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Nuclear association states of rat uterine oestrogen receptors as probed by nuclease digestion

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The solubilization of oestrogen receptors from uterine nuclei by micrococcal nuclease and deoxyribonuclease I was examined after the injection of oestradiol or Nafoxidine into castrated female rats. At 1 h after an injection of oestradiol, 30% (0.18 pmol/mg of DNA) of the nuclear oestrogen receptors was solubilized by 5 min of mild digestion with either nuclease. No further receptor release occurred, although DNA hydrolysis continued throughout a 20 min interval. The limitation in receptor solubilization was not due to an artifact of digestion conditions or insufficient nuclease concentrations. Similar patterns of receptor solubilization and DNA hydrolysis were obtained with both nucleases whether the animals had been injected with oestradiol 1 h before death or if the uteri from uninjected animals were incubated with [^{3}H]oestradiol for 1 h *in vitro*. When uterine nuclei were digested with these enzymes 12 h after the animal was injected with oestradiol there was little change in the quantity of nuclease-sensitive sites (0.11 pmol/mg of DNA); however, the quantity of nuclease-resistant sites decreased 10-fold. These values correspond quantitatively to the changes in salt-resistant and salt-extractable sites observed over a 12 h interval after oestradiol treatment. Nuclease digestion of uterine nuclei obtained 16 h after Nafoxidine treatment gave a pattern qualitatively and quantitatively similar to that observed 1 h after oestradiol treatment, a result consistent with the agonist/antagonist action of this compound. An analysis by sucrose-density-gradient centrifugation of the time course of nuclease-dependent receptor solubilization indicated that the solubilized receptors were not associated with discrete nucleosomal fragments. We believe that these data indicate that only a portion of the receptors translocated to the nucleus become associated with chromatin, and this association may occur on regions of chromatin that are preferentially susceptible to nucleolytic cleavage.

In a previous report from this laboratory (Schoenberg & Clark, 1979), it was demonstrated that crude uterine nuclei contain an endogenous nucleolytic activity whose action resulted in the solubilization of oestrogen receptors through DNA hydrolysis. The intercalating drugs ethidium bromide and actinomycin D were found to inhibit the breakdown of DNA and the concomitant production of soluble oestrogen–receptor complexes by this enzyme(s). At the time of that study, we discovered that highly purified nuclei did not contain the endogenous nuclease activity and hence showed no release of nuclear oestrogen receptors under the conditions employed with the crude nuclear preparations. We also made two observations that suggested to us that the endogenous nucleases might be recognizing some structural feature(s) of chromatin with which oestrogen receptors might be associated. First, we found that the concentration of ethidium bromide that was maximally effective in inhibiting the nuclease-catalysed receptor solubilization was also that which saturated the binding sites for intercalating drugs in the internucleosomal linker region of chromatin. Secondly, the endogenous nuclease solubilized chromatin fragments that correspond to the oligomers produced by digestion of nuclei with micrococcal nuclease (Kornberg, 1977). We therefore decided to examine the nuclear association states of the oestrogen–receptor complex by digesting highly purified nuclear preparations with DNAase I and micrococcal nuclease, enzymes whose actions on chromatin have been well characterized.

Several reports have appeared in the recent

Abbreviation used: DNAase, deoxyribonuclease.

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literature in which DNAase I or micrococcal nuclease was used to fractionate uterine chromatin to study the resultant partitioning of the nuclear oestrogen receptor. Senior & Frankel (1978) reported that oestrogen receptors were equally partitioned between the core particle and the internucleosomal linker region. Andre *et al.* (1978) used micrococcal nuclease to extract up to 80% of the nuclear oestrogen receptors from lamb endometrial nuclei, and reported that most of these were 4S and 6S particles, although some receptors were associated with nucleosome monomers. In a related study Massol *et al.* (1978) found that micrococcal-nuclease digestion of hen oviduct chromatin gave an oestrogen–receptor complex with a sedimentation rate of 13–14S, slightly faster than the 11S nucleosome monomer. In contrast, Rennie (1979) found that the androgen receptor from the rat prostate gland was preferentially associated with the internucleosomal linker region. Our data correlate with a paper by Scott & Frankel (1980) in which they found nuclear oestrogen receptors associated with a transcriptionally active fraction of chromatin in MCF-7 mammary-gland tumour cells.

Materials and methods

Animals

Female rats (45 days old) were obtained from Holtzman (Madison, WI, U.S.A.) and were castrated within 5 days of arrival. They were kept for at least 1 week after surgery in a controlled environment at 22°C with constant relative humidity and a light/dark cycle of 12h each. Food and water were provided *ad libitum*. Oestradiol was dissolved in 0.9% NaCl/2% (v/v) ethanol and injected at a 5μg dose subcutaneously. Nafoxidine (U11100-A) was dissolved in aq. 5% (v/v) ethanol and injected as a 50μg dose subcutaneously. The animals were killed by cervical dislocation, and the uteri were quickly excised and stripped of connective tissue. Uteri were slit lengthwise with iris scissors and, depending on the protocol, placed into ice-cold 0.9% NaCl or, for experiments *in vitro*, placed into Eagle's minimal essential medium at 25°C. In all of the following experiments, purified nuclei were prepared by the modified hexylene glycol procedure (Hardin *et al.*, 1976).

