Exclusive nuclear location of estrogen receptors in *Squalus* testis

(steroid action/DNA affinity/osmolarity/elasmobranchs)

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ABSTRACT An estrogen (E)-binding molecule having both occupied and unoccupied sites is restricted to nuclear subfractions in the testis of the spiny dogfish (*Squalus acanthias*). We investigated the hypothesis that a species characterized by high body-fluid osmolarity (1010 mosM) has an estrogen receptor (ER) that binds to chromatin with high affinity and consequently resists redistribution during tissue processing. Although the steroid binding and sedimentation properties of the *Squalus* nuclear ER conformed to those of classical ER, its elution maximum from DNA-cellulose was unusually high (0.55 M NaCl). A tendency to adhere tightly to cell nuclei was reflected in the high salt concentration (0.43 M KCl) required to extract 50% of the receptors from the nuclear compartment during homogenization and in the stability of the nuclear ER population in the presence of high concentrations of a nonionic solute (urea) or increased buffer volume. Mixing and redistribution experiments showed that nuclear ER could be quantitatively and qualitatively measured in cytosolic extracts, ruling out the possibility that soluble receptors were being masked. Although *Squalus* oviduct ER was similar to that of testis, ER in the testis and liver of a related elasmobranch (*Potamotrygon*) that maintains osmotic equilibrium at 300 mosM more closely resembled mammalian ER in its elution maximum from DNA-cellulose (0.22 M NaCl) and cytosolic/nuclear ratios in low-salt buffers. We conclude that *Squalus* testis has a single ER pool located exclusively in the nuclear compartment. These observations support a revised concept of steroid action and further indicate that the chromatin affinity of the hormone-ER complex is an important factor in determining subfractional distribution during tissue processing.

The classical model of steroid hormone action includes both nuclear and extranuclear receptors (1). Although this concept has been supported by the majority of studies in mammals, birds, reptiles, and amphibians (1-5), recent reports claim that mammalian estrogen receptors (ER) are restricted to cell nuclei when assay methods avoid redistribution artefacts inherent in homogenization procedures (6, 7). However, despite conventional methods of tissue processing, there are several systems in which steroid receptors are detectable only in nuclear subfractions. These include ER in unstimulated chicken (8) and frog (9) liver and in hormone-free tumor cells from pituitary (10) and breast (11); 1,25-dihydroxyvitamin D₃ (secosteroid) receptors in the intestinal mucosa of chicks (12-15); and the ecdysterone receptor in *Drosophila* imaginal discs (16). The basis for these exceptions is not readily apparent. Recently, we identified an estradiol (E)-binding component having both occupied and unoccupied sites in nuclear subfractions of dogfish testis (*Squalus acanthias*; subclass, Elasmobranchii)*. In contrast to other systems, this model offers a testable hypothesis. A striking feature of marine elasmobranchs like *Squalus*, as compared to mammals and most other vertebrates, is an exceptionally high body-fluid osmolarity (1010 vs. 300 mosM), enabling them to equilibrate with seawater (17). Total solutes and their major constituent (urea = 350 mosM) are equally high inside and outside the cell. These conditions, namely high solute and/or low free-water concentration, have been used experimentally in mammalian systems to disrupt the relatively weak, noncovalent bonding between chromatin and receptors (12, 18, 19). We reasoned, therefore, that the *Squalus* ER would require a higher affinity for nuclei in order to counteract effects of the internal milieu and, as a consequence, would be more resistant to extraction from the nuclear compartment during tissue processing.

In the experiments reported here, our major purpose was to assess the DNA- and nuclear-adhering ("affinity") properties of the *Squalus* testicular ER. At the same time, it was necessary to establish that the ER of *Squalus* testis conformed to the essential features of steroid receptors (high affinity, limited capacity, estrogen specificity) and to rule out the possibility that unknown factors somehow interfered with ligand binding and, hence, detection of ER in cytosol. Since the elasmobranchs also are represented by species that have evolved for long periods in freshwater and exhibit a body fluid composition similar to mammals (20), we had a unique opportunity to evaluate the importance of physiological adaptation in determining ER characteristics in phylogenetically related groups.

