DEVELOPMENT OF YEAST BASED STEROID BIOASSAY FOR SCREENING SOME NATURAL ENDOCRINE DISRUPTORS

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

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in

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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **DEVELOPMENT OF YEAST BASED STEROID BIOASSAY FOR SCREENING SOME NATURAL ENDOCRINE DISRUPTORS** in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from January, 2006 to August, 2009 under the supervision of Dr. Partha Roy, Assistant Professor, Department of Biotechnology and Dr. C. B. Majumder, Assistant Professor, Department of Chemical Engineering, Indian Institute of Technology Roorkee, Roorkee, India.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other Institute.

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This is to certify that the above statement made by the candidate is correct to the best of our knowledge.

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Supervisor

The Ph.D. Viva-Voice Examination of Mr. **Shamba Chatterjee**, Research Scholar, has been held on _____

Signature of Supervisors

Signature of External Examiner

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complex of multiple polypeptides, although each of them is represented as a single polypeptide for simplicity.

Fig 2.7 Models for corepressor and coactivator regulation of nuclear receptors. For simplicity, only one of the two receptor dimers is illustrated. 19

A) Corepressors interacting with nuclear receptor (NR) in the absence of ligand. Their interaction with chromatin modifiers, e.g., HDACs leads to histone states unfavorable for transcription and expression of target gene is blocked.

B) Coactivators interacting with NR in the presence of agonist. Agonist occupation of the NR ligand binding domain (LBD) leads to exchange of corepressors for coactivators, and in this scenario, recruitment of chromatin modifiers e.g., histone acetyl transferases (HAT) which acetylate lysine side chains. The remodeled, open chromatin structure allows addition proteins positively regulating transcription (not shown) to be recruited and promoter activity to ensue.

C) Agonist-dependent corepression. REA competes with SRC for the AF-2 of ligand-bound receptor reducing the opportunity for transcription-enhancing proteins, e.g., HAT, to participate in the transcription complex. Further loss of transcription-favorable chromatin structure may be lost by REA's direct association with HDACs and subsequent reduction of acetylated histones.

D) Generalized version of agonist-dependent corepression. Transcriptional repressors in addition to or instead of HDACs may participate in blocking expression. AF-1: activating function 1, AF-2: activating function 2, DBD: DNA binding domain of NR, Lig: ligand, Ac: acetylated residues on histones comprising nucleosome, CoAct: coactivator, HAT: histone acetyl transferase, HDAC: histone deacetylase, REA: Repressor of Estrogen Activity, SRC: steroid receptor coactivator (Adapted from Gurevich et al.,

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LIST OF ABBREVIATIONS

Symbols		Description
DNA	:	Deoxyribonucleic acid
RNA	:	Ribonucleic acid
AR	:	Androgen Receptor
PR	:	Progesterone Receptor
ER	:	Estrogen Receptor
GR	:	Glucocorticoid Receptor
MR	:	Mineralocorticoid Receptor
hAR	:	Human Androgen Receptor
hPR	:	Human Progesterone Receptor
hER	:	Human Estrogen Receptor
ARE	:	Androgen Response Element
PRE	:	Progesterone Response Element
ERE	:	Estrogen Response Element
EDC	:	Endocrine Disrupting Chemical/Compound
EDCs	:	Endocrine Disrupting Chemicals/Compounds
PCR	:	Polymerase Chain Reaction
RT-PCR	:	Reverse Transcriptase-Polymerase Chain Reaction
cDNA	:	Complementary DNA
RNase	:	Ribonuclease
DNase	:	Deoxyribonuclease
ppm	:	Parts Per Million
T_m	:	Melting Temperature
U	:	Units
v/v	:	Volume/ Volume
w/v	:	Weight/ Volume
EC_{50}	:	Half-Effective Concentration
IC_{50}	:	Half-Inhibition Concentration

α	:	Alpha
β	:	Beta
D	:	Dalton
kD	:	KiloDalton
m	:	Meter
°C	:	Degree Centigrade
%	:	Percentage
~	:	Approximately
bp	:	Basepair
ng	:	Nanogram
µg	:	Microgram
pg	:	Picogram
mg	:	Milligram
g	:	Gram
kg	:	Kilogram
µl	:	Microlitre
ml	:	Millilitre
l	:	Litre
M	:	Molar
mM	:	Millimolar
µM	:	Micromolar
nM	:	Nanomolar
pM	:	Picomolar
h	:	Hour
min	:	Minute
sec	:	Second
Fig.	:	Figure
GC-MS	:	Gas Chromatography-Mass Spectrometry
NCBI	:	National Centre for Biotechnology Information
SGD	:	Saccharomyces Genome Database
RDI1	:	Rho GDP dissociation inhibitor

AF1	:	Activating Function-1
GRIP-1	:	Glucocorticoid Receptor Interacting Protein 1
p300/CBP	:	p300/CREB binding protein

CHAPTER 1

Introduction

1.1 General Introduction

Evidence has been accumulating which ind icates that humans and domestic and wildlife species have suffered adverse health consequences from exposure to environmental chemicals that interact with the endocrine system. To date, these health problems have been identified primarily in domestic or wildlife species with relatively high exposures to organochlorine compounds, including 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane (DDT) and its metabolites, polychlorinated biphenyls (PCBs) and dioxins, or to naturally occurring plant estrogens. It is not known if similar effects are occurring in the general human population, but again there is evidence of adverse effects in populations with relatively high exposures. Several reports (Medical Research Council, 1995) of declines in the quality and decreases in the quantity of sperm production in humans over the last four decades and reported increases in incidences of certain cancers (breast, prostate, testicular) that may have an endocrine related basis have led to speculation about environmental etiologies. However, considerable scientific uncertainty remains regarding the causes of these reported effects. Nevertheless, it is known that the normal functions of all organ systems are regulated by endocrine factors, and small disturbances in endocrine function, especially during certain stages of the life cycle such as development, pregnancy, and lactation, can lead to profound and lasting effects. The critical issue is whether sufficiently high levels of endocrine-disrupting chemicals exist in the ambient environment to exert adverse health effects on the general population. Current methodologies for assessing human and wildlife health effects (e.g., the generation of data in accordance with testing guidelines developed by the U.S. Environmental Protection Agency [U.S. EPA]) are generally targeted at detecting effects rather than mechanisms, and may not adequately evaluate effects on the endocrine system. This is particularly true for exposures that occur during critical developmental periods when the endocrine system plays a key role in regulating essential physiological and morphological processes. Given the potential scope of the problem, the possibility of serious adverse effects on the health of human and wildlife populations, and the broad occurrence and persistence of some endocrine-disrupting agents in the environment, it is important to focus on the available resources for research on the most critical gaps in our knowledge base so that more informed regulatory and public health decisions can be made in the future. The broad

nature of the problem necessitates a coordinated effort on both the national and the international levels. In response to the growing public health concerns related to chemicals in the environment that have the potential to act as endocrine disruptors, the Office of Research and Development of the U.S. EPA held a workshop on April 10-13, 1995, in Raleigh, North Carolina, to begin developing a research strategy related to endocrine-disrupting chemicals.

An environmental endocrine disruptor was broadly defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes" (adapted from EDSTAC, 1998). This definition reflects a growing awareness that the issue of endocrine disruptors in the environment extends considerably beyond that of exogenous (anti)estrogens and includes (anti)androgens and agents that act on other components of the endocrine system such as the thyroid and pituitary glands. In many cases, these endocrine disruptors share no apparent structural similarities to traditional steroids. Endocrine disruptors include natural products (phytoestrogens, e.g., genistein) (Martin et al., 1978; Miksicek et al., 1993; Verdeal et al., 1979), pharmaceuticals (i.e., diethylstilbestrol, ethynyl estradiol) (McLachlan et al, 1984), environmental pollutants (i.e., DDT, polychlorinated biphenyls, dioxins, polyaromatic hydrocarbons) (Clemons et al., 1996; Fieldenet al., 1997; Jansen et al., 1993; Kelce et al., 1995; Safe, 1995), and industrially relevant chemicals (i.e., alkylphenols, bisphenol A) (Korach, 1993; Krishnan et al., 1993; Nimrod et al., 1996; Wilcox et al., 1995; Fisch et al., 1996; Fisch et al., 1996). However, recent scientific evidences reveal the existence of newly defined class of environmental contaminants which may have variable chemical structures and can alter the normal endocrine physiology (Cargouët et al., 2004). Endocrine disrupting compounds can be categorized as (anti)androgenic, (anti)progestagenic and (anti)estrogenic based on with which steroid it's alike or mimicking. Mostly they enter the animal and/or human system through oral ingestion of their diet as well as contaminated water and possibly generate an agonistic and/or antagonistic effect. Endocrine disrupting compounds generally exert their effects either by indirectly targeting the arylhydrocarbon receptor (AhR) (Indarto and Izawa, 2001) or by direct binding of these compounds to steroid receptors (Sanderson and Vanden

Berg, 2003; Rice et al., 2006). Thereby, they upregulate and/or downregulate the promoter of the target gene (Lin et al., 2006; Lyssimachou et al., 2006). Evidences also suggests that various environmental as well as therapeutic compounds acts as endocrine disruptors yielding sex hormone disease or disorders (Satoh et al., 2001; Roy et al., 2005; Sone et al., 2005; Guillete, 2006; Buck et al., 2006; Darbre, 2006; Massart et al., 2006; Maffini et al., 2006; Chen et al., 2007). Chronic exposure in a very low dose of these compounds may disturb the delicate hormone balance and results in serious reproductive anomaly (Ralph et al., 2003). Different types of chemicals and their byproducts are being discharged daily to surface water streams and it retains after the rigorous effluent treatment process through effluent treatment plant (Chatterjee et al., 2007; Chatterjee et al., 2008, Kumar et al., 2008). Hence there is an increasing need of potential and robust *in vitro* bioassay system for mass screening of these steroidogenic compounds. This would help in their identification and potential remediation.

1.2 Aims & Objecctives:

1. Development of yeast based *in vitro* β-gal assay for screening environmental (anti)androgen compounds driven by Probasin promoter as hormone response element.
2. Development of yeast based *in vitro* yEGFP assay for screening environmental (anti)progestagenic compounds driven by Prolactin promoter as hormone response element.
3. Development of multidrug transporter deleted yeast based *in vitro* yEGFP assay for screening environmental (anti)estrogen compounds driven by 3xERE.
4. Elucidation of *RDI1* mediated modulation of the in vitro estrogen receptor transactivation response in *Saccharomyces cerevisiae* and the role of AF-1 domain.

CHAPTER 2

Review of literature

2.1 Introduction

Research described in this thesis was aimed at developing *in vitro* yeast based system capable of detecting the illegal use of androgenic, progestagenic, and estrogenic compounds, the presence of potential endocrine disruptors in various environmental matrices and finally some brief insights on potential modulators involved in *in vitro* yeast based estrogen assay. The system was intended to be capable of integrating the effects of modulating compound as well. Furthermore gaining insights into the mechanism underlying yeast estrogen assay via receptor-modulator interaction and receptor activation was an important goal. These would help us to develop potential assay system for (anti) androgenic, (anti) progestagenic and (anti) estrogenic environmental compounds leading to endocrine disruption as well as potential insights on yeast based estrogen assays (Galbraith and Topps, 1981; Sonnenschein and Soto, 1998; Simon, 2001; Su et al., 2001 and 2002, Bovee et al., 2004; Bovee et al., 2007; Sanseverino et al., 2009).

2.2 Steroid hormone

With the exception of retinoic acid, the steroid hormones are all derived from cholesterol. Moreover, with the exception of vitamin D, they all contain the same cyclopentanophenanthrene ring and atomic numbering system as cholesterol. The conversion of C_{27} cholesterol to the 18-, 19-, and 21-carbon steroid hormones (designated by the nomenclature C with a subscript number indicating the number of carbon atoms, e.g. C_{19} for androstanes) involves the rate-limiting, irreversible cleavage of a 6-carbon residue from cholesterol, producing pregnenolone (C_{21}) plus isocaproaldehyde. Common names of the steroid hormones are widely recognized, but systematic nomenclature is gaining acceptance and familiarity with both nomenclatures is increasingly important. Steroids with 21 carbon atoms are known systematically as pregnanes, whereas those containing 19 and 18 carbon atoms are known as androstanes and estranes, respectively. The important mammalian steroid hormones are shown in Fig 2.2 depicting their biochemical synthesis pathway along with the precursor, pregneolone. Retinoic acid and vitamin D are not derived from pregnenolone, but from vitamin A and cholesterol respectively (Schulman & Heyman, 2004; Germain et al., 2006; Lu et al., 2006).

All the steroid hormones exert their action by passing through the plasma membrane and binding to intracellular receptors. Both the steroid and thyroid hormone-receptor complexes exert their action by binding to specific nucleotide sequences in the DNA of responsive genes. These DNA sequences are identified as hormone response elements (HREs). The interaction of steroid-receptor complexes with DNA leads to altered rates of transcription of the associated genes.

Steroid hormones are crucial substances for the proper function of the body. They mediate a wide variety of vital physiological functions ranging from anti-inflammatory agents to regulating events during pregnancy. They are synthesized and secreted into the bloodstream by endocrine glands such as the adrenal cortex and the gonads (ovary and testis). Steroid hormones are all characterized by the steroid nucleus which is composed of three six member rings and one five member ring, ingeniously labeled A, B, C, and D respectively. The steroid nucleus structure has been shown in Fig 2.1.

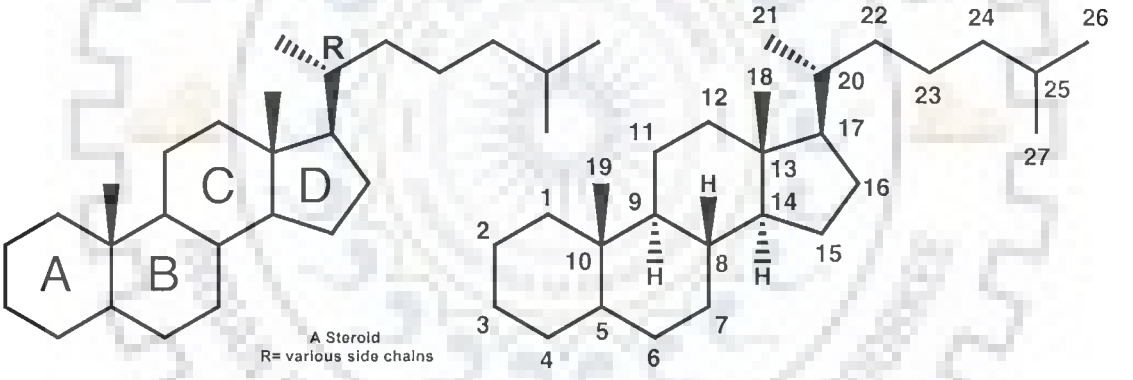


Fig 2.1 Steroid nucleus structure.

This structure, which has six asymmetric carbons, provides many possible stereo isomers, which one would expect since steroid hormones have an array of functions. Furthermore at C-17, there is a substituent which varies from hormone to hormone, depending on its function.

2.2.1 Steroid Biosynthesis

Steroid hormones are derived from cholesterol through the actions of enzymes that modify the sterol side-chain and ring structure. The steroidogenic machinery is composed of:

1. Proteins participating in the acquisition of cholesterol by cells;
2. The cytochrome P450s that catalyzes cleavage of carbon side-chains from the sterol nucleus, introduce hydroxyl groups and convert the sterol A ring to an aromatic structure;
3. The oxido-reductases, or hydroxysteroid dehydrogenases, which catalyze the oxidation or reduction of alcohols and ketones, respectively, at carbons 3, 11, 17 and 20;
4. The double bond reductases which irreversibly reduce the Δ^{4-5} double bond of the sterol A ring of Δ^{4-3} ketosteroids;
5. The steroid hormone sulfonating enzymes and sulfatases which participate, respectively, in the inactivation of steroid hormones and the release of active hormone or hormone precursor by removal of the sulfate group.

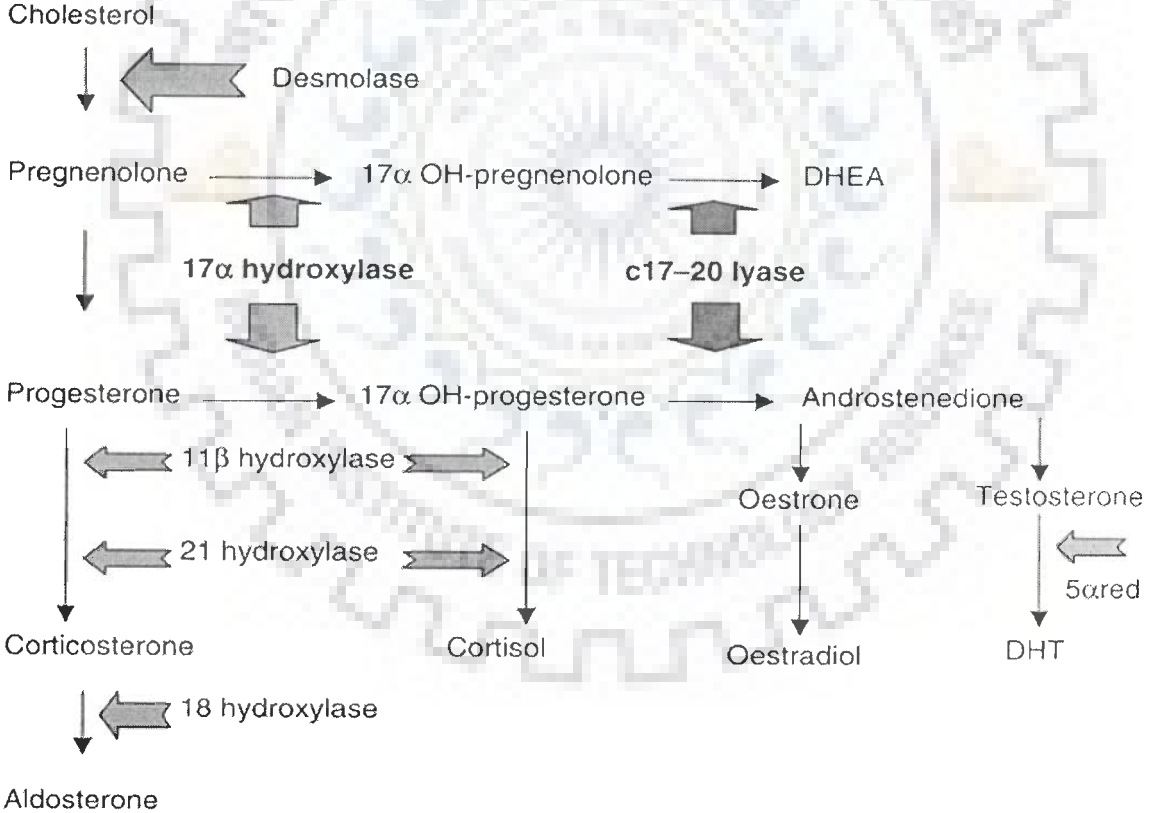


Fig 2.2 Biochemical pathway for the synthesis of steroid hormones.

2.2.2 Domain structure of Steroid Hormone Receptors (SHRs)

The cloning of the receptors for glucocorticoids (GR) and estrogens (ERα) were followed by identification and extensive characterization of the receptors for androgens

(AR), progestins (PR) and mineralocorticoids (MR). Afterwards, a second estrogen receptor (ERß) and two estrogen-related receptors (ERRa/ERR1) and (ERRß/ERR2) have been characterized. Steroid hormone receptors (SHRs) are designated by a central DNA-binding domain (DBD), that targets the receptor to the HREs, and a ligand-binding domain (LBD), required for switching the receptors' functions (Beato, 1989) (Fig 2.3).

All steroid hormone receptors are modular proteins composed of distinct regions as shown in Fig 2.4 After the availability of chicken estrogen receptor cDNA, the comparison with the human estrogen and glucocorticoid receptor sequences led to the nomenclature of region A–F for all members of the whole nuclear receptor superfamily till today (Krust *et al.*, 1986; Green an d Chambon, 1987; Kumar *et al.*, 1987). Functional and structural analyses clearly revealed that these distinct regions correspond to functional and structural units called domains. Region C, the DNA binding domain, and region E, the ligand binding domain, display a high degree of sequence conservation, whereas no significant conservation was detected between the paralogous SHRs for the regions A/B, D and F. Region D is considered as a flexible hinge region between the DNA and ligand binding domains. Its very amino terminus is an integral part of the DNA-binding domain and involved in DBD dimerization. The F region is essential for hormone binding in the PR, GR and AR but not in the ERα. Afterwards, work exposes that this domain is also important for the discrimination between agonistic and antagonistic hormone ligands (Nichols et al., 1998). The A region is highly conserved only between chicken and human estrogen receptors, but this distinction is much less clear in the other steroid receptors. Therefore, regions A and B are combined into an A/B region in most cases. Regions C and E are not only responsible for DNA- and ligand binding respectively, but also encode other functions as well (Claessens et al., 2008).

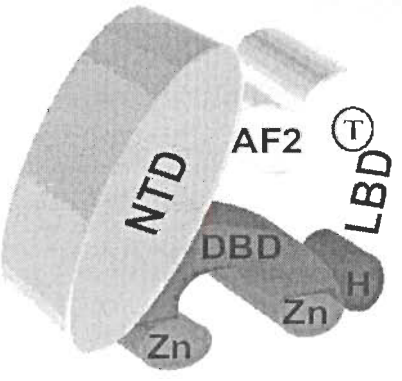


Fig 2.3 Model of conformation of the activated Androgen Receptor showing multiple interactions within the receptor protein. Abbreviations: NTD, N-terminal domain; DBD, DNA-binding domain, LBD, ligand-binding domain; T, testosterone, AF2, activation function 2 of LBD, Zn, zinc finger [(reproduced from U. Karvonen (Karvonen, 2003)].

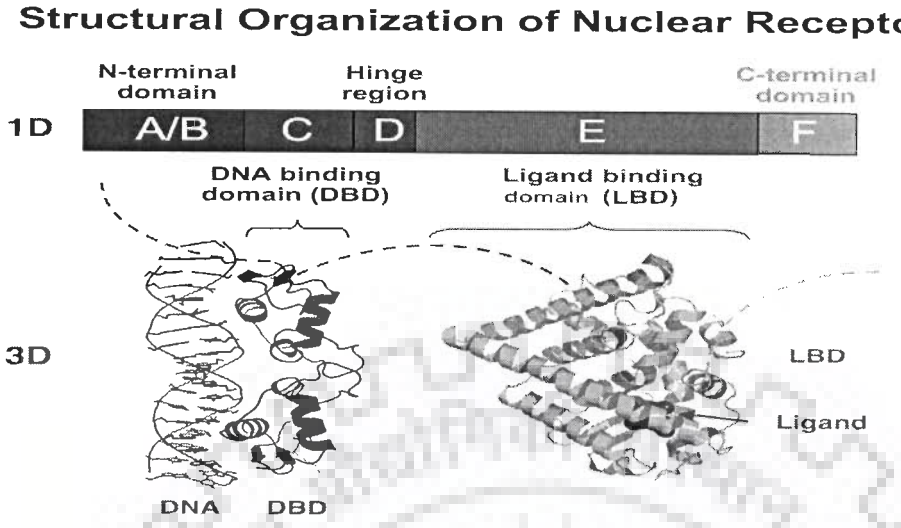


Fig 2.4 Schematic representation of a nuclear receptor. A typical nuclear receptor is composed of several functional domains. The variable NH2-terminal region (A/B) contains the ligand-independent AF-1 transactivation domain. The conserved DNA-binding domain (DBD), or region C, is responsible for the recognition of specific DNA sequences. A variable linker region D connects the DBD to the conserved E/F region that contains the ligand-binding domain (LBD) as well as the dimerization surface. The ligand-independent transcriptional activation domain is contained within the A/B region, and the ligand-dependent AF-2 core transactivation domain within the COOH-terminal portion of the LBD (Courtesy: http://commons.wikimedia.org).

2.2.3 Receptor isoforms and variants

The first steroid hormone receptor which has been reported to exist in two common isoforms generated by differential promoter usage was progesterone receptor (PR) (Kastner et al., 1990). Interestingly, one promoter initiates transcription at positions +1 and +15 of the gene which gives rise to the longer isoform B, PR-B. On the other hand, the second promoter initiates human PR transcripts between +737 and +842 encoding the 164 amino acid residues shorter hPR form A, PR-A. Both of them show high affinity for the natural ligand progesterone and the synthetic agonist R5020. The only substantial difference is PR-B harbours a third activation function at its specific amino terminus termed AF-3, which fun ctions in a promoter and cell-specific manner (Sartorius et al., 1994). Both isoforms thus display differential target gene specificity. Although this was initially

regarded as an exception within the family, but later differential promoter usage and alternative splicing were found in all the members.

The human ERα was cloned from a cDNA expression library produced from the breast cancer cell line MCF-7 (Walter et al., 1985) and subsequently sequenced (Greene et al. 1986). Thereafter, a second estrogen receptor, ERβ was characterized which is very similar to ERα in terms of structure and function but also shows subtle and important functional differences (Kuiper et al., 1996; Mosselman et al., 1996; Ogawa et al., 1998). Both ERα and ERβ receptors bind the ligands like estradiol, diethylstilboestrol, estriol, and estrone with high affinity (Paech et al., 1997) and can form heterodimers on HREs (Pettersson et al., 1997). Next, cDNA clones for the estrogen-related receptors α and β were isolated from a human testis cDNA library usin g the human hERα DNA-binding domain as a probe. Breast cancer cells as well as other transformed estrogen target cells contain truncated variants of hERα whose relation to the progression of the malignancy is still unraveled (Pfeffer et al., 1995; Vladusic et al., 1998).

Two different classes of glucocorticoid receptor (GR) cDNAs have been described in humans, hGR α and β which are the result of alternative splicing from a single gene transcript (Hollenberg et al., 1983; Bamberger et al., 1995; Oakley et al., 1996). hGR α and β isoforms are identical up to amino acid 727 and then diverge with hGRα being slightly larger (777 amino acids) than hGRβ (742 amino acids). In coparison to hGRα, that is commonly referred to as the *bona fide* hGR, hGRβ was long dismissed as a cloning artefact but is now shown to be expressed at modest but varying levels in a wide range of tissues. As we know, hGRβ does not bind hormone and is transcriptionally inactive, it acts as a ligand-independent negative regulator of glucocorticoid action in transfection experiments. Also, the ß-isoform is not conserved across species and the relative expression of both isoforms is not known. However, hGRα shows high affinity for the artificial glucocorticoid dexamethasone moderate affinity for the physiological steroids cortisol and corticosterone, and low affinity for mineralocorticoids and progesterone. In contrast to all other SHRs, GRα dimerizes only weakly, which is generally believed to be due to the lack of a strong dimerization interface within the LBD. Interestingly, the monomeric form of GRα is involved in gene repression via interaction with other sequence-specific transcription factors, such as AP1.

Several years after that human mineralocorticoid (hMR) and androgen receptors (AR) were cloned (Arriza et al., 1987; Chang et al., 1988; Lubahn et al., 1988; Patel et al., 1989; Faber et al., 1991). Interestingly, hAR also exists in two isoforms hAR-A and hAR-B which are structurally analogous to the two hPR isoforms (Wilson and McPhaul, 1996). However, in contrast to hPR-A/B, hAR-A is expressed at substantially lower levels than the B-form, and its contribution to androgen action is not clear. The hAR binds the two naturally occurring ligands dihydrotestosterone and testosterone with high affinity whereas the hMR shows high and equivalent affinity for aldosterone, physiological corticosteroids, and progesterone.

2.2.4 Hormone Response Elements

Nuclear receptors regulate transcription by binding to specific DNA sequences in target genes known as hormone response elements (HREs). These elements are located in regulatory sequences normally present in the -59-flanking region of the target gene. Although often the HREs are found relatively close to the core promoter, in some cases they are present in enhancer regions several kilobases upstream of the transcriptional initiation site. It has been revealed upon analysis of a large number of naturally occurring as well as synthetic HREs that a sequence of 6 bp constitutes the core recognition motif. Later on, two consensus motifs have been identified: the sequence AGAACA is preferentially recognized by steroid class III receptors, whereas AGG/TTCA serves as recognition motif for the remaining receptors of the superfamily (Beato et al., 1995). It should be noted that these motifs represent consensus idealized sequences and that naturally occurring HREs can show significant variation from the consensus. Although some monomeric receptors can bind to a single hexameric motif, most receptors bind as homo- or heterodimers to HREs composed typically of two core hexameric motifs. On the other hand, for dimeric HREs, the half-sites can be configured as palindromes (Pal), inverted palindromes (IPs), or direct repeats (DRs). Steroid hormone receptors typically bind to palindromes of the AGAACA sequence separated by three nucleotides, with the exception of the ERs that recognize the consensus AGGTCA motif with the same configuration. On the basis of the analysis of glucocorticoid receptor/ER chimeras, the first zinc finger has been identified as the one responsible for the discrimination of the DNA

motif (Green et al., 1988). Studies have shown that mutation of three residues in the P box, which are identical in the glucocorticoid, progesterone, androgen, and mineralocorticoid receptors that recognize the same HRE, was sufficient to switch the sequence recognized by glucocorticoid receptors and ERs. Furthermore, cocrystal structures of receptor DBDs with DNA have shown that P box residues, which are contained within the recognition helix 1 of the DBD, were indeed involved in interaction with specific bases of the recognition motifs (Glass et al., 2000; Brosems et al., 2004). In contrast to steroid receptors that almost exclusively recognize palindromic elements, nonsteroidal receptors can bind to HREs with different configurations. In this case, the arrangement as well as the spacing between the motifs is determinant to confer selectivity and specificity. Some of these response elements are capable of mediating transcriptional responses to more than one ligand. This is the case of the palindromic element AGGTCATGACCT that confers regulation by both thyroid hormones and retinoic acid (Umesono et al., 1988). Consequently, both ligands can control overlapping gene networks as demonstrated by the regulation of the rat growth hormone gene by the two hormones via a common HRE (Bedo et al., 1989). Similarly, IPs can also mediate transcriptional responses to both ligands as well as to vitamin D. However, a careful analysis of natural and synthetic HREs has shown that the most potent HREs for nonsteroid receptors are configured as DRs. Analysis of variably spaced DRs suggested that the length of the spacer region is an important determinant of the specificity of hormonal responses. DRs separated by 3, 4, and 5 bp (i.e., DR3, DR4, and DR5) mediate preferential regulation by vitamin D, thyroid hormone, and retinoic acid, respectively (Naar et al., 1991, Umesono et al., 1991). The subsequent demonstration that DR1 serves as the preferred HRE for the RXR or for the PPAR and that RARs can also activate transcription through a DR2, expanded the model from a 3-to-5 rule to a 1-to-5 rule (Mangelsdorf et al., 1995). Furthermore, a DR0 sequence can also act as a receptor binding site, and widely spaced DRs can act as promiscuous response elements for different nonsteroid receptors and even for ERs (Kato et al., 1995). It has been shown that in addition to spacing, small differences in the half-site sequence and the sequence of the flanking extension of the response elements also appear to be important parameters in determining receptor binding efficiency (Mader et al., 1993; Klinge, 2001; Barbulescu et al., 2001; Haelens et al., 2003; Ramsey et al., 2004; Geserick et al., 2005).

2.2.5 Heat shock proteins and steroid hormone receptors

It has been known for more than two decades that steroid hormone receptors are associated with other proteins upon isolation from target tissues under low salt conditions. In the isolated large complexes (molecular masses of approximately 300 kDa and with sedimentation constants of 8-9 S on sucrose gradients) it appears that several different heat shock proteins (hsp90, hsp70 and hsp 56) together with other proteins are complexed with steroid receptors. The stoichiometry in the complex of the different proteins and the receptor molecule depends on the conditions for the isolation. In intact target cells in the presence of hormone and at physiological temperatures, these complexes rapidly dissociate. Several functions have been attributed to the association of heat shock proteins with steroid hormone receptors. Folding of the correct hormone binding pocket upon synthesis of the receptor molecule at the ribosome has been suggested for the glucocorticoid receptor in studies with yeast mutants lacking hsp90 or with diminished levels of hsp90 (Bohen et al., 1995). Another possible function is the prevention of the interaction of the receptor molecule with DNA in the absence of hormone. No association with heat shock proteins has been found for the receptors for retinoic acid, thyroid hormone and vitamin D. These receptors appeared to be more tightly bound in the nucleus even in the absence of hormone.

2.2.6 Steroid hormone receptor phosphorylation

Steroid hormone receptors are phosphoproteins in the absence of of ligand, and they become hyper phosphorylated in the presence of hormone. The hormone induced extra phosphorylation, which is 2 to 7 fold more than basal phosphorylation, is a rapid process. All steroid receptors are phosphorylated at more than one single site. Most phosphorylation sites are located in the N-terminal domain, and phosphorylation occurs mainly on serine residues. In only a few cases, phosphorylation on threonine residues occurs. Tyrosine residue phosphorylation occurs only in the estrogen receptor. Phosphorylation of this tyrosine residue in the ligand-binding domain is needed to maintain the estrogen receptor in a transcriptionally inactive state in the absence of ligand. Six different kinases (estrogen receptor kinase, PKA, PKC, casein kinase II, DNA dependent kinase, Ser-Pro directed kinases) have so far been reported to phosphorrylatee steroid receptors. The following

receptor functions or activities linked to phosphorylation have been suggested: receptor associaton with heat shock proteins, activation of hormone binding, nuclear import, subnuclear localization, nucleocytoplasmic shuttling, modulation of bindings to hormone response elements, receptor dimerization, interactions with other transcription factors and receptor half-life (e.g. receptor turnover and recycling).

2.2.7 Nuclear import of steroid hormone receptors

As steroid receptors are synthesized in the cytoplasm, they need to be transported in some way into the nucleus before steroid hormone action take place. The mechanism till date is largely unknown but appears to be energy dependent. One important structural aspect in the nuclear import mechanism is a nuclear localization signal that is remarkably identical in all nuclear receptors and which was first identified in the protein nucleoplasmin. This nuclear localization signal has a bipartite character, with two basic amino acid residues from another set of four or five basic amino acid residues (Table 2.1). This signal is highly conserved among the members of the superfamily and it has been found to be active for the progesterone and androgen receptor. For the glucocorticoid and mineralocorticoid receptor it has been found that these receptors are associated with microtubules and the actin network and that the movement of receptor molecules along the cytoskeletal network in association with hsp90 is essential during their nuclear import (Guiochon-Mantel *et al,* 1991; Kumar et al., 2006).

Table 2.1 Nuclear localization signal in various proteins. Basic residues thought to be important for the signal are shown in bold capital. Amino acid residues are indicated as one letter symbol.

Protein	Species	Sequence
Simian virus 40 (SV 40) large T antigen	SV 40	pKKKRK
Nucleoplasmin	Xenopus	kavKRpaatkkagqaKKKKl
Androgen receptor	human	crlRKcyeagmtlgaRKlKKl
Progesterone receptor	human	crlRKccqagmvlggRKfKKf
Glucocorticoid receptor	human	cryRKclqagmnleaRKtKKk
Mineralocorticoid receptor	human	crlqKclqagmnlgaRKsKKl
Estrogen receptor	human	crlRKcyevgmmkggiRKdRRg

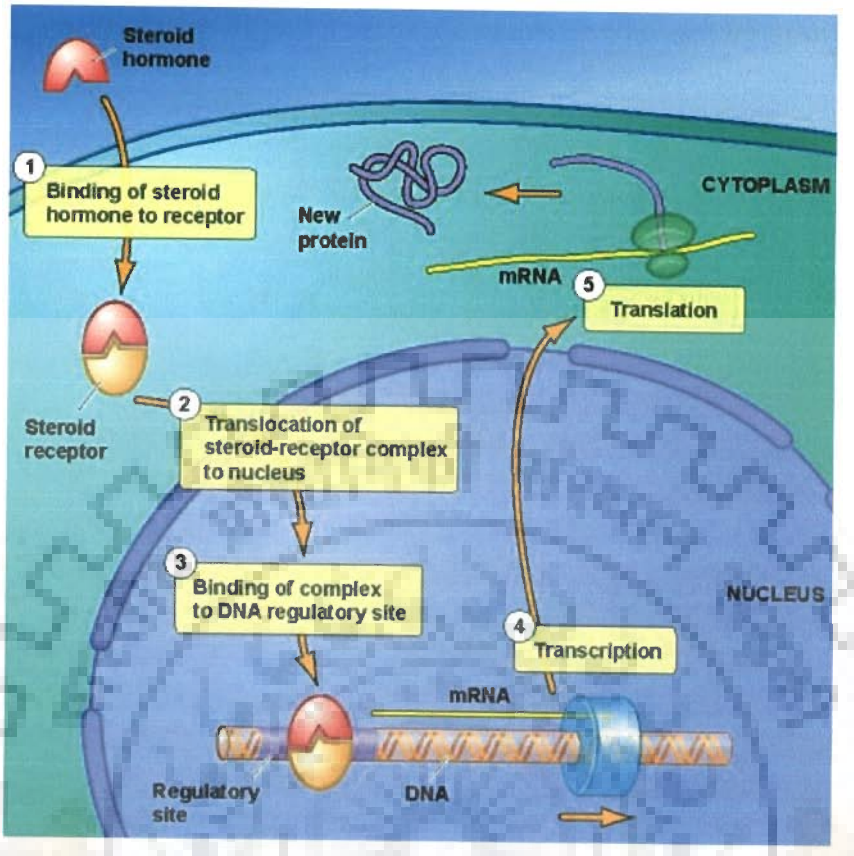


Fig 2.5 Schematic representation of the steroid hormone mechanism of action (Courtesy: http://www.thepepproject.net).

2.3 Steroid hormone receptor co-activators

Genetic studies implicated that transcription cofactors with no specific DNA-binding activity are essential components of transcriptional regulation, which ultimately led to identify a series of nuclear receptor-interacting coregulatory proteins (Horwitz et al., 1996). Thus far, these proteins have been shown to have a few characteristic features, as summarized in Fig 2.5. Firstly, they bind to target transcription factors in a ligand dependent manner. Secondly, many of them are capable of directly interacting with the basal transcriptional machinery. Thirdly, some of them exhibit enzymatic function intrinsically linked to gene regulation, such as the nucleosomal remodeling histone acetyl transferase (HAT) or deacetylase (HDAC) activities. Thus, these proteins appear to function by either remodeling chromatin structures and/or acting as adapter molecules

between nuclear receptors and the components of the basal transcriptional apparatus.

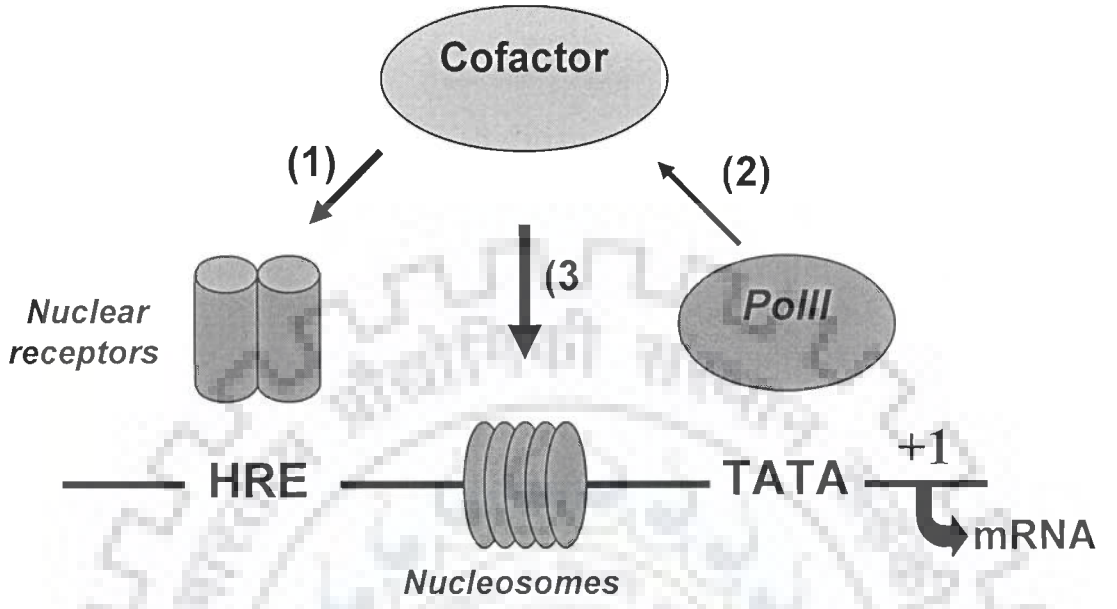


Fig 2.6 The role of transcriptional cofactors. Three general functions of known receptor cofactors are denoted as (1), (2), and (3). HRE and +1 denote hormone response elements and transcription initiation site, respectively. Nuclear receptors, nucleosomes, cofactor and RNA polymerase II bound to TATA sequences are schematically depicted. Notably, RNA polymerase II and most cofactors exist as steady-state complex of multiple polypeptides, although each of them is represented as a single polypeptide for simplicity.

The p160 Family

A group of related proteins were found to enhance ligand-induced transactivation function of several nuclear receptors, named the p160 family (Lonard & O'Malley, 2005). These proteins are grouped into three subclasses based on their sequence homology: 1) SRC-1/NCoA-1 (Hong et al., 1997; Torchia et al., 1997; Voegel et al., 1998), 2) TIF2/GRIP1/NCoA-2 (Hong et al., 1997; Voegel et al., 1998), and 3) p/CIP/ACTR/AIB1/xSRC-3 (Anzick et al., 1997; Chen et al.,1997; Kim et al., 1998; Torchia et al., 1997).

