Eur J Cancer Clin Oncol, Vol. 22, No. 2, pp. 181–190, 1986.
Printed in Great Britain.

0277–5379/86$3.00+0.00

Binding of Estrogen Receptor from *N*-Nitrosomethylurea-Induced Rat Mammary Tumors to Nuclei

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Abstract—*The binding of the cytoplasmic estrogen receptor (ER_c) from* N-*nitrosomethylurea (NMU)-induced rat mammary tumors to the nucleus using a cell-free system is described. All tumors studied were estrogen-receptor-positive and most of them were hormone-dependent. Sixty-two percent of all tumors investigated* (n = 134) *decreased in size more than* 30% 4–5 days *after ovariectomy. Brief heating of the cytosol loaded with tritiated estradiol induced activation of the ER_c measured by an increase of nuclear binding activity. Temperature-dependent activation was evident in every case. The optimal time and temperature of activation were* 15–60 min *at* 30°C. *After denaturation of the ER_c by heating for* 20 min *at* 56°C *only small parts of free estradiol could be bound to nuclei. Mg^{2+} ions and EDTA inhibited the nuclear binding of the receptor. The nuclear binding assay was performed for* 1 hr *at* 0–4°C. *After this time the activated ER_c was bound nearly maximally to nuclei. Under optimized conditions* 50–60% *of the ER_c could be bound to nuclei maximally. Using the same medium for the preparation of crude and purified nuclei the binding of the receptor to both kinds of nuclei was similar.* Na_2MoO_4 *prevented the activation of the ER_c from NMU-tumors completely but did not influence the binding of the previously activated receptor to nuclei.*

INTRODUCTION

STEROID hormone receptors in the cytosol of human mammary tumors are measured for the prediction of the hormone responsiveness of these carcinomas. Patients with estrogen-receptor-positive mammary tumors were found to have a better prognosis and a higher response rate to endocrine therapy than patients with receptor-negative tumors [1–3]. Hormone-dependent tumor growth presumes an intact hormone receptor system. The steroid action in the target cell involves the binding of the steroid to the cytoplasmic receptor, the translocation of the receptor to the nucleus, the transcription of certain DNA-segments and the translation of specific m-RNAs into specific proteins. One of the early steps of the estrogen action is the activation of the occupied receptor and its binding to nuclei. Several methods were used to measure the ability of the ER_c and other steroid receptors to bind to nuclei, for example the direct *in vivo* labeling by injection of animals with tritiated estradiol [4, 5], the nuclear exchange after injection of cold estradiol [6, 7], the translocation *in vitro* (the incubation of tissue slices or whole tissue with tritiated estradiol [8, 9]) and cell-free systems [10–12].

In the case of cell-free systems it is necessary to activate steroid receptor in cytosol to the nuclear binding form. Some methods are described for the activation of the receptor, for instance the temperature-dependent activation after warming the cytosol up to 15–30°C [10, 12, 14, 15], the salt activation after incubation of the cytosol with high concentrations of salt, e.g. 0.3–0.4 M KCl [13–15], and the activation by simple dilution of the cytosol [16]. Also, after a long incubation at 0–4°C the receptor becomes activated [10]. The activated receptor is measured by its binding rate to nuclei [10, 12, 13, 15] or polyanions such as DNA–cellulose or DNA [14, 15, 17], ATP–Sepharose [15, 18] or phosphocellulose [18]. Molybdate, a phosphatase inhibitor, prevents the activation of steroid receptors under cell-free conditions [15, 19].

The NMU-induced mammary tumor of the rat, firstly characterized by Gullino *et al.* [20], is used as a model tumor for human mammary carcinoma [21]. NMU-tumors contain receptors for estradiol [22–24], progesterone [22, 23], glucocorticoids [25] and prolactin [23]. Some authors found that NMU-tumors are able to metastasize [20]. Others could not confirm this observation [26]. Most of the NMU-tumors were hormone-dependent, the tumors decreasing after ovarectomy. Therefore an intact estradiol receptor system might be postulated.

The purpose of this study was to investigate the binding of the cytoplasmic estrogen receptor from NMU-induced mammary tumors of the rat to the

Accepted 12 August 1985.

nucleus after temperature-dependent activation of the ER_c using a cell-free system.

MATERIALS AND METHODS

Chemicals

[17β-(2,4,6,7)^{3}H] estradiol (E^3H, sp. act. 3.3–4.3 MBq/nmol) was obtained from New England Nuclear, Boston (MA). It was stored in absolute ethanol at −20°C. Its purity was checked by thin-layer chromatography. *N*-Nitrosomethylurea was synthesized by Dr D. Ziebarth (Central Institute for Cancer Research, Berlin, G.D.R.).

