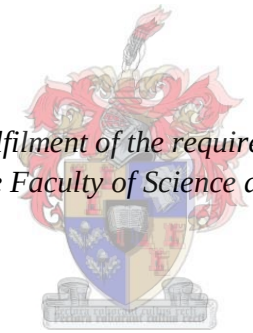
The Production and Purification of Functional Steroid Hormone Receptor Ligand Binding Domains towards the Development of a Biological Endocrine Disruptor Detection System

by
Timo Tait



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Supervisor: Prof Pieter Swart

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Declaration

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Timo Tait

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When I first entered the study of hormone action, some 25 years ago, there was a widespread feeling among biologists that hormone action could not be studied meaningfully in the absence of organized cell structure.

However, as I reflected on the history of biochemistry, it seemed to me there was a real possibility that hormones might act at the molecular level.

— *Earl W. Sutherland, Nobel Address, 1971*

SUMMARY

During the last two and a half decades a large body of research has accumulated indicating the presence of various natural and synthetic chemical compounds within the environment capable of inducing hormone-like responses in humans and animals. Such compounds, termed endocrine disruptors, have been implicated in a variety of developmental, reproductive and physiological abnormalities which have been shown to converge on the endocrine system. Given that endocrine disrupters are comprised of a diverse group of molecules with dissimilar chemical structures, general screening techniques are not feasible for effective environmental monitoring. A primary method of action by which these exogenous molecules affect the homeostatic regulation of the endocrine system is believed to be via the modulation of gene transcription. It is now well established that many endocrine disrupting compounds act upon a principal group of transcription factors, the nuclear receptors, by chance interaction with the ligand binding domains of these proteins.

With a view to ultimately design a portable kit for the detection of endocrine disrupting compounds in water based on the bio-specific immobilisation of nuclear receptor ligand binding domains to a stationary membrane matrix, this study specifically describes:

1. The effects on recombinant protein expression by the addition of small molecules to the cultivation media of bacteria.
2. The optimisation of conditions for the lysis of bacterial cells to increase the solubility of heterologously expressed proteins.
3. The purification of recombinant proteins from bacterial cell lysates by means of a two-step chromatographic methodology.
4. The cloning of the genes for the human androgen and estrogen receptors' ligand binding domains into baculovirus transfer plasmids.
5. Transfer of genetic material from the created baculovirus transfer plasmids to a linearised baculovirus genome for the generation of recombinant viruses.
6. The cultivation, and baculoviral infection, of *Spodoptera frugiperda* and *Trichoplusia ni* cell lines.
7. Expression and purification of N-terminal hexahistidine-tagged human nuclear receptor LBDs from insect cell lysates by means of immobilised metal affinity chromatography.

OPSOMMING

Die teenwoordigheid van natuurlike en sintetiese chemiese middels wat oor die vermoë beskik om die aksies van hormone in die mens en dier na te boots het toenemend aftrek gekry in navorsing gedurende die laaste twee en ’n halwe dekades. 'n Verskeidenheid van ontwikkelings-, reproduktiewe- en fisiologiese abnormaliteite ontstaan as gevolg van die aksies van hierdie molekule, genaamd endokriene-ontwrigters, op die natuurlike funksionering van die endokriene-sisteem. Gegewe dat die groep chemiese middels waaruit endokriene-ontwrigters bestaan van diverse oorsprong afkomstig is lei dit daartoe dat algemene analitiese tegnieke nie in alle gevalle geskik is vir effektiewe omgewingsmonitering is nie. Die modulasie van geentranskripsie is een van die metodes wat voorgestel word as ’n metode waarop hierdie eksogene molekule die homeostatiese regulering deur die endokriene-sisteem omverwerp. ’n Algemene metode waarop vele endokrien-ontwrigtende stowwe geentranskripsie beïnvloed, is deur interaksie met die hormoon-bindende gedeeltes van ’n belangrike groep transkripsiefaktore, die nukluêre reseptore.

Hierdie studie, met die uiteindelike ontwikkeling van ’n draagbare toetsstelsel vir die opsporing van endokrien-ontwrigtende-stowwe in water, gebasseer op die bio-spesifieke immobilisering van nukluêre reseptor ligand bindingsdomeins op ’n stasionêre membraanmatriks, het ten doel om die volgende te beskryf:

1. Die effek wat die byvoeging van klein molekule tot die groeimedium van bakteriële het op die uitdrukking van rekombinante proteïene.
2. Die optimisering van bakteriese sel-lisering in terme van verhoging in die oplosbaarheid van heteroloë proteïene.
3. Die suiwering van rekombinante proteïen vanuit bakteriese sellisate deur middel van ’n twee-stap chromatografiese sisteem.
4. Die klonering van die gene vir die menslike androgeen en estrogeen reseptore se ligand bindingsdomeine in bakulovirus oordragplasmiede.
5. Die oordrag van genetiese materiaal vanaf hierdie bakulovirus oordragplasmiede na ’n gelineariseerde bakulovirus genoom deur middel van homoloë rekombinasie vir die produksie van rekombinante virusse.
6. Die groei en infeksie van *Spodoptera frugiperda* en *Trichoplusia ni* sellyne wat lei tot die uitdrukking van menssoortgelyke nukluêre reseptor ligandbindingsdomains.
7. Suiwering van N-terminaal heksahistidien-etiket-gekoppelde menslike nukluêre reseptor ligandbindingsdomeins vanuit inseksellisate deur middel van geïmmobiliseerde metaal affiniteitschromatografie.

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My ouers, Jan en Alida, asook my broers, Wikus, Jan, Déan en Richardt. Die storie tot dusver het begin met julle. Baie dankie vir julle insette tot die rykdom van my lewe. Ek is ontsetlik lief vir julle.

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LIST OF ABBREVIATIONS

6xHis	A protein fusion tag containing six sequential histidine residues used for the purification of proteins via IMAC
AcMNPV	*Autographa californica* multiple nucleopolyhedrovirus
AD	Activation domain
AF-1	Activation function 1
AF-2	Activation function 2
AP-1	Activation protein 1
AR	Androgen receptor
ARE	Androgen response element
BBAs	Biologically based assays
BEVS	Baculovirus expression vector system
bLB	Buffered LB
bLBg	Buffered LB containing glucose
BmNPV	*Bombyx mori* nucleopolyhedrovirus
BPA	Bisphenol A
BSA	Bovine serum albumin
BV	Budded virus
CAF	Central Analytical Facility
CALUX	Chemical activated luciferase gene expression
CAV	Caveolin
CBP	CREB binding protein
CBZ	Carbamazepine
CDK	Cyclin-dependent protein kinase
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate
ChIP	Chromatin immunoprecipitation
CM-Asp	Carboxymethyl aspartate
CPM	Counts per minute
CTE	Carboxy-terminal extension
DAX-1	Dosage-specific sex reversal-adrenal hypoplasia congenital critical region on the X chromosome-1
DBD	DNA binding domain
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DHT	5α-Dihydrotestosterone

DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dpi	Days post infection
Dsbs	Disulphide bond formation catalysts
E_1	Estrone
E_2	17β-Estradiol
E_3	Estriol
EDCs	Endocrine disrupting chemicals
EDTA	Ethylene diamine tetra-acetic acid
EGFP	Enhanced GFP
ELISA	Enzyme Linked Immuno-Sorbent Assays
ELRA	Enzyme Linked Receptor Assays
EMA	European Medicines Agency
eNOS	Epithelial nitric oxide synthase
ERE	Estrogen response element
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FBS	Foetal bovine serum
FX	Fluoxetine
GFP	Green fluorescent protein
GlcNAc	Acetylglucosamine
GPC	Gel permeation chromatography
GPCR	G-protein coupled receptor
GPER1	G-protein coupled ER 1
GRAS	Generally regarded as safe
HCD	Higher-energy collisional dissociation
HCH	Hexachlorocyclohexane
HDAC	Histone deacetylase complex
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid
hpi	Hours post inection
Hsc70	Heat-Shock Cognate 70
HSP	Heat shock protein
Hsp40	Heat-Shock Protein 40
IDA	Iminodiacetic acid
IFN-β	Interferon β
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria Bertani

LBD	Ligand binding domain
LBP	Ligand binding pocket
LEF	Late expression factors
LRP	Lac repressor protein
mAb's	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MBP	Maltose binding protein
MISS	Membrane-initiated steroid signalling
MOI	Multiplicity of infection
MTT	3-(4,5,-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCoA	Nuclear receptor coactivator
N-CoR	Nuclear receptor corepressor
NICE	Nisin-inducible controlled gene expression system
NMBS	Sodium metabisulphite
NMDR	Non-monotonic dose responses
NP	Nonylphenol
NP40	Nonidet P-40
NTA	Nitrilotriacetic acid
NTD	N-terminal domain
OB	Occlusion bodies
OB	Occlusion body
OC	Organochlorine
ODV	Occlusion derived virus
OHT	4-Hydroxytamoxifen
PBDEs	Polybrominated diphenyl ethers
PDB	Protein Data Bank
PDI	Protein disulphide isomerase
PI3K	Phosphatidylinositol 3-kinase
PIPES	Piperazine-N,N′-bis(2-ethanesulphonic acid)
PKA	Protein kinase A
PKC	Protein kinase C
POPs	Persistent organic pollutants
PPCP	Pharmaceuticals and personal care products
QSAR	Quantitative structure-activity relationships
RAL	Raloxifene
SCR	Steroid receptor coactivator
SDS-PAGE	Sodium dodecyl sulphate poly-acrylamide gel electrophoresis
SEAP	Secreted alkaline phosphatase

SERDs	Selective estrogen receptor downregulators
SERMs	Selective estrogen receptor modulators
SF21	*Spodoptera frugiperda* clonal isolate
Sf9	*Spodoptera frugiperda* clonal isolate of Sf21
SHBG	Sex hormone binding globulins
SHP	Short heterodimeric partner
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptors
SP-1	Stimulating protein 1
T	Testosterone
T. ni	*Trichoplusia ni* BTI-TN-5B1-4
TBA	Trenbolone acetate
TCEP	Triscarboxyethyl phosphine
TFA	Trifluoroacetic acid
TGCT	Testicular germ cell tumours
TIEG	Transforming growth factor β-inducible early gene-1
TIF	Transcription intermediary factor
TREN	17β-Trenbolone
UNEP	United Nations Environmental Programme
US FDA	United States Food and Drug Administration
VLF-1	Very late factor-1
VSMc	Vascular smooth muscle cells
WHO	The World Health Organisation

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CHAPTER 1

INTRODUCTION

Numerous natural and synthetic chemical compounds, which are ubiquitous in the environment, have been discovered to bring about hormone-like responses. Such compounds have been implicated in a variety of developmental, reproductive and physiological abnormalities which have been shown to converge on the endocrine system (1). This large group of molecules, aptly termed endocrine disrupting chemicals (EDCs), consists of a heterogeneous mixture of pharmaceuticals, pesticides, industrial chemicals, fuels, heavy metals, plastics and plasticisers; with the particular identities of many still remaining unknown. The environmental concentrations of these chemicals are generally very low. Nevertheless, some have been shown to be highly persistent in nature (2, 3) and since the effects of EDCs might be seen at very low concentrations, concerns have arisen about the effects of complex mixtures of chemicals occurring at such levels (4). Moreover, since many of these molecules are of a hydrophobic nature it has been suggested that bio-accumulation may occur within trophic webs, leading to higher than environmental exposure to wildlife and humans (5). Initial reports of EDC impact on wildlife and the subsequent studies on the aetiology of multiple human physiological states, including cancers of the breast and prostate, cryptorchidism, endometriosis and metabolic disorders have led to the scientific understanding that EDCs pose a prominent threat to human and animal health. Consequently, several thousand articles have been written prior to, and subsequently to, the original coining of the term 'endocrine disruptor' at the Wingspread Conference Centre in Racine, Wisconsin, in 1991 (6). In its simplest form an endocrine disruptor is "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations"; and a potential endocrine disruptor is "an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub)populations" (7).

The presence of EDCs is a global environmental issue. Certain persistent organic pollutants (POPs), such as organochlorine (OC), various pharmaceuticals and personal care products (PPCP), polybrominated diphenyl ethers (PBDEs), dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexane (HCH) have been reported as far as the Arctic and Antarctic regions (8–10). Many of these compounds have also been identified in the Lake Tanganyika basin in Tanzania (11), while measurable concentrations of the EDCs nonylphenol (NP), bisphenol A (BPA) and phenolic steroids hormones such as estrone, 17α-estradiol, 17β-estradiol and 17α-ethinylestradiol was found to be present at concentrations varying from 20 pg/L to 135 ng/L in a study of drinking water, sewage treatment effluent and river systems in Germany (12). Recently, high concentrations of the malarial vector controlling agent DDT, and its metabolites, have been identified within the blood and breast milk of nursing mothers in South Africa (13). Subsequently, these compounds have also been detected at levels ranging between 0.8 ng/g to

123 ng/g of sediment at locations in southern Africa's largest natural freshwater lake, Lake Sibaya, in northern KwaZulu-Natal (14). These results are of critical concern, since studies suggest an association between pre- and post natal exposure to DDT and urogenital malformations in new-born boys (15). Furthermore, mounting evidence supports a link between non-occupational exposure to DDT and reduced semen quality in men (16), as well as altered retinoid and thyroid hormone profiles (17).