A distinguishing structural feature of the p160 family is the presence of multiple LXXLL signature motifs (Heery et al., 1997; Torchia et al., 1997; Leo & Chen, 2000). The

AF2 core (helix 12) was recently shown to undergo a conformational change upon ligand binding, forming part of a charged clamp and thus accomodates p160 coactivators within a hydrophobic cleft of the receptor LBD, through direct contacts with these LXXLL motifs (Darimont et al., 1998; Nolte et al., 1998; McKenna et al., 2002). These factors can also interact with CREB-binding protein (CBP)/p300 via a separate domain (Kamei et al., 1996; Yao et al., 1996). On the other hand, a weak intrinsic HAT activity has been reported in SRC-1 and ACTR, suggesting that a function of this factor may involve chromatin remodeling (Chen et al., 1997; Spencer et al., 1997). It has recently been shown that SRC-1 also mediates transactivation by a series of other transcription factors, including AP-1, NFkB, SRF, and p53 (Lee et al., 1998; Na et al., 1998; Kim et al., 1998a; Lee et al., 1999). Specifically saying, SRC-1 and p/CIP were strong coactivators for p53, whereas AIB1 and xSRC-3 were repressive in nature (Lee et al., 1999).

p/CAF

The N-terminus of p/CAF interacts with CBP and members of the p160 family (Grant et al., 1997; Blanco et al., 1998; Korzus et al., 1998; Ogryzko et al., 1998). It has been suggested that there might be a possible link between the p/CAF complex and the RNA polymerase II core machinery. This p/CAF complex bears resemblance to the GCN5/SAGA complex in yeast. Other subunits of the complex facilitate p/CAF to acetylate histones in the context of nucleosomes, although p/CAF alone is inert.

TRAP/DRIP complexes

The thyroid hormone receptor associated proteins (TRAPs) (Fondell et al., 1996) has shown potentiated transactivation function of liganded thyroid receptor (TR) in reconstituted in vitro transcription assays utilizing naked DNA templates. On the other hand, DRIP (vitamin D3 receptor (VDR) interacting protein) complex, having high homology to the earlier, was also isolated using VDR as the affinity matrix (Rachez et al., 1998) This potentiated ligand-dependent transactivation function of VDR on a chromatinized template in vitro (Rachez et al., 1999). A newly discovered ARC (activator recruited cofactor) complex, which is identical constituents of DRIP complex has been found to be essential for a number of other transcription factors such as SREBP, NFκB and

VP16 (Naar et al., 1999; Rachez et al., 1999; Rachez et al., 2000). Recruitment of this TRAP/DRIP complex to the LBD AF2 core following ligand-binding occurs through a single subunit (DRIP205/TRAP220/TRIP2) via a single LXXLL motif (Lee et al., 1995; Naar et al., 1999; Rachez et al., 1999). All these, strongly suggests their direct connection to the RNA polymerase II core machinery (Kim et al., 1994).

CBP/p300

CBP and p300 have been implicated in functions of a large number of regulated transcription factors (Goldman et al., 1997). For nuclear receptors, the interaction with CBP/p300 is ligand- and AF2-dependent, although this direct interaction does not appear to be essential with many nuclear receptors (Westin et al., 1998; Li et al., 2000). Interestingly, CBP and p300 harbor HAT activity (Bannister et al., 1996; Ogryzko et al., 1996). Purified p300 has shown potentialization of ligand induced ER function only on chromatinized template, strongly supports that a major function of CBP/p300 could be to modify chromatin structure via histone acetylation (Kraus and Kadonaga, 1998). However, it is notable that CBP/p300 can also acetylate and functionally modulate, either in a negative or positive manner, non-histone proteins, including TFIIEb, p53, hematopoietic transcription factor GATA-1 and erythroid Krüppel-like factor (Imhof et al., 1997; Gu and Roeder, 1997; Boyes et al., 1998; Zhang and Bieker, 1998; Lee et al., 2001). These results suggest that CBP/p300 may also target different aspects of gene activation, in addition to their roles in chromatin remodeling.

ASC-1

Recently a novel nuclear receptor interacting coactivator, termed activating signal cointegrator-1 (ASC-1) has been discovered (Kim et al., 1999). ASC-1 harbors an autonomous transactivation function and binds to basal t ranscription factors TBP and TFIIA and transcription integrators SRC-1 and CBP/p300. The ASC-1 binding sites involve the hinge domain of nuclear receptors. ASC-1 may have more active roles in replacing NCoR/SMRT from receptors upon ligand binding. ASC-1 may also act as an adaptor to coordinate pre-mRNA splicing and transcriptional activation of class II genes (Ge and Wolfe, 1998).

ASC-2

Activating signal cointegrator-2 (ASC-2) is another novel transcriptional coactivator molecule of nuclear receptors (Lee et al., 1999a). Similar or identical molecules has also been reported, viz., TRBP, PRIP, and RAP250 (Ko et al., 2000; Zhu et al., 2000; Caira et al., 2000). ASC-2 binds to basal transcription factors TBP and TFIIA and transcription integrators SRC-1 and CBP/p300. It enhances the receptor transactivation, either alone or in conjunction with SRC-1 and p300. ASC-2 was also recently found to mediate transactivation by a series of mitogenic transcription factors, including SRF, AP-1, NFκB and E2F (Lee et al., 2000).

2.4 Steroid hormone receptor co-repressors

Two well characterized co-repressors are, NCoR (Chen et al., 1995) and SMRT (Horlein et al., 1995), which were originally isolated as factors associated with the hinge domain of these nuclear receptors in the absence of ligand. The molecular basis of corepressor recruitment involves cooperative binding of two h elical interaction motifs within the NCoR carboxyl terminus to both subunits of an RAR-RXR heterodimer (Hu and Lazar, 1999; Perissi et al., 1999) (Fig 2.6). In the absence of bound agonist, NRs bound to their response elements on DNA interact with a corepressor via the so-called corepressor NR (CoRNR "corner") box (Perissi and Rosenfeld, 2005). Their receptor interaction motifs have a consensus sequence of LXXI/HIXXXI/L, which represents an extended helix in comparison to the coactivator LXXLL helix (Heery et al., 1997; Torchia et al., 1997).

It interacts with the corepressor docking surface on helices 3 through 5 of the receptor ligand-binding domain (LBD) (Privalsky, 2004). When the bound agonists are absent, these receptors are held in the cytoplasm in a complex with heat shock protein 90 (steroid receptors) or 72 (PPARα) through multiple chaperone proteins (Huang et al., 1994; Pratt et al., 2004). The receptor LBD has several interaction sites shared by coactivators and corepressors but amongst them helix 12 (also referred to as activation function 2 [AF-2]) is most critical in implementing coregulator docking, i.e., a corepressor or coactivator. While Nuclear Receptors (NRs) are in unliganded stat, helix 12 remains in an extended conformation helping the corepressor to bind. Alternatively, in agonist bound state, helix

12/AF-2 is repositioned close to the LBD and this three dimensional change in the NR results in conformational change of the three turn α helix of the CoRNR box making it unfit for corepressor-binding area. The two-turn α-helix of a coactivator NR box, having a sequence of LXXLL, has been favored by helix 12/AF-2 repositioning for proper interaction. Once coactivators interact with the NR, helix 12 forms a charge clamp with helix 3 thus locking the coactivator into place on the agonist-bound receptor (Privalsky, 2004). Fig 2.7 as already mentioned shows general models for corepressor and coactivator regulation of nuclear receptors.

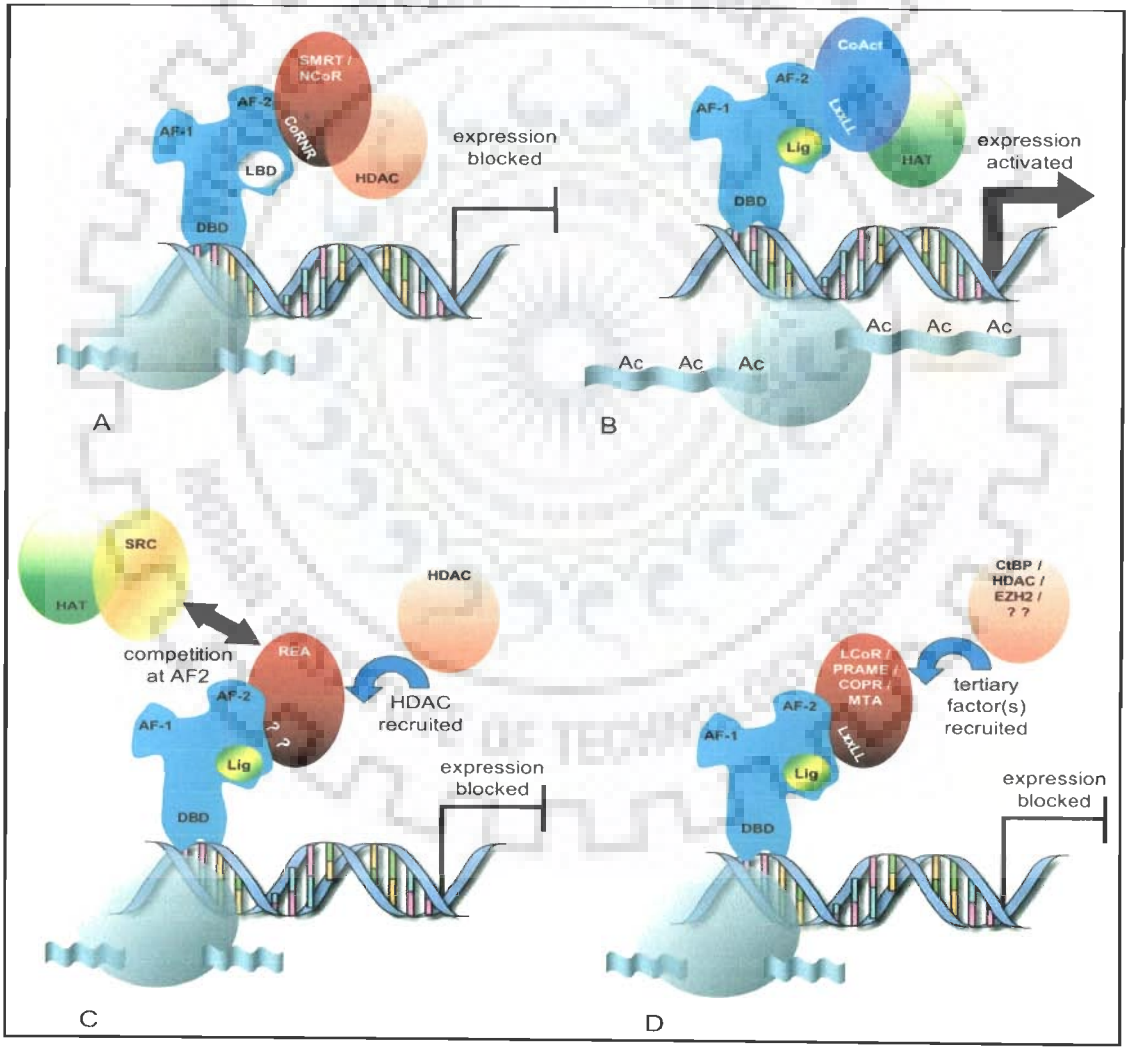


Fig 2.7 Models for corepressor and coactivator regulation of nuclear receptors. For simplicity, only one of the two receptor dimers is illustrated. (continued...)

A) Corepressors interacting with nuclear receptor (NR) in the absence of ligand. Their interaction with chromatin modifiers, e.g., HDACs leads to histone states unfavorable for transcription and expression of target gene is blocked.
B) Coactivators interacting with NR in the presence of agonist. Agonist occupation of the NR ligand binding domain (LBD) leads to exchange of corepressors for coactivators, and in this scenario, recruitment of chromatin modifiers e.g., histone acetyl transferases (HAT) which acetylate lysine side chains. The remodeled, open chromatin structure allows addition of proteins which positively regulates transcription (not shown) to be recruited and promoter activity to ensue.
C) Agonist-dependent corepression. REA competes with SRC for the AF-2 of ligand-bound receptor reducing the opportunity for transcription-enhancing proteins, e.g., HAT, to participate in the transcription complex. Further loss of transcription-favorable chromatin structure may be lost by REA's direct association with HDACs and subsequent reduction of acetylated histones.
D) Generalized version of agonist-dependent corepression. Transcriptional repressors in addition to or instead of HDACs may participate in blocking expression. AF-1: activating function 1, AF-2: activating function 2, DBD: DNA binding domain of NR, Lig: ligand, Ac: acetylated residues on histones comprising nucleosome, CoAct: coactivator, HAT: histone acetyl transferase, HDAC: histone deacetylase, REA: Repressor of Estrogen Activity, SRC: steroid receptor coactivator (Adapted from Gurevich et al., 2007).

2.5 Endocrine disruptors: an overview

Many chemical substances of natural or anthropogenic origin are suspected or known to be endocrine disruptors, which can influence the endocrine system of life. This observation has led to increased interest on the part of the public and the media, as well as to a steep rise of research activities in the scientific community.

Environmental "endocrine disruptors" have been defined by the US Environmental Protection Agency as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behavior" (EPA Final Report, 1998).

The endocrine system and nervous system are the two major modes of communication that coordinate and control body function. In contrast to the nervous system that conducts electrical impulses along precise nerve cell circuits, the endocrine

system communicates in a more diffuse fashion, transporting information through the circulation from one tissue to another via chemical messengers or hormones. Endocrine disruption refers to the interference of endocrine system function by environmental chemicals. The endocrine system participates in virtually all important functions of an organism, such as sexual differentiation before birth, sexual maturation during puberty, reproduction in adulthood, growth, metabolism, digestion, cardiovascular function, and excretion. Hormones are also implicated in the etiology of certain cancers of hormone-dependent tissues, such as those of the breast, uterus, and prostate gland. Therefore, endocrine disruption can potentially produce widespread effects. However, for reasons to be made obvious later, most examples of endocrine disruption involve reproductive abnormalities.

The European Union, through its adherence to the "Precautionary Principle" as the basis for regulating chemicals, is paying special attention to possible endocrine-disrupting chemicals because of the association between substances of this type and "serious human health effects" such as breast and prostate cancer, decreased sperm production, and genital deformities (Commission of the European Communities, 2001). Since hormones play an integral part in reproduction, endocrine disruption is also likely to emerge as an issue with respect to the reproductive toxicity provisions of California Proposition 65. As well as the issue of endocrine disruption was incorporated into the Food Quality Protection Act (FQPA) and amendments to the Safe Water Drinking Act (SWDA) in 1996. The U.S. Environmental Protection Agency (EPA) formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), composed of representatives from academia, industry, and public health advocacy groups. The task of EDSTAC was to develop and implement a screening program (Endocrine Disruptor Screening Program or EDSP) that was to be underway by 1999. Over 87,000 chemicals (e.g., pesticides, industrial chemicals, food additives, cosmetics, and naturally occurring substances) and select mixtures (human breast milk contaminants, those common to hazardous waste sites, and pesticide fertilizer mixtures) were candidates for testing (EDSTAC Final Report, 1999). Furthermore, the National Research Council, USA (1999) recommended that more research is needed on endocrine disruption since not many information are available about the reproductive and developmental effects of hormonally active agents.

2.5.1 Historical background

The association between chemical contamination of the natural environment and the onset of disease, reproductive failure and death of wildlife species began noticed as early as from 1947 in US (Tarin, 1972). During that period, ornithologists began to observe a sharp decline in east coast populations of the American bald eagle, a phenomenon that was associated with changes in courtship behavior and the maternal care of chicks. Since then, a growing number of cases confirmed that the contamination of lakes and rivers with sewage from local manufacturing industries and chemical plants and also from agricultural run-off was the root cause for these developmental and reproductive problems in certain aquatic species. Further due to their higher position in the food chain- avian, reptilian and mammalian species were also facing the problem. For example, in 1960s, the mink industry around the Great Lakes region in the US suffered economic hardship due to reproductive failure of these animals, a repercussion of its heavy reliance on local fish that were later found to be contaminated with, among other chemicals, polychlorinated biphenyls (PCBs) along with other chemicals. In this same decade, a high incidence of tumors was observed in fish that resided within Californian waters receiving sewage from local industries. In 1970s, the herring gull population of Lake Ontario in Canada was observed to suffer from developmental deformities such as club feet, missing eyes, twisted bills and the presence of adult feathers in chicks instead of down, phenomena that were attributed to dioxin exposure. In 1980s, alligators in Lake Apopka, Florida were observed to exhibit micro-phallus and other reproductive-related disorders, a consequence of a local industrial accident and, more recently, agricultural run-off of chemicals including 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (DDT). Thus it became lucidly clear that certain industrial and agricultural chemicals could cause developmental and reproductive anomalies in wildlife, an incident that was beautifully depicted by Rachel Carson in her 1962 book "Silent Spring" (Carson, 1987), and one that indicated the same kind of fear in humans. Finally, the agricultural and industrial use of DDT and PCBs was banned in 1973 and 1977, respectively, within the US. Nonetheless, new and different cases of wildlife disturbances continued to emerge over the next few decades, due to bio-persistence of the banned chemicals (DDT has a half-life of 57 years) and the production of a plethora of new chemicals.

Motivated by these outcomes, Dr. Theo Colborn, World Wildlife Fund, convened the 1991 Wingspread Conference in Racine, Wisconsin. This scientific meeting emphasized the notion that these chemicals might be inducing their observed effects as a result of their hormonally active nature. The conference addressed the issue that "a large number of man-made chemicals that have been released into the environment . . . have the potential to disrupt the endocrine system of animals, including humans," and made the observation that some of the effects documented in the genital tracts of wildlife were comparable to those seen in the daughters and sons of women who had been exposed during pregnancy to the synthetic estrogen diethylstilbestrol (DES). DES was administered to pregnant women between the years 1948 and 1971 as an anti-abortive therapy in the US, Europe and Australia. However, it was found that fetal exposure to this potent estrogen induced a rare cancer, clear cell adenocarcinoma of the vagina, and other severe reproductive disorders that became apparent after puberty. In some instances these malformations manifested even when these women tried to become pregnant (Herbst etal, 1988; Mittendorf et al, 1995). The conference participants recognized that the human DES syndrome was an extreme expression of the plasticity of the fetus in response to environmental cues, and further, it provided a template for the potential effects that other hormonally active chemicals could have on human health.

Carlsen et al. (1992) analyzed results of semen quality in papers published between 1938 and 1991, and concluded that there was "a significant decrease in me an sperm concentration between 1940 and 1990 from 113×10^6/ml to 66×10^6/ml". It was suggested that "some common prenatal influences could be responsible for the decline in sperm density and for the increase in cancer of the testis, hypospadias and cryptorchidism", and this concept was recently formulated as the testicular dysgenesis syndrome (TSD) (Skakkebaek et al., 2001; Devi et al., 2006; Majumdar et al., 1997; Nagarajan et al., 2005; Kumar et al., 2008). These all incidents have made the pavement for increasing research concern for endocrine disruptors. Fig 2.8 shows examples of just a few distinct environmental chemicals found capable of altering estrogen levels in aquatic organisms. For some EDCs, the parent compound may have no endocrine disrupting activity whereas the metabolites of the same chemical may be biologically active.

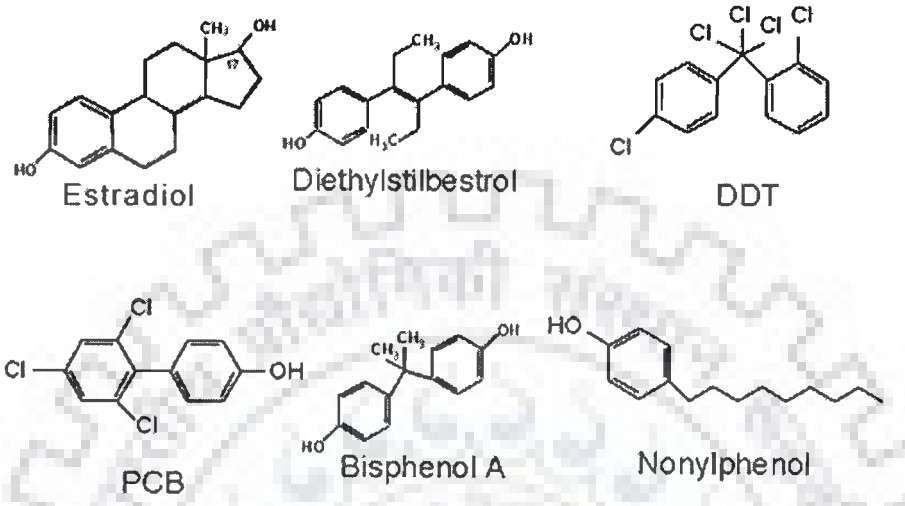


Fig 2.8 Structures of some estrogenic compounds in comparison to estradiol as model.

2.5.2 Impact of endocrine disruptors on male reproductive health

2.5.2.1 Sperm quality deterioration

Conclusion has been drawn from a meta-analysis that a decrease in sperm quality of 40% worldwide since the 1940s was observed (Carlsen et al., 1992) and was confirmed by Swan et al., 1997. Substantial support was gathered by mono- and multilaboratory studies in Belgium (Van Waeleghem et al., 1996), Finland (Pajarinen et al., 1997), France (Auger et al., 1997) and Danmark (Andersen et al., 2000) which were only differed by regional differences following environmental factors. A decrease of ejaculate volume, sperm concentration and of the percentage of normal motile spermatozoa was recorded in sons of women, who were treated with diethylstilbestrol during pregnancy (Bibbo et al., 1978). It was also observed that heavy exposure to PCBs resulted in negative effects on sperm morphology and motility, but not on sperm concentration (Guo et al., 2000; Dallinga et al., 2002).

2.5.2.2 Increased incidence of testicular cancer

At present, testicular cancer is the most common malignant tumour in young males of western countries. The main risk factor for testicular cancer is cryptorchidism, followed by hypospadias (Sharpe, 2003). There has been a significant increase in the prevalence of testicular cancer, albeit with clear racial and geographical differences in recent decades (Dearnaley et al., 2001). Profound regional differences in occurrence and the association with birth cohorts indicate a possible role of environmental factors in the development of testicular cancer (McKiernan et al., 1999; Takamiya et al., 2007). A significantly increased risk of seminoma among plastic workers exposed to polyvinyl chloride (PVC) was also reported (Ohlson et al., 2000). Incidence rates for testicular cancer in 20th centuries were significantly correlated with the per-capita consumption of coffee and pigmeat, the principal dietary sources of potential mycotoxin, ochratoxin A (Schwartz, 2002). Identification of such endocrine disruptors causing testicular cancer as well as its therapeutics is underway (Jana et al., 2004; Kumar et al., 2008; Sinha et al., 2006).

2.5.2.3 Increase in cases of cryptorchidism and hypospadias

Cryptorchidism, the most common congenital condition in babies, takes place when the testis fails to descend into its normal position in the scrotum (Akre et al., 1999). The risk for cryptorchidism was higher in sons of women, working with pesticides (Weidner et al., 1998), as well as a significantly higher concentrations of hexachlorbenzene and heptachlorepoxide were found in adipose tissues of boys with testicular maldescent, compared to those of a control group (Hosie et al., 2000).

Hypospadias is a birth defect of the urethra in the male that involves an abnormally placed urinary meatus (opening). Instead of opening at the tip of the glans of the penis, a hypospadic urethra opens anywhere along a line (the urethral groove) running from the tip along the underside (ventral aspect) of the shaft to the junction of the penis and scrotum or perineum. Suspected endocrine disruptors behind it include phthalates, DDT, and polychlorinated biphenyls. In the Seveso accident, 1976, exposure of fathers to dioxins resulted in an increased incidence of hypospadias among their sons (Baskin et al., 2001).

Side by side, an increased transgenerational risk of hypospadias in sons of women that were exposed in utero to diethylstilbestrol (DES) has also been reported (Klip et al. (2002).

2.5.2.4 Alteration in sex ratio

Several studies reported a small but significant decrease in this sex ratio in Canada and the United States (Davis et al., 1998), the Netherlands, Denmark and several other European countries (Martuzzi et al., 2001). A remarkable decrease in the number of sons of fathers who were exposed to PCBs before (but not after) the age of 19 during the Yu-Cheng disaster has been reported in recent times (Del Rio-Gomez, 2002). Exposure of dioxin during the Seveso accident resulted in a dose-dependent decrease in the sex ratio of the offspring of males that were younger then 19 years of age at the moment of accident (Mocarelli et al., 2000).

2.5.3 Impact of endocrine disruptors on female reproductive health

2.5.3.1 Puberty and breast development

An early sign of puberty is breast development, which in humans is triggered by an increase in the ratio of estrogen to androgen. Among many factors, metabolism of androgen may play a principal role in induction for the onset of breast development. When an enzyme (CYP3A4) responsible for testosterone hydroxylation, an inactivating step in testosterone metabolism, is found as the homozygous alleles for a high-activity variant of the enzyme (CYP3A4*1B) there is an early onset of breast development (Kadlubar et al, 2003). Girls (aged 9.5±0.3 years) found to be homozygous for CYP3A4*1B, 90% exhibited breast development (Tanner score 2B or higher), whereas only 40% of the girls homozygous for the low-activity variant (CYP3A4*1A) exhibited breast development at the same age. Other means of increasing the estrogen-to-androgen ratio, such as increased estrogen due to over-expression of aromatase (the enzyme that converts T–E2), have been shown to cause premature breast development in girls and gynecomastia in boys (Stratakis et al., 1998; Braunstein, 1999). The findings of Hayes' group, showed that the pesticide atrazine can increase aromatase in frogs to a functionally estrogenizing level; which can also be linked to human as well. Laboratory studies on rodents showing that pre-pubertal

exposure to environmental estrogens can advance puberty in females suggest that environmental estrogens may be causing this change. A survey of parents at four US Army pediatric clinics, found that 64% of African Americans (but only 6.9% of Caucasians) use hair-care products containing hormones or placenta, and that half of those parents also use the products on their children (Tiwary, 1997). When in a case study mothers ceased using estrogen- or placenta-containing hair products on the girls, incidents of premature thelarche and/or pubic hair development at early puberty resolved, thus suggesting a transient effect of exogenous estrogen rather than a central neuroendocrine effect (Tiwary, 1998). Studies from an early puberty cohort in Puerto Rico showed a link between phthalic esters used as plasticizers and premature thelarche (Colon et al., 2000). Phthalates, which acts generally as antiandrogens, may account for the connection between early thelarche and elevated plasma levels of phthalic esters in the Puerto Rican girls (Gray et al., 2000).

2.5.3.2 Breast disease

EDCs could potentially affect females by lactation suppression. It is well known that exogenous estrogens such as DES (diethylstilbestrol) will effectively suppress lactation. Gladen and Rogan evaluated the role of estrogenic organochlorine compounds on lactation and found a striking adverse effect (Gladen et al., 1995).

2.5.3.3 Uterine disease

It has been shown that nonhuman primates exposed to the widespread environmental contaminant TCDD (dioxin) have a high rate of endometriosis. The recent evaluation of the cohort of women exposed to massive doses of dioxin after a chemical accident in Seveso, Italy (Wiess et al., 2003), does not support these earlier finings in non-human primates. Since dioxin is not an estrogenic contaminant its effect on the uterus may not be profound. Another benign uterine disease, leiomyomas or fibroids, has both high prevalence (affecting from 25% of white women to 70% of black women) and morbidity (including infertility and abnormal bleeding) and is dependent on circulating estrogen. Onset of fibroids occurs after puberty, and these benign tumors regress after menopause. It has been shown that fibroids are hypersensitive to the effects of estrogen (Baird et al,

2005). Because of its dependence on estrogen for growth, a role of environmental estrogens in fibroid disease should be considered.

2.6 Endocrine-Disrupting Chemicals: Modus operandi

2.6.1 Modulation of Steroid Hormone Metabolism

Human steroid and xenobiotic receptor/ r odent pregnane X receptor (SXR/PXR) (Blumberg et al., 1998, Kliewer et al., 1998) and constitutive androstane receptor (CAR) (Forman et al., 1998; Xie et al., 2000), are important regulators of xenobiotic and steroid hormone metabolism. They are highly expressed in the liver and intestine, mediating the induction of cytochrome P450 enzymes (e.g. CYP3A, CYP2B, and CYP2C (Pascussi et al., 2003), conjugation enzymes (e.g. UGT1A1) (Xie et al., 2003), and transporters (e.g. P-glycoprotein, multidrug resistance-associated proteins, and organic anion transporter peptide 2) (Staudinger et al., 2003) in response to xenobiotic ligands and steroid hormones. On the other hand, they also function in the regulation of bile acid synthesis and cholesterol metabolism (Guo et al., 2003). Unusually SXR/PXR has broad ligand specificity amongst nuclear receptors and is activated by a large number of Endocrine disrupting chemicals (EDC). The organochlorine pesticides, di-(2-ethylhexyl) phthalate and nonylphenol, were found to be mouse PXR activators and CYP3A inducers (Schuetz et al., 1998; Masuyama et al., 2000). Bisphenol A, an estrogenic compound used in the manufacture of plastics, activates human SXR (Takeshita et al., 2001). A more extensive analysis of 54 xenobiotics of environmental concern found that alachlor, benzophenone, benzene hexachloride, methoxychlor, nonylphenol, trifluralin, and vinclozolin activated rat PXR and induced the expression of CYP3A (Mikamo et al., 2003). *Trans*-nonachlor, a component of the banned pesticide chlordane, repressed the basal activity of mouse CAR (Moore et al, 2002). Activation of SXR/PXR and CAR and up-regulation of their target genes by the many compounds mentioned above can increase the levels of endocrine-disrupting metabolites, thus provides a route through which Endocrine disrupting chemicals (EDC) can alter steroid receptor activity without directly binding to steroid receptors.

2.6.2 Species Specificity

Some xenobiotic compounds results into species-specific effects on SXR/PXR activation and target gene induction. Polychlorinated biphenyls (PCBs) are one such group of compounds and as a result banned worldwide on their production and use. In addition to their effects on endocrine receptors (Bonefeld-Jorgensen et al., 2001; Kester et al., 2000; Iwasaki et al., 2002., Portigal et al., 2002), some PCBs were also able to activate mouse PXR (Schuetz et al., 1998).

2.6.3 Modulation of Nuclear Receptor Coactivators

Changes in the expression levels of transcription coactivator mRNAs and proteins would be expected to modulate receptor activity. Upon drug treatment, a steady state increase in nuclear receptor coactivator levels has been recorded which in turn increases transcriptional activation of ERα in the presence of xenobiotics (Lonard et al., 2004). Similarly, an increased expression level of the coactivator thyroid hormone receptor activator protein 220 and increased expression of ERβ in mouse uterus have been recorded by bisphenol A, whereas in Ishikawa endometrial cells, it only increased the expression of ERβ (Inoshita et al., 2003). These outcomes reveal that an Endocrine disrupting chemical (EDC) can modulate target gene expression by altering coregulator and transcription factor levels, and that this modulation may be tissue specific. Constitutive androstane receptor (CAR) can inhibit ER-mediated transcriptional activity without binding to an estrogen response element (Min et al., 2002). Overexpression of CAR results into a dose dependent reduction of ER activity. This effect was potentiated by further activating CAR with 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, whereas the CAR antagonist, androstenol, relieved the CAR-mediated repression of ER transcriptional activity. CAR repression of ER was relieved by increasing amounts of the coactivator GRIP-1 (Min et al., 2002). This indicates CAR as well as SXR/PXR may have effects on steroid hormone receptor by limiting coactivator availability.

2.6.4 Effects on Proteasome Mediated Degradation of Nuclear Receptors

The ubiquitin-proteasome pathway takes major part in degradation of most of the nuclear receptor superfamily members through ligand-dependent manner, which in turn

prevents cells from overstimulation by endogenous hormones or other activating signals and may also reset the transcriptional apparatus in preparation for a subsequent response (Dennis et al., 2001). It has been reported that down-regulation in transcriptional activity of progesterone receptor (PR) and androgen receptor (AR) occurs following inhibition of the ubiquitin proteasome degradation pathway (Syvala et al., 1998; Sheflin et al., 2000; Lin et al., 2002). ERα in the presence of Estrogen Receptor (ER) agonists, antagonists, and selective ER modulators, illustrates that transcriptional activity can be affected by modulating receptor stability as a result of different rates of proteasome-mediated degradation (Wijayaratne et al., 2001). Thus the above points help us to hypothesize that EDCs impose drastic effects by acting on proteasome-mediated degradation of the earlier mentioned receptors or coregulatory proteins in the normal hormonal response magnitude as well as duration. In a special case, bisphenol A activated ER-mediated transcription, but did not enhance the interaction between ERβ and SUG1 (suppressor for Gal 1), thus slower its degradation followed by ERβ accumulation resulting in endocrine disrupting effect (Masuyama et al., 2004). Substantially, transcriptional activity of other nuclear receptors, such as SXR/PXR, is also regulated by proteasome degradation. Phthalic acid causes blocking of normal proteasome –mediated degradation of PXR in comparison with the endogenous PXR ligand, progesterone, thus raising the possibility of increase in PXR protein levels foll owed by alteration the expression of PXR target genes as potential endocrine disruptor (Masuyama et al., 2002). In turn, this could affect the clearance of endogenous hormones. p1 60 family coactivators, such as GRIP1 and SRC-1, are also degraded via the proteasome (Lonard et al., 2004; Hoang et al., 2004), potentially broadening this research area.

2.6.5 Hormone sensitizing activity

Recent work shows that valproic acid and methoxyacetic acid (MAA) do not mimic endogenous hormones, rather, increase hormone receptor (ERα, ERβ, AR, progesterone receptor, and thyroid hormone receptor β) activity by altering cell signals that activate protein kinases or inhibit histone deacetylases (Jansen et al., 2004). Valproic acid is a commonly prescribed anticonvulsant and mood stabilizer (Lammer et al., 1987; Dansky et al., 1991) and in this case acts as a histone deacetylase inhibitor while also increasing the

expression of the cell cycle regulator p21 (Zhu et al., 2004). Metabolites of Ethylene glycol monomethyl ether (EGME), a commonly used solvent in paints, dyes, and fuel additives, as well as MAA depicted earlier, can potentiate the effects of testosterone and increase the expression of ERβ (Tirado et al., 2003; Tirado et al., 2004). Studies revealed that the above mentioned short-chain fatty acids function as activators of p42/p44 mitogen-activated protein kinase as well as inhibitors of histone deacetylases. Specifically saying, result of increased activation of MAPK is the phosphorylation of coactivators (Font de Mora et al., 2000), whereas the inhibition of histone deacetylase activity has more general effects on nuclear receptor activity. Thus, people who were exposed to these chemicals could likely to have side effects following exogeneous estrogen and progestin administration as in oral contraception and postmenopausal hormone replacement therapy.

2.6.6 Transgenerational effects on fertility by reprogramming DNA methylation

Recently, methoxychlor (a replacement for DDT) and vinclozolin (a fungicide used in the wine industry), were shown to alter the spermatogenic capacity of male germ cells and sperm viability via their effects on DNA methylation. A transient embryonic exposure to these chemicals during gonadal sex determination in the rat reduced fertility and sperm development in the adult testis (Staudinger et al., 2003). Exposure levels in the rat studies were higher than a typical environmental exposure, but the epigenetic effects on male fertility resulted by these EDCs points to an important new mechanism for EDC disruption of gene expression.

2.6.7 Effects as obesogens

Research revealed that EDC effects potentially via ER on the growing obesity epidemic (Heindel, 2003). Tributyltin (TBT) represents, to our knowledge, the first example of an environmental EDC that promotes adipogenesis through RXR and PPARγ at nanomolar level (Kanayama et al., 2005) and chronic exposure could acts as chemical stressors or obesogens that activate RXR and/or RXR:PPARγ signaling to promote long-term changes in adipocyte number and/or lipid homeostasis.

2.7 Endocrine disruptors: Various sources and biological relevance

A large number of environmental pollutants including phthalates, alkylphenolic compounds, polychlorinated biphenyls (PCBs), organochlorine pesticides, bisphenol A have been shown to disrupt endocrine function in animals. Because hormonally mediated events play a central role in central nervous system (CNS) development and function, there is speculation that some of the cognitive deficits that arise from developmental exposure to environmental chemicals may be the result of endocrine disruption. Prominent disturbances caused by these chemicals are their contrary effect on the endocrine system, which is also known as endocrine disruption (Roy et al., 2004; Buck et al., 2006; Guillette, 2006; Maffini et al., 2006; Massart et al., 2006). For example, thyroid hormone is important for proper neuronal proliferation, cell migration, and differentiation in the developing mammalian brain (Porterfield et al., 1998). A list of synthetic chemicals and their effects on endocrine function were grouped in Table 2.2.

Table 2.2 List of few synthetic chemicals and their effects on endocrine function

Compound	Estrogen/Androgen	Thyroid	Glucocorticoids	References
Alachlor	E+	$\downarrow T_4$; $\uparrow T_3$; $\uparrow$TSH; G	?	Al-Hachim et al. 1973
Aldrin	A–	?	?	Lemaire et al. (2004)
Alkylphenols (p-Nonylphenol)	A+;E+	?	?	Schantz et al. 2001
Atrazine	A–; E+	?	?	Thibaut & Porte (2004)
Bisphenol A	A–; E+	?	?	Schantz et al. 2001
Carbendazim	E+	?	?	Morinaga et al. (2004)
Carbofuran	A–;E+	?	?	Goad et al. (2004)
Chlordane	A–	G	$\downarrow$C (females) $\uparrow$C (males)	Lemaire et al. (2004)
Chlordecone (Kepone)	E+	?	Mixed ($\downarrow$C or no change	Tilson et al. (1987)
Cyproconazole	A+;E–	?	?	Trosken et al. (2004)
2,4-D	A–	$\downarrow$PBI; I uptake	?	Kim et al. (2005)

DDT	A–;E+	↑T3; ↓PBI; G	↓C; ↓response to ACTH	Lemaire et al. (2004)
DDE	A–;E+	G; ↓I uptake	↓C; ↓response to ACTH	Lemaire et al. (2004)
Endosulfan	E+	↑T4; ↓T3; G	?	Lemaire et al. (2004)
Endrin	A–	?	?	Lemaire et al. (2004)
Heptachlor	A–	?	↓C	Fang et al. (2003)
Lindane	E+/–	↓T4; ↓T3; ↑TSH; ↓PBI; G	?	Rivera et al. (1998)
Mancozeb	?	↓T4; ↓T3	?	Fang et al. (2003)
Methoxychlor	A–;E+	?	?	Eriko et al. (2003)
Oxychlordane	?	G	?	Schantz et al. 2001
PCBs	E+/–; A–	Mixed (↓T4; unchanged or ↓T3; unchanged or ↑TSH)	↓C	Rice et al. (1997a) Rice et al. (1997b) Schantz et al. (1997)
Prochloraz	A–;E–	?	?	Vinggaard et al. (2006)
Phthalates	A–; E+	?	?	Schantz et al. 2001
Vinclozoline	A–;	?	?	Eriko et al. (2003) Fang et al. (2003)

Abbreviations: A+: androgenic; A–: antiandrogenic; ACTH: adrenocorticotropic hormone; C: corticosterone; 2,4-D: 2,4-dichlorophenoxyacetic acid; E+: estrogenic; E–: antiestrogenic; G: goiter; I: iodine; PBI: protein-bound iodine; TSH: thyroid-stimulating hormone; ?: unknown.

2.8 In vitro bioassays for the assessment of endocrine disruptors

2.8.1 Receptor binding tests

These tests are used to detect compounds, which interact directly with an endocrine receptor. The binding affinity of the receptor for selected ligands is being measured in

Receptor binding assays. Here assays are based on the competition between a non-radioactive compound and a well-established radioactive hormone (Lambright et al., 2000; Scippo et al., 2004; Yamasaki et al., 2004; Viswanath et al., 2008), which are present in cellular or nuclear extracts of a target tissue containing that receptor (Wong et al., 1995; Evans, 1988; Traish et al., 1986; Zava et al., 1979). The major drawback of these assays is that they do not provide information regarding hormonal activity, and thus, they cannot discriminate between agonistic and antagonistic actions of a particular substance (Branham et al., 2002).

2.8.2 Cell proliferation assays

The best-known example of this type of assay is the E-Screen assay and based on a human breast cancer cell line (MCF-7), proliferating in response to estrogens or estrogenic active agents (Lippman, 1976; Shafie et al., 1979; Soto, 1995). Following the high responsiveness of the cells to estrogens, the E-screen is one of the most sensitive assays to analyse the estrogenicity of compounds (Rasmussen et al., 2002; Körner et al., 2004). It is highly suitable to distinguish between estrogen agonists and antagonists. This assay is sensitive to culture conditions (different subclones, serum batch), and thereby the inter- and intralaboratory variability of results is rather high (Zacharewski, 1997). Additionally, this assay is difficult to be used for environmental samples containing toxic constituents.