Buffers and solutions

PD-buffer: 10 mM potassium phosphate, 1 mM dithiothreitol (DTT) pH 7.4; buffer 1: 10 mM potassium phosphate, 330 mM sucrose, 3 mM $MgCl_2$, pH 7.4; buffer 2: 10 mM potassium phosphate, 250 mM sucrose, 3 mM $MgCl_2$, pH 7.4; solution 3: 2.4 M sucrose, 3 mM $MgCl_2$; solution 4: 2.0 M sucrose, 3 mM $MgCl_2$; buffer 5: 10 mM potassium phosphate, 250 mM sucrose, 3 mM $MgCl_2$, 0.05% Triton × 100, pH 7.4; and buffer 6: 10 mM potassium phosphate, 250 mM sucrose, 1 mM DTT, pH 7.4.

Animals

Tumors were induced by *N*-nitrosomethylurea (5 mg/100 g body wt) in female Sprague–Dawley rats (51–53 days of age). NMU was given by two intravenous injections, with the second injection 4 weeks after the first injection. The appearance of tumors was monitored by weekly palpation of the animals and appearing tumors were measured using calipers. The size of tumor was calculated after measurement of two orthogonal dimensions and calculation of volume assuming the tumor to be a prolate spheroid (vol. = $ab^2\pi/6$) [27]. Animals were bilaterally ovarectomized (hexobarbital narcosis) when the sizes of the tumors were 2–4 cm^3. The rats were killed 4–5 days after ovariectomy and the tumors utilized immediately. Necrotic tissues were discarded.

Subcellular fractionation

All procedures for subcellular fractionation were performed at 0–4°C. Tumor tissue was minced with scissors and homogenized in 4 vol. (v/v) PD-buffer using glass–Teflon homogenizers (4 × 5 strokes by a loose fitting homogenizer followed by 3 × 5 strokes by a tight-fitting glass–Teflon homogenizer). The homogenate was centrifuged at 1000 ***g*** for 20 min, the pellet rehomogenized (2 × 5 strokes by a tight fitting homogenizer) centrifuged at 1000 ***g*** for 10 min and the combined supernatants centrifuged at 100,000 ***g*** for 60 min to obtain the cytosol. The pellet was washed again twice and suspended in PD-buffer to obtain the crude nuclear fraction. Nuclei were purified as previously described [24]. Briefly, the tissue was homogenized in 4 vol. of buffer 1. The 800 ***g*** pellet was washed once with buffer 2, resusupended in buffer 2 and was well mixed with 2 vol. (v/v) of solution 3. The aliquots of this suspension (about 15 ml) were layered on 10 ml of solution 4 and centrifuged at 40,000 ***g*** for 60 min. The pellets were suspended by a loose-fitting glass–Teflon homogenizer, washed once with buffer 2, filtered through nylon gauze and incubated with about 4 ml/g fresh wt of buffer 5 for 5 min at 0–4°C. Finally, the purified nuclei were washed once in buffer 2 and resuspended in buffer 6. The purity of the nuclear preparation was checked by light microscopy.

Labeling of cytosols with ^{3}H-labeled estradiol and nuclear binding assays

In most experiments NMU-tumor cytosol in PD-buffer (protein content 3–4 mg/ml) was incubated for 2.5 hr at 0–4°C (non-activated ER_c) or for 2 hr at 0–4°C and 30 min at 30°C (activated ER_c) with 4 nmol [17β-^{3}H]estradiol in the absence or presence of a 100-fold excess of diethylstilbestrol (DES) to determine the total and the non-specific binding capacities. Other incubation times and temperatures or further treatments of the cytosol are noted in the figure legends. After incubation the cytosol was cooled (if necessary), incubated with a pellet from the same volume of dextran-coated charcoal (DCC) suspension (0.5% Norit, 0.05% dextran, 10 mM phosphate buffer, pH 7.4) for 20 min at 0–4°C and centrifuged at 3000 ***g*** for 10 min. The supernatant was measured by liquid scintillation counting (LSC: efficiency 40–50%) to obtain the estrogen receptor concentration in the cytosol. For nuclear binding assays, 0.2-ml aliquots of tritium estradiol-labeled and of DCC-treated activated or non-activated cytosols were incubated with 0.4 ml nuclear suspension in PD-buffer (crude nuclei) or buffer 6 (purified nuclei), usually at 0–4°C for 1 hr with intermittent shaking. The cytosol/nuclei ratio in the assay was normally 1/2, in comparison with the ratio 1/1 in the homogenates. For estimation of the unspecific binding to nuclei in parallel samples the binding of [^{3}H]estradiol + 100 × DES-labeled and DCC-treated cytosol to nuclei was determined. Samples were then centrifuged for 10 min at 3000 ***g***. The radioactivity of the supernatant was measured by LSC, the nuclei were washed three times with PD-buffer and extracted with 1 ml absolute ethanol overnight. The radioactivity of the ethanol extract was measured by LSC. The DNA-content of nuclei per sample was 100–200 μg DNA in most experiments. All samples were processed in duplicate or triplicate. Only specific binding is presented in

Results. The percentage of unspecific binding to nuclei in the case of activated estrogen receptor was lower than 20% in all experiments.

