represents the mean of three determinations from 10 pooled flasks (experiment repeated three times).

10 nM oestradiol. At this concentration oestradiol binds to R_c , translocating most of the complex ER_c into the nucleus, simultaneously with direct binding of oestradiol to R_n (see Fig. 3). Fig. 2 shows that neither ER_n nor ER_c could be detected after incubation with [^3H]oestradiol at 0°C up to 20 h. However, at 30°C exchange took place, reaching a maximal value after 3 h of incubation, which remained stable up to 6 h.

Oestrogen-receptor character of nuclear and cytoplasmic binding sites

The dissociation constants of the nuclear and cytoplasmic receptors were determined in untreated cells and in cells 1 h after exposure to oestradiol. Only one type of nuclear receptor was detected. The binding affinities calculated were similar: $K_d = 0.64\text{ nM}$ at 0°C for the unoccupied nuclear receptor in the untreated cells; $K_d = 0.90\text{ nM}$ at 30°C for the occupied nuclear receptor in the cells after 1 h exposure to oestradiol. Similarly, one type of cytoplasmic receptor was found. The K_d values calculated were 0.33 nM at 0°C for the unoccupied cytoplasmic receptor in the untreated cells and 2.00 nM at 30°C for the occupied cytoplasmic receptor, which did not translocate to the nucleus during 1 h exposure to oestradiol (see Fig. 3).

The specificity of oestradiol binding was checked by incubating the nuclear or cytoplasmic fraction with [^3H]oestradiol in the absence or presence of excess unlabelled competitors (Table 1). Com-

Fig. 3. Effect of oestradiol dose on receptor distribution. Cells at confluency were exposed for 1 h to a medium fortified with increasing concentration of $[^3\text{H}]$ oestradiol (0.1–10 nM). R_c , R_n , ER_n and ER_c were measured by incubation with 7.5 nM- $[^3\text{H}]$ oestradiol as described in the Materials and methods section. Abbreviation: C, unexposed cells at confluency. Each point on the curves represents the mean of three determinations from two pooled flasks (experiment repeated three times).

petition for unoccupied binding sites was assessed at 0°C in untreated cells, whereas competition for occupied binding sites assessed at 30°C in cells 1 h after exposure to oestradiol. Table 1 shows that the specific binding of $[^3\text{H}]$ oestradiol to nuclear and cytoplasmic receptors was greatly inhibited by the presence of a 100-fold excess of diethylstilboestrol, oestradiol and oestriol but not by similar concentrations of the non-oestrogenic steroids cortisol, progesterone and dihydrotestosterone, suggesting that the binding is specific for oestrogens.

R_n is not a contamination product of R_c

Direct evidence: We measured the distribution of unoccupied receptors in cells frozen and thawed before homogenization in TED buffer. As shown in Table 2 about 74% of the unoccupied receptor content in the MCF-7 cells were found in the first cytoplasmic extract. The second cytoplasmic extract contained an additional 2–4% from the unoccupied receptors. Additional extraction either by resuspension or by rehomogenization of the remaining pellet could not extract more receptors, leaving 22–24% of the unoccupied receptors in the nuclear fraction. Light-microscopic examination of the stained cells before homogenization showed that in most of the cells the cytoplasmic membrane was disrupted and the cytoplasm partially detached from the nucleus. After the first homogenization most of the nuclei were intact and deprived of cytoplasm. Further homogenization resulted in distortion of the nuclear integrity.

Although we have shown that unoccupied receptors could not be further extracted, we did not eliminate the possibility that insoluble receptors are

bound either to cytoplasmic material attached to the nucleus (but not revealed by light microscopy) or to membrane fractions that sediment at 800g. To eliminate this possibility we purified nuclei by two different procedures: (1) centrifugation through 1.8 M-sucrose the treatment with 0.7% Triton X-100; (2) centrifugation through 50% glycerol containing 0.1% Triton X-100 as described in detail in the Materials and methods section. Examination of the purified nuclei by light microscopy showed essentially clean and intact nuclei. Under the scanning electron microscope the nuclear membrane appeared well preserved and mostly free of adhering cytoplasmic components (Plate 1). In addition the overall nuclear preparation was clean, with very little amorphous extranuclear material. As shown in Table 3 the receptor concentration found in the purified nuclei by both methods and in the nuclear fraction was similar.