Preparation and determination of nuclear oestrogen–receptor complexes

For studies *in vivo* on the interaction of the receptor with the nucleus, animals received oestradiol 1 or 12h before death, or Nafoxidine 16 or 40h before death. The resultant fractions obtained from nuclease digestion (see below) were assayed at least in duplicate by an exchange reaction at 37°C with 8nM-[^{3}H]oestradiol in the presence or absence of a 100-fold excess of diethylstilboestrol to determine non-specific binding (Anderson *et al.*, 1972). These values have been corrected to give the specific binding activity. All samples were adsorbed on hydroxyapatite and were washed three times with 10mM-Tris/HCl/1.5mM-EDTA buffer, pH7.4. Hydroxyapatite was also added to the nuclear pellets after the exchange assay to trap any oestradiol–receptor complexes that might otherwise be lost in the washing procedure. For studies in which nuclear oestrogen–receptor complexes were generated *in vitro*, uteri from five rats were placed in 8.7ml of Eagle's minimal essential medium in a 25ml Erlenmeyer flask containing 20nM-[^{3}H]oestradiol in the presence or absence of 2μM-diethylstilboestrol to determine non-specific binding. This was gassed with O_2/CO_2 (19:1) and incubated on a shaking water bath at 37°C for 1h. The uteri were then placed into ice-cold 0.9% NaCl and processed by the hexylene glycol procedure for nuclear purification. The parallel preparations were adjusted to equal DNA concentrations in the appropriate digestion buffers, so that the results obtained were equivalent to those obtained by the exchange assay. Samples processed in this manner could be counted for radioactivity directly or adsorbed on hydroxyapatite for receptor assay. In all preparations total DNA was determined by the diphenylamine procedure of Burton (1956). The production of DNA fragments soluble in 0.4M-$HClO_4$ was used to monitor the extent of nuclease digestion of chromatin.

Nuclease digestions

The purified nuclei were washed and resuspended in the appropriate digestion buffer before the addition of nuclease. The basic buffer A contained 50mM-Tris/HCl buffer, pH7.4, 25mM-KCl, 5mM-$MgCl_2$ and 0.25M-sucrose, and was used for studies with DNAase I. Buffer B contained in addition 0.5mM-$CaCl_2$ for studies with micrococcal nuclease. DNAase I (EC 3.1.21.1) was prepared fresh in 0.9% NaCl before each experiment. Micrococcal nuclease (EC 3.1.31.1) was initially dissolved at 1mg/ml in distilled water and was stored frozen until use. Enzyme units were as defined by the supplier. One unit of DNAase I causes an increase of 0.001A_{260}/min per ml at 25°C in a solution of highly polymerized DNA at pH5.0. One unit of micrococcal nuclease causes an increase of 1.0 in the A_{260} of acid-soluble oligonucleotides produced by 30min digestion of calf thymus DNA at 37°C at pH8.0. Nuclear digestions were performed at 20°C or at 2°C as indicated in the Figure legends and were stopped by the addition of 10mM-EDTA. The nuclei were pelleted by centrifugation at 1000*g* for 10min, and the resulting supernatant (SF1) was removed. The nuclear pellet was resuspended in Tris/EDTA buffer for 30min and centrifuged again to yield the

final pellet fraction and the second supernatant fraction (SF2). Most of the solubilized oestrogen-receptor activity was present in fraction SF1 along with the smaller chromatin fragments. The sum of the receptor assays in fractions SF1 and SF2 is presented at each point. Before analysis on sucrose density gradients, some of the supernatant fractions were treated very briefly with dextran-coated charcoal to remove any unbound [^{3}H]oestradiol that may be present.

Sucrose density gradients

Linear 5–20% (w/v) sucrose gradients were prepared in Tris/EDTA buffer in polyallomer tubes with a Beckman gradient-forming apparatus. For studies on the size distribution of chromatin fragments, 0.5ml samples were layered on to 12ml gradients made in Tris/EDTA buffer and were centrifuged at 230000 $g_{max.}$ for 16h at 2°C in an SW40 rotor of a Beckman L5-65 ultracentrifuge. The markers used in these gradients were ^{14}C-labelled immunoglobulin G (7S) and nucleosome monomers (11S). Fractions (0.5ml) were collected at 2°C by pumping from the bottom, and the chromatin fractionation patterns were analysed by u.v. absorbance at 260nm before radioactivity determination. For studies on the released receptor only, 0.25ml samples were layered on 10–30% (w/v) sucrose gradients (5ml) made in Tris/EDTA buffer or Tris/EDTA buffer containing 0.4M-KCl and were centrifuged at 243000 $g_{max.}$ for 20h at 2°C in an SW50.1 rotor of a L3-50 ultracentrifuge. The markers used in these gradients were ^{14}C-labelled ovalbumin (3.7S) and ^{14}C-labelled immunoglobulin G. Fractions (0.2ml) were collected dropwise.