Protein and DNA determination

Protein concentration was measured by the method of Lowry *et al.* [28] using bovine serum albumin as a reference standard. DNA was assayed by the method of Burton [29] using calf thymus DNA as a reference standard.

RESULTS

Hormone-dependence and cytoplasmic estrogen receptor in NMU-induced mammary tumors of the rat

The response of NMU-tumors shortly after ovariectomy was determined. The tumor volume was measured before and 4–5 days after ovariectomy (Table 1). The tumors are mostly hormone-dependent. Sixty-two percent of all tumors examined decreased in size more than 30% 4–5 days after ovariectomy. Only 13% of the tumors grew further after ovariectomy. The growing tumors were not used for the experiments. It should be noted, however, that this short-time response did not allow an absolute statement about hormone sensitivity or hormone insensitivity of the NMU-tumors. Some tumors increased in size after ovariectomy for some days and decreased later (data not shown).

The cytoplasmic estrogen receptor of NMU-tumors was characterized as previously described [24]. The ER_c-content was similar in all tumors assayed: 42.8 ± 12.3 fmol/mg cytosol protein (mean ± S.D., n = 42). All tumors were estrogen-receptor-positive. The equilibrium dissociation constant (K_D) was calculated using Scatchard analysis and was found to be 2.2 ± 1.1 × 10^{-10} M(mean ± S.D.; n = 10).

Table 1. *Response of NMU-tumors shortly after ovariectomy*

	Decrease >50%	Decrease 50–30%	Decrease 30%–stable	Increase
response/n	54/134	29/134	33/134	18/134
%	40	22	25	13

The tumor volume was determined before and 4–5 days after ovariectomy (Materials and Methods). n = No. of tumors investigated.

Influence of time and temperature on the activation of the ER_c

Figure 1 shows the binding of the ER_c to nuclei after warming the cytosol several times to 30°C. The warming of the cytosol before the nuclear binding assay induced a rapid activation of the ER_c measured by an increase of receptor binding to nuclei. An incubation of the cytosol for 15 min at 30°C after saturation of the receptor with tritiated estradiol was sufficient for the activation of the ER_c. Longer incubation times at 30°C did not further increase the portion of nuclear-bound ER_c. The optimal activation time of the cytosol was 15–60 min at 30°C. An activation time of 30 min at 30°C was used generally.

Similar behavior could be obtained after activation at 23°C for 60 min (not shown).

Influence of buffers on the activation of the ER_c from NMU-tumors

Several buffers were tested to obtain the optimal medium for the activation of the ER_c. Dithiothreitol was generally added to the homogenization buffer because SH-groups seem to be necessary for the binding of the steroid to the receptor [30, 31] and for the activation of the steroid receptors [16, 31]. As shown in Table 2, the nuclear binding of the activated estrogen receptor was similar using Tris buffer and phosphate buffer. A significantly higher amount of receptor was already bound to nuclei without activation in the case of Tris buffer.

EDTA in phosphate buffer and Mg^{2+} ions in both buffer systems tested significantly inhibited the nuclear binding of the activated receptor. (The influence of EDTA in Tris buffer was not investigated.)

As shown in Table 2, the buffer systems used had no effect on the temperature stability of the ER_c. Nearly the same receptor content could be measured before and after incubation of the cytosol for 30 min at 30°C in all cases.

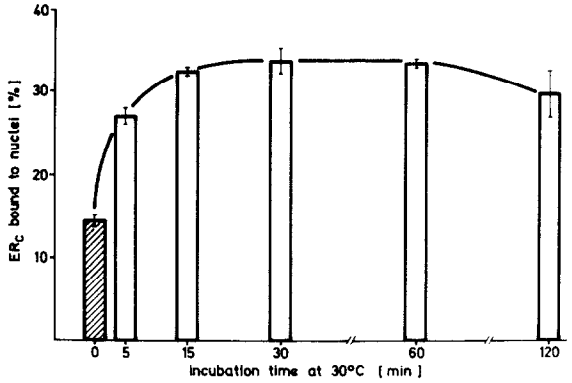

Fig. 1. *Influence of different times of incubation at* 30°C *on the activation of the ER_c from NMU-tumors. Cytosol and nuclei were prepared in PD-buffer and the cytosol was incubated with tritiated estradiol for* 2.5 hr *at* 0–4°C (▨) *or for* 2 hr *at* 0–4°C *plus the indicated times at* 30°C (□). *The excess of* E^3H *was removed by DCC and the cytosol incubated with nuclei for* 1 hr *at* 0–4°C. *The values are the mean* ± *S.E.M. of three independent experiments* (100% = 6338 ± 680 dpm/0.2 ml *cytosol*). ▨ *non-activated ER_c;* □ *activated ER_c.*

