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COMPARISON OF ESTROGEN RECEPTOR SYSTEMS IN ESTROGEN-RESPONSIVE (ISHIKAWA) AND UNRESPONSIVE (HEC-50) HUMAN ENDOMETRIAL ADENOCARCINOMA CELLS

by

Sharon Deborah Kassan

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the City University of New York.

1988

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The City University of New York

Abstract

COMPARISON OF ESTROGEN RECEPTOR SYSTEMS IN
ESTROGEN-RESPONSIVE (ISHIKAWA) AND UNRESPONSIVE (HEC-50)
HUMAN ENDOMETRIAL ADENOCARCINOMA CELLS

by

Sharon Deborah Kassan

Advisor: Dr. Erlio Gurpide

Cells from the Ishikawa and HEC-50 human adenocarcinoma cell lines differ in their responsiveness to estrogens; the former, but not the latter, respond to estradiol. This dissertation describes studies conducted in order to investigate possible alterations in the estrogen receptor system that could underlie an insensitivity to estrogens.

Saturation analysis of cytosolic estrogen binders was performed over a wide range of [3H]estradiol concentrations and equilibrium dissociation constants (Kd) were determined graphically from Scatchard plots. No significant differences were noted in the Kd of the high affinity specific binder (approximately 0.7nM) present in each of the two cell lines and in normal endometrium.

The estrogen receptor monoclonal antibody, JS 34/32, was used to search for structural differences in the specific estrogen binders

in cytosol and nuclear extracts of the 2 cell lines and endometrial tissue that could be present without affecting binding parameters. Interaction of the antibody with the estrogen receptor of Ishikawa cells and normal endometrium was demonstrated by immunoprecipitation with Protein A-Sepharose beads and by the increase in the sedimentation rate of specifically bound [3H]estradiol in glycerol density gradients. None of these effects were noted with the HEC-50 cells.

The ability of [3H]estradiol receptors to be transformed to nucleophilic forms by interaction of the hormone was assessed by binding to DNA-cellulose. In contrast to the clear transformation of some of the specific binders observed in the estrogen-responsive Ishikawa cells, the HEC-50 cells could not be transformed by estradiol.

These results indicate that the lack of responsiveness of the HEC-50 cells to estrogens might be due to structural alterations resulting in a modification or masking of the antigenic domains of the estrogen receptor and in a loss of its capability to undergo estrogen-directed conformational changes leading to increased affinity for DNA.

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This thesis is dedicated in memory of Granny,
whose love, energy and strong spirit are
an everlasting inspiration.

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LIST OF ABBREVIATIONS

ATPP	aqueous two-phase partitionig
BSA	bovine serum albumin
DCC	dextran-coated charcoal
DIT	dithiothreitol
E_2	estradiol
ER	estrogen receptor
EDTA	ethylenediamine N,N,N,N'tetra acetic acid
GR	glucocorticoid receptor
HRE	hormone response element
hsp90	90kDa heat shock protein
Kd	equilibrium dissociation constant
Mr	molecular weight
Na_2MoO_4	sodium molybdate
nt-	nuclear transfer deficient GR phenotype
nt^i	nuclear transfer increased GR phenotype
OVA	ovalbumin
PAGE	polyacrilamide gel electrophoresis
PMSF	phenylmethylsulfonylfluoride
PR	progesterone receptor
RE_2	receptor-estradiol complex
SHBG	sex-steroid hormone binding globulin
SDS	sodium dodecyl sulfate
TAZ	tamoxifen aziridine

I. GENERAL BACKGROUND

The purpose of this section is to review the information that has accumulated over the past 25 years on the mechanism of action of estradiol in target cells. A synopsis of the current status of these concepts is schematically presented in Fig. 1: (a) The lipophilic steroid hormones are distributed within both the cytoplasmic and nuclear compartments. (b) unoccupied receptors (R) exist in a dynamic equilibrium between the cytoplasmic and nuclear compartments but are primarily concentrated in the nuclei, loosely bound to the nuclear matrix and/or chromatin,. (c) steroid binding to the receptor results in the conversion of what is probably a transient ([RS]) occupied receptor species into the biologically active occupied transformed (R*S) receptor form. The affinity of the transformed receptor for polyanions is increased favoring receptor binding to the chromatin-localized acceptor sites and activation of a class of transcriptional enhancers which result in the induction (or occasionally repression) of mRNA transcription, protein synthesis and regulation of cell function (1).

1. Estrogen entry and binding to receptor.

In blood, the majority of estrogen is bound to serum proteins by dissociable, non-covalent bonds (2). The interaction of these blood binders for estradiol varies from very weak and nonspecific (serum albumin;Kd=10^{-5}-10^{-4}M) to very strong and specific (steroid binding globulin,SHBG;Kd=10^{-9}-10^{-8}M) (3). The mode of entry of estrogen into target cells is a controversial issue. The

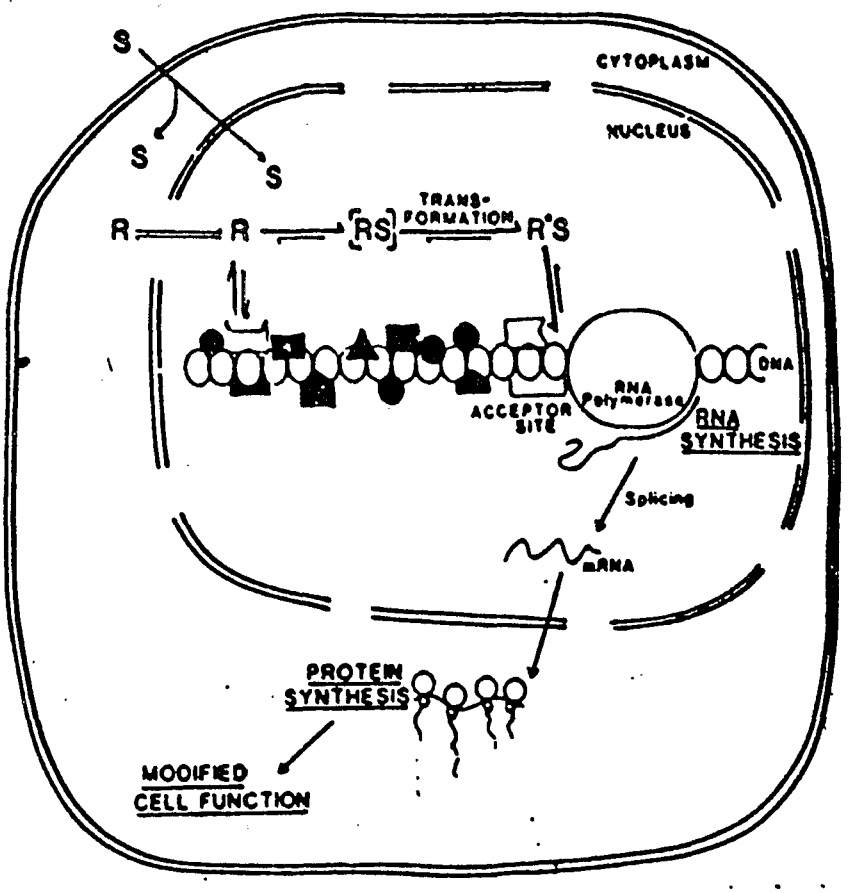


Fig. 1 Scheme depicting some events involved in the mechanism of action of estradiol in a target cell. (1)

passage via the cell membrane most likely occurs by passive diffusion due to the lipophilic nature of steroids but may also involve facilitated transport at physiologic concentrations of the steroid hormone. Work by a number of investigators indicates that steroid uptake is a nonsaturable process that is not only determined by specific binding to receptor at physiologic concentrations of steroid hormones (4,5), but also by non-specific binding and metabolism (6). This mechanism is further supported by superfusion studies of human endometrium (7) or endometrial cancer cells in suspension (8) and measurement of unidirectional flow of steroid tracers.

Evidence for carrier systems mediating the entry of estradiol has been suggested by Milgrom et al. (9) based on saturability in the range of physiologic hormone concentrations and differential effects of sulfhydryl blockers on estradiol entry and estradiol binding. However Milgrom's proposal has been criticised by a number of investigators. Gurpide et al. (10) have suggested that since the inhibitor was added to the tissue prior to cell fractionation and addition of [3H]E_2, the observed lack of inhibition of estradiol binding to the cytosolic receptor may have been due to incomplete diffusion of the inhibitor through the thickness of the tissue. This criticism is supported by the fact that inhibition of nuclear accumulation of estradiol-receptor complexes was observed when [3H]E_2 and inhibitor were added simultaneously to the tissue. Peck et al. (11), using similar techniques to determine the mechanism of E_2 uptake did not observe saturation and found no effect of

N-ethylmaleimide (an SH-blocking reagent not used by Milgrom's group) on [3H]E_2 entry.

However, the existence of carrier systems is supported by the finding of specific estrogen-binding proteins on the surface of target cells, i.e. rat uterine cells (12), breast cancer cells (13,14), and rat pituitary membranes (15). Furthermore, immunofluorescent detection of plasma sex-steroid hormone binding globulin (SHBG) in tissues of the reproductive tract (16) and in MCF-7 cells exposed to SHBG in vitro (16), has led to proposals for a role of plasma proteins as carriers of estrogens and other steroids into cells. This is supported by recent evidence (17) using radioiodinated SHBG to study binding of SHBG to MCF-7 cell membranes and to follow its internalization.

A. Subcellular Localization of ER

It is widely accepted that specific intracellular receptor proteins are involved in the estrogenic regulation of gene expression and growth in reproductive tissues. The binding of estrogen to receptor proteins in target cells was first proposed by two separate groups (18,19). They proposed a two-step model for steroid hormone receptor action based on evidence from studies involving the uptake and binding of radioactive estrogen to receptors in rat uterine tissue, followed by subcellular fractionation and autoradiography. According to this model the hormone is required for translocation of the cytoplasmic receptors to the nucleus and for its binding to the DNA-responsive elements of target genes. However, using

immunocytochemical (20) as well as enucleation techniques (21,22) it was recently demonstrated that all estrogen receptors are localized, but weakly bound, in the nucleus even in the absence of hormone. Cytoplasmic receptors are therefore an artifact due to cell fractionation procedures. These studies also indicated that ER is not located in the plasma membrane since the cytoplasts did not contain ER and none of the immunocytochemical studies indicated localization of receptor to the periphery of the cell. Additional evidence challenging the classical view include the observation of "inactive" 8-9S forms of ER in nuclei isolated from rat uterus (23) and the immunocytochemical detection with monoclonal antibodies of ER in nuclei but not in cytosol of breast tumors from post-menopausal women (24).

A significant amount of evidence has accumulated which suggests that a small proportion of membrane-bound, high affinity E_2 binders may mediate non-genomic effects of estrogens, including:

-direct effects of estradiol at the cell surface as reported in brain neurons, which were very rapidly hyperpolarized by estradiol, even in the presence of cycloheximide or actiniomycin D (25).

-changes in Ca++ flux in suspensions of cells isolated from uteri of ovariectomized rats in less than 3 min after the addition of estradiol (10^{-9}M) to the medium (26).

-estradiol-induced increases in adenylate cyclase activity in plasma membranes isolated from human secretory endometrium (27)

-changes in the binding of domaminergic agents in membranes isolated from rat pituitary cells (28).

Based on experiments to date, one can not rule out the possibility that ER may not be located at all times in a single subcellular compartment, but rather exist in a dynamic equilibrium between the plasma membrane, cytoplasm and nucleus. Gorski et al. (29) have proposed a model involving low affinity hydrophobic interactions of unoccupied receptors and chromatin, leaving no free receptor because of the almost infinite capacity of the chromatin surface to adsorb unoccupied receptor. Ligand binding to the unoccupied receptor causes a conformational change that disrupts the random, hydrophobic interactions with chromatin while subsequently inducing additional conformational changes in the receptor that result in the receptor's increased affinity for DNA. The most appealing aspect of this hypothesis is that it predicts the classically observed extraction of unoccupied steroid receptors upon homogenization in low salt aqueous buffers into the cytoplasmic environment.

It is important to realize that the recent findings do not invalidate the two-step model since there are still two identifiable states of ER: an active (estrogen-associated) and an inactive (unliganded) one. The real significance of the original two-step idea is retained; steroid-receptor complexes are nuclear regulatory elements wherever they originate in the cell.

B. Heterogeneity of Estradiol Binding

Specific estrogen receptor proteins are present in target cells in a limited quantity (approximately 15,000 sites/cell) and possess a high affinity (Kd 10^{-10}-10^{-9}M) and specificity for estradiol. Cells that are deficient in ER or contain altered ER have been shown to be unresponsive to estradiol (see section I-4).

Cytosolic estrogen binders differing in their affinity for estradiol have been demonstrated in a number of systems (30,36-39). At least two populations of estrogen binders have been identified in these systems: high affinity (Kd $\sim 10^{-10}$M) and low affinity (Kd $\sim$ 4-20 X 10^{-9}M). The biological relationship between these two populations of estrogen binders is an unresolved issue. Clark's group suggests that the high affinity sites (Type I) represent the "true", functional estrogen receptors, whereas cytosolic, low affinity sites (Type II) represent "non-translocatable" binders which may be involved in retention of estrogens in the uterus, by creating an estrogen-rich environment for subsequent binding to type I sites (30). The steroid specificity of binding to Type II sites has been reported to be similiar to that of ER, demonstrating that these sites are not receptors for another steroid hormone (31). Nuclear Type II sites have been shown to be "highly stimulated" in neoplastic tissues (32-33) and it has been suggested that this may be related to the rapid rate of proliferation in these cell populations. These sites are different from cytoplasmic Type II sites by virtue of their inability to bind the classical antiestrogens nafoxidine or clomiphene and by their sensitivity to sulfhydryl reagents (35).

2. ER Transformation

It is generally accepted that after binding hormone, the receptor undergoes a process called "activation" or "transformation", which results in an alteration(s) in its physiochemical properties. Such changes are essential to high affinity nuclear binding and biological activity of receptor-estrogen complexes (RE_2). To date, it has not been resolved whether this transformation is due simply to a change of conformation or whether more complex alterations occur. The exact biochemical processes involved in receptor transformation in vivo are unknown. Transformation of RE_2 may be mimicked in vitro by exposure to : heat (40), high ionic strength (41,42), ammonium sulfate (43,44), DNA-cellulose (45,46), phosphocellulose (47), DEAE-cellulose (48), ribonuclease (49), ribonucleotides (50), dilution, extended incubation times at $4^{o}C$ (51) and alkaline pH (52). These experimental manipulations have the potential of producing artifactual changes in ER which may or may not have any direct relevance to in vivo processes.

The most extensively studied ER transitions in vitro have been those changes in RE_2 that accompany heating or salt treatment of the low-temperature stabilized, non-transformed estrogen-receptor complex. Essential to these studies has been the use of molybdate, a reversible inhibitor of the salt or temperature-induced transformation of RE_2 (48,43). Molybdate fails to have any effect once receptors have been previously transformed.

Numerous temperature or salt-induced, molybdate inhibitable changes in RE_2 have been documented. These changes include

alterations in: size, as reflected in density sedimentation profiles (54), binding affinities for nuclei, DNA and other polyanions (53-56), ligand-dissociation kinetics (42,44), partition coefficients in aqueous two phase systems (57,58) and interaction with anti-ER monoclonal antibodies (59,60). However, recent studies indicate that these physicochemical changes may not represent a common biochemical event. An open question in this repertoire of changes is their biologic significance. This section summarizes the physicochemical and immunochemical characteristics of untransformed and transformed RE_2 followed by their molecular composition and subunit structure. Finally I shall describe several mechanisms which have been proposed to explain the biochemical and molecular events that occur during receptor transformation.

A. Size Characteristics

Studies on the size of both untransformed and transformed ER have been based upon the hydrodynamic properties of crude or partially purified receptor preparations (Table 2). Both untransformed and transformed ER species are highly asymmetric proteins as evidenced by their frictional and axial ratios.