Chemicals

[2,4,6,7-^{3}H]Oestradiol (95Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Diethylstilboestrol, oestradiol, Tris, EDTA (disodium salt) and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nafoxidine (U11100-A; 1-{2-[*p*-(3,4-dihydro-6-methoxy-2-phenylnaphth-1-yl)phenoxy]ethyl}pyrrolidine hydrochloride) was provided by Dr. J. Wilkes of the Upjohn Co. (Kalamazoo, MI, U.S.A.). Pipes (1,4-piperazinediethanesulphonic acid) (sodium salt monohydrate) and dithiothreitol were obtained from Calbiochem (Los Angeles, CA, U.S.A.). 2-Methylpentane-2,4-diol (hexylene glycol) was obtained from Eastman (Rochester, NY, U.S.A.). Eagle's minimal essential medium without glutamine was obtained from Grand Island Biological Co. (Grand Island, NY, U.S.A.). Sucrose (ribonuclease-free) was obtained from Bethesda Research Laboratories (Bethesda, MD, U.S.A.). Micrococcal nuclease and DNAase I (ribonuclease-free) were obtained from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Hydroxyapatite was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals were of the highest quality available.

Radioactivity determinations

[^{3}H]Oestradiol was extracted from hydroxyapatite pellets with 1ml of ethanol, and this extract was added to 4ml of ACS (Amersham/Searle, Arlington Heights, IL, U.S.A.). Sucrose gradient fractions received 0.4ml of distilled water and 5ml of ACS. Radioactivity was determined in a Beckman LS233 liquid-scintillation spectrometer with approx. 30% counting efficiency.

Results

Limited digestion of nuclei prepared 1h after oestradiol treatment in vivo

Within 1h after a 5μg injection of oestradiol virtually all of the uterine oestrogen receptors are localized in the nucleus. We have previously reported that only a fraction of these receptors (approx. 25%) is required for the full uterotropic response (Clark & Peck, 1976). When highly purified nuclei obtained 1h after oestradiol were digested with low concentrations of DNAase I (50units/ml), 30% of the oestrogen receptors were solubilized within 5min, along with 1–2% of the DNA (Fig. 1). At this point the production of soluble receptors ceased, although production of acid-soluble DNA continued throughout the interval. The recovery of solubilized receptors and those remaining in the nucleus was stoicheiometric, and the addition of the proteinase inhibitor phenylmethanesulphonyl fluoride did not affect the distribution. Identical results were also obtained with micrococcal nuclease when the digestion was performed in buffer B, containing 0.5mM-Ca^{2+}. If a second batch of nuclease was added at $t = 10$min, no change occurred in the receptor distribution; however, there was an additive increase in the production of acid-soluble DNA.

Limited digestion of nuclei after oestrogen-receptor translocation by [^{3}H]oestradiol in vitro

One of the standard procedures for the preparation of radiolabelled nuclear oestrogen–receptor complexes is to incubate whole uteri (or in some cases uterine endometrium) at 37°C in tissue-culture medium containing [^{3}H]oestradiol. The resultant cytoplasmic depletion and nuclear accumulation of oestrogen receptors has been taken to reflect the situation observed after an injection *in vivo*; however, there has been little information to support this assumption in the literature. To determine if receptors translocated *in vitro* were associated with nuclease-sensitive and -resistant sites, as observed *in*

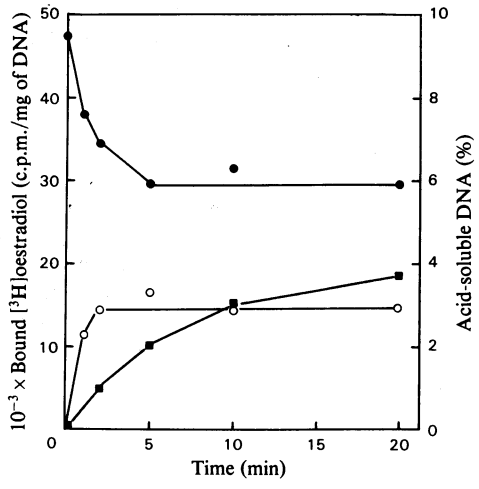

Fig. 1. *Time course for nuclear receptor solubilization with respect to DNA (1 h after oestradiol treatment)*
Purified rat uterine nuclei were prepared 1 h after an injection of oestradiol, suspended in buffer A and digested with 50 units of DNAase I/ml. After termination, the nuclear pellet (●) and supernatant (○) fractions were separated and assayed independently by exchange at 37°C with 8 nM-[^{3}H]-oestradiol ± 800 nM-diethylstilbeostrol to determine specific binding activity. Both the supernatant and pellet fractions were adsorbed on hydroxyapatite, washed three times with Tris/EDTA buffer, and extracted with ethanol. (The hydroxyapatite was added to the pellet fraction to adsorb any receptors that might be lost in the washing procedure.) Parallel nuclear samples received an equal volume of cold 0.8 M-$HClO_4$, and the A_{260} of these supernatant fractions was measured after 30 min at 2°C to determine the yield of acid-soluble DNA (■).