2.8.3 Reporter gene assays

The main theme of reporter gene expression assays is to measure the induction of gene transcription after hormone receptor activation. Mammalian cell lines (MCF7, COS1 etc.) or yeast strains such as *Saccharomyces cerevisiae* are popularly used having transfected with a reporter plasmid. The plasmid consists of a hormone response element (HRE) coupled to a reporter gene, such as β-galactosidase or luciferase (Hoogenboom et al., 2001; Arnold et al., 1996; Gaido et al., 1997; McDonnell et al., 1991; Metzger et al., 1988; Roy et al., 2004; Bovee et al., 2004; Bovee et al., 2007; Chatterjee et al., 2007; Sanseverino et al., 2009). Yeast strains contain an additional expression vector, which encodes for a specific hormone receptor (e.g. estrogen or androgen receptor). Following exposure to a hormonally active compound in turn resulted in the binding of this

compound with its receptor. The ligand-receptor complex forms dimers, which interact with the HRE-element, resulting in the activation of the transcription and expression of the β-galactosidase gene (Routledge and Sumpter, 1996). Subsequently, β-galactosidase metabolizes a substrate, generating a red colour that can be measured with a spectrophotometer (De Boever et al., 2001), and where luciferase is the reporter, the substrate luciferin and this generates a flash/glow of light, which can be determined by luminometer (Roy et al., 2004). Moving a step forward, GFP has also been used as reporter where the generated fluorescence can be detected by multiplate reader (Bovee et al., 2004; Bovee et al., 2007; Chatterjee et al., 2008). These assays are widely used in the pharmaceutical industry for drug screening and protocols are relatively well standardized (Ankley, 1998).

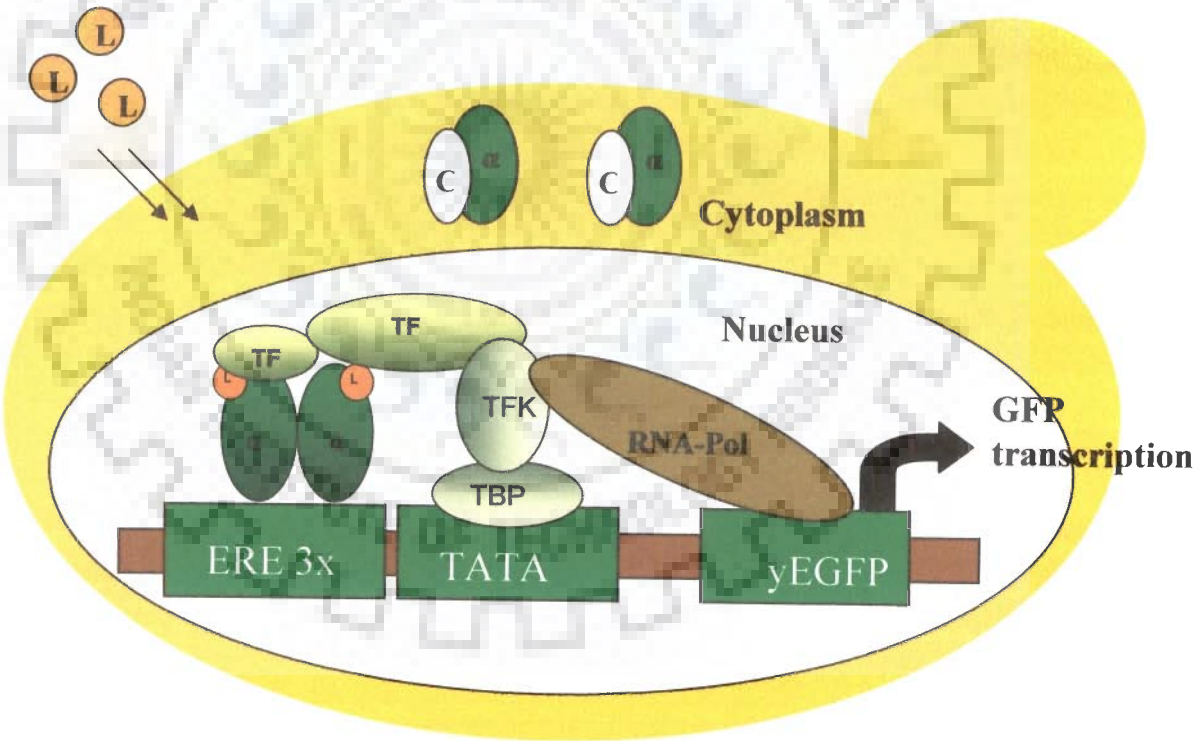


Fig 2.9 Schematic diagram of reporter based transactivation assay using estrogen receptor as model in yeast system. Where, C: Chaperon, ERE: Estrogen Response Element, L: Ligand, TBP: TATA Binding Protein, TF: Transcription Factor, yEGFP: yeast Enhanced Green Fluorescent Protein.

Yeast cells remain permanently transformed if grown under the appropriate conditions and are therefore, easy to use. Some other advantages are quantification without the use of radioactive compounds, the relatively short-term duration of the assay, the possibility to adapt for other hormone receptors (e.g. androgen and progesterone receptor) and the assessment of chemical mixtures (Arnold et al., 1996; Tyagi et al., 2003). However, testing of environmental samples may be hampered by toxicity or needs proper extraction procedure.

2.9 In vivo bioassays

2.9.1 Rodent 3-day uterotrophic assay

In 1930s, the rodent uterotrophic assay has been introduced, which screens for (anti-) estrogenic activity of chemicals, to evaluate the in vivo binding to the estrogen receptor. As we know, estrogens or estrogenically active compounds can induce hypertrophy of the uterus of immature female rats and of mature ovariectomized rats. Test animals are subcutaneously injected with a compound for three consecutive days. After the experiment, the animals are killed, followed by the immediate removal and weighing of the uterus. The performance of this test was evaluated by Owens et al. (2002) and they concluded that it is reliable and can detect compounds with estrogenic properties.

2.9.2 Rodent 5–7 day Hershberger assay

This assay measures the (anti-) androgenic activity of chemicals. In this assay, the capacity of chemicals to restore the weight of the prostate and seminal vesicles of castrated male rodents is determined (Hershberger et al., 1953). Later on, Dorfman validated this test in 1962 (Dorfman et al., 1962).

2.9.3 The rodent pubertal female assay (20 days)

The assay screens for estrogenic and thyroid activity in immature female animals exposed to chemicals during puberty. It examines abnormalities associated with development of the female sex organs and secondary sex characteristics. This test is undergoing standardization and validation by the EPA (Gray et al., 2002).

2.9.4 The rodent pubertal male assay (20 days)

The assay is based on the same principle of the rodent pubertal female assay. It determines the androgenic and thyroid properties of compounds in immature male rodents during puberty. The rodent pubertal male assay examines abnormalities associated with development of the male sex organs and secondary sex characteristics. This test is also undergoing standardization and validation by the EPA (Gray et al., 2002).

2.9.5 Fish reproduction assay

The fish reproduction assay is a 42 days assay and generally used to test agents for estrogenic and androgenic effects. Adult, reproductively active test animals are exposed to a chemical for 21 days. It examines abnormalities associated with survival, reproductive behaviour, secondary sex characteristics, and fecundity (number of spawns, number of eggs per spawn, fertility, and development of offspring). Fish reproduction and developing assays can be included in higher tier screening programs (Hutchinson et al., 2000).

2.9.6 Frog metamorphosis assay

Frog metamorphosis assay is based on the assessment of tail absorption of tadpoles (which metamorphose into frogs) in Xenopus laevis and examines thyroid hormone-like effects. Metamorphosis in frogs is regulated by thyroid hormones and reflects potential thyroid effects in humans. This test is also undergoing standardization and validation by the EPA (Yaoita et al., 1997; Tietge et al., 2000).

2.10 Conclusion

Hormones act at extremely low levels (parts per trillion); therefore, in theory, even exposures to low levels of hormonally active agents may be of concern, particularly during sensitive periods of fetal development. Furthermore, endocrine-mediated effects may be subtle and manifest primarily in populations rather than in individuals. For example, slight overall declines in sperm density or IQ may have little relevance for an individual but important adverse implications for the population.

Low-level exposures to endocrine-disrupting chemicals are ubiquitous in today's environment. Persistent chemicals such as DDT, PCBs and dioxins are detectable in a large

population of human blood samples, and even some of the shorter-lived potential endocrine disruptors are frequently detected in general population surveys of residues in blood or urine. The ubiquitous nature of the exposures combined with the nontrivial potential health effects justifies further research, education and preventive action to reduce human exposures to endocrine disruptors. A great deal of work on endocrine disruptors is under way in government agencies, nongovernmental organizations and international organizations. Improved monitoring of disease and exposure is essential for tracking trends in subtle, delayed effects of environmental exposures.

CHAPTER 3

Development of a yeast-based *in vitro* β-gal assay for screening (anti)androgenic natural endocrine disruptors

3.1. Introduction

Androgens are the hormones that play a pivotal role in the development and maintenance of the male sex characteristics. The biological effects are mediated by the ubiquitously expressed androgen receptor (AR). The levels of AR change in different pathological conditions such as malignancies or in response to physiological changes of the endocrine system. AR belongs to the nuclear receptor super family comprising receptors for steroid hormones, vitamin D3, thyroid hormones and retinoids. These receptors have conserved DNA- and ligand binding domains (DBD and LBD, respectively) and variable hinge and N-terminal regions (Mangelsdorf et al., 1995). In the case of AR, the N-terminal region encompasses the primary transcriptional activation domain. Once the ligand binds to AR, the cytosolic AR translocates to the nucleus where it binds to the regulatory regions of androgen-responsive genes and subsequently stimulates their transcription (Keller et al., 1996; Blankvoort et al., 2001). In addition to endogenous steroid hormones, an increasing number of natural products, industrial effluents, pesticides, fungicides, have been identified as AR agonists and antagonists. These chemicals having the capability to alter male and female sexual functions are generally considered as endocrine disrupting chemicals (EDC) and have become an important environmental concern (Kelce and Wilson, 2001; Roy et al., 2004). Numerous reports exist on testing of the estrogenic activity of these chemicals, but limited information is available on their androgenic or anti-androgenic nature. Chemical analyses of androgenically active substances or binding experiments to the receptors are not always suitable to assess the biological effects of these compounds. Thus, some in vitro reporter gene test systems have been developed that imitate the mechanism of androgen action via the androgen receptor pathway and that are suitable to assess the androgenic potential of a variety of substances. The existing test systems have been mainly reported in human prostate cancer cell lines LNCaP cells (Horoszewicz et al., 1980), PC-3 cells (Sultan et al., 2001) but none of them are able to discriminate between androgenic and anti-androgenic compounds. Therefore the latter cell line needs to be co-transfected with AR and the androgen responsive reporter genes. AR co-transfection assay had also been performed in näive cells like HEK293, COS1, CHO, COS7, CV1 (Raivio et al., 2001; Kemppainen et al., 1999; Lobaccaro et al., 1999; Anderson et al., 2002; Roy et al., 2004; Roy et al., 2006; Durhan et al., 2006; Sun et

al., 2007). Another mode of AR transactivation assay is by recombinant yeast strains such as *Saccharomyces cerevisiae* where the yeast cells are cotransfected with steroid receptors and a reporter gene, which is usually β-galactosidase (β-gal). The majority of the yeast-based transactivation systems are based on the study of estrogen receptor function by Metzger et al. (1988). Although there are several reports on screening estrogenic chemicals using yeast reporter systems, there are only few reports for androgenic chemicals.

The main aim of this study was firstly, to develop a yeast reporter-based, rapid, efficient and economic assay system, suitable for screening multiple numbers of samples simultaneously for their androgenic or anti-androgenic activities. Towards this end, the yeast strain *Saccharomyces cerevisiae*, was transformed with human AR (hAR) and androgen response element (ARE) and minimal yeast-based promoter (CYC1) driving β-gal reporter gene in the corresponding yeast-based expression vectors. The recombinant yeast strain was then used to test the androgenecity of different steroids and synthetic chemicals. Finally, the developed assay was also used to analyze the pulp and paper mill effluents for their in vitro androgenicity. To the best of our knowledge, there is only one more report from a Swedish group on the analysis of paper mill effluents using recombinant yeast-based reporter system (Svenson and Allard, 2004). But our X-gal based microtiter plate assay requires minimum hands-on time (~16h) in comparison to the CPRG based assay of the Swedish group (3-4 days). The developed assay can thus be used to screen (anti) androgenic chemicals from various environmental sources.

3.2 Materials and methods

3.2.1 Chemicals

Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were obtained from Himedia (Mumbai, India), while Trizol reagent, L -leucin and uracil were from Sigma (St. Louis, MO, USA). Ammonium sulphate, chloroform, isoamyl alcohol, isopropanol, ethanol absolute and dimethyl sulfoxide were obtained from Merck (Merck, Mumbai, India). Deoxyribonuclease I, Ribonuclease inhibitor and restriction endonucleases were all purchased from Promega (Madison, WI, USA). Testosterone (T), Dihydrotestosterone (DHT), estrogen, progesterone, all trans retinoic acid, dexamethasone,

hydroxy flutamide, cyproterone acetate, spironolactone, p,p'-DDE (99% purity) and vinclozolin were kindly provided by Professor Ilpo Huhtaniemi, Imperial College, London, UK.

3.2.2 Sample collection and preparation

Water effluents were sampled from the outlets of five different pulp and paper industries of northern India. These samples (2 L) were extracted immediately after collection by using solid phase extraction. Water samples were filtered through 0.1μm glass fibre filters (Type GMF5, Rankem, Mumbai, India), acidified with concentrated sulphuric acid to pH 2.0, and divided into two 1 L samples. One litre of sample was then extracted using reverse phase C18 solid phase extraction columns (RP-C18 SPE, Rankem, Mumbai, India) and dissolved in 1000μL of dimethylsulfoxide (concentration factor of 1000). Further, a 1:100 dilution in medium for samples extracted with C18 solid phase extraction columns resulted in the highest test concentration of 1mL eq./well.

3.2.3 Yeast strain

The yeast strain *Saccharomyces cerevisiae* YPH499 (*Mat a, Ura 3-52, leu-2-Δ1, his3-Δ200, trp1-Δ63, ade2-101, lys2-801*) was a kind gift from Dr. A. Bachhawat (Institute of Microbial Technology, Chandigarh, India).

3.2.4 Plasmids

The pRS425-Leu2-ARS and pLG669Z-CYC1-lacZ (2 micron) yeast expression vectors (both kindly provided by Dr. A. Bachhawat, Institute of Microbial Technology, Chandigarh, India) were used to make hAR and reporter gene constructs, respectively. For creating yeast compatible hAR construct, hAR in pSG5 expression vector was digested with *Bam*HI and *Bgl*II, followed by end filling with Klenow polymerase (Promega, Madison, WI, USA). Then pRS425-Leu2-ARS was digested with *Sma*I and the cleaved product of *Bam*HI and *Bgl*II was ligated into it to form the recombinant hAR construct pRS425-Leu2-ARS-hAR.

For creating the reporter plasmid, the vector pLG669Z was digested with *Xho*I and end-filled with Klenow polymerase. A 0.3 kb fragment of probasin promoter was used as

androgen response element (ARE) which was digested with *Bgl*II and *Hind*III from pGL3-Probasin-Luciferase expression vector endfilled with Klenow polymerase and both the blunt ended products were then ligated using T4 DNA Ligase with appropriate blunt end ligation buffer to form pLG669Z-ARE-CYC1-β-gal and was termed as ARE-lacZ.

3.2.5 Transformation and culture of yeast cells

Plasmids encoding hAR and ARE-lacZ were cotransformed into *Saccharomyces cerevisiae* YPH 499 using the lithium acetate protocol (Sambrook et al., 1989). Transformants were grown in synthetic dextrose (sd) medium without uracil and leucine. Selective sd medium contained 1.7 g/L yeast nitrogen base without amino acids and ammonium sulphate, 5 g/L ammonium sulphate and 20 g/L dextrose. Glycerol stocks of the two recombinant strains were preparedand stored at −80°C.

3.2.6. Isolation of RNA and RT-PCR

RNA was isolated according to the method described by Ausubel et al. (1995). The yeast cells were grown in 10mLof desired medium till it reached the mid exponential phase (OD600 = 1.0). The culture was centrifuged at 1500×g for 3 min, 4°C. After the supernatant was discarded, the pellet was resuspended in 1mL ice-cold water. It was then centrifuged for 10 s at 4°C, and the supernatant was removed. The cell pellet was resuspended in 400µL TES buffer (10 mM Tris–Cl, pH 7.5; 10 mM EDTA, 0.5% (w/v) SDS) followed by the addition of 400µL acid phenol. This was then incubated for 30–60 min at 65°C with occasional vortexing. It was cooled on ice for 5 min followed by centrifugation for 5 min and this was repeated twice. The aqueous phase was collected and 400µL chloroform was added to it, vortexed and centrifuged for 5 min. The aqueous layer obtained was transferred to a new tube and 40µL of 3M sodium acetate (pH 5.3); 1mL of ice-cold 100% ethanol was added to facilitate precipitation.

RNA pellet was washed briefly in ice-cold 70% ethanol and centrifuged to get RNA pellet. The pellet was resuspended in 50µL of DEPC treated water and stored at −70°C for future use. One microgram of total RNA was used as template for reverse transcription by using the MMLV reverse transcriptase and oligo-dT primers (Promega, Madison, WI, USA). Oligonucleotide primers (20 mers) were d esigned from areas conserved in the

published sequences of hAR cDNA sequences as follows: forward, 5′-ACCATGTTTTGCCCATTGAC-3′; reverse, 5′-GCTGTACATCCGGGACTTGT-3′; product size 715 bp. Amplification was achieved using 28 PCR cycles with Taq polymerase (94°C for 30 s, 50°C for 75 s and 72°C for 90 s). PCR product was separated on 1.8% agarose gel in 0.045 mol/L Tris–borate EDTA buffer (pH 8.0) and visualized by ethidium bromide staining.

3.2.7 Western blot

Western blot analysis was performed according to an adapted protocol described by Laemmli (1970). Briefly, the proteins from yeast cytosensors with/without pRS425-Leu2-ARS-hAR expression vectors were isolated by centrifuging 1mL of the yeast culture and re-suspending the cell pellet in 0.5mL sample buffer (20 mM Tris/HCl, pH 6.8; 0.8% (w/v) SDS, 3.5% (v/v) glycerol, 0.002% (w/v) bromophenol blue, 2% (v/v) β-marcaptoethanol). Samples were shaken for 45 min and 0.25 g glass beads were added (425–625μm, acidwashed, Sigma). Samples were vortexed three times for 1 min, heated at 95 °C for 5 min, centrifuged at 13,000×g for 5 min and 20μL of the supernatant was loaded on a 10% SDS polyacrylamide gel. Proteins were transferred to a nylon membrane and the blot was incubated overnight with a polyclonal antibody for hAR (1:200) in the presence of blocking buffer. This was followed by incubation with alkaline phosphatase labeled secondary antibody (1:1000). Colour development was performed in 30mL AP-buffer (100mM Tris/HCl pH 9.5, 100mM NaCl, 5mM $MgCl_2$), with 200μL NBT (50 mg/mL) and 100μL BCIP (50 mg/mL). The extracted protein samples from LNCaP cells were used as positive control, which was processed similarly as that of yeast proteins.

3.2.8 β-galactosidase assay

For yeast cells with a β-gal reporter construct, 96-Well X-gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) plates were prepared with the YPD/-Ura/-Leu medium containing X-gal at 40μg/mL concentration. Once the plates were dried, the recombinant yeast cells (yeast cells transformed with pRS425-Leu2-ARS-hAR and ARE-LacZ) were inoculated on each wells of the plate at a volume of 200μL. Thereafter the cells were treated with varying concentrations standards (testosterone, DHT), non-androgenic steroids

(estradiol, progesterone, retinoic acid, dexamethasone), test chemicals (hydroxyflutamide, cyproterone acetate, vinclozolin, DDE, paper mill effluents) in the presence or absence of testosterone depending on experiments and incubated at 30°C.

The use of a β-gal reporter gene complements growth selection and offers an easy means to measure the reporter gene activity in yeast two-hybrid and related genetic screening systems (Fields and Song, 1989; Fashena et al., 2000; Serebriiskii and Golemis, 2000).

The β-gal activities were detected by the production of blue colour after incubation for 16 h. The intensity of the blue colour was correlated with the amount of β-gal activity and to quantitate this, the following equation was used:

(1000×A615)/(time×volume×OD546) = β-galactosidase activity (expressed as relative β-gal activity).

3.2.9 Statistical analysis

The statistical analysis of the data was carried out using the Student's T-test. The acceptance level was set at $p < 0.05$.

3.3 Results

3.3.1 Establishment of yeast strain and level of expression of AR

The present chapter describes the development of a recombinant yeast-based bioassay for screening different environmental pollutants from pulp and paper mill effluents. Towards this end, *Saccharomyces cerevisiae* YPH 499 was co-transformed with pRS425-Leu2-ARS-hAR and ARE-LacZ. In this recombinant yeast strain harboring lacZ reporter plasmid under the control of CYC1 promoter, the androgen-dependent interaction between hAR and ARE can be detected by the expression of reporter gene β-gal.

Transcriptional activity of the transformed hAR cDNA was determined by isolation of total RNA and RT-PCR amplification. The amplicon of the desired size confirmed the exact transcription of the AR gene within the recombinant yeasts, while there was no band in non-transformed YPH 499 host cells (Fig 3.1).

Fig 3.2 shows the Western blot of two different positive yeast cells that contain a single copy of the ARE-LacZ and that were transformed with the pRS425-Leu2-ARShAR receptor expression constructs. Proteins isolated from clones were blotted using AR antibody. As expected, an immunoreactive protein band with apparent molecular weight of 110 kDa was observed in all transformants tested with slight variation in their level of expressions and was in accordance with band patterns from LNCaP cells, used as positive control. The band was absent in untransformed yeast cells. The 110 kDa band confirmed the exact translation of the AR protein in the transformed yeast cells.

3.3.2 Dose-dependent induction of β-galactosidase by androgen

Analysis of androgen-response capacity of the transformed yeast strain was carried out in the yeast liquid β-gal assay with increasing concentrations of androgens (Fig 3.3). The β-gal activity was induced by testosterone (T) and dihydrotestosterone (DHT) in a dose dependent manner with the saturation of β-gal induction at around 10nM in both the cases. The developed assay showed a half maximal effect (EC_{50}) in the transformed yeast strain at 16nM for T and 4 nM for DHT, which is consistent with the relative AR binding affinity and agonist activity of these androgens in mammalian cells (Kemppainen et al., 1999; Terouanne et al., 2000) and yeast based assays (Lee et al., 2003; Michelini et al., 2005).

3.3.3 Ligand specificity in the transformed yeast cells

The induction of β-gal activity in the transformed yeast strain was tested for its specificity with steroidal and nonsteroidal hormones. Transformed recombinant yeast strain was incubated with increasing concentrations (0.01–100 μM) of T, DHT, estradiol, progesterone, all-trans-retinoic acid and dexamethasone and measured for their β-gal activity (Fig 3.4). At a concentration of 0.1 μM, T and DHT significantly induced β-gal activity (about 25-folds over control), while the other hormones, except estradiol, failed to induce significant enzymatic activity at the same concentration. Regarding estradiol, it has been shown to bind AR and exert partial agonist activity at high concentrations in mammalian cells (Kemppainen et al., 1992, Jenster et al., 1997). A similar pattern seems to take place in transformed yeast cells used by us where estrogen resulted in a significant

elevation of β-gal activity at 0.1μM concentration and this continued further till 100 μM, followed by no further elevation in its activity.

However, this fact can be ignored because the concentration used here is supra physiological and the effect is about three- to four-folds lesser than the effect caused by T and DHT, respectively at the same concentration. The relative lack of specificity of the hAR assay has already been reported by some authors (Gaido et al., 1997; Michelini et al., 2005).

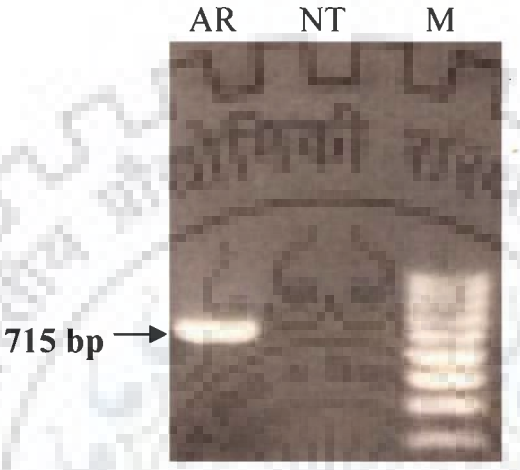


Fig 3.1 RT-PCR analysis of progesterone receptor mRNA expression in yeast cells. The total RNA isolated from the yeast cells and LNCaP cell line, reverse transcribed and cDNA obtained was subjected to PCR. (NT) Nontransformed yeast cells; (AR) yeast cells transformed with androgen receptor and (M) DNA marker.

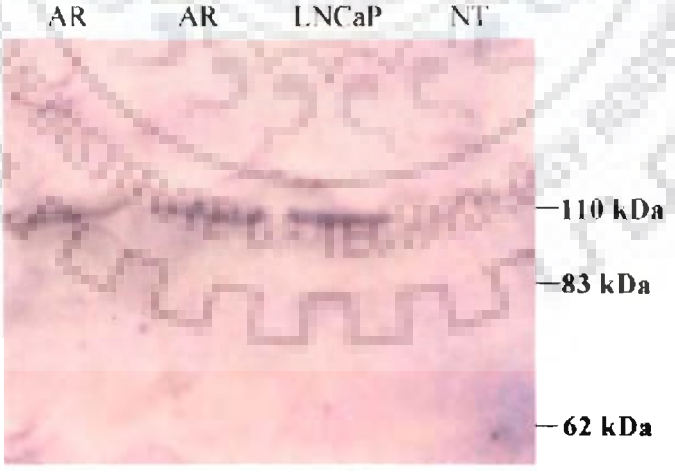


Fig. 3.2 Immunoblot analysis for the expression of AR in transformed yeast cells. The first two lanes represents AR proteins in transformed cells (AR), the third lane is from LNCaP cells used as positive control (LNCaP) and fourth lane is nontransformed yeast cell extract used as negative control.

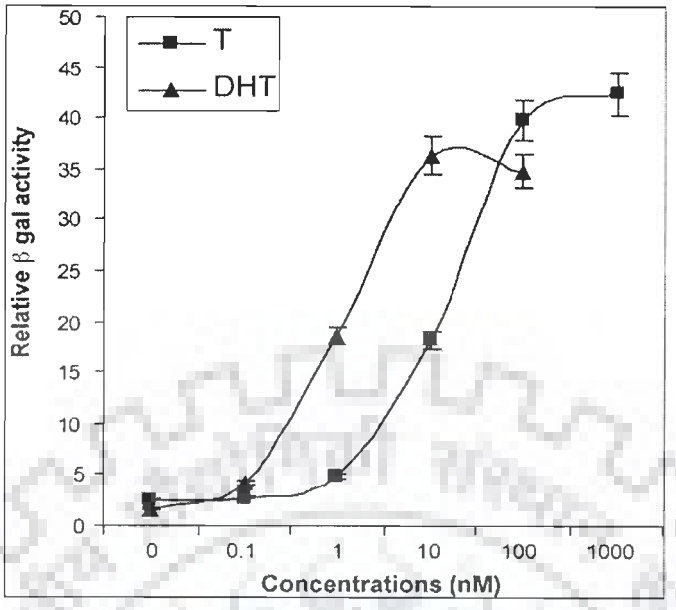


Fig 3.3 Dose-dependent β-galactosidase induction by increasing concentrations of testosterone (T) and dihydrotestosterone (DHT) in transiently cotransformed yeast cells. The β-galactosidase activity in the absence of ligand was set as zero. The values represent the mean±S.E.M. of four independent experiments each performed in quadruplicates.

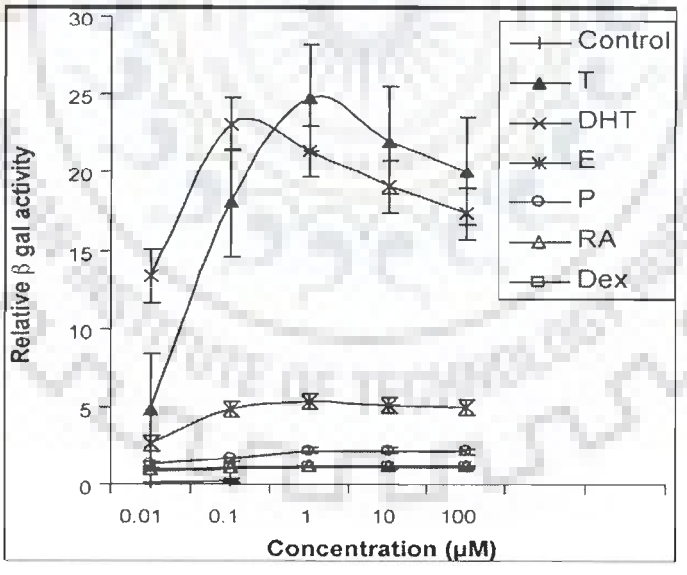


Fig 3.4 Determination of ligand specificity in recombinant yeast strains in response to nonandrogenic steroids. Yeast cells were incubated with various androgenic and nonandrogenic steroids at increasing concentrations (0.01–100μM) followed by the determination of β-galactosidase activity. T: Testosterone; DHT: Dihydrotestosterone; E: 17β-estradiol; P: Progesterone; RA: all-trans-retinoic acid; Dex: Dexamethasone. The values represent the mean±S.E.M. of four independent experiments each performed in quadruplicates.

3.3.4 Activity of AR antagonists in transformed yeast strain

The recombinant yeast strain was tested for its response to several antagonistic compounds for androgen. Fig 3.5 shows their activity at different concentrations (0.01–100μM) in the absence or presence of 10nM testosterone. Dose dependent inhibition of testosterone-induced β-gal expression with-nonsteroidal and steroidal compounds, flutamide and cyproterone acetate, respectively show potent androgen antagonism.

The steroidal compound spironolactone was less effective when competed with testosterone. Effective antagonistic activity in transformed yeast strain was also shown by the environmental anti-androgen p,p′-DDE and fungicide vinclozoline. Their IC_{50} values were estimated approximately as 6.1 μM for flutamide, 7.67 μM for cyproterone acetate (CPA), 10.23 μM for p,p′-DDE and 34.41 μM for vinclozolin ($p < 0.05$) using our yeast-based assay and this corroborated earlier reports (Lee et al., 2003; Michelini et al., 2005). When all these compounds were tested alone for their androgen agonistic activities, all the compounds except CPA failed to transactivate the β-gal expression. CPA alone showed a slight agonistic activity by displaying a mild elevation in the β-gal activity. At a concentration of 100 μM CPA showed almost 2.5-folds elevation of transcriptional activity (Fig. 3.5), this finding proves that CPA is a partial agonist and antagonist which has also been reported earlier in mammalian cell line by Roy et al. (2004).

3.3.5 Detection of androgenic activity of pulp and paper mill effluents on 96-well X-gal Plates

The sound response with androgenic and anti-androgenic compounds of our recombinant yeast strain (YPH 499 cotransformed with pRS425-Leu2-ARS-hAR and ARE-LacZ) in yeast liquid β-gal assay allowed us to test its responses on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) plates. Identification of β-gal activity by blue-colour appearance of cells on X-gal plates is now a well-established, easy and rapid method (Lee et al., 2003). Recombinant yeast cells were incubated on 96-well X-gal plates applied with 1:100 dilution of solid phase extracted water sample of pulp and paper mill effluents at 30°C and colour change was monitored in a time course manner (16–24 h) (Fig 3.6). The data showed detectable levels of androgen receptor mediated transcriptional activity at levels significantly greater than the background ($p < 0.05$). The results suggest

that the solid phase extracted water samples of all the five pulp and paper mill effluents contain potential androgen mimicking compounds and they are in the range of 0.1 to 1.2µg/L eq. of T.

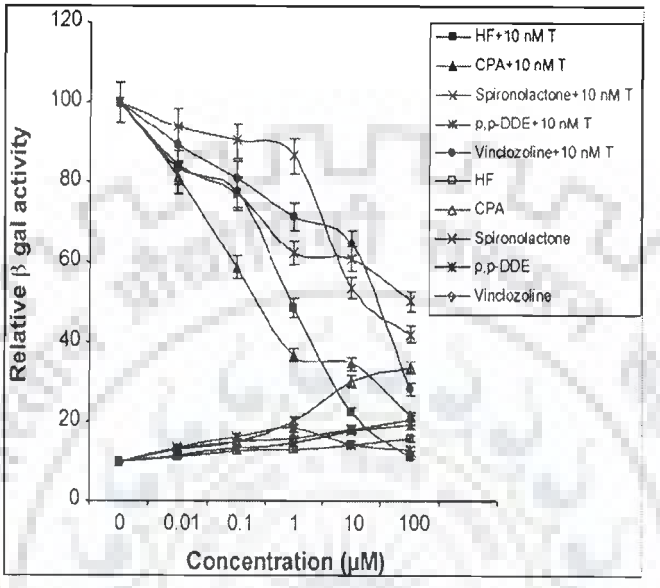


Fig 3.5 Anti-androgenic activities of some androgen receptor antagonists in recombinant yeast cells. The cells were treated either with increasing concentrations (0.01–100µM) of hydroxyflutamide (HF), cyproterone acetate (CPA), spironolactone, *p,p'*-DDE and vinclozolin alone or in the presence of 10 nM T. The β-galactosidase activities were compared with 10 nM testosterone only, which was set as 100. The values represent the mean±S.E.M. of four independent experiments each performed in quadruplicates.

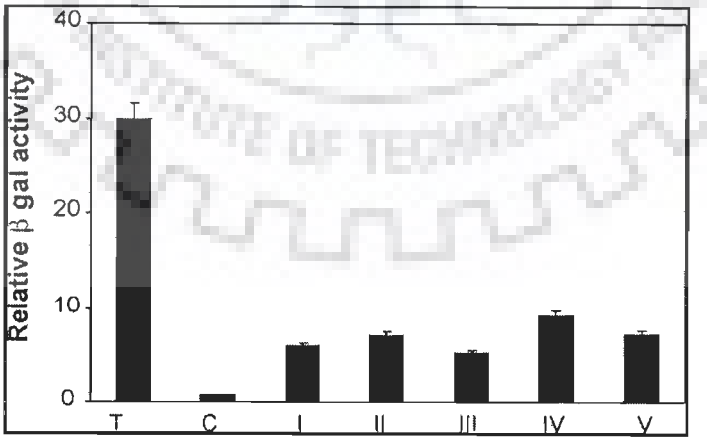


Fig 3.6 Detection of androgenic effects of solid phase extracted effluents from five different paper and pulp mills (I–V) with respect to β-galactosidase induction by transiently cotransformed yeast cells using 96-well X-gal plates. The β-galactosidase activities were compared with that obtained with control (vehicle treated). The values represent the mean±S.E.M. of four independent experiments each performed in quadruplicates.

3.4 Discussion

Many androgenic EDC identified so far are persistent organochlorine pesticides (e.g. DDT, methoxychlor and dieldrin). In addition, the (anti) androgenic activities of various commonly used compounds like industrial wastes, fungicides and pesticides has not yet been studied or analyzed properly. Therefore, robust and user-friendly assay systems are required for the screening of any potentially harmful compounds. Some methods are already available for screening, each having some advantages and disadvantages. For example in *in vivo* screening, castrated and intact animals have been used (Shimamura et al., 2002; Ashby et al., 2004). The (anti) androgenic activities *in vitro* have been assessed using cellular models in the same fashion as has been developed earlier for studying agonistic and antagonistic properties of ligands in other steroid receptors. Transactivation assays with AR and androgen-responsive reporter genes have been performed using DU-145, COS, CHO, CV-1, MCF-7 and HepG2 (Kemppainen et al., 1999; Lobaccaro et al., 1999; Vinggaard et al., 1999; Roy et al., 2004; Roy et al., 2006; Xu et al., 2005; Sun et al., 2007). The sensitivities of these mammalian assays have been better than any other assays reported so far. On the other hand, the biggest challenge with this assay system is the expression of other non-specific endogenous receptors, for example the estrogen receptor in MCF-7 cells, the progesterone receptor in T47D cells and the glucocorticoid receptor in PC-3, and CHO cells (Reyes-Moreno et al., 1995; Roy et al., 2004). So chances of interferences can never be ignored in these assay systems. In addition, maintenance of mammalian cells and analysis of reporter expression (luciferase assay) require time, labor and cost, this may limit the mass screening of such compounds. Another potential assay is the yeast-based assay. Though there are several yeast-based assays reported for screening estrogenic compounds (Bovee et al., 2004a, b; Sievernich et al., 2004; Wozei et al., 2005), not much has been known for screening androgenic or anti-androgenic compounds except some scanty reports on screening different synthetic chemicals like pesticides, fungicides using β-gal or GFP as reporter genes (Sohoni and Sumpter, 1998; Lee et al., 2003; Bovee et al., 2004a, b; Bovee et al., 2007; Beck et al., 2005; Michelini et al., 2005). We report here the development of a rapid and robust yeast based assay for identifying the (anti)androgenic chemicals from pulp and paper industries using a transactivation assay. This system is based on transient co-transformation of two recombinant yeast DNA

constructs having androgen receptor and the respective androgen response elements that can detect androgenic and antiandrogenic compounds in aqueous solution without the need of breaking cells or washing steps before the measure. Prior to analyzing the unknown pulp and paper mill effluents, the specificity of the assay was demonstrated by determining the responses of known androgens and anti-androgens. As shown in the results sections, they were in accordance with earlier reported assays (Sohoni and Sumpter, 1998; Lee et al., 2003; Michelini et al., 2005; Christiaens et al., 2005) since there can be some variations in activity of compounds like EC_{50} values and sensitivity, these can be attributed to several parameters like constructs used, background of the yeast strains, time of incubations used for transactivation assays, and so on. The present assay system reported here is obviously not as sensitive as reported earlier by Sohoni and Sumpter (1998) but it is in accordance with others reported earlier using almost similar assay conditions (Lee et al., 2003; Michelini et al., 2005). This yeast-based system has several advantages like: first, little chance of interference by other hormone receptors as in mammalian cell lines; second, yeast culture is relatively simple and cost effective allowing the use of media that does not contain steroids (Arnold et al., 1996; Gaido et al., 1997; Leskinen et al., 2003; Bovee et al., 2004a, b; Roy et al., 2004; Michelini et al., 2005a; Michelini et al., 2005b; Roy et al., 2006; Eldridge et al., 2007; Sanseverino et al., 2005; Sanseverino et al., 2009) in comparison to mammalian cells; third, analysis of the effect of androgenic or anti-androgenic compounds can be done quantitatively or semi-quantitatively by the liquid β-gal assay and the colour change can be measured by the intensity in X-gal plates. To the best of our knowledge, this is the only report, other than Svenson and Allard (2004), where androgenecity of chemicals in pulp and paper mills effluents has been evaluated using a recombinant yeast-based model in 96 well plate format. As the first application of yeast androgen bioassay, we analyzed some pulp and paper mill effluents in the northern region of India, which is one of the major producers of pulp and paper products in the country. Basically, the role of paper and pulp mill effluents as endocrine disruptor is a controversial issue. While some investigators report that the hormonal profile of sex steroids (mainly testosterone) are reduced (McMaster et al., 1992; Van der Kraak et al., 1992; Rana et al., 2004), others have reported no change (van den Heuvel and Ellis, 2002). The reduction of steroid hormone production is a complex end point other than any method of testing

androgenic or anti-androgenic activities since the effect can be initiated at multiple sites (Van der Kraak et al., 1992). For example, negative feedback at the pituitary will reduce levels of gonadrotrophins, resulting in the rapid shutdown of steroid hormone production in the gonads, likely associated with apoptosis. Indeed, ovarian apoptosis has been observed in association with pulp and paper effluent exposure (Janz et al., 1997). Direct effects on the gonads are also likely, and the best documented mechanisms has been the apparent ability of phytosterols to inhibit the first step in the conversion of cholesterol to steroids by preventing cholesterol from entering the mitochondria (Leusch and MacLatchy, 2003). Another potential mechanism for reduction of gonadal steroid synthesis is handling stress (van den Heuvel et al., 1995; Bandelji et al., 2006). However, our data showed detectable levels of androgen receptor mediated transcriptional activity at levels significantly greater than the background recorded in the effluents ($p < 0.05$) indicating its androgenic activity, which is in accordance with earlier report. Contaminants with specific androgenic activity, without any estrogenic characteristic can be present in the samples, since no information is available about the estrogenic nature of paper and pulp mill effluents. Parks et al. (2001) have reported masculinization of female mosquitofish in river water, contaminated with pulp mill effluent, which has been further supported by a recent study in New Zealand (Bandelji et al., 2006). This pulp mill effluent contained a chemical, or a mixture of chemicals, that binds to AR and induces androgen dependent gene expression in vitro. It has been hypothesized that this pulp-mill effluent possibly contained testosterone-like molecules, produced from microbial metabolism of phytosterols (Parks et al., 2001; Christiaens et al., 2005). Previous studies have lent credence to the hypothesis that the masculinizing compound(s) in pulp and paper mill effluent are androstenedione and androstadienedione (Jenkins et al., 2001, 2004). Our study also demonstrated that the average levels of androgen like compounds in various pulp and paper mill effluents was about 1 µg/L equivalent of testosterone as estimated by our assay.

Earlier Jenkins et al. (2001) in their studies reported that the river water receiving paper and pulp industry effluents contained approximately 40 ng/L of androgen equivalents of compounds. The inconsistency in the results obtained may be due to several factors like, type of process used in the paper manufacture and the kind of wastewater treatment plant (if installed) at the paper mills, the volume of effluent discharged, the level

of dilution and the composition of the effluents. Another reason for the differences in results could be day-to-day variability in the effluent quality. Concentrations of some compounds can change in very short periods depending on mill operations and treatment system performance (Rana et al., 2004). Thus, all these data indicates the need of identifying the compounds from the industrial sources for further understanding their mode of action as (anti) androgenic compounds. The volume of the sample extracts obtained was too limited to enable further characterization of the chemical composition.