Untransformed ER: Numerous studies have been performed to determine the size of the untransformed RE_2. Since the untransformed ER is very sensitive to salt-induced dissociation into 4S monomers, analysis of this form has been performed in hypotonic buffers in the presence of the transition metal oxyanion, molybdate ($MoO_4^{=}$). Essential to these studies is the fact that molybdate stabilizes the

Table 1. Physicochemical Properties of ER

Parameter	Untransformed	Transformed		Reference
		Monomeric	Oligomeric	
HYDRODYNAMIC PROPERTIES:				(48,62)
Sedimentation Coefficient	8-9S	4S	5S	
Stokes radius (nm)	7.4	4.0	5.7	
Molecular Weight (kDa)	303	60-65	129	
Frictional Ratio(f/fo)shape	1.53	1.34	1.57	
Axial Ratio	11	7	13-16	
CHARGE PROPERTIES:				
Isoelectric Point	4.8	6.3-6.9	5.4	(63)
CHROMATOGRAPHIC BEHAVIOR DETERMINED BY ELUTION WITH KCl:				
DEAE-cellulose	0.13-0.3M	0.06-0.15M	0.06-0.15M	(48)
DNA-cellulose	not adsorbed	0.2-0.4M	0.2-0.4M	(45,51)

untransformed steroid-receptor complexes. The molecular mechanism whereby molybdate stabilizes the untransformed ER remains a controversial issue, but probably involves the inhibition of subunit dissociation and/or receptor proteolysis. Molybdate inhibits both heat- and salt-mediated transformation of the RE_2 to its DNA-binding form (61). Since both heat and salt can destabilize the weak bonding forces involved in subunit interactions, certain investigators suggest that molybdate interacts directly with the "8-9S" receptor forms to strengthen subunit interactions and thereby inhibit or prevent subunit dissociation (64). It has also been proposed that the molybdate divalent anion may neutralize opposing positive charges at one or more sites of contact between the interacting subunits. It has been suggested that molybdate stabilization of the 8S form involves a direct interaction with sulfhydryl groups in steroid receptors or an indirect interaction involving formation of a bridging structure between it and a receptor-associated protein (hsp90) (112). Others have suggested that it is associated with inhibition of phosphatases (64). Another possibility is that molybdate is inhibiting proteolysis of the untransformed receptor, by interacting directly with sites which may be vulnerable to proteolysis (110).

Since the majority of work on "8-9S" ER forms is based on evidence from molybdate-stabilized receptor in vitro, one may question the physiological relevance of these findings. The recent report on molybdate actions on ER in cultured GH_1 rat pituitary cells (66), supports the view that the large, oligomeric receptor is

present in vivo and that transformation involves protein dissociation. The results clearly showed that cells treated with molybdate have more cytosolic and less nuclear receptor than control NaCl-treated cells and supports the concept that molybdate acts to slow the rate at which cytosolic ER accumulates in the nucleus.

Transformed Receptor: It is generally accepted that the transformation of the hetero-oligomeric "8-9S" RE_2 involves the dissociation of proteins that do not bind steroids such as hsp90 (see section I-2D). Evidence exists for at least two transformed ER species; a monomeric "4S" form and an oligomeric "5S" form which is probably a homodimer consisting of two 4S monomers. To date, it has not been firmly established which one(s) is the gene regulatory receptor species.

B. Charge Characteristics of Untransformed and Transformed ER.

The most widely used method for determination of the charge characteristics of ER has been ion-exchange chromatography. When cytosol from calf uterus is chromatographed on diethylaminoethyl (DEAE)-cellulose at 0-4^{o}C, two peaks of estrogen binding activity are obtained. The predominant ER peak (untransformed) elutes at elevated salt concentration, while a second (transformed) form elutes at lower ionic strength (Table 2). When the untransformed ER is stabilized by molybdate, all of the receptor elutes at the higher salt concentration (48). In addition to cation-exchange chromatography, anion-exchange chromatography has been used to

analyze ER structure. Receptor transformation yields a protein that binds to anionic-exchange columns and is eluted at elevated salt concentrations (Table 2). No adsorption of the untransformed ER occurs when it is chromatographed on phosphocellulose or DNA-cellulose. Cation-exchange chromatography has been routinely used to determine the amount of transformation of ER.

These results support the concept that the transformed receptor is a less acidic protein than the untransformed species. Other studies have shown that the ability of RE_2 to bind to DNA-cellulose is associated with the exposure of a localized region of positive charges involving lysyl (67-68,70), arginyl (69) and histidyl (57) residues residues on the receptor's surface, which are inaccessible in the nontransformed species. Analysis of ER by isoelectric focusing has generated information in support of this hypothesis (Table 2).

An alternative approach to analyze the charge characteristics of ER forms is aqueous two-phase partitioning (ATPP). This method partitions ER in a biphasic polymer system in which the distribution of macromolecules is independent of the molecular weight, but dependent on both the net electrostatic properties as well as surface hydrophobic content (71). ATPP has the distinct advantage over previously described chromatographic methods in the speed and sensitivity with which it can detect modifications in protein structure. Using ATPP to analyze rat uterine ER, Hansen and Gorski have reported the existence of two independent conformational transitions that occur within the receptor monomer (57,58). The

first structural change is due to ligand binding to the unoccupied ER, resulting in decreased hydrophobicity without alterations in net surface charge. The second structural change is induced by heating, and has been shown to be a hormone-independent, irreversible structural change that apparently begins to occur immediately upon homogenization at 0-4^{o}C and subsequent extraction of receptors into dilute cytosol.

Recently, the partioning properties of ER were further analyzed by ATPP in experiments in which triazine dyes were conjugated to the polyethylene glycol in the system since they have been reported to interact with nucleotide binding domains of proteins by mimicking the natural ligands NAD^{+}, ATP and DNA (72). The results indicated changes in the partioning behavior such that estrogen binding increased the affinity of the receptor for the triazine dyes. Heating the occupied ER had no effect on its affinity for the dyes. Based on these results Hansen and Gorski presented a model in which unoccupied ER possesses a hydrophobic domain(s) that becomes less apparent or "buried" upon ligand binding resulting in decreased hydrophobicity of the receptor and in increased affinity for DNA. This conclusion is consistent with a recent analysis of the ER primary amino acid sequence (73-75), which demonstrated that the carboxyl-terminal half of the protein (including the putative steroid binding site) posesses considerable hydrophobicity.

C. Immunochemical Properties of Untransformed and Transformed ER.

Monoclonal antibodies which recognize antigenic determinants which reflect conformational changes of the receptor due to the transformation process have been described.

Differences in reactivity of monoclonal antibodies with different forms of ER has been described for an IgG-class antibody, D547Sp , which was raised against a preparation of extranuclear ER from MCF-7 human breast cancer cells (76). The antigenic determinant recognized by this antibody has been reported to be localized in an intermediate region of the MCF-7 receptor, neither close to the steroid-binding domain, nor close to the DNA-binding domain (77). Two forms (α and β) of the cytosol ER from fetal guinea-pig uterus were differentiated on high salt sucrose gradients by their selective binding to the antibody (59). Evidence that the form, which is recognized by the antibody is the transformed form of the ER was based on the following correllations:

1. The β to α conversion was a time, temperature and salt dependent process.
2. Molybdate completely blocked the transformation induced by time and temperature but had little effect on that induced by high ionic strength.
3. The appearance of the α form was paralleled by an increase in receptor binding to nuclei and DNA-cellulose.

However, no differences have been reported in the binding of the D547Spδ monoclonal antibody to transformed or untransformed forms of the human ER, except for a slight difference in the

sedimentation rate of the receptor-antibody complex. The authors suggest that the guinea-pig receptor may be different enough from the human receptor so that the same antibody will selectively bind only to the transformed form.

Borgna et al. (60), have documented differences in reactivity of an IgM-class monoclonal antibody, B_{36}, with transformed and molybdate-stabilized calf uterine ER. This antibody was raised against the purified calf uterine nuclear ER and interacts more strongly with the nuclear than with the cytosolic ER (78). They demonstrated that the B_{36} antibody reacted more strongly with the heat-transformed form of the cytosol ER, than with the molybdate-stabilized complex. Also, this antibody partially (60%) inhibited the binding of transformed ER to DNA-cellulose. However, while molybdate totally prevented the interaction of ER with DNA-cellulose, it decreased but did not suppress the ER-B_{36} interaction. The authors concluded that the conformational changes induced by estradiol involve not only the hormone-binding and DNA-binding domains, but also the B_{36}-binding domain.

D. Molecular Structure of Untransformed ER, Transformed ER and Meroreceptor.

As was previously discussed, the untransformed receptor is a large oligomeric complex, while the transformed receptor can exist in an oligomeric (5S) and a monomeric (4S) form. In this section the molecular structure of these forms shall be discussed.

Untransformed ER. Several molecular models of the "8-9S" oligomeric form have been proposed. Discussion as to whether untransformed RE_2 is made up of similar or dissimilar subunits has not been completely resolved, although, most of the data support the latter.

One model postulates the participation of two nonsteroid binding components of 58kDa and 13.7kDa which are destroyed by trypsin but not DNase or RNase (79). By using various combinations of these components (receptor binding factors, RBF's) in association with the monomeric 4S ER subunit the differently sedimenting forms of the ER could be generated. Addition of these two components to the transformed 4S ER yielded 5S, 6S or 8S forms which are unable to bind to nuclei.

A model consisting of a hetero-oligomer containing a dimer of the 65kDa binding subunits interacting with a nonsteroid binding moiety has also been proposed. Redeuilh et al.(62) have reported the purification of the molybdate-stabilized, untransformed form of the calf uterus ER and presented biochemical and immunochemical evidence that the untransformed ER is a heteroligomer consisting of two steroid-binding subunits with possibly two molecules of a nonsteroid-binding 90kDa protein. This 90kDa protein has been shown to be a serine phosphoprotein that reacts immunologically with several polyclonal and monoclonal antibodies directed against the 90kDa heat shock protein (hsp90). This hsp90 has also been shown to be associated with receptors for progesterone, androgens, and glucocorticoids (80-82), as well as with the Rous sarcoma oncogene product, $pp60^{v\text{-}src}$ (83). While the function(s) of this protein

remains unknown, some of its properties have been defined through various experimental manipulations. It is one of a select group of proteins whose synthesis is stimulated during heat shock treatment of cells or after exposure to several cytotoxic agents, such as arsenite or canavanine (84). At the present time, there are no clear-cut functional relationships between steroid hormone action and cellular stress reponses or the activity of oncogenic proteins such as $pp60^{v-src}$. It has been proposed that the hsp90 has a role in maintaining the untransformed state of steroid receptors (80).

Other proteins have been identified which could be associated with the nontransformed ER and in some cases converted into a nonsteroid-binding form. Protein phosphorylation seems to be important in the association of these proteins with ER (85,86).

These reports have been critisized with regard to the in vitro procedures used (87). Since it is generally accepted that in vivo, most unliganded receptor is in the nucleus, it has been suggested that the cytosolic forms detected after homogenization in the presence of molybdate may not be biologically relevant. This is further supported by immunocytochemical studies which have shown that hsp90 is essentially cytoplasmic whereas the estrogen receptor is intranuclear even in the absence of hormone. Secondly, the majority of evidence on hsp90 associating with steroid binding units relies on the use of molybdate-stabilized preparations. Housely et al.(88), reported an interaction of hsp90 with glucocorticoid receptors only in the presence of molybdate. Studies showing changes in the levels of nontransformed receptor in target cells exposed to heat shock

should be done in order to explain the biological relevance of hsp90 in steroid hormone action.

Barnett et al.(89) have characterized a macromolecular inhibitor of glucocorticoid receptor (GR) transformation. This factor does not bind dexamethasone, is destroyed by heating, prevents GR from binding to DNA-cellulose and can be separated from the transformed GR via DEAE-cellulose chromatography. This factor is 10-14kDa in size and is resistant to trypsin, chymotrypsin and RNase A. These observations have led to the proposal that it is a polypeptide.

Transformed 5S ER. Several models have been proposed for the 5S transformed species including either a homodimer of two identical hormone binding subunits or an oligomer of dissimilar subunits.

The following observations support the homodimeric model: (a) in vivo labeling of MCF-7 cells with deuterium-labeled amino acids suggests that the transformed nuclear receptor is an oligomer of at least 2 monomers closely related in size, which are metabolized at the same rate (90,91), (b) immunochemical studies show that the 5S form is able to bind more than one anti-ER monoclonal antibody (92,93) and (c) molecular weight analysis by gel filtration showed that the 5S form is approximately 2x larger than the 4S form (62). A model which interprets these observations was proposed by Vedeckis (64). He suggests that in vitro conditions such as high ionic strength promote the rapid dissociation of the 8S RE_2 into artifactual 4S monomers. Also, high salt conditions may promote a configurational change in the monomer which will exhibit ionic

properties considered to be consistent with transformation, but hamper a dimerization which the author believes is the physiologically relevant species. This proposal is supported by the following observations: (a) the 5S oligomeric species is relatively stable in high ionic strength buffers, (b) it is the 5S species which is isolated from nuclei after in vivo transformation and nuclear binding and (c) the 5S form is presumed to be the thermodynamically-favored state. The inhibition of 5S formation by high salt suggests that ionic interactions may play a role in the stabilization of this complex.

Evidence supporting the dissimilar subunit model has been presented by Thampan and Clark (94) who have described a nonhormone-binding protein which interacts with the 4S ER and causes an increase in the sedimentation to 5S. This protein is basic, of relatively small size (2.9S) and stimulates binding of the 5S RE_2 to both nuclei and DNA-cellulose.

Other investigators suggest that ER may exist in a ribonucleoprotein complex. This suggestion is based on evidence that the treatment of crude receptor preparations with ribonuclease (RNase) increases receptor binding to DNA in contrast to its inhibition by the addition of cellular RNA (49,50).

Vedeckis et al. (95,96) have reported the presence of a non-hormone binding factor associated with GR in AtT-20 cells which can be separated from transformed GR by DEAE-cellulose chromatography. This factor is of low molecular weight, is resistant to boiling for 20min. and is destroyed by treatment with RNase A.

They suggest that the factor is transfer RNA or an RNA similiar to it as evidenced by a discrete shift in sedimentation of the transformed GR from 3.8 to 6.6S upon the addition of total RNA or transfer RNA. The physiological relevance of this apparent RNA-binding activity of steroid receptors is unknown since it may be due to the increased affinity of transformed receptors for anionic substances. However it has been suggested that this may represent a mechanism for turning off gene expression when RNA levels become very high (97).

Transformed 4S ER. This form of the receptor represents a monomer of the hormone binding unit as determined by molecular weight analysis as well as by the existence of only one antigenic determinant (89,90). Limited proteolysis of crude and purified receptors using a variety of proteolytic enzymes revealed three domains within the receptor protein which are relatively resistent to protease action, and are separated by protease-sensitive regions (64,102,110). The availability of cloned receptor cDNAs for ER (73-75,114) GR (98) and PR (99-101) from cells containing normal as well as mutant receptors together with site-directed mutation analysis and amino acid sequence analysis of the translated receptor protein has enabled identification of the structure and function of these domains at the DNA and protein levels (102-108) (Figure 2).