A hallmark of these contaminants, and other EDCs, is the ability to elicit endocrine responses at extremely low concentrations (parts per billion to parts per trillion). The modes of action by which EDCs affect molecular physiology involve divergent pathways. These mechanisms include regulatory actions mediated by the nuclear receptors for estrogens, androgens, thyroid hormone, retinoids, and other hormones. Moreover, steroidogenic enzymes, neurotransmitter receptors and many other pathways, which are highly conserved in wildlife and humans, are targets for disruption by exogenous molecules. Given that there are numerous EDCs that can negatively affect humans and wildlife, general screening techniques based on chemical structures are not feasible for effective environmental monitoring. The impracticality of analysing samples for all possible known EDCs, even neglecting unknown EDCs, necessitates the production of a system that may selectively concentrate specific molecules by some property, such as analogous molecular responses. At concentrated levels such molecules may be readily detected by standard colorimetric techniques. Biologically based assays (BBAs) may provide major advantages by estimating the total effects of many EDCs in the environment (18). These techniques may potentially be adapted for field portable biologically directed sampling and analyses.

In light of the phenomenon of endocrine disruption via the nuclear receptors, and a requirement for the development of rapid, sensitive and economical means of EDC detection, the current study was initiated with the following aims in mind:

1. to gain a thorough understanding of the structure and functions of nuclear receptors with regards to gene expression and the maintenance of cellular homeostasis from available literature;

2. to identify and discuss the mechanisms by which homeostatic control over such processes may be disrupted by exogenous molecules;

3. to review the most prominent existing approaches of heterologous protein production in various expression systems;

4. the synthesis of the human androgen and estrogen receptor ligand binding domains, in soluble forms, using heterologous expression systems;

5. the purification of the expressed recombinant proteins from complex cell lysates via affinity chromatography;

6. and the validation of steroid binding activity of the purified proteins towards natural endogenous ligands.

The endocrine system is involved in various aspects of cellular regulation. Hormones secreted by the endocrine glands, or which act as paracrine or autocrine signals, interact with multiple cellular pathways to exert control over diverse processes involved in growth, adaptation and proliferation. Within the classical context of endocrinology small lipophilic molecules serve as chemical messengers that alter the expression of specific genes via cognate nuclear receptors. In the current study, chapter two discusses the classification and organisation of the nuclear receptor superfamily, with specific reference to the family of steroid receptors. The mechanisms involved in the activation of nuclear receptors, as well as more recent ideas on the hormonal management of cellular processes by endogenous ligands to the estrogen receptor are presented. It is now well established that EDCs may interfere with homeostatic control by mixed mechanisms, including the activation of signalling cascades, the disruption of regulatory feedback loops and hormonal transport systems, and the alteration of gene expression via complementary interaction with nuclear receptors. Consequently, there is a growing need for the detection of chemical pollutants within the environment, especially within aquatic systems. Several of the methods commonly used for EDC detection are introduced, along with some of the inherent drawbacks associated with such techniques. The construction of a rapid, on-site monitoring system, based on the interaction of EDCs with specific nuclear receptors, may serve as an auxiliary means to identify whether water sources are contaminated with specific classes of EDCs. Consequently, the major recombinant expression systems used for the production of heterologous proteins are presented, with the intention of producing human-like proteins for the design and fabrication of a novel EDC detection kit.

Escherichia coli is the most widely used expression host for both structural analysis and screening of protein targets (19–21). Consequently, the bacterial expression of the genes for the ligand binding domains of human androgen and estrogen receptors are discussed in the third chapter. Within the cytosol of human cells the relatively hydrophobic nuclear receptor proteins are associated with stabilising protein complexes. However, eukaryotic proteins expressed heterologously in prokaryotic cells are not afforded the added stability of these molecular chaperones. Therefore, a challenging step in the expression of any protein or protein fragment is predicting whether such a construct can be produced in large, active quantities; considering many heterologous proteins express poorly or fold improperly in bacterial expression systems. Thus, the efforts to increase the solubility of human-like proteins capable of binding natural steroid ligands and exogenous compounds, which have been expressed in *E. coli*, are conveyed. Furthermore, attempts to purify these proteins in functional forms are addressed by the use of immobilised metal affinity chromatography (IMAC) and immobilised ligand affinity chromatography.

A contrasting system to prokaryotic heterologous protein production, the baculovirus expression vector system (BEVS), is presented in chapter four. Baculoviruses form a big family of insect pathogens with large, double-stranded, circular genomes packaged in rod-shaped, enveloped nucleocapsids. These viruses produce lethal infections in their hosts by appropriation of cellular transcriptional and translational machinery for viral replication, followed by cellular degeneration (22). In the natural environment synthesised virions are protected after death of the insect host within crystalline matrices

known as occlusion bodies (OB). BEVS relies on the infection of insects, or insect cells in culture, with an engineered vector capable of high level recombinant protein expression. Within the confines of tissue culture experiments the formation of OB are not a prerequisite for viral replication or the production of infectious particles. As a result, the viral genes encoding for the major proteins of OB, polyhedrin (nucleopolyhedroviruses) or granulin (granuloviruses), may be exchanged for heterologous genes. The generation of recombinant baculoviruses expressing the human androgen and estrogen receptor ligand binding domains, in lieu of polyhedrin, is described in chapter four. These recombinant proteins were subsequently purified by means of IMAC and subjected to radioligand binding assays to establish functionality. A comparison between the two expression systems, BEVS and *E. coli*, is presented at the end of the chapter.

In the final chapter the conclusions drawn from the results obtained in this study, and the implications for future work, are discussed. The ability to detect EDCs within the environment is paramount in the development of strategies to reduce the effects these exogenous chemical compounds on human and animal health. Therefore, a supplementary approach to the detection methods described in chapter two, based on the molecular conformation changes that occur upon receptor-ligand interaction, is also described.

CHAPTER 2

OVERVIEW OF EXISTING LITERATURE PERTAINING TO THE ENDOCRINE SYSTEM, CHEMICAL DISRUPTION AND HETEROLOGOUS PROTEIN EXPRESSION

The neuroendocrine system is an intricately connected web of control elements, signal transducers and effectors, of which diverging and converging pathways are in continual communication. The specialised tissues and organs of multicellular organisms, such as human beings, require the essential integration of hormonal signals for the coordination of metabolic activities. Receptors to various classes of lipophilic and hydrophilic ligands serve as binding sites for endocrine, paracrine and autocrine hormones. Binding of peptides or small lipophilic molecules to cognate receptors initiate cascades of molecular events which direct the biological fate of the cells. Tissue responsiveness to specific hormones are constrained and defined by the particular combination of membrane bound and cytosolic receptors available to cells. Moreover, hormonal action in different cells housing the same receptors may vary widely due to alternate intercellular targets. Structural complementarity of a ligand to the three-dimensional arrangement of the receptor ligand binding pockets (LBP) results in high affinity coordinating binding between the two molecules. As a result, hormones elicit cellular events at very low concentrations. The molecular events that occur upon ligand binding are extremely selective, thus structurally similar hormones may elicit alternative responses within the same cell. Accordingly, the receptors of the neuroendocrine system are choice targets for the development of pharmaceutical agents to address aetiologies relating to hormonally controlled bioprocesses.

A current focus for endocrinologists is the potential of chemicals, discarded into human water treatment facilities or present in everyday life, to affect the molecular functioning of the endocrine system. Several scientific reviews published by, for example, the Endocrine Society (1), the European Commission (23, 24), the European Environment Agency (25) and the Endocrine Disrupters Expert Advisory Group (26) have discussed advances in the evaluation of potential EDCs, with specific focus on exposure risks, adverse effects on human and animal health, mechanisms of endocrine disruption and applied screening systems. Since the first formal definition of endocrine disruption, the effects of these exogenous compounds have been linked to a multitude of disease states at different periods of the life cycle, including cancer, genetic modification, organ malfunction, metabolic syndromes and reproductive dysregulation. It is now well established that EDCs may interfere with the maintenance of cellular homeostasis by mixed mechanisms, including the activation of signalling cascades, the disruption of regulatory feedback loops and hormonal transport systems, and the alteration of gene expression via complementary interaction with nuclear receptors.

2.1 DISRUPTERS OF THE ENDOCRINE SYSTEM

Concern about the prevalence of hormones in drinking water (27), as well as the environmental effects of chlorinated hydrocarbons and other pesticides, have been raised as early as the 1960. Spurred by Rachel Carson's book, *Silent Spring*, a report by the President's Science Advisory Committee that focused on pesticides and other substances, specifically including steroid hormones, proposed that "there must be an awareness of long-range and possibly synergistic effects of low-level amounts of toxic or physiologically active substances" (28). However, already prior to that time, several studies indicated that exogenous compounds could interact and modulate the mechanisms governing the functions of the endocrine system by mimicry of endogenous ligands (29–32).

Initial reports of the presence of several human hormones and pharmaceuticals in aquatic environments and water treatment facilities were published during the 1970s and 1980s (33, 34). Apart from a few publications (35–38), these early reports did not attract significant investigation due to mere trace level detection, and a lack of knowledge of the possible toxicology of these substances (39, 40). Nevertheless the small contributions have multiplied, and steadily a picture has emerged in which there may be astounding credence to the earliest statements that focused our attention on the phenomena of endocrine disruption (41). It is now known that trace concentrations of EDCs can significantly alter the reproductive cycles and cellular homeostatic control mechanisms of animals by affecting the biological systems governing gene transcription, molecular transport and metabolism. Numerous EDCs are present in the environment, the majority of which may exhibit only mediocre hormonal activity; yet certain molecules have been developed to specifically act upon the endocrine system. Furthermore, EDCs that are present at low levels may elicit endocrine actions with physiological or reproductive relevance by non-monotonic dose responses (NMDR) (4).

Recently, the effects of 17β-trenbolone [17β-17-hydroxyestra-4,9,11-trien-3-one; TREN] were shown to persist in the model organism *Danio rerio*, the zebrafish, following discontinuation of exposure to the potent androgen (2). The zebrafish is sensitive to changes in its hormonal milieu and therefore it has routinely been employed as an indicator of reproductive endocrine disruption (42). Known side-effects resulting from the abuse of potent synthetic androgens such as TREN, including neuropsychiatric disorders, sterility, gynaecomastia and testicular atrophy in males, and virilisation in females, are prevalent in athletes desiring increased muscle mass and strength (43, 44). Yet in developed countries, the parent drug trenbolone acetate [17β-acetoxyestra-4,9,11-triene-3-one; TBA], is routinely used as a livestock growth promoter (45–47) to increase lean muscle mass with higher feed conversion efficiency. Consequently, the metabolites of TBA (TREN, 17α-trenbolone [17α-hydroxy-estra-4,9,11-trien-3-one] and trendione [estra-4,9,11-trien-3,17-dione]) have become environmental androgens due to urinary and faecal excretion with increased run-off to soil and surface water. With relative binding affinity to the androgen receptor similar to DHT (48), the modulation of the AR by exogenous growth promoting agents, such as TREN, have been shown to induce endocrine, developmental, morphological, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects in aquatic organisms

associated with concentrated animal feeding operation sites (45, 49–51) and epidemiological studies have provided evidence in support of a link between the amount of red meat and other animal-products consumed and the aetiology of certain forms of hormone-dependent cancers (52–54).

There are, however, multiple ways of entry for steroid hormones and other EDCs to soil, aquatic systems and dietary sources. The growing and aging global population has increased demand for intensive food production and has stimulated a need for escalated pharmaceutical utilisation. It is estimated that several thousand tonnes of pharmacologically active substances are consumed and released into the environment every year due to pronounced use in animal husbandry and aquaculture, along with veterinary and medical practices (55, 56). Many of these chemical compounds are distributed to land surfaces owing to the application of dry sewage biosolids for fertiliser or aqueous sewage effluent for irrigation (56). A range of such pharmaceuticals have been shown to be persistent in the environment due to low biodegradability. For example, the antidepressant fluoxetine (FX), the active ingredient in the most widely prescribed psychoactive drug, Prozac®, has an estimated dissipation half-life in receiving water of 112 – 113 days (3). FX is commonly detected downstream from point-sources of municipal waste effluent and, because of the ubiquitous use of the drug, such sampling areas are continually exposed to renewed insults of the drug. Hazelton *et al.* (57) investigated the behaviour and physiology of the freshwater wavy-rayed lampmussel (*Lampsilis fasciola*) upon chronic exposure to FX and found significant alterations in the movement patterns and rates of lure display between treated and untreated mussels (57). Furthermore, the presence of FX in aquatic and marine waters, at environmentally relevant concentrations, have been linked to detrimental effects in the Mediterranean mussel (*Mytilus galloprovincialis*) by affecting cAMP/PKA signalling and gene transcription regulation (58).