Table 2. Influence of buffers on the binding of activated and non-activated ER_c to nuclei

Buffer	ER_c content (fmol/mg protein)		Nuclear binding (% ER_c)		
	2.5 hr, 0–4°C*	2 hr, 0–4°C and 30 min, 30°C*	2.5 hr, 0–4°C*	2 hr, 0–4°C 30 min, 30°C*	n
PD	40 ± 2.1	41 ± 2.6	14.8 ± 1.0	41.8 ± 1.5	17
PED	39 ± 5.6	36 ± 4.0	14.1 ± 2.0	33.3 ± 3.0§	7
PDMg	33 ± 3.2	32 ± 2.4	13.5 ± 1.5	30.5 ± 5.0§	4
TD	37 ± 2.4	37 ± 6.2	27.5 ± 4.4†	43.0 ± 1.5	3
TDMg	31 ± 3.3	35 ± 2.1	24.0 ± 2.1‡	29.9 ± 2.2‖	3

Cytosol and crude nuclei were prepared in buffers indicated above. $MgCl_2$ was added after fractionation of the tissue. Cytosol was incubated with 4nM [^{3}H]estradiol ± 400 nM DES for 2.5 hr at 0–4°C or 2 hr at 0–4°C and 30 min at 30°C. After cooling the cytosol was incubated with a pellet of DCC and thereafter incubated with nuclei for 1 hr at 0–4°C, as described in Materials and Methods. The ratio cytosol/nuclei was about 1/2 in comparison with the ratio in the homogenate. The results are the mean ± S.E.M. PD: 10 mM potassium phosphate, 1 mM DTT, pH 7.4; PED 10 mM potassium phosphate; 1 mM DTT, 1 mM EDTA, pH 7.4; PDMg: 10 mM potassium phosphate, 1 mM DTT, 3 mM MgCl, pH 7.4; TD: 10 mM Tris–HCl, 1 mM DTT, pH 7.4; TDMg: 10 mM Tris–HCl, 1 mM DTT, 3 mM MgCl, pH 7.4. n = No. of independent experiments.

*Incubation time and temperature of cytosol.

§$P < 0.01$ vs PD-buffer (activated ER_c).

‖$P < 0.01$ vs TD-buffer (activated ER_c).

†$P < 0.001$ vs PD-buffer (non-activated ER_c).

‡$P < 0.01$ vs PDMg-buffer (non-activated ER_c). Statistical significance was determined using Student's t test.

The optimal incubation time for the nuclear binding assay

Activated and non-activated ER_c in NMU-tumor cytosol were incubated with nuclei at 0–4°C for different times. Figure 2 shows a typical assay. The activated receptor bound very quickly to nuclei and the binding process was nearly finished after a 1-hr incubation of cytosol with nuclei. Only a very slight increase of nuclear binding of the activated ER_c could be observed after a 2-hr incubation of cytosol and nuclei in comparison with the 1-hr incubation (Fig. 2b). We generally used a 1-hr incubation in the following assays. An overnight incubation of cytosol and nuclei resulted in a decrease of the nuclear binding in the case of the activated receptor and in an increase of nuclear binding in the case of 'non-activated' receptor (Fig. 2b incubation time 18 hr), in comparison with a 1- or 2-hr incubation. Figure 2a shows the ER_c remaining in the cytosol after the nuclear contact of the activated receptor. Most of the activated ER_c which disappeared from the cytosol was bound to nuclei (Fig. 2a vs b). About 85–90% of the total receptor previously seen in cytosol could be found in the sum ER_c bound to nuclei and remained in the cytosol.

The activated estrogen receptor could be maximally bound to nuclei after activation once for 30 min at 30°C

Figure 3 shows the binding of the activated receptor, the binding of the non-activated receptor and the binding of the free steroid (after temperature denaturation of the ER_c) to increasing amounts of nuclei. Maximally 50–60% of the total receptor content could be activated and bound to nuclei using the conditions mentioned. The ratio cytosol/nuclei in the assay was maximally 1/8–1/10 in comparison with the ratio in the homogenate. Using larger amounts of nuclei in the same volume, it becomes more and more difficult to handle the suspension. A further dilution of the cytosol resulted in less precise results because NMU-tumors contain a relatively low ER_c content. As shown in Fig. 3, only small parts of free steroid could be bound to nuclei. A linear dependence could be obtained between the binding of free steroid to nuclei and the quantity of nuclei available in the sample.