The hormone-binding domain (E), located in the carboxy-terminal half of the protein, is generated by trypsin treatment and exhibits considerable hydrophobicity (73-74). Widespread substitutions within this domain have marked effects on the receptor's steroid-binding

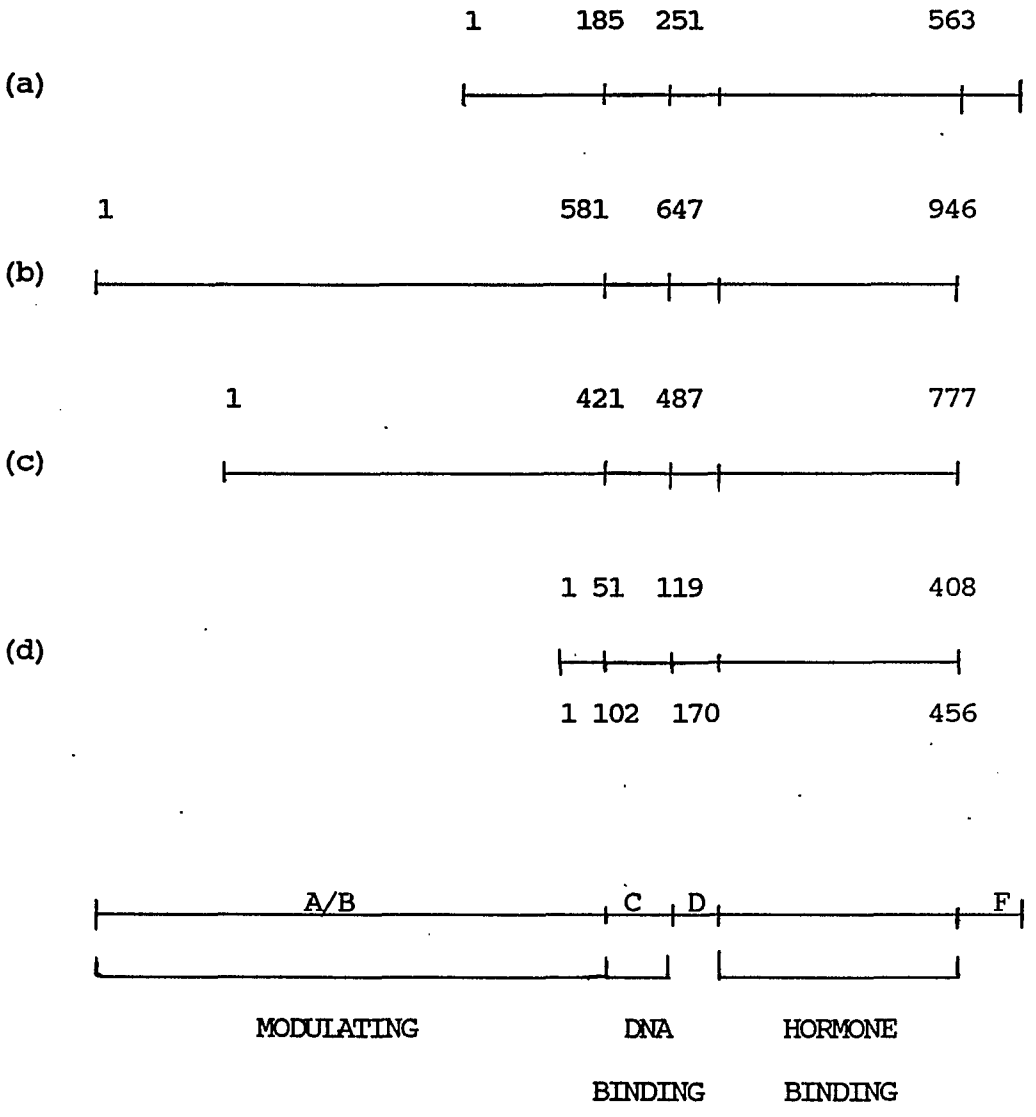


Fig 2 Schematic alignment of steroid and thyroid hormone receptor amino acid sequences as deduced from their complementary DNA sequences: (a) human ER, (b) chicken PR (c) human GR and (d) chicken (1-408) and human (1-456) c-erb A proteins (111).

capacity. Point-mutations in the hormone-binding region can lead to receptors lacking hormone-binding activity (107,108). Based on these studies, Danielsen et al (107) proposed that the steroid-binding domain normally represses receptor function. This proposal has been confirmed by studies by Godowski et al (109) which demonstrated the following:

-deletion of 29 C-terminal residues in the GR destroys its steroid-binding activity and results in constitutive DNA-binding activity.

-residues 407-566 gave rise to a product with constitutive DNA-binding activity that was capable of regulating transcriptional activity.

These results have recently been confirmed by Pratt et al (112) who identified a small region of homology within this domain which is directly involved in the binding of the GR to a receptor-associated, nonhormone binding protein in the 8S complex (hsp90). Since deletion of this site results in constitutively active GR the authors have suggested that it may be critical for transducing the steroid binding event into dissociation of the receptor from other components of the 8S complex to yield a free receptor that is capable of DNA binding (112).

Chymotrypsin treatment generates a receptor form which contains both the hormone and DNA-binding domains (110,113). Further digestion of this fragment with trypsin reveals an intact DNA-binding domain (C). Mutations within this domain result in alterations in the receptor's DNA-binding capacity (107-109). The DNA-binding domain

corresponds to the region of the ER protein that exhibits a very high degree of homology with other steroid receptors, with other DNA-binding proteins, and with the product of the v-erb-A gene of the avian erythroblastosis virus (114). This region contains high amounts of cysteine, arginine and lysine and has the potential to form at least two zinc-stabilized "DNA-binding fingers" analgous to those proposed for the Xenopus transcription factor TFIIIA (115), but involving two pairs of cysteines instead of pairs of cysteines and histidines (98). The actual existence of fingers has been demonstrated only for TFIIIA with X-ray absorption spectroscopy (251), confirming the identity of the amino acids suspected to chelate zinc. There is strong evidence for the steroid receptors that DNA binding is a property specifically conferred by the region containing the "zinc finger". In a so-called "finger-swap", Green and Chambon (116) replaced the putative finger region of the ER with that of the GR and showed that DNA binding specificity had been converted. Also, mutations within the finger region of the human GR destroy its ability to bind to specific DNA sequences in vitro (103).

Interestingly, several groups have presented indirect evidence supporting the presence of metal atoms in steroid receptors (117-119). Recently, Sabbah et al (120) reported that the binding of the ER to DNA-cellulose is lost following exposure to metal chelators, but is restored upon addition of zinc (120). The effect of zinc on ER binding to DNA-cellulose indicates that the role of this metal is not necessarily related to the receptor's interaction with specific DNA sequences of hormone-regulated genes. Therefore,

the direct demonstration of zinc fingers in steroid receptors as well as their function in the interaction of steroid receptors with specific DNA sequences has yet to be elucidated.

Recently, a cDNA sequence of human c-erb-A, the cellular counterpart of the v-erb-A, has been cloned. It was shown that c-erb-A shared an overall structural homology to steroid hormone receptors notably in the putative DNA-binding domain of these proteins. In vitro translation was used as a tool to characterize the binding activity of the cloned chicken (121) and human (122) c-erb-A cDNA and showed that the c-erb-A protein is a high-affinity receptor for thyroid hormone. The fact that the chicken c-erb-A product is localized in the nucleus supports the proposal that this protein is the thyroid receptor. The fact that steroid and thyroid hormones, which are neither structurally nor biosynthetically related, have receptors that have evolved from a common ancestor gene has suggested to many investigators that this is representative of a large superfamily of genes whose products are transcriptional regulatory proteins. Green and Chambon (111) have speculated that because of other mutations, v-erb-A has acquired the ability to bind constitutively to responsive elements of target genes and may be important in oncogenic transformation by interfering with the transcriptional regulation of crucial target genes. This idea is supported by the recent report by Munoz et al. (123) who demonstrated the presence of several point mutations in the carboxy-terminal half of the retrovirus-encoded form ($P75^{gag\text{-}v\text{-}erb\text{-}A}$) which may contribute to its defective ligand

binding capacity. The authors suggested that the hormone-independent activity of the v-erb-A protein provided a selective advantage to the avian erythroblastosis virus during its evolution towards an acutely oncogenic retrovirus. Additional studies involving transcriptional activation of thyroid receptor-responsive genes using cloned c-erb-A will be required in order to positively demonstrate that they correspond to functional thyroid hormone receptors.

A third domain (A/B) located at the amino-terminal end of the protein contains the remainder of the molecule, carries the major antigenic determinants (124) and most likely plays a role in modulating DNA-binding activity, since it has been shown that lack of this domain results in a nonfunctional receptor protein (125). Whether this region is required for activating transcription is not clear. Mutagenesis of ER (106), GR (103,104,112), and PR (105) in the A/B domain have demonstrated the importance of this domain in retention of the receptor's transcriptional regulatory function.

Between the hormone-binding region and the DNA-binding region there exists a hydrophilic region, which is variable in both length and sequence, and may correspond to a hinge between these two domains. It has been proposed that steroid binding and subsequent transformation result in a conformational change in the protein in this region (73,75,102).

An additional region (F) is only found in ER and is located at the carboxy-terminal end of the protein. Its function is not clear (111).

Mero-Receptor. Sherman et al. (126) were the first to identify the existence of a small (2-3S;Mr=19-29kDa), hormone-binding fragment resulting from the action of proteases on the monomeric ER called the mero-receptor. This fragment is the smallest nondenatured form of the receptor which retains hormone-binding activity. Calcium-activated proteases have been demonstrated in calf uterus (127) and chick oviduct (128) which are able to generate the mero-receptor. The mero-receptor is the most basic receptor form, having an isoelectric point of 6.2-8.3 (129) and has been characterized by its ability to bind to hydroxyl apatite and its inability to bind to DNA-cellulose (130).

E. Mechanisms of Estrogen Receptor Transformation.

A number of mechanisms for the transformation process of ER have been proposed. The sometimes conflicting reports from various laboratories of ER transformation have prevented the formation of a consensus model. This is partially due to the fact that transformation is produced by more than one physical or chemical means at 0°C including such varied treatments as salt, dialysis, gel filtration and even simple dilution. Secondly, the extent of receptor transformation has been assessed by different physiochemical changes in receptor structure which do not represent a common biochemical event i.e. increased sedimentation rates in high salt density gradients (4S to 5S), decreased estradiol dissociation rates and increased affinity for DNA and polyanions. It is most likely that there is no single mechanistic model for receptor

transformation. I shall present the following mechanisms which involve biochemical changes which are not necessarily mutually exclusive events, including; dimerization of receptor subunits, conformational changes, dissociation of nonhormone binding subunits, dissociation of low molecular-weight inhibitors, limited site-specific proteolysis and dephosporylation.

1. Dimerization of ER subunits.

The following evidence from in vitro studies supports a dimerization mechanism for receptor transformation: (a) the kinetics of 4S to 5S conversion are consistent with a second order reaction (131), (b) the biphasic [3H]estradiol dissociation kinetics whereby heat promotes the conversion of RE_2 from a state with fast to one with slow estradiol dissociation kinetics requires 4S to 5S conversion (42) and (c) the formation of the 5S species is associated with a positive cooperative estradiol-binding reaction at sufficiently high concentrations in hypotonic buffers (132). These investigators proposed a model in which the combined effects of enhanced steroid affinity and dimer formation manifest themselves with steroid binding kinetics that exhibit positive cooperativity.

a. $R + E_2 \rightleftharpoons R\text{-}E_2$ (low affinity 4S monomer)

b. $2(R\text{-}E_2) \rightleftharpoons RE_2\text{-}RE_2$ (high affinity 5S dimer)

2. Conformational change without a change in mass.

Subsequently, many investigators questioned the proposal that the 4S to 5S dimerization is synonomous with the acquired affinity of RE_2 for DNA. Bailly et al. (133) showed that the processes of 4S to 5S dimerization and of acquired affinity for DNA-cellulose occur independently of each other and at different rates with different kinetics. Muller et al. (67) presented evidence that both 4S and 5S forms of RE_2 exhibited the same sensitivity to salt extraction from DNA-cellulose. Studies with molybdate showed that once a transformed $4SRE_2$ is formed, molybdate is unable to inhibit the 4S to 5S dimerization (67). These investigators suggested that the inhibitory action of molybdate on heat-promoted 8S to 5S transformation was an indirect effect due to the inhibition of the initial 8S to 4S dissociation. In further support of this proposal was the following evidence demonstrating that the dimerization and conformational change of RE_2 as detected by the decrease in the rate of estradiol dissociation are separate events: (a) $4SRE_2$ immobilized on hydroxylapatite exhibited biphasic estradiol dissociation rates (134,135), and (b) the existence of low affinity receptor dimers bound to estradiol (69), estriol (136) and tamoxifen (138). To explain these findings the following model was proposed:

a. $R + E_2 \rightleftharpoons R\text{-}E_2$ (low affinity 4S monomer)

b. $R + E_2 \rightleftharpoons R^*\text{-}E_2$ (high affinity 4S* monomer with DNA-binding ability)

c. $2(R^*E_2) \rightleftharpoons R^*E_2\text{-}R^*E_2$ (high affinity 5S* dimer)

This model suggests that under the influence of estradiol and thermal energy, transformation involves conformational changes in the 4S monomers due to the disruption of ionic bonds whereby three distinct receptor domains are altered independently: one domain is associated with the exposure of lysyl (67,68) arginyl (69), cysteinyl (250) and histidyl (57) residues on the receptor surface thereby increasing its affinity for negatively charged DNA; a second domain involving lysyl residues (70), is associated with the conversion of RE_2 into a state with increased affinity for estradiol and a third domain which mediates the association of 4S monomers into 5S dimers. This model is compatible with the claim that transformation is a first order reaction and that it is hormone-dependent and reversible and results in a thermodynamically-stable end-product.

This model is also consistent with that proposed by Gorski et al. (29), in which unoccupied, untransformed ER possesses a hydrophobic domain(s) that becomes less apparent or "buried" upon ligand binding, resulting in decreased hydrophobicity of the receptor and in increased affinity for DNA.

3. Dissociation of a heteroligomeric receptor.

An alternative, and most widely accepted model is that ER transformation is similiar to that described for other classes of steroid hormone receptors in that it involves subunit dissociation, with an oligomeric, 8-9S untransformed moiety being converted to an oligomeric, transformed 5S receptor and/or 4S transformed monomer.

Baulieu's group have proposed that ligand binding induces the dissociation of nonreceptor protein(s) from the untransformed receptor (such as hsp90) (62). By measuring functional GR by its ability to induce the mouse mammary tumor virus fusion gene this group demonstrated that mutated GR which was constitutively active did not contain hsp90 and sedimented at 4S in contrast to triamcinolone acetonide-inducible forms which contained hsp90 and sedimented at 9S (112).

4. Low-molecular inhibitors of transformation

Based on the observation that spontaneous receptor transformation occurs in hypotonic buffer at low temperatures by a variety of experimental procedures including, gel filtration, dialysis or dilution, it has been suggested that the dissociation of a low molecular weight cytosolic component from untransformed receptor complexes is a prerequisite for transformation.

Sato et al. (139) have demonstrated the involvement of a low molecular weight (dialyzable) inhibitor of ER transformation and 4S to 5S conversion. Bailly et al. (140) demonstrated the presence of a heat-stable low molecular weight inhibitor of GR transformation with a size of less than 500 daltons based on membrane ultrafiltration. The molecule was not a steroid based on solubility characteristics and was positively charged since it was adsorbed by anionic resins. Leach (141) characterized a heat-stable factor(s) that causes a stabilization of the hormone-binding activity as well as inhibition of GR transformation. This factor(s) was acid labile but resistant

to a wide variety of hydrolytic enzymes and was negatively charged. Recent studies have demonstrated that thioredoxin and NADPH are required for efficient hormone-binding activity in the mouse GR system (142). Vedeckis (143) has suggested that perhaps NADP(H) is the low molecular weight inhibitor of transformation since reduced sulfhydryl groups have been implicated in the process of receptor transformation.

5. Receptor Phosphorylation.

The following observations support the concept that dephosphorylation of the untransformed 8-9S steroid-receptor or some other component in cytosol is responsible for conversion to the 5S salt-stable, transformed species: (a) in vivo and in vitro studies have demonstrated that ER (85,86,143,144), GR (145,146) and PR (147,148) are phosphoproteins, (b) phosphatase inhibitors such as molybdate, tungstate and vanadate effectively inhibit ER transformation (149), (c) addition of calf intestinal alkaline phosphatase to cytosol GR results in transformation as assayed by an earlier elution of the receptor from DEAE-cellulose (150) and by 9S to 5S conversion (64) and (d) sodium fluoride, glucose 1-phosphate and levamisol (which are all effective phosphatase inhibitors but poor protease inhibitors) can block endogenous receptor transformation (64). The putative substrate for this dephosphorylation reaction has not been identified.

Despite these results, controversy exists as to whether or not dephosphorylation promotes receptor transformation. This is due to

the observation that molybdate can inhibit certain proteases (151), that leupeptin, a protease inhibitor, blocks alkaline phosphatase-promoted receptor transformation (64) and that certain calf uterine alkaline phosphatase preparations were shown to contain contaminating protease activity (152). However, Vedeckis et al. (64) have reported that neither of the two common AtT-20 cell GR proteolytic fragments are detected when alkaline-phosphatase transformed receptor is analyzed by high-salt, high performance gel exclusion chromatography.

6. Receptor Proteolysis.

As a result of the reported inhibition of proteolytic activity by molybdate, and the observed sensitivity of ER to proteases, an exquisitely specific receptor proteolysis has been suggested as a possible mechanism of receptor transformation. Puca et al.(152) have reported that the ER itself has proteolytic activity and that it is responsible for its own transformation. This proposal is supported by the following findings: (a) serine protease inhibitors (aprotinin, diisopropylfluorophosphate, leupeptin) and substrates impair transformation of calf uterine ER, (b) that ER acquires upon transformation a serine binding site for aprotinin, and (c) protease activity is devoid of catalytic activity in the absence of hormone. The authors suggest that the dissociation of the untransformed oligomeric receptor is a consequence of and not the mechanism of this process.

4. Nuclear Binding of RE_2 and Induction of Gene Transcription.

Purification of steroid hormone receptors and the availability of cloned fragments of genes regulated by steroid hormones have enabled the investigation of the specific interaction of steroid-receptor complexes with DNA. Purified GR, ER and PR have been shown to bind in vitro with high affinity to defined regions of DNA near regulated promoters (153). The sequences essential for these interactions are functional in vivo as hormone-dependent transcriptional elements and are called hormone-response elements (HRE). Deletion of these sequences abolishes hormone induction of transcription (153). The results of numerous studies have shown that the HRE's contain no promoter activity, however they increase transcriptional activity by acting as an enhancer element i.e. they exert an influence on nearby promoters that is independent of orientation of the receptor-binding region and relatively independent of distance to the promoter, at least up to several kilobase pairs. The activity of the HRE is strictly hormone dependent and is not functional in cells containing nonfunctional receptors (153).