3.4 Conclusion

In conclusion, the yeast-based assay developed by us can be applied to rapid and efficient screening of androgenic and anti-androgenic compounds from different environmental contaminants in aqueous sources. The use of X-gal screening further makes the assay user-friendly and robust since it does not need cell lysis and minimum hands-on time (~16h), which makes it also practical for mass screening of environmental samples. The initial compounds identified by this assay will help to identify chemicals, which can be taken up for in vivo analysis for their potential adverse effects on male reproduction.

CHAPTER 4

Development of a yeast-based *in vitro* GFP assay for screening (anti) progestin natural endocrine disruptors

4.1 Introduction

Progesterone is a hormone that plays a pivotal role in the estrogen primed animals, preparing the uterine endometrium for implantation and maintenance of the embryo. Further, this hormone regulates the characteristic transformation of uterine epithelium from proliferative to the secretory state. Its biological effects are mediated by the ubiquitously expressed progesterone receptor. Estrogens and progestagens are the two key regulators of proliferation and differentiation in reproductive tissues (Graham and Clarke, 1997), and progestagens are known to oppose the biological effects of estrogens in many systems (Zhu et al., 2000; Guo et al., 2001). Such functional interplay between estrogen and progesterone is fundamental to maintain a significant physiological process (Rose, 1996). Progesterone receptor (PR) belongs to the nuclear receptor super family comprising receptors for steroid hormones, vitamin D3, thyroid hormones and retinoids. These receptors have conserved DNA- and ligand-binding domains (DBD and LBD, respectively) and variable hinge and N-terminal regions (Mangelsdorf et al., 1995). Upon ligand-binding the ligand-receptor complex binds to the regulatory regions of progesterone-responsive genes and subsequently stimulates their transcriptions.

Anti-progestative compounds, respectively bind to the PR, but block its transcriptional activity. PR is unique within the family of steroid hormone receptors since it exists as two isoforms, PR-A (~94 kDa) and PR-B (~116 kDa). Basically, PR-A is truncated form of PR-B lacking the first 164 N-terminal amino acids. Two distinct promoters within the single copy gene for PR have been shown to independently regulate the expression of PR isoforms (Kastner et al., 1990; Pieber et al., 2001). The expression of pure homodimers of PR-A and PR-B has shown that they act as repressors and activators of transcriptions, respectively (Mohamed et al., 1994). Further an inhibitor function region (IF) has also been characterized lying 292 amino acids upstream of activation function element 1 (AF1) which in known to inhibit the function of both activation function elements (AFI and AFII). Since PR-B contains an additional activation function element (AFIII) which is not inhibited by IF, that could explain the functional difference between these receptor isoforms (Hovland et al., 1998). Various chemicals such as pesticides, plasticizers and persistent pollutants are highly suspected to display endocrine disrupting effects in animals and humans. In recent years, public and researchers have expressed concern on this aspect

as is evidenced by a large number of reports. To give some examples, in animals, environmental EDC could be the cause of reproductive and teratogenic effects in bald eagles of the Great Lakes (Bowerman et al., 2000). Reproductive disorders have been described in animals and humans (Vos et al., 2000; Guillette and Gunderson, 2001; Sharpe, 2001). In humans, endometriosis (Lebel et al., 1998) and testicular cancer (Ohlson and Hardell, 2000) have been linked to exposure to organochlorine and plasticizers, respectively. A general decline of the male and female reproductive health has been observed in recent times which could also be attributed to EDC (Scippo et al., 2004). The probable modes of action of these EDC are by either inhibiting or mimicking the endogenous hormone which ultimately effects the production of endogenous hormone. Majority of EDC that have been studied so far are supposed to act as agonist or antagonist by their direct interaction with the receptor (Molina-Molina et al., 2006). Numerous reports exist on testing of the estrogenic and (anti)androgenic activities of these chemicals which have been shown to exert their effects through interaction with estrogen and androgen receptors, respectively (Anderson et al., 2002; Kojima et al., 2004; Lemaire et al., 2004; Roy et al., 2004; Scippo et al., 2004). However, much less is known about the interaction of these compounds with PR.

Previous studies indicated that indeed environmental chemicals, which are able to inhibit PR binding, may also have an important impact on abnormalities associated with the developing reproductive system (Vonier et al., 1996; Pickford and Morris, 1999). All these information posed a need to identify these environmental progestative compounds. Scippo et al. (2004) reported the screening of several EDC by utilizing hormone binding assay for PR that have been produced in genetically modified bacteria. Earlier some authors reported progesterone receptor binding by various chemicals using duck, hen, rabbit, and alligator PR (Lundholm, 1988; Vonier et al., 1996).

Though all these data and chemical analysis of progestative substances provide some clue about the mode of action of these chemicals yet they are not always suitable to assess the biological effects of these compounds. Thus some in vivo and in vitro reporter gene test systems have been developed that imitate the mechanism of progesterone action via the PR pathway and that are suitable to assess the progestative potential of a variety of substances. Pesticides like DDT have been tested for their anti-progestative activity in rats

by estimating the inhibition of progesterone induced enzymes (Hrdina et al., 1975) and the decreased frequency of ova implantation upon exposure of this chemical in mice (Lundberg and Kihlstrom, 1973).

The first yeast based transcription assay for progesterone was reported by Mak et al. (1989) where chicken oviduct progesterone receptor was expressed in *Saccharomyces cerevisiae*. This was followed by several other yeast based assays using β-galactosidase as reporter gene (Tran et al., 1996; Jin et al., 1997; Koltz et al., 1997; Death et al., 2004; Wang et al., 2005; Li et al., 2006) where they analyzed the role of various environmental chemicals like pesticides, soot from fossil fuel combustion emissions, air particulate matters, and drinking water by their ability to interact with PR expressed in yeast. Using almost similar strategy several mammalian cell based transactivation assays were developed simultaneously which became popular due to high sensitivity as compared to yeast based assays using luciferase as reporter gene unlike b-galactosidase as was used in yeast transactivation assays (Molina-Molina et al., 2006; Koltz et al., 1997; Schoonen et al., 1998; Dijkema et al., 1998; Schreurs et al., 2005; Hamers et al., 2006).

The main aim of the present study was to develop a yeast based, rapid, efficient and economic assay system suitable for screening multiple number of samples simultaneously for their (anti)progestative activities. To address this issue, we attempted to design a fluorescent based assay system to screen progesterone receptor agonists and antagonists in yeast, Saccharomyces cerevisiae. Towards this end, the yeast strain was transformed with hPR and progesterone response element (PRE) and minimal yeast based promoter driving yeast optimized green fluorescent protein (yEGFP) reporter gene in the corresponding yeast based expression vectors. The recombinant yeast strain was then used to test the progesterone like activity of different steroids and synthetic chemicals in a 96 well format. Finally, the developed assay was also used to analyze the leather industry effluents for their in vitro progesterone receptor interactions. To the best of our knowledge, this is the first report on screening the PR interacting ligands using green fluorescent protein as the reporter gene in yeast based model. Since the assay is optimized in a 96 well format, it can be used to screen multiple samples having progesterone receptor agonist or antagonist like activities from various environmental sources.

4.2 Materials and methods

4.2.1 Chemicals

Dextrose and yeast nitrogen base without amino acids and without ammonium sulphate were obtained from Himedia (Mumbai, India) while Trizol reagent, L-leucin, uracil were from Sigma (St. Louis, MO, USA). Ammonium sulphate, chloroform, isoamyl alcohol, isopropanol, ethanol absolute and dimethyl sulfoxide were obtained from Merck (Merck, Mumbai, India). Deoxyribonuclease I, Ribonuclease inhibitor and restriction endonucleases were all purchased from Promega (Madison, WI, USA). Progesterone, testosterone, estrogen, dexamethasone, RU486, o,p'-DDT, p,p'-DDT, o,p'-DDE, p,p'-DDE, vinclozolin, nonylphenol, α-endosulfan (all were about 99% purity) were kindly provided by Professor Ilpo Huhtaniemi, Imperial College London, UK.

4.2.2 Sample collection and preparation

Samples were collected monthly from May to November, 2006, in amber colored glass bottles, rinsed initially with acetone and MilliQ water (Millipore, India) from the site receiving effluent from a number of leather industrial units, stored at 4°C till extraction and the extraction was performed within 48 h of sampling. All the glassware to be used in the experiments was rinsed with dichloromethane (DCM) with 0.6% concentrated hydrochloric acid to prevent the clinging of steroids on the glass walls. The water samples were filtered and subjected to organic phase extraction by adding DCM according to the method described earlier (Soto et al., 2004) with slight variations as per our laboratory conditions. DCM was added to each aliquot at the ratio of 60 mL/L of crude sample and mixed thoroughly for 2 min, left for 10 min for settling down of organic phase and finally the aqueous phase was separated by separating funnel. The procedure was repeated thrice to extract organic phase and all aqueous phase was poured off. Extracted organic phases were mixed and concentrated under reduced pressure on a Bucchi rotatory evaporator to 2 mL/L of crude sample and concentrated organic phase was solvent exchanged with 10% ethanol. In the final preparation, each 2 μL of prepared sample in the highest test concentration was equivalent to 400 mL eq/well of 96 well plate which was further serially diluted with same 10% ethanol to 200, 100 and 50 mL equivalent samples per well. Prepared dose was stored at -20°C until used for the assay.

4.2.3 Yeast strain

The yeast strain *Saccharomyces cerevisiae* YPH499 (*Mat a, Ura 3–52, leu-2-Δ1, his3-Δ200, trp1-Δ63, ade2-101, lys2-801*) was a kind gift from Dr. A. Bachhawat (Institute of Microbial Technology, Chandigarh, India).

4.2.4 Plasmids

The pRS425–Leu2–ARS and pLG669Z–CYC1 (2 μm) yeast expression vectors were used to make hPR and reporter gene constructs, respectively. For creating yeast compatible hPR construct, hPR-B in pSG5 expression vector was modified and digested with *Eco*RI and *Bgl*II followed by end filling with Klenow polymerase (Promega, Madison, WI, USA). Then pRS425–Leu2–ARS was digested with *Sma*I and the cleaved product was ligated into it to form the recombinant hPR construct pRS425–Leu2–ARS–hPR. The reporter plasmid was created in two steps, firstly, PRE was introduced into the vector and secondly, within the same vector yEGFP gene was introduced. For the former step, the vector pLG669Z was digested with *Xho*I and end filled with Klenow polymerase. A 0.39 kb fragment of prolactin promoter containing 2X PRE was digested with *Hind*III from pGL3–PRL–Luciferase expression vector end filled with Klenow polymerase and both the blunt ended products were then ligated using T4 DNA Ligase with appropriate blunt end ligation buffer to form pLG669Z–PRE–CYC1 vector and was termed as PRE–CYC1. In the second step, yEGFP, obtained from the vector was cloned in the corresponding *Sal*I/*Hind*III sites of the pLG669Z–PRE–CYC1 reporter constructs created earlier and renamed as PRE–CYC1–yEGFP.

4.2.5 Transformation and culture of yeast cells

Plasmids encoding hPR and PRE–CYC1–yEGFP were co-transformed into *S. cerevisiae* YPH 499 using the lithium acetate protocol (Sambrook et al., 1989). Transformants bearing both the plasmids, pRS425–Leu2–ARS–hPR and PRE–CYC1–yEGFP, were grown in synthetic dextrose (sd) medium without uracil and leucine. Selective sd medium contained 1.7 g/L yeast nitrogen base without amino acids and ammonium sulphate, 5 g/L ammonium sulphate and 20 g/L dextrose. A batch of cells was also transformed with PRE-less construct (CYC1–yEGFP) to determine the specific

interaction of PR with PRE in co-transformed cells. Glycerol stocks of the two recombinant strains were prepared and stored at -80°C. Before running the assay, an agar plate containing the selective medium was inoculated with the transformed yeast from a frozen -80 °C stock (20% glycerol v/v). The plate was incubated at 30°C for 24–48 h. The day before the assays, a single colony of yeast from the agar plate was inoculated in 5 mL of the selective sd medium. This culture was grown overnight at 30°C with vigorous orbital shaking at 225 rpm. At the late log phase, the culture was diluted (1:10) using the same medium and set for assay.

4.2.6 Yeast optimized green fluorescent protein assay

The yEGFP assay was performed as per the method described earlier (Bovee et al., 2004). Briefly, 200 µL of the diluted culture (1:10) as prepared above, was added per well of 96 well plate. There after the cells were treated with 2 µL of varying concentrations of standard (progesterone), non-progesterogenic steroids (estradiol, testosterone, dexamethasone), known anti-progestin (RU486), and test chemicals (o,p'-DDT, p,p'-DDT, o,p'-DDE, p,p'-DDE, vinclozolin, α-endosulfan, nonylphenol and leather industry effluents) in the presence or absence of progesterone depending on experiments and incubated at 30°C. Only ethanol controls were included in each experiment and each sample was assayed in quadruplicate. The exposure was performed for 24 h. The fluorescence at the end of incubation was measured directly in spectroflourimeter (Cary Eclipse, Varian, Palo Alto, USA) using excitation wavelength at 485 nm and measuring emission at 530 nm. The fluorescence signal was corrected with the signals obtained with selective sd medium containing only ethanol. For the estimation of cytotoxicity, the same plate was again measured at 620 nm in a spectrophotometer to measure turbidity as an indicator for cell growth (Fent et al., 2006) and further confirmed by observing them microscopically.

4.2.7 Isolation of RNA and RT-PCR

RNA was isolated according to the method described by Ausubel et al. (1995). The yeast cells were grown in 10 mL of desired medium until it reached the mid exponential phase (OD600=1.0). The culture was centrifuged at 1500 x g for 3 min, 4°C. After the

supernatant was discarded the pellet was resuspended in 1 mL ice-cold water. It was then centrifuged for 10 s at 4°C, and the supernatant was removed. The cell pellet was resuspended in 400 µL TES buffer (10 mM Tris-Cl, pH 7.5; 10 mM EDTA, 0.5% (w/v) SDS) followed by the addition of 400 µL acid phenol. This was then incubated for 30–60 min at 65°C with occasional vortexing. It was cooled on ice for 5 min followed by centrifugation for 5 min and this was repeated twice. The aqueous phase was collected and to it 400µL chloroform was added, this was vortexed and centrifuged for 5 min. The aqueous layer obtained was transferred to a new tube and 40 µL of 3 M sodium acetate (pH 5.3); 1 mL of ice-cold 100% ethanol was added to facilitate precipitation. RNA pellet was washed briefly in ice-cold 70% ethanol and centrifuged to get RNA pellet. The pellet was resuspended in 50 µL of DEPC treated water and stored at -70°C for future use. One microgram of total RNA was used as template for reverse transcription by using the MMLV reverse transcriptase and oligo-dT primers (Promega, Madison, WI, USA). Oligonucleotide primers (20 mers) were designed from conserved areas as was in the published sequences of hPR cDNA (accession number: AY212933) as follows: forward, 5′-AAATCATTGCCAGGTTTTCG-3′; reverse, 5′-TGTGAGCTCGACACAACTCC-3′; product size 404 bp. Amplification was achieved using 32 PCR cycles with Taq polymerase (94°C for 30 s, 55°C for 75 s and 72°C for 90 s). PCR product was separated on 1.8% agarose gel in 0.045 mol/L Tris-borate EDTA buffer (pH 8.0) and visualized by ethidium bromide staining.

4.2.8 Western blot

Western blot analysis was performed according to an adapted protocol described by Laemmli (1970). Briefly, the proteins from yeast cytosensors with/without pRS425–Leu2–ARS–hPR expression vectors were isolated by centrifuging 1 mL of the yeast culture and re-suspending the cell pellet in 0.5 mL sample buffer (20 mM Tris/HCl, pH 6.8; 0.8% (w/v) SDS, 3.5% (v/v) glycerol, 0.002% (w/v) bromophenol blue, 2% (v/v) β-marcaptoethanol). Samples were shaken for 45 min and 0.25 g glass beads were added (425–625 µm, acid washed). Samples were vortexed three times for 1 min, heated at 95°C for 5 min, centrifuged at 13,000 x g for 5 min and 20 µL of the supernatant was loaded on a 10% SDS polyacrylamide gel. Proteins were transferred to a nylon membrane and the

blot was incubated overnight with a polyclonal antibody for hPR (1:200) (kindly provided by Dr. A. Bandyopadhyay, Indian Institute of Chemical Biology, Kolkata, India) in the presence of blocking buffer. This was followed by incubation with alkaline phosphatase labeled secondary antibody (1:1000). Colour development was performed in 30 mL AP-buffer (100 mM Tris/HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), with 200µL NBT (50 mg/mL) and 100 µL BCIP (50 mg/mL). The extracted protein samples from T47D human breast cancer cell line was used as positive control which was processed similarly as that of yeast proteins.

4.2.9 Chemical analysis

One liter of collected effluent water sample was extracted with DCM, concentrated to 1ml and then evaporated to dryness in a 1.5 ml vial. Then the extract was derivatized in same vial by addition of *N,O* bis(trimethylsilyl) trifluoroacetamide (BSTFA) (Sigma, USA) as a silylation agent. The derivatization was performed by reconstituting the dried elute sample with 110 µl of acetone:hexane (1:1, v/v) and 20 µl of BSTFA. The vials were then capped and placed in a water bath at 65°C for 30 min to ensure complete derivatization of the compounds of interest. The derivatization leads to silylation of all the target compounds except hexachlorobenzene. On completion of derivatization, 2µl of the reaction mixture was injected (in splitless mode) into GC–MS system. GC–MS analysis was performed using the protocols described earlier (Leusch et al., 2006; Liu et al., 2004) with some modifications. The analysis was performed on a Perkin-Elmer Clarus 500 gas chromatograph coupled with a mass spectrometer and an autosampler. An Elute-1 Crossbond® (5% Diphenyl–95% Polysiloxane) column of 30 m×0.25 mm i.d.×0.25 µm film thickness was used. The GC column temperature ranged from 75°C (initial equilibrium time for 2min) to 155°C at a temperature increase of 10°C/min, 155–260°C at a temperature increase of 15°C/min and 260–300°C at a temperature increase of 8°C/min. The mass spectrometer was operated in the full acquisition electronic impact mode (70 eV). The presence of the compounds were confirmed by matching the retention time of the standards with that of corresponding peaks in the chromatogram of sample and further by analyzing mass spectra of the matching peaks. Quantification of contaminants was performed using selected ion monitoring mode (SIM); the *m/z* values of the ions monitored

are listed in Table 4.1. Quantification was done using the external standards of nonylphenol, hexachlorobenzene, 4-aminobiphenyl and benzidine which were spiked in acetone:hexane (1:1, v/v) in concentrations ranging from 0.2 to 2.0 µg/ml. Calibration curves were prepared by linear regression of peak areas of standard solutions against their respective concentrations.

4.2.10 Statistical analysis

The statistical analysis of the data was carried out using the Student's T-test. The acceptance level was set at $p < 0.05$. The detection limit is defined as the progesterone concentration that corresponds to the blank plus three times the standard deviation. The median effective concentration (EC_{50}) value of progesterone and the median inhibitory concentration (IC_{50}) value of test chemicals, and RU486 were calculated from dose response curve considering best-fit values using GraphPad Prism software (Graphpad Software Inc., USA).

4.3 Results

4.3.1 Establishment of yeast strain and level of expression of PR

The present study describes the development of a recombinant yeast based bioassay for screening different environmental pollutants and industrial effluents like that from leather industry. Towards this end, *S. cerevisiae* YPH 499 was co-transformed with pRS425–Leu2–ARS–hPR and PRE–CYC1–yEGFP constructs. In this recombinant yeast strain harboring yEGFP reporter plasmid under the control of CYC1 promoter and PRE, the progesterone dependent interaction between hPR and PRE can be detected by the expression of yeast optimized green fluorescent protein as reporter gene. The cells transformed with PR and yEGFP construct lacking PRE did not show any response in the presence of progesterone indicating further the need of PRE for this crosstalk (data not shown). Transcriptional activity of the transformed hPR cDNA was determined by isolation of total RNA followed by RTPCR amplification. The amplicon of the desired size confirmed the exact transcription of the progesterone receptor gene within the recombinant yeasts while there was no band in non-transformed YPH 499 host cells (Fig 4.1).

Fig 4.2 shows the Western blot of progesterone receptor protein from a positive yeast cell (lane B) that were cotransformed with PRE–CYC1–yEGFP and pRS425–Leu2–ARS–hPR receptor expression constructs in comparison to a strain not transformed with hPR construct (lane A). Proteins isolated from clones were blotted using PR antibody. As expected, an immunoreactive protein band with apparent molecular weight of 105 kDa was observed in all transformants tested with slight variation in their level of expressions and was in accordance with band patterns from T47D cells (lane C), used as positive control. The band was absent in untransformed yeast cells (lane A). The corresponding band at lane B and lane C confirmed the exact translation of the PR protein in the transformed yeast cells.

4.3.2 Dose dependent induction of yeast optimized green fluorescent protein by progesterone

Analysis of progesterone response capacity of the transformed yeast strain was carried out in the yeast based assay with increasing concentrations of progesterone in 96 well plates (Fig 4.3). The yEGFP expression was induced by progesterone in a dose dependent manner with the saturation of reporter gene induction at around 100 nM resulting in about 9 folds of induction at this concentration. The developed assay showed a half maximal effect (EC_{50}) in the transformed yeast strain at 1 nM for progesterone.

4.3.3 Ligand specificity in the transformed yeast cells

The induction of yEGFP expression in the transformed yeast strain was tested for its specificity with non-progesterone steroidal compounds. Transformed recombinant yeast strain was incubated with increasing concentrations (0.001 to 10 μM) of progesterone, testosterone, estradiol, and dexamethasone and measured for their induction of fluorescent protein expression (Fig 4.4). At a concentration of 0.001 μM, progesterone significantly induced the reporter gene expression (about 6.5 over vehicle control), while the other hormones failed to induce significant levels of yEGFP at the same concentration and above.

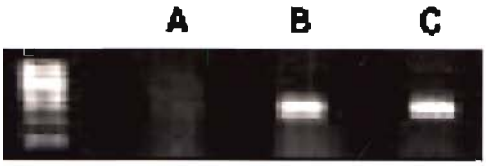


Fig 4.1 RT-PCR analysis of progesterone receptor mRNA expression in yeast cells. The total RNA isolated from the yeast cells and T47 D cell line, reverse transcribed and cDNA obtained was subjected to PCR. (A) Nontransformed yeast cells; (B) yeast cells transformed with progesterone receptor and (C) T47D cell line.

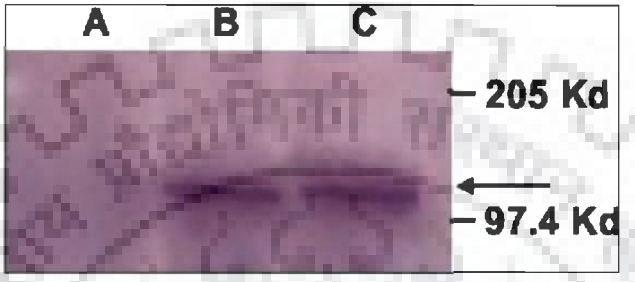


Fig 4.2 Immunoblot analysis for the expression of hPR in transformed yeast cells (A) the non-transformed yeast cells, used as negative control; (B) the yeast cells transformed with hPR and (C) proteins isolated from T47 D cells used as positive control. hPR, human progesterone receptor.

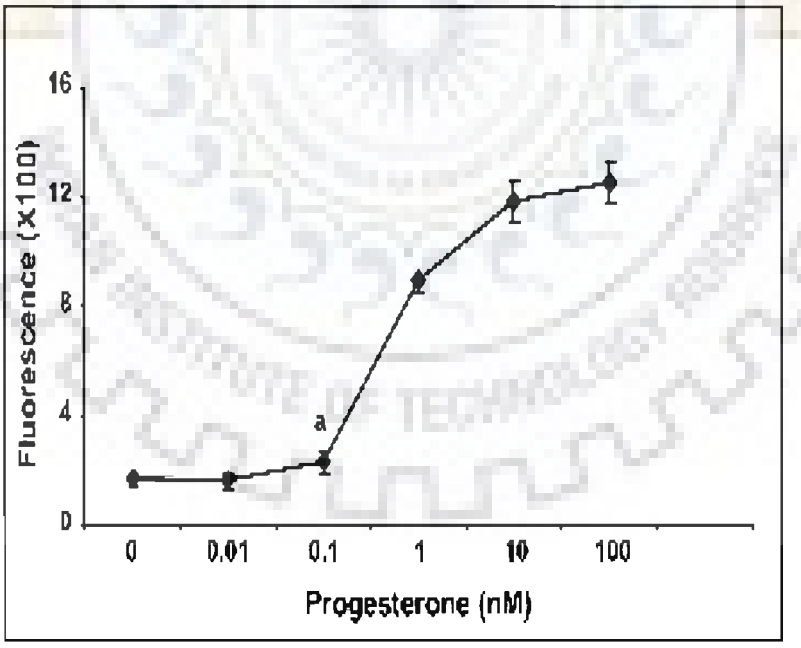


Fig 4.3 Dose dependent yEGFP induction by increasing concentrations of progesterone in hPR and PRE–yEGFP co-transformed yeast cells. The fluorescence signal was corrected with the signal obtained with the sd medium containing only ethanol. The values represent the mean ± SEM of four independent experiments each performed in quadruplicates. a, indicates the significant level of difference in fluorescence induction as compared to vehicle treated group ($p < 0.05$).

There was a slight increase in the level of fluorescence induction by testosterone and estradiol at 10 μM concentration, which was not statistically significant. However, this fact can be ignored because the concentration used here is supra physiological and the effect is no way related to the induction caused by progesterone at the same concentration. Further, several authors have already reported that at a high concentration the steroid hormones lose their specificity towards the hormone specific receptors (Gaido et al., 1997; Michelini et al., 2005a; Koltz et al., 1997).

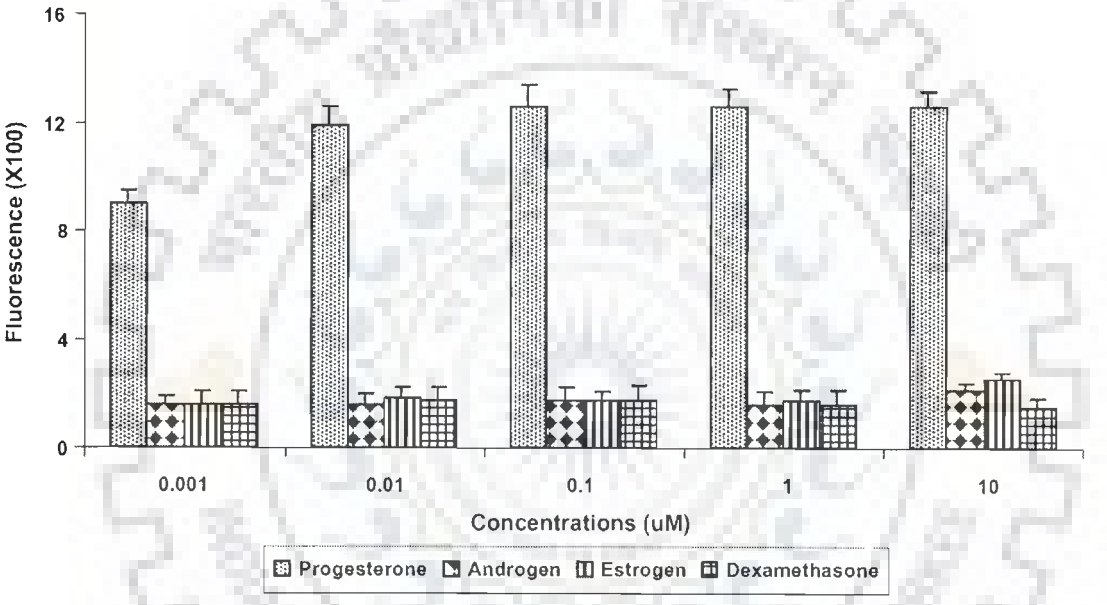


Fig 4.4 Determination of ligand specificity in recombinant yeast strains in response to non-progestative steroids. Yeast cells were incubated with progesterone and non-progestative steroids (estradiol, testosterone, and dexamethasone) at increasing concentrations (0.001–10 μM) followed by the determination of yEGFP expression. The values represent the mean ± SEM of four independent experiments each performed in quadruplicates.

4.3.4 The effect of known potent anti-progestin on expression of yeast optimized green fluorescent protein in transformed yeast strains

The effect of RU486, a potent anti-progestin, was tested in the recombinant yeast strain (Fig 4.5). The anti-progestin activity was checked by treating the cells with increasing concentrations of RU486 in the presence of 1 nM progesterone. Fig 4.5 shows their activity at different concentrations (0.001–10 μM) in the absence or presence of

testosterone. Dose dependent inhibition of progesterone induced yEGFP expression with RU486 demonstrated a potent progesterone antagonism. The IC_{50} for RU486 was found to be 0.1 μM.

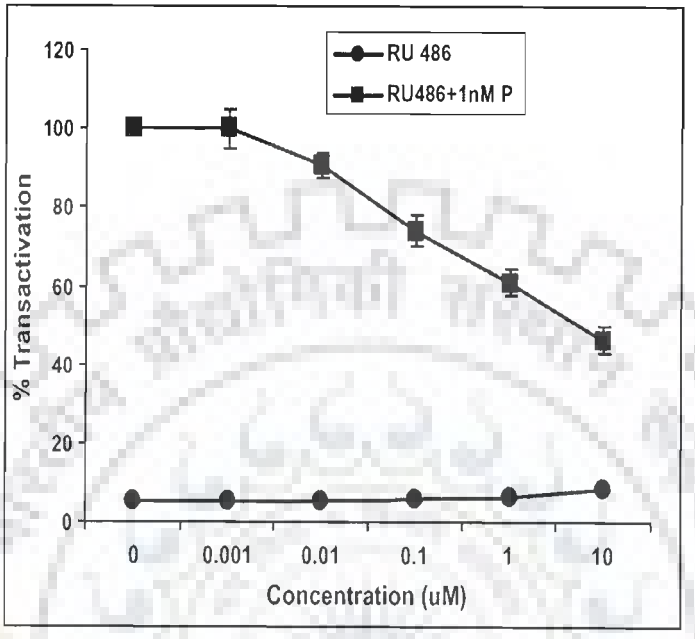


Fig 4.5 Anti-progestative activity of RU486, a potent progesterone receptor antagonist, in recombinant yeast cells. The cells were treated either with increasing concentrations (0.001–10 μM) of RU486 alone or in the presence of 1 nM progesterone. The mean fluorescence obtained with 1 nM progesterone was given the value of 100%. The values represent the mean ± SEM of four independent experiments each performed in quadruplicates. P, progesterone.

4.3.5 Screening various synthetic chemicals for their antiprogestin activity using hPR and yeast optimized green fluorescent protein expressing recombinant yeast

Several compounds were tested for their anti-progestin activities (Fig 4.6). The activities of all the compounds tested were measured against 1 nM progesterone. The compounds tested were of various classes and they can be in general called anthropogenic chemicals showing endocrine disrupting activities. Using this assay we tested: o,p′-DDT, p,p′-DDT, o,p′-DDE, p,p′-DDE, α-endosulfan, vinclozolin, and nonylphenol. All these chemicals have been tested earlier for their (anti)estrogenic and (anti)androgenic activities, however, not much information is available for their interaction with progesterone receptors or transactivation assays. All the chemicals resulted in significant reduction in progesterone induced transactivation at 1 μM concentration (Fig 4.6). The IC_{50} values of the tested chemicals in our assay were within the range of 3–20 μM for all the chemicals.

However, in our assay, none of the compounds appeared to have any progestative activity, when tested alone (results not shown).

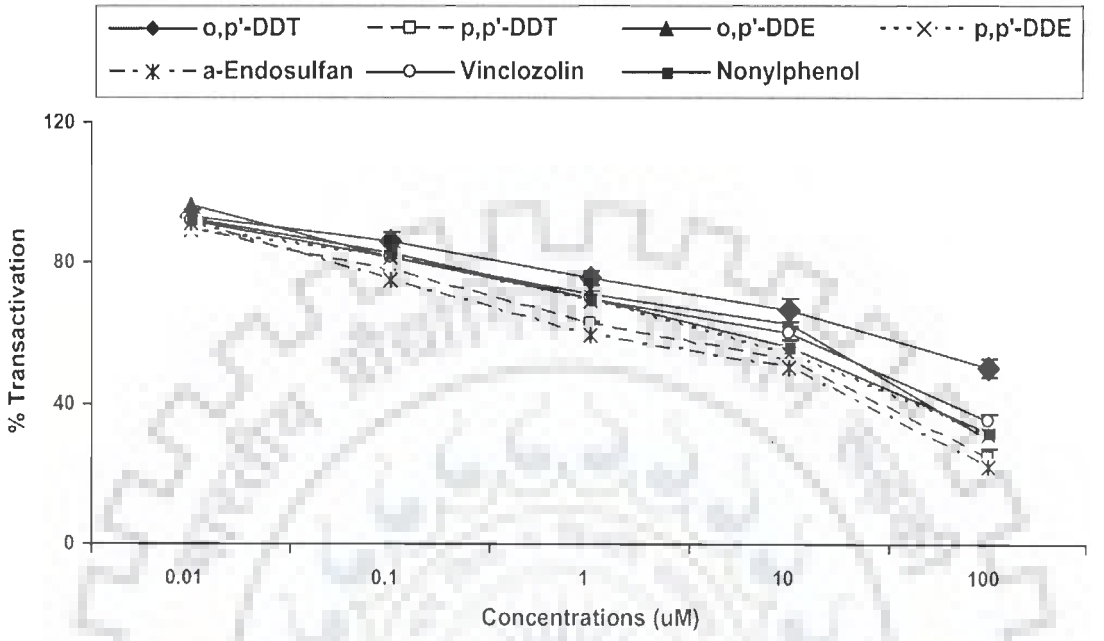


Fig 4.6 Determination of anti-progestative activity of some endocrine disrupting chemicals with clear endocrine disrupting nature. Transformed cells were treated with various concentrations of the chemicals (0.01–100μM) in the presence of 1 nM progesterone. The fluorescence was expressed as percentage of that obtained with 1 nM progesterone which was given the value of 100%. The values represent the mean ± SEM of four independent experiments each performed in quadruplicates.

4.3.6 Detection of anti-progestin activity of leather industry effluents on 96-well plate assay

Recombinant yeast cells were incubated on 96-well plates in the presence of 1 nM progesterone and varying dilutions (50, 100, 200 and 400 mL equivalent) of extracted water sample from leather industry effluents at 30°C and the expression profile of yEGFP was observed on completion of the incubation (Fig 4.7). Our data showed a clear inhibition of progesterone induced transactivation which was about 20 and 30% with 200 and 400 mL equivalent of leather industry effluents, respectively with respect to 1 nM progesterone ($p < 0.05$). However, 50 and 100 mL equivalent water samples did not cause any significant change in (anti)progestative activity probably due to lower concentration of anti-progestins in the water samples. To determine further the anti-progestin components

in the water samples, GC–MS analysis was performed which revealed the presence of four prominent compounds viz., 4-nonylphenol, hexachlorobenzene, 4-aminobiphenyl and benzidine having retention times 11.25, 11.03, 15.71 and 16.78 min, respectively (Fig 4.8) (Table 4.1). Thus at this point, the antiprogestin activity of the effluent could be attributed to the presence some or all of the contaminating chemicals in the water samples which needs further analysis to pinpoint the exact compounds.

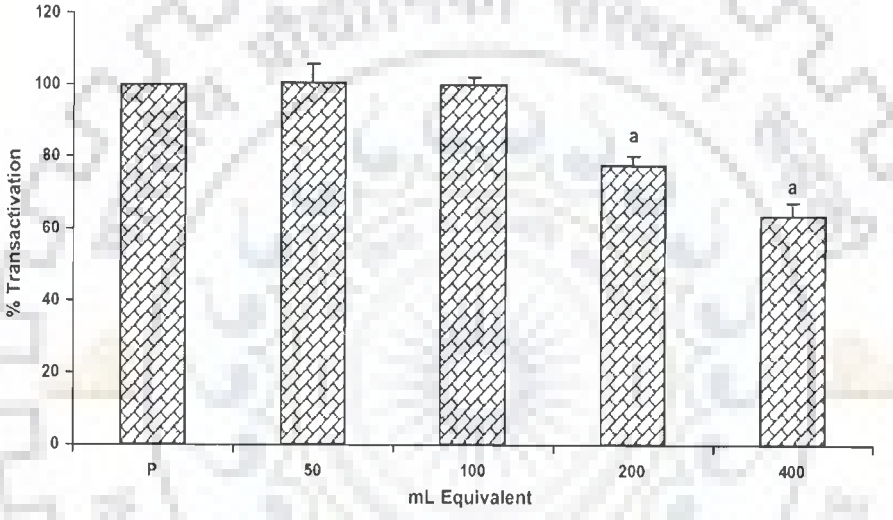


Fig 4.7 Detection of anti-progestative effects of various dilutions of extracted effluents from leather industry. The transformed cells were treated with various dilutions of effluents (50, 100, 200 and 400 mL equivalent) in the presence of 1 nM progesterone. The induction of fluorescence was expressed as percentage of that obtained with 1 nM progesterone which was given the value of 100%. The values represent the mean ± SEM of four independent experiments each performed in quadruplicates. a, indicates the significant level of difference in transactivation as compared to only progesterone treated group ($p < 0.05$).

Table 4.1

Concentration (μg/l) of 4-nonylphenol, hexacholrobenzine, benzidine and 4-aminobiphenyl in 300 ml equivalent leather water sample determined by the *selected ion monitoring mode* (SIM) GC–MS analysis

	Concentration (μg/l)	Molecular mass	Retention time (min)	Selected ions (*m/z*)
Nonylphenol	4.6	220.35	11.25	162
Hexachlorobenzene	3.3	284.8	11.03	284
4-Aminobiphenyl	2.1	169.20	15.71	184
Benzidine	2.2	184.25	16.78	169

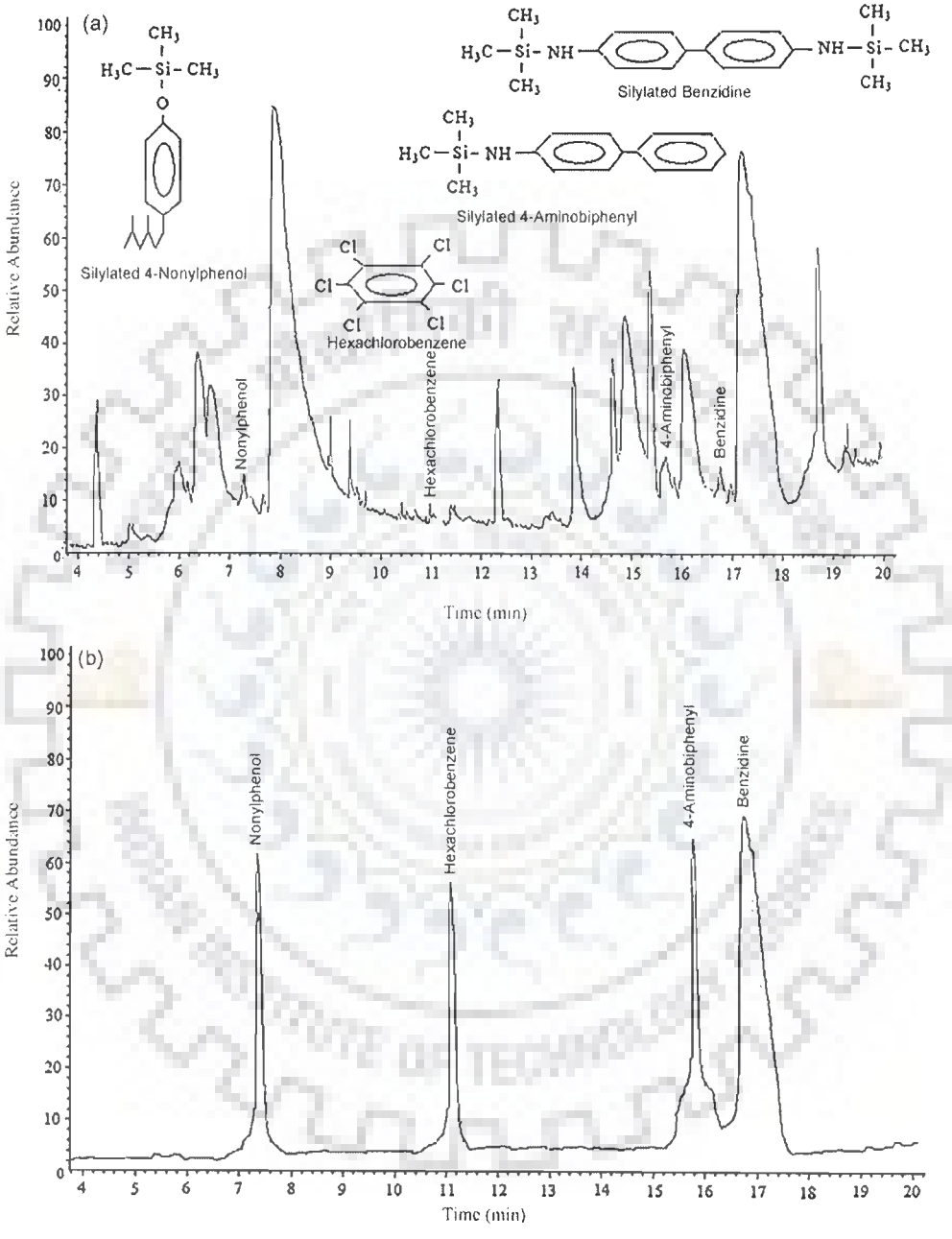


Fig 4.8 Representative GC–MS chromatograms of the extracted leather industry effluent sample (a) and its comparison with solutions of knownstandards (b) in full acquisition mode. The leather industry effluents were derivatized as described in Section 4.2.9 and analyzed by GC–MS. The insets in (a) shows the structure of derivatized (silylated) forms of target chemicals except hexchlorobenzene that is not derivatized by BSTFA.