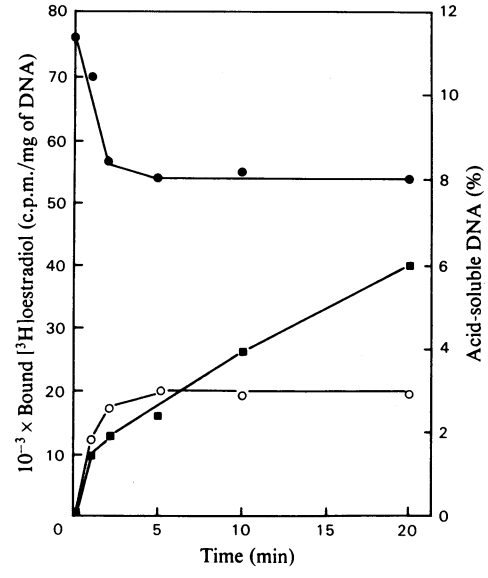

Fig. 2. *Nuclear receptor solubilization by DNAase I after translocation in vitro with [^{3}H]oestradiol ± a 100-fold excess of diethylstilboestrol*
Uteri from 7-day-castrated rats were incubated for 1 h at 37°C in Eagle's minimal essential medium containing 20 nM-[^{3}H]oestradiol or 20 nM-[^{3}H]-oestradiol + 2 μM-diethylstilboestrol. The nuclei from these preparations were purified separately, adjusted to the same DNA concentration, and digested at 2°C with 250 units of DNAase I/ml. The nuclear pellet (●) and supernatant (○) fractions were separated and placed directly into scintillation vials for radioactivity determination. Parallel samples were assayed as in Fig. 1 for acid-soluble DNA production (■). The same result was obtained if the samples were adsorbed on hydroxyapatite, washed and extracted with ethanol. The data are corrected to reflect the specific binding activity.

vivo, uteri from untreated animals were incubated for 1 h at 37°C *in vitro* with 20 nM-[^{3}H]oestradiol ± 2 μM-diethylstilboestrol. The nuclei from these preparations were purified separately and, in the first experiments, digested at 20°C with 50 units of DNAase I or micrococcal nuclease/ml to yield a pattern identical with that obtained from injected animals in Fig. 1 (results not shown). We also digested these nuclei at 2°C with 250 units of DNAase I or micrococcal nuclease/ml to increase our flexibility of reaction conditions and to control for possible temperature-induced artifacts of receptor aggregation. The data in Fig. 2 indicate that the pattern of differential solubilization of receptor and DNA by exogenous nucleases was retained under these conditions. The limited release of approx. 26% of the nuclear oestrogen receptors at 2°C was virtually identical with that obtained from rat uterine nuclei 1 h after an injection *in vivo*. Therefore, by these criteria, receptors translocated *in vitro* appear to recognize the same sites in the nucleus as those translocated by an injection of oestradiol *in vivo*.

Limited digestion of nuclei after prolonged uterine stimulation with oestradiol and Nafoxidine

A single injection of either oestradiol or the triphenylethylene compound Nafoxidine results in a uterotropic response that culminates after 24 and 48 h respectively in DNA synthesis. It has been well established by this laboratory and by others that oestrogen receptors that accumulate in uterine nuclei after an injection of oestradiol are subsequently 'processed' (Clark & Peck, 1976, 1979; Horwitz & McGuire, 1978), so that by 12 h they reach a substantially lower steady-state value than that at 1 h. After an injection of Nafoxidine, however,

oestrogen receptors accumulate in the nucleus and remain there for prolonged periods of time, perhaps owing to a block in receptor processing.

The data presented in Fig. 3 show that most (78%) of the nuclear receptors remaining 12h after an injection of oestradiol are rapidly solubilized by mild nuclease digestion. This result is in sharp contrast with the situation seen 1h after oestradiol injection (Fig. 1), where a much smaller percentage of the receptors was solubilized. It is noteworthy that the total number of nuclear receptor sites [9×10^3c.p.m. (0.22pmol)/mg of DNA] present 12h after oestradiol treatment is much lower than that 1h after oestradiol [47×10^3–76×10^3c.p.m. (1–1.2pmol)/mg of DNA]. This is in accord with the data noted previously on processing of the nuclear receptor–oestrogen complex.

When animals were injected with Nafoxidine 16h before being killed, the digestion pattern obtained with mild nuclease treatment is different from that observed 12h after oestradiol. In Fig. 4, the overall solubilization of receptors was virtually identical with that observed 1h after oestradiol (31%), and it did not approach the extent observed 12h after oestradiol treatment (Fig. 3). An elevated nuclear receptor content [82×10^3c.p.m. (1.3pmol)/mg of DNA] was maintained over a 40h period and the pattern of nuclease-catalysed receptor solubilization remained unchanged. These data are consistent with the idea that Nafoxidine affects a block in nuclear receptor processing.

Sucrose-density-gradient analysis of receptors solubilized by nuclease digestion

The above results indicate that the nuclease-sensitive oestrogen receptor sites were preferentially solubilized with respect to the bulk of the total chromatin. However, these data gave little indication of the physical association state(s) of these proteins with chromatin. To examine this, the soluble fractions generated by nuclease digestion were centrifuged through sucrose density gradients that were designed to resolve large chromatin fragments (13ml, SW40 rotor). In the following experiments, uteri were incubated *in vitro* with [^{3}H]oestradiol ± a 100-fold excess of diethylstilboestrol, and nuclei purified from these preparations were digested at 2°C with 500 units of micrococcal nuclease/mg of DNA per ml as in Fig. 3.