Carter *et al.* (59) highlights the concern expressed over the detection of pharmaceuticals in soil as a number of studies have indicated the presence of human and veterinary medication, including antidepressants, anticonvulsants, nonsteroidal anti-inflammatory drugs and antibiotics in crops and plants. In a study on the fate and uptake of compounds covering a diverse range of physicochemical properties certain pharmaceuticals, notably the anticonvulsant carbamazepine (CBZ), FX, and propranolol, a sympatholytic nonselective beta blocker, were found to persist in soil unchanged for at least 40 days (59). Pharmaceuticals and chemical compounds that enter the municipal waste stream, including personal care products and other EDCs, and the subsequent application of treated water and biosolids to land as plant nutrients have also raised concern about intake by soil- or sediment-ingesting organisms or possible bioaccumulation in plants, with subsequent transferral to higher trophic levels (5). For example, higher levels of two antimicrobial agents, triclocarban and triclosan, were detected in the soil and earthworms at a site treated with biosolids, as compared to a control site. Moreover, the concentrations of these compounds were significantly higher in the eggs of European starlings (*Sturnus vulgaris*) nesting on biosolid-applied land while nesting success of another bird species, the American kestrel (*Falco sparverius*), was significantly lower compared to an untreated site (60); indicating that antimicrobial agents can be transferred from soil to secondary and tertiary consumers where it impacts not only on

reproduction, but also on behavioural success. Yet with an estimated half-life of 18 days (59, 61), triclosan, and other pharmaceutical products, do not persist in soil or water to the extent exhibited by drugs such as FX and CBZ. Nevertheless, their continual release to surface waters results in their classification as pseudo-persistent and some studies have indicated an apparent link between low-level chronic exposure and observed detrimental effects in aquatic and terrestrial organisms (62). Moreover, these emerging pollutants are of concern not only because of the harmful effects that they inflict on the endocrine system, but also because of possible increases in the development of antibiotic resistance in bacteria due to their ubiquitous and constant presence (63). Such concerns are sure to be intensified in light of a growing global human population; and emerging pollutants are likely to increase within the environment, thus supplementing the deleterious effects on human and animal endocrine health posed by traditional EDCs, including natural and synthetic hormones, phenol-derived compounds, phthalates and pesticides.

Ultimately, the challenge is to elucidate the mechanism of actions of EDCs thus providing a basis of assessment of isolated, and possible synergistic, effects on human and animal well-being. Rigorous clinical trials and regulatory bodies in the pharmaceutical industry enforce the assessment of medication influence on human physiology. However, the ecological risk and impact of thousands of drugs, or drug metabolites, upon wildlife populations following excretion is not well researched. A further challenge is the determination of causal links, rather than effects (64), between initial exposure to EDCs and subsequent adverse effects; which may occur years or decades later in mammalian life, or within the offspring of exposed individuals, as in the well-known case of diethylstilboestrol (65–70). Historically, much of the focus has been on the developmental and reproductive effects of EDCs. Accordingly, much more is known about the effects of compounds identified at the inception of the endocrine disruption research era which are known to inappropriately activate or antagonise the sex steroid receptors (71). For example, owing to its omnipresence the estrogenic and antiandrogenic activities of bisphenol A and its analogues have been widely cited (72–77). As a result, most of the initial research into the phenomena of endocrine disruption has been centred on the interruption or modulation of the processes governing gene transcription, with a specific focus on nuclear receptors; mostly with particular reference to the androgen, estrogen and thyroid hormone receptors. From this early work the realisation sprouted that EDCs may "interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that is responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour" (78). Consequently, many other proteins have also been implicated as targets for disruption. For instance, entrance of a molecule to the cell interior is dependent on its concentration within the extracellular environment. In serum the concentration of androgens and estrogens are controlled by sex hormone binding globulins (SHBG). Various EDCs capable of binding to the AR, ER or α-fetoprotein can also interact with the SHBG, thus inhibiting the binding of endogenous sex steroids to these transport proteins (79). Consequently, sex steroid mediated gene transcription is interrupted, resulting in perturbation of negative feedback loops governing secretion of the key regulatory hormones of the hypothalamic-pituitary-gonadal axis (177). Furthermore, localised within the cell membranes of certain

reproductive tissues are receptors to which SHBG can bind. The interaction of the hormone conjugated SHBG is proposed to initiate cAMP mediated signalling cascades (81–84). Binding of EDCs to SHBG may therefore activate signal transduction pathways, thus further modulating steroid hormone action.

Moreover, transcriptional regulation is governed by a complex web of protein-protein interaction and post-translational modification of cofactors by second messenger mediated pathways. Rapid non-genomic responses to hormones include the activation of kinases, phosphatases, and phospholipases that govern calcium-dependent signalling and phosphorylation cascades. Cytoplasmic cross-talk between classical nuclear receptor transcriptional pathways and membrane-associated signalling pathways can enhance the mechanisms involved in cell cycle control, cell survival and regulation of modifications to the epigenome (85). Several studies have concluded that the recently identified GPER can bind to phytoestrogens and flavonoids (86, 87), synthetic estrogens (88), SERMs and selective estrogen receptor downregulators (SERDs) (89, 90), atrazine (91), bisphenol A (92), and the polychlorinated toxins methoxychlor, DTT and its metabolites (93), among other compounds. Consequently, the G-protein coupled receptor has been implicated in the formation of various physiological malignancies, including cancers of the reproductive (88, 94, 95) and non-reproductive tissues (96–98). However, the mechanisms of cross-talk and feedback involved in GPER regulated signalling pathways are not fully understood (99) and the possible means of GPER-mediated function-disruption by exogenous molecules, therefore, requires further investigation. Of course, in light of the evidence presented by Chaudhri *et al.* (100), disruption of rapid estrogenic signalling may involve alternative pathways requiring splice variants of the ER, for example ERα36.

Risk assessment (101) of possible EDCs requires resolution on definitions of what constitutes an adverse effect and what constitutes a biological indicator. The diversities of potential endocrine-active chemicals, coupled to the complexity of the regulatory system with which they interact, makes the evaluation of combined effects of EDCs a daunting task. The 2013 WHO/UNEP state of science report on endocrine disruption (102), although criticised as biased in their approach to EDC data interpretation (64), especially with regards to NMDR, called for more research to improve global knowledge on the associations between the environmental impacts of EDCs and possible risks to humans and wildlife. Methods of identifying known and unknown EDCs are required to discover routes of exposure, while models of synergistic effects of such compounds necessitate clarification. Improved data sharing between countries and research groups, with particular emphasis on developing countries and emerging economies, will help to close the gaps in knowledge on the underlying mechanisms of endocrine effects by exogenous chemicals.

2.1.1 Monitoring of endocrine disruptors

The detection of chemicals, with the ability to modulate physiological processes, is important in the substantiation of cause and effect chains between the presence of EDCs within the environment, and their perceived detrimental effect on human and animal life. Heterogeneity is a hallmark of EDCs, yet this chemically diverse group of molecules can elicit similar or related disruptive biological effects. Traditional methods of detection based solely on chemical structure are therefore not sufficient. Several techniques have thus been developed for the detection of select or heterogeneous molecules within *in vivo* or *in vitro* contexts. However, the extremely low concentrations at which most EDCs occur often severely hamper the assessment of such molecules within an environmental context. Furthermore, the proposition that exogenous materials may effect physiologically relevant alterations to gene transcription and other regulatory mechanisms by NMDR places further impetus on the requirements for increasingly sensitive testing systems, capable of low-level EDC detection.

Several classical *in vivo* systems have been designed for the prediction of estrogenicity or androgenicity of specific compounds. These testing methods include uterotrophic and vitellogenin assays for measurements of estrogenic and anti-estrogenic effects, while the Herschberger assay assesses the maintenance of androgen dependent organs. Several species of fish, such as the Japanese medaka (*Oryzais latipes*) and the zebrafish, along with certain amphibian creatures, including the South African clawed toad (*Xenopus laevis*), have been exploited as biomarkers for compounds with reproductive relevance (103–105). These bioassays rely on the detection of serum proteins or the assessment of organ development and generally require the sacrifice of test animals. Alternatives to adult animal utilisation, in line with the 3R principles (106), include the use of fish embryonic and early larval stages since most organs are functional at the end of embryogenesis. Embryos express endocrine markers such as vitellogenin and aromatase, enabling strategies to evaluate cellular effects by monitoring of the transcriptome, with regards to the expression patterns of genes such as *aromatase b*, *vitellogenin 1*, *11β-hydroxylase*, *3β-hydroxysteroid-dehydrogenase* and *gonadotropin-releasing hormone receptor 2* (107), amongst others (103, 104, 108).

The incorporation of a multitude of biochemical processes within cellular and reproductive cycles, all with different temporal windows of sensitivities, poses significant challenges to environmental EDC assessment. *In vivo* approaches towards confirmation of the endocrine activity of specific compounds are therefore invaluable. However, low sensitivity, high costs, animal husbandry, the requirements for technical expertise and labour intensity of end-point measurements complicates the application of *in vivo* techniques for environmental monitoring. As such, *in vivo* techniques often require additional *in vitro* methods for validation of environmentally relevant results. These methodologies are performed under experimental conditions by the utilisation of unicellular organisms, immortalised cell lines in tissue culture or heterologous proteins. Many EDCs affect cellular bioprocesses by direct interaction with endogenous hormone receptors. Therefore, several *in vitro* techniques, such as competitive ligand binding assays and derivatives thereof (e.g. surface plasmon resonance sensors), enzyme linked receptor

assays (ELRA), enzyme linked immuno-sorbent assays (ELISA), cell proliferation screens (e.g. the E-screen), as well as yeast based receptor/reporter assays and human chemical activated luciferase gene expression (CALUX), are based on receptor-ligand interactions (18). There are, however, certain drawbacks to such *in vitro* techniques. For example, competitive ligand binding assays offer an excellent means to assess the affinity of a test compound to a particular receptor under *in vitro* conditions. However, these assays cannot distinguish between agonist and antagonist activity, nor can they elucidate the effect of receptor binding on further biochemical events such as transactivation or repression of multiple genes, which relies on the presence of a large cohort of transcription cofactors and regulatory molecules. Furthermore, these techniques are not indicative of the environmental milieu and, as a result, fail to take into account possible synergism between various molecules present at a specific location.

Therefore, in view of the complexity of the endocrine system, several authors have indicated that a battery of complementary tests, comprised of *in vivo*, *in vitro* and chemical analysis by analytical methods such as GC/MS or LC/MS, may be beneficial in the prediction of developmental and reproductive toxicology of single compounds or heterogeneous mixtures found in the environment (109–111). Invariably these tests involve multiple pre-defined endpoints which collectively provide information about the system as a whole. In addition, *in silico* methods, including quantitative structure-activity relationships (QSAR) or parallel screening, which includes 3D- or 2D descriptors, are powerful approaches for the rapid qualification of suspected EDCs (112–114). However, in light of environmental monitoring of molecules with endocrine disruptive capabilities, rapid, simple and economical assessment of specific locations require the development of *in vitro* biological or physicochemical detection systems. As a result, *in vitro* techniques still offer the simplest and most cost-effective mechanisms to assess whether environmental systems have been compromised by chemicals capable of acting as exogenous ligands to hormone receptors which act as signal transducing molecules or genetic transcription factors (115, 116).

2.2 NUCLEAR RECEPTORS

The nuclear receptors form a large superfamily, encompassing proteins which bind small lipophilic molecules such as steroid and thyroid hormones, vitamin D and retinoids. Nuclear receptors are involved in the regulation of cellular metabolism, growth and differentiation by acting as transcription factors, thus modulating the activation or repression of gene transcription. As a result, these receptors are involved in a myriad of diverse physiological processes including, but not limited to, the regulation of the reproductive system, biosynthesis of cholesterol and bile and control over the pre- and postnatal developmental programs.

The human genome contains genes encoding 48 nuclear receptors, thought to be evolutionarily derived from a common ancestor. Within this large group, different orphan receptors, many of which are constitutively active transcription factors (117), are also included. Ligands to this class of nuclear receptor have recently been identified as products of lipid metabolism, including prostaglandins, leukotrienes, fatty acids and cholesterol derivatives. The vast majority of ligands toward the orphan receptors remain unknown (118).

Early classification grouped nuclear receptors into four categories based on ligands, DNA binding properties or other functional characterization (119). More recent work, based on amino acid sequence alignment and phylogenetic tree construction, distributed these transcription factors into six subfamilies and resulted in the acceptance of systematic nomenclature based on designations similar to that of the P450 enzymes (Figure 2.1 and Table 2.1) (120).

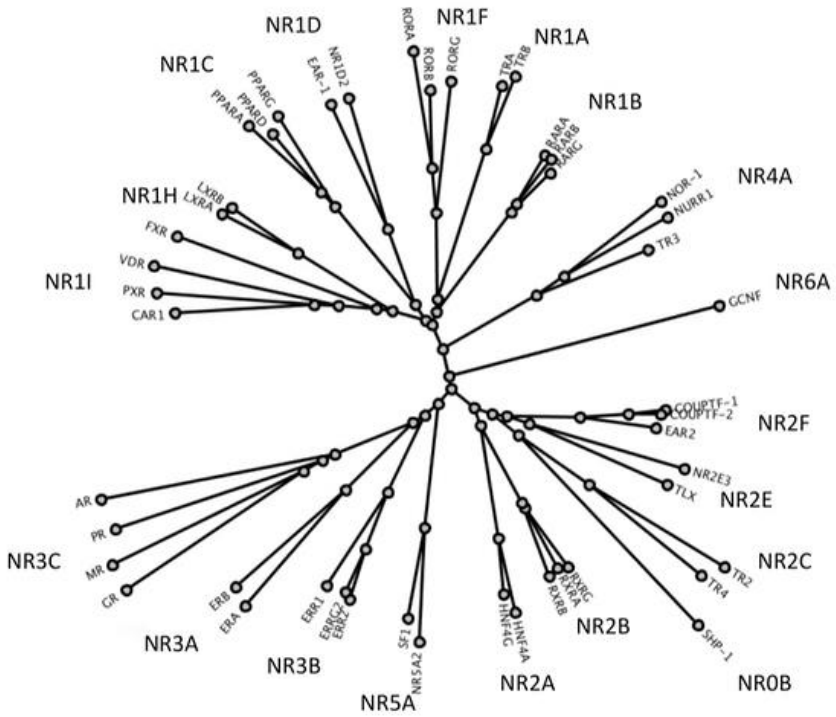

Figure 2.1 Neighbour joining tree of nuclear receptors phylogeny indicating the common evolutionary ancestry of the receptor superfamily (118).