The mechanism whereby HRE's exert their effect is unclear. There have been reports indicating that there is an inverse correlation between the extent of DNA methylation and the expression of steroid hormone regulated genes (154). Jost et al. (155) have shown that the HRE for the chicken vitellogenin II gene contains an estrogen-dependent hypomethylated site .

It has been documented that receptor binding to the HRE is accompanied by changes in chromatin structure that render a region of 200 base pairs surrounding the HRE hypersensitive to DNase I digestion (153). Whether this altered structure is a prerequisite for receptor binding or rather a consequence of binding is not known.

Several mechanisms for HRE action have been postulated:
-the binding of the hormone-receptor complex to the HRE enables specific transcriptional factors to gain access to that region and increase transcriptional activity (157,158).
-induction of specific folding of the DNA resulting in the placement of the enhancer sequence in the vicinity of the promoter (159).
-the transcription factors enter at the HRE and slide along the DNA to the promoter region (153,160).

Although it is well accepted that steroid hormone receptors can recognize specific DNA sequences in vitro as well as in vivo, the exact chemical nature of the productive nuclear acceptor site, as well as the mechanism by which transcription is activated is still unknown. A significant amount of data tends to substantiate the hypothesis that although the initial specificity of steroid action is conferred by the hormone-receptor complex, the nuclear acceptor is the proposed mediator of the ultimate effect of the steroid hormone on the genome (161,162) and that factors involved in the regulation of gene expression by steroid hormones involve higher order gene structure. Studies from Spelsberg's group (161) have demonstrated that the tissue specificity of the acceptor sites was transferrable by the nonhistone chromosomal protein fraction and that the actual

nuclear acceptor site for the receptor is a DNA-protein complex of chromatin-associated nonhistone proteins and the DNA-backbone. They showed that these nucleoacidic proteins exhibit specific, high affinity and saturable binding sites for the steroid-receptor complexes.

Such nonhistone chromosomal proteins have been partially characterized for the ER system in calf uterus (162) and hen oviduct (163). In addition, chromatin acceptor sites have been shown to interact differently with receptors bound by estrogens vs. antiestrogens (163,164). Recent studies suggest that the nuclear matrix, a nucleic acid-depleted nuclear fraction which may be the site of nucleic acid synthesis (165) contains acceptor sites (166,167).

5. Altered Steroid Receptors

Significant levels of ER have been detected in more than 50% of human breast cancers. Approximately 70% of these ER-positive tumors respond to anti-estrogen therapy compared with only 5% of ER-negative tumors, suggesting a strong correllation between the growth of breast tumors in vivo and the presence of ER (168). However, 30% of ER positive tumors do not respond to hormonal therapy. This may be due to alterations in ER which do not involve the hormone-binding domain assuming that other steps involved in the action of the horone are not affected. In addition, the loss of estrogen dependence in some human breast cancers is often associated with increased malignancy (169). It has been demonstrated that transfection of an oncogene

(v-ras^{H}) into MCF-7 cells results in cells which are estrogen independent in terms of cell growth or tumorigenicity (170).

Lack of responsiveness to estrogens could also be a result of other nuclear binding defects, or of the presence of inhibitory factors which mask the receptor's DNA-binding domain. The recent development of receptor monoclonal antibodies which recognize aspects of the protein that are not necessarily involved in hormone binding have enabled investigators to identify alterations in receptor structure which may be distinct from the hormone-binding domain. The implications of the use of receptor antibodies in determination of functional ER may be important in the ability to predict the response of a patient to hormone therapy.

In this section I shall review studies involving the characterization of mutant steroid receptors derived from target cells which are altered in their sensitivity to steroids or antisteroids.

A. GR mutants

An extensive series of mouse lymphoma cell mutants have been isolated which fail to respond to the cytolytic action of glucocorticoids (S49;171,172, P1798;173,174, and W7;175). These variants are characterized by distinct receptor defects:
-receptor-deficient(r-) mutants lack steroid-binding activity.
-nuclear-transfer deficient (nt-) lines produce a receptor which exhibits normal hormone-binding activity but interacts with reduced affinity with both nonspecific (176,177) and specific (178)

DNA-sequences and do not accumulate in the nucleus to as great an extent as the wild type (179). The monomeric form of this mutant receptor has normal size characteristics (4S;Mr=94kDa). Recently Danielsen et al. (107) have reported the sequencing of wild type and nt- mouse GR cDNAs. Sequence comparisons revealed a point mutation in the mutant receptor's DNA-binding domain (107).
-nuclear-transfer increased (nt^i) mutants contain a hormone-binding species that lacks a major antigenic determinant within the A/B domain (125,180), associates with nonspecific DNA with increased affinity (177) and binds specific DNA sequences with reduced affinity (181). The monomeric form of this mutant receptor is smaller (180-183) than the wild type protein (3.5S;Mr=58kDa), and is produced from a 5'-truncated mRNA (184,185). This nt^i GR is indistinguishable from a partially proteolyzed form of the wild type mouse (186) and rat liver cytosolic (124) GR.

Schmidt et al (187) have described a unique class of GR mutants which are derived from a steroid resistant variant of a human leukemic cell, CEM-C7, which are "activation-labile". These cells contain GR which appears to be completely normal until they are subjected to transforming conditions (warming), upon which they lose hormone binding activity as well as the ability to accumulate in the nucleus. The structural modification responsible for this mutant GR has not been identified.

Hawkins et al. (188) have reported differences in the properties of GR in corticosteroid-sensitive and -resistant lines of transplantable hamster melanomas. They showed that cytosols from

sensitive tumors contained two forms of GR analyzed in the absence of molybdate which sedimented at 7S and 13S, while only the 7S form was detected in cytosol from resistant tumors. Transformed complexes from sensitive and resistant tumors were indistinguishable by centrifugation in molybdate buffer; both sedimented at 4-5S. Different DEAE-cellulose patterns were obtained in which the resistant tumors had a higher affinity for DNA-cellulose, suggesting that the resistant tumors expressed the nt^i phenotype.

B. ER Mutants

Studies comparing the properties of ER derived from transplantable rat mammary adenocarcinomas which differ in their sensitivity to ovariectomy, demonstrated a decreased ability of the resistant tumor to bind to DNA-cellulose (189). Different charge characteristics of ER from these two tumors were demonstrated by differences in their chromatofocusing patterns (190).

Studies from Lippman's group have described an estrogen-independent variant (R3) which was selected by cloning wild type MCF-7 cells in soft agar in the presence of tamoxifen (191). Altered characteristics of the ER system of the R3 cells compared to the MCF-7 wild-type cells included: mimimal induction of PR, minimal nuclear ER processing and the presence of ER which exhibited an increased affinity for DNA-cellulose. Equilibrium binding studies in these mutant cells did not reveal differences in the affinity or number of specific binding sites for estradiol. Receptor from these mutant cells exhibited similiar hydrodynamic properties to those of

the wild type ER. Based on previous studies in MCF-7 cells from McGuire's group (192) which demonstrated a direct correlation between the extent of ER processing (depletion of ER as a function of increasing doses of E_2 in vivo) and PR induction, Lippman's group proposed that altered processing mechanisms could contribute to the estrogen independence of the R3 cells. However, this proposal maybe criticized due to the unclear nature of receptor processing. Several proposals regarding the nature of the processing step have been suggested, including:

-an active state in which a new equilibrium between degradation and synthesis is achieved (193).

-redistribution of receptor within nuclear binding sites of differing affinities (194) or specificities (195) is achieved.

- sequestration of receptor to sites inaccessible to salt extraction (196).

The ER derived from an estrogen-resistant mouse mammary cancer (C3H) was compared with ER derived from the estrogen-sensitive DMBA-induced rat mammary tumor (197). It was reported that ER in the resistant tumor was present in a lower concentration compared to the sensitive tumor, but had a higher affinity for DNA-cellulose. The authors suggested that the reason for the inefficiency of ER in the C3H tumor may be related to its increased affinity for nonspecific DNA sites as previously proposed by Sibley amd Tomkins (171,172) for nt^i GR mutants.

An alternative mechanism for lack of DNA-binding has been associated with proteolysis of the receptor by endogenous proteases (198). However, the differences in DNA-binding observed may not reflect differences in the receptor per se but might rather be due to certain factors in the cytosol which would bind to ER and directly influence the DNA-binding site. Such substances have been reported to influence receptor binding in a number of different systems (199,200).

Studies from Witliff's group have documented the existence of several ER isoforms in human breast cancers based on size, shape and surface charge properties using various HPLC techniques (201-203). They correlated the incidence of remission in patients with breast cancer and the presence of the 8S ER form. Using the monoclonal antibody D547SP they showed that some of the 4S species of ER from human breast cancer did not react with the antibody suggesting that a battery of monoclonal antibodies will be necessary to measure ER in a clinically valid form (204).

II. INTRODUCTION TO THESIS RESEARCH

In contrast to the clear responsiveness to ovarian hormones observed in primary cultures of epithelial cells derived from normal endometrium (205), cells of most human endometrial adenocarcinoma lines are not responsive to estrogen or progestins in vitro. Since it has recently been demonstrated that one of the endometrial cancer lines (Ishikawa) responded to the addition of estradiol (E_2) to the culture medium by increasing cell density, progesterone receptor levels or alkaline phosphatase and DNA polymerase α activities (206-208), investigation of altered biochemical determinants of hormone action in unresponsive endometrial cancer cells became possible. The purpose of the studies described below was to compare characteristics of the estrogen receptor system in Ishikawa cells and in cells of an estrogen-unresponsive endometrial adenocarcinoma line (HEC-50). Levels and affinities of specific hormone binders were measured in 3H-E_2 binding experiments and the presence of altered high-affinity estrogen binding proteins in the unresponsive cells was sought by a variety of approaches:

-using a monoclonal antibody against purified calf uterine estrogen receptor which cross-reacts with the human receptor.

-applying molecular weight electrophoretic analysis under denaturing conditions of ER covalently labeled with [3H]tamoxifen aziridine.

-evaluating the ability of specific E_2 binders to undergo transformation by measuring their binding to DNA in DNA-cellulose preparations.

Normal human endometrium or calf uterus were used as positive controls.

III. MATERIALS AND METHODS

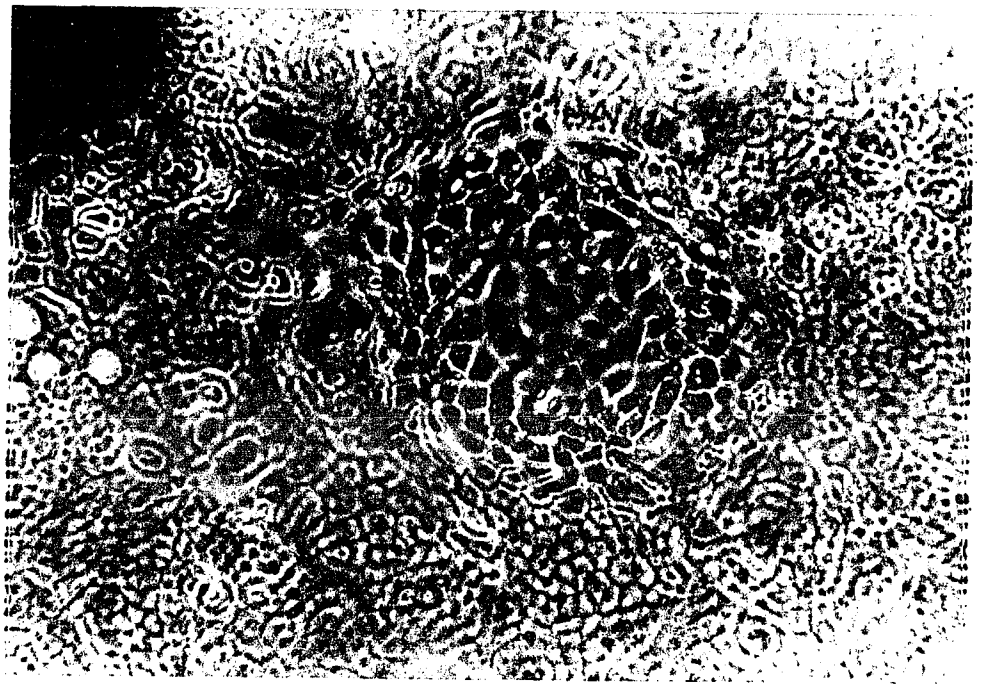
1. Cell Culture and Tissue Preparation.

A. Ishikawa and HEC-50 cells (Fig. 3).

The Ishikawa cell line was established by Nishida et al. (209) at Tsukuba Univ., Japan from a well-differentiated endometrial adenocarcinoma of a 39 y.o. patient; the HEC-50 cell line was established by Kuramoto et al. (210), Kitasato Univ., Kanagawa-Ken, Japan, from a poorly-differentiated endometrial adenocarcinoma of a post-menopausal woman. Both cell lines were originally made available by Dr. H. Kuramoto. Cells used for these studies were grown in 10cm diameter tissue culture dishes (Falcon Plastics) in MEM (Minimum Essential Medium) with Earle's salts (Flow Laboratories) supplemented with 15% fetal bovine serum previously treated with a dextran-charcoal (0.05%0 dextran-0.5% charcoal w/v, DCC) suspension (SFBS). Cultures were maintained at 37°C in a humidified atmosphere of 95% air-5%CO_2 until densities of about 10-15 X 10^6 cells/dish were achieved. Cells were then harvested by treatment with 0.05%trypsin-0.02% EDTA solution (GIBCO) for 10-20 min. at 37°C. The effect of trypsin was stopped by addition of 7% SFBS in MEM. The cells were pooled and washed with Hank's balanced salt solution (HBSS,GIBCO). Cell pellets were stored at 0-4°C.

Ishikawa cells have been shown to respond to estrogens by increasing a number of parameters: cell proliferation, progesterone receptor levels, alkaline phosphatase, and DNA polymerase α activities (206-208). The HEC-50 cell line did not show responsiveness to estradiol.

(A)



(B)

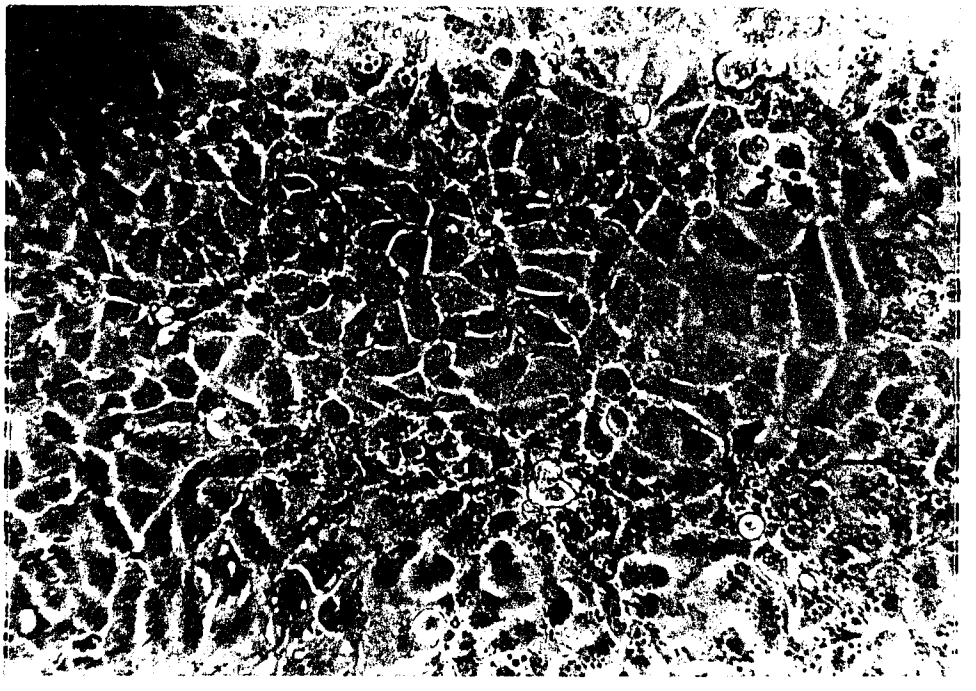


Fig. 3 Photomicrographs of Ishikawa and HEC-50 cells.

Ishikawa (A) or HEC-50 (B) cells were grown to confluence and examined at 100X (A) or 200X (B) magnification using a phase contrast microscope.