When the digestion was performed under these conditions no solubilized receptors could be detected at $t = 0$ on the gradients. However, by 5min of digestion a peak of bound radioactivity that sedimented more slowly than 7S was observed (Fig. 5), along with some heavy pieces of chromatin that were found at the bottom of the gradient. By $t = 10$–20min the amount of 'free' receptor ceased to change; however, substantial changes occurred in

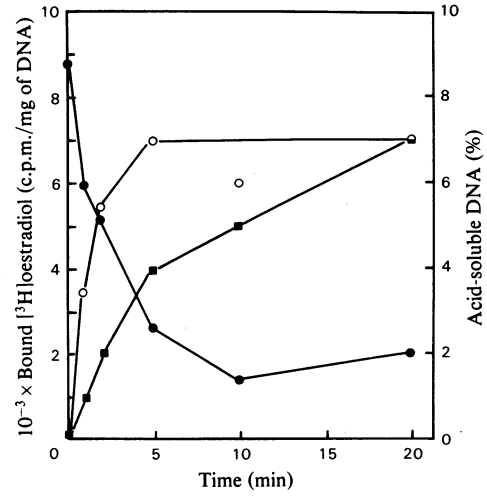

Fig. 3. *Time course for nuclear receptor solubilization by DNAase I (12h after oestradiol treatment)*
Purified rat uterine nuclei were prepared 12h after an injection of oestradiol, suspended in buffer A and digested with 50 units of DNAase I/ml at 20°C. The supernatant (○), nuclear pellet (●) and acid-soluble DNA (■) fractions were assayed as described in Fig. 1 legend, and the data represent the specifically bound radioactivity.

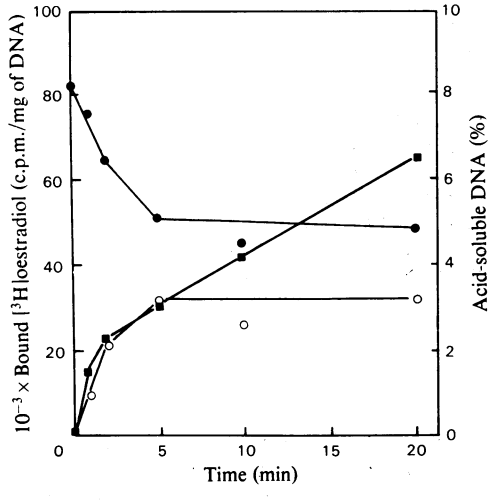

Fig. 4. *Time course for nuclear receptor solubilization by micrococcal nuclease (16h after Nafoxidine treatment)*
Purified rat uterine nuclei were prepared 16h after an injection of Nafoxidine, suspended in buffer B and digested with 50 units of micrococcal nuclease/ml at 20°C. The supernatant (○), nuclear pellet (●) and acid-soluble DNA (■) fractions were assayed as described in Fig. 1 legend, and the data represent the specifically bound radioactivity.

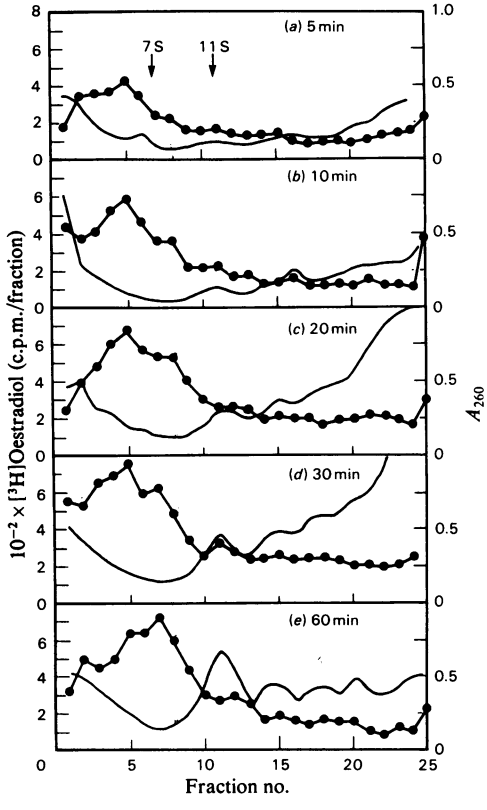

Fig. 5. *Sucrose-density-gradient analysis of receptor and chromatin solubilization by micrococcal nuclease*
Radiolabelled nuclear oestrogen–receptor complexes were generated by the '*in vitro*' methodology described in Fig. 2 legend. Purified nuclei were then suspended in buffer B and digested with micrococcal nuclease at 2°C for the following times: (*a*) 5 min; (*b*) 10 min; (*c*) 20 min; (*d*) 30 min; (*e*) 60 min. The supernatant fractions obtained from these preparations (0.5 ml) were layered on linear 5–20% sucrose density gradients in Tris/EDTA buffer and centrifuged at 230000 *g* for 16 h in an SW40 rotor; 0.5 ml fractions were collected, and the data represent specific binding activity. ●, [^{3}H]oestradiol; ——, A_{260}.