Comparison of the binding of ER_c to crude and purified nuclei from NMU-tumors

To exclude the possibility that the receptor binds rather to eventually existing contaminations of crude nuclear pellets than to pure nuclei, the nuclei of NMU-tumors were purified further (Materials and Methods). The yield of purified nuclei was about 40–50%. As shown in Fig. 4a, a greater portion of activated and also of non-activated ER_c bound to crude nuclear pellets than to purified nuclei. Using nuclei containing 300 μg DNA per sample, about 50% of the activated ER_c bound to

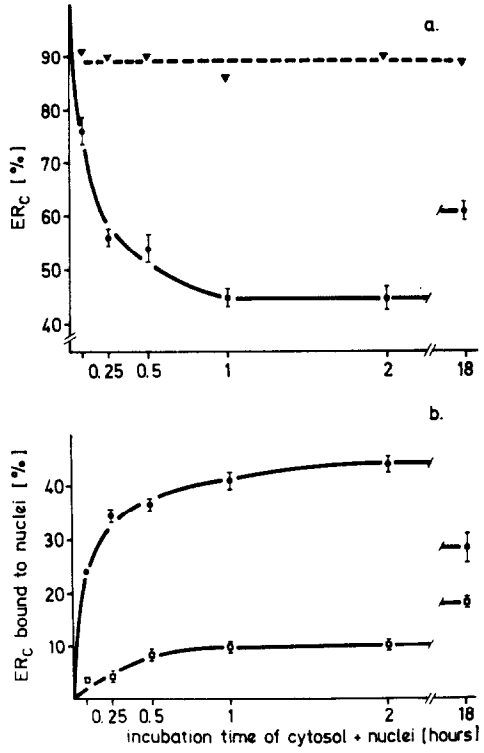

Fig. 2. Variation of the incubation time for the nuclear binding assay. Cytosol was incubated with tritiated estradiol for 2.5 hr *at* 0–4°C (□—□) *or for* 2 hr *at* 0–4°C *and* 30 min *at* 30°C (●—●). *The nuclear binding assay was performed for different times as indicated in the figure. (a) The portion of receptor remaining in cytosol after the nuclear binding assay in the case of activated ER_c. (b) The binding of activated or non-activated receptor to crude nuclei.* □—□ *non-activated ER_c bound to nuclei (b);* ●—● *activated ER_c bound to nuclei (b) and ER_c remaining in cytosol after the nuclear binding assay (a);* ▼—▼ *sum of activated receptor bound to nuclei and receptor remaining in cytosol after the nuclear binding assay (a). All values are the mean ± S.E. of a typical experiment.* (100% = 10189 dpm/0.2 ml *cytosol*).

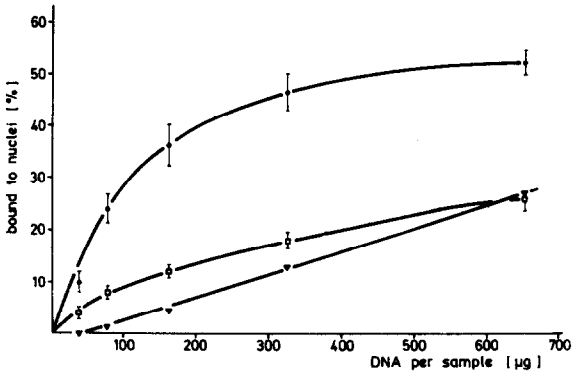

Fig. 3. Binding of non-activated ER_c, activated ER_c and remaining steroid after temperature denaturation of the ER_c to increasing amounts of crude nuclei. Cytosol was incubated with E^3H in the following manner before the nuclear binding assay performed for 1 hr *at* 0–4°C. □—□ 2.5 hr, 0–4°C + *DCC-pellet (non-activated ER_c);* ●—● 2 hr, 0–4°C *and* 30 min, 30°C + *DCC-pellet (activated ER_c);* ▼—▼ 2.5 hr, 0–4°C + *DCC-pellet and* 20 min, 56°C *(denatured ER_c). The values for the binding of the receptor to the nuclei are the mean ± S.E.M. of* 5 *independent experiments* (100% = 7336 ± 748 dpm/0.2 ml *cytosol*). *The last point,* i.e. 650 µg *DNA per sample, resulted from only* 3 *of the* 5 *experiments. The assay for the binding of tritiated estradiol to nuclei after denaturation of the receptor was performed in two independent experiments.*