Indirect evidence. (a) Unoccupied and occupied receptors for oestradiol were measured in nuclear fraction and in cytosol of cells at preconfluency and during the following 5 days. Table 4 summarizes a representative pattern of receptor distribution in MCF-7 cells. Both unoccupied and occupied receptors were found in the nuclear fraction and cytosol. Although in the cytosol the amount of unoccupied receptors increased continuously from 3654 fmol/mg of DNA at preconfluency to 10968 fmol/mg of DNA 5 days thereafter, the amount of R_n did not change considerably (1513 fmol/mg of DNA at preconfluency and 1690 fmol/mg of DNA 5 days thereafter). The amount of occupied receptors in the cytosol varied from 428 fmol/mg of DNA at preconfluency to 847 fmol/mg of DNA 5 days later, whereas the amount of occupied receptors in the nuclear fraction found at preconfluency was 289 fmol/mg of DNA and 325 fmol/mg of DNA 5 days later.

(b) Cells at confluency were exposed for 1 h to medium fortified with increasing concentrations of $[^3\text{H}]$ oestradiol (0.1–10 nM). The distribution of oestrogen receptors is shown in Fig. 3. With the increase in $[^3\text{H}]$ oestradiol concentration R_c is depleted and a concomitant increase in ER_n occurred. Despite the R_c depletion R_n values did not change considerably up to 1 nM- $[^3\text{H}]$ oestradiol, which negates the possibility that R_n is a cytoplasmic contaminant. At concentrations greater than 1 nM it was probable that a free penetration of oestradiol into the nucleus occurred and that oestradiol also charged the R_n .

Discussion

This is the first report in which nuclear oestradiol receptors of MCF-7 cell line were measured directly in the resuspended nuclear fraction. We found no degradation of either cytosolic or nuclear receptors

EXPLANATION OF PLATE 1

Scanning electron micrographs of nuclei from MCF-7 cells

Nuclei were detached from their cytoplasm in a preparation of thawed cells before homogenization (A), $\times 4500$; (B), $\times 3200$. Nuclei were also purified as described in the Materials and methods section, showing well preserved nuclear membrane: (C), $\times 6000$; (D), $\times 5000$.

Table 1. *Percentage inhibition of [³H]oestradiol binding to nuclear and cytoplasmic receptors by unlabelled competitors*
Nuclear and cytoplasmic fractions were incubated with 7.5 nM-[³H]oestradiol in the absence or presence of 750 nM unlabelled competitors. Inhibition of [³H]oestradiol binding to unoccupied receptors was measured at 0°C for 4 h. The exchange of the occupied receptors (measured only in the cells after 1 h exposure to oestradiol) was determined at 30°C for 3 h. Each value represents triplicate determinations from four pooled flasks.

Competitor	Inhibition of binding (%)			
	Untreated cells		Cells 1 h after exposure to oestradiol	
	Nuclear	Cytoplasmic	Nuclear	Cytoplasmic
Diethylstilboestrol	90	93	92	95
Oestradiol	92	92	92	96
Oestriol	82	87	86	89
Cortisol	5	4	8	6
Progesterone	2	1	5	3
Dihydrotestosterone	6	1	9	8

Table 2. *[³H]Oestradiol binding to cytoplasmic extracts and nuclear fraction*

Thawed cells were homogenized (60–70 strokes) in TED buffer (3 ml/flask) and centrifuged at 800 g. The pellet was divided in two equal parts. Part A was further extracted by resuspension and sedimentation at 800 g, whereas part B was further extracted by rehomogenization (10–20 strokes) and sedimentation at 3000 g. Portions of cytoplasmic extracts or nuclear fraction were incubated in triplicate with 7.5 nM-[³H]oestradiol at 0°C for 4 h. Non-specific binding, measured with 750 nM-diethylstilboestrol, was subtracted. Values in parentheses are percentages.

Extract procedure	Extract ...	Radioactivity c.p.m.				Nuclear fraction
		I	II	III	IV	
A		20 393 (74)	480 (2)	0	0	6727 (24)
B		20 393 (74)	1209 (4)	0	0	5988 (22)

Table 3. *Distribution of oestrogen receptors between cytosol and nuclear preparations*

Thawed cells were homogenized in Tris/sucrose buffer and centrifuged at 800 g. The supernatant was used for cytosol preparation, and the pellet was divided into three parts. One part was treated with TED buffer for nuclear fraction preparation and the second and third parts were used for purification of nuclei as described in the Materials and methods section. Each preparation was further divided into two parts; one was used for DNA estimation and the other part for the receptor assay as described in the Materials and methods section.