Table 2.1 **Nomenclature of mammalian nuclear receptors and selected ligands** (121, 122). **The phylogeny groups the nuclear receptors into six subfamilies. The three digit identifier after 'NR' designates respectively the subfamily, group and individual gene. More information may be obtained from these references** (123–125).

Trivial name	Common abbreviation	Unified nomenclature	Ligands
Thyroid hormone receptor	TRα	NR1A1	Thyroid hormones
	TRβ	NR1A2	Thyroid hormones
Retinoic acid receptor	RARα	NR1B1	Retinoic acid
	RARβ	NR1B2	Retinoic acid
	RARγ	NR1B3	Retinoic acid
Peroxisome proliferator-activated receptor	PPARα	NR1C1	Fatty acids, leukotriene B4, fibrates
	PPARβ	NR1C2	Fatty acids
	PPARγ	NR1C3	Fatty acids, prostaglandin J2
Reverse erbA	Rev-erbα	NR1D1	Haem†
	Rev-erbβ	NR1D1	Haem†
RAR-related orphan receptor	RORα	NR1F1	Cholesterol, cholesteryl sulphate
	RORβ	NR1F2	Retinoic acid, sterols†
	RORγ	NR1F3	Retinoic acid, sterols†
Liver X receptor	LXRα	NR1H3	Oxysterols
	LXRβ	NR1H2	Oxysterols
Farnesoid X receptor	FXRα	NR1H4	Bile acids, Fexaramine
	FXRβ‡	NR1H5	Lanosterol
Vitamin D receptor	VDR	NR1I1	1α,25-dihydroxy vitamin D3, litocholic acid
Pregnane X receptor	PXR	NR1I2	Xenobiotics, pregnenolone 16α-carbonitrile
Constitutive androstane receptor	CAR	NR1I3	Xenobiotics, phenobarbital
Hepatocyte nuclear factor 4	HNF4α	NR2A1	Fatty acids†
	HNF4γ	NR2A2	Fatty acids†
Retinoid X receptor	RXRα	NR2B1	9-cis retinoic acid and docosahexanoic acid
	RXRβ	NR2B2	9-cis retinoic acid and docosahexanoic acid
	RXRγ	NR2B3	9-cis retinoic acid and docosahexanoic acid
Testicular orphan receptor	TR2	NR2C1	Orphan
	TR4	NR2C2	Orphan
Tailless homolog orphan receptor	TLX	NR2E1	Orphan
Tailless	TLL	NR2E2	Orphan
Photoreceptor-cell-specific nuclear receptor	PNR	NR2E3	Orphan
Chicken ovalbumin upstream promoter-transcription factor	COUP-TFα	NR2F1	Orphan
	COUP-TFβ	NR2F2	Orphan
ErbA2-related gene-2	COUP-TFγ / EAR2	NR2F6	Orphan
Estrogen receptor	ERα	NR3A1	17β-Estradiol, tamoxifen, raloxifene
	ERβ	NR3A2	17β-Estradiol, various synthetic compounds
Estrogen receptor-related receptor	ERRα	NR3B1	Orphan
	ERRβ	NR3B2	DES, 4-OH tamoxifen
	ERRγ	NR3B3	DES, 4-OH tamoxifen
Glucocorticoid receptor	GR	NR3C1	Cortisol, dexamethasone, RU486
Mineralocorticoid receptor	MR	NR3C2	Aldosterone, spirolactone
Progesterone receptor	PR	NR3C3	Progesterone, medroxyprogesterone acetate, RU486
Androgen receptor	AR	NR3C4	Testosterone, flutamide
Nerve growth factor-induced gene B	NGFIB	NR4A1	Orphan
Nur-related factor 1	NURR1	NR4A2	Orphan
Neuron-derived orphan receptor 1	NOR1	NR4A3	Orphan
Steroidogenic factor 1	SF1	NR5A1	Orphan
Liver receptor homologous protein 1	LRH1	NR5A2	Phospholipids†
Germ cell nuclear factor	GCNF	NR6A1	Orphan
Dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1	DAX-1	NR0B1	Orphan
Short heterodimeric partner	SHP	NR0B2	Orphan

† These ligands appear to be constitutively bound to receptors

‡ FXRβ exists only as a pseudogene in humans

2.2.1 Canonical structural organisation of nuclear receptors

Nuclear receptors, despite the vast and diverse regulatory roles which they govern, share remarkable structural and functional organisation. In general, these molecules are modular in nature (126) and consist of a single polypeptide chain that may be delineated into four or five segregate domains (Figure 2.2). The centrally located DNA binding domain (DBD) recognises highly specific DNA sequences which, upon binding of the receptors, mediate activation or repression of gene transcription via an array of transcription factor recruitment. Within the C-terminal half is located a ligand binding domain (LBD) which recognises particular hormonal and non-hormonal ligands that directs the specificity of the biological response. These two domains are linked by a hinge domain of variable length while being flanked on either side by variable N-terminal and C-terminal domains.

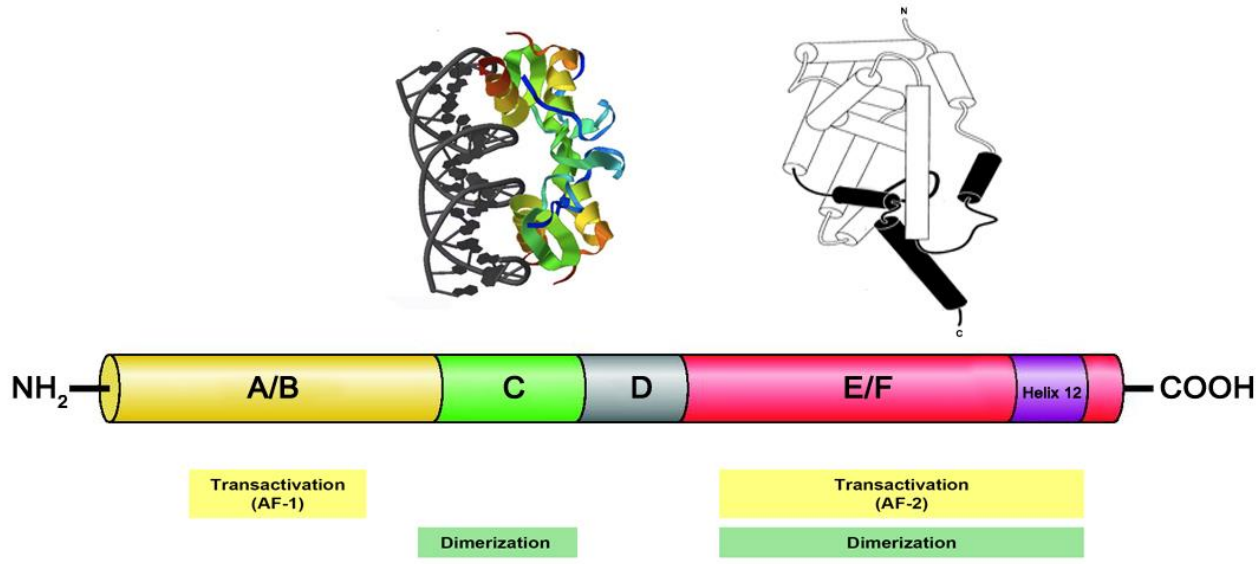

Figure 2.2 **General modular structure of nuclear receptors. The N-terminal A/B domain, including the activation function 1 (AF-1), is important for the constitutive transcription activity. The central DNA binding domain (C) is followed by a hinge region (D) and the ligand binding domain (E) that includes the activation function 2 (AF-2) within the twelfth helix, crucial for the ligand-dependent cofactor interaction. A few nuclear receptors feature an additional F domain at the C-terminal of which the exact function is still poorly understood.**

2.2.1.1 N terminus (A/B)

The domain towards the N-terminal domain (NTD) of the receptor is known as the modulator, hypervariable or A/B domain (118). This acidic domain is constitutively functional and varies in length between different receptors or receptor subtypes. The ligand independent transactivation activity exhibited by the A/B domain is facilitated by activation function 1 (AF-1) which contains a variety of kinase recognition sites. Phosphorylation at these sites may modulate the activity of the receptor, possibly by affecting the interaction between transcription co-activators and AF-1 (127). Furthermore, the many secondary start sites and alternative splicing loci contained within this domain may be a determining factor in the receptor-, cell type, and species-specific variations exhibited by different isoforms of receptors binding to the same ligand.

2.2.1.2 DNA-binding domain (C)

The DNA binding domain (DBD) is the most highly conserved region among the various nuclear receptors and facilitates binding of the receptor to hormone response elements within the major groove of the DNA double helix (128). In response to ligand binding two zinc finger motifs within the DBD, containing characteristic four-cysteine motifs which coordinate tetrahedrically with one zinc ion each (129), interact with specific sequences located in the promoter regions of target genes. These hormone response elements, consisting of two half-sites, function as palindromic repeats of six base pairs separated by one to ten non-conserved nucleotides. Each molecule of the receptor dimer interacts with one half-site; of which two variants exist, those recognised by steroid receptors, excluding the ER (5'-AGAACA), and others by the ER and other nuclear receptors (5'AGGTCA) (130). Response elements may consist of direct repeats (DR_x: e.g. AGGTCA-N_x-AGGTCA), everted repeats (ER_x: e.g. ACTGGA-N_x-AGGTCA) or inverted repeats (IR_x: e.g. AGGTCA-N_x-ACTGGA); N representing any nucleotide and x being a number between three and five (IR_3: GR, PR, AR and ER / DR_3: VDR / DR_4: TR / and DR_5: RAR) (121). Some residues of the proximal zinc finger, between the last two cysteine residues (P-box), are located in the first helix of the DBD and are involved in discrimination of the core half site of the hormone response element. The dimerization interface (D-box) of the nuclear receptor DBD is formed by residues of the second distal zinc finger located in the second α-helix of the DBD, perpendicular to the P-box. The only exceptions to this method of receptor-DNA interaction within the entire family are dosage-specific sex reversal-adrenal hypoplasia congenital critical region on the X chromosome-1 (DAX-1) and short heterodimeric partner (SHP) (122). These receptors lack DBDs, yet function as corepressors via interaction with other transcriptional factors, thus assisting in the regulation of their target genes (131, 132). Depending on class of receptor, nuclear receptors bind DNA as either monomers (RVR, ROR, TLX, ERR, NGFI-B, FTZ-F1), homodimers (GR, PR, ER, AR and MR) or heterodimers (TR, RAR, VDR, PPAR and most orphan receptors) with the retinoid X receptor, recognising a variety of palindromic response elements. The different three dimensional structures of heterodimers create different DNA binding affinities.

2.2.1.3 Hinge domain (D)

The hinge domain is located between the DBD and the LBD and is of an ill-defined function. It seems as if the carboxy-terminal extension (CTE) of the DBD may recognise the extended 5'-end of the HRE; the A and T boxes, located within the CTE is critical to monomeric binding (133) of receptors to response elements via contacts with the DNA backbone. Moreover, the hinge domain may contain nuclear localisation signals, mediate protein-protein interactions or allow for spatial arrangement between the DBD and LBD, thus allowing protein conformational change upon ligand binding (118, 134).

2.2.1.4 Ligand binding domain (E/F)

Unlike the DBD, the LBD is slightly less conserved and substantial variation occurs between the domains of different nuclear receptors, such as to make ligand recognition distinct. Four structurally discrete surfaces may commonly be observed: a dimerization interface facilitating interaction with partner LBDs, a helix towards the C-terminal involved in ligand dependent transactivation, termed activation function 2 (AF-2), a surface that mediates coregulators interaction, which may modulate transcriptional activity in a positive or negative manner, and a hydrophobic ligand binding pocket of which specific residues mediates binding of the receptor with potential lipophilic ligands.

To date, the LBDs of all the traditional nuclear receptors, along with the already adopted orphan receptors, have been resolved (135). The molecular organisation of the majority of the orphan receptors, however, remains unknown. Generally, the LBD of nuclear receptors consists of twelve highly structured α-helices arranged in a three layered anti-parallel sandwich (127), eleven of which are actively involved in the formation of a ligand binding pocket (LBP) at the narrower end of the domain. Helix 12 (H12), comprising the activation domain of AF-2, extends away from the LBD. Interaction of an agonistic ligand with the LBD results in a conformational change within the protein structure: in the apo-state, H11 is in a position of near perpendicularity towards H10 (Figure 2.3, panel A). In agonist mode H11 shifts to a position in continuation with H10 (Figure 2.3, panel B). This repositioning allows H12 to translocate to a position underneath H4 and above the now formed hydrophobic LBP. This repositioning creates the defined surface of AF-2, consisting of H3, H4, loop L3-4 and H12, thus forming a shallow surface accessible hydrophobic groove. This arrangement allows for interaction with co-activator elements containing a helical nuclear receptor LxxLL motif called a nuclear box. Agonists generally stabilise the LBD by tight-fitting interaction with the amino acid residues of the LBP. Contrarily, antagonists cannot be accommodated within the LBP due to the presence of extended side chains from the molecule which exists between H3 and H11. When an antagonist is present within the LBP, H11 becomes slightly unwound while H12 is unable to translocate to the position underneath H4 and, instead, rotates approximately 120° in a clockwise direction towards the N-terminus of the LBD. This conformational change results in the obstruction of the hydrophobic cleft to which the LxxLL nuclear box of a nuclear cofactor would bind, thus inhibiting activation of the receptor. This molecular configuration is stabilised by a degenerated LxxLL motif within H12. Consequently, the receptor itself inhibits the binding of co-activators to the activation domain (AD) of the LBD by competition for binding to the groove formed between H3, H4, L3-4 and H12.