B. Human Endometrium (Fig. 4)

Surgical specimens of human endometrial tissue were obtained from patients undergoing dilatation and curettage procedures or after hysterectomy. Only histologically normal proliferative specimens were used in these studies. Endometrial tissue was transferred from the operating room at 4°C in MEM (Eagle's,containing Earle's salts, Grand Island Biological Co.,Grand Island, NY, GIBCO) containing 1% antibiotic-antimycotic mixture (GIBCO). Tissue was trimmed to remove blood clots, mucus and debris, cut into small fragments in HBSS and stored at -70°C.

A sample of fresh tissue was fixed in 10% formalin and stained with hematoxylin and eosin. The day of the menstrual cycle was determined for each tissue according to Noyes et al. (211).

C. Calf Uterus

Immature calf uteri were obtained from the slaughterhouse and transferred on ice. The uteri were trimmed of fat frozen in liquid nitrogen, and and stored at -70°C.

2. Preparation of Cytosol and Nuclear Extracts.

Adenocarcinoma cells or endometrial tissue were disrupted at 0-4°C by glass/glass homogenization in 2-4 volumes of GTEDP buffer (10% glycerol w/v, 20mM Tris-HCl,pH 7.4, 1.5mM EDTA, 1mM DTT, 0.5mM PMSF) using a motor driven pestle. Calf uterine tissue was suspended in 4-6 volumes of GTEDP and homogenized using 3x5sec bursts of a Polytron set at speed 5. The homogenate was centrifuged at

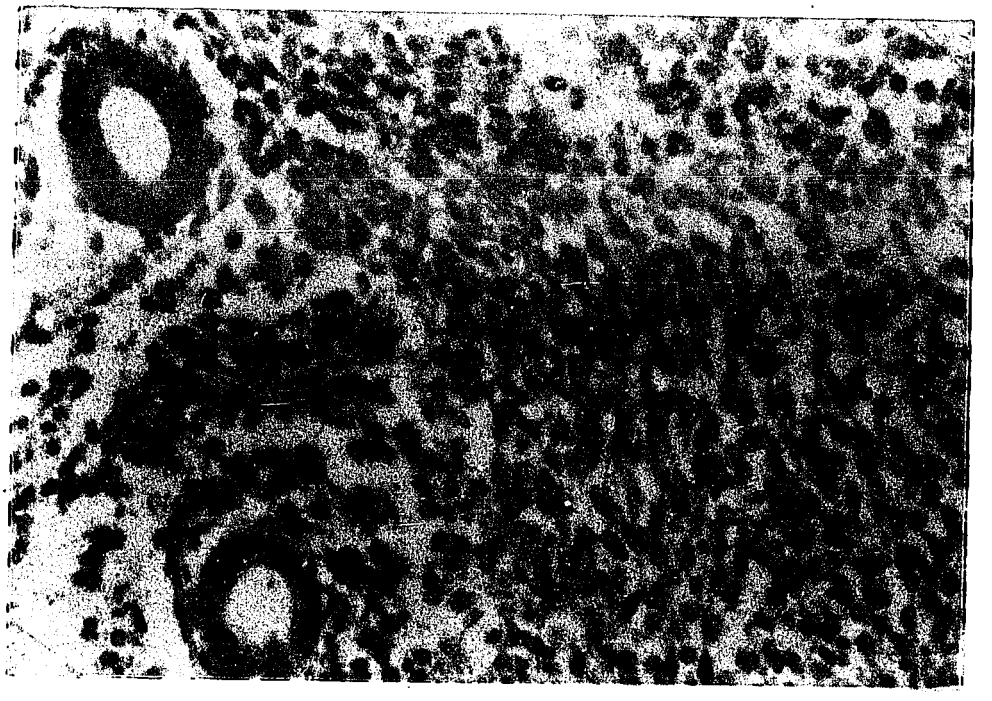


Fig. 4 Photomicrograph of Proliferative Endometrium.

Human proliferative endometrium was obtained and processed as described in Materials and Methods and was examined at 100X magnification using a phase contrast microscope.

800 x g for 5 min and the supernatant was centrifuged in a Ti50.3 rotor at 105,000xg for 60min and the supernatant was used as cytosol. The low speed pellet (nuclear pellet) derived from the cancer cells or endometrial tissue was washed twice with GTEDP and then resuspended in 1ml GTEDP containing 0.6M KCl and incubated for 45min at 0°C. During this incubation the nuclear pellet was resuspended and homogenized at 10 min. intervals. At the end of the incubation the nuclear suspension was diluted to 0.1M KCl and was centrifuged at 1500xg for 15 min. The supernatant was used as nuclear extract.

3. Protein Determination

Protein concentrations in cytosol preparations from human endometrium, calf uterus and adenocarcinoma cells were determined with a Bio-Rad protein assy kit based on the colorimetric method of Bradford (212) using bovine serum albumin as standard.

4. Estradiol Binding Assay

Aliquots of cytosol or nuclear extracts were incubated at 0-4°C for 2 hours with (2,4,6,7) 17β-[3H]E_2, (100Ci/mmol, Amersham) at concentrations ranging from 10-20nM as specified in each experiment, with or without a 300-fold excess of unlabeled E_2. Free steroid was removed by incubation with a suspension of DCC for 10min. at 0-4°C, followed by centrifugation at 1500 x g for 15 min. Radioactivity was measured in an aliquot of the supernatant mixed with Liquiscint (National Diagnostics Co.) using a Beckman LS 9000

counter. Specific binding was calculated by subtracting from the total [3H]E_2 bound the amount of radioactive ligand bound in the presence of excess unlabeled E_2. Levels of specific binding were expressed as fmol [3H]E_2 bound/mg protein.

A. Saturation Analysis of E_2 Binding

Aliquots of cytosol were incubated with [3H]estradiol in the concentration range 0.1-40nM for 2-4hr at 0°C (time required for binding to reach plateau). Free steroid was removed by DCC adsorption. The affinity constants (Kd) and number of sites (N) were determined using graphic procedures: Scatchard plots, Rosenthal analysis or curve fitting implemented by computer with and RS/1 program.

a. Scatchard analysis.

In this method the nonspecifically bound ligand is calculated by extrapolation of the slope of the line (total bound versus total ligand added) observed at points of high concentration of ligand. Specifically bound [3H]E_2 is determined by subtraction of the nonspecific binding from the total bound ligand. The ratio of specifically bound/unbound ligand was plotted against specifically bound ligand according to Scatchard (213) and the Kd was determined from the slope of the line best fitting the resulting points.

b. Rosenthal analysis.

In this method the specifically bound ligand is determined graphically as described by Rosenthal(214) and Chamness and McGuire (215). A plot of the total bound/free ligand versus total ligand

bound is drawn and specific binding is obtained by subtracting from total bound the amount corresponding to nonspecific binding (asymptotic part of the curve), at a constant concentration of free ligand. The Kd is determined from the slope of the line best fitting the generated points, according to Scatchard (213).

c. RS/1 Computer program.

This program, provided by Dr. Roman Osman, Department of Physiology and Biophysics, Mount Sinai School of Medicine, determines the Kd and concentration of binding sites by fitting the data to an equilibrium binding equation consisting of saturable and unsaturable binders, e.g.:

$B_T = B_{sp\ max}\ [F]/(Kd+[F])$ + (slope at high conc. of ligand) [F], where B_T is total bound concentration, $B_{sp\ max}$ corresponds to the number of specific binding sites and [F] is the concentration of unbound ligand.

5. Thin Layer Chromatography.

Stock solutions of radiolabeled steroid were periodically checked for purity by chromatography on silica gel GF plates (ANALTECH). Aliquots of $[3H]E_2$ and $[14C]E_2$ were mixed in a ratio of 10:1. Unlabeled estradiol (500ug) and unlabeled estrone (500ug) were added as carriers, dissolved in methanol and applied to each plate. The solution was air dried and eluted for 45 min. with chloroform:ethylacetate (4:1). Steroids were localized on the plates by u.v. light. The plates were scraped at the origin, the zone of

unlabeled E_2 and the zone of unlabeled estrone, followed by elution with methanol. Following centrifugation at 1500 x g, aliquots of the supernatant were counted for radioactivity and the 3H/14C ratio was determined in each zone.

6. Immunochemical Interactions.

A. Preparation of the monoclonal antibody.

The monoclonal antibody JS 34/32 was a gift from Dr. Indu Parikh, Glaxo Inx., Research Triangle Park, N.C. This antibody was obtained from hybridoma cells produced by fusing SP 2/O-Ag/4 mouse myeloma cells with spleen cells from a mouse immunized with a purified preparation of estrogen receptor from calf uterine cytosol (206). The antibody is of the immunoglobulin G (IgG) class and cross-reacts with cytoplasmic and nuclear forms of calf receptor as well as with rat and human receptors. The equilibrium dissociation constant of the antibody-receptor complex is 6.5×10^{-11}M in solid phase and 5.6×10^{-10}M in solution (216). This antibody recognizes both occupied and unoccupied ER (92) as well as molybdate-stabilized ER (62). This antibody cross-reacts specifically with a protein that gives a single band of approximately 65,000 daltons on SDS-PAGE. Two molecules of this antibody are able to simultaneously interact with the native, 8S "low-salt " form of the receptor, indicating two antigenic determinants, in contrast to the one antigenic determinant in the "high-salt, transformed" 4S form (92). Dissociating agents that induce the dissociation of the 8S form to smaller forms also induce the dissociation of the two antigenic determinants. It has

also been shown that the JS34/32 antibody recognizes the receptor after phosphorylation with [γ 32P]ATP as well as after dephosphorylation (217,218). Recent studies have shown that ER purified from rat uterus binds with high affinity and specificity to anti-phosphotyrosine antibodies suggesting that ER is phosphorylated on tyrosine (143).

B. Immunoprecipitation of Antibody-Receptor Complexes.

Aliquots of [3H]estradiol-receptor complexes were incubated at 0-4^{o}C for 2-4 hr with or without various dilutions of JS34/32. The immune complexes were precipitated as follows using either Pansorbin cells (Calbiochem-Behring Corp.) or Protein-A Sepharose 6MB (Pharmacia). Both methods rely on the ability of Protein-A, a 42,000 dalton protein, to bind two IgG molecules. Protein-A has a strong and highly specific affinity for the Fc region of IgG molecules. [3H]estradiol-receptor-JS34/32 immune complexes bound to either Pansorbin cells or Protein-A Sepharose beads were isolated from solution by centrifugation and washing of the pellet followed by elution with methanol.

Pansorbin Cells are derived from formaldehyde-inactivated Staphylococcus aureus cells (SAC, Cowan Type I) whose outer coat contains Protein-A (219). Aliquots of 25ul of a 5% suspension of SAC in phosphate-buffered saline containing 1mg/ml BSA, PBS/BSA, (binding capacity: 1mg human IgG/ml of cell suspension) were incubated with [3H]estradiol receptor-JS34/32 immune complexes (300ul) for 2-4hr at 0^{o}C with shaking. The 2500xg pellet was washed 3 times in 2ml

PBS/BSA followed by extraction with 2ml methanol. The methanol extract was dried down under nitrogen and liquiscent was added.

Protein-A Sepharose beads are formed by coupling of Protein-A to macrobeads of sepharose using cyanogen bromide (220). ER-JS34/32 immune complexes (300ul) were incubated with 50ul of swollen gel, for 2-4hr. at 0°C (binding capacity: 5mg human IgG/ml bed volume) with shaking in 2ml conical microfuge tubes, followed by centrifugation at 12,000 x g in a microfuge for 90 sec. The pellet was extracted with methanol, dried down under nitrogen gas and liquiscent was added. Specific binding to Protein-A was calculated after subtraction of the radioactivity bound in the absence of antibody.

C. Glycerol Density Gradient Analysis of Immune Complexes.

Analysis of immune complexes by centrifugation on high salt glycerol gradients was based on the observation by Moncharmont et al. (216) that [3H]ER-JS34/32 immune complexes sediment more rapidly (8-9S) than [3H]ER complexes (3.5-4.5S). Continuous 15-40% (w/v) glycerol gradients (4.4ml) were prepared in a 20mM Tris-HCl, pH7.4, 12mM monothioglycerol, 1.5mM EDTA, 0.5mM PMSF, 0.4M KCl buffer. Aliquots of [3H]estradiol-immune complexes (200-500ul) were layered on the top of the gradient and centrifuged at 4°C for 17 hours at 250,000 x g in an SW 55 rotor (Beckman). Four drop fractions were collected by upward displacement using an Auto Densi Flow IIC (Haake-Buchler) and assayed for radioactivity. [14C]bovine serum albumin (4.6S) and [14C]gamma globulin (7S) standards (Amersham) were

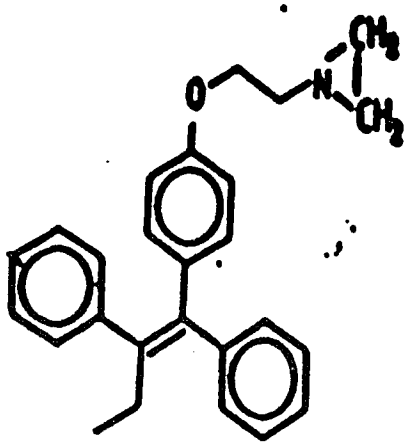
used as marker proteins in parallel runs. The data were plotted in the direction of increasing glycerol density (1.03-1.08 g/cm^3). Density gradients were prepared with glycerol rather than sucrose since glycerol has been reported to have a greater stabilizing effect on a variety of enzymes and receptors (221). Glycerol might therefore decrease the dissociation of steroid from receptor over the prolonged centrifugation time and increase the sensitivity of the measurement.

7. SDS-PAGE of ER labeled with 3H-tamoxifen aziridine.

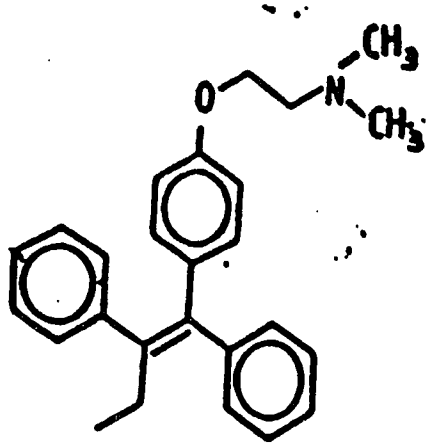
[3H]tamoxifen aziridine ([3H]TAZ) was used to covalently label cytosolic ER for subsequent molecular weight analysis on SDS-PAGE. 3HTAZ was obtained from Amersham at a specific activity of 22 Ci/mmol (Fig. 5). This compound is an electrophilic analog of the antiestrogen, tamoxifen and has been shown to covalently label ER with high efficiency and selectivity (222). Wei et al. (223) demonstrated that TAZ displayed a bioactivity profile similiar to that of tamoxifen, the reversibly binding ligand, in vitro and in vivo.

Incubation of cytosol with [3H]TAZ at final concentration of 20nM (prepared in dimethylformamide at a final concentration of 7%), was carried out for 45 min. at 25^{o}C. Determination of nonspecific labeling was accomplished by presaturation of ER with 5uM radioinert estradiol for 60 min. prior to the additon of [3H]TAZ. At the end of the incubation, excess ligand was removed by incubation with DCC for 15 min at 25^{o}C followed by centrifugation at 1500 x g for 15 min.

(A)



(B)



(C)

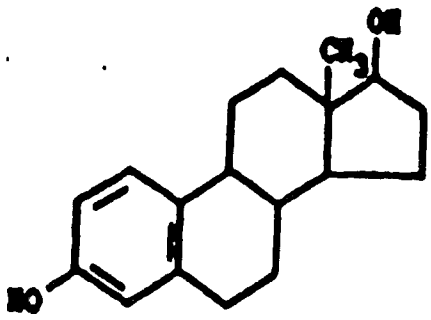


Fig. 5 Structure of (A) tamoxifen aziridine, (B) tamoxifen and (C) estradiol.

Samples were solubilized in buffer containing 0.01M Tris pH 7.4, 8% glycerol w/v, 1% 2-mercaptoethanol, 1% SDS and 0.01% bromophenol blue and heated at 100°C for 5min.

Disc gels were prepared in cylindrical tubes (6mm x 125mm) according to Laemmli (224), except that all buffers contained 0.1% SDS. The separating gel consisted of 7.5% acrylamide prepared in 0.37M Tris buffer pH 8. Gels were polymerized by the addition of TEMED (Biorad) and ammonium persulfate. The running buffer consisted of 0.025M Tris buffer pH8.3 and 0.197M glycine. Gels were run in an apparatus (Haake Buchler) which contained a cold water jacket, at a constant current of 1.5mA/gel. Following electrophoresis the gels were assayed for radioactivity by freezing them on dry ice and slicing into 2mm sections. Each slice was treated with 0.5ml of NCS solubilizer (Amersham) overnight at 24°C. The gels were counted for radioactivity in 5ml Liquiscint containing 1% glacial acetic acid to quench the fluorescence. [14C]BSA (67kDa) and [14C]OVA (45kDa) were run in parallel gels and served as molecular weight markers.