4.4 Discussion

Many endocrine disrupting compounds identified so far are persistent organochlorine pesticides (e.g. DDT, methoxychlor and dieldrin). However, the endocrine activity of numerous commonly used compounds like industrial wastes, fungicides, pesticides has not yet been studied in detail so far. Therefore, there is a need of good and user friendly assay systems for the screening of any potentially active endocrine disruptor using both mammalian and yeast based models. Although mammalian cell lines are more sensitive than yeast and may be able to identify progestagens that require human metabolism for activation in their active state, yet it has several drawbacks associated with it. The biggest challenge with mammalian cell based assay system is the expression of other non-specific endogenous receptors, for example the estrogen receptor in MCF-7 cells, the progesterone receptor in T47D cells and the glucocorticoid receptor in PC-3, and CHO cells (Reyes-Moreno et al., 1995; Roy et al., 2004). So chances of interferences can never be ignored in these assay systems.

In addition, maintenance of mammalian cells and analysis of reporter gene expression (luciferase assays) require time, labor and cost, which may limit the mass screening of such compounds (Chatterjee et al., 2007). On the other hand, yeast based assays in spite of having low sensitivity as compared to mammalian cell based assays still have several advantages. These include robustness, low costs, lack of known endogenous receptors that may compete with the receptor activity under investigation (no crosstalk) and the use of media that are devoid of steroids (Bovee et al., 2004, 2007). These qualities make a yeast based bioassay a promising tool for high throughput screening of relatively more contaminated samples, requiring little or no sample clean up, or of complex matrices, in which there are more endocrine active substances than steroids only (Arnold et al., 1996; Gaido et al., 1997; Bovee et al., 2004; Roy et al., 2004; Michelini et al., 2005a; Chatterjee et al., 2007). Until now, yeast based progesterone and majority of estrogen and androgen bioassays are dependent on an extra chromosomal reporter construct with β-galactisidase (β-gal) as a substrate induced reporter protein. This method of bioassay although have several advantages, yet it has several problems associated with it in terms of screening compounds, most prominent of it being the cell lysis step. The other reporters like luciferase and green fluorescent protein though have been used extensively in animal and

bacterial cells, did not receive much attention in yeast system except some recent studies (Leskinen et al., 2003; Bovee et al., 2004, 2006, 2007; McNabb et al., 2005;Michelini et al., 2005a,b).

This chapter describes the development of new yeast based bioassays for screening (anti)progestins that use yeast enhanced green fluorescent protein (yEGFP). This system is based on co-transformation of two recombinant yeast DNA constructs having progesterone receptor and progesterone response element driving the expression of yEGFP that can detect progestin and (anti)progestin compounds in aqueous solution without the need of breaking cells or performing washing steps before the measurement. This system is well suited for in vitro screening of the progestin and anti-progestin activity of various chemicals due to its high sensitivity and simplicity. An additional advantage is that the assay could be adapted for the 96-well format without any cell lysis step thus making the assay suitable for high throughput screening, reducing the cost of expensive substrate. Prior to the analysis of leather industry effluents, the specificity and sensitivity of the assay was checked by determining the response of known (anti)progestin using the developed assay. As shown in the results sections the corrected fluorescence signal was proportional to the progesterone concentration ranging from 0.1 to 100 nM. Progesterone significantly induced yEGFP expression at concentration of 0.1 nM (lower limit of detection) and the maximum activity was obtained at 100 nM thereafter it leveled off with an EC_{50} value of 1 nM. Although the EC_{50} value does not match exactly with the earlier reports yet is within in the range as described earlier by others. This variations in the activity of compounds like EC_{50} values and sensitivity could be attributed to several factors like constructs used, background of the yeast strains, time of the incubations used for transactivation assays, type of reporter genes, and so on (Chatterjee et al., 2007). Each factor contributes individually towards the transactivation of the reporter genes. For example, different vectors induce the expression of receptor genes at various levels. Same is true for each type of yeast strains with varying backgrounds. All these acts in synergism to determine parameters like sensitivity and EC_{50} values for an assay. The non-transformed cells did not show any response to progesterone which further indicated that the activation was through PR. RU486, a potent anti-progestin also exhibited antagonistic activity at concentration of 0.01 μM and greater. There is a discrepancy in IC_{50} value of RU486 as found by us and by

Schreurs et al. (2005). This discrepancy could be attributed to the use of different cell systems by them and us. Bioavailability of test chemicals has always been a problem with the yeast based assays and the same may have happened with our assay system resulting in higher IC_{50} for RU486 as compared to that reported by Schreurs et al. (2005). Further the fluorescence activity could not be induced by estradiol, testosterone and dexamethasone, which suggested that the assay system is highly specific to progesterone without crosstalk to any other steroid receptors. The assays showed acceptable repeatability to progesterone with intra and inter assay CVs were <10% and <22%, respectively at high concentration which is at par with other sensitive assays reported earlier (Michelini et al., 2005b; Xu et al., 2006). The main aim of this study was to develop a model to monitor different progestin and anti-progestins using in vitro cell model. In the first phase of it, we analyzed 7 different compounds representing pesticides, fungicide or organochemical class in this model. All of the seven compounds tested by us: DDT and its metablolites, nonylphenol, vinclozolin and endosulfan, were found to be antiprogestins and this was in accordance with earlier reports by several authors (Laws et al., 1996; Koltz et al., 1997; Scippo et al., 2004; Molina-Molina et al., 2006). Majority of these chemicals like DDT, vinclozolin, endosulfan have been reported to be estrogenic and/or anti-androgenic both in vitro and in vivo. At the same time these chemicals demonstrating direct interaction with PR in vitro suggests that they may exhibit multiple hormonal activities, which renders difficult the interpretation of their mechanism of action (Molina-Molina et al., 2006). These results reconfirm the effects of these compounds in our yeast based transactivation assay. Of course there could be variations in the IC_{50} values of chemicals tested here and those reported earlier in other cell lines and that can be attributed to the presence of coactivators in different cell lines and to interference through other signal transduction pathways (Roy et al., 2004; Michelini et al., 2005b). In the second phase of our study the (anti)progestative activities of leather industry effluents were evaluated using our assay. To the best of our knowledge, this is the first report where leather industry effluents have been tested in vitro for their (anti)progestative activities. The effluents were collected from an area, housing more than 100 major and minor leather industries in Northern part of India. The leather tanning industry has a major relevance in Indian economy covering about 30% of leather demand in India and Asian subcontinent. However, this industry is recognized as

a prime pollution source in terms of conventional toxic parameters, which include sulfide, chromium, oil and grease, polychlorinated phenols, dimethyl formamide and so on. With the development of industrialization, an extensive body of evidence has associated a number of health hazards with the leather and tanning industry, including both occupational exposures, water and land contamination (Bianchi et al., 1997; Chang et al., 2004; Meriç et al., 2005; Veyalkin and Gerein, 2006). This has resulted in a number of health effects, affecting crops, aquatic and terrestrial biota, and humans (Barnhart, 1997), as well as acute toxicity on certain bacteria (Jochimsen and Jekel, 1997), crustaceans (Tisler et al., 2004; Ju´nior et al., 2007), sea urchin (Meriç et al., 2005), and fish cell lines (Riva et al., 2005). Though there are several reports on the presence of various classes of compounds in the leather industry effluents and majority of them have been shown to have toxic effects but none of them reported the effects of these chemicals on endocrine system except two studies by Chang et al. (2004) and Fail et al. (1998) where they reported them to affect the fertility in leather industry employees. All these reports emphasizes on the fact that the leather industry effluents be analyzed to assess the degree of adverse effects caused by the contaminating chemicals as endocrine disruptors. Since some reports were already available for these effluents to interact with AR and resulting in reproductive failures, we decided to check its effects on PR. Our data showed a significant reduction in the progesterone induced transactivation on treating the recombinant yeast cells with 200 mL equivalent of water samples which further declined steadily at 400 mL equivalent samples also ($p < 0.05$). However, there was no significant reduction in the transactivation of PR at 50 and 100 mL equivalent concentrations which could be attributed to the presence of low concentration of the contaminants in the samples. In order to gain further insight into the exact interfering components in the samples, we performed the GC–MS analysis of the water extracts and they were found to contain several aromatic compounds also like nonylphenol, hexachlorobenzene, aminobiphenyl and benzidine in addition to some other inorganic compounds. All these chemicals have been known to be used at various phases of leather processing and some of them or their derivatives like nonylphenol have been known to act via binding through PR (Scippo et al., 2004) and thus further supported our findings. At this point though it is difficult to speculate the exact amount of these contaminating chemicals within the effluents, however, according to some earlier reports,

androgenic and estrogenic chemicals demonstrating similar type of effects are within the range of 10–1000 ng/mL (Chatterjee et al., 2007). This wide variation in the amount of chemicals could be attributed to several factors like, type of process used in industries, volume of effluents discharged, the level of dilution and composition of effluents and of course the day to day variation of the effluent quality. Thus all these data indicates the need for further understanding their mode of action as (anti)progestative compounds. According to some authors, even low concentration of these chemicals in the environment (10 ng/mL) should not be ignored since it may show effects in long run due to gradual accumulation of these chemicals in the body.

4.5 Conclusion

To conclude, although disruption of progestative activities was rarely addressed in the past, in contrast to estrogenic and anti-androgenic activities, it could as well contribute in triggering off a wide range of hormonal and/or anti-hormonal effects in vivo. The yeast based assay developed by us can be applied to rapid and efficient screening of progestative compounds from various environmental contaminants in aqueous sources. The use of yEGFP as reporter for screening further makes the assay user friendly and robust since it does not need cell lysis and minimum hands-on time, which also makes it practical for mass screening of environmental samples. Test chemicals, steroids, and extracted water samples at all the doses tested by us in the assay did not affect the viability and proliferation of yeast cells which further enhanced the bioassay's analytical robustness. However, further work is needed to fine tune this assay in order to reduce the background and increase the sensitivity of the developed assay, since high background is one of the limitations of fluorescence based bioassays. The compounds demonstrating anti-progestative activities as identified by this newly developed assay needs to be taken up for in vivo analysis for their potential adverse effect on pregnancy and reproduction.

CHAPTER 5

Development of a multidrug transporter deleted yeast-based *in vitro* GFP assay for screening (anti)estrogenic natural endocrine disruptors

5.1 Introduction

Estrogens are an important class of signaling molecules, regulating a diverse range of physiological processes in animals and humans. The human estrogen receptors α (hERα) and β (hERβ) belong to the steroid-thyroid-retinoic acid super family of nuclear receptors. The actions of these hormones are mediated by intracellular receptor proteins (ERs) that on dimerization act as ligand-activated transcription factors by binding of the DNA-binding domain (DBD) to specific DNA sequences [estrogen responsive elements (ERE)] in the regulatory regions of target gene promoters. The hERβ receptor was identified a decade ago (Kuiper et al., 1996) and the tissue distribution of ERα and ERβ also differs substantially (Kuiper et al., 1997; Cullen et al., 2000). ERβ is very important in the bone, urogenital tract, cardiovascular system, central nervous system and the developing brain (Enmark et al., 1999; Gustafsson, 1999; Wang et al., 2003). ERα seems the more important receptor type in the mammary gland and the uterus. It has been hypothesized that ERβ is important for the protection against hyperproliferation and carcinogenesis in breast, prostate and the gastrointestinal tract (Enmark et al., 1999; Arai et al., 2000; Saji et al., 2002; Weihua et al., 2002; Forster et al., 2002; Ogawa et al., 2003). These observations led to the hypothesis of potential differences in the biological function and tissue-selective actions of the two receptors. These observations also suggest the existence of two previously unrecognized pathways of estrogen signaling: via the ERβ subtype in tissues exclusively expressing this subtype and, since estrogen receptors are known to function as dimers (McDonnell et al., 1995), via the formation of heterodimers in tissues expressing both subtypes. The differences in tissue distribution may also be very important from a pharmaceutical point of view, as hormone replacement therapy in postmenopausal women is an increasingly significant health issue (Gustafsson, 1999).

Saccharomyces cerevisiae cells do not contain members of the steroid-thyroid-retinoic acid receptor super family but have been used to study respective receptor expression (McEwan, 1999) and structure-function relationships (Wrenn et al,. 1993; Graumann et al., 1996; Almlof et al., 1997) and for the identification of signaling pathway components (Kimura et al., 1995; Caplan, 1997; vom Baur et al., 1998; DeFranco et al., 1999). Such approaches used that by expression of the receptor proteins and on ligand exposure the receptor-mediated signaling can be reconstituted and measured using a

reporter gene driven by DNA responsive elements. In the absence of hormone, steroid receptors are found in a complex with molecular chaperones, such as Hsp90, in the cytoplasmor nucleus (DeFranco et al., 1999). Yeast Hsp82 and YDF1 as counterparts of mammalian molecular chaperones function to maintain steroid receptors in a hormone-binding conformation and to assist activation of the downstream pathway (Caplan, 1997). The introduction of *S. cerevisiae* based screens for estrogenic activity (Arnold et al., 1996; Shiau et al., 1996; Chen et al, 1997; Jungbauer et al., 2002; Bovee et al., 2004a & b; Sievernich et al., 2004) has also demonstrated the applicability of this type of assay in such diverse disciplines as environmental monitoring and analysis of food components. Besides classical hormone testing such assays served to detect estrogenic activity of natural (e.g. phytoestrogens) and synthetic compounds (the latter widely referred as to xenoestrogens among the endocrine disruptors) (Graumann et al., 1999; Payne et al., 2000; Agradi et al., 2001; De Boever et al., 2001; Witters et al., 2001). Furthermore, toxicity of samples to yeast or mammalian cells is a potential problem in assessing estrogenic activity in complex samples. As cytotoxicity occurs more frequently in mammalian cell assays than in yeast assays (Graumann et al., 1999, Witters et al., 2001) and because yeast is more resistant to environmental contaminants, such as heavy metals and bacterial endotoxins (Breithofer et al., 1998), yeast assays can best be used to study the estrogenic activity in such samples.

Here, we report on the transactivation assay toward hERα and hERβ receptor expression coupled to the 3xERE-GFP-reporter in a genetically modified yeast strain, devoid of three endogenous xenobiotic transporters (*PDR5, SNQ2,* and *YOR1*), and comparative analysis of natural and synthetic (anti) estrogenic compounds. Among the natural compound was the endogenous estrogens estradiol-17β (E_2); the phytoestrogen genistein, and the synthetic compounds with clinical application ethylene estradiol, tamoxifen citrate were tested. Transcriptionally activated GFP fluorescence emissions were normalized to cell growth determined by absorption and correlated to internal reference standards. Obtained dose-response curves served for EC_{50} value calculation. Assay protocol optimization comprised conditions like agitation, temperature, and pH value to determine the most practical test handling conditions.

5.2 Materials and methods

5.2.1 Sample collection and preparation

Samples were collected in amber colored glass bottles, rinsed initially with acetone and MilliQ water (Millipore, India) from the site receiving Sewage Treatment Plant (STP) effluent in northern India, stored at 4°C till extraction and the extraction was performed within 48 h of sampling. All the glassware to be used in the experiments was rinsed with dichloromethane (DCM) with 0.6% concentrated hydrochloric acid to prevent the clinging of steroids on the glass walls. The water samples were filtered and subjected to organic phase extraction by adding DCM according to the method described earlier (Soto et al., 2004) with slight variations as per our laboratory conditions. DCM was added to each aliquot at the ratio of 60 mL/L of crude sample and mixed thoroughly for 2 min, left for 10 min for settling down of organic phase and finally the aqueous phase was separated by separating funnel. The procedure was repeated thrice to extract organic phase and all aqueous phase was poured off. Extracted organic phases were mixed and concentrated under reduced pressure on a Bucchi rotatory evaporator to 2 mL/L of crude sample and concentrated organic phase was solvent exchanged with 10% ethanol. In the final preparation, each 2 µL of prepared sample in the highest test concentration was equivalent to 400 mL eq/well of 96 well plate which was further serially diluted with same 10% ethanol to 200, 100 and 50 mL equivalent samples per well. Prepared dose was stored at -20°C until used for the assay.

5.2.2 Plasmids and yeast strains

The plasmid pcDNA3-hERβ, harboring the human short beta receptor ($hER\beta_{\Delta aa1-43}$), was kindly provided by Dr. K. Korach (National Institutes of Health). For expression in *S. cerevisiae*, the hERβ receptor was excised as *Bam*HI-*Xho*I fragment with the protruding *Xho*I end filled with T4-DNA polymerase (MBI Fermentas) and gel purified. The episomal vector backbone was the high copy *Escherichia coli*/yeast shuttle vector pYEX-BX (Clontech, Palo Alto, CA) in which the *URA* cassette was replaced with the *TRP* selection marker. After *Eco*RI linearization, filling in of the protruding end and subsequent *Bam*HI digest both fragments were ligated to yield the 8.8 kb pYEX-hERβ plasmid. Plasmid

pERE-CYC1-GFP was described before (Sievernich et al., 2004). Plasmid YEpE12 (hERα) was a gift from Dr. A. Jungbauer (University of Vienna).

For the SNQ2 gene deletion cassette, the pUG6 plasmid with the kanamycin marker gene (Güldener et al., 1996) was used as template. For the deletion cassettes of the *S. cerevisiae* YOR1 and PDR5 genes, the pUG6 plasmid (Güldener et al., 1996) was modified by exchange of the kanamycin with the *LEU2* marker. The isopropyl-malate dehydogenase gene was amplified with *S. cerevisiae* wild-type (S288C) genomic DNA as template by PCR using the oligonucleotides 5′-*gagaagatct*gagttcgaatctcttagcaacc-3′ and 5′-*gagagagctc*caaattaggaatcgtagtttcatg-3′ with primer encoded *Bgl*II and *Sac*I restriction sites. The PCR fragment was cleaved with *Bgl*II/*Sac*I and ligated with the *Bgl*II/*Sac*I-digested pUG6 to yield plasmid pUG6 (LEU). *PDR5* and *YOR1* gene replacement cassettes were PCR generated comprising the *LEU2* (*PDR5* and *YOR1*) or kanamycin resistance (*SNQ2*) gene flanked by ~500 bp gene specific homologous 5′- and 3′-targeting regions (Hasenbrink et al, 2006). The oligonucleotides used for the *SNQ2*, *YOR1*, and *PDR5* gene replacement cassettes are listed in Table 5.1. All gene replacement cassettes were subcloned to pBSK (*Sma*I). All recombinant plasmids recovered from transformed *E. coli* XL1-blue cells were mapped by restriction analysis and confirmed by sequencing (GeneART). Computer analysis of nucleotide and amino acid sequences was performed using the Vnti software (Informax).

The yeast strains used throughout this study are summarized in Table 5.2. The *S. cerevisiae pdr5 snq2 yor1* disruption strain was derived from PLY232 (Bertl et al., 2003), using the Cre-loxP recombination system described by Güldener et al., except that for *PDR5* and *YOR1* the LEU2 marker was used for replacement. After yeast transformation, in LEU2 positive or G-418 (200 mg/l) resistant single cell derived colonies the ORF::loxP-kanMX/LEU2-loxP introduced markers were rescued on expression of the Cre recombinase with plasmid pSH47. Final pSH47 plasmid loss was achieved by growth on YPD medium for 3 days. The eventually obtained triple mutant strain *S. cerevisiae pdr5 snq2 yor 1* served as host for expression of the hERα-ERE-GFP, hERβ-ERE-GFP plasmids. The plasmids were obtained by standard DNA manipulations according to Sambrook et al., 1989 and used to transform *S. cerevisiae pdr5 snq2 yor1* cells to tryptophan, uracil, and leucine prototrophy by standard methods (Rothstein, 1991).

Table 5.1 Oligonucleotides

Oligonucleotide	Sequence (5′-3′)
PvuII-PDR5	5′Target-s *gagacagctg*cttctacgccgtggtacgatatc
SalI-PDR5	5′Target-as *gagagtcgac*ttgtctaaagtctttcgaacgagcg
SpeI-PDR5	3′Target-s *gagaactagt*gaattttgaatttggttaagaaaagaaac
SacII-PDR5	3′Target-as *gagaccgcgg*ctatatccattgcgtcctttctttta
PvuII-YOR1	5′Target-s *gagacagctg*ccgcgaggcggaaatggcaca
SalI-YOR1	5′Target-as *gagagtcgac*attcgtatatagcaacggctttactc
SpeI-YOR1	3′Target-s *gagaactagt*tttatattatttgttgcatgatttttctc
SacII-YOR1	3′Target-as *gagaccgcgg*ctctttaaacaagaatggctttttcc

Table 5.2 Haploid yeast strains

	Genotype	Source
PLY232	*MATa his3-200 leu2–3,112 trp1-901 ura3–52 suc2-9*	Bertl et al., 2003
SCTD-10/04	*MATa his3-200 leu2–3,112 trp1-901 ura3–52 suc2-9 pdr5-1::loxP snq2::loxP yor1–1::loxP*	Hasenbrink et al., 2006
SCTD-10/04 hERα	SCTD-10/04 [pERE-CYC1-gfp] [YEpE12]	This study
SCTD-10/04 hERß	SCTD-10/04 [pERE-CYC1-gfp] [pYEX-hERß short]	This study

5.2.3 Media and growth conditions

All yeast cells were grown aerobically at 30°C. Nutritional requirements appropriate for selection and maintenance of mutants and plasmids in the transformed strain were scored on either liquid complete synthetic SDAP medium (Rodriguez-Navarro and Ramos, 1984) plus 0.5% d-glucose or minimal YNB media consisting of 0.67% yeast nitrogen base (YNB) with $(NH_4)_2SO_4$, amino acids, and 0.5% D-glucose without uracil, tryptophan, and leucine (the latter to use the LEU2-d function of the pYEX-BX vector to increase for plasmid copy numbers) adjusted to pH 6.4 or 4.5 with NaOH and, where indicated, buffered with 50 mM citric acid monohydrate.

5.2.4 Western blot

Yeast cells were grown to an OD of ~1 (corresponding to 1.2 x 10^7 cells/ml). 10 ml of yeast cells were harvested by centrifugation and washed with distilled water. Protein extraction was carried out using the adapted protocol by Laemmli, 1970. Protein content was determined using the Bio-Rad protein assay kit (Bio-rad Laboratories). For detection of hERα and hERβ proteins, ~20 µg of total protein were separated by SDS–PAGE (10% polyacrylamide). The gel was run at 120 V until the loading dye ran off the gel (approximately 90 min). Proteins were transferred to a nitrocellulose membrane (Amersham) at 120 V for 60 min at approximately 4°C by wet blotting. Blots were incubated with anti-hERα and hERβ rabbit polyclonal antibody (1:200) (Santacruz) respectively and with a secondary alkaline phosphatase-conjugated goat anti-rabbit antibody (1:1000) (Santacruz). Colour development was performed in Fast Red Solution (Sigma).

5.2.5 Assay conditions and fluorescence monitoring

For quantitative assessment of growth phenotypes and fluorescence development, logarithmic growing cells (70% budding) were diluted to a start optical density (OD) 600 of 0.4 (Pharmacia Ultrospec 2000 Spectrophotometer) corresponding to 3.25 x 10^6 cells/ml. For each tested compound, at least three tests were carried out on different days. Each experiment consisted of two replica test cultures with respective controls and minimum four test concentrations. Test compounds were dissolved in DMSO and added to the test cultures of yeast strains transformed with the respective expression-reporter system in a total volume of 200 µl. The final concentration of the DMSO solvent did not exceed 0.5%. The growth was estimated by end point OD600 measurements after 16.5 h incubation in transparent 96-well microtitre plates using a microplate reader (Tecan, Spectrofluor Plus). Tests were considered as valid when the turbidity of the control cultures increased at least fivefold during the incubation period. For fluorescence development read-outs, the excitation wavelength was adjusted to 485 nM and emission was observed at 535 (25nM bandwidth).

5.2.6 Data capture and evaluation

After 16.5 h incubation, obtained end point fluorescence (FL) values (corrected for blanks) were divided by growth determined as OD 600 (corrected for blanks, OD) for each replica well to normalize fluorescence for cell number (FL/OD). To increase the reproducibility of results, the FL/OD values obtained for a test compound at a given concentration were expressed as fractional values of the maximal response of a saturating concentration of the reference compound E_2. The fractional values (response relative to the maximal E_2 ($R_{relβÖ}$) at a given concentration of test compound (ct) were calculated according to $R_{relβÖ}$ (ct) = (FL/OD(ct)-bottom)/(top-bottom), with "top" corresponding to the fitted FL/OD at saturating E_2 concentration and "bottom" to the fitted FL/OD for the negative (solvent) control.

The top and bottom values were obtained by Hill equation fit:

$$y(x) = \text{bottom} + \frac{(\text{top-bottom})}{1+10^{(LEC50-x)*hill\_slope}}$$

with y(x) =FL/OD at the actual compound concentration, x = the decadical logarithm of compound concentration, LEC_{50} = d ecadical logarithm of EC_{50}, top = fitted maximal FL/OD at saturating concentrations, bottom = fitted maximal FL/OD of negative control and hill_slope as the hill steepness parameter to the FL/OD values for each E_2 concentration using the R function *nls* (The R Foundation for Statistical Computing, http://www.r-project.org/). Dose-response data for test compounds were obtained from the $R_{relβÖ}$ (ct) for each test compound using the analogous fitting algorithm. EC_{20}, EC_{50}, and EC_{90} values were calculated from the fitted dose-response curves, confidence intervals were determined using the R function *confint*.

5.2.7 Microscopy

Yeast strains were cultured with exposure to E_2 for 16.5 h at 30°C with shaking at 950 rpm and 1 ml of each culture conditions were washed with MilliQ, aliquoted and fixed with 3.7% formaldehyde for 1 h. Cells were then harvested and washed twice with PBS + BSA (1 mg/ml) + 0.1% (v/v) Triton X-100. Next, the pellet was resuspended in 50 µl PBS/BSA buffer. Finally, Cells were washed twice with PBS + BSA (1 mg/ml) (w/o

Triton X-100) and were examined under Olympus Fluoview FV-1000 (Olympus, Japan) with a 60x oil-immersion objective.

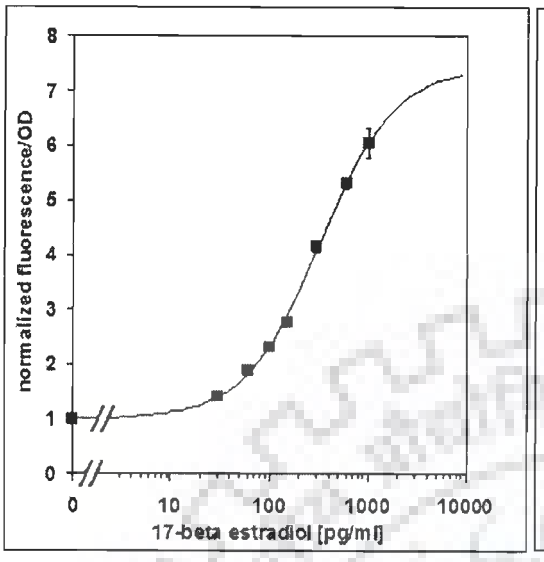
5.3 Results

A triply *PDR5*, *SNQ2*, and *YOR1* deleted *S. cerevisiae* yeast strain was used as host for the heterologousexpression of hERα and hERβ. The expression deployed a two plasmid strategy, the episomal expression of the individual receptors whereby the receptor cDNA was set under control of the constitutive *CUP1* promoter and the episomal expression of the cis-acting estrogen hormone-responsive element (ERE) fused to the yeast-optimized green fluorescent protein (yEGFP; Cormack et al.,1996). The advantage of using a *S. cerevisiae* mutant devoid of the three major transporters conferring pleiotropic drug resistance and thus inability to efficiently export small, hydrophobic molecules.

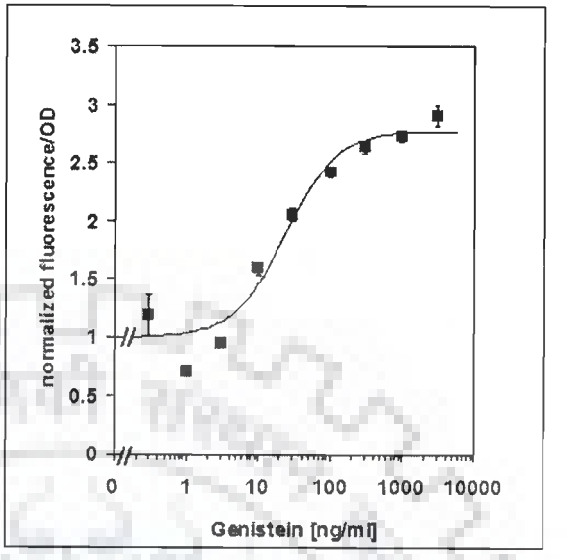
For dose-response curves, fluorescence readouts were obtained by measuring emission at 535 nm at the end of the 16.5 h incubation period. In this way, possible strain specific and compound-induced altered growth characteristics with variations of maximum cell density in the stationary phase were taken into account, enabling consistent curve fits. Blank and negative control cultures served for the correction following the accumulation of oxidized flavines in the late growth phase. At least five fold increase in turbidity with distinguishable fluorescence emission were observed with different concentrations of E_2, Ethylene estradiol, Genistein and Tamoxifen citrate on hERα and hERβ in YNB-minimal medium having 0.5% glucose and histidine (lacking tryptophan, leucine and uracil for selection). All ligand exposure was at pH 6.4 and in 50 mM citric-acid buffered YNB growth medium and this has been routinely adopted for subsequent assays. Temperature condition was fixed at 30°C with constant shaking of the test microplates.

The sensitivity of the hERα and hERβ bioactivity assay in wild type strains represented by the calculated EC_{50} values as obtained from the fit of the Hill function by nonlinear regression. Among the natural hormonal compounds for E_2 as perfect ligand, EC_{50} values of wild type strains of hERα and hERβ 322.9 (Fig 5.1A), 191.6 pg/ml (Fig 5.2A) respectively. In case of ethylene estradiol, it was 98.6 and 77.9 pg/ml for the

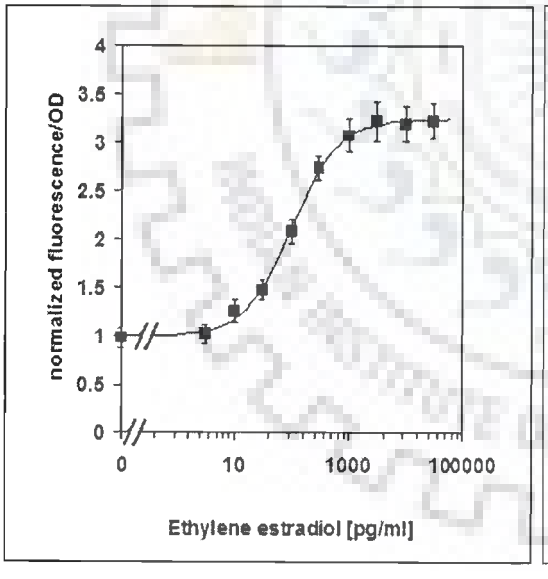
5.1 A



5.1 B



5.1 C



5.1 D

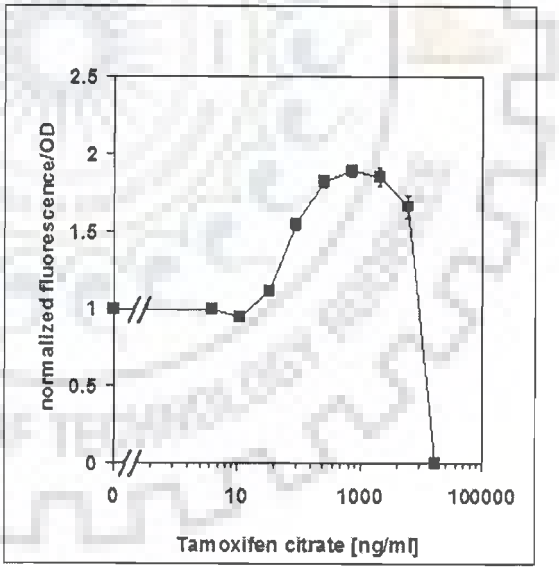
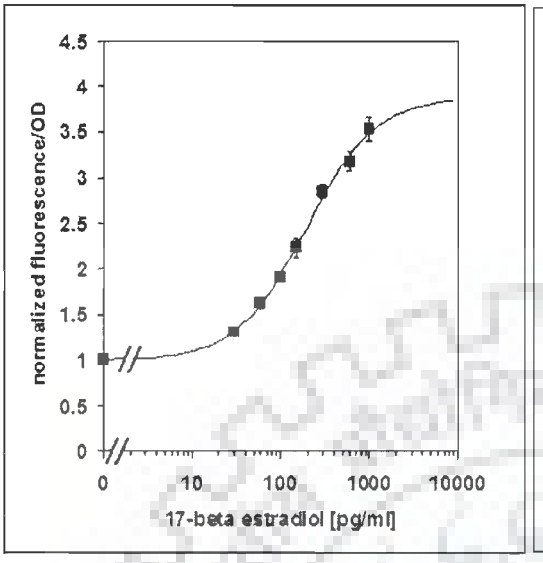
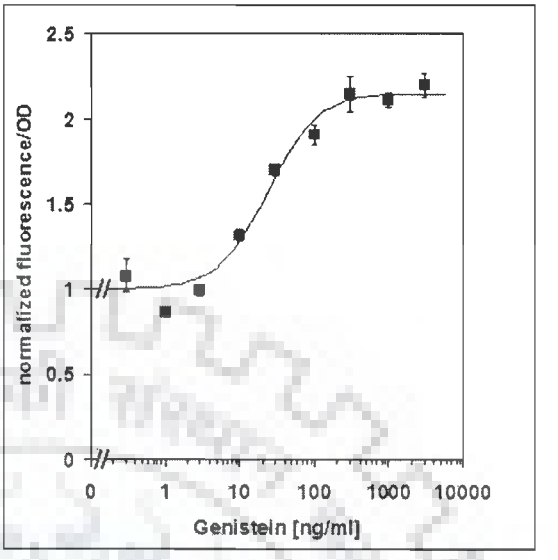


Fig 5.1 Dose response curve fit of *S. cerevisiae* Δ*pdr5,snq2,yor1* hERα wild-type strain expressing the hERα receptor and ERE-GFP reporter constructs on exposure to increasing E_2 (A), Genistein (B), Ethylene estradiol (C) and Tamoxifen citrate (D) concentrations. Fluorescence emission was measured (excitation at 485 nM and emission at 535 nM) and normalized to Fluorescence/OD without test compound. Values are mean of duplicate samples of 3 independent experiments ±SE.

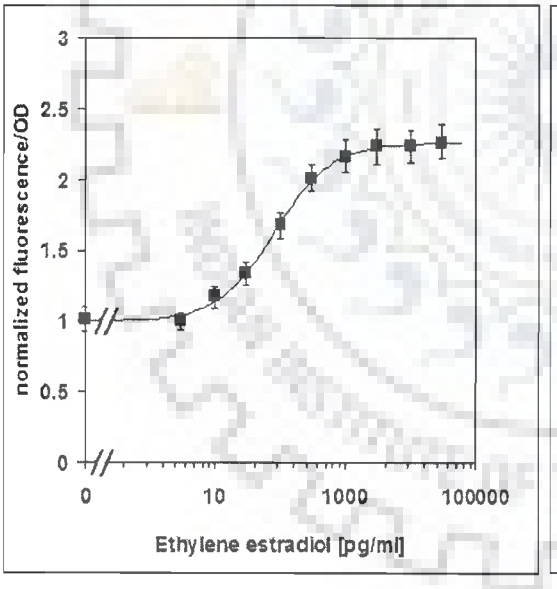
5.2 A



5.2 B



5.2 C



5.2 D

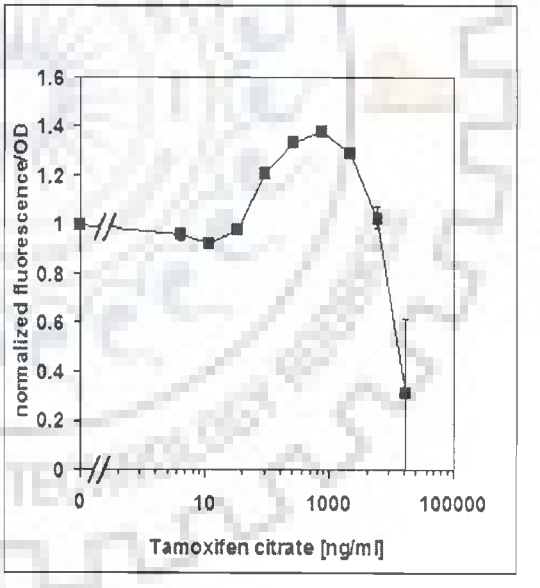


Fig 5.2 Dose response curve fit of *S. cerevisiae* Δ*pdr5,snq2,yor1* hERβ wild-type strain expressing the hERβ receptor and ERE-GFP reporter constructs on exposure to increasing E_2 (A), Genistein (B), Ethylene estradiol (C) and Tamoxifen citrate (D) concentrations. Fluorescence emission was measured (excitation at 485 nM and emission at 535 nM) and normalized to Fluorescence/OD without test compound. Values are mean of duplicate samples of 3 independent experiments ±SE.

Table 5.3 EC_{20}, EC_{50} and EC_{90} values for different compounds in case of hERα (Wild)

		hERα (Wild)		
Class	Compound	EC_{20}	EC_{50}	EC_{90}
Natural Estrogen	17ßE$_2$ (pg/ml)	96.5	322.9	2192.3
Synthetic Estrogen Pharmaceuticals	Ethylene estradiol (pg/ml)	25.9	98.6	788.9
Natural Phytoestrogen	Genistein (ng/ml)	8.5	21.7	140.1
Synthetic Antagonist	Tamoxifen citrate	n.d.	n.d.	n.d.

Table 5.4 EC_{20}, EC_{50} and EC_{90} values for different compounds in case of hERβ short (Wild)

		hERβ short (Wild)		
Class	Compound	EC_{20}	EC_{50}	EC_{90}
Natural Estrogen	17ßE$_2$ (pg/ml)	54.1	191.6	1378.2
Synthetic Estrogen Pharmaceuticals	Ethylene estradiol (pg/ml)	21.2	77.9	721.8
Natural Phytoestrogen	Genistein (ng/ml)	9.9	22.1	128.5
Synthetic Antagonist	Tamoxifen citrate	n.d.	n.d.	n.d.

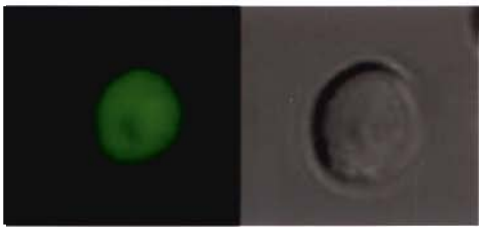
hERα (Fig 5.1C) and hERβ (Fig. 5.2C). Among the plant secondary metabolites for genistein as isoflavone, EC_{50} values of wild type strain of hERα (Fig 5.1B) and hERβ (Fig 5.2B) were 21.7 and 22.1 ng/ml respectively. No EC values could be determined for the ER-receptor antagonist tamoxifen citrate (Fig 5.1D and 5.2D). From the EC_{50} values, it is clearly evident that hERβ short isoform is more sensitive than hERα constructs in this multidrug transporter deleted yeast strains. EC_{20}, EC_{50}, and EC_{90} values were given in Table 5.3 and 5.4.

Western blotting revealed no considerable change in hERα (Fig 5.4 A) and hERβ (Fig 5.4 B) protein production. Confocal microscopy established successful expression of GFP reporter system (Fig 5.3 A & B). Recombinant yeast cells were incubated on 96-well plates in the presence of 1 nM estrogen and varying dilutions (50, 100, 200 and 400 mL equivalent) of extracted water sample from STP effluent at 30°C and the expression profile of yEGFP was observed on completion of the incubation (Fig 5.5 A and B). Our data showed a clear induction of estrogen induced transactivation which was about 20 and 30% with 200 and 400 mL equivalent of STP effluents, respectively with respect to 1 nM estrogen ($p < 0.05$). However, 50 and 100 mL equivalent water samples did not cause any significant change in estrogenic activity probably due to lower concentration of estrogen in the water samples. The results suggest that the solid phase extracted water samples of STP effluent contain potential estrorogen mimicking compounds and they are in the range of 0.005 to 0.037 ng/mL eq. of E_2.