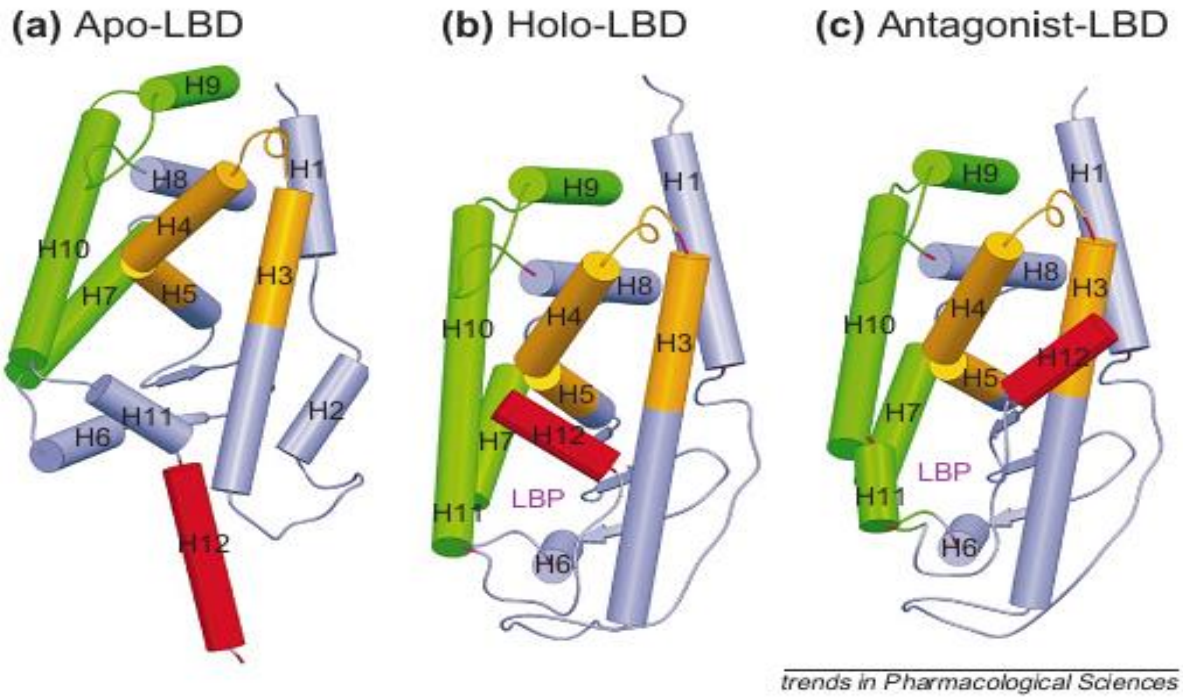

Figure 2.3 **Graphical representations of the retinoid X receptor LBD existing in three different conformational states. The unliganded (apo) RXRLBD in panel A indicates destention of H1. In panels B and C, which respectively represents the agonist (holo) and antagonist forms of the LBD, H12 is repositioned by between 120° and 270°. The various regions of the LBD have been coloured depeding on function. The activation function, the coactivator/corepressor binding motif of the LBD is coloured orange. Helix 12, which contains the core activation function activation domain, AF-2, is shown in red, while the LBD dimerisation interface is coloured green. Other structural elements are shown in purple** (127).

2.2.2 Receptor classes and general transcriptional control

Based on their respective DNA-binding and dimerization properties the nuclear receptor superfamily may be subdivided into four distinct classes (119). Class I receptors bind DNA as ligand induced homodimers by recognising half-sites organised as inverted repeats. This class includes all the receptors to the steroid hormones and, in the absence of ligand, are generally sequestered to the cytosol where the LBD is often associated with heat shock proteins (HSP) (136, 137). In this state HSPs, notably HSP90, HSP70 and HSP56, interact directly with the LBD and, in some instances the CTE of the DBD (138), thus repressing receptor activity. The association with HSP90, at least for some nuclear receptors (GR, MR and DR), seems essential for the stabilisation of the LBD in a conformation that can bind ligand (136). Upon binding of an agonistic ligand, the conformational changes introduced in section 2.2.1.4 lead to dissociation of the HSP complex, thus relieving receptor repression. As a result, the receptor translocates to the nucleus and binds to DNA as homodimers to the relevant hormone response elements. Within the nucleus the AF-2 is recognised by transcriptional co-activator proteins via the α-helical LxxLL motif. The co-activator peptide is held in place within the hydrophobic groove of the nuclear receptor by hydrogen bonds formed between the hydrophobic residues of the LxxLL motif and two critically conserved receptor amino acid residues. This charge clamp is formed between the three co-activator leucine residues and a lysine at the C-terminus of H3 and a glutamate in H12 of the LBD, resulting in

further stabilisation of the association between the two molecules. A plethora of coactivator proteins that mediate nuclear receptor functioning have been characterised, including the p160 transcription intermediary factor/NR-coactivator/steroid receptor coactivator (TIF/NCoA/SRC) family of proteins (139). Such co-activators generally contain multiple nuclear receptor box motifs and may exhibit variable cooperative or non-cooperative binding to nuclear receptors due to differing affinities for AF-2. Regardless, a key feature of nuclear receptor co-activators is their transcriptional-enhancing properties, which include the ability to recruit additional general or receptor/ligand-specific transcription factors, histone acetylation and chromatin remodelling via ATPase activity (140); resulting in the formation of the basal transcription machinery complex with RNA polymerase II which facilitates the polymerisation of mRNA.

Class II receptors, exclusive of the steroid hormone receptors, contain all receptors that are known to be ligand dependent. In contrast to the receptors of class I, these receptors heterodimerize with the RXR and bind to DNA via direct repeats or, to a lesser extent, via symmetrical repeats (119). Moreover, in the absence of ligand, receptors of class II are constitutively bound to their respective HREs and are therefore retained within the nucleus. In this state, H12 of the receptor stably interacts with the N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) containing corepressor complexes via two nuclear receptor interacting domains, ID1 and ID2, located at the C-terminus of the repressor. These interactions are mediated by motifs within each ID similar to the LxxLL nuclear receptor box called LxxxIxxxI/L motif (CoRNR box) (120). Unliganded, the DNA-bound nuclear receptor-corepressor complex recruits transcriptional determinants containing specific histone deacetylase complexes (HDACs) which enforce target gene repression. A critical determinant in the interaction between the nuclear receptor and co-activator is the formation of the charge clamp between AF-2 and the nuclear box. Agonist binding promotes repositioning of H12 and disrupts the hydrophobic groove of AF-2. The slight difference in length between the NR box and CoRNR box means that co-repressors cannot be accommodated in the hydrophobic groove of the receptor in the liganded state, resulting in an exchange of co-repressor for co-activator (141).

Class III receptors primarily bind to DNA as homodimers via direct repeats, while receptors of class IV tend to bind extended core sites as monomers. Some orphan receptors are categorised within class II and therefore heterodimerize with the RXR, yet the majority tend to fall into these last two groups. Orphan receptors of class III are typically constitutive transactivators or transrepressors and can bind to either palindromic or direct repeat response elements which may overlap with recognition sequences of other hormone receptors. These qualities suggest that they may be ligand independent transcription factors which may modulate known hormone signalling pathways (142). The monomeric orphan receptors of class IV binds to extended half-sites of the estrogen receptor class (AGGTCA). Determination of the specificity towards individual receptors of this class is determined by sequence of A/T rich nucleotides 5' of this binding site (143, 144). Similar to receptors of class III, many of these orphan receptors display constitutive activity.

One group of orphan receptors are not readily placed in any of the categories mentioned above as they contain members that are lacking of either a DBD, as is the case with DAX-1, or an LBD. For reviews on orphan receptor structure, function, discovered ligand and physiological roles, please refer to these references (145–148).

2.2.2.1 Further control measures and nongenomic effects

The central dogma of nuclear receptor mediated control over gene transcription involves interaction of the liganded receptor with its cognate response element, followed by recruitment of cofactors and chromatin remodelling, with subsequent assembly of the transcriptional machinery. A pertinent question, therefore, is what happens to the activated receptors following these processes? In contrast with a conventional view of nuclear receptor action (149, 150), the interaction between receptor and HRE does not appear to be static. The recruitment of cofactors to the transcription machinery complex following binding of the receptor to the HRE occurs in a systematic, yet cyclical manner (151). *In vivo*, chromatin immunoprecipitation (ChIP) has indicated that in the presence of continual hormonal stimulation regular assembly and disassembly of the transcriptional machinery occurs, providing a mechanism for continual assessment of the external environment (152, 153). Phosphorylation of RNA polymerase II may lead to dissociation of the transcription complex, since inhibition of the cyclin-dependent protein kinases CDK7 and CDK9, which are known to act upon the C-terminal domain of the large subunit of polymerase II, stabilises transcription of the ERα (152).

Furthermore, nuclear receptors are themselves phosphoproteins. Post-translational phosphorylation of the nuclear receptors by protein kinase A (PKA), protein kinase C (PKC), proline dependent kinases or mitogen-activated protein kinases (MAPKs) occur mainly in the A/B region, however, several residues within the DBD and LBD are also amenable to phosphorylation. Such modifications may modulate transcriptional activity by influencing dimerization ability or co-activator recruitment (154–159). Yet phosphorylation events may also be involved in the termination of the ligand response by the induction of DNA dissociation, decreasing receptor affinity for the ligand or by eliciting receptor degradation, since CDK7 protein kinase is also able to phosphorylate nuclear receptors (160, 161). The phosphorylation of proteins is often a precursor to ubiquitylation (162) and subsequent degradation within the proteasome. Moreover, the regulatory cofactors to nuclear receptors may also be phosphorylated, impacting on their ability to exert control over transcriptional activity. For example, phosphorylation of the corepressors SMRT and N-CoR results in the export of these proteins from the nucleus, making it unavailable to repress steroid receptor transcriptional activity (163).

Over and above the regulation imposed on nuclear receptors by covalent modification such as phosphorylation, ubiquitylation, acetylation and sumoylation, other forms of nuclear receptor cross-talk are also known to occur. As of late it has become apparent that extranuclear steroid receptors may activate non-nuclear pathways in various cell types via signalling cascades involving several kinases,

including phosphatidylinositol 3-kinase (PI3K), PKC and cAMP/PKA-dependent mechanisms, along with calcium and other second messengers (164, 165).

According to Levin, additional steroid hormone receptor pools actively involved in such processes have been detected not only in cytosol, but also localised to the plasma membrane (166, 167). Membrane bound ER, for example, is proposed to be involved in the rapid increases in cAMP levels and calcium responses observed in ovariectomised rats and endometrial cells following administration of 17β-estradiol [1,3,5(10)-estratriene-3,17β-diol; E_2] (168, 169), which is not a function of the nuclear localised ER (170). Such fast-acting membrane-initiated steroid signalling (MISS) has been implicated in reproductive organ development and function, cardiovascular responsiveness, and cancer biology (171–173). MISS impacts on the regulation on gene expression via a variety of mechanisms of which the full integrative nature remains elusive.

2.2.3 Focus on the sex steroid receptors

Clearly the nuclear receptor superfamily is intricately involved in the regulation of the cellular environment in response to external stimuli, predominantly via particular cognate ligands. However, due to the large number of nuclear receptors governing countless biological processes, the focus here will predominantly be on the sex steroid receptors. Although the progesterone receptor is integral to the regulation of multiple reproductive processes, including follicular growth, oocyte maturation, ovulation, implantation and the maintenance of gestation, specific discussion will be limited to the androgen and estrogen receptors.

2.2.3.1 Estrogen receptor

The estrogens (Figure 2.4) are predominantly concerned with the development and maintenance of the female reproductive system and secondary sexual characteristics. Moreover, they are also involved in a range of non-reproductive functions, including growth and bone density regulation, nervous system maturation and endothelial responsiveness. The two major biologically active estrogens, E_2 and estrone [3-hydroxy-1,3,5(10)-estratrien-17-one; E_1], are synthesised via P450-mediated oxidative removal of the C-19 methyl group from the androgens testosterone and 4-androstene-3,17-dione, with subsequent aromatization of the A ring by the P450-dependent aromatase enzyme. These two hormones can be interconverted by a redox reaction whilst estriol [1,3,5(10)-estratriene-3,16-α,17β-triol; E_3], a third bioactive estrogen, is formed by the 16-hydroxylation and 17-reduction of E_1. E_3 is the main estrogen during pregnancy, but plays no significant role in men or non-pregnant women.