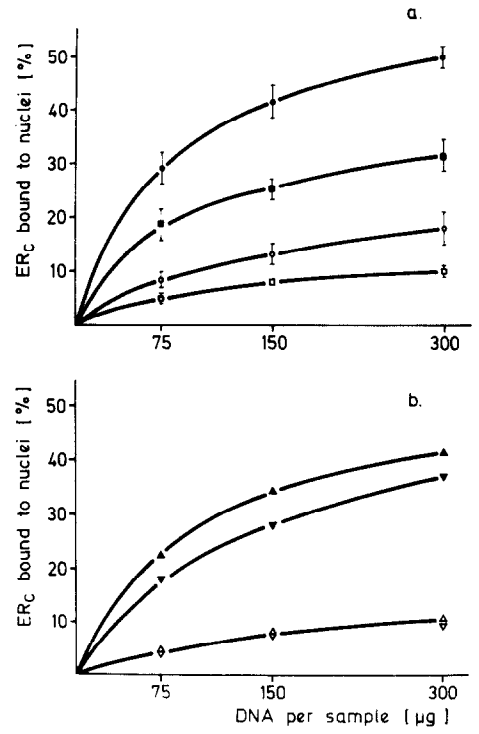

Fig. 4. Binding of the activated and of the non-activated estrogen receptor from NMU-tumors to crude and purified nuclei. Cytosol was prepared in PD-buffer and the activated or non-activated ER_c was bound to increasing amounts of nuclei. (a) Crude nuclei (in PD-buffer) and purified nuclei were isolated as described in Materials and Methods. Non-activated ER_c bound to crude (○—○) *and purified nuclei* (□—□) *and activated ER_c bound to crude* (●—●) *and purified nuclei* (■—■). *All values are the mean ± S.E.M. of three independent experiments* (100% = 7681 ± 1124 dpm/0.2 ml *cytosol*). *(b) Purified nuclei were isolated as described in Materials and Methods. The Triton X-100 washing step was omitted. Crude nuclei were prepared in buffer* 2 *and suspended in buffer* 6 *before the nuclear binding assay. Non-activated ER_c bound to crude* (△—△) *and purified nuclei* (▽—▽) *and activated ER_c bound to crude* (▲—▲) *and purified nuclei* (▼—▼). *All values are the mean of two independent experiments* (100% = 6678 *or* 9927 dpm/0.2 ml *cytosol*).

crude nuclei and 32% bound to purified nuclei. In the case of non-activated receptor, 18 and 10% were bound to crude and purified nuclei respectively. Whereas crude nuclei were prepared in PD-buffer, the purified nuclei were isolated using buffers containing $MgCl_2$ and suspended in isotonic phosphate buffer without $MgCl_2$ before the nuclear binding assay was started (Materials and Methods). $MgCl_2$ inhibited the nuclear binding of the ER_c as shown in Table 2. To standardize the conditions and to obtain a direct comparison of the receptor binding to crude and purified nuclei we prepared crude nuclei in buffer 2 (10 mM phosphate buffer, 250 mM sucrose, 3 mM $MgCl_2$, pH 7.4) and incubated these nuclei with cytosol containing activated or non-activated receptor using the same conditions as described for purified nuclei (Fig. 4b). The Triton X-100 washing step was omitted during the preparation of these purified nuclei as shown in Fig. 4b because it is well known

that Triton X-100 influences at the very least the binding of steroid receptor to nuclei [32]. Using nuclei containing 300 μg DNA per sample, about 41% of activated ER_c bound to crude nuclei and 37% to purified nuclei. Of the non-activated ER_c, 11 and 10% bound to crude and purified nuclei respectively (Fig. 4b). These results are similar. It seems likely that the nuclear isolation procedure performed in the presence of Mg^{2+} influenced the binding of the ER_c to nuclei. The washing of purified nuclei with Triton X-100 using a low concentration (i.e. 0.05%, see Materials and Methods) before the nuclear binding experiments had a very little effect on the binding of the ER_c to nuclei. The nuclear isolation procedure to obtain purified nuclei had also apparently only a slight effect on the binding of the receptor.

Influence of Na_2MoO_4 *on the nuclear binding of the* ER_c

It is well known that molybdate stabilizes the steroid receptor [19, 33] and prevents its activation [15, 19, 33]. We tested the influence of molybdate on the activation and nuclear binding of the ER_c from NMU-tumors. The results obtained are shown in Fig. 5. If molybdate was present during the temperature-dependent activation process nearly the same portion of the receptor was bound to nuclei as in the case of nonactivated receptor. If molybdate was added to the cytosol after the activation of the ER_c the same portion of the activated receptor bound to nuclei as without molybdate. Thus whereas molybdate inhibited the activation of the ER_c, it did not affect the nuclear binding of the activated estrogen receptor.