5.4 Discussion

Current investigations focused on sensitized *S. cerevisiae* strains expressing either the hERα or hERβ receptor and p3xERE-GFP reporter plasmids to identify and assess (anti) estrogenic bioactivity. Since yeast do not contain endogenous steroid receptors, the indicator strains expressing the full functional hERs enable quantification of both the DNA binding and transcriptional activation function because the receptors are estrogen induced and bind their own response element. Investigations comprised both sensitivity and specificity of the system. Implementation of a *S. cerevisiae* host strain devoid of three endogenous xenobiotic-transporting plasma membrane ATPases enhanced the sensitivity for steroids. Thus most of the tested natural and synthetic compounds appeared to be the

5.3 A



5.3 B

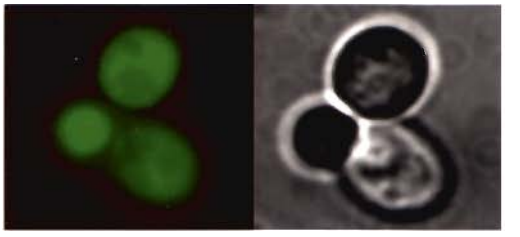
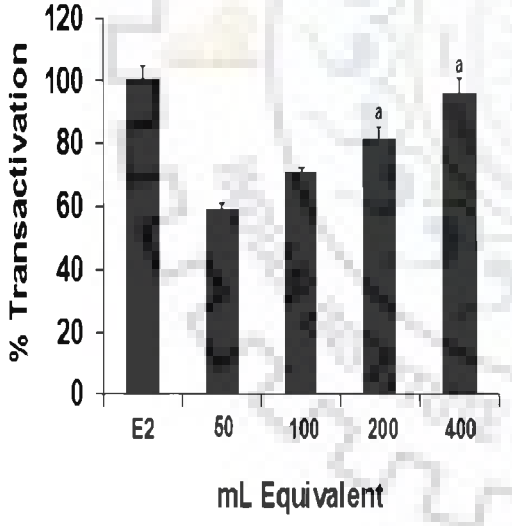


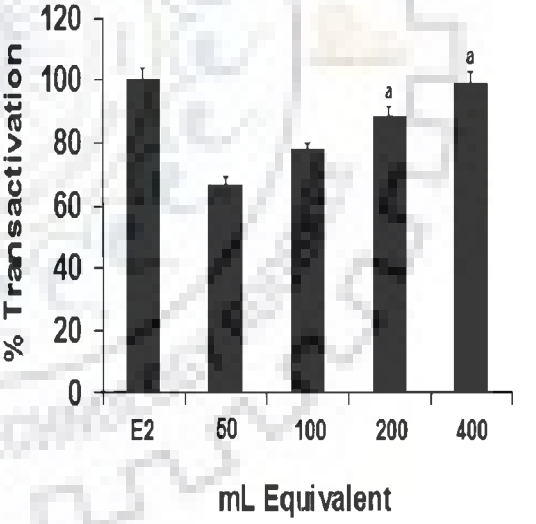
Fig 5.3 Confocal microscopy image showing functional GFP expression upon ligand exposure to the recombinant yeast construct of (A) hERα and (B) hERß short receptor.

5.4 A

5.4B

Fig 5.4 Western blotting of (A) hERα (~67 kDa) and (B) hERß (~53kDa) short receptor. First lane in each case comprising wild type strain without receptor/reporter construct and the second lane comprising wild type strain with receptor/reporter construct also in each cases.





5.5 A

5.5 B

Fig 5.5 Detection of estrogenic effects of various dilutions of extracted effluents from Sewage Treatment Plant (STP) for hERα (5.5 A) and hERβ short (5.5 B) receptor/reporter strain. The transformed cells were treated with various dilutions of effluents (50, 100, 200 and 400 mL equivalent) and in the presence of 1 nM estrogen (17-β E_2). The induction of fluorescence was expressed as percentage of that obtained with 1 nM estrogen (17-β E_2) which was given the value of 100%. The values represent the mean ± SEM of four independent experiments each performed in quadruplicates. a, indicates the significant level of difference in transactivation as compared to only estrogen treated group ($p < 0.05$).

substrates for either or all of Pdr5p, Snq2p, or Yor1p.

5.4.1 Applicability

Growth in YNB met the defined validity criteria as fitness parameter. High speed culture shaking (950 rpm) was identified as an essential factor serving both oxygen supply and even cell distribution within the well and thus eventually growth and, if induced, GFP production. Since no significant differences of E_2 induced hERα and hERβ responses at external pH values of 4.5, 6.4, and buffered 6.4 were observed, we conclude the robustness of the assay.

5.4.2 Specificity and sensitivity

The specificity of the assay was confirmed by the negligible transactivation response on application of the ER receptor antagonist tamoxifen citrate with both hERα and hERβ. The specificity of this system was also tested by the application of the phytoestrogen, viz., genistein as well as the synthetic estrogen ethylene estradiol. All of these yielded a substantially lower biological estrogenic activity as the true agonist 17β-E_2.

In humans and other mammalians the response of different organs, tissues, and cell types on estradiol is dependent on the differential expression of the two estradiol receptors (and coactivators) and their intrinsic (binding) properties. The sensitivity of the yeast GFP system was tested by serial dilutions of 17β-E_2 with kinetic measurements of specific fluorescence development in a *S. cerevisiae* wild type *pdr5 snq2 yor1* mutant. In the current test, the presence of the pleiotropic resistance mediating transporters Pdr5, Snq2 and Yor1 did influence 17β-E_2-induced GFP transactivation in both strains when applied in DMSO. Subtle differences were observed between the hERα and hERβ mediated responses for the endogenous hormones E_2. In this assay approximately EC_{50} values for hERα and hERβ were 322.9 and 191.6 pg/ml respectively. Obtained values were in good agreement with other yeast assays (Jungbauer et al., 2002; Schultis et al., 2004; Bovee et al., 2004a & b) and appeared considerably more sensitive than receptor binding assay results (Gutendorf et al., 2001) but less sensitive than the E-screen-assay with MCF-7 cells (Gutendorf et al., 2001). For the natural compound, viz., genistein sensitivity of hERα and hERβ, were almost same, i.e., 21.7 and 22.1 ng/ml respectively. The synthetic estrogen ethylene

estradiol having more sensitivity than natural estrogen E_2, i.e., 98.6 and 77.9 pg/ml for hERα and hERβ respectively. But in both the cases, hERβ was more sensitive than hERα.

In the second phase of our study the estrogenic activities of sewage treatment plant (STP) effluents were evaluated using our assay. To the best of our knowledge, this is the first report where STP effluents have been tested with the *S. cerevisiae* strain deleted in the multidrug transporter genes *PDR5, SNQ2 AND YOR1 in* vitro for their estrogenic activities. The effluents were collected from a sewage treatment plant effluent, housing a major population in Northern part of India. To date the natural steroid, environment borne 17β-estradiol has been proved to act as an endocrine disruptor in male vertebrate organisms, e.g. fish (Anderson et al., 1996; Christiansen et al., 1998; Lim et al., 1991; Miles-Richardson et al., 1999; Panter et al., 2000). Most of these chemicals are released into surface waters via municipal and industrial sewage treatment plant (STP) effluents. By means of chemical analysis, major activities in untreated municipal STP effluents could be attributed to the strong steroidal estrogens estrone, 17β-estradiol and 17α-ethinylestradiol (Desbrow et al., 1998). Chemical analyses of STP effluents across Europe and North America identified estrone, 17β-estradiol and 17α-ethinylestradiol at environmentally relevant concentrations within the ng/L range (Belfroid et al., 1999; Baronti et al., 2000; Desbrow et al., 1998; Huang and Sedlak, 2001; Joss et al., 2004; Johnson et al., 2007; Kuch and Ballschmiter, 2000; Stumpf et al., 1996; Ternes et al., 1 999c). Using dual approach comprising gas chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry, and compared chemical and biological analysis confirmed that most of the estrogenic activity occurring in STP, which receives mainly domestic sewage, resulted from sex hormones (Muller et al., 2008). More recently, six estrogens including diethylstilbestrol, estrone, β-estradiol, estriol, 17α-ethynylestradiol and β-estradiol 17-valerate have been detected by gas chromatography–mass spectrometry in surface water and sediment sampled from three rivers having constant input of STP effluent in Tianjin area, northern China (Lei et al., 2009). Also in recent decades, an additional attention has been paid to the occurrence and fate of phytoestrogens in municipal/industrial waste water in which they were detected in high frequencies. Coumestrol, a least discharged phytoestrogen, has also been reported existing in municipal wastewater treatment plants (WWTPs) (Bacaloni et al., 2005). In addition, based on the fact that natural estrogen

conjugates occurred both the influent and effluent of WWTPs (D'Ascenzo et al., 2003; Erbs et al., 2007; Isobe et al., 2003; Komori et al., 2004; Lagana et al., 2004; Nakada et al., 2006; Reddy et al., 2005), phytoestrogen conjugates may also be present in influent and effluent of WWTPs with high frequency. So, both for natural and synthetic estrogenic substance development of potential assay system is an urgent environmental concern.

Research across the world presently are actively engaged in detection of these (anti)estrogenic substances from various environmental point sources by chemical or biological means, especially in surface and river waters which day by day are getting a huge discharge from WWTPs. Chemical analyses, however, do not consider additive or synergistic effects of structurally different xenoestrogens present in sewage effluent and river water samples; in contrast, bioassays can do so (Arnold et al., 1997; Graumann et al., 1999; Islinger et al., 1999; Körner et al., 1999; Pawlowski et al., 2000a,b; Smeets et al., 1999). Among various bioassays developed to elucidate estrogenic activities of individual chemicals and environmental water samples (Feldman and Krishnan, 1995; Flouriot et al., 1993; Jobling et al., 1995; Jugan et al., 2009; Miège et al., 2009; Pawlowski et al., 2000a,b), the yeast estrogen screen (YES, Arnold et al., 1996) received particular attention as a rapid and comparatively simple screening assay in laboratory and field experiments (Garcı´a-Reyero et al., 2001; Harries et al., 1997; Johansson, 1999; Murk et al., 2002; Routledge and Sumpter, 1996; Sanseverino et al., 2005; Sanseverino et al., 2009; Soto et al., 1995; Svenson et al., 2003; Vermeirssen et al., 2006). Here we have used *Saccharomyces cerevisiae pdr5 snq2 yor1* mutant indicator strain for more sensitive and rapid screening. This study clearly documents the presence of elevated levels of estrogenic activity in municipal STP effluents. Measured concentrations of steroidal estrogens up to the tens of ng/L in the STP effluent indicate a potential relevance for aquatic organisms including fish (Länge et al., 2001; Panter et al., 1998). Particularly ethylene estradiol has been documented to be estrogenic to fish at levels down to 1 ng/L (Jobling et al., 2003; Pawlowski et al., 2003a, b), and E_2 has been shown to severely interfere with the hormone system of various fish species at the level of tens of ng/L (Routledge et al., 1998; Miles-Richardson et al., 1999; Rose et al., 2002). Therefore, further in vitro and in vivo/in situ work over prolonged experimental periods and at different times of the year are required to be done to estimate the possible hormonal impact of STP effluents.

5.5 Conclusion

To avoid the complexity with mammalian systems and in view to both, understanding the molecular mechanisms underlying hormone-dependent activation and assessment of a large number of natural and xeno-estrogens a variety of *in vitro* (Serrano et al., 1986) including yeast (anti)estrogenic screens have been developed (Almlof et al., 1997; Bovee et al., 2004a & b; Gustafsson, 1999; McEwan, 1999; Paech et al., 1997; Passos et al., 2009; Wrenn et al., 1993). Many of these employ the transcriptional activation of β-galactosidase, and upon cell lysis, the measurement of the dye reaction. The green fluorescence protein production of hER-transformed *S. cerevisiae* cells in relation to controls with and without 17β-estradiol (E_2) was successfully established as a simple screening system for the quantitative determination of estrogenic bioactivity of steroids samples. With a detection limit for E_2 of 5 pg/ml (0.018 nM) in the *S. cerevisiae pdr5 snq2 yor1* mutant indicator strain, thus this screening system offers a sensitive, fast and reliable method for potential routine testing of estrogen bioactivity.

CHAPTER 6

RDI1 mediated modulation of the in vitro estrogen receptor transactivation response in *Saccharomyces cerevisiae* requires a functional AF-1 domain

6.1 Introduction

The human estrogen receptors (ERα and ERβ) belong to the nuclear receptors (NR), a large family of ligand dependent transcription factors responsible for translating environmental changes directly into the modulation of transcriptional activity and, thus, phenotypic alterations. In the absence of hormone, the receptors are tethered in a multiprotein inhibitory complex in the cytoplasm. The binding of ligand induces the release of the complex and thus conformational change that promotes dimerization and, subsequently, high-affinity binding to specific estrogen-response elements (EREs) located within the regulatory regions of target genes. hERα and hERβ exhibit modular properties whereby individual domains were subscribed to distinct functions. Both receptors share common protein structures with independent but interacting domains of various degrees of similarity (12-97% amino acid homology): the N-terminal A/B domain, the central DNA-binding domain (DBD, C-domain) and the C-terminal ligand-binding domain (LBD, D/E/F domain). The C-domain is important for receptor dimerization and, via the cysteine-rich, zinc-coordinated structure, binding to DNA. The C-terminal domain, organised in 10-13 α-helices comprises besides the LBD one of the transcriptional regulatory functions, the ligand dependent activation function AF-2 (core in helix 12). The second, N-terminal constitutive activation function AF-1 is present only in hERα implicating that synergism between these AFs for full transcriptional activity pertains to hERα and AF-2 as sole transcriptional regulator of hERß which might account for the functional distinct receptor roles. The transcription activations functions (AFs), AF-1 and AF-2 provide surfaces which upon estradiol binding to estrogen receptor (ER) results into conformational changes and thus providing interaction capability with general transcription factors (GTFs) and additional coactivators. However, the recruitment of coregulatory proteins to ERs is required for ER-mediated transcriptional and biological activities. Many cofactors have been identified that participate in estrogen receptor (ER) transcriptional regulation. The best characterized p160 family of proteins (SRC1, GRIP1, and RAC3) was initially known to accelerate ER transactivation through AF2 and AF1 in ligand-dependent and independent manner, respectively (Hong et al., 1997; Onate et al., 1995; Chen et al., 1997; Webb et al., 1998; Heery et al., 1997). Additionally they do the same by recruiting other transcriptional regulatory factors through two activation domains (AD1 and AD2) which in

turn interact with CREB binding protein (CBP)/p300 and coactivator associated arginine methyl transferase 1 (CRAM1) (Voegel et al., 1998; Chen et al., 1999a; Watanabe et al., 2001). CBP/p300 have cross-talks with basal transcriptional machineries like RNA Pol II, TBP and $TF_{II}B$ alongwith other coregulators (p/CAF) which in turn stimulates ER transactivation (Yang et al., 1996; Yao et al., 1996). The histone acetyl transferase activity (HAT) of CBP/p300 also aids acetylation of ER (Wang et al., 2001; Chen et al., 1999b). This multiple signal transduction pathway is also connected with Rho guanine nucleotide dissociation inhibitor alpha (Rho GDIα). A link between ER- and the Rho mediated signaling was first demonstrated when Brx, a hER interacting protein containing a domain virtually identical to the Rho guanine nucleotide exchange factor Lbc (Rho-GEF, these activate Rho-GTPases by accelerating GDP/GTP exchange) was identified (Rubino et al., 1998). Rho GDI signal is transduced to ER by CBP/p300 through GRIP-dependent and independent pathways (Su et al., 2002). Altho ugh *S accharomyces cerevisiae* cells are devoid of the steroid-thyroid-retinoic acid receptor super family yet they have been used to study respective receptors (Caplan et al., 1997; Arnold et al., 1996). The *modus operandi* behind this approach is the expression of the receptor proteins and, upon ligand exposure, reconstitution of the receptor mediated signalling by using a reporter gene preceded by DNA responsive elements. *S. cerevisiae* based screens for estrogenic activity have proved the applicability of this type of assay in diverse disciplines, *viz.*, environmental monitoring and analysis of food components (Shiau et al., 1996; Bovee et al., 2004a & b). In recent years, assays were developed utilizing a yeast-optimised green fluorescent protein (yEGFP) fused to the cis-acting ERE, which has been regarded as advantageous over the cell lysis procedures (Bovee et al., 2004a & b, Hasenbrink et al., 2006). Previously, we have reported construction and characterisation of the hERα and hERß short isoform coupled to the 3xERE-GFP-reporter test system in *S. cerevisiae* to assess (anti)estrogenic compounds. Subsequently, it was being used for sewage treatment plant effluent (STP) assessment. The present study was undertaken to identify yeast factors necessary to participate in the transcriptional activation. Rho GDIα, the mammalian homolog of Rho GDI specifically increases the transcriptional activity of ERα and ERß as well as the glucocorticoid receptor (GR) and androgen receptor (AR) and that this activation is mediated via repression of Rho GTPases (Su et al., 2001). The yeast Rho guanine

nucleotide dissociation inhibitor (Rho GDI, *RDI1*) is the yeast homologue of the mammalian Rho GDIα, a cytoplasmic protein originally identified as a negative regulator of the Rho family of GTP binding proteins, *viz.*, RhoA, Rac1, and Cdc42 (Masuda et al., 1994, Coso et al., 1995; Narumiya et al., 1996; Koch et al., 1997; Clark et al., 2000). This Rho family of GTPases are best known for their ability to regulate actin cytoskeleton remodelling in response to extracellular signals, thereby resulting in changes in cellular morphology, adhesion, and motility (Hall et al., 1998). Additionally, they affect multiple signalling pathways and thereby regulate gene transcription, cell cycle progression and proliferation (Watanabe et al., 2001; Yang et al., 1996; Yao et al., 1996; Wang et al., 2001). Rdi1 as sole yeast Rho GDI can display three biochemical activities: inhibiting the dissociation of GDP from Rho GTPases (Chuang et al., 1993), inhibiting the intrinsic and GAP-stimulated GTPase activity of Rho GTPases (Hancock et al., 1993; Su et al., 2002), and, specifically in yeast, interacting only with Rho GTPases Cdc42, Rho1 and Rho4 by distinct mechanisms thereby extracting these Rho GTPases from cellular membranes to the cytosol (Nomanbhoy et al., 1996; Tiedje et al., 2008). To investigate potential cross-talks in the Rho/*RDI1*-ER signal transduction pathways in yeast we analyzed deletion and overexpression of yeast *RDI1* affecting estrogen receptor transactivity, receptor expression as well as actin cytoskeleton organization. Functional analyses demonstrate that Rdi1p modulation of ligand induced hER transcriptional activity requires a functional AF-1 and full N-terminal domain in hERα and hERß, respectively. This effect is dosage dependent but not related to reduced receptor protein amount, directly accompanied by visible changes in the appearance of disordered actin patches at the periopheral cortex of mutant hER cells and, thus, indicative for cooperative hER and Rho mediated signalling in transcriptional regulation by involving the AF-1 function for recruitment of additional coactivators.

6.2 Materials and methods

6.2.1 Yeast strains and growth conditions

Haploid *S. cerevisiae* yeast strains used throughout this study are listed in Table 6.1. Strains were grown at 30°C. All medium components were dissolved in ultrapure MilliQ

water. Nutritional requirements appropriate for selection and maintenance of mutants and plasmids in the transformed strains were scored on liquid minimal YNB media consisting of 0.67% yeast nitrogen base (YNB) with $(NH_4)_2SO_4$ and 0.5% carbon source (D-glucose) adjusted to pH 6.4 with 50 mM citric acid buffer and supplemented with appropriate amino acids except tryptophan, leucine and the pyrimidine base uracil to utilize auxotrophic marker selection. The plasmids were obtained by standard DNA manipulations according to Sambrook et al. (Sambrook et al, 1989) and used to transform FYAK26/8-10B1 cells (Kolaczkowski et al., 1998) to tryptophan, uracil and leucine prototrophy by standard methods (Rothstein, 1991). All amino acids were purchased from Fluka; YNB from Difco and DMSO from Sigma–Aldrich. All components were of analytical quality.

6.2.2 Strain and plasmid construction

Plasmid pYEX-hERβ short isoform and pERE-CYC1-GFP were described before (Chapter 5). Plasmid YEpE12 (hERα) was a gift from Dr. A. Jungbauer (University of Vienna). For the gene replacement cassette a pUG6 plasmid (Güldener et al., 1996) derivative with the *HIS3* auxotrophic marker gene served as template. For the construction of *RDI1* replacement cassette gene specific homologous 5′ (205 bp) and 3′ (262 bp) flanking regions (Chr. IV, 219288-218680) were amplified using adaptamers (set iii, iv, v, and vi, respectively in Table 6.2) with *Pvu*II/*Sal*I and *Spe*I/*Sac*I restriction sites for ligation to correspondingly digested pUG6 (*HIS3*). The complete *RDI1* replacement cassette was amplified with primers (xi, xii), gel purified and used to transform FYAK26/8-10B1 for histidine prototrophy. Integration confirmation PCR with FYAK26/8-10B1 *rdi1::loxpHIS3loxp genomic* DNA as template used the primer pairs set vii, viii and ix, x, respectively against FYAK26/8-10B1 genomic DNA as negative control. Elimination of *HIS3* was performed by transformation with pSH47 (Güldener et al., 1996) and induction of the recombinase by shift to 2% galactose. pSH47 plasmid loss was scored on YPD full medium supplemented with 5 μg/ml 5FOA.

For the overexpression cassette of *RDI1*, the gene (629 bp) was amplified with adaptamers (set xiii and xiv) including restriction sites (5′: *Sal*I; 3′: *Bam*HI) from genomic *S. cerevisiae* BY4741 DNA. Ligation to vector pHT-P_{Pma1} (Christianson et al., 1992) set

RDI1 expression under control of the *PMA1* promoter. Following linearization by *Not*I and transformation the plasmid integrated into the *TOK1* locus (Chr. X, 256807-254732) by homologous recombination, replacing the complete *TOK1* gene sequence in FYAK26/8-10B1 host strain after selection for histidine prototrophy. Control PCR served to confirm integration in FYAK26/8-10B1 *tok1::*P_{PMA1}*/RDI1* with the primer pair xv and xvi against BY4741 genomic DNA as negative control template. For the hERα AF-1 mutant, a region coding for amino acid residues 15–63 comprising the AF1 core helix (35–47) was deleted by an annealed oligonucleotide mutagenesis approach (primer set xvii and xviii). Plasmid YEpE12 was digested with *Nhe*I and *Not*I, the 9552 bp fragment gel purified and ligated to compatible protruding ends of the annealed 18mer. For the hERß long isoform, the N-terminal lacking region was amplified from pBluescript vector containing full length human hERβ cDNA (Imagene) using suitable adaptamers (viz., set xix and xx) having *Bam*HI/*Msc*I compatible ends. The PCR product was digested with *Bam*HI/*Msc*I and cloned into pYEX-TRP-hER beta short compatible ends (earlier one) to generate pYEX-TRP-hER beta long. For the final pYEX-TRP-v2-hER beta full length plasmid with leu2d deletion (to increase the copy number), *Bam*HI/*Nde*I fragment from pYEX-TRP-hER beta long containing the complete hER beta cDNA sequence was cloned into pYEX-TRP-v2 vector. All oligos are listed in Table 6.2.

Recombinant plasmids recovered from transformed *E. coli* XL1-blue cells were mapped by restriction analysis and confirmed by sequencing (GeneART). Computer analysis of nucleotide and amino acid sequences was performed using the Vnti software (Informax). Constructed *Δrdi1* and P_{PMA1}*/RDI1* strains were cotransformed with pYEX-hERβ long or pYEX-hERß short and pERE-CYC1-GFP or YEpE12 (hERα) or YEpE12-mutAF1 and pERE-CYC1-GFP sets (Table 6.1) and grown under conditions depicted in 6.2.1.

6.2.3 Western blot

Yeast cells were grown to an OD of ~1 (corresponding to 1.2 x 10^7 cells/ml). 10 ml of cells were harvested by centrifugation and washed with distilled water. Protein extraction was carried out using the adapted protocol by Laemmli, 1970. Protein content was determined using the Bio-Rad protein assay kit (Bio-rad Laboratories).

Table 6.1 Haploid yeast strains

	Genotype	Source
FYAK26/8-10B1	*MATa, ura3-52, trp1Δ63,leu2 Δ1, his3Δ200,GAL2+,pdr5- Δ1::hisG,snq2::hisG, yor1-1::hisG*	Kolaczkowski et al,1998
FYAK26/8-10B1/ *Δrdi1*	*MATa, ura3-52, trp1Δ63,leu2 Δ1, his3Δ200,GAL2+,pdr5- Δ1::hisG,snq2::hisG, yor1-1::hisG, Δrdi1*	This study
FYAK26/8-10B1/*RDI1**	*MATa, ura3-52, trp1Δ63,leu2 Δ1,his3Δ200,GAL2+,pdr5-Δ1::hisG,snq2::hisG, yor1-1::hisG,tok1::P_{PMA1}::RDI1*	This study
SC1	FYAK26/8-10B1/ *Δrdi1* [pERE-CYC1-GFP]	This study
SC2	FYAK26/8-10B1/ *Δrdi1* [pERE-CYC1-GFP] [YEpE12]	This study
SC3	FYAK26/8-10B1/ *Δrdi1* [pERE-CYC1-GFP] [pYEX-hERβ short]	This study
SC4	FYAK26/8-10B1/*RDI1* [pERE-CYC1-GFP]	This study
SC5	FYAK26/8-10B1/*RDI1* [pERE-CYC1-GFP] [YEpE12]	This study
SC6	FYAK26/8-10B1/*RDI1* [pERE-CYC1-GFP] [pYEX-hERβ short]	This study
SC7	FYAK26/8-10B1/ *Δrdi1* [pERE-CYC1-GFP] [YEpE12-del af1]	This study
SC8	FYAK26/8-10B1/*RDI1* [pERE-CYC1-GFP] [YEpE12-del af1]	This study
SC9	FYAK26/8-10B1 [pERE-CYC1-GFP] [YEpE12-del af1]	This study
SC10	FYAK26/8-10B1/ *Δrdi1* [pERE-CYC1-GFP] [pYEX-hERβ long]	This study
SC11	FYAK26/8-10B1/ *RDI1* [pERE-CYC1-GFP] [pYEX-hERβ long]	This study
SC12	FYAK26/8-10B1 [pERE-CYC1-GFP] [pYEX-hERβ long]	This study

*** Overexpression strain**

Table 6.2 Oligonucleotides

Oligonucleotide	Sequence (5′-3′)
i. pUG6-His s	gagatctagaaattcccgttttaagagcttggtgag
ii. pUG6-His a	gagactcgagcgagttcaagagaaaaaaaaagaaaaagc
iii. RDI1_5′_s	gagacagctgcctccccaacgctttaatcattctg
iv. RDI1_5′_a	gagagtcgacgctcaatactcgagttattcgtgtggct
v. RDI1_3′_s	gagaactagtcttgaaatgcacaatgtagatatggcctg
vi. RDI1_3′_a	gagaccgcggcaccaaacggcaatttattttattagtgatc
vii. RDI1_out_5_s	tccgtaagaccaggtgaacctagcg
viii. His_cass_a	agagtgtactagaggaggccaagagt
ix. His_cass_s	ccaaaggtgttcttatgtagtgacacc
x. RDI1_out_3_a	ctttgaccaaattcgaagcttccc
xi. RDI1_cass_s	cctccccaacgctttaatcattctg
xii. RDI1_cass_a	caccaaacggcaatttattttattagtgatc
xiii. RDI1_cds_s	gagagtcgacccaccatggccgaagaaagtaccgacttt
xiv. RDI1_cds_a	gagaggatccttatttttgacaatttcgacccccc
xv. T317	agttgggtaacgccagggttttcc
xvi. T187	aagagggccgctgctctctg
xvii. hera_daf1_s	ctagcgggatggccctagccgc
xviii. hera_daf1_a	ggccggccgtagggccatcccg
xix. her_bh1_ad_s	gagaggatccaccatggatataaaaaactcaccatctag
xx. herb_int_a	tagcgatcttgcttcacaccagg

For detection of hERα and hERβ proteins, ~20 μg of total protein were separated by SDS–PAGE (10% polyacrylamide). The gel was run at 120 V until the loading dye ran off the gel (approximately 90 min). Proteins were transferred to a nitrocellulose membrane (Amersham) at 120 V for 60 min at approximately 4°C by wet blotting. Blots were incubated with anti-hERα and hERβ rabbit polyclonal antibody (1:200) (Santacruz) respectively and with a secondary alkaline phosphatase-conjugated goat anti-rabbit antibody (1:1000) (Santacruz). Colour development was performed in Fast Red Solution (Sigma).

6.2.4. Assay conditions and fluorescence monitoring

For quantitative assessment of growth phenotypes and fluorescence development logarithmic growing cells (70 % budding) were diluted to a start OD_{600} of 0.4 (Pharmacia Ultrospec 2000 Spectrophotometer) corresponding to 3.25 $x10^6$ cells/ml. For each tested compound at least 3 independent tests were carried out in duplicate on different days. Test compounds were dissolved in DMSO (final concentration did not exceed 0.5 %) and added to the test cultures of yeast strains in a total volume of 200 μl. The growth was estimated by endpoint OD_{600} measurements following 16.5 h incubation in transparent 96-well microtitre plates using a microplate reader (Tecan, Spectrofluor Plus). Tests were considered as valid when the turbidity of the control cultures increased at least five fold during the incubation period. For fluorescence development read-outs, the excitation wavelength was adjusted to 485 nm and emission was observed at 535 (25 nm bandwidth). Blank and negative control cultures served for the correction of medium background and the accumulation of oxidized flavines in the late growth phase, respectively.

6.2.5. Data capture and evaluation

After 16.5 h incubation, obtained end point fluorescence (FL) values (corrected for blanks) were divided by growth determined as OD 600 (corrected for blanks, OD) for each replica well to normalize fluorescence for cell number (FL/OD). To increase the reproducibility of results, the FL/OD values obtained for a test compound at a given concentration were expressed as fractional values of the maximal response of a saturating

concentration of the reference compound E_2. The fractional values (response relative to the maximal E_2 ($R_{relβÖ}$) at a given concentration of test compound (ct) were calculated according to $R_{relβÖ}$ (ct) = (FL/OD(ct)-bottom)/(top-bottom), with "top" corresponding to the fitted FL/OD at saturating E_2 concentration and "bottom" to the fitted FL/OD for the negative (solvent) control.

The top and bottom values were obtained by Hill equation fit:

$$y(x) = bottom + \frac{(top-bottom)}{1+10^{(LEC50-x)*hill\_slope}}$$

with y(x) =FL/OD at the actual compound concentration, x = the decadical logarithm of compound concentration, LEC_{50} = d ecadical logarithm of EC_{50}, top = fitted maximal FL/OD at saturating concentrations, bottom = fitted maximal FL/OD of negative control and hill_slope as the hill steepness parameter to the FL/OD values for each E_2 concentration using the R function *nls* (The R Foundation for Statistical Computing, http://www.r-project.org/). Dose-response data for test compounds were obtained from the $R_{relβÖ}$ (ct) for each test compound using the analogous fitting algorithm. EC_{20}, EC_{50}, and EC_{90} values were calculated from the fitted dose-response curves (Table 4.3 and 4.4), confidence intervals were determined using the R function *confint*. Relative potencies (E_2 = 1) were determined as ratio of EC_{50} of E_2 and the compound EC_{50}.

6.2.6 Microscopy

Yeast strains were cultured under various conditions (w/o any exposure and with exposure to E_2) for 16.5 h at 30°C with shaking at 950 rpm and 1 ml of each culture conditions were washed with MilliQ, aliquoted and fixed with 3.7% formaldehyde for 1 h. Cells were then harvested and washed twice with PBS + BSA (1 mg/ml) + 0.1% (v/v) Triton X-100. The pellet was then resuspended in 50 µl PBS/BSA buffer and 3U of rhodamine phalloidin (Molecular Probes) were added, followed by an incubation of 30 min in the dark at room temperature. Cells were then washed twice with PBS + BSA (1 mg/ml) (w/o Triton X-100) and were examined under Olympus Fluoview FV-1000 (Olympus, Japan) with a 60x oil-immersion objective.

6.3 Results

The previously described *S. cerevisiae Δpdr5 Δsnq2 Δyor1* mutant was used as host for the construction of the *Δrdi1* and *RDI1* overexpression yeast strains and subsequent heterologous expression of the hERα and hERβ receptor/reporter system. The *Δrdi1* deletion and *RDI1* overexpression host strains were constructed via homologous recombination of genomic DNA replacing the target gene with the *HIS3* marker. Elimination of *HIS3* was performed by transformation with pSH47 in both the cases. For overexpression of *RDI1* the cDNA was set under control of the constitutive plasma membrane ATPase promoter P_{PMA1} and the construct replaced the single non-essential gene *TOK1* (Chr. X). The expression of hERα and hERβ were based on a two plasmid strategy, the episomal expression of the individual receptors alongwith the receptor cDNA was controlled by the constitutive CUP1 promoter and the cis-acting estrogen hormone responsive element (ERE) fused to the yeast enhanced green fluorescent protein (yEGFP) respectively. The advantage of using the *S. cerevisiae* mutant devoid of three major transporters conferring pleiotropic drug resistance is to enhance the sensitivity of the assay in a five fold increased manner in comparison to wild type strain.

Both strains, *S. cerevisiae Δpdr5 Δsnq2 Δyor1 Δrdi1 and S. cerevisiae Δpdr5 Δsnq2 Δyor1 tok1Δ1::* P_{PMA1}*/RDI1* overexpression served as hosts for the expression of the hERα and hERß receptor/reporter constructs. The sensitivity of the hERα and hERβ bioactivity assay in deletion and overexpression background of *RDI1*, represented by the calculated EC_{50} values as obtained from the fit of the Hill function by nonlinear regression. Among the natural hormonal compounds for E_2 as perfect ligand, EC_{50} values of wild type, deletion and overexpression strain of *RDI1* 324.9, 109.9, 226.6 pg/ml respectively for hERα ; 193.7, 41.8 and 71.9 pg/ml respectively for hERβ short isoform. For ethylene estradiol, 100.3, 29.5, 84.0 pg/ml respectively for hERα; 80.9, 32.3 and 30.0 pg/ml respectively for hERβ short isoform. Among the plant secondary metabolites for genistein as isoflavone, EC_{50} values of wild type, deletion and overexpression strain of *RDI1* 23.6, 14.8, 13.5 ng/ml respectively for hERα; 23.9, 2.1 and 0.8 ng/ml respectively for hERβ short isoform. No EC values could be determined for the ER-receptor antagonist tamoxifen citrate.

On the other hand, in case of newly constructed hERα N-terminal activation domain (AF-1) mutant ($hER\alpha_{\Delta aa15\text{-}63}$), EC_{50} values of wild type, deletion and overexpression strain of *RDI1* 774.88, 12.89, 32.75 pg/ml respectively for E_2 and for the same compound hERß long isoform showed 154.98, 609.42 and 306.14 pg/ml respectively. For ethylene estradiol, 74.93, 226.44, 12.05 pg/ml respectively for $hER\alpha_{\Delta aa15\text{-}63}$; 54.34, 78.61 and 47.8 pg/ml respectively for hERβ long isoform. Upon genistein exposure EC_{50} values of wild type, deletion and overexpression strain of *RDI1* 12.89, 1.18, 0.44 ng/ml respectively for $hER\alpha_{\Delta aa15\text{-}63}$; 13.54, 14.73 and 10.39 ng/ml respectively for hERβ long isoform. Similar as earlier no EC values could be determined for the ER-receptor antagonist tamoxifen citrate. All EC_{20}, EC_{50}, EC_{90} values has been listed in Table 6.3 and 6.4.

In case of natural estrogen E_2, *RDI1* deletion as well as overexpression strain of hERα and hERβ short isoform were more sensitive than wild type strain (Fig. 6.1A and D). But for hERβ short isoform, both deletion and overexpression strain of *RDI1* have shown lower transactivation response. For synthetic estrogen ethylene estradiol, again *RDI1* deletion as well as overexpression strain of hERα and hERβ were more sensitive than wild type strain, whereas hERβ has shown much lower transactivation response for both deletion and overexpression strain of *RDI1* in comparison to E_2. Upon genistein exposure, all strains of hERα has shown lower sensitivity, but deletion and overexpression ones are even lower than wild one, though the difference was not that much. Responses for hERβ deletion and overexpression strains were also not sound for this compound. Tamoxifen citrate has failed to induce any considerable response for all the strains of hERα and hERβ (Fig. 6.2A and D).

Western blotiing revealed no considerable change in hERα (Fig.6.3A) and hERβ short isoform (Fig.6.3B) protein production following deletion and overexpression of *RDI1* in comparison to nontransformed host cells as control. So, neither deletion nor overexpression has resulted into depleted level of hER protein expression, thus proving functional aspect of the system.

For E_2, $hER\alpha_{\Delta aa15\text{-}63}$ *RDI1* deletion as well as overexpression strain showed high sensitivity in comparison to full length normal hERα, but having low sensitivity for wild type strain. But surprisingly for hERβ long isoform, *RDI1* deletion as well as overexpression strain sensitivity get decreased, but transactivity regained as normal hERα.

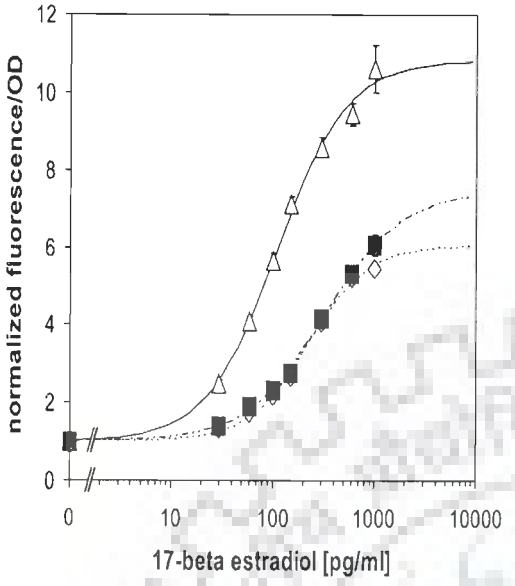


Fig 6.1A

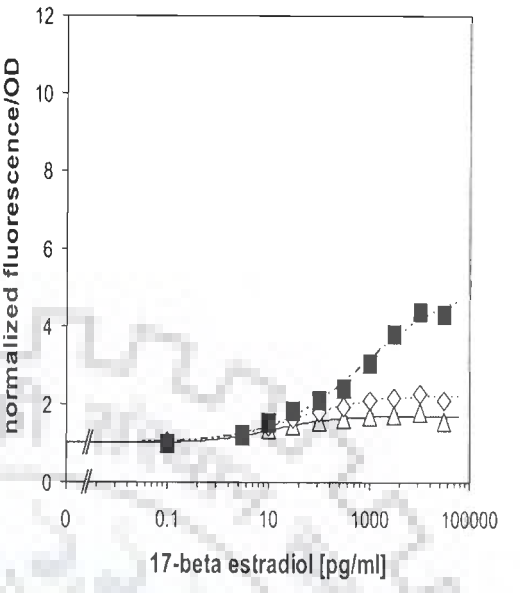


Fig 6.1B

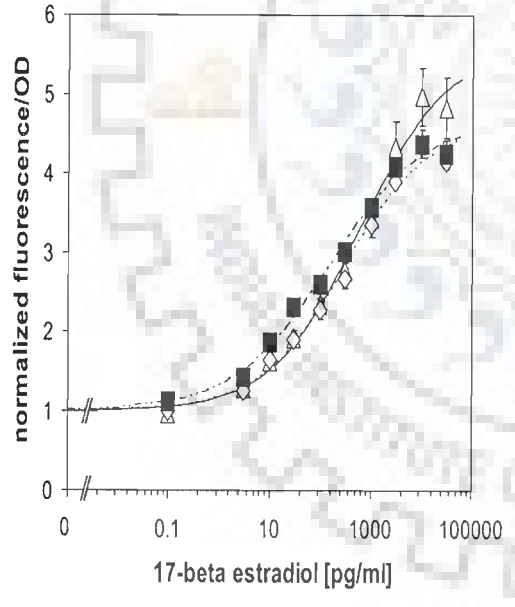


Fig 6.1C

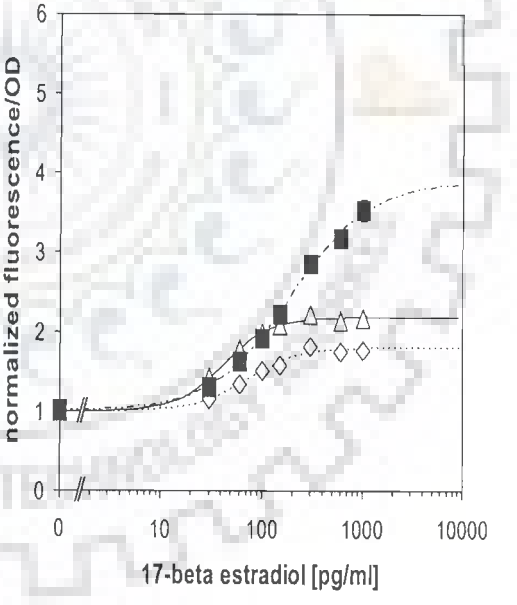


Fig 6.1D

Fig 6.1 Dose response curve fit of *S. cerevisiae* Δ*pdr5,snq2,yor1* hERα normal (6.1A), $hER\alpha_{\Delta 15\text{-}63}$(6.1B) and hERß long isoform(6.1C), hERß short isoform(6.1D) wild-type (■), deletion (Δ) and overexpression (◊) strains expressing the hERα receptor and ERE-GFP reporter constructs on exposure to increasing E_2 concentrations. Fluorescence emission was measured (excitation at 485 nM and emission at 535 nM) and normalized to Fluorescence/OD without test compound. Values are mean of duplicate samples of 3 independent experiments ±SE.