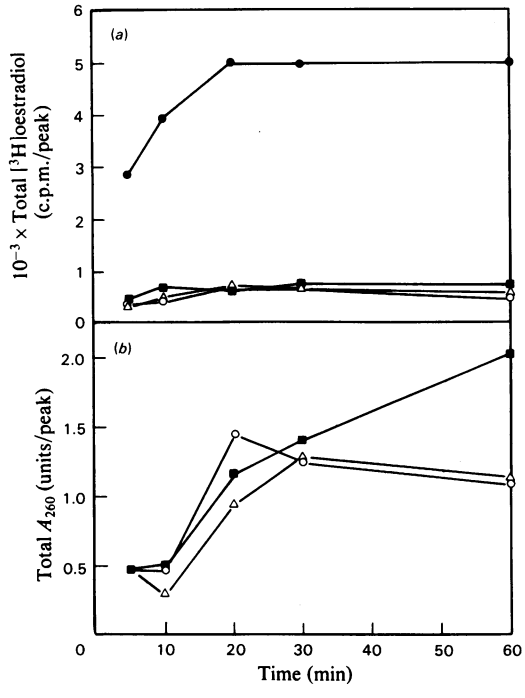

Fig. 6. *Time course for receptor solubilization with respect to the generation of chromatin subunits by micrococcal nuclease*
(*a*) The total areas under the pattern for [^{3}H]-oestradiol distribution in Fig. 5 were determined for those fractions that corresponded to each of the chromatin subunits (■, mono-, △, di-, and ○, tri-nucleosomes) and for free receptors (●) (< 11 S). These are plotted as a function of the time of digestion. (*b*) The total areas under the pattern for chromatin distribution (A_{260}) in Fig. 5 were determined for those fractions that corresponded to each of the chromatin subunits (■, mono-, △, di-, and, ○, tri-nucleosomes). These are plotted as a function of the time of digestion.

the size distribution of the chromatin fragments. At $t = 20$ min dinucleosomes were evident, along with a large amount of heavier pieces at the bottom of the gradient. At $t = 30$–60 min, the latter fractions appeared to be converted into smaller oligomeric forms, such that by 60 min the predominant species was the mononucleosome. At no time in the digestion process were nuclear oestrogen receptors observed in association with discrete chromatin fragments. Rather, they were always found as a diffuse peak that sedimented somewhat more slowly than 7S. Similar results were obtained for digestion with DNAase I, although in this case digestion continued past the mononucleosome form to produce small chromatin fragments, which were found at the top of the gradient (results not shown).

To assess more accurately the relative production of free oestrogen receptors with respect to the various chromatin fractions, the areas under the curves in Fig. 5 were determined and plotted as a function of the time of digestion. In Fig. 6(*a*) the relative amount of [^{3}H]oestradiol associated with chromatin subunits remained constant throughout the interval; however, the quality of 'soluble' (less than 7S) receptors increased up to 20 min, after which it remained constant. The data in Fig. 6(*b*)

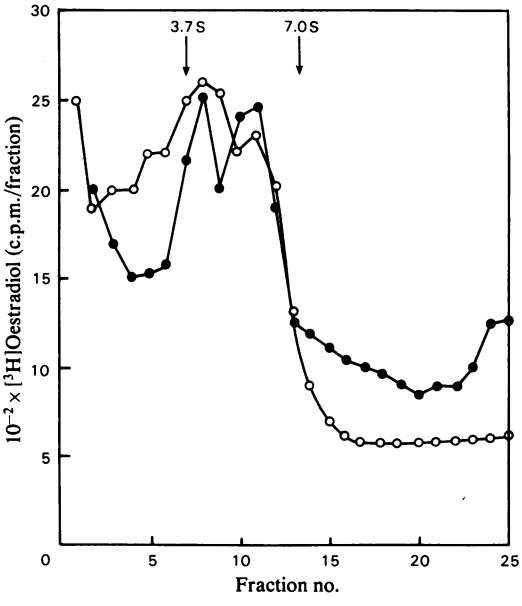

Fig. 7. *Sucrose-density-gradient analysis of receptors solubilized by micrococcal nuclease*

Radiolabelled nuclei were prepared and digested with micrococcal nuclease for 20min under the same conditions as described in Fig. 5 legend. Samples (0.25ml) were then layered on linear 10–30% sucrose gradients in Tris/EDTA buffer (●) or Tris/EDTA buffer containing 0.4M-KCl (○) buffer and centrifuged at 243000*g* for 20h in an SW50.1 rotor; 0.2ml fractions were collected, and the data represent specific binding activity.

show that little change occurred in the chromatin fractions between 5 and 10min; however, after this interval, there were substantial alterations in the amount and distribution of these fragments. As expected, the net effect was a conversion of chromatin into oligomeric forms and ultimately into mononucleosomes.

The nature of the diffuse peak of solubilized radioactivity became apparent when the soluble fraction generated by a 20min digestion with micrococcal nuclease was sedimented through a smaller and steeper sucrose density gradient. The data in Fig. 7 indicate that the solubilized receptors consist of a doublet of 4.5S and 5.7S forms, very much like the 4S and 6S doublet reported by Andre *et al.* (1978) for a similar preparation from lamb endometrium. They found that these forms were all converted into a single 4S peak in 0.4M-KCl; however, in the present study the 4.5S and 5.7S doublet was retained on sedimentation into 0.4M-KCl. The reason for the discrepancy in these results is not known, but may be due to differences in experimental conditions.