8. DNA-cellulose Binding Assay.

Binding of [3H]E_2-binders to DNA-cellulose was evaluated by: (A) DNA-cellulose column chromatography as described by Kovacs et al. 225) or (B) batchwise incubation as described by Evans et al. (226). Native, calf thymus DNA-cellulose (227) or unsubstituted cellulose powder purchased from Sigma Chemical Co. was washed before use in buffer without EDTA (GTDP). The studies were conducted in the absence of EDTA since it has been recently shown that this compound

may prevent transformed ER from binding to DNA-cellulose by chelation of receptor bound metal (120).

A. Labeled E_2-binders in cytosol were transformed by incubation at 25°C for 25min. Since molybdate has been reported to maintain hormone receptor complexes in an untransformed state (48,53), cytosol containing Na_2MoO_4 at a final concentration of 25mM served as untransformed control. Molybdate had no effect on the DNA-cellulose binding if it was added after the activation step. Aliquots of DCC-treated cytosol (400ul) containing [3H]ER complexes were washed with 5ml buffer and eluted with 0.4MKCl and methanol. 0.5ml fractions were collected and assayed for radioactivity.

B. Aliquots of DCC-treated cytosol (400ul) containing [3H]-ER complexes were added to 40mg DNA-cellulose (containing 200ug DNA) or to cellulose and incubated for 15-17 hr at 0-4°C with gentle agitation. Molybdate (25mM) was added in parallel incubations. In these experiments receptor activation and DNA interaction occurred simultaneously during the incubation of cytosol at 0°C in the presence of DNA. After centrifugation, the supernatants were discarded and the pellets were washed in 3 x 1ml GTDP buffer. The pellets were resuspended in 7ml of Liquiscint and counted.

The specific binding of labeled-ER complexes to DNA-cellulose was expressed as the fraction of the specifically bound [3H]E_2 in the test aliquot, specifically retained by DNA. Comparison of results obtained in the presence or absence of molybdate indicate the extent of ER activation in the samples.

IV. RESULTS

1. Saturation analysis of cytosolic estrogen binders.

Saturation analysis of cytosolic E_2 binders was performed at concentrations ranging from 0.1 to 40nM for 4hr at 0-4°C. As shown in Fig. 6, specific estrogen binding reached equilibrium between 2-4 hr at the indicated temperatures. Since the specific binding was higher at the low temperature at which unoccupied ER is more stable, all subsequent experiments were performed at 0-4°C.

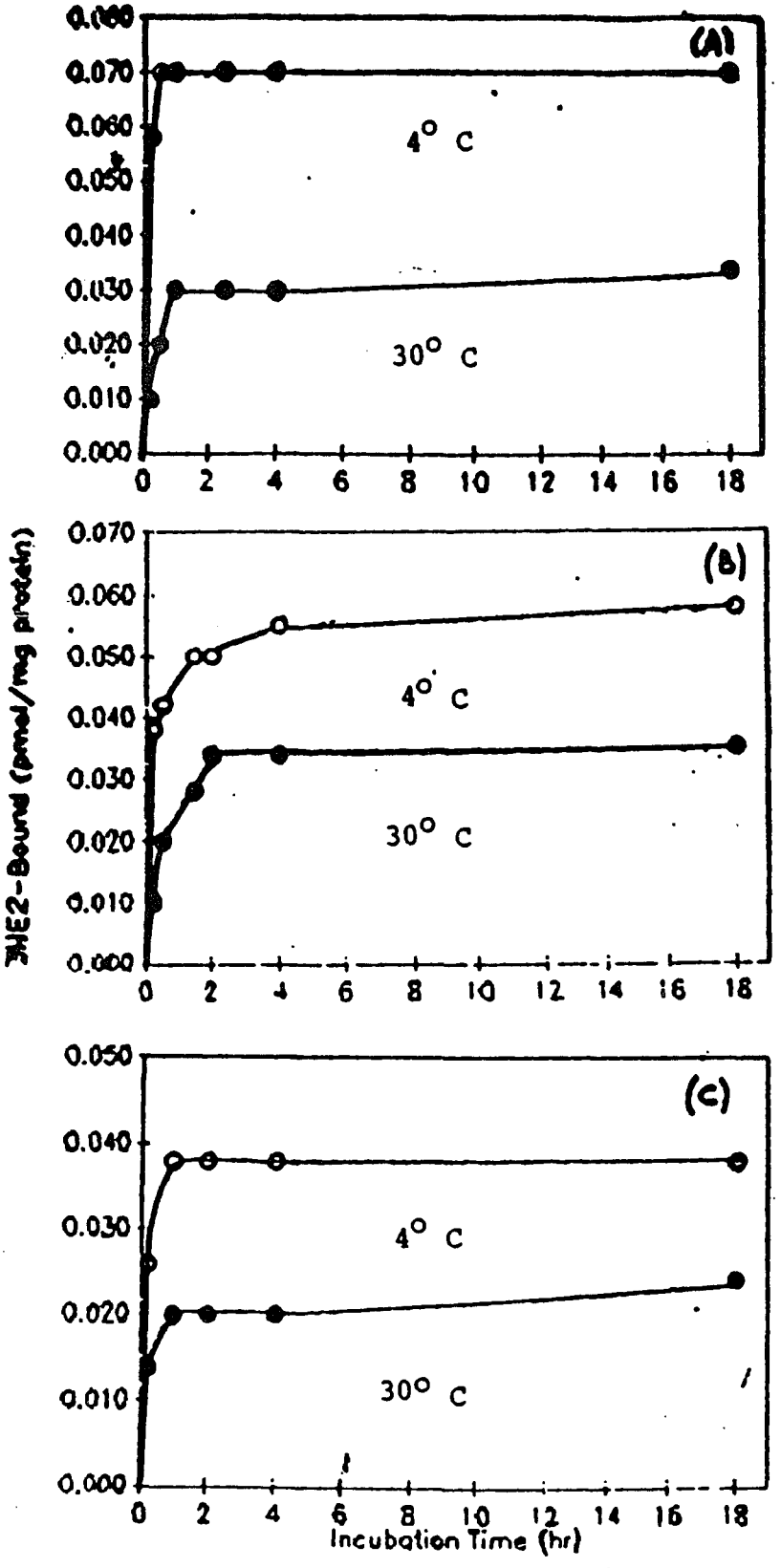
Fig.7 shows a typical [3H]E_2 binding curve in Ishikawa cell cytosol.

Scatchard plots corresponding to the specific binding of [3H]E_2 in cytosol from Ishikawa cells and HEC-50 cells are shown in Fig. 8. As shown in Table 2, the presence of a high affinity E_2 binder was evident in both cell lines and human endometrium in all three methods of analysis of the binding data. No significant differences were observed between the values obtained for the affinity constant (Kd) and number of binding sites ($B_{sp\ max}$) for the E_2 binders in the two cell lines (Kd approximately 0.7nM) as determined by t-test analysis of the means. Despite the observed variation in the values of the Kd and $B_{sp\ max}$ among the different methods employed to analyse the binding data no differences were observed in these values between the two cell lines. It can therefore be concluded that specific estradiol binders with equal, or at least similar affinities for the ligand are present in the estrogen-unresponsive HEC-50 cells, the estrogen-responsive Ishikawa cells and endometrial tissue.

Fig. 6 Effect of incubation time and temperature on specific [3H]estradiol-binders.

Aliquots of cytosol from (A) Human endometrium (B) Ishikawa cells or (C) HEC-50 cells were incubated with 10nM [3H]estradiol in the absence (total binding) or presence (nonspecific binding) of 3uM unlabeled E_2 for 15min-18hr at either 4°C (open circles) or 30°C (closed circles). Free steroid was removed by DCC adsorption. Radioactivity was measured in an aliquot of the supernatant and specific binding was determined as described in Materials and Methods (section III-4).

Fig.6



Human endometrial cytosol

Ishikawa cytosol

HEC-50 cytosol

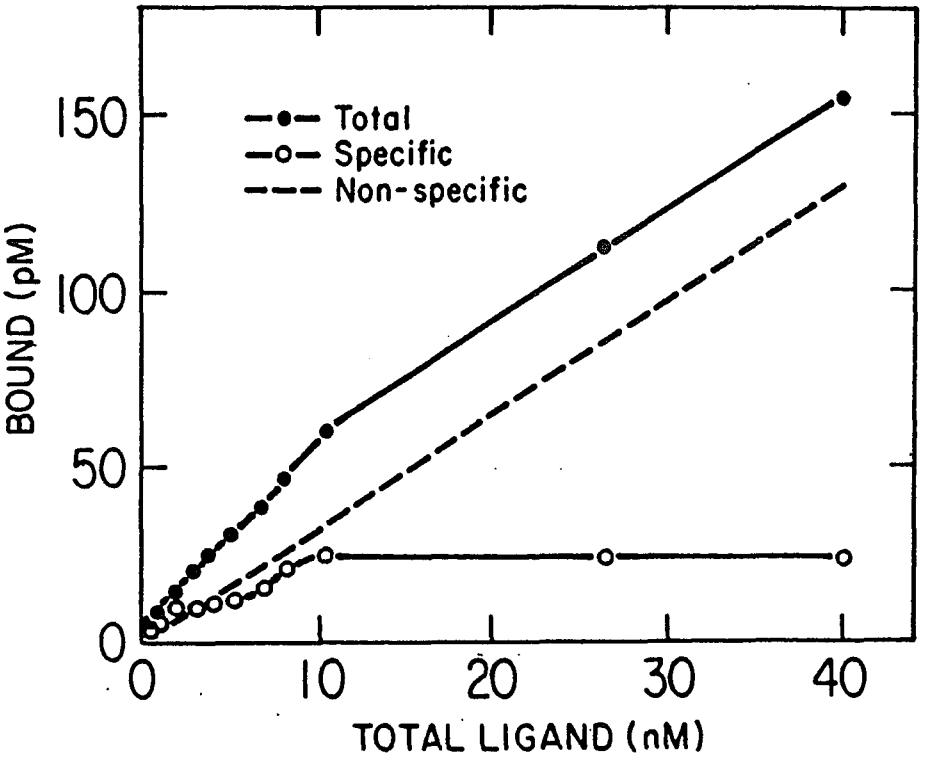


Fig. 7 [3H]Estradiol binding in cytosol from Ishikawa cells. Aliquots of cytosol were incubated with [3H]E_2 in the concentration range 0.1nM to 40nM for 4hr at 4°C. Free steroid was removed by DCC adsorption and radioactivity was measured in an aliquot of the supernatant as described in Materials and Methods (Section III-4A). Total bound (open circles), non-specific binding (dashed line) and specifically bound (closed circles) [3H]E_2 was determined as described in Materials and Methods (Section III-4A-a).

Fig. 8 Scatchard analysis of specific [3H]estradiol binders in cytosol from Ishikawa cells and HEC-50 cells.

Cytosol was labeled with [3H]estradiol in the concentration range 0,1nM to 40nM for 4 hr at 4°C. Unbound steroid was removed by DCC. Specific binding (B_{sp}) was estimated as described under Fig. 7 and in Materials and Methods (Section III 4A-a). The data was plotted according to Scatchard and the dissociation constants (Kd) as well as the amount of specific binder ($B_{sp\ max}$) were determined from the slope and x-intercept of the best fitting straight line:

$$B_{sp} / F = -(1/Kd)\ B_{sp} + B_{sp\ max} / Kd$$

Fig. 8

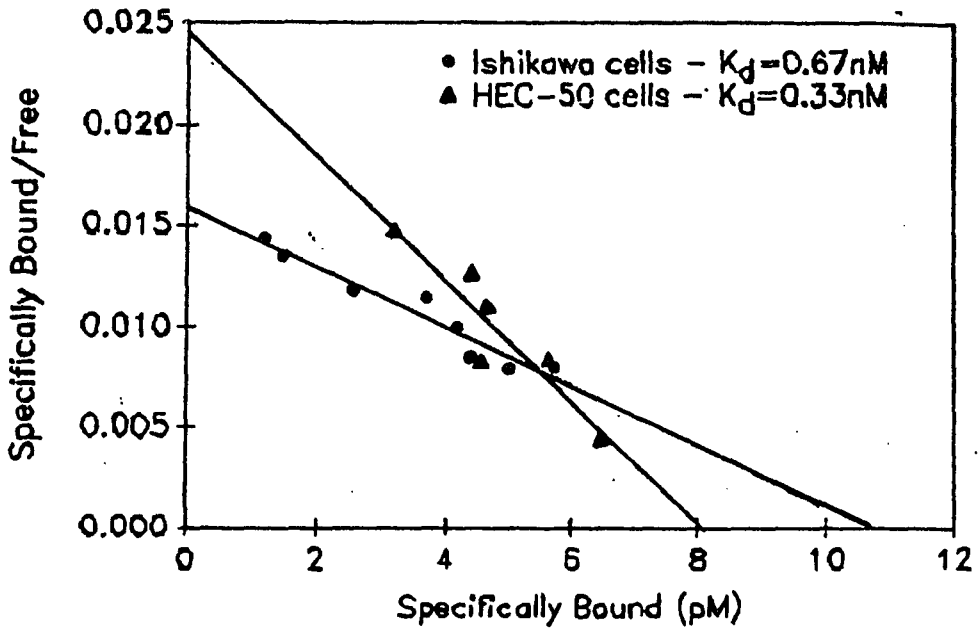

0.025
0.020
0.015
0.010
0.005
0.000
Specifically Bound/Free
• Ishikawa cells – K_d=0.67nM
▲ HEC-50 cells – K_d=0.33nM
0
2
4
6
8
10
12
Specifically Bound (pM)

Table 2 Summary of estimated equilibrium dissociation binding constant and concentration of high affinity binder in cytosol from [3H]estradiol saturation curves.

Cell or Tissue	Grahic Methods				RS/1 Computer Method	
	Scatchard		Rosenthal			
	Kd*	Bsp max**	Kd*	Bsp max**	Kd*	Bsp max**
Ishikawa	0.93	17	0.22	1.3	1.3	8
cells	0.81	7	0.17	2.0	8.0	9
	0.67	6	0.53	4.0	5.1	16
Mean±SE	0.80±0.08	10±3.5	0.31±0.11	2.4±0.81	4.8±1.9	21±9.2
HEC-50	0.55	12	0.10	2.0	8.5	45
cells	0.33	4	0.25	2.0	0.3	6
	1.25	4	0.18	0.5	2.8	2
Mean±SE	0.71±0.28	6.7±2.7	0.18±0.04	1.50±0.5	3.9±2.4	18±13
Human Endometrium	1.0	25	1.2	26	0.55	15

* nM

** fmol/mg protein

Concentrations of binding sites per mg cytosol protein were lower in the 2 cell lines than in the proliferative endometrium (Table 2). In agreement with previous reports (217), significant differences in the number of binding sites per cell were observed within the same line.

These results revealed the presence of a high affinity, specific estradiol binder in Ishikawa and HEC-50 cells as well as in endometrial tissue. They failed to indicate, however, any qualitative differences in binding affinities of E_2 to the ER that could account for the lack of responsiveness of HEC-50 cells to the hormone.

2. Interactions with the JS 34/32 monoclonal antibody.

The monoclonal estrogen receptor antibody JS 34/32 was used to search for structural or functional differences in the specific estrogen binders in cytosol and nuclear extracts of the two cell lines. In was previously reported that JS 34/32 recognizes estrogen binders from proliferative endometrium and calf uterus (208). Therefore, estrogen binders from these sources were used as positive controls. Initially the interaction of estrogen binders with the antibody was tested using Pansorbin cells. Fig. 9 indicates that 0.75ug of JS 34/32 antibodies were sufficient to obtain maximal formation of the complex in cytosol from calf uterus.