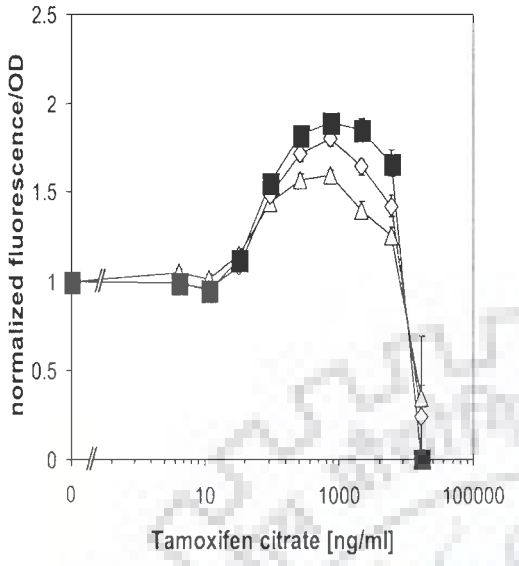


Fig 6.2A

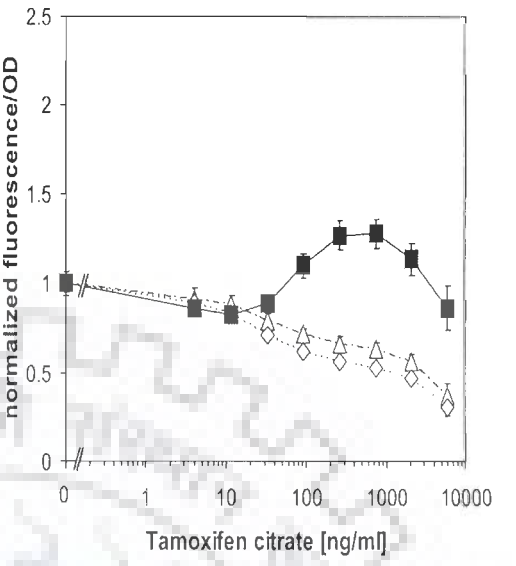


Fig 6.2B

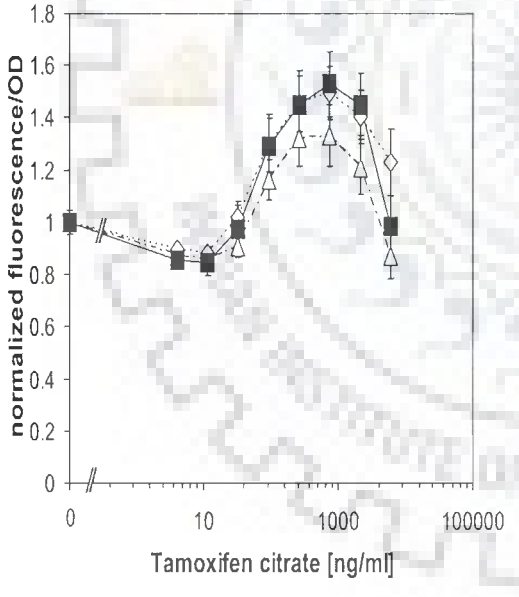


Fig 6.2C

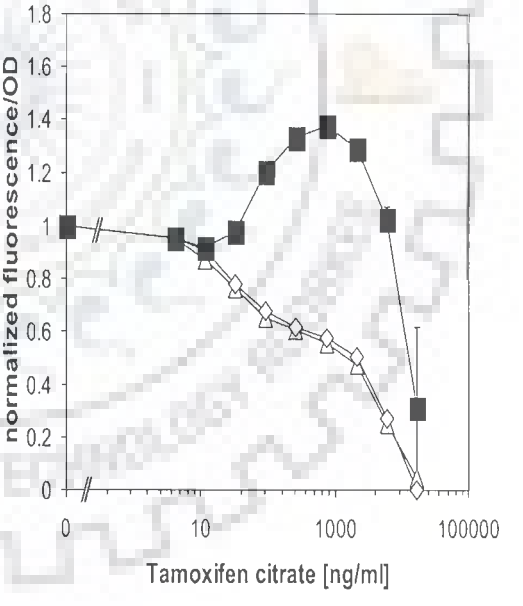


Fig 6.2D

Fig 6.2 Dose response curve fit of *S. cerevisiae* Δ*pdr5,snq2,yor1* hERα normal (6.2A), $hER\alpha_{\Delta 15\text{-}63}$(6.2B) and hERß long isoform(6.2C), hERß short isoform(6.2D) wild-type (■), deletion (Δ) and overexpression (◊) strains expressing the hERα receptor and ERE-GFP reporter constructs on exposure to increasing Tamoxifen citrate concentrations. Fluorescence emission was measured (excitation at 485 nM and emission at 535 nM) and normalized to Fluorescence/OD value without test compound. Values are mean of duplicate samples of 3 independent experiments ±SE.

Table 6.3 EC_{20}, EC_{50} and EC_{90} values for different compounds in case of hERα (Wild type and deletion as well as overexpression strain of *RDI1*), hERβ short (Wild type and deletion as well as overexpression strain of *RDI1*).

		hERα (Wild)			hERα (Deletion)			hERα (Overexpression)		
Class	Compound	EC_{20}	EC_{50}	EC_{90}	EC_{20}	EC_{50}	EC_{90}	EC_{20}	EC_{50}	EC_{90}
Natural Estrogen	17ßE$_2$ (pg/ml)	97.41	324.9	2194.19	37	109.9	619	88.57	226.6	1004.60
Synthetic Estrogen Pharmaceuticals	Ethylene estradiol (pg/ml)	27.26	100.3	790.81	9	29.5	211	24.82	84.0	580.43
Natural Phytoestrogen	Genistein (ng/ml)	7.63	23.6	141.71	6	14.8	66	4.77	13.5	70.20
Synthetic Antagonist	Tamoxifen citrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

		hERß short (Wild)			hERß short (Deletion)			hERß short (Overexpression)		
Class	Compound	EC_{20}	EC_{50}	EC_{90}	EC_{20}	EC_{50}	EC_{90}	EC_{20}	EC_{50}	EC_{90}
Natural Estrogen	17ßE$_2$ (pg/ml)	56.11	193.7	1380.16	20.11	41.8	133.30	32.05	71.9	258.54
Synthetic Estrogen Pharmaceuticals	Ethylene estradiol (pg/ml)	20.30	80.9	720.13	6.64	32.3	396.22	7.42	30.0	275.52
Natural Phytoestrogen	Genistein (ng/ml)	8.38	23.9	126.49	0.23	2.1	64.54	0.05	0.8	53.65
Synthetic Antagonist	Tamoxifen (pg.ml) citrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.= No response detected.

Table 6.4 EC_{20}, EC_{50} and EC_{90} values for different compounds in case of $hER\alpha_{\Delta aa15\text{-}63}$ (Wild type and deletion as well as overexpression strain of *RDI1*), hERβ long (Wild type and deletion as well as overexpression strain of *RDI1*).

		$hER\alpha_{\Delta aa15\text{-}63}$ (Wild)			$hER\alpha_{\Delta aa15\text{-}63}$ (Deletion)			$hER\alpha_{\Delta aa15\text{-}63}$ (Overexpression)		
Class	Compound	EC_{20}	EC_{50}	EC_{90}	EC_{20}	EC_{50}	EC_{90}	EC_{20}	EC_{50}	EC_{90}
Natural Estrogen	17ßE$_2$ (pg/ml)	46.04	774.88	68002.73	1.87	12.89	273.76	3.26	32.75	1266.5
Synthetic Estrogen Pharmaceuticals	Ethylene estradiol (pg/ml)	15.12	74.93	947.46	1.39	226.44	725977.9	4.19	12.05	64.33
Natural Phytoestrogen	Genistein (ng/ml)	3.79	12.89	89.79	0.105	1.18	55.47	0.12	0.44	3.35
Synthetic Antagonist	Tamoxifen (ng/ml) citrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

		hERß long (Wild)			hERß long (Deletion)			hERß long (Overexpression)		
Class	Compound	EC_{20}	EC_{50}	EC_{90}	EC_{20}	EC_{50}	EC_{90}	EC_{20}	EC_{50}	EC_{90}
Natural Estrogen	17ßE$_2$ (pg/ml)	7.83	154.98	17597.21	38.69	609.42	48154.55	21.63	306.14	20418.78
Synthetic Estrogen Pharmaceuticals	Ethylene estradiol (pg/ml)	10.72	54.34	711.65	18.7	78.61	759.19	8.0	47.8	811.33
Natural Phytoestrogen	Genistein (ng/ml)	3.26	13.54	128.97	3.51	14.73	142.96	2.56	10.39	95.98
Synthetic Antagonist	Tamoxifen citrate	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.= No response detected.

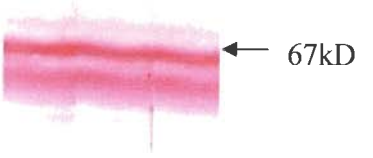


Fig 6.3 A

Fig 6.3 B

Fig 6.3 Western blotting of (6.3A) hERα (~67 kDa) and (6.3B) hERß short (~53 kDa) receptor. Last 3 lanes are showing overexpression, deletion and wild type strains comprising receptor/reporter construct in each case (A & B). First 3 lanes in each case are the negative controls (overexpression, deletion and wild type strain w/o receptor/reporter construct). The extracted protein samples from FYAK26/8-10B1, FYAK26/8-10B1 Δrdi1, FYAK26/8-10B1 *RDI1* (overexpression) were used as negative control.

Both deletion and overexpression strain of $hER\alpha_{\Delta aa15\text{-}63}$ *RDI1* have shown lower transactivation response as of hERβ short isoform earlier (Fig. 6.1B and C). For synthetic estrogen ethylene estradiol, $hER\alpha_{\Delta aa15\text{-}63}$ *RDI1* wild type and overexpression strain showed high sensitivity against low one for deletion strain. Repeatedly hERβ long isoform has shown decrease in sensitiveness for deletion and overexpression strain against increased sensitivity for the wild type, whereas transactivity response for all strain regained as depicted for E_2. Upon genistein exposure, all strains of hERα has shown higher sensitivity, but having lower transactivity. Sensitivity response for hERβ long isoform deletion strain increased but overexpression one is just having opposite outcome and expectedly wild type strain has shown high one. Tamoxifen citrate has failed to induce any considerable response for all the strains of $hER\alpha_{\Delta aa15\text{-}63}$ and hERβ long isoform as earlier (Fig. 6.2B and C). Over all result indicates the importance of functional AF1 domain for interaction with cofactors followed by a sound transactivation response.

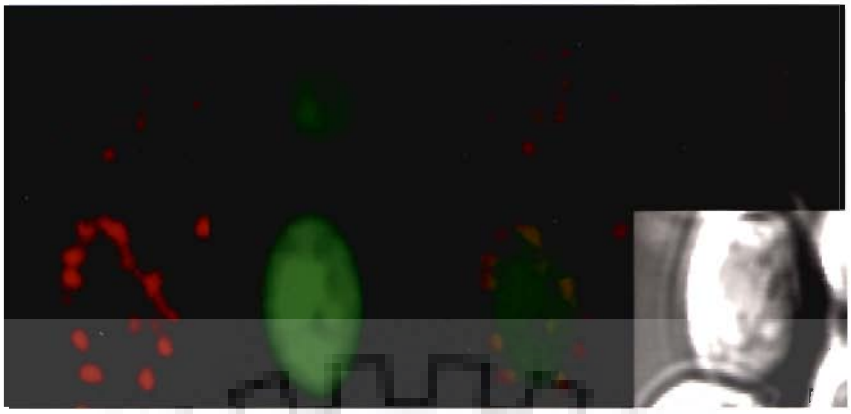


Fig 6.4A

Fig 6.4B

Fig 6.4C

Fig 6.4 Organization of actin cytoskeleton upon rhodamin phalloidin staining. The different conditions are as follows: A) hERα wild type, Upper panel: w/o E_2, Lower panel: with E_2; B) Δ*rdi1*/hERα, Upper panel: w/o E_2, Lower panel: with E_2; C) *RDI1*/ hERα, Upper panel: w/o E_2, Lower panel: with E_2. All panels are printed at the same magnification.

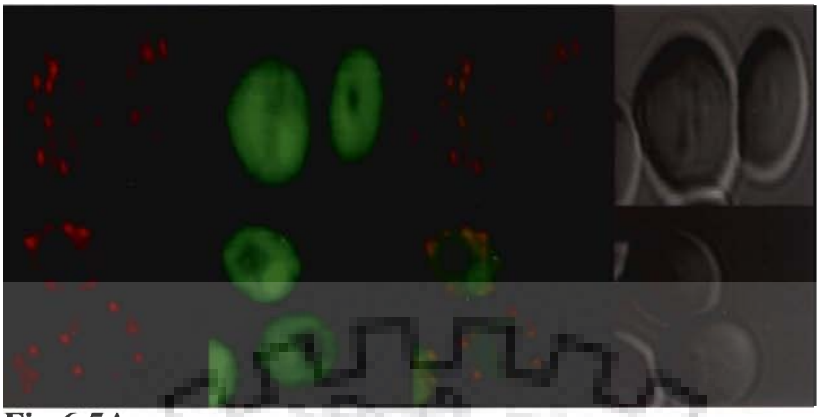


Fig 6.5A

Fig 6.5B

Fig 6.5C

Fig 6.5 Organization of actin cytoskeleton upon rhodamin phalloidin staining. The different conditions are as follows: A) hERβ short isoform wild type, Upper panel: w/o E_2, Lower panel: with E_2; B) Δ*rdi1*/hERβ short isoform wild type, Upper panel: w/o E_2, Lower panel: with E_2; C) *RDI1*/hERβ short isoform wild type, Upper panel: w/o E_2, Lower panel: with E_2. All panels are printed at the same magnification.

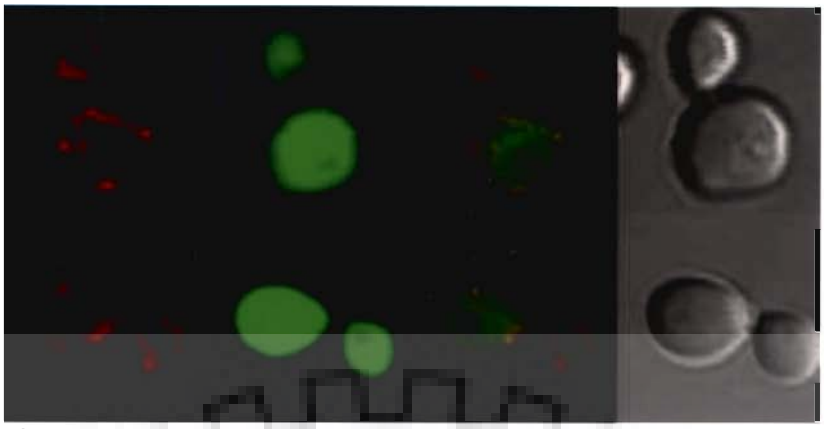


Fig 6.6A

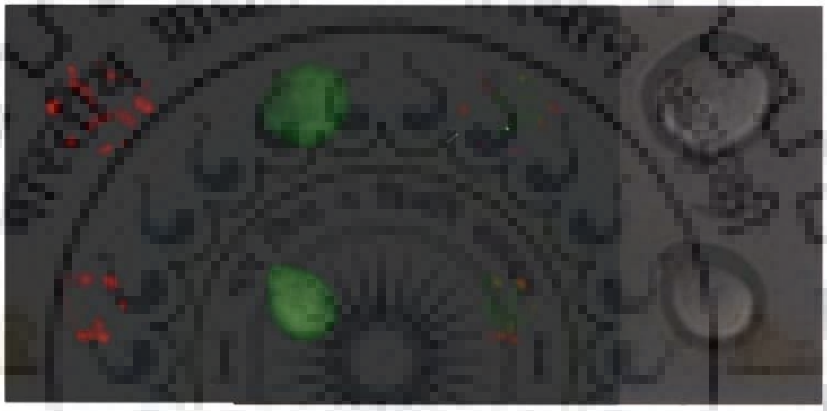


Fig 6.6B

Fig 6.6C

Fig 6.6 Organization of actin cytoskeleton upon rhodamin phalloidin staining. The different conditions are as follows: A) hER$\alpha_{\Delta aa15\text{-}63}$ wild type, Upper panel: w/o E_2, Lower panel: with E_2; B) Δ*rdi1*/ hER$\alpha_{\Delta aa15\text{-}63}$, Upper panel: w/o E_2, Lower panel: with E_2; C) *RDI1*/ hER$\alpha_{\Delta aa15\text{-}63}$, Upper panel: w/o E_2, Lower panel: with E_2. All panels are printed at the same magnification.

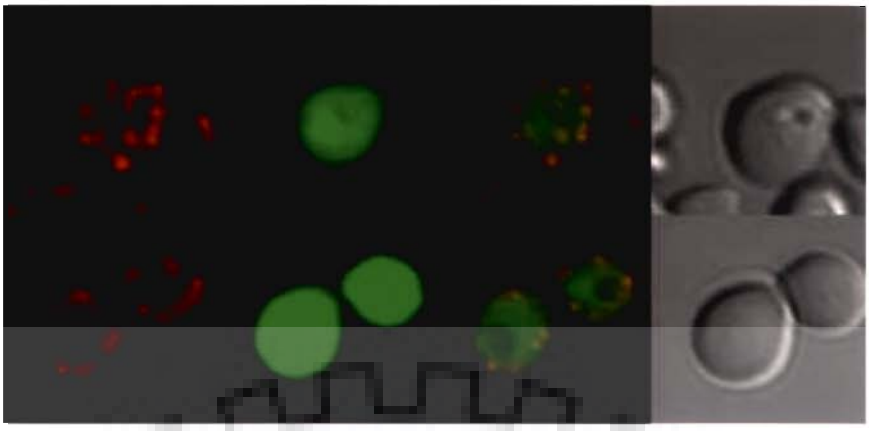


Fig 6.7A

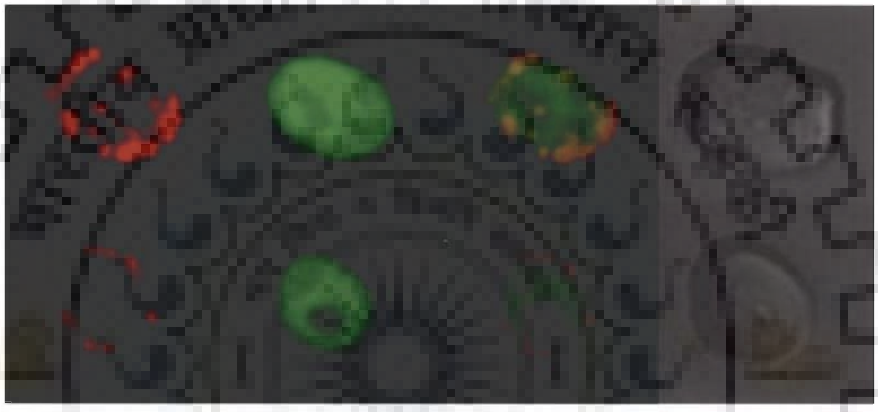


Fig 6.7B

Fig 6.7C

Fig 6.7 Organization of actin cytoskeleton upon rhodamin phalloidin staining. The different conditions are as follows: A) hERβ long isoform wild type, Upper panel: w/o E_2, Lower panel: with E_2; B) Δ*rdi1*/hERβ long isoform wild type, Upper panel: w/o E_2, Lower panel: with E_2; C) *RDI1*/hERβ long isoform wild type, Upper panel: w/o E_2, Lower panel: with E_2. All panels are printed at the same magnification.

Microscopy work in all strains of hERα and hERβ for Rhodamin Phalloidin, has shown both peripheral and random distribution of actin patches following with or without exposure of E_2 respectively (Fig 6.4A-C; Fig 6.5A-C; Fig 6.6A-C and Fig 6.7A-C).

6.4 Discussion

We investigated the effect of *RDI1* as modulator of ER dependent transactivation assays in *S. cerevisiae* to hypothesize and characterize how *RDI1* deletion and overexpression affects ER dependent transactivation assays and affects on actin distribution. We observed overexpression causes no better transactivation rate and deletion causes increased sensitivity in case of normal hERα (Fig 6.1A). On the other hand, both deletion and overexpression have deleterious effects on hERβ short isoform transactivation in comparison to wild type strain, but in this case also the deletion strain has shown better sensitivity than overexpression one (Fig 6.1D). Our results showed that upon overexpression also the transactivity could not surpass the transactivation response of the wild type strain in case of both normal α and short β form of estrogen receptor as well as it has deleterious affect on specifically β form. On the other hand deletion of *RDI1* has increased the sensitivity for hERα and hERβ, though in case of hERβ both deletion and overexpression showed decreased transactivation rate. These results antagonizes the report of Su et al, 2001, where they have shown Rho GDIα, mammalian homologue of Rho GDI, increases the tranactivation response of ER (both α and β isoforms) upon overexpression in ER-negative U20S cell line.

This suggests that there may be some other cofactors which are involved in this multiple cross talks in yeast. Several such possibilities can be plausible. In one model, it has been presented that both ER AF-1 and AF-2 are necessary, but individually not sufficient for the cooperative effect of Rho GDIα and GRIP1 on ER transcription activation. It has been noted that Rho GDIα overexpression increases the number of CBP/p300 molecules recruited to ER, either directly or indirectly through GRIP1 binding, thereby enhancing the functional interaction between AF-1 and AF-2. As MAPKs regulate p160 and p300/CBP activity, a link between the MAPK pathway and Rho GDI-dependent increase in ER transactivation appears possible (Su et al., 2002).

GRIP1 and CBP/p300 may be regulated by other common effectors of the Rho signaling pathway, such as the PAK family of serine/ threonine kinases. As yeast do not have a homologue for GRIP1, it can be speculated that Rho signaling might be occuring place through CBP/p300 via GRIP1-independent mechanism which is dependent on both AF-1 and AF-2 domain of estrogen receptor. Since CBP and p300 modulate the activity of a large number of transcription factors, induction of CBP/p300 activity by Rho GDI could result in vast changes in gene expression. Although the overall consequences of ER activation by Rho GDI is currently unknown, the interplay between Rho signaling and ER function may prove particularly important during normal development when regulation of cellular proliferation by ER may need to be coordinated with Rho-regulated events, such as cellular migration. Therefore, these down stream signaling events may be responsible in differential expression for hERα and hERβ as there is considerable variation in their AF-1 domain in comparison to AF-2 domain (59% homologous). Thereby, we designed a new strategy to unveil whether AF-1 domain of short isoform of hERβ is responsible for the decreased transactivity or not. To achieve this goal we constructed the $hER\alpha_{\Delta aa15\text{-}63}$ by annealed oligo deletion mutagenesis approach and also constructed hERβ long isoform by substituting the lacking N-terminal part through cloning strategy (described earlier in plasmid construction part). When we repeated the assay for same compounds with the newly designed constructs, interesting and supporting result came out. This time the hERβ long isoform regained its transactivity level (Fig 6.1C) and AF-1 mutant of hERα has shown loss of transactivity and had similar nature like hERβ short isoform (Fig 6.1B). Thus, we speculate that *RDI1* mediated modulation of *in vitro* estrogen receptor transactivation response in *S. cerevisiae* requires a functional AF-1 domain as well as full N-terminal domain as it is crucial in ER transactivation in a GRIP1-independent manner through CBP/p300.

Besides activation of multiple signaling pathways, Rho GTPases are responsible for actin cytoskeletal rearrangements. So, either of these two aspects could be responsible for Rho mediated effects on ER. One such key player in polarity establishment is Cdc42 GTPase, which is involved in actin organization, septin organization, and exocytosis (Adams et al., 1990; Johnson, 1999; Longtine et al., 2003). Cdc42, which belongs to the Rho subfamily of the Ras superfamily GTPases, is highly conserved from yeast to humans

at both the sequence (80 to 95% identity in the predicted amino acid sequence) and functional (Johnson, 1999) levels. Cdc42 activity is regulated by at least three types of regulators: the GEF Cdc24; the GAPs Bem3, Rga1, Rga2, and Bem2; and the GDI Rdi1. The Guanine nucleotide exchange factors (GEF) and perhaps the GTPase activating protein (GAPs) are likely to mediate the regulation of Cdc42 by most internal and external signals. Once activated, Cdc42 regulates the organization of the actin cytoskeleton and the septins and interacts with components of the exocytic machinery. The polarized actin cytoskeleton guides exocytosis, leading to polarized cell growth.

However, the crystallographic structure of the Cdc42/Rho GDI complex reveals that the switch I and switch II domains and the geranylgeranyl moiety of Cdc42 interact with Rho GDI (Hoffman et al., 2000; Scheffzek et al., 2000). Yeast *RDI1*, can efficiently extract Cdc42 and Rho1 from the vacuolar membrane (Eitzen et al., 2001) and can also extract Cdc42 from other membranes including the plasma membrane (Richman et al., 2004; Tcheperegine et al., 2005). Activated Cdc42 binds to Bem1, which, in turn, binds to Cdc24 and Cla4, and Cla4 phosphorylates Cdc24 (Bose et al, 2001; Butty et al., 2002; Gulli et al., 2000; Irazoqui et al., 2003). This cascade of events may result in the accumulation of more Cdc24 and Cdc42 at sites of polarized growth. A recent study also indicates that Cdc42 does not have to interact directly with actin patch components (Aguilar et al., 2006, Gao et al., 2003).

There are three classes of Cdc42 effectors: the formin Bni1 (Evangelista et al., 1997); the PAKs Ste20 (Simon et al., 1995, Zhao et al., 1995), Cla4 (Cvrckova et al., 1995), and Skm1 (Martin et al., 1997); and the pair of CRIB motif-containing, yeast-specific proteins Gic1 and Gic2 (Brown et al., 1997; Burbelo et al., 1995; Chen et al., 1997). The formins Bni1 and Bnr1 are required for actin cable formation during polarized growth (Evangelista et al., 2002; Sagot et al., 2002) and are required for actin ring formation during cytokinesis (Tolliday et al., 2002; Vallen et al., 2000). In *S. cerevisiae*, the GBD (GTPase-binding domain) of Bni1 has been shown to interact with Cdc42 (Evangelista et al., 1997) and Rho1 (Kohno et al., 1996), while the GBD of the formin Bnr1 has been shown to interact with Rho4 (Imamura et al., 1997; Kamei et al., 1998). In addition to the direct interaction between Cdc42 and Bni1, Cdc42 also regulates the phosphorylation of Bni1 through its

effector, the PAK Ste20 (Goehring et., 2003). The functional significance of this phosphorylation is not known. So, multiple crosstalks are involved behind this.

Although the association of Rdi1 with Cdc42 and Rho1 has been demonstrated (Koch et al., 1997; Eitzen et al., 2001; Richman et al., 2004; Tcheperegine e t al., 2005), it remained unclear whether Rdi1 also interacts with other Rho GTPases. Recently by using co-immunoprecipitations and membrane extraction assays it was demonstrated that Rdi1 only binds to Cdc42, Rho1 and Rho4 (Tiedje et al., 2008).

Rho1 regulates actin organization via two distinct pathways: the CWI pathway and the formin Bni1. Thus, it appears that Rho1-Pkc1 controls transient actin depolarization during heat stress (Delley et al., 1999) and also controls formin-mediated actin cable formation at 37°C (Dong et al., 2003).

The PAK-family kinase and Cdc42 effector Cla4 negatively regulates binding between Rdi1 and Cdc42 as well as Rho1. The regulation of Rdi1 by Cla4 is mediated via an unknown protein. Till date it is unclear how Cla4 interferes with Rdi1 function. Cla4 could either inhibit the formation of a Rho GTPase-Rdi1 complex or alternatively disrupt an already existing complex. Interestingly, Cla4 also phosphorylates Cdc24 (Bose et al., 2001; Gulli et al., 2000) and it was suggested that both proteins restrict Cdc42 activation via a negative feedback loop (Gulli et al., 2000). A dual role of Cla4 is observed, like In the early stages Cla4 may be involved in the plasma membrane recruitment and subsequent activation of Cdc42, whereas at later stages Cla4 terminates polarity via Cdc24. Importantly, Cla4 also has an effect on Rho1. Thus, Rho1 activity is indirectly regulated by Cdc42. Cla4 has no effect on the interaction between Rho4 and Rdi1. Thus, Cla4 is highly specific for Cdc42 and Rho1.

Rdi1 removes only active Rho4 from the membrane and targets it for destruction to terminate Rho4 activity. The depletion of Rho4 causes the depolarization of actin patches and the loss of actin cables (Adamo et al., 1999; Matsui et al., 1992). It has been shown that Rho4 interacts with Bnr1, but not with Bni1, by two-hybrid and *in vitro* protein-binding assays (Brennwald et al., 1994). So, these multiple cross talks can also be involved at the down stream signaling pathway of *RDI1*, which in turn can affect transactivation rate as well as actin organization.

Here, in our case wild type normal α and ß short isoform strains upon actin staining by fluorescent phalloidin has shown random organization of actin patches all over the cell, but in presence of E_2 progressive localization of actin towards the edge of the cell membrane was observed, where cortical actin complex or actin patches are formed. In case of hERα, both deletion and overexpression strains have shown the same kind of orientation as wild type hERα. But for hERß short isoform, in both the cases upon estrogen induction distribution was not even at peripheral cortex. On the other hand, with the newly constructed $hER\alpha_{\Delta aa15\text{-}63}$ and hERβ long isoform the result is just opposite, i.e., AF-1 mutant α strain showed the distribution pattern like β short isoform. Whereas hERβ long isoform having the orientation as normal α strain earlier. So, in both the cases, whether deletion or overexpression, the order of organization of actin patches to the peripheral cortex are more pronounced for hERα in comparison to hERß short isoform. This may have resulted following the above discussed multiple crosstalks in between Rho GTPases and actin depolymerizing proteins.

On the other hand, suppression of ER transactivation could result either from releasing an ER corepressor which is associated with free G-actin or from binding a coactivator to actin filaments, thus preventing its interaction with the ER. One such actin regulated factor is SWI/SNF complex. Yoshinaga et al., (1992) reported that transcriptional activation by GR or ER is dependent on SWI1, SWI2 and SWI3 gene function in yeast. Hence, further investigations are required to test whether SWI/SNF regulates the effects of Rho GTPases on ER transactivation.

6.4.1 Specificity & Sensitivity

The specificity of this yEGFP based yeast assay system was confirmed by the negligible transactivation response on application of the ER receptor antagonist tamoxifen citrate with all hERα and hERβ. Slight difference were observed between normal wild type hERα and hERβ short isoform mediated responses for the endogenous hormone E_2 for which in this assay EC_{50} values with 324.9 and 193.7 pg/ml for wild type strains respectively, were observed. The value determined were with good parity in comparison to other yeast based biological assays (Bovee et al., 2004a & b; Schultis et al., 2004) and it's more sensitive than receptor binding assay (Gutendorf et al., 2001) but somehow less

sensitive than E-screen assay with MCF-7 cells (Gutendorf et al., 2001). For newly constructed hER$\alpha_{\Delta aa15\text{-}63}$ and hERβ long isoform, obtained EC_{50} values were 774.88 and 154.98 pg/ml with wild type strains respectively. So, as expected hER$\alpha_{\Delta aa15\text{-}63}$ appeared to be less sensitive, whereas sensitivity has slightly increased for hERβ long isoform.

For synthetic estrogen ethylene estradiol, EC_{50} values showed a little bit more sensitivity for both hERα and hERβ. The natural compound genistein showed almost 17 fold higher sensitivity for hERα and 8 fold for hERβ short isoform which is comparable also with the other yeast tests and receptor binding tests (Bovee et al., 2004a; Gutendorf et al., 2001). Newly constructed strains have shown slight increase in sensitivity.

The EC_{50} values for all deletion strains suggest that those strains are more sensitive than the wild type and overexpression strains. But for hER$\alpha_{\Delta aa15\text{-}63}$ and hERβ short isoform the transactivation response for both deletion and overexpression strains are drastically minimal in comparison to normal hERα and hERβ long isoform. It clearly depicts that both deletion and overexpression are detrimental for hER$\alpha_{\Delta aa15\text{-}63}$ and hERβ short isoform.

6.4.2 Relative potency

Based on the relative potencies (Fig 6.8A, B, C and D), a ranking of compounds with the normal hERα deletion and overexpression strain was determined as E_2, ethylene estradiol and genistein. For hERβ short isoform, and hERβ long isoform deletion and overexpression strain the compound ranking was the same; but for, hER$\alpha_{\Delta aa15\text{-}63}$ the ranking was ethylene estradiol, E_2 and genistein. In case of normal hERα, overexpression strain has shown higher relative potency for ethylene estradiol, but lower for genistein and the deletion strain was having just opposite. For hERβ short isoform, both ethylene estradiol and genistein were having higher relative potency for deletion strain and it was just opposite for hERβ long isoform. hER$\alpha_{\Delta aa15\text{-}63}$ is having the same relative potency characteristics as hERβ short isoform. This spectrum of estrogenic activity may characterize the estrogen receptor subtypes, potentially indicative for the different crosstalks following overexpression and deletion of *RDI1*.

6.5 Conclusion

The yEGFP based yeast estrogen bioassay are presently a profound area of interest

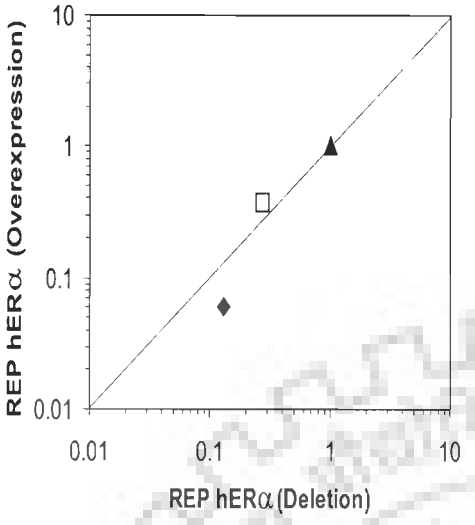


Fig 6.8A

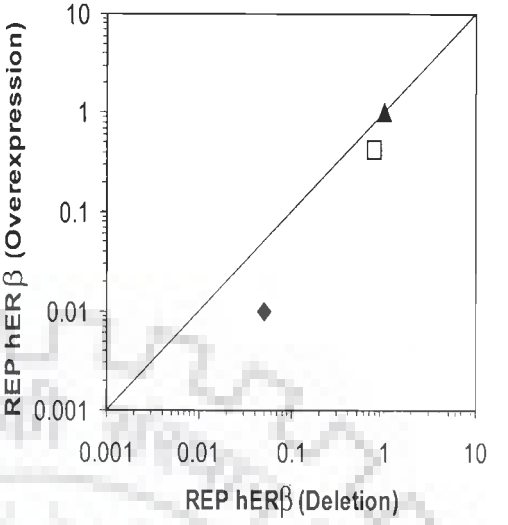


Fig 6.8B

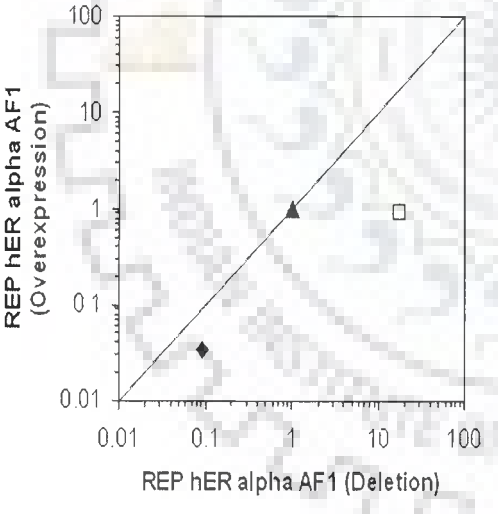


Fig 6.8C

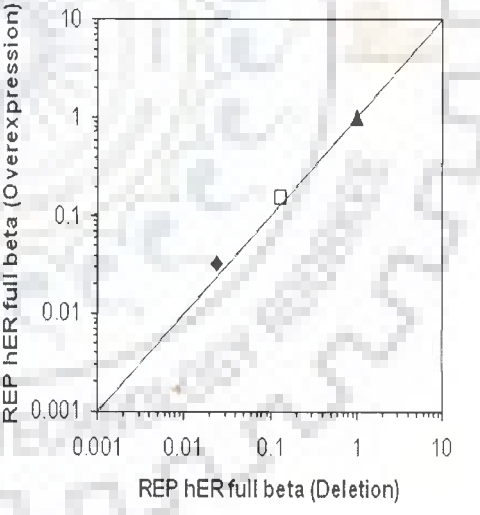


Fig 6.8D

Fig 6.8 Relative potencies (REP, relative to E_2) of natural estrogen E_2 (▲), the synthetic estrogen ethylene estradiol (□), and natural phytoestrogen genistein (♦) for deletion vs. overexpression strain for hERα (6.8A) hERβ short isoform (6.8B), $hER\alpha_{\Delta aa15\text{-}63}$ (6.8C) and hERβ long isoform (6.8D). Diagonal line with a slope of 1 indicates differences in relative potency of single compounds between *RDI1* deletion and overexpression strain of hERα and hERß.

as a directly measurable format within short time tenure. But till date it was not well explored that how the transactivation assay has been affected with *RDI1* as modulator. Though several research have been carried out about the role of *RDI1* in mammalian cell lines to see the effect on ER transactivation, but no such reports are available with the effect for ER transactivation assay in yeast. Most recently, Tiedje et al., (2008) has reported some mechanism behind regulation of Rho GTPases by Rdi1, but that does not comprise any correlation with ER transactivation. Our data showed differential response from hERα and hERβ with deletion and overexpression background of Rdi1 in comparison to wild type. Our data revealed that hERα and hERβ transactivation was severely affected by *RDI1* in different mode and amongst them hERβ short isoforrm is having drastic effect. Finally, with our obtained data we speculated that AF-1 domain is responsible for ER transactivation assays in yeast *S. cerevisiae*.

We've also found this differential effect is not following lower expression of both ER proteins. On the other hand actin cytoskeletal analysis has revealed, whether deletion or overexpression, the order of organization of actin patches to the peripheral cortex are more pronounced for hERα and hERß long isoform in comparison to $hER\alpha_{\Delta aa15\text{-}63}$ and hERß short isoform. So, it can be assumed that there may be some cross talk in between signal transduction pathways through actin polymerizing proteins; and/or involvement of Cdc42/Rho1/Rho4 and/or involvement of other cofactors like SWI/SNF complex. Taken together, it can be speculated that *RDI1* influences ER transcriptional activation in *S. cerevisiae* through CBP/p300 action, which in turn affects estrogen receptor transactivation via GRIP1-independent mechanism and requires a functional AF-1 domain as well as full N-terminal domain.

CHAPTER 7

Summary

Summary

The budding yeast, *Saccharomyces cerevisiae*, has indeed proved to be a powerful model for studying the functions of more complex metazoan eukaryotes. The advantages of yeast arise in part from the relatively short doubling time in culture, the ability to use defined culture conditions and the availability of well-established genetic tools for analysing yeast mutants. In addition, a number of fundamental processes, such as transcription initiation and regulation, appear to have been conserved in yeast (Guarente et al., 1992). It is therefore, not surprising that yeast has proved to be an extremely useful experimental model system for investigating various biological activities like steroid receptor function. Following this reason Arthur Kornberg has mentioned: ``The yeast cell, responsible for the birth of modern biochemistry ... is now budding again. With its rich legacy of genetic and biochemical data, yeast appears to be the most accessible object for current studies of the nucleus, mitochondria, and other complex features of eukaryotic cells" (For the Love of Enzymes, The Odyssey of a Biochemist, Harvard University Press). The first steroid receptors were expressed in yeast over ten years back. Till date, the activity of all the classical steroid receptors as well as a number of non-steroid members of the nuclear receptor superfamily have be en reconstituted in yeast, *S. cerevisiae*. At the same time this model was explored for the development of steroid based bioassay like transactivation assay. Although human cell lines are more sensitive than yeast and may also be able to identify steroidal compounds that require human metabolism for activation into their st eroidogenic state (Legner et al., 1999; Hoogenboom et al., 2001), yet yeast based assays have several advantages. These include robustness, low costs, lack of known endogenous receptors and the use of media that are devoid of steroids. The different steps in steroid receptor signaling could be reconstituted in yeast cells by co-expression of the receptor protein and a reporter gene driven by the appropriate hormone response element (Metzger et al., 1988; Schena et al., 1988, Bovee et al., 2004a; Bovee et al., 2004b; Michelini et al., 2005; Bovee et al., 2007; Sanseverino et al., 2005; Sanseverino et al., 2009). In this way, functional analysis in yeast has facilitated the identification and characterization of a number of genes that are involved at different steps in nuclear receptor signaling.

The present thesis depicts the development of yeast based steroid bioassay approach for the natural endocrine disruptors with major focus on (anti)androgenic, (anti)progestagenic and (anti)estrogenic EDCs and finally a brief insight has been drawn for *RDI1* mediated transcriptional activation of yeast estrogen assay. Towards this end the yeast based assays are being developed for screening and identification of different endocrine disruptors for androgen receptor (β-Gal based), progesterone receptor (yEGFP based) and estrogen receptor (yEGFP based). The androgen receptor based system revealed the presence of androgen mimicking compounds in pulp and paper mill effluents (Chatterjee et al, 2007). On the other hand, the progesterone based system exposes the anti progestagenic effect of leather industry effluents (Chatterjee et al, 2008). A yeast based multidrug transporter deleted estrogen bioassay was also developed for (anti)estrogenic substance screening as well as it was also used for sewage treatment plant effluent assay. Finally, *RDI1* was analyzed to identify its role in the improvement of yeast based estrogen bioassay. In our studies *RDI1* was found to be a crucial effector and modulator of yeast based estrogen bioassays (yEGFP based).