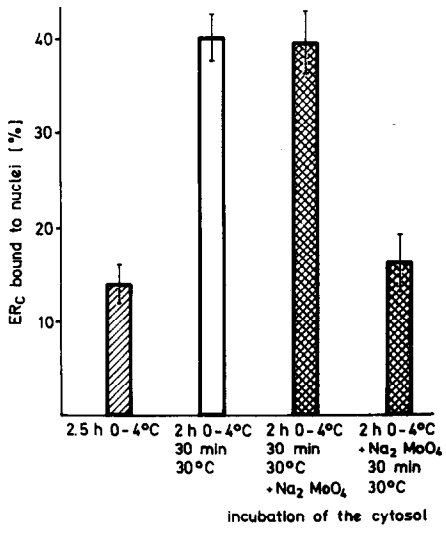

Fig. 5. Influence of 10 mM Na_2MoO_4 *on the activation and nuclear binding of the* ER_c. *Cytosol was prepared and incubated with tritiated estradiol and molybdate as indicated in the figure. The nuclear binding assay using crude nuclei was performed as described in Materials and Methods. All values are the mean ± S.E.M. of five independent experiments* (100% = 7193 ± 988 dpm/0.2 ml *cytosol*).

DISCUSSION

Activation and nuclear binding of the cytoplasmic estrogen receptor from NMU-induced mammary tumors of rats were investigated using a cell-free system. There are some methods available to activate steroid receptor to the nuclear binding form. In this paper only the temperature-induced activation of the ER_c was used. After warming the cytosol before contact with nuclei the receptor became activated and its ability to bind to nuclei increased. In the experiments described the nuclear binding assay was always performed at 0–4°C, although authors have used a temperature of 20–30°C [12, 34, 35]. Comparing the results obtained in both methods, a higher amount of activated ER_c bound to nuclei could be found using a common incubation of cytosol and nuclei at higher temperatures [35]. However, using such conditions it is difficult to distinguish between the activation process, i.e. alteration of the receptor molecule at higher temperatures, and the process of nuclear binding of the receptor. Both processes could be influenced using a nuclear binding assay at higher temperatures. By using a nuclear binding assay at 0–4°C a better comparison of the binding of the non-activated and the activated ER_c to nuclei should be possible. On the other hand, some authors and ourselves described a distinct degradation of the ER_c by nuclei during a common incubation of cytosol and nuclei at higher temperatures in the case of uteri [34, 36] and NMU-tumors [24].

The removal of the excess of tritiated estradiol before the nuclear binding assay was performed to improve the precision of the assay and to reduce the non-specific binding of steroid to nuclei. The receptor content of NMU-tumors is relatively low, 42.8 ± 12.3 fmol/mg cytosol protein (mean ± S.D., $n = 42$), and a large proportion of the non-specific bound steroid could be obtained if the nuclear binding assay was performed in the presence of an excess of tritiated estradiol (not shown).

Comparing Tris buffer and phosphate buffer, similar amounts of activated ER_c were bound to the nuclei in both systems. Using Tris buffer a relatively large amount of non-activated receptor (cytosol kept at 0–4°C) bound to nuclei compared with the phosphate buffer. This effect cannot be explained yet. The phosphate buffer seems to be a better system to show the temperature dependent activation of the ER_c from NMU-tumors. Mg^{2+} ions and EDTA inhibited the binding of the receptor to nuclei. This confirms the observations published by others [32, 35].

The optimal incubation time for the occupied ER_c to become activated was 15–60 min at 30°C. Using such conditions maximally 50–60% of the ER_c could be bound to nuclei (Fig. 3). It is generally accepted that not all of the receptor

present in cytosol can be activated [37]. Some inhibiting factors preventing the nuclear binding of the receptor are described in the crude cytosol [37].

A distinct part of 'non-activated' ER_c could also be bound to nuclei. It is well known that the receptor becomes activated also after a long incubation at 0–4°C [10]. An incubation time of 2.5 hr was used to saturate the receptor with tritiated estradiol. During this time an activation of a small part of the receptor could not be excluded.

The receptor is necessary for transmitting the steroid to the nucleus because only a small amount of free steroid after denaturation of the receptor could be bound to nuclei (Fig. 3). A linear binding curve of binding of free estradiol to increasing amounts of nuclei could be observed, in contrary to the saturation curves of binding of non-activated and activated ER_c to nuclei (Fig. 3).

The activated ER_c bound quickly to nuclei at 0–4°C, the binding process being nearly completed after an incubation time of cytosol and nuclei for 60 min (Fig. 2). An overnight incubation of cytosol containing activated ER_c with nuclei resulted in a distinct decrease of nuclei-bound ER_c in comparison with an incubation time of 1 or 2 hr. This finding can be explained by a loss of nuclear binding capacity which could be seen during overnight storage of nuclei at 0–4°C and also at −30°C (unpublished results). On the other hand, a loss of nuclear binding capability of the activated ER_c or a dissociation of the steroid from the DCC-treated ER_c during an overnight incubation with nuclei cannot be excluded.

Similar amounts of activated and non-activated ER_c bound to purified or crude nuclei when both kinds of nuclei were isolated using the same medium (Fig. 4b). Washing of nuclei with 0.05% Trition X-100 before the nuclear binding assay showed only a very small effect on the binding of ER_c to nuclei. The results confirm the findings of Hemminki [32].