MATERIALS AND METHODS

Animals. Mature dogfish (*Squalus acanthias*) were collected at the Mount Desert Island Biological Laboratory, Salsbury Cove, ME, or the Marine Biological Laboratory, Woods Hole, MA, and were held in running seawater aquaria without feeding for no longer than 1 week. Two immature male freshwater stingrays (*Potamotrygon*, ssp.), which are native to South America (20), were purchased locally. Animals were decapitated, and the testes or other tissues were removed to iced dishes. Because of the undeveloped state of *Potamotrygon* testis, it was not separable from the larger mass of surrounding epigonal (lymphoid) tissue; however, the testicular component was verified histologically. Epigonal tissue itself is ER-negative*.

Chemicals and Reagents. [2,4,6,7-³H]E (specific activity, 93-105 Ci/mmol; 1 Ci = 37 GBq), ¹⁴C-labeled bovine serum albumin (specific activity, 20 Ci/mg), and 11 β -methoxy-19-nor-17 α -pregna-1,3,5(10)-trien-20-yne-3,17 β -diol (moxestrol, R2858) were purchased from New England Nuclear. [³H]E was further purified by TLC with ether/hexane, 3:1 (vol/vol). Radioinert steroids were obtained from Sigma or Steraloids, Wilton, NH. Bovine serum albumin (Pentex) was

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Abbreviations: E, 17 β -estradiol; ER, estrogen receptor(s); DHT, 5 α -dihydrotestosterone.

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purchased from Miles; calf thymus DNA, from Worthington; Cellex 410 cellulose, from Bio-Rad; Sephadex LH-20 gel, from Pharmacia; and PPO (2,5-diphenyloxazole) and POPOP 1,4-bis[5-phenyl(oxazolyl)]benzene, from Research Products International, Mt. Prospect, IL. Other chemicals were reagent grade. The buffers used were as follows: tissue homogenizing buffer [buffer H: 50 mM Tris-HCl/1 mM EDTA/12 mM monothioglycerol/30% (vol/vol) glycerol, pH 7.5] containing 0, 0.25, 0.5, or 1.0 M KCl or 0, 0.5, or 1.0 M urea; washing buffer for nuclear pellets [buffer W: 10 mM Tris-HCl/3 mM MgCl₂/2 mM monothioglycerol/0.25 M sucrose, pH 7.5]; extraction buffer for nuclear pellets [buffer E: 0.7 M KCl in buffer H, pH 7.5]; elution buffers for DNA-cellulose affinity chromatography [TEMG buffer: 10 mM Tris-HCl/1 mM EDTA/1 mM 2-mercaptoethanol/10% (vol/vol) glycerol, pH 7.5] containing 0.05, 0.4, or 1.0 M NaCl or 0.5 or 1.0 M urea, each with 0.2 mg of bovine serum albumin per ml; and elution buffers for Sephadex LH-20 columns, which contained the same KCl concentration as in the samples, 0 M (for cytosol) or 0.5 M (for nuclear extracts), in TEMG buffer.

Tissue Preparation. This and all subsequent procedures were carried out at 4°C. Tissues were washed and homogenized (1:4 tissue weight per final volume) in buffer H containing 0 M KCl (or as specified) by three 5-sec bursts with a Polytron PT-10/35 homogenizer (Brinkmann). The homogenate was centrifuged at 1000 × g for 15 min, and the resulting supernatant was centrifuged at 100,000 × g for 1 hr (cytosol). The crude pellet from centrifugation at 1000 × g was washed three times with buffer W (10 vol), incubated with buffer E for 1 hr with vigorous mixing every 15 min, and centrifuged at 100,000 × g for 1 hr (nuclear extract). Samples were stored frozen (−70°C) no longer than 1 month. No degradation was observed during this time.