	Receptor (concn. fmol/mg of DNA)			
	Cytosol	Nuclear fraction	Purified nuclei	
			Sucrose/Triton X-100	Glycerol/Triton X-100
Unoccupied	4543	1611	1341	1531
Occupied	538	438	522	326

Table 4. *Distribution of oestradiol receptors in MCF-7 cells as a function of growth stage*

Oestrogen receptors were measured in MCF-7 cells at preconfluency and during the following 5 days. Nuclear and cytoplasmic receptors were determined as described in the Materials and methods section. Each value represents triplicate determinations from two pooled flasks.

Days of culture ...	Receptor (concn. fmol/mg of DNA)					
	0*	1	2	3	4	5
R _c	3654 (62)	5531 (70)	5778 (72)	6665 (72)	8752 (74)	10 968 (79)
ER _c	428 (7)	500 (6)	515 (6)	826 (9)	1220 (10)	847 (6)
R _n	1513 (26)	1547 (20)	1488 (19)	1421 (15)	1537 (13)	1690 (12)
ER _n	289 (5)	329 (4)	252 (3)	316 (4)	383 (3)	325 (3)
Total	5884 (100)	7907 (100)	8033 (100)	9228 (100)	11 892 (100)	13 833 (100)

* Day of preconfluency.

at 30°C, which enabled us to measure them directly. The K_d values and specificity of oestradiol binding to the oestrogen receptors measured by this method are similar to those published previously (Zava & McGuire, 1977; Geier *et al.*, 1979b). In other reports nuclear receptors in MCF-7 were measured in the high-salt extract by adsorption to protamine sulphate (Zava *et al.*, 1977; Zava & McGuire, 1977). However, Clark & Peck (1976) have indicated that only a part of the nuclear oestrogen receptors can be extracted by KCl. According to the authors the residual sites represent the oestrogen receptors that are associated to specific acceptor sites. Not all the nuclear receptors in MCF-7 cells are extracted by 0.4 M-KCl, on the basis of the results obtained by Brooks *et al.* (1978). They showed that in MCF-7 cells incubated with [³H]oestradiol there remained a fraction of oestrone and oestradiol that is not extractable by 0.4 M-KCl.

Several experiments exclude the possibility that R_n might be due to a contamination of the nuclear fraction by cytosol that is high in unoccupied receptors. First, multiple extraction with TED buffer released about 75% of the receptor content, leaving the rest unextractable in the nuclear fraction (Table 2). Secondly, nuclei purified by two different procedures and the crude nuclear fraction contained similar amounts of unoccupied receptors (Table 3). Third, this was also supported by the following observations. (a) In cells cultured during 5 days after preconfluency a 3-fold increase in the amount of R_c occurred, whereas the amount of R_n did not change considerably (Table 4). (b) Conversely, in cells exposed to increasing concentrations of oestradiol, R_c was continuously depleted but no considerable change in R_n was found (Fig. 3). These experiments suggest that the cytoplasmic unoccupied oestrogen receptors, which are defined as receptors easily solubilized and separated from particulate components of the cells, are not randomly trapped by the nuclear pellet.

McGuire's group, which originally published data on the unoccupied nuclear receptors in the MCF-7 cell line, more recently reported that the subcellular distribution of unoccupied oestrogen receptor observed in their later passages differed from their original findings. In the earlier studies (Zava *et al.*, 1977; Zava & McGuire, 1977) they found 75% of the cell's unoccupied receptors in the crude nuclear fraction, whereas in the more recent study (Edwards *et al.*, 1980) they found 24–30%, which by further purification was decreased to 3%. They also reported that the later passages of their cell line contained 6 pmol of oestrogen receptors per mg of DNA compared with 2 pmol/mg of DNA found previously. The possibility might be considered that the changes that occurred in the later passages of the MCF-7 cells in McGuire's laboratory included also

a loss of the high level of R_n that was present in the earlier passages.

We reported previously (Geier *et al.*, 1979a) that the MCF-7 cells in our possession contained about 6 pmol of oestradiol receptors/mg of DNA, 25% of which were contained in the nuclear fraction. These results are similar to the receptor distribution at the beginning of confluency, which we report in the present study.

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