Discussion

The results obtained in the present study can be summarized in three main points. (1) When uterine nuclei are digested with exogenous nucleases after oestrogen-receptor translocation, only a portion of the receptors are solubilized. (2) The rate of receptor solubilization is independent of the rate of DNA hydrolysis. (3) The actual percentage of the receptors that is solubilized is dependent on the regimen of steroid (or antagonist) administration; however, the total number of nuclease-sensitive receptor sites (on a per-mg-of-DNA basis) remains fairly constant under all treatment conditions.

When purified nuclei that were obtained 1h after an injection of oestradiol were digested with low concentrations of DNAase I (Fig. 1) or micrococcal nuclease, receptor solubilization was rapid and usually reached a plateau by 5min of reaction. Usually 30% of the nuclear receptors were solubilized at this point, and this occurred with the conversion of 1% of the DNA into acid-soluble fragments. Although DNA hydrolysis continued throughout the 20–30min interval under study, no further receptor release occurred, even after addition of a second batch of nuclease at a delayed time point. The receptors released by nuclease digestion did not reassociate with either undigested control nuclei or nuclei that had been digested with either enzyme (results not shown).

Since it appeared from these data that only a certain fraction of nuclear receptors was associated with nuclease-sensitive sites, we chose to examine the relative distribution of receptors with respect to such sites after translocation *in vitro* by [^{3}H]-oestradiol. The data in Fig. 2 indicate that receptors that were translocated *in vitro* by a 1h incubation of uteri with [^{3}H]oestradiol in the presence or absence of a 100-fold excess of diethylstilboestrol associate with nuclease-sensitive and -resistant sites in a manner that is both qualitatively and quantitatively similar to that seen with injection *in vivo*. By the criterion of nuclease-dependent solubilization it therefore appears that the receptors translocated *in vivo* or *in vitro* become associated with the same target molecules in the nuclear milieu.

To extend this approach we examined the association states of nuclear oestrogen receptors produced by oestradiol and the oestrogen antagonist Nafoxidine. Previous studies have shown that an apparent excess of nuclear oestrogen receptors accumulates within the first 1h after an injection of oestradiol, and that those molecules not associated with sites that are required for the full uterotropic response are subsequently 'processed' out (Clark & Peck, 1976; Horwitz & McGuire, 1978). In this way, only those receptors that remain in the nucleus 12h after an injection of oestradiol should be

associated with the putative 'acceptor' sites, and they should also correspond to the salt-resistant (or non-extractable) sites reported in the literature. When nuclei were obtained from rats that had received oestradiol 12h before death, most (78%) of the oestrogen receptors were nuclease-sensitive (Fig. 3). A comparison of the overall quantity of nuclease-sensitive and -resistant sites 1h and 12h after oestradiol proved revealing. At 1h after oestradiol there was 1.00pmol of resistant sites/mg of DNA and 0.18pmol of sensitive sites/mg of DNA. By 12h after oestradiol the quantity of resistant sites declined to 0.10pmol/mg of DNA, whereas the nuclease-sensitive sites only declined to 0.11pmol/mg of DNA. These data suggest that the 'processing' of nuclear oestrogen receptors occurs on those sites that are resistant to nucleolytic breakdown. The nuclease-sensitive sites may very well reside on chromatin and correspond to the salt-resistant sites described above; however, no attempt was made in the present study to examine this point.

The triphenylethylene derivative Nafoxidine exhibits mixed agonist/antagonist effects on the oestrogen-responsiveness of the uterus. When given as a single injection to an oestrogen-deficient animal, Nafoxidine serves to hyperoestrogenize the uterus, exerting a prolonged stimulation of water imbibition, RNA polymerase activity, growth and nuclear oestrogen-receptor retention (Clark *et al.*, 1974, 1978; Capony & Rochefort, 1975). The latter effect is believed to be due to the inhibition of receptor processing, and the concomitant deficit (or lack of replenishment) in cytoplasmic oestrogen receptors renders the uterus intractable to subsequent stimulation by oestradiol. These effects may, in part, be due to a long half-life for Nafoxidine in the body, as has been reported for the related triphenylethylene derivative compound CI-628 (Katzenellenbogen *et al.*, 1978). When nuclei were obtained from rats that had been injected with Nafoxidine 16 or 40h before death, nuclease digestion yielded a pattern of receptor solubilization (Fig. 4) that was both qualitatively and quantitatively similar to that observed in Fig. 1 with animals that had been treated with oestradiol for 1h. The total number of nuclear oestrogen receptors is similar after 1h of oestradiol or 16h of Nafoxidine treatment. Digestion of nuclei after the latter treatment solubilized virtually the same percentage (31%) as that observed 1h after oestradiol treatment. The data therefore suggest that the nuclease-sensitive sites obtained with Nafoxidine correspond quantitatively to those seen 1h and 12h after oestradiol and may be a reflection of genomic loci in a transcriptionally active state. The prolonged maintenance of this distribution of nuclease-sensitive and -resistant sites by Nafoxidine supports the idea that members of this class of steroid antagonists exert a block in the processing of nuclear oestrogen receptors.