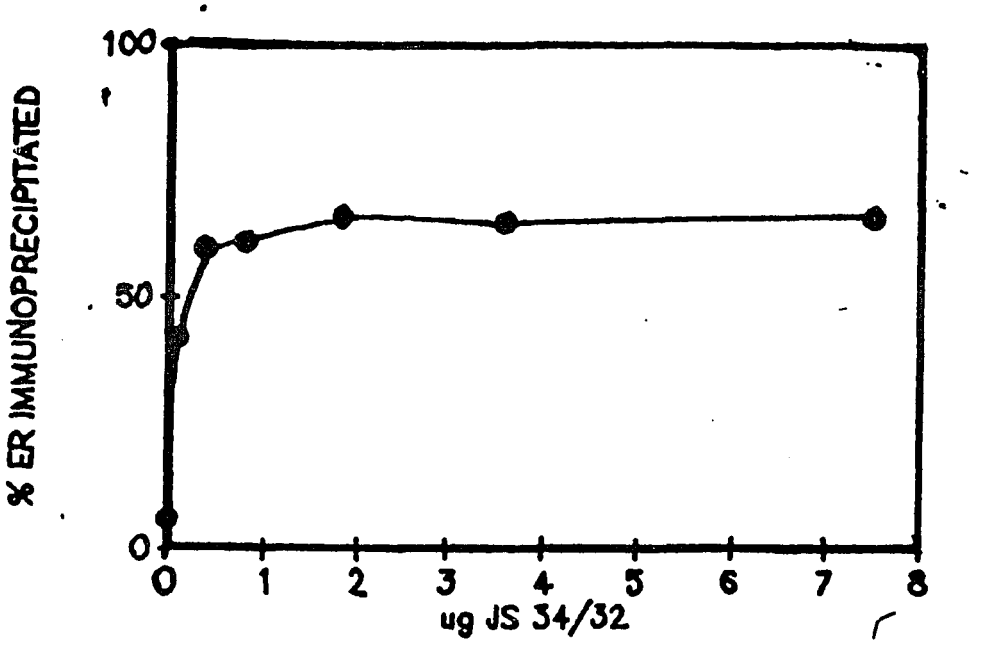


Fig. 9 Immunoprecipitation of cytosolic [3H]estradiol binders from calf uterus with increasing concentrations of JS 34/32.

Aliquots of calf uterus cytosol were labeled with 10nM [3H]E_2 for 2 hr in the presence or absence of 3uM unlabeled E_2. Free steroid was removed by DCC. [3H]E_2-receptor complexes (0.4pmoles) were incubated with increasing amounts of JS 34/32 for 2 hr and were precipitated with Pansorbin. All steps were performed at 0-4°C. The radioactivity adsorbed by Protein A in Pansorbin was determined as described in Materials and Methods (Section III-6B).

Fig. 10 demonstrates that the antibody recognized only a fraction of the number of specific binding sites detected with the DCC method. Partial precipitability of the specific [3H]estradiol binders was not due to insufficient Pansorbin concentration since the amounts of Pansorbin were in excess of its capacity to adsorb IgG.

As shown in Fig. 11, 7ug of JS 34/32 antibody is sufficient to obtain maximal precipitation of the specifically-bound (.06pmoles) [3H]E_2-receptor complexes in endometrial cytosol (Ab:ER molar ratio of approximately 800). A summary of the results of experiments analysing the ability of JS 34/32 to recognize specific E_2 binders in cytosol and nuclear extracts from the two cell lines and human endometrium are shown in Table 3. Approximately 30% of the labeled specific binders in proliferative endometrium and about 20% of those in Ishikawa cells were precipitated. In contrast, no precipitation of binders in HEC-50 cells could be detected under these conditions. Similar lack of antibody recognition was observed in ER present in the nuclear extract from HEC-50 cells.

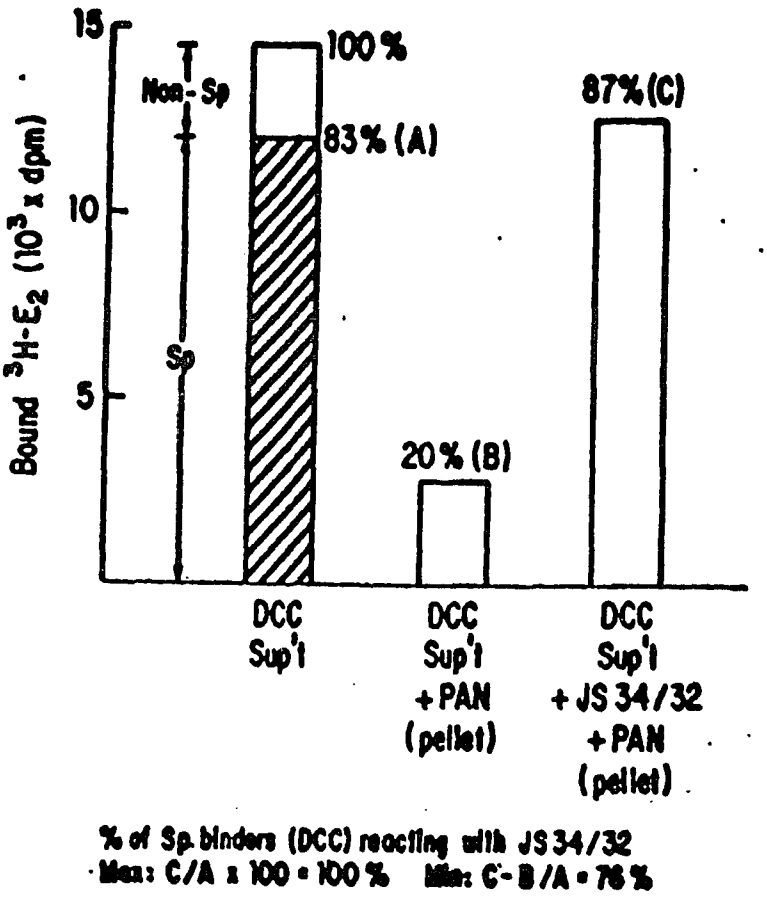


Fig. 10 Immunoprecipitation of [3H]estradiol-receptor-JS 34/32 complexes in calf uterine cytosol with Pansorbin.

Aliquots of calf uterus cytosol were labeled with 1nM [3H]E_2 for 2hr at 4°C in the presence or absence of 0.3uM unlabeled E_2. Free steroid was removed with DCC and the supernatant (0.05pmoles of specifically-bound [3H]E_2) was incubated with or without 0.3ug JS 34/32 antibody for 2hr. Immune complexes were precipitated with Pansorbin as described in Materials and Methods (Section III-6B).

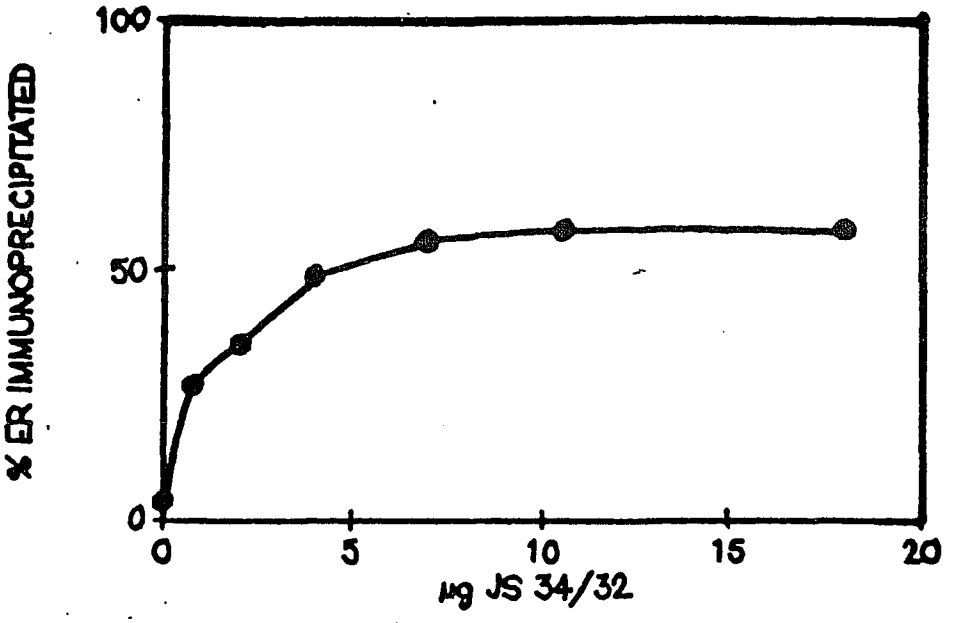


Fig. 11 Immunoprecipitation of specific [3H]estradiol-binders in human endometrial cytosol with increasing concentrations of JS 34/32.

Aliquots of endometrial cytosol were incubated with 20nM [3H]E_2 for 2hr at 4°C in the presence or absence of 6uM unlabeled E_2. Free steroid was removed by DCC. Aliquots of labeled specific binders (.06pmoles) were incubated with the specified quantity of JS 34/32 (ug protein). Immune complexes were precipitated with excess Protein A Sepharose beads as described in Materials and Methods (Section III-6B).

Table 3 Immunoprecipitation of ^{3}H-estradiol receptor-JS 34/32 antibody complex with Protein A-Sepharose.

Cell or Tissue	Number of samples tested	Specific binding (fmol/mg protein) ($\bar{X}\pm SE$)	% of sp. binding immunoprecipitable
Endometrium:			
cytosol	21	90±10	>30
nuclear extract	5	130±40	>25
Ishikawa cells:			
cytosol	4	22±9	>20
nuclear extract	6	50±20	>24
HEC-50 cells:			
cytosol	4	34±16	not detectable
nuclear extract	4	43±8	not detectable
Calf uterus:			
cytosol	3	84±12	>61

The lack of interaction of the JS 34/32 antibody with the E_2 specific binders of HEC-50 cells is also apparent from the results shown in Fig. 12. This graph depicts the distribution of [3H]estradiol specific binders in high-salt glycerol gradients, in the presence or absence of JS 34/32 antibody. Addition of the antibody provoked a shift in the sedimentation value of the specifically bound radioactivity from approximately 4S to 8-9S in cytosol or nuclear extracts of the Ishikawa cells and endometrial tissue. A similiar shift in the sedimentation value of the specifically bound radioactivity was observed in calf uterine cytosol (208). In contrast, no shift was observed in cytosol of the HEC-50 cells.

These results indicate the absence of immunoprecipitable estrogen receptor in the non-responsive HEC-50 cells and its presence in the estrogen-responsive Ishikawa cells.

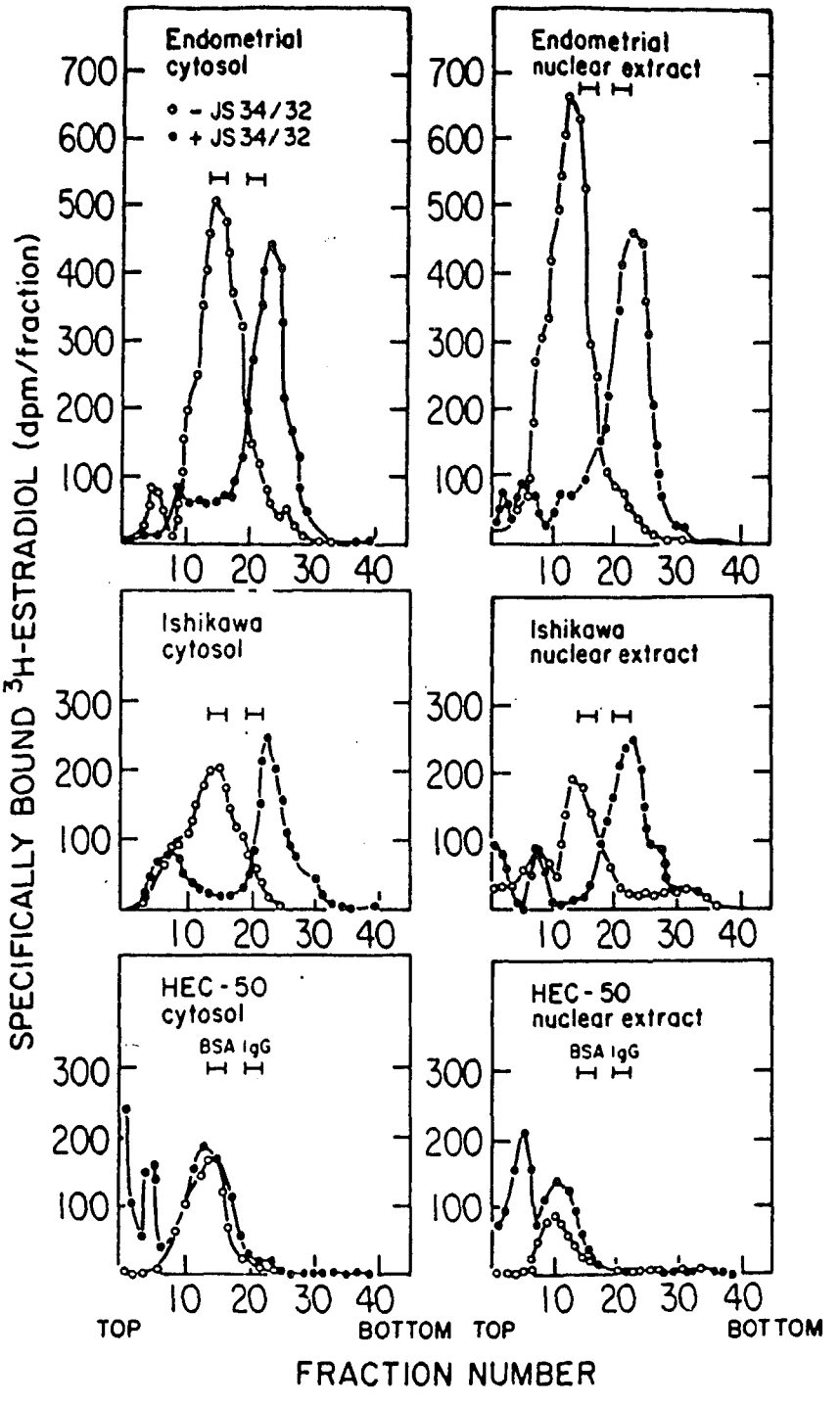
3. Size characteristics.

The size of specifically bound [3H]E_2 recognized by the antibody did not appear different in the cells and tissue as determined from high salt glycerol gradients (Fig.12). However, in the cells and endometrium there was evidence of specifically bound E_2 binders which sedimented more slowly and were not immunoreactive (2-3S). This may represent meroreceptor resulting from the activity of endogenous proteases in crude preparations during the extended incubation times. These receptor fragments were more evident in the adenocarcinoma cells. This could be due to the presence of larger

Fig. 12 Glycerol density gradient analysis of [3H]ER-JS 34/32 immune complexes.

Cytosol from human endometrium, Ishikawa cells or HEC-50 cells was incubated with 20nM [3H]E_2 in the presence or absence of 6uM unlabeled E_2 for 2hr at 4^{o}. Free steroid was removed by DCC. Aliquots of 3H-E_2 binders were incubated in the presence (closed circles) or absence(open circles) of JS 34/32 at an Ab/R molar ratio of 800 for 2hr. The immune complexes were centrifuged in 15-40%(w/v) glycerol gradients in the presence of 0.4M KCl and analyzed as described in Materials and Methods. Bars show migration of [14C]labeled standards: Bovine serum albumin (BSA),4.5S and gamma globulin (IgG), 7S (Section III-6C).

Fig. 12


Endometrial cytosol
○ - JS 34/32
● + JS 34/32
Endometrial nuclear extract
Ishikawa cytosol
Ishikawa nuclear extract
HEC-50 cytosol
BSA IgG
HEC-50 nuclear extract
BSA IgG
SPECIFICALLY BOUND ³H-ESTRADIOL (dpm/fraction)
TOP
BOTTOM
TOP
BOTTOM
FRACTION NUMBER

amounts of endogenous proteases in these cells as compared to the tissue, since it has been reported that certain mammary cancers contain increased protease activity (198). Alternatively, the E_2-binders in the cell lines may be more suceptible to proteases.

4. Interaction of specifically bound [3H]estradiol to DNA-cellulose preparations.

Retention of specific [3H]E_2 cytosolic binders by DNA in DNA-cellulose was measured in the absence or presence of 25mM Na_2MoO_4, a compound reported to prevent receptor transformation by stabilization of the untransformed species. As shown in Table 4, warming of [3H]ER complexes (30min at 25°C) or extended time (15hr at 4°C) did not increase the amount of specific binding to DNA over that observed by 3hr incubation at 4°C. These results suggested that ligand binding alone resulted in receptor transformation. Molybdate inhibited the transformation and DNA-binding when added at the same time or prior to addition of ligand. Molybdate had no effect if added after the 3hr incubation with ligand. The lack of effect of heat on the transformation process has been reported for ER derived from MCF-7 cytosol (43).

Since the specific E_2-binders in cytosol from endometrium and adenocarcinoma cells were previously shown to be labile when the incubation temperature was elevated to 30°C (Fig. 6), transformation of these complexes was performed simultaneously with DNA-cellulose binding i.e. overnight incubation at 4°C in the presence of DNA.

Table 4 Effects of temperature, time and sodium molybdate on estrogen receptor binding to DNA-Cellulose.