Sephadex LH-20 Column Chromatography. Cytosolic or nuclear extracts (0.4 ml) were incubated with 10 nM [³H]E with or without a 100-fold excess of radioinert E at 4°C for 15–18 hr (cytosol) or at 22°C for 1 hr (nuclear extract). Free and bound [³H]E were separated on LH-20 columns at 4°C by procedures previously described for measuring ER in lower vertebrates (4, 5). Specific ER binding was calculated as the difference between [³H]E bound in the absence (total) and presence (nonspecific) of 100-fold excess radioinert E.

DNA-Cellulose Affinity Chromatography. These methods have been used routinely in this laboratory to separate ER from sex steroid-binding proteins (2–5). For the prelabeling assay, tissue extracts were incubated with 10 nM [³H]E with or without a 100-fold excess of radioinert E at 4°C for 15–18 hr. For nuclear extracts or cytosol prepared in high-ionic-strength homogenizing buffers, the final molarity was adjusted to 0.1 M KCl prior to chromatography. Then samples were applied to the columns and incubated for 1 hr at 22°C followed by 15 min at 4°C. After an overnight washing with 0.05 M TEMG buffer to remove free steroid and non-DNA-adsorbing components, ER was eluted stepwise with 0.4 and/or 1.0 M TEMG buffer or with a linear NaCl gradient (0.05–1.0 M) in TEMG buffer. The concentration of NaCl in the eluates was determined by using a conductivity meter (CDM 3, Copenhagen). For the postlabeling assays, tissue extracts were applied to the columns and allowed to incubate at 4°C for 30 min. After being washed with 0.05 M TEMG buffer (6–8 bed volumes) to remove non-DNA-adsorbing components including endogenous free steroid, ER was labeled *in situ* with 10 nM [³H]E with or without a 100-fold excess of unlabeled E at 22°C for 1 hr followed by 15 min at 4°C. Subsequent elution procedures were the same as in the prelabeling technique to obtain specifically bound radioactivity.

Sucrose Gradient Centrifugation. Bound fractions (0.4 ml) obtained from LH-20 columns were mixed with ¹⁴C-labeled

bovine serum albumin (2 μl) and layered onto linear sucrose gradients (5–20%; 4 ml) prepared in TEMG buffer containing 0.5 M NaCl. Gradients were centrifuged at 219,000 × g for 4.5 hr (50,000 rpm in a Sorvall OTD-65 ultracentrifuge equipped with a W²dt integrator with a Sorvall TV 865B vertical rotor). Fractions (0.22 ml) were collected from the top by using an Auto Densi-Flow Unit (Buchler Instruments, Fort Lee, NJ).

RESULTS

Characteristics of ER in Nuclear Extracts. When nuclear extracts were incubated with [³H]E at 22°C, specific binding activity was maximal after 30 min and remained constant for 2 hr. Binding approached saturation at 5 nM E, and Scatchard plot analysis gave a K_d value of 1.5×10^{-9} M (Fig. 1A). Maximal binding capacity in the experiment shown was 82 fmol/g of whole testicular tissue but ranged from 40 to 250 fmol/g depending on the time of year and testicular region. E was found to be the best competitor, displacing 88% of the total [³H]E binding when present in 100-fold excess (Fig. 2). Estrone, moxestrol, and 5α-androstane-3β,17β-diol were somewhat less effective in displacing [³H]E, whereas estradiol, 5α-androstane-3α,17β-diol, testosterone, 5α-dihydrotestosterone (DHT), and progesterone were poor competitors. When [³H]DHT with or without a 100-fold excess of radioinert DHT was used under the same assay conditions, no specific binding was detected. When nuclear extracts were further analyzed on high-salt sucrose gradients, a specific E-binding macromolecule sedimented at 4.7–5 S (Fig. 3A).