Some of the physical association states of the nuclease-sensitive receptor sites were examined by sucrose-density-gradient centrifugation under conditions that resolve nucleosome multimers. We found that there was no detectable receptor release in the absence of digestion; however, the nucleases catalysed the solubilization of a broad slowly sedimenting peak of bound [^{3}H]oestradiol (Fig. 5). This material sedimented more slowly than 7S, and its appearance did not appear to correlate with that of the various nucleosome fragments. At no time were solubilized oestrogen receptors found in association with chromatin subunits under these experimental conditions. This result was more graphically demonstrated by integrating the areas under the receptor and chromatin peaks and plotting these data as a function of time of digestion (Fig. 6). It is clear from these data that the rate of receptor solubilization does not correspond to the rate of nucleosome fractionation, a result that supports the previous data (Figs. 1–4) comparing the rate of receptor solubilization with the production of acid-soluble DNA fragments.

The broad peak of solubilized nuclear oestrogen receptors observed in Fig. 5 is composed of a doublet of 4.5S and 5.7S entities (Fig. 7). This result corresponds closely to that obtained by Andre *et al.* (1978), when they digested nuclei from lamb endometrium under somewhat more vigorous conditions. They also reported the conversion of the two receptor forms into a single 3.8S species in 0.4M-KCl; however, we did not obtain any change in the sedimentation properties of the doublet under these conditions. We believe that the 5.7S species may be equivalent to the 6S form that can be extracted from nuclei by salt. It may consist of a 4.5S 'monomer' stably associated with a chromatin component that causes it to sediment faster. Preliminary results suggest that DNA may be involved in this aggregate, since digestion with DNAase I can produce the 4.5S peak without the 5.7S form (D. R. Schoenberg & J. H. Clark, unpublished work). This result also suggests that the 6S receptor extracted by salt from the nucleus of oestrogen-treated rat uteri is not identical with the so-called 'activated' 6S complex that can be generated by warming cytosol in the presence of [^{3}H]oestradiol (Clark & Peck, 1979).

The preceding discussion has dealt primarily with those receptor molecules that could be solubilized by nucleases. It is clear that this represents only one class of receptors; those remaining belong to the class of nuclease-resistant sites. The results obtained in the present study shed little light on the nature of these sites. The data suggest that most of the nuclear oestrogen receptors that are processed between 1 and 12h after oestradiol belong to this class.

Furthermore, Nafoxidine induces the prolonged maintenance of these sites, a result that is in accord with the inhibition of receptor processing caused by this drug. It is quite possible, therefore, that the nuclease-resistant sites correspond to those that are not essential for the uterine growth response, but may play a role in some of the other uterotropic effects of oestrogens. Qualitatively and quantitatively, this situation resembles previous results reported by our laboratory that were obtained by differential steroid regimens and salt extractions (Clark *et al.*, 1974; Clark & Peck, 1976). The physical nature of the nuclease-resistant sites remains an enigma. Reports by Barrack *et al.* (1977) and work from our own laboratory (results not shown) indicate that these sites may be associated with the nuclear matrix. No attempts were made in the present study to examine this thoroughly; however, this concept offers some interesting possibilities for the mechanism of oestrogen-receptor action.

Finally, we would like to speculate on the nature of the nuclease-sensitive sites. A considerable literature exists on the preferential sensitivity of actively transcribed genes to digestion by low amounts of exogenous nucleases (Weintraub & Groudine, 1976; Gorel & Axel, 1976; Bloom & Anderson, 1978). Studies on both globin and ovalbumin genes have shown that such preferential digestion not only occurs on actively transcribing loci, but also reflects the 'transcriptional potential' of a given cell type. Recent reports in fact demonstrate that preferential nuclease-sensitivity extends to non-transcribed flanking regions of both the globin (Stalder *et al.*, 1980) and ovalbumin gene families (Lawson *et al.*, 1980). We believe that only a fraction of the receptors translocated to the nucleus by oestradiol associate with chromatin; the rest may associate with other non-chromatin targets. All of the chromatin-bound receptors may be associated with regions that are in an altered conformation so as to have an enhanced potential to be transcribed, and this conformation renders these loci preferentially susceptible to nuclease attack. This idea is supported by the paper of Scott & Frankel (1980), in which nuclear oestrogen receptors from MCF-7 mammary-gland tumour cells were found in association with a transcriptionally active region of chromatin. It should be noted that the quantity of nuclease-sensitive receptor sites shows a relatively small decrease during the interval from 1 to 12h after oestradiol. It has been well established that the uterine growth response to oestradiol requires the prolonged maintenance of a similar quantity of nuclear oestrogen receptors.

The solubilization of nuclear oestrogen receptors by the nucleases used in the present study occurs at a rate much faster than that for the hydrolysis of DNA (Fig. 1) or the fractionation of chromatin subunits (Figs. 5 and 6). Furthermore, the solubilized receptors were never found associated with chromatin fragments. These data indicate that receptors most likely are associated with a chromatin structure that is rapidly, and perhaps completely, degraded. Were the receptors bound to the internucleosomal linker region, as reported by Rennie (1979) for the androgen receptor, we would expect to have found them on higher oligomers of chromatin. On the other hand, random distribution of receptors should yield fractions bound to both the nucleosome core particle and the linker region, as reported by Senior & Frankel (1978). Since we obtained neither of these results, it is our belief that the nuclear oestrogen receptors observed in the present study associate with a limited, specific, set of chromatin loci, and these loci may be located in a transcriptional 'domain' that is specific for a given cell type. These receptors are not 'processed' like those associated with the nuclease-insensitive loci, but rather remain in place throughout the rather prolonged interval required for the uterine growth response.

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