In our first effort, we have constructed an efficient and reliable yeast-based detection system to evaluate the androgenic activity of different natural endocrine disruptors as well as we identified (anti)androgenicity of pulp and paper mill effluents (PPME). This system consists of human androgen receptor and androgen response elements driven β-galactosidase genes transformed in yeast, *Saccharomyces cerevisiae*. The transcriptional activation by known androgens, correlated with androgenic activities as measured by other assay systems. This assay system when applied to evaluate anti-androgenic activities, the known anti-androgens effectively inhibited reporter gene induction by testosterone. The specificity of the assay was tested by incubating the transformed cells with supraphysiological concentrations of non-androgenic steroids and none of them gave a significant response. To the best of our knowledge, there is only one more report from a Swedish group on the analysis of paper mill effluents using recombinant yeast-based reporter system (Svenson and Allard, 2004). But our X-gal based microtiter plate assay requires minimum hands-on time (~16h) in comparison to the CPRG based assay of Swedish group (3-4days). These results suggest that PPME are

rich in androgenic chemicals and the employed detection system could be applicable to primary screening for effectors on androgen receptor functions.

The second study was aimed to develop a sensitive, fast and user friendly progesterone receptor transactivation assay using recombinant yeast cells, *Saccharomyces cerevisiae*, modified to express human progesterone receptor (PR) and progesterone response element (PRE) driving the expression of green fluorescent protein. Stimulation of cells with increasing concentrations of progesterone resulted in significant elevation in fluorescence activity, with the minimum effective dose of progesterone being 0.1 nM. RU486, significantly inhibited progesterone induced transactivation and non-progesterogenic steroids failed to transactivate PR till 10 μM concentrations. About 7 different chemicals (mostly pesticides or their metabolites) like DDT and its metabolites, nonylphenol, endosulfan were screened in this assay system for their role in transactivation and they were all found to be anti-progestative and IC_{50} values within the range of 3–20 μM. Further, the assay was used to analyze the endocrine disrupting activity of extracted water samples from leather industries which are known for their high content of various chemicals and it was found to be rich in anti-progestative compounds. It resulted in about 30% reduction in progesterone induced transactivation. To the best of our knowledge, there is no other report on the analysis of leather industry effluents using yEGFP based yeast progesterone assay. Assays generated here could be used for screening of other EDCs. In conclusion, we demonstrated that this yeast based bioassay provides a rapid and robust assay for high throughput screening of (anti)progestative compounds from various sources.

In our third study, we reported the transactivation assay toward hERα and hERβ receptor expression coupled to the 3xERE-GFP-reporter in a genetically modified yeast strain, devoid of three endogenous xenobiotic transporters (*PDR5, SNQ2,* and *YOR1*), and comparative analysis of natural and synthetic (anti) estrogenic compounds. Among the natural compound, the endogenous estrogen: 17-βE_2; the phytoestrogen genistein, and the synthetic compounds with clinical application ethylene estradiol, tamoxifen citrate were tested. In order to demonstrate the practical application of this assay, some Sewage Treatment Plant (STP) effluent were also examined for their (anti)estrogenicity. Transcriptionally activated GFP fluorescence emissions were normalized to cell growth

determined by absorption and correlated to internal reference standards. Obtained dose-response curves served for EC_{50} value calculation. Assay protocol optimization comprised conditions like agitation, temperature, and pH value to determine the most practical test handling conditions. To the best of our knowledge, this is the first report where STP effluents have been tested with the *S. cerevisiae* strain deleted in the multidrug transporter genes *PDR5, SNQ2 AND YOR1 in vitro* for their estrogenic activities.

Finally, we have explored the *RDI1* mediated estrogen receptor transactivation response in *Saccharomyces cerevisiae*. It is already known that the human estrogen receptors hERα and hERβ mediate transcriptional activation of a large variety of target genes and in a tissue specific manner. To elucidate the role of Rho-mediated signaling in ligand induced hERα and hERß transcriptional activation in *Saccharomyces cerevisiae* we investigated the Rdi1p component. Rdi1p is the yeast homologue of the mammalian Rho guanine nucleotide dissociation inhibitor (RhoGDIα), a cytoplasmic protein identified as negative regulator of the Rho GTPases in mammalian cells and yeast. A hERα N-terminal activation domain (AF-1) mutant ($hER\alpha_{\Delta aa15\text{-}63}$) and a hERß short isoform ($hER\beta_{\Delta aa1\text{-}43}$) in the *rdi1::loxp* background exhibited complete abolishment of the transactivation response that was not restored by overexpression of *RDI1*; whereas normal transactivation response in full length hERα and hERß long isoform strongly supports the involvement of AF-1 domain through GRIP-1 independent mechanism (Su et al., 2002). Simultaneously, a disordered actin organization at the peripheral cortex of mutant hER expressing cells upon estradiol exposure suggest a link between *RDI1* bound Rho signaling by actin polymerization in transmitting signals to the nucleus and hER transactivation, possibly as cooperative recruitment factor for additional transcriptional cofactors via AF-1. So, it can be assumed that there may be some cross talk within signal transduction pathways through actin polymerizing proteins; and/or involvement of Cdc42/Rho1/Rho4 and/or involvement of other cofactors like SWI/SNF complex. Taken together, it can be speculated that *RDI1* influences ER transcriptional activation in *S. cerevisiae* through CBP/p300 action, which in turn affects estrogen receptor transactivation via GRIP1-independent mechanism and requires a functional AF-1 domain as well as full N-terminal domain.

In conclusion, in the present thesis, three potential yeast based *in vitro* steroid bioassay system have been developed for screening some natural (anti)androgenic, (anti)progestagenic and (anti)estrogenic EDCs and a brief attempt was made to unravel the modulation of estrogen receptor by *RDI1* in case of yeast based steroid receptor bioassay. The successfully established three yeast based bioassay could be implied to assay other resources of (anti)androgenic, (anti)progestagenic and (anti)estrogenic EDCs in natural environment as well as they could be used for the assessment of potential side effects of drugs having hormonal side effects. The holistic approach as depicted in this thesis in understanding the *RDI1* mediated estrogen receptor transactivation response shows that it requires a functional AF-1 domain as well as full N-terminal domain. Further studies with deep insights to the downstream signaling mechanism could be undertaken.

CHAPTER 8

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APPENDIX-A

APPENDIX-A

ATCC 77106

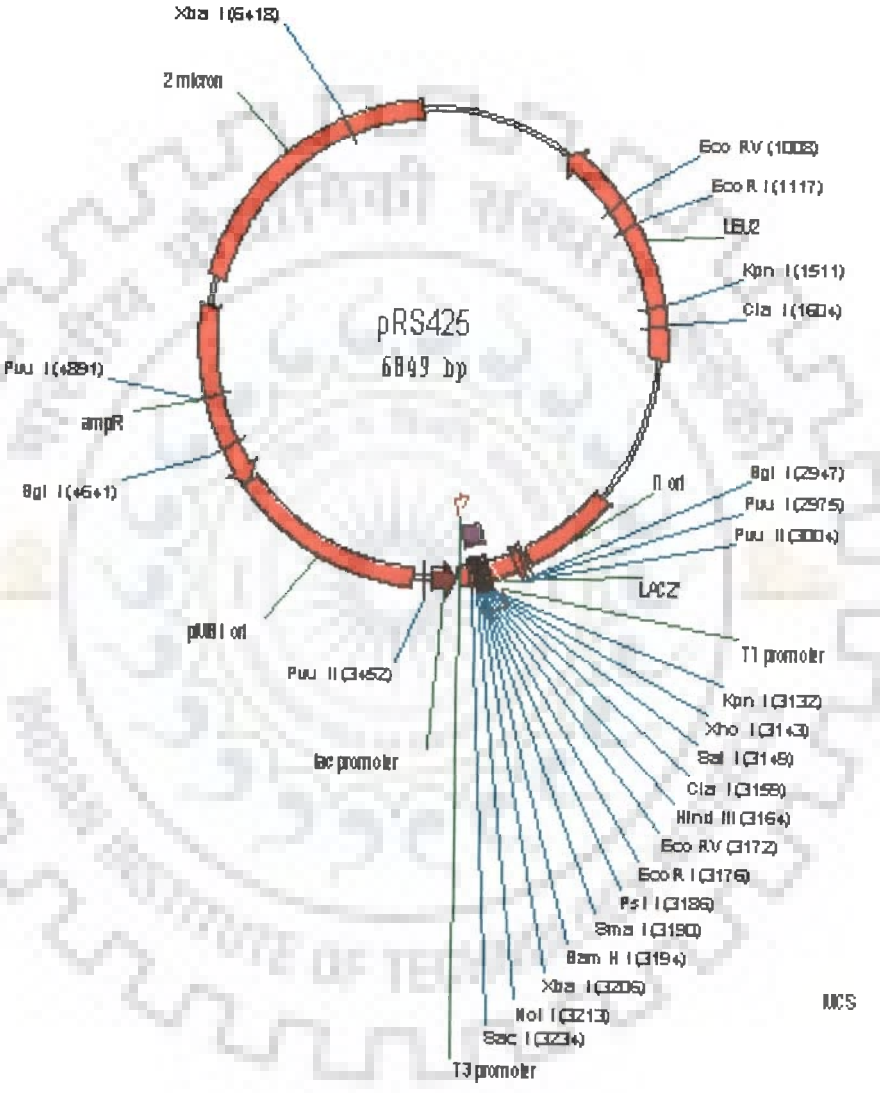

Fig A1 The plasmid pRS425-Leu2-ARS [YE-type (episomal) shuttle vector], the parent for androgen and progesterone receptor constructions, is shown. The locations of all the restriction sites used in making the constructions are shown.

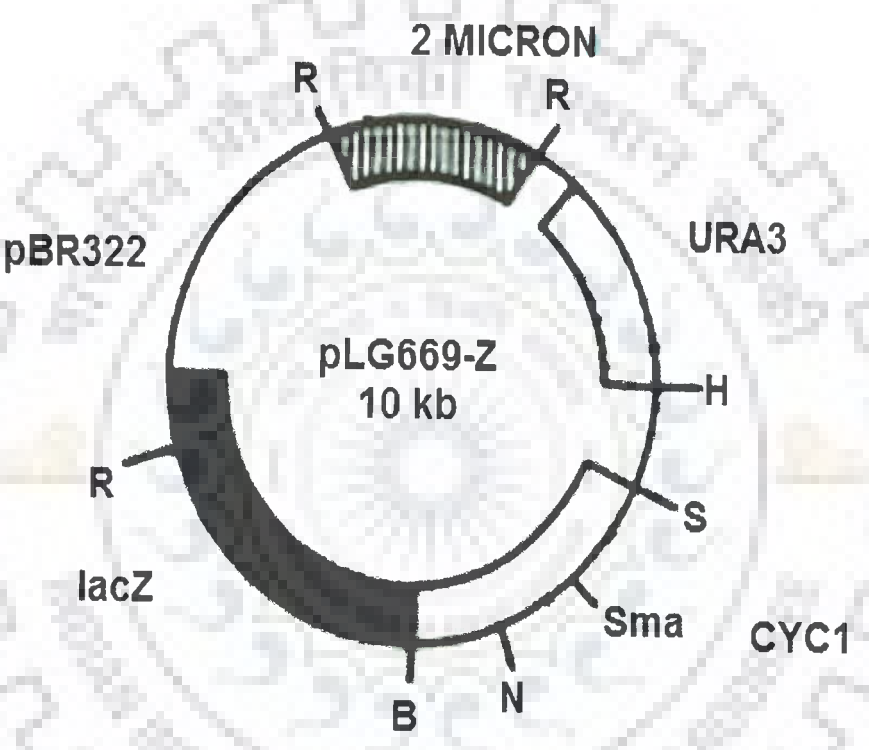

Fig A2: The plasmid pLG669-Z, the parent for β-gal and yEGFP reporter system for androgen as well as progesterone bioassay, is shown. The CYCI portion (open box) has been expanded for clarity. The locations of the restriction sites used in making the constructions are shown. Other restriction sites: B, *Bam*HI; H, *Hind*III; N, *Nde* I; R, *EcoR*I; S, *Sal* I.

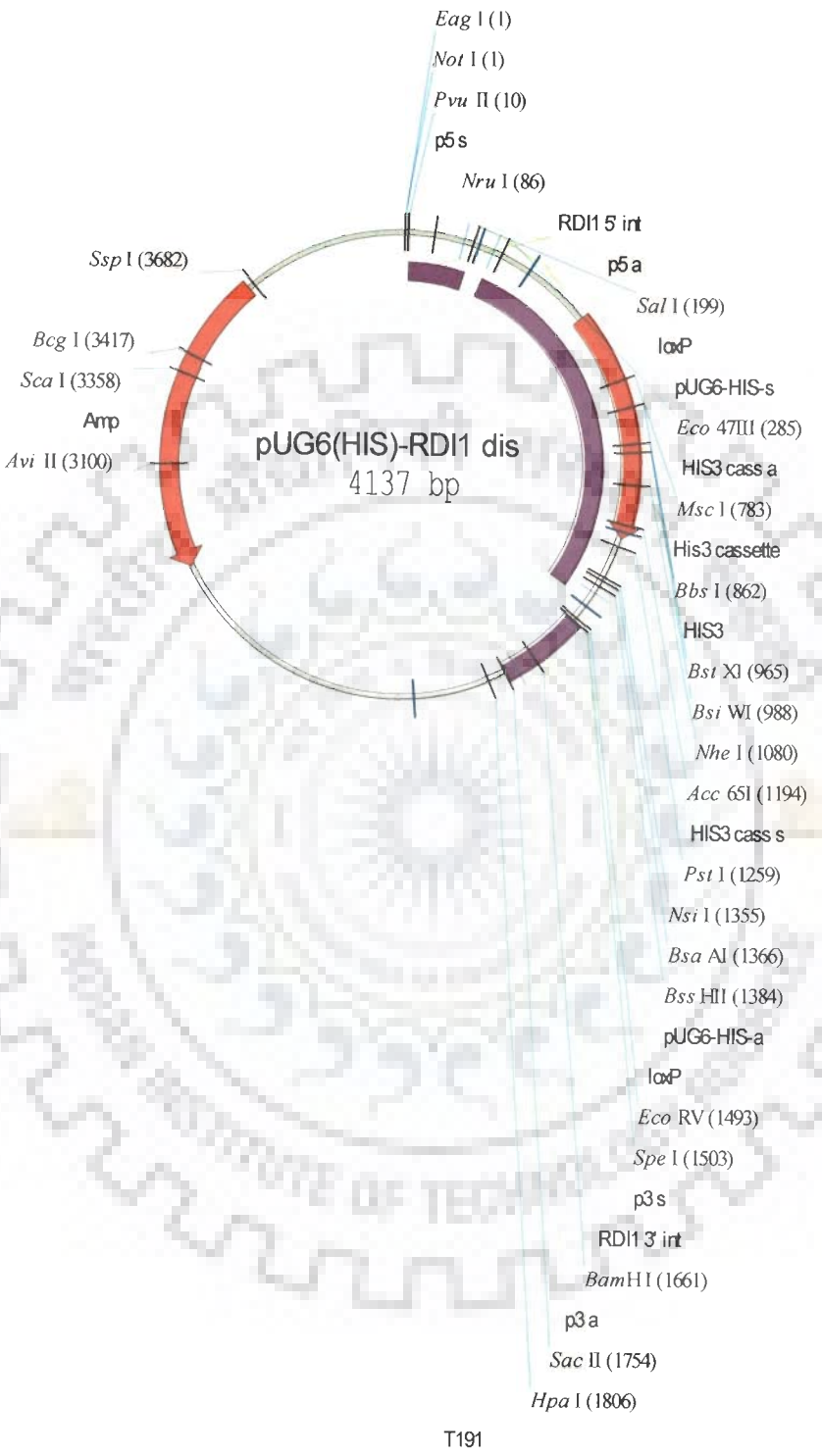

Fig A3 The plasmid pUG6(His)RDI1 dis, the parent for the *Δrdi1* strain constructions, is shown. The complete *RDI1* replacement cassette was picked up by PCR through suitable adaptamers and subsequent transformation in host *S. cerevisiae* yielded *Δrdi1* strain.

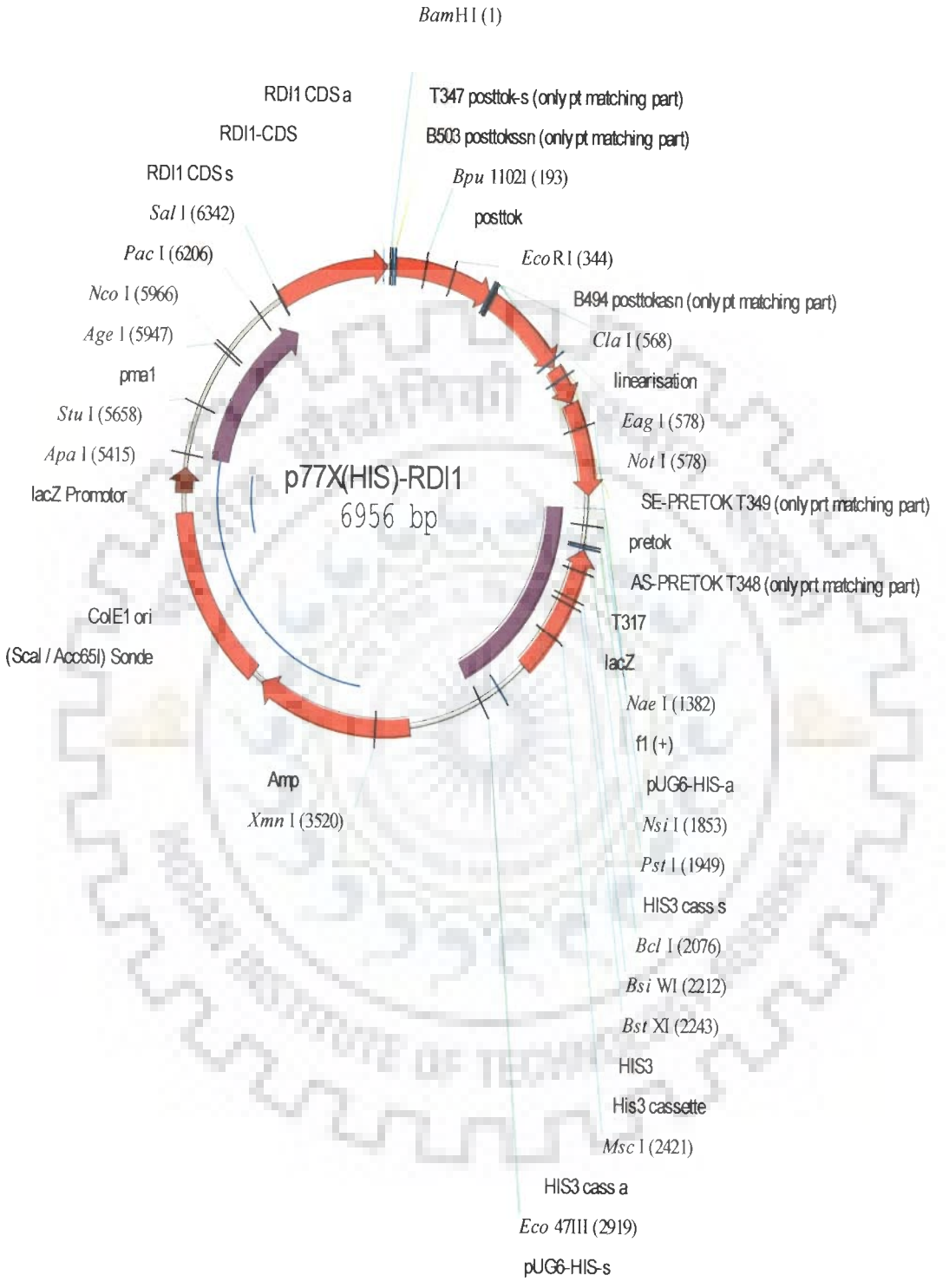

Fig A4 The plasmid p77X(His)-*RDI1* (also designated as pHT-P_{Pma1}-*RDI1*), the parent for the *RDI1* strain constructions, is shown. Following linearization by *Not*I and subsequent transformation in host *S. cerevisiae* yielded *RDI1* stra in.

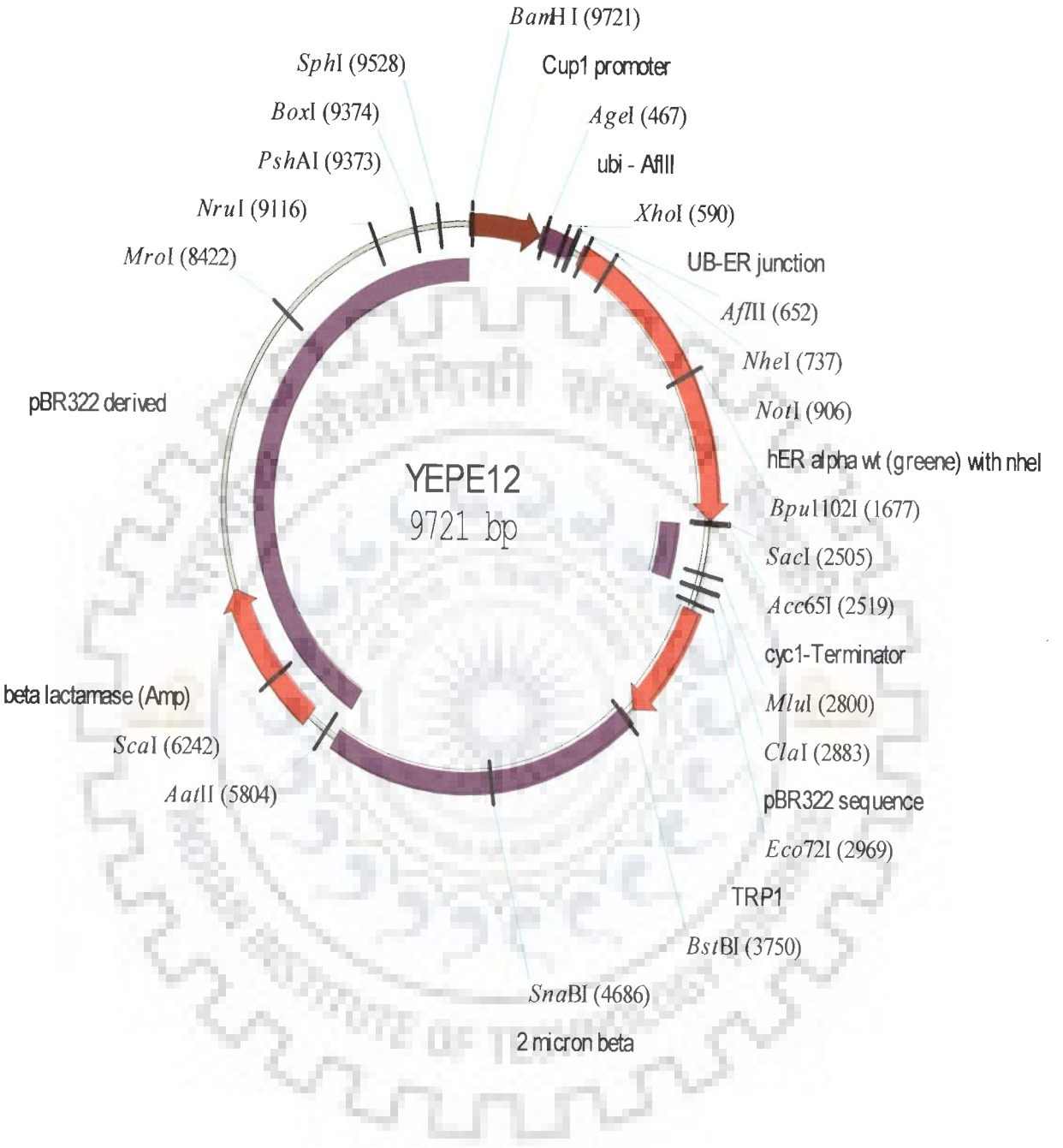

Fig A5 The plasmid YEpE12, the parent hERα construct, is shown. The locations of all the restriction sites used in making the constructions are shown.

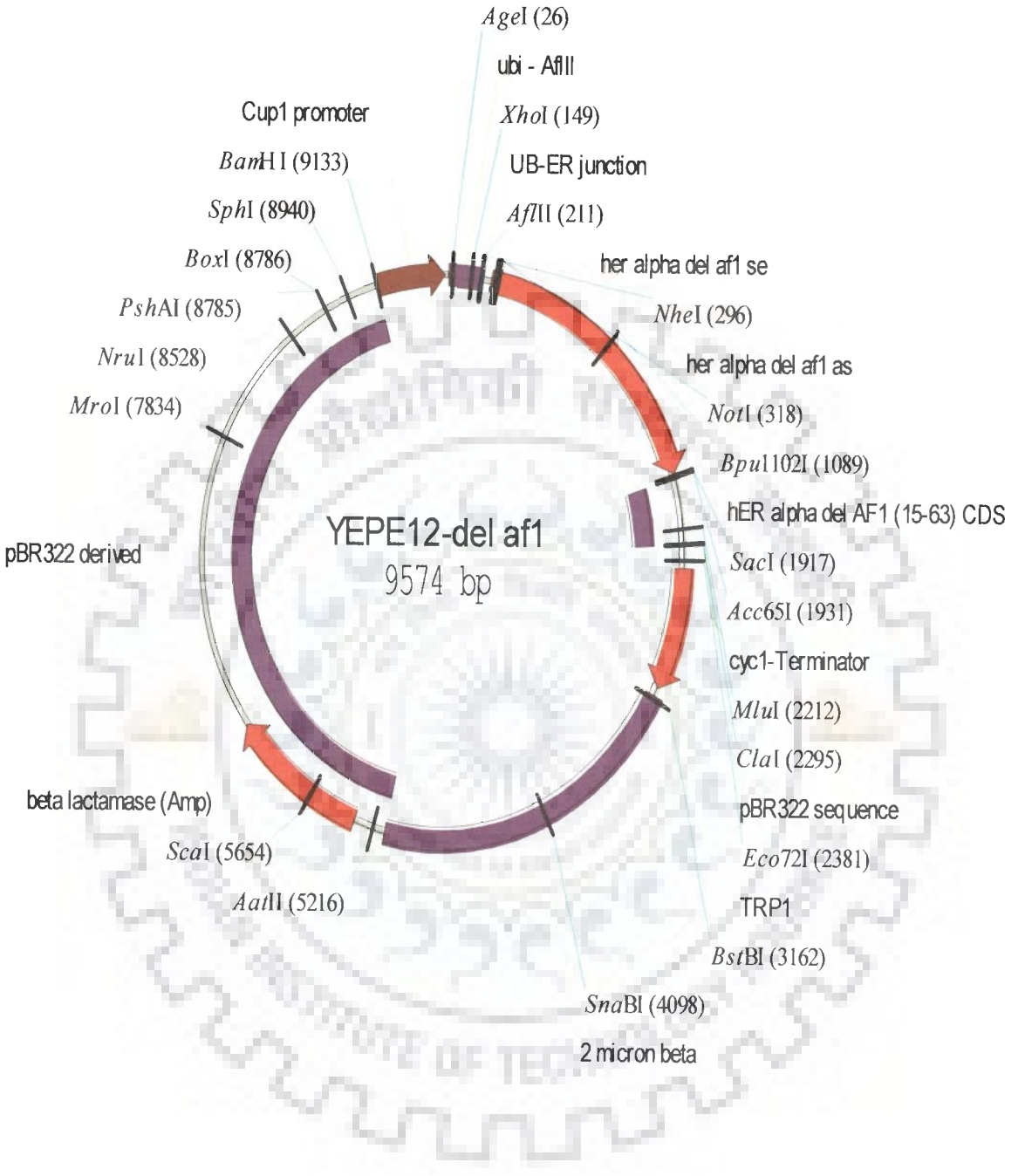

Fig A6 The plasmid YEpE12 del AF1, the mutated hERα construct, is shown. The locations of all the restriction sites used in making the constructions are shown.

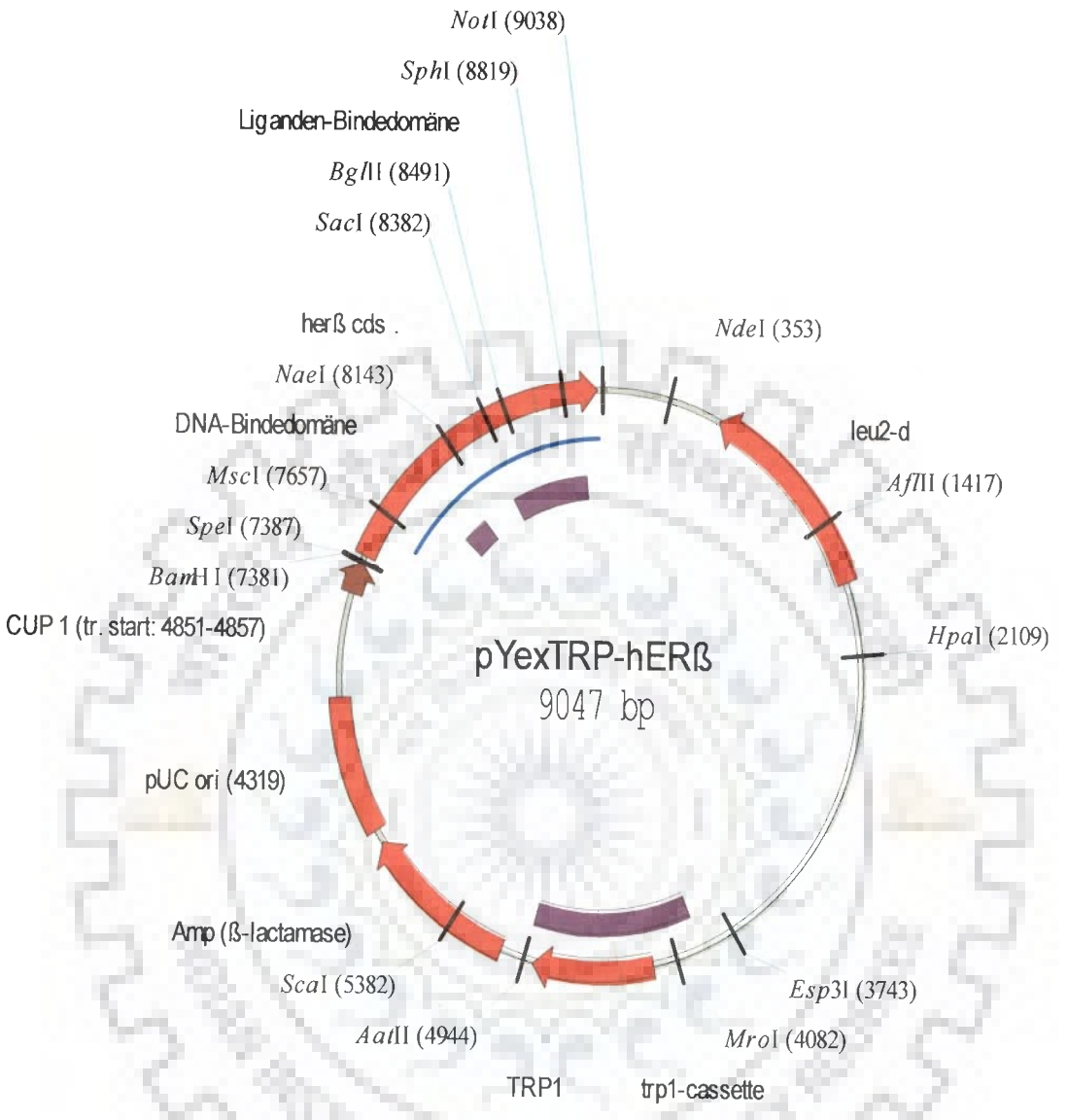

Fig A7 The plasmid pYEX-TRP-hERβ short ($hER\beta_{\Delta 1\text{-}43}$), the hERβ short construct, is shown. The locations of all the restriction sites used in making the constructions are shown.

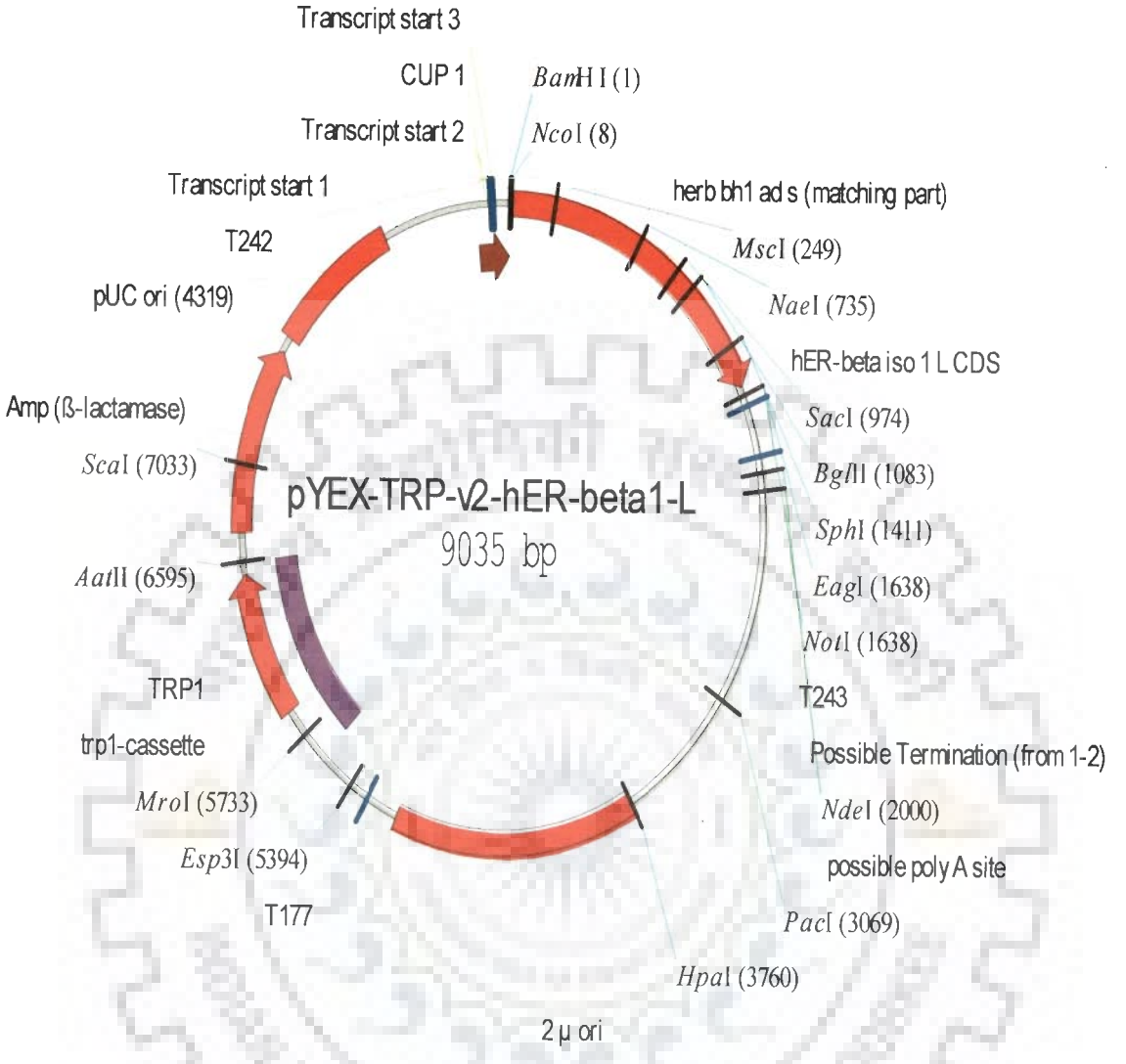

Fig A8 The plasmid pYEX-TRP-v2-hERβ 1-L, the hERβ long construct, is shown. The locations of all the restriction sites used in making the constructions are shown.

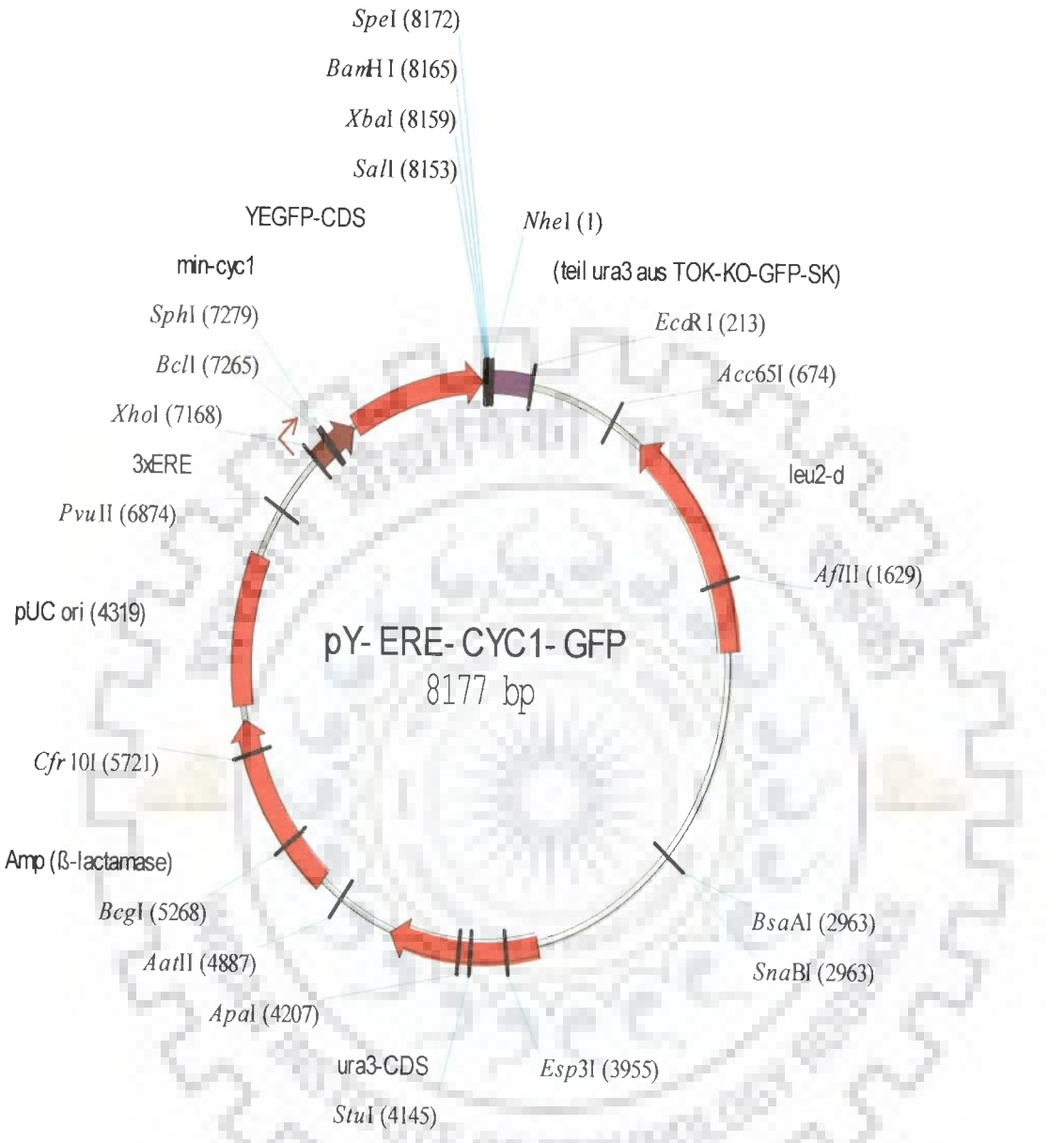

Fig A9 The plasmid pY-ERE-CYC1-GFP, the reporter construct for all estrogen receptor assays, is shown. The locations of all the restriction sites used in making the constructions are shown.

APPENDIX-B

APPENDIX B

Publications:

INTERNATIONAL JOURNALS:

1. **"Development of a yeast-based assay to determine the (anti)androgenic contaminants from pulp and paper mill effluents in India."** Shamba Chatterjee, Chandrajeet B. Majumder and Partha Roy. ***Environmental Toxicology and Pharmacology***, Volume 24, Issue 2, September 2007, Pages 114-121.

2. **"Screening of some anti-progestin endocrine disruptors using a recombinant yeast based in vitro bioassay".** Shamba Chatterjee, Vikas Kumar, Chandrajeet B. Majumder and Partha Roy. ***Toxicology in Vitro***, Volume 22, Issue 3, April 2008, Pages 788-798.

3. **"*RDI1* mediated modulation of the in vitro estrogen transactivation response in *Saccharomyces cerevisiae* requires a functional AF-1 domain." (*Manuscript in preparation*).**

CONFERENCE PUBLICATIONS:

National:

- **Shamba Chatterjee**, Vikas Kumar, Chandrajeet B. Majumder, Partha Roy. "Screening of some anti-progestin endocrine disruptors using a recombinant yeast-based in vitro bioassay." 23rd National Convention of Chemical Engineers under the auspices of The Institution of Engineers (India) Roorkee Local Center, Department of Chemical Engineering, Indian Institute of Technology Roorkee, Roorkee, Uttarakhand, October 5-7, 2007.

International:

- Chatterjee S., Hasenbrink G., Ludwig J., Höfer M., Partha R. and Lichtenberg-Fraté H. "*RDI 1* as modulator of the in vitro estrogen transactivation response in *Saccharomyces cerevisiae*." 26th SMYTE (Small Meeting on Yeast Transport and Energetics), Molecular And Environmental Biology Center (CBMA), Dept of Biology, University of Minho, Braga, Portugal; September 6-9 2008.