Absence of ER in Cytosol. When crude cytosolic extracts prepared by homogenization in low-salt buffer were incubated with [³H]E in the presence or absence of radioinert E at 4°C for 18 hr or at 22°C for 1 hr, large amounts of bound

FIG. 1. (A) Saturation analysis of ER in nuclear extracts of *Squalus* testis. Samples were incubated with [³H]E with and without a 100-fold excess of radioinert E, and free and bound steroid were separated by LH-20 chromatography. (B) Saturation analysis of nuclear ER redistributed to cytosolic subfractions after homogenization of tissues in buffer H with 1.0 M KCl (for details see the legend to Fig. 5A). Specifically bound [³H]E was assayed by DNA-cellulose affinity chromatography (prelabeling conditions). The saturation analyses include saturation curves (Left) and Scatchard analysis of the same data (Right). Values were similar for three separate tissue preparations.

FIG. 2. Steroid-binding specificity of ER in nuclear extracts of *Squalus* testis. Samples (0.4 ml) were incubated for 1 hr at 22°C with [3 H]E (10 nM) with or without 1-fold, 10-fold, or 100-fold excess radioinert competitors: E₂, estradiol-17 β ; E₁, estrone; E₃, estriol; Mox, moxestrol; 3 β Diol, 5 α -androstane-3 β ,17 β -diol; 3 α Diol, 5 α -androstane-3 α ,17 β -diol; Prog, progesterone; DHT; and T, testosterone. Free and bound steroid were separated by LH-20 chromatography. Total binding in the absence of competitor was set at 100%. Points are values obtained from replicates (2-3) of a single tissue preparation. Two other preparations tested with competitor in 100-fold excess gave similar results.

[3 H]E (623 fmol/g of tissue) were eluted from LH-20 columns; however, this binding could be displaced equally well by radioinert E (73%), estrone (73%), testosterone (76%), and progesterone (68%). Estriol (39%) and DHT (54%) were somewhat less effective competitors. The E-binding component in crude cytosol sedimented at 9-10 S on a high-salt sucrose gradient and, thus, was distinctly different from ER (Fig. 3B). Since these characteristics resembled those of sex steroid-binding proteins previously reported in *Squalus* plasma (21), cytosolic extracts were analyzed by DNA-cellulose affinity chromatography to eliminate non-DNA-adhering components (2-5). No specific E-binding activity was detected in cytosol when the columns were eluted stepwise with elution buffers containing 0.05 and 1.0 M NaCl. This was true whether cytosol was analyzed under prelabeling or postlabeling conditions at exchange (22°C) or nonexchange (4°C) temperatures (data not shown). In order

FIG. 3. Sedimentation analysis of ER in nuclear extracts and crude cytosol of *Squalus* testis. (A) Nuclear extracts: total (●—●) and nonspecific (○—○). (B) Crude cytosol: total (▲—▲) and nonspecific (△—△). The arrow indicates the position of the internal marker bovine serum albumin (4.7 S). Three tissue preparations were tested.

FIG. 4. DNA-cellulose affinity chromatography of nuclear ER from two elasmobranchs, *Squalus* and *Potamotrygon*. Nonspecific binding is shown for *Squalus* testis only (□—□). Total estrogen binding: *Squalus* testis (■—■); *Potamotrygon* testis/epigonal tissue (●—●). Determinations were made on testis/epigonal organ and liver from two *Potamotrygon* specimens; results from *Squalus* testis were identical using three separate preparations.

to rule out the presence of proteolytic enzymes or other factors which might preclude detection of cytosolic ER in ligand-binding assays, nuclear extract containing a known amount of ER was mixed with an equal volume of crude cytosol. All ER was recovered by using either LH-20 or DNA-cellulose column chromatography (data not shown). Finally, possible inhibitory effects of urea on the binding assay *per se* were tested by adjusting nuclear extracts to a final urea concentration exceeding that in *Squalus* cytoplasm (1.0 or 5.0 M). No differences in specific binding were seen at saturating concentrations of [3 H]E.