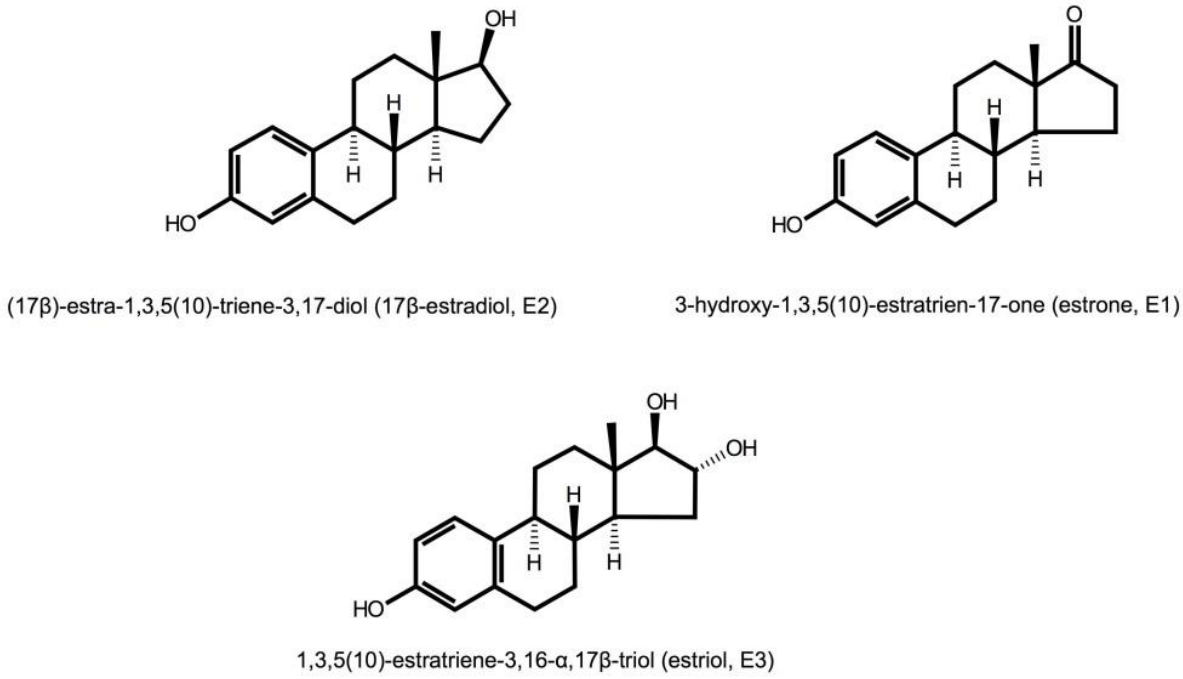

Figure 2.4 Structures of the major estrogens, natural ligands to the estrogen receptor.

The aromatisation of testosterone to E_2 occurs primarily in ovaries and testes. Small amounts, however, are also formed in the adrenal glands and some peripheral tissues, most notably adipose tissue. By contrast, most of the circulating E_1 is derived from the peripheral aromatisation of androstenedione originating in the adrenals. E_2 demonstrates far greater biological potency, as compared to E_1, and is present in higher concentrations within the circulation of premenopausal, non-pregnant women. Following the onset of menopause, however, ovarian production of E_2 ceases, resulting in a reversal of the ratio of E_2:E_1.

The estrogens elicit their biological actions by binding to the estrogen receptor family of receptors. Traditionally, two ligand-activated transcription factors, the nuclear receptors ERα (174) and ERβ (175), were thought to be the soul mediators of estrogenic activity. These two ERs are encoded by two genes located on different chromosomes (176) and exhibit diverging tissue distributions (177) with slightly altered affinities to E_2 (178), and different ligand binding characteristics (179). ERα is predominantly expressed in the uterus and mammary glands, and is intricately involved in reproductive events. Conversely, the distribution of ERβ is more general and seems to be relevant to the functioning of the central nervous system, the cardiovascular system and the skeletal system. Its expression is also highly prevalent in the lungs, kidneys, colon, ovaries, testes and urogenital tract (175). However, ERα and ERβ share significant homology in their DNA binding domains (95%) with identical P-box sequences; they therefore interact with the same EREs (180). The ligand binding domains are less alike with only 56% homology of structure, yet the LBPs of these receptors are remarkably similar, differing by only two amino acid residues; Leu_{384} in the ERα is replaced by Met_{336} in ERβ, while Met_{421} in ERα corresponds to Ile_{373} in ERβ (181).

Synthetic estrogens (Figure 2.5), such as the SERMs raloxifene (RAL) or 4-hydroxytamoxifen (OHT), which exhibit agonism in a tissue-dependent manner, or the non-steroidal full agonist diethylstilbestrol (DES) bind to the ERα and ERβ in the same position as estrogen, resulting in similar molecular events. As with all the nuclear steroid receptors, binding of ligands results in the dissociation of ERs from their cytoplasmic chaperone partners, with a concomitant translocation to the nucleus. Once bound to DNA, agonistic ligands promote the recruitment of cofactors which transmit regulatory information to the transcription complex, leading to transactivation. ERα and ERβ, however, differ in their ability to activate genes when bound to the same ligands due to multiple reasons including alternative conformation upon ligand binding, distinct cofactor interaction (182), and discrete ERE binding patterns (183).

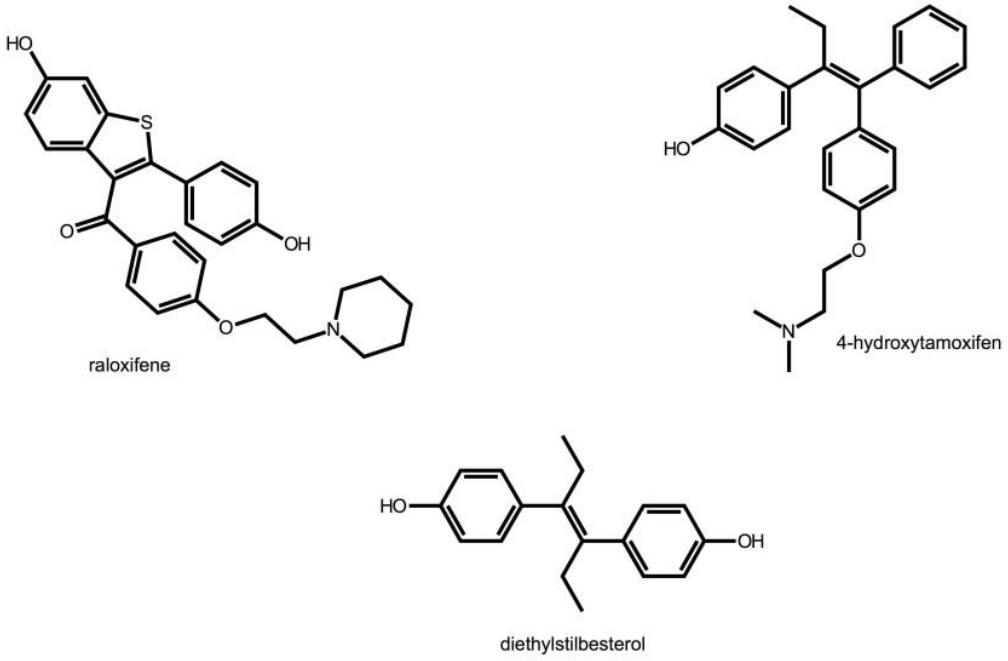

Figure 2.5 **Structures of synthetic compounds that interact with the estrogen receptor. The actions of SERMs such as raloxifene and 4-hydroxytamoxifen is distinguisable from pure receptor agonists and antagonists. These compounds selectively activate or repress the estrogen receptor, depending on tissue type involved. Raloxifene and 4-hydroxytamoxifen both exhibit agonism in bone and antagonism in breast tissue. However, whereas raloxifene acts as an antagonist in the uterus, the actions of 4-hydroxytamoxifen in this tissue is agonistic. Conversely, diethylstilbestrol is a non-selective full agonist to the ER.**

Furthermore, the A/B domain, containing the ligand-independent AF-1, of the 595 amino acid ERα protein, shares a mere 17% homology at amino acid level with the 530 residue ERβ. Transcriptional activity of ERα relies on a synergistic effect between AF-1 of the N-terminal domain and the AF-2 located within the C-terminal LBD. The constitutively active AF-1 of ERα can bind to histone acetyltransferase-active transcription coactivators such as the CEBP binding protein/p300 and p160 subfamilies, which include steroid receptor coactivator-1 (SRC-1) and the transcription intermediary factor TIF2 (184, 185). Binding of E_2 to ERα promotes an interaction between the B-domain at the N-terminal of the receptor, and the LBD (184), a conformational change which directs the two domains closer together. In response to agonist binding, p160 transcriptional factors also interact with the ERLBD

by docking to AF-2 via the nuclear box motif, resulting in enhanced transcriptional synergy between AF-1 and AF-2. For ERα, this synergy seems to be a requirement for the recruitment of RNA polymerase II to the transcription complex, as an inhibitory fragment of SRC-1, in the presence of E_2, blocked RNA polymerase II activation (177). The same does not hold true for ERβ and the same ligand-independent SRC-1-binding ability seen in murine ERβ AF-1 is not mirrored in the human homologue of the receptor (186). However, E_2-regulated genes may contain imperfect and nonpalindromic EREs that are still recognisable by activated ERs (187, 188). As such, ERα and ERβ interaction with co-activators may be dependent on the specific sequence of the ERE that they are bound to. Furthermore, ERs may indirectly interact with response elements other than the ERE by tethering to DNA bound transcription factors such as activation protein 1 (AP-1) and stimulating protein 1 (SP-1). For example, in complex with SP-1, bound to the SP-1-directed promoter of the transforming growth factor β-inducible early gene-1 (*TIEG*) gene in human osteoblasts, ERβ is able to bind SRC-1, leading to transcription from *TIEG* in response to E_2 via a nuclear ERE-independent mechanism (189).

The classical receptor-DNA interaction of ERα and ERβ to EREs remains the best-characterised signalling mechanism for estrogen-mediated effects on cellular homeostasis. However, within the last two decades it has become evident that estrogen can mediate rapid cellular responses by non-nuclear mechanisms in a variety of cell types, particularly in non-reproductive tissues such as the vascular smooth muscles, endothelial cells, neuronal tissue, osteoblasts and osteoclasts (190–193). Subsets of ERs, capable of high affinity interaction with estrogenic compounds are localised not only in the nucleus or sequestered to the cytoplasm in association with HSP complexes, but also in, or proximal to, the plasma membranes of certain cells and distinct cytoplasmic organelles such as mitochondria and the endoplasmic reticulum (165, 194). A variant of ERα, ERα36, cloned by Wang *et al.* (195) has been reported to be involved in cell proliferation due to cross-talk with the epidermal growth factor receptor pathway (196). This 36 kDa truncated ER that lacks both activation domains AF-1 and AF-2 observed in its 66 kDa counterpart. The absence of these transactivation domains means that ERα36 has no intrinsic transactivation activity (195), thus highlighting the role of E_2 in non-genomic modulation of cell cycle control. Other ER variants, such as the N-terminally truncated 46 kDa ERα isoform are known to be expressed in numerous cell types, including endothelium (197), osteoblasts (198), bone marrow stromal cells (199), endometrium (200) and breast tissue (173). Gorosito *et al.* (201) reported a 52 kDa splice variant of ERα in hypothalamic tissue which localised to the plasma membrane upon treatment with E_2; a result that was subsequently corroborated by Bondar et al. (202) and Dominguez and Micevych (193).

The localisation and palmitoylation-status of ERs can potentially regulate ER ability to interact with intracellular kinases. Trafficking of ER variants to the membrane occurs by association with membrane bound proteins within caveolae rafts (203). It seems as if E_2 can regulate the level of palmitoylation at a single amino acid residue in the ERα, Cys447 (204), modulating association with the membrane protein caveolin-1 (CAV1) and caveolin-3 (CAV3). Within hippocampal neurons caveolin-bound ER is involved in two segregated signalling pathways (205, 206). CAV1-bound ERα interacts with the

metabotropic glutamate receptor mGluR1a, stimulating activation of the Gq domain of this GPCR, which in turn leads to the phosphorylation of CREB (cAMP activated regulatory protein) via PLC/IP3/MAPK signalling. Conversely, ERα or ERβ can activate the second pathway in which the CAV3-linked receptors stimulate mGluR2/3 to activate Gi/o signalling. Consequently, inhibition of PKA occurs which facilitates the attenuation of calcium channel-dependent CREB phosphorylation by a reduction in L-type calcium channel currents.

Non-nuclear ER can therefore act upon signal transduction pathways via G-protein coupled receptors (GPCRs), phosphatases or signalling cascades, stimulating nongenomic effects (100, 196, 207). Work by Garrido *et al.* (208) implicates rapid signalling by E_2 in MCF-7 breast cancer cells in the translocation of GLUT4 to the plasma membrane. The interaction of E_2 with ERα46 may therefore indirectly stimulate glucose metabolism by exerting control over key steps modulated by p85 in the PI3K/Akt-signaling cascade, suggesting a link between glucose uptake and E_2-mediated breast cancer proliferation. Conversely, the truncated splice-isoforms of ERα appear to activate multiple signalling cascades, including PI3K/Akt, in vascular smooth muscle cells (VSMc), all seemingly converging on the induction of epithelial nitric oxide synthase (eNOS) (209). The resulting release of cardioprotective nitric oxide antagonises leukocyte adhesion and promotes endothelial cell growth and migration, while attenuating the growth and migration of VSMc (210). Moreover, Kim *et al.* (192) proposed the localisation of ERα46 to the plasma membrane as a transmembrane receptor with an N-terminal ecto-domain. Transmembrane orientation seems to rely on critically specific amino acid residues since mutation of Ile386, located within the proposed transmembrane domain, to cysteine dramatically impaired E_2-induced signalling in mutant ERα46Ile386Cys expressing cells. Transmembrane receptors which influence the functioning of E_2 activated intercellular pathways may be of great value as drug targets. Molecules that cannot traverse the plasma membrane, yet alter cellular activities mediated by E_2 signalling, could selectively influence the mechanisms controlling the initiation and progression of certain cancers and other undesirable physiological conditions.