Molybdate — a phosphatase inhibitor — inhibited the binding of the receptor to nuclei. This effect of molybdate was shown in cell-free systems of several tissues [15, 19, 33]. Also, in the case of the ER_c obtained from NMU-tumors it was evident that molybdate inhibited the activation of the cytoplasmic estrogen receptor but did not influence the binding of the activated ER_c to nuclei (Fig. 5). Dephosphorylation of the receptor protein as the method of activation, producing the ability of binding to nuclear sites, has been postulated [38, 39]. On the other hand, other phosphatase inhibitors have been described which did not influence the activation of steroid receptors [19, 40]. Previously Naray [41] showed that the glucocorticoid receptor in crude cytosols could be activated *in vitro* only in the absence of molybdate. The *in vivo* nuclear binding and the activation of partially purified glucocorticoid receptor was not inhibited by molybdate.

The physiological significance of receptor activation is not fully understood. It could be argued that 'activation' was confined to the test tube and had little relevance to physiology since the temperature of the cell would be about 37°C, resulting in an immediate activation of receptor after ligand binding. However, some results suggest that activation may be of physiological significance [42]. Munck and Foley [43] demonstrated a time-dependent appearance of the activated steroid–receptor complex in thymic cells maintained under tissue culture conditions. Marcovic and Litwack [44] quantified non-activated and activated steroid–receptor complexes in rat liver following an intraperitonal injection of [^{3}H]triamcinolone acetonide. In both papers cited firstly the non-activated receptor appeared followed by its decline and concomitant appearance of the activated form. Welshons *et al.* [45] and King and Greene [46] recently showed that the cytoplasmic estrogen receptor may be an artifact obtained by disruption of the tissue and that the estrogen receptor is situated *in vivo* in the nuclear compartment. The so-called 'translocation' of the receptor would be an alteration of the affinity of the receptor to the nuclear binding sites [46]. However, if this is so, the activation of the receptor which could be observed *in vivo* [43, 44] and *in vitro* nevertheless may be of physiological significance. In this case the activation measured *in vitro* in the prepared cytosol may demonstrate an alteration of nuclear affinity of the (*in vivo* nuclear) estrogen receptor released into the cytosol by homogenization.

The ability of the ER_c to become activated and bound to nuclei should be one supposition for an intact hormone receptor system in tumor tissues. But as yet it is not clear whether this supposition is sufficient to explain the hormone-dependency of mammary tumors. On the one hand, Khan *et al.* [47] showed that the activated ER_c from hormone-dependent mammary tumors bound to DNA-cellulose significantly more than ER_c from hormone-independent mammary tumors. On the other hand, Vignon and Rochefort [48] clearly showed the direct translocation of the ER_c from the estrogen-independent C3H mammary tumor into the nucleus by *in vivo* labeling of the estrogen receptor with tritiated estradiol injected into the animals and protamine sulfate or hydroxylapatite precipitations of the receptor. The authors concluded that the estrogen-independence of C3H mammary tumors cannot be explained by a defect of cytosol binding or nuclear translocation of the receptor. Using tumor slices incubated with tritiated estradiol in Krebs–Ringer–bicarbonate buffer,

Hawkins *et al.* [49, 50] found an accumulation of the ER_c in the nucleus of all hormone-dependent and -independent human and rat mammary tumors studies.

In this study only hormone-dependent NMU-tumors are included. It would be of great interest to study the behavior of ER_c-positive but hormone-independent mammary tumors with respect to the activation of the receptor using the conditions described here. The NMU-tumor does not seem to be a very useful model for such studies for the following reasons: (1) NMU-tumors are mostly hormone-dependent. Only a small number of tumors grow progressively after ovariectomy (Table 1); and (2) to distinguish exactly between hormone-sensitive and autonomous tumors the tumor growth must be observed for longer times after ovariectomy (as mentioned above, some tumors increased in size after ovariectomy for some days and decreased later). This is difficult because the NMU-tumors become necrotic relatively quickly after appearing and large tumors are more frequently necrotic than small ones. Since necrotic tumors cannot be used for receptor studies, appearing tumors should be used as soon as possible.

It is clear that the results discussed here do not allow the conclusion that the receptor has been bound to specific binding sites inside the genome of the purified nuclei. It is, however, evident that the estrogen receptor extracted from hormone-dependent NMU-tumors can be activated *in vitro* similarly to the receptor from normal tissues like uterus. The demonstration of the ability of the ER_c to become activated and bound to nuclei suggests that it may be used for the valuation of the steroid receptor system studied rather than just to show that there are specific binding sites for the steroid in the cytosol [34].

Acknowledgements—We thank Monika Binder for excellent technical assistance.

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