Chromatographic Behavior of Nuclear ER on DNA-Cellulose. ER in nuclear extracts of *Squalus* testis were eluted at 0.55 M NaCl (Fig. 4). Buffers in which molarity was adjusted with urea (0-1 M) were not effective in eluting ER from DNA-cellulose (not shown). Nuclear extracts prepared from the testis/epigonal organ of the freshwater stingray (*Potamotrygon*) had a major component with an elution maximum of 0.22 M and a minor peak corresponding to 0.55 M NaCl. E-binding activity in nuclear extracts of liver from the same animal also was eluted at 0.22 M (not shown).

Effects of Salt, Urea, and Buffer Volume on Redistribution of ER. The adhering properties of the receptors to cell nuclei *in situ* were tested by varying the osmolarity and volume of buffer H. When *Squalus* testes were homogenized in KCl-free buffer at a tissue weight/final volume ratio of 1:4 (final tissue concentration, 0.25 g/ml), essentially all ER was found in the nuclear compartment (Fig. 5A). As the concentration of KCl in the homogenizing buffer increased from 0 to 1 M, this pool of receptors progressively decreased in the nuclear compartment and was recovered quantitatively in the cytosolic compartment. The concentration of KCl required to extract 50% of the nuclear receptors into the cytosol was $\approx$ 0.43 M. By contrast, homogenizing buffers adjusted to 0.5 and 1.0 M with urea were relatively ineffective in reducing nuclear-associated receptor (respectively, 92% and 71% of control levels) (not shown). By itself, dilution of the original homogenates a further 1:10 in salt-free buffer had little effect on ER associated with nuclei; however, a dilution effect became apparent and was potentiated by the addition of increasing amounts of KCl (Fig. 5B). As nuclear ER decreased progressively with increasing salt and buffer volume, binding activity was recovered in corresponding cytosols (not shown). Saturation analysis of the cytosols obtained with high-salt buffers revealed a compo-

FIG. 5. Effects of KCl concentration and volume of buffer H on the subfractional distribution of ER in *Squalus* testis. (A) Tissues were homogenized (1:4 tissue weight per final volume) in buffer H containing 0, 0.25, 0.5, or 1.0 M KCl and processed to give nuclear extracts (—) and cytosol (---). (B) Tissues were homogenized at 1:4, 1:8, 1:20, and 1:40 tissue weight per final volume in buffer H containing 0 M (■), 0.25 M (▲), or 1.0 M KCl (●), and nuclear extracts were prepared. Nuclear and cytosolic ER were measured by LH-20 and DNA-cellulose chromatography, respectively. Results were essentially the same for two separate tissue preparations.

ment with an affinity for E resembling that of the nuclear ER ($K_d = 7.7 \times 10^{-9}$ M) (Fig. 1B).

Table 1 compares nuclear and cytoplasmic ER levels in various elasmobranch tissues after homogenization in 0 M or 0.5 M KCl buffer. Although small amounts of E-binding activity were detected in *Squalus* oviduct cytosol prepared in low-salt buffers, nuclear ER predominated and 50% remained adherent to nuclei after processing in 0.5 M KCl. By contrast, testis and liver from *Potamotrygon* displayed E-binding activity in cytosolic subfractions of KCl-free homogenates, and in liver cytosol levels exceeded those in nuclei.