Several reports have, however, indicated that the rapid increase of second messengers following E_2 stimulation is mediated by a member of the largest group of cell-surface molecules, the GPCR superfamily (211). Extremely diverse arrays of ligands, including neurotransmitters, hormones, lipids, a plethora of small molecules and even light stimulate cognate GPCRs to change conformation, leading to the exchange of an α-subunit bound GDP molecule for GTP (212). GTP-bound GPCR α-subunits interact with a variety of intercellular signal transduction pathways which regulate the biochemistry of basal metabolism, cognition, environmental sensing and cell proliferation control. The effects of GPCR signalling are mediated via second messengers that impact on phosphorylation and other regulatory activities. The orphan receptor, G-protein coupled ER 1 (GPER1), or GPR30 as it was originally named (125), is thought to induce rapid non-genomic actions upon binding to estrogen. The exact cellular location of this estrogen associated GPCR is still under dispute. Some analyses indicate membrane or cytosolic association (213–216), while others point towards the localisation of the GPER1 to the

endoplasmic reticulum (217–219). However the case may be, cloning of the *GPER* gene by Carmeci *et al.* (220) from an ER-positive breast carcinoma cell line (MCF7) and the subsequent transfection of this gene to estrogen-unresponsive MDA-MB-231 cells (221) substantiated a non-nuclear role of estrogen in signal transduction. The underlying mechanisms of estrogen-GPER1 signalling is still disputed, although it is has been reported that the α-subunit of the receptor activates adenylyl cyclase which results in the production of cAMP (222). Moreover, matrix metalloproteinase is activated to degrade pro-HB-EGF by the G-protein βγ/Src/integrin-α5β1/Shc complex (223). HB-EGF in turn binds to the EGFR leading to the mobilisation of intracellular calcium and the activation of PI3K and Erk-1/-2 pathways (224–227). Reportedly, GPER1 is involved in mediating the actions of estrogen during testicular development and spermatogenesis. It is expressed in the somatic cells (Sertoli and Leydig) as well as the spermatogonia, spermatocytes and spermatids. However, GPER1 also seems to be prevalent in the development and growth of testicular cancers (211) and testicular germ cell tumours (TGCT) (95). Several reports have challenged the possible roles that GPER1 plays during estrogen-mediated non-nuclear activities (171, 219, 228). Kang *et al.* (196) found that ERα36 expression was augmented by GPER1 signalling by activation of the ERα36 promoter, and the authors concluded that the non-genomic activities ascribed to GPER1 are in fact mediated via this ER variant.

The multiple mechanisms by which estrogen influences cellular homeostasis form an intricately connected network that converges at the regulation of gene transcription. These numerous pathways are involved not only in normal reproductive physiology and other biological processes, but also impact on various human disease states, including breast and endometrial cancers, cardiovascular disease, osteoporosis, and Alzheimer's disease.

2.2.3.1 Androgen receptor

Male fertility, indeed the whole of the vertebrate masculine phenotype, is primarily influenced by the cellular actions of androgenic hormones. In mammals, some androgens are produced by the zona reticularis of the andrenal cortex (229). However, in males androgenic steroid hormones are predominantly synthesised in the Leydig cells of the testes, while substantial amounts are produced by the ovarian theca and stromal cells of females; which prominently influence female secondary sexual characteristics and cellular differentiation. Steroidogenesis of androgens from cholesterol-derived precursors may be accomplished via several cytochrome P450 controlled pathways (230, 231), resulting in the synthesis of molecules with divergent potencies towards the androgen receptor (AR). Classically, testosterone (T) and its 5-alpha reduced form, 5α-dihydrotestosterone (DHT), have been identified as the endogenous ligands to the androgen receptor (232). However, many other physiologically relevant androgenic hormones have been discovered which may influence cellular homeostasis via interaction with the AR, including the 11-keto or 11-hydroxylated forms (233) of these and other androgens, such as 11β-hydroxyandrostenedione, which serves as a precursor to recognised and novel androgenic steroids (Figure 2.6) (234).

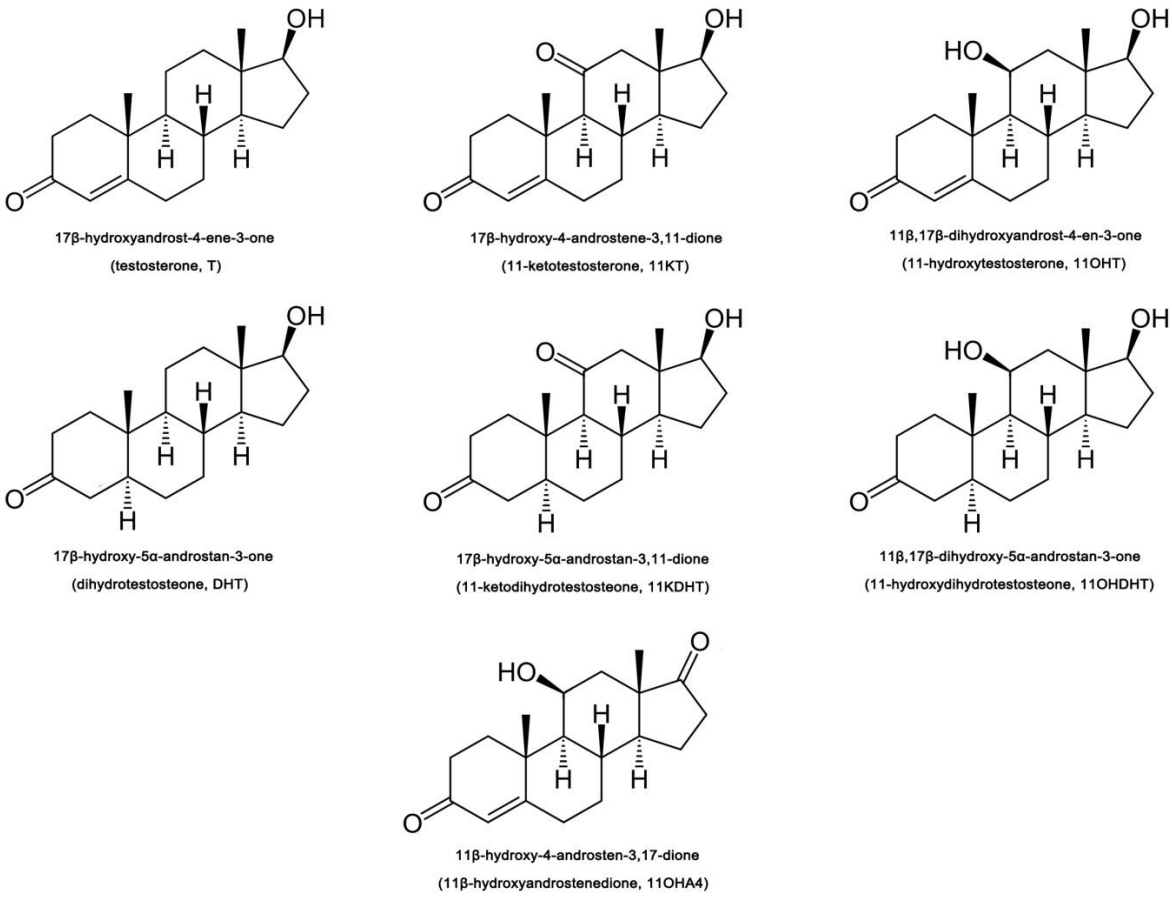

Figure 2.6 **Some of the endogenous ligands known to activate the androgen receptor. These molecules have varying potencies, indicating that small alterations to the molecular structure of agonists can induce slightly different conformational changes to the LBD of a nuclear receptor, thus affecting the interaction between AF-2 and co-activator molecules.**

Androgens are well-known to play an important part in male embryonic differentiation, male sexual development and maturation, maintenance of spermatogenesis (235) and masculinisation of the brain (236). Furthermore, several other biological processes, over and above the development and regulation of male reproductive physiology, are also enacted by the androgens. These diverse actions include control over the hair follicle growth cycle (237), regulation of adipogenesis, lipolysis and fat distribution (238), bone remodelling and lean muscle maintenance. However, due to the involvement of androgens in such processes, various physiologically-altered and pathological states, including male pattern hair loss, acne, spinal and bulbar atrophy, benign prostatic hyperplasia, androgen-insensitivity syndrome and the progression of prostate cancer are arbitrated via the AR (239).

The AR is a ligand-dependent transcription factor which, like other steroid receptors, undergoes a conformational change upon ligand binding, translocates to the nucleus and interacts as homodimers with cognate hormonal response elements in the promoters of target genes. Recruitment of coregulators and chromatin remodelling complexes at genomic loci results in the formation of the basal transcription machinery and transcription of AR controlled genes. Canonical AREs are composed of 3-nt-spaced inverted repeats of 5'-TGTTCT-3' half-sites and are essential for AR-mediated transcriptional activation

(240). The domain structure of the AR is similar to the other members of the nuclear receptor superfamily. The highly conserved DNA binding domain recognises androgen response elements (ARE) by means of two zinc-finger motifs.

As with the ER and other nuclear hormone receptors, the autonomous AF-1 region is located within the N-terminal domain. Unique to the AR, however, AF-1 contributes the majority share of activity to the ligand-bound-AR and is constitutively active when removed from the apo-protein *in vitro*. Interaction between the NTD and the activation function, located within the LBD, seems to be required for agonist-induced gene transcription or repression when bound by an antagonist (232). The molecular alteration which occurs when the AR interacts with ligand is similar to the ER. Upon ligand binding the receptor translocates to the nucleus where it forms homodimers, binds to ARE and regulates transcription in cooperation with a range of cofactors. The conformational change involves translocation of H12 to a position covering the LBP which stabilises receptor-ligand interaction. Along with H3 and H4, the hydrophobic groove of AF-2 is thus formed on the surface of the LBD (241). This groove is crucial for coactivator recruitment as well as the intermolecular interaction of the NTD with the LBD. In this regard, the AR varies from other steroid receptors due to the ability of AF-1 to regulate ligand-bound receptor activity in the process of N/C interaction (242). Within the A/B domain of the AR, two LxxLL-related sequences, have been found to mediate the molecular interchange between the NTD and LBD when bound by ligand. Therefore, N/C interaction is required for full transcriptional activation of the AR and is enhanced by multiple coactivators, including CREB binding protein (CBP), the proto-oncogene c-Jun, and the p160 family of cofactors (241). However, in contrast to other steroid receptors, the AF-2 of the AR exhibits relatively low affinity to most LxxLL motifs (243). The agonist bound AR transforms its conformation in such a way that the hydrophobic groove which is AF-2 is elongated, deeper and narrower than those of its homologue receptor counterparts. As a result, AF-2 is recognised preferentially by aromatic residue-containing LxxLL-related motifs. Two such motifs, FxxLF and WxxLF are located in the A/B domain and can bind to AF-2. AR specific coregulators, such as ARA54 and ARA70, also feature the FxxLF recognition motif.

Dissociation of the ligand is slowed due to stabilisation of H12 by N/C interaction (244, 245). Bound to DNA, however, the importance of this association is weakened (246), thus providing a mechanism for transient binding to the ARE of AR controlled gene promoter regions. Depending on its nature, a ligand to the AR can influence the binding stability of the AR/DNA. Consequently, loss of the bound ligand leads to nuclear export of the AR to the cytoplasm where reassociation with stabilising heat shock proteins occurs. AR antagonists such as bicalutamide and hydroxyflutamide induce the recruitment of corepressors due to their inability to facilitate N/C interaction. However, continued exposure to such substances has been shown to result in the development of androgen resistance due to cellular modifications or the inclusion of point mutations in the *AR* gene.

Furthermore, the AR can be covalently modified to influence its agonist or antagonistic behaviour. Various non-genomic actions of the AR include modulation of multiple signalling molecules at the plasma membrane. The PI3K pathway is activated by direct interaction of the AR with the p85 regulatory subunit of the kinase. Numerous other biochemical routes, including Src, ERK1/2, MAPK and Akt pathways are impacted upon by ligand binding to the AR or act upon the AR via extracellular stimulation by cytokines, mitogens, hormones or possibly disruptive exogenous molecules (247).

Regulation of these intricately connected pathways is an important function towards the maintenance of controlled cellular differentiation and homeostasis. The androgens present during foetal development direct sexual development because of its proportionality with estrogen, following aromatisation by P450 aromatase. Disruption of the androgen/estrogen ratio, by mutation or by exogenous chemical modulation, may lead to a reduction in developmental prowess or the manifestation of hermaphroditism. Furthermore, with over 500 putative protein kinase and more than 90 annotated tyrosine kinase genes in the human genome (248) it is clear that phosphoproteins, such as the AR, are subject to myriads of biochemical signals which may alter its activity towards oncogenic or tumour-producing functions. Such an intricate network of interconnected signalling cascades allows for the dysregulation of protein-protein interaction via multiple mechanisms.