Aliquots of ER derived from calf uterus cytosol were incubated with 10nM $3HE_2$ for 3hr at 4^oC in the presence or absence of 25mM Na_2MoO_4. Sample 3 was warmed at 25^oC for 30min. Samples 4 and 5 were incubated for 15hr in the presence or absence of molybdate. Free steroid was removed by DCC. At the end of the incubation samples 1, 3 and 4 were made 25mM sodium molybdate and added to DNA-cellulose columns. Nonspecific binding was determined by addition to plain cellulose columns. Samples were assayed for DNA-cellulose binding as described in Materials and Methods (Section III-8A). The fraction bound to DNA-cellulose was corrected for the cellulose background.

Experimental Conditions	Amount of [3H]ER fmol/mg protein	Fraction Bound to DNA-Cellulose.
1. 4^oC 3hr	320	44%
2. +Mo 4^oC 3hr	332	2%
3. 4^oC 3hr +25^oC 30min.	320	31%
4. 4^oC 15hr	446	32%
5. +Mo 4^oC 15hr	446	3%

As shown in Table 5, approximately 15% of labeled specific binders in Ishikawa cytosol bound to DNA whereas no binding to DNA-cellulose above background levels was detected in HEC-50 cells. This observation can be interpreted to indicate that the specific binders in HEC-50 cells cannot be transformed by E_2, in contrast to the clear transformation of some of the specific binders observed in the estrogen-responsive Ishikawa cells.

These results indicated that the lack of responsiveness to estrogens of the HEC-50 cells might be due to an alteration in the binding protein that prevents its activation by estradiol to a DNA-binding form.

Table 5 Binding of [3H]estradiol-receptor to DNA-Cellulose.

Aliquots of cytosol were incubated with 20nM [3H]E_2 for 3hr at 4°C in the presence or absence of 25mM Na_2MoO_4. Samples were added to DNA-cellulose pellets and incubated for 16hr at 4°C with gentle agitation. Non-specific binding was determined in parallel incubations with cellulose. Binding to DNA was determined as described in Materials and Methods (Section III-8B).

Cell Type	Amount of [3H]ER fmol/mg protein	Fraction Bound to DNA-Cellulose +Molybdate		-Molybdate
Ishikawa	32	3		18
	11	7		22
	11	7		19
	31	4		17
Mean ± SD	21.2±11.8	5.2±2.0	p<0.001	19±2.1
HEC-50	7	6		5
	9	5		3
	10	5		7
	15	1		1
Mean ± SD	10.2±3.4	4.2±2.2	not sig.	4.0±2.6

V. DISCUSSION

The presence of estrogen receptors measured by radiolabeled ligand-binding assays has been routinely used as a marker for the prediction of responses to endocrine therapy in patients with breast cancer (168) and endometrial cancer (229). However, only about one-half of the patients with ER-positive mammary cancer achieve remission through hormone-related treatments (199). This underscores the need for ER assays which can determine the quality of the ER system in the tumors. The recent development of receptor monoclonal antibodies which recognize aspects of the protein that are not necessarily involved in hormone binding have enabled investigators to identify alterations in receptor structure which may be distinct from the hormone-binding site (124,125). The implications of the use of receptor antibodies in determination of functional ER may be important in the ability to predict the response of a patient to hormone therapy.

Despite the demonstration that immunocytochemical techniques provide additional information on the distribution and cellular localization of ER among cells or regions within a lesion, significant correlations have been reported between immunocytochemical and steroid-binding assays for ER in human breast tumors (230) and human endometrial adenocarcinoma (231). The majority of immunocytochemical studies have utilized ER monoclonal antibodies generated from the Ben May Cancer Research Laboratory (77) which are sold as part of kits for immunocytochemical detections of ER in tumor specimens by Abbot Labs. Unfortunately these

antibodies were never made available to us. Recently, significant correlations were reported between immunocytochemical assays and steroid-binding assays of ER in human breast tumor specimens (232) using the JS 34/32 monoclonal antibody

We are not aware of any report describing an estrogen-unresponsive cell line derived from a patient with breast cancer or endometrial cancer which exhibits high affinity, specific estradiol-binders which are not recognized by anti-ER monoclonal antibodies.

The HEC-50 human endometrial adenocarcinoma cell line represents a model system to study the biochemical process that underlies an insensitivity to estrogens, since in spite of the presence of specific [3H]E_2-binders (233), these cells are unresponsive to estradiol.

In the present study it was demonstrated that the nature of the cytosolic and nuclear high affinity E_2 binders from E_2-responsive Ishikawa cells or human endometrium and E_2-unresponsive HEC-50 cells are different; while they all exhibit a high affinity, specific E_2 binder (Kd approximately 0.6nM), those present in HEC-50 cells are not recognized by an ER monoclonal antibody and are unable to bind to DNA under transforming conditions. These results suggest that the high affinity specific binders in HEC-50 and Ishikawa cells differ in the structure or accessibility of both their antigenic and/or DNA-binding domains.

This section discusses the possible reasons for the unresponsiveness of the HEC-50 cells, as proposes additional

experiments which could be performed to elucidate the nature of the receptor lesion(s) responsible for the observed E_2-insensitivity.

1. Low ER levels

The majority of cell variants which are resistant to the effects of steroids are of the receptorless phenotype by virtue of their inability to bind radiolabeled ligand. Recent evidence indicates the existence of immunologically reactive GR which is unable to bind hormone due to a point mutation in the ligand-binding domain (107,183).

The levels of specific binders were usually lower in HEC-50 cells and the possibility that hormone unresponsiveness may be related to the lower receptor levels cannot be ruled out since minimal levels necessary to obtain biologic responses have not been determined. However the significance of specific binding levels as a parameter characterizing a cell line is questionable since variability of the number of binding sites in cultured cells has been previously reported (228,233,234) and suggested to be due to differences in metabolic factors which may result in changes in cGMP/cAMP concentration ratios, which have been shown to vary during the cell cycle in synchronized cell cultures (235). Also, in contrast to the well-documented decline in PR levels with loss of differentiation in endometrial adenocarcinoma (236), average levels of ER in poorly differentiated adenocarcinomas were found by various authors either not to differ or to be much lower than those in well-differentiated tumors (236,237). It is interesting to note that

the HEC-50 cells were derived from a specimen of poorly differentiated endometrial adenocarcinoma, whereas the Ishikawa cells were derived from a specimen of well-differentiated adenocarcinoma.

Alternatively, since the ER is very sensitive to experimental manipulations, vigorous homogenization could release lysosomal enzymes which may degrade the receptor and reduce its hormone-binding ability (Dr. N. Dayani, personal communication).

2. Point mutations or deletion mutations in the ER protein.

Mutations within the functional domains of steroid receptors which result in the loss of biological activity have been demonstrated for both GR and ER using polyclonal and monoclonal antibody technology. The production of monoclonal antibodies to ER of calf (216) and human (76-78) origin and to GR of rat origin (227-231) which recognize several unique receptor determinants and show negligible cross-reactivity with other proteins have advanced the study of altered receptors. Greene and his colleagues have prepared a library of ER monoclonal antibodies, which react with different regions of the receptor molecule. Using a variety of proteolytic enzymes including: papain, chymotrypsin and trypsin the determinants for these different monoclonal antibodies have been identified. The majority of Greene's monoclonal antibodies recognize determinants located in the steroid-binding domain of the receptor and they do not interfere with the interaction of the receptor with the hormone. One of the antibodies, H226, recognizes a determinant in the DNA-binding domain. Another antibody, H222, appeared to be

located in the steroid binding domain near the "dimerization" region of the receptor protein (93). Unfortunately, such studies have not been done to determine the antigenic determinant of the JS 34/32 monoclonal antibody. However, despite the fact that the JS 34/32 antibody only recognizes native receptor (216), it has been reported to immunoreact with both transformed and molybdate-stabilized nontransformed ER (57).

In contrast, the majority of antibodies to the GR have been reported to recognize an epitope of the receptor molecule which is distinct from the steroid and DNA-binding domains. This domain has been termed the "modulating domain" since its absence results in a truncated receptor molecule which is biologically inactive. Two groups have reported GR antibodies which recognize determinants in the DNA-binding domain (241,242).

Using GR antibodies a number of alterations in receptor structure have been identified which contribute to the observed steroid-unresponsiveness. Stevens et al. (186) reported that the lack of antibody recognition of GR in a corticosteroid-resistant, mouse lymphoma variant of the nt^i phenotype was a result of a deletion of a large portion of the receptor protein corresponding to the antigenic domain and was reflected in a truncated protein which exhibited an increased affinity for non-specific DNA-sequences. As of the writing of this dissertation no other group has demonstrated alterations in antibody recognition of ER which can be related to estradiol-unresponsiveness.

The physicochemical characteristics of the ER in the HEC-50 cells reported here do not appear to conform to the nt^i phenotype but rather display characteristics similiar to those described for the nt- phenotype (176-177) i.e.:

-similar sedimentation rates of specific [3H]E_2 binders were demonstrated in the two cell lines and endometrium (Fig. 11).

-ER in the HEC-50 cells exhibited a decreased affinity for nonspecific DNA sequences (Table 5).

However, altered immunoreactivity of GR derived from cells exhibiting the nt- phenotype with monoclonal antibodies has not been reported.

It has been recently reported that the nt- phenoytpe is due to a point mutation in the DNA-binding domain of the GR protein (106,107). Danielsen et al. (107) isolated the point mutation by cloning, sequencing and expression of the mutant receptor protein However, Drs. T. Garcia and B. Schacter, Dept. OB-GYN & Reproductive Sci., MSSM, were unable to detect any point mutations or deletions in the ER mRNA of the HEC-50 cells (unpublished observation). Their results were based on the analysis of the HEC-50 messenger RNA (ERmRNA) using an RNase protection assay which had been successfully used by Perrucho and colleagues (243,244) for the detection of point mutations in the c-K-ras oncogene, which were correlated with human colon tumorigenesis and tumor invasiveness. However, the possibility exists that point mutations are present in the HEC-50 receptor but were undetected due to the limitations of the technique i.e. mutations resistant to the RNase A digestion or mutations in regions that were not analyzed (143,244). These limitations could be

overcome by cloning of the ER cDNA from the HEC-50 cells and amino acid sequence analysis of the expressed protein.

It would be interesting to be able to confer E_2-responsiveness to the HEC-50 cells by transfection of cloned receptor cDNA from Ishikawa cells into HEC-50 cells. Such experiments would illustrate the presence of altered ER in the HEC-50 cells. Recently, Miesfield et al (245) have reported stable transfection of cloned cDNA from intact rat liver GR into a hormone-unresponsive, receptor deficient (r-) mouse lymphoma cell line and produced functional complementation of the receptor defect. Functionality of the transfected GR was measured by dexamethosone induction of transcription of a GRE-linked promoter from the mouse mammary tumor virus. Also Lai et al. (246) have reported the expression of the estrogen-regulated chicken ovalbumin gene in MCF-7 cells.

3. Size characteristics of [3H]TAZ-ER.

In order to search for differences in the size of the ER protein present in the two cell lines and endometrium, cytosolic ER was labeled with the covalent affinity ligand, [3H]TAZ, followed by molecular weight determination by SDS-PAGE. Two peaks of radioactivity were observed in the SDS-PAGE patterns of endometrial cytosol: one peak which migrated just ahead of BSA corresponding to approximately Mr=60-65kDa and a second peak which migrated just behind OVA corresponding to approximately 45-50kDa. No differences in the SDS-PAGE pattern were observed between the two cell lines,

however, in contrast to the two peaks observed in endometrial cytosol, only the second peak was present in the two cell lines. Unfortunately, we could not demonstrate binding specificity under the incubation conditions used since, unlabeled E_2 did not compete with the irreversible binding of [3H]TAZ to cytosolic ER derived from human endometrium or endometrial cancer cells. In similar studies using ER derived from calf uterine cytosol specificity was observed. The reason for the lack of specificity of [3H]TAZ binding to ER from endometrial cancer cells and endometrium may reside in the kinetics of reversible binding of E_2 and irreversible binding of [3H]TAZ.

The existence of two peaks of [3H]TAZ labeled ER has been reported for ER derived from calf uterus (212) and MCF-7 cells (232). The second peak was believed to be a product of proteolytic degradation. It has been reported that in vitro translation of the MCF-7 ER poly(A)RNA, gives two proteins of 65kDa and 46kDa (104,105).

Since the JS 34/32 monoclonal antibody does not recognize denatured receptor immunological identification of the receptor could not be performed. However the identification of these peaks could be determined using the anti-ER monoclonal antibodies from the Ben May Cancer Research laboratory (77) since they have been reported to recognize denatured receptor (248).

It would be very informative to examine the ability of the various monoclonal antibodies from the Ben May Research laboratory to recognize ER in HEC-50 cells and to compare the results to those reported here using JS 34/32.

4. Altered DNA-binding of ER.

The inability of the HEC-50 ER to bind to DNA are indicative of alterations in the surface charge of the protein. There are a number of available methods which have been used to examine the molecular charge of ER:

-Chromatofocusing is an ion-exchange procedure which separates proteins largely on the basis of isoelectric point (249). This technique has been used to demonstrate differences in the charge properties of ER derived from estrogen responsive and unresponsive rat mammary tumors (189,190) and was correlated with the affinity of ER for nonspecific DNA.

-ATPP is a method which has been used to determine the charge characteristics of ER based on its hydrophobic content. This method has been used to demonstrate charge differences between occupied and unoccupied ER (57,58).

-DEAE-cellulose chromatography has been routinely used to determine the charge characteristics of ER. Heterogeneity of ER has been demonstrated in breast tumors using this method (201-203).

-Isoelectric focusing in polyacrilamide gels could be used to analyze the charge characteristics of ER in the HEC-50 cells (129).

Charge differences in the ER from the HEC-50 cells compared to the Ishikawa cells may result from post-translational modifications of the receptor such as glycosylation or phosphorylation. This possibility could be examined by two dimensional gel electrophoresis in which the first dimension is isoelectric focusing and the second is SDS-PAGE (247).

5. Inhibitory Factors.

The differences in the two cell lines may reflect differences in levels of "inhibitor" substances in the cytosol which could bind to ER and directly influence its properties. A number of investigators have described low molecular weight inhibitors of ER (139) and GR (140-142) binding to isolated nuclei (139,140) or to DNA-cellulose preparations (141-142). The mechanism whereby these factors inhibit transformation is not known but it has been suggested that they mask the receptor's DNA-domain. A mechanism which involves interation with essential sulfhydryl groups in the receptor protein has also been proposed (110).

Experiments which would test the "inhibitor hypothesis" are as follows:

-partial purification of the HEC-50 ER by DEAE-cellulose chromatography or glycerol density gradients followed by determination of the ability of the receptor to bind to DNA-cellulose or to be recognized by JS 34/32.

-mixing experiments using cytosol of HEC-50 and Ishikawa cells followed by immunoprecipitation with JS 34/32 or incubation with DNA-cellulose.

It has also been suggested by Sherman et al. (198) that the activity of endogenous proteases on the ER could affect its ability to bind to DNA by removing essential receptor sites. In fact, an increased proteolysis of specifically bound [3H]E_2 in cytosol and nuclear extracts of the HEC-50 and Ishikawa cells was observed in the density gradient studies by the presence of a peak which sedimented

at approximately 2-3S which was not immunoreactive and may represent meroreceptor.

The results of these studies suggest that it is important to evaluate the quality of the ER in addition to the quantity of ER in tumor specimens. This can be achieved by assessing ER by various immunochemical methods as well as by measuring the ability of the ER to bind to DNA. These types of studies could enhance the prognostic value of ER measurements. The HEC-50 endometrial adenocarcinoma cell line represents a model system to examine the biochemical properties of the ER in cells which are ER positive but unresponsive to estradiol. More studies must be done to determine the location of the lesion(s) responsible for the insensitivity of these cells to estradiol.

VI. CONCLUSIONS

The studies reported here are the first demonstration of an ER-positive human adenocarcinoma cell line which is unresponsive to estradiol due to alterations in the quality of its ER system. The HEC-50 cell line represents a model system to study the biochemical determinants of altered receptor function.

In summary, altered ER in HEC-50 cells was demonstrated by the following:

-the inability of specifically-bound [3H]E_2 to be recognized by the JS 34/32 ER monoclonal antibody.

-the inability of specifically-bound [3H]E_2 to be activated to a high affinity DNA-binding state.

Whether these observations are related is not clear.

This is the first report of immunochemical alterations in E_2-unresponsive human adenocarcinoma cells using a monoclonal antibody. The results of these studies underscore the importance of determination of the quality of the ER system in predicting the response to endocrine therapy in patients with endometrial cancer. The quality of the ER system may be determined by using a variety of immunochemical techniques and DNA-cellulose binding assays.

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