DISCUSSION

The estrogen-binding macromolecule present in nuclear subfractions of dogfish testis has the essential physicochemical properties of a classical ER. It binds E with high affinity and limited capacity, adheres to DNA-cellulose, and sediments at 4–5 S in a high-salt sucrose gradient. Although estrogens are more effective ligands than progesterone, testosterone, or DHT, a significant interaction occurs with 5α -androstane- 3β , 17β -diol. This androgen is capable of binding and translocating rat ER (22), suggesting that the conformation of the steroid binding site has been highly conserved through evolution. We previously have reported

Table 1. Comparison of ER in nuclear versus extranuclear compartments of elasmobranch tissues

Tissues	KCl in buffer H	ER, fmol/g of tissue	
		Nuclei	Cytosol
<i>Squalus</i> testis	0 M	77	ND*
	0.5 M	32	39
<i>Squalus</i> oviduct	0 M	107	27
	0.5 M	59	80
<i>Potamotrygon</i> testis	0 M	44	10
<i>Potamotrygon</i> liver	0 M	18	24

Tissues were homogenized in buffer H containing 0 M or 0.5 M KCl. Nuclear extracts and cytosol were assayed by using LH-20 and DNA-cellulose chromatography, respectively. Values shown are representative of two or more tissue preparations.

*Nondetectable. Limit of detection for binding activity is 8 fmol/g of tissue.

that ER is undetectable in epigonal tissues and free sperm from the same animals, thus exhibiting tissue specificity*.

As predicted, a unique property of *Squalus* ER is an unusually high affinity for DNA and nuclei. This binding property manifests itself experimentally as a resistance to disruption by salt, urea, or increased buffer volume. When nuclear extracts obtained from low-salt homogenates are chromatographed on DNA-cellulose, the elution maximum for specific E binding (0.55 M NaCl) is more than twice that reported for various mammalian and reptilian ER assayed under the same conditions (3, 4, 23, 24). Since DNA itself can activate ER and thus enhance affinity, these results were confirmed by testing receptor adherence to nuclei *in situ*. When buffers of increasing KCl concentration were used for tissue homogenization, ER was redistributed progressively from nuclear to cytosolic subfractions. Quantitative recovery of total ER number at each salt concentration is good evidence for a single receptor pool. The characteristics of the solubilized ER (steroid-binding affinity, adherence to DNA) substantiate its identity with the nuclear form. It should be noted that the KCl concentration required to extract 50% of the ER population from the nuclear compartment (0.43 M) is similar to the elution maximum for ER on DNA-cellulose. In the vitamin D₃-chick mucosa system, salt concentrations as low as 0.11–0.16 M are effective in redistributing the majority of nuclear receptors (12, 13). Moreover, these same secosteroid receptors exhibit elution maxima on DNA-cellulose of 0.18–0.28 M NaCl, depending on their state of occupancy and activation (15). Thus, even when compared to a system in which receptors apparently have an exclusive nuclear location, *Squalus* testicular ER displays remarkable nuclear-adhering properties.

This conclusion is further reinforced by the stability of the nuclear receptor population (<10% reduction) over a wide range of tissue homogenate dilutions (0.25–0.025 g/ml), providing buffers contain no salt. This contrasts with mammalian and avian steroid receptors which, even in the absence of salt, exhibit marked redistribution effects as a consequence of small dilutions. For example, the chick mucosa secosteroid receptor is 50% extracted at a tissue concentration of 0.05 g/ml (12). Similarly, ER in immature rat uterus appears mainly in the cytosol after dilution to 0.08 g/ml (recalculated from ref. 18). Although it can be argued that a higher-affinity ER might artificially "stick" to chromatin during tissue processing, this seems unlikely in the case of *Squalus* ER, where >90% is nuclear-associated at exceedingly high homogenate dilutions (0.025 g of tissue per ml). Since all measurements of DNA/nuclear adherence in these studies as well as those cited in other publications (3–5, 23, 24) were carried out under conditions known to activate receptors *in vitro*, the unique affinity characteristics of *Squalus* ER cannot be ascribed solely to activating effects of salt, dilution, or exposure to estrogen *in vivo*; however, endogenous ligand is inferred by the presence of some occupied binding sites and aromatase (estrogen synthetase) in the testis itself*.

Using several different assay methods, we were unable to detect ER in low-salt cytosolic subfractions of *Squalus* testis. Despite the well-known denaturing effects of urea on protein, even in urea-rich fishes (25), the presence of urea during incubation of nuclear ER with saturating concentrations of [³H]E neither enhanced nor diminished binding. The use of a postlabeling procedure and DNA-cellulose chromatography enabled us to rule out possible interference from endogenous E, nonreceptor binding proteins, and [³H]E-metabolizing enzymes, all of which might be expected to lower effective ligand concentrations. Moreover, this method of analysis proved to be satisfactory for *Squalus* nuclear ER that had been mixed with cytosol directly or extracted into cytosol by high-salt homogenizing buffers. We

conclude that crude cytosol derived from *Squalus* testis contains nothing that might obscure detection or accurate measurement of receptors, were they present.

From limited observations, the essential features of the *Squalus* oviduct receptor seem to be the same as in the testis: a predominant nuclear location and a high resistance to salt extraction. Although some ER is detectable in oviduct cytosols homogenized in low-salt buffers and also has been reported in brain cytosol of another dogfish (*Scyliorhinus canicula*) (26), minor tissue-to-tissue variations in cytosolic receptor number occur with the secosteroid system as well (12). Presumably, receptor synthesis occurs in the extranuclear compartment, and turnover rates would be reflected in cytosolic levels at any one time.

What is particularly striking as an example of biological adaptation is the comparatively low DNA-adhering properties of ER in *Potamotrygon*. Both liver and testis ER display an elution maximum from DNA-cellulose closely resembling that of mammals, birds, and reptiles (≈ 0.22 M NaCl) (3, 4, 23, 24). This genus and other freshwater elasmobranchs are believed to have left the sea to return secondarily to rivers and lakes (20). In contrast to *Squalus*, they maintain osmotic equilibrium at 300 mosM and have negligible urea (20). It is also relevant that *Necturus maculosus*, a freshwater amphibian that maintains osmolarity at 200 mosM (27), has a testicular ER that is eluted at 0.15 M under the same assay conditions (5). Thus, body-fluid osmolarity is closely correlated with the DNA affinities of various vertebrate ER. Although we can only speculate about selection pressures during receptor evolution, intracellular Na^+ , K^+ , and Cl^- concentrations are relatively stable throughout eukaryotes despite marked variations in nonionic constituents (25). In marine elasmobranchs, for example, high osmotic pressure inside the cell is maintained by solutes of no net charge such as urea, amino acids, and trimethylamine oxide (17). This implies that osmolarity *per se* or a particular osmotic agent rather than ionic strength was the operative variable during evolution.

These investigations support and extend the equilibrium hypothesis of steroid receptor distribution originally presented by Williams and Gorski (ref. 28; see also refs. 6, 7, 12–15, 18). According to this model, each of the receptor species (unoccupied/unactivated; occupied/activated) is in equilibrium between the nuclear and extranuclear compartments. Determinants of the equilibrium are the relative volume and free-water concentration of the two compartments, as well as the affinity of the receptor for chromatin. If it is assumed that the numbers of specific binding sites within the nucleus are limited, then total receptor number also becomes a factor. Clearly, the composition of the intracellular fluid with respect to ions that perturb the association between receptor and chromatin has a powerful controlling influence on receptor distribution, and this is independent of their effects as solutes. A corollary of the equilibrium hypothesis is that receptors having lower chromatin affinities are readily solubilized during tissue processing, whereas those with higher affinities remain associated with chromatin. This would account for the differential distribution into two compartments of unoccupied/non-activated/lower-affinity receptors versus occupied/activated/higher-affinity receptors in mammalian systems (6, 7, 18, 28) and also would explain the exclusive or predominant nuclear location of *Squalus* ER by the same methodology. Although these findings challenge the concept of an essential translocation step in the mechanism of steroid action, they

do not negate functionally important transformation or activation steps that can occur entirely within the nucleus (15). On the contrary, the high-affinity *Squalus* ER suggests an approach that may be useful for characterizing numerous salt-resistant receptor forms reported in the literature.

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