2.3 A BRIEF OVERVIEW OF MAJOR HETEROLOGOUS PROTEIN EXPRESSION SYSTEMS

The development of protein production platforms have become an important cornerstone of therapeutic applications in pharmacology, academia and biotechnology. Proteins are being characterised at the molecular level by exploiting recombinant techniques in the expression and purification of biological macromolecules. The ability to compare large datasets at the genomic level in the delineation of a diverse protein cohort has been a valuable tool in the study of protein interaction. However, structural analyses by various biophysical methods require substantial quantities of protein (249). Purification of many proteins from their native hosts is often impeded by the low amounts present in such source materials. However, knowledge of how to manipulate cells for the overexpression of heterologous proteins has been steadily compiling since the first bacterial transformation with a recombinant plasmid by Cohen *et al.* in 1973 (250) and the subsequent production of somatostatin from a transgenic bacterium by Genetech, Inc. (251), followed by the market release of bacterially produced human insulin, Humulin™, in 1982 (252). Advances in expression technology have increased the recovery of heterologously expressed proteins in a variety of host systems, leading to a surge in biotechnological and therapeutic applications (253). The success of any biological construction, however, relies heavily on the approach taken and several considerations must be met in selection of an expression platform (254). Depending on the application and scale of the intended project specific attention must be given to

productivity, process economics, product quality and safety, production stability, lead times, scalability, and regulatory acceptance.

2.3.1 Bacterial expression systems

Expression of recombinant proteins in bacterial systems, of which *Escherichia coli* is the most well-known, is often the preferred choice in the synthesis of non-glycosylated proteins for a variety of reasons. The host metabolic needs are well characterised and cultures can rapidly achieve high biomass, it is inexpensive and has the ability to generate high protein yields.

Apart from *E. coli*, other prokaryotic organisms have also been employed in heterologous protein expression. *Bacillus* is the second most utilised prokaryote for expression studies and are often successfully utilised in the production of pharmaceutically relevant proteins. In part, this is because these Gram-positive organisms do not contain lipopolysaccharides in their outer membranes, conferring to them generally regarded as safe (GRAS) status (255). What is more, however, is that the complete genomes of certain *Bacillus* have been sequenced (256, 257). Organisms such as *Bacillus subtilis*, *Brevibacillus choshinensis* and *Bacillus megaterium* all exhibit naturally high secretion capacity and are therefore well-suited to the generation of toxins and exo-enzymes. Many of the excreted proteins are correctly folded, soluble and active and, under certain conditions, yields of up to 3 g recombinant protein per litre have been reported (258). Furthermore, some bacilli inherently have low levels of extracellular proteases (259), in the case *of B. subtilis* efforts to characterise or reduce endospore formation resulted in the generation of protease-deficient strains, thus increasing the stability of secreted proteins (260). Some of the regulatory elements of these species have also been characterised to potentiate transcript stabilisation (261). However, compared to *E. coli*, the transformation of bacilli have been arduous, often requiring polyethylene glycol-mediated protoplasting (262), use of minimal-media protocols, or host strain alterations (263). The lack of suitable expression vectors and producer strains has been addressed in the last decade (264), yet plasmid instability and misfolding in more complex proteins has maintained reservations to the use of bacilli as expression hosts.

In theory, all bacterial cells could be used for the production of heterologous proteins. Many examples of alternative prokaryotic cells as expression hosts exists, including *Staphylococcus carnosus* (265), *Pseudomonas* spp. (266, 267), *Ralstonia eutropha* (268) and *Methylobacterium extorquens* (269), have been shown to be capable of effective recombinant protein production. As with *Bacillus* strains, the nisin-inducible controlled gene expression system (NICE) of *Lactococcus lactis* have also recently emerged as an attractive alternative due to its GRAS status, lack of endotoxins and ability towards extracellular protein production (270). However, compared to *E. coli*, these systems are still poorly characterised in literature and little support with regard to vectors and promoter are available. As a result, such hosts are rarely investigated as a means of protein synthesis alternatives and only 1% of therapeutic protein products are produced in bacteria other than *E. coli* (271). However, as more information is gathered regarding the growth requirements and genetic regulation of these organisms, with concomitant

availability of commercial support, a new generation of prokaryotes may come to rival *E. coli* as expression systems.

Until such time, however, *E. coli* will most likely remain the most prevalent prokaryotic host for the heterologous expression of proteins. In 2013, Férnandez and Vega estimated that 88.6% of distinct protein chain entries in the Protein Data Bank (PDB) indicated *E. coli* as expression host (264). Decades of research pertaining to the genetics of *E. coli*, and its use as propagation host for DNA used in nearly all cloning and expression systems, have secured this bacterium as a firm favourite of many protein biochemists. However, as the complexity of proteins increases, as is commonly seen with mammalian proteins, the use of *E. coli* is faced with certain constraints. Proteins which require post-translational modification, such as glycosylation, acetylation or even phosphorylation, or those that depend on correct disulphide bond formation for structure or function are often challenging in *E. coli* expression systems. Furthermore, the evolutionary selective use of codon triplets in different organisms further complicates the overexpression of protein in any heterologous system. Nevertheless, multiple strategies have been designed to counter such problems. Codon bias has been successfully addressed by site-directed mutagenesis, the use of specialised strains such as Rosetta-gami (272) or the incorporation of plasmids such as pRIG, pRARE, pACYC or pSC101 (273). Directing protein expression to the periplasm and the co- or pre-expression of protein disulphide isomerase (PDI), sulphydryl oxidases or disulphide bond formation catalysts (Dsbs) have, in some cases, relieved issues arising from the reductive nature of the *E. coli* cytoplasmic environment (274, 275). Even the appreciable inability of *E. coli* to produce glycosylated proteins has been addressed following the identification, and subsequent transfer, of N-linked glycosylation systems in the Gram-negative bacterium *Campylobacter jejuni* (276) and, recently, the δ-proteobacterium *Actinobacillus pleuropheumoniae* (277). Structurally the bacterial N-glycans differ from their eukaryotic counterparts. Still, they provide the basis for the engineering of a bacterial glycosylation system capable of replicating mammalian glycan structures.

2.3.2 Yeast expression systems

With a mere 35% of heterologous proteins produced in *E. coli* being sufficiently soluble and correctly folded for biological studies (278), the sometimes limited success with bacterial expression systems restricts the use of these organisms as expression host for certain proteins. Increasing knowledge of the cellular regulation at the transcriptional and translational levels of different organisms presents a means of selecting appropriate expression hosts on the basis of codon usage and a possible necessity for post-translational modifications.

As with bacterial cells, the development time from gene to final product is significantly shorter than higher eukaryotic expression systems. Yet the expression of soluble, active protein is often achievable to higher degrees in yeast than in *E. coli*, especially if the product is secreted from the host cell to the cultivation media (279). Furthermore, many eukaryotic proteins fail to produce properly in *E. coli* due to particular requirements for key interaction partners which facilitate proper folding and effective structural

additions to peptide chains. Unicellular, eukaryotic fungi contain many of the cellular machinery necessary for the overexpression of a broad range of proteins with such requirements. Modifications to the protein structure, carried out in the endoplasmic reticulum following translation, are almost identical in lower and higher eukaryotes, since the processing reactions in this organelle are highly conserved. However, restructuring of N-glycan moieties in the Golgi apparatus differs between species and is cell specific (280, 281). As a result the glycosylation patterns of proteins, heterologously synthesised in yeast, may differ from their human or mammalian counterparts, thus affecting biological activity or eliciting immunogenic responses in therapeutic milieus. Considerable effort have therefore been exerted in engineering yeast strains that produce aglycosylated peptide chains such as the recombinant human serine protease ocriplasmin, produced under the brandname Jetrea® (ThromboGenics) (282). This biotherapeutic product, for treatment of symptomatic vitreomacular adhesion, is expressed in the methylotropic yeast *Pichia pastoris*. Moreover, N-glycan engineering in yeast was pioneered in *P. pastoris* (syn. *Komagataella pastoris*) during the last decade (283) by limiting the yeast ability to mannosylate while introducing glycosyltransferases which can produce complex oligosaccharide terminal sugars such as galactose, sialic acid and acetylglucosamine (GlcNAc) (284). The humanisation of glycosylation in *P. pastoris* has facilitated the production of various yeast-derived therapeutic proteins and antibodies as heterologous products. Furthermore, other yeast strains, including *Saccharomyces cerevisiae* (285, 286), *Hansenula polymorpha* (syn. *Ogataea parapolymorpha*) (287–289), *Yarrowia lipolytica* (290, 291) and *Kluyveromyces lactis* (292), have since been reported as systems capable of synthesising heterologous proteins. According to Bill (293) 11% of publications entered into the PubMed Central database relating to recombinant gene expression during 2013 indicated *P. pastoris* as expression host. Currently, 20% of biopharmaceutic proteins are synthesised in yeasts (294).

2.3.3 Mammalian expression systems

However, within the pharmaceutical sector mammalian expression systems are still the dominant choice for the production of bio-pharmaceutically relevant proteins. With market approved therapeutic proteins produced in such systems exceeding 50% (253) there is a clear mandate for stringent mimicry of human-like biologicals. Recent optimisations to media formulation, population selection and process control have improved cell line development to the point where upwards of 10 g/l mAb/Fc-fusion proteins have been produced in 100 litre bioreactor cultures of Chinese hamster ovarian cells (CHO) (295). The production of complex protein therapeutics, with monoclonal antibodies being the most prevalent and rapidly developing sector, are preferably performed in mammalian systems due to highly human compatible posttranslational modifications and pharmacokinetic functionalities (296). Predominantly, the attraction to mammalian culture is centred on the capability to add composite carbohydrates to synthesised proteins. The extensive posttranscriptional metabolic machinery, required to functionalise humanoid proteins, are simply unavailable in prokaryotic bacteria and are mostly inadequate in lower eukaryotic cells such as fungi or unmodified yeasts (297, 298). CHO cells are therefore readily used to produce about 35% of the therapeutic proteins on the market (271) since the glycosylation patterns

produced by these cells are similar to those in humans (299). CHO, and its derivatives, and the murine derived myeloma cell line NS0 have traditionally been the predominant choices for heterologous gene expression in mammalian cells due to known productivity profiles and safety concerns (296, 300). Other cell types include human embryonic kidney (HEK-293) cells, baby hamster kidney (BHK-21) cells, and the myeloma lymphoblastoid-like cell line Sp2/0-Ag14.

2.3.4 Baculovirus and insect cell expression systems

Ten years ago the pharmaceutical companies GlaxoSmithKline (301, 302) and Merck (303–305) both invested in and produced vaccines to protect against cervical cancer. These vaccines, licenced under the trade names Cervarix™ and Gardasil®, respectively, were constructed by producing the L1 protein of the human papillomavirus viral capsid in heterologous systems. While Merck produced these viral proteins (HPV-6/11/16/18 L1) in yeast, the bivalent HPV-16/18 L1 vaccine by GlaxoSmithKline was a product of the baculovirus expression vector system (BEVS). The 2007 European Medicines Agency (EMA) and 2009 US Food and Drug Administration (US FDA) market authorisations of Cervarix™ was a critical milestone in the approval of baculovirus-derived vaccines. Various other BEVS vaccines have been approved for use in humans or animals, and many clinical trials for the introduction of new vaccines are currently underway (306).

Following the expression of the first foreign gene, human beta interferon β (IFN-β) (307), in *Spodoptera frugiperda* cells, the BEVS gained prominence for the production of eukaryotic protein targets for a variety of reasons. Baculoviruses are insect pathogens which exhibit considerable host-tropism. Their limited transmission range therefore means that they are considered safe for use in vertebrates. Wild-type and recombinant viral particles cannot replicate in eukaryotes other than their insect cell hosts. An increase of laboratory biosafety levels is generally not required. Furthermore, lepidopteran cell lines are relatively easy to culture and can be adapted to growth in suspension. The system is therefore easily amenable to bioreactor scale expansion.

The large, double-stranded, circular DNA genomes of baculoviruses are generally stable and can accommodate large heterologous inserts. With few exceptions, baculovirus mRNAs do not require intron-splicing and mammalian cDNA is generally used for heterologous expression. Notwithstanding, examples of the expression of efficiently spliced mammalian transcripts in insect cells have been published (308, 309). Chen constructed a baculovirus polyhedrin promoter within an artificial intron and inserted it into a coding sequence, resulting in functional expression of genes with overlapping open reading frames (310). A major attraction, however, is the post-translational capacity exhibited by the system. Insect cells, being eukaryotes, follow the same initial steps during glycosylation of proteins. Within the endoplasmic reticulum Asn-X-Ser/Thr glycosylation sites are recognised on the nascent peptide-chain and oligosaccharide branches are added to the asparagine residue in this motif, as with mammalian cells. Yet carbohydrate additions to these branches following mannosidase trimming differ

slightly between insects and mammals. Native insect proteins contain simple oligo-mannose moieties, while mammalian cell proteins may contain complex sugars with terminal sialic acid groups (311).

Two baculoviruses, *Autographa californica* multiple nuleopolyherovirus (AcMNPV) and *Bombyx mori* NPV (BmNPV) are commonly used in BEVS for heterologous gene expression. AcMNPV are generally used to infect cells derived from the fall army worm, *Spodoptera frugiperda*, or the cabbage looper, *Trichoplusia ni*, while BmNPV is used, to a lesser extent, during the infection of *Bombyx mori* silk worm larvae (312). The genus Alphabaculovirus, to which AcMNPV, the genus type species, and BmNPV belong, produce two different virion types during its infectious cycle (313, 314). Budded virus (BV) disseminates the infection in the host organism by budding from the cell membrane of one cell and fusing with the membrane of another. This type of virus is produced early in the infection and is enclosed within a viral protein-modified envelope. Conversely, occlusion derived virus (ODV) is entirely encased in a proteinaceous matrix within the nucleus of infected host cells. MNPV ODV comprises of multiple viral particles within a polyhedral shaped occlusion body (OB) that consists of the viral protein polyhedrin. Contrary to BV, parts of the nuclear membrane are used during the construction of the ODV envelope at the nuclear stages of infection and are more complex in its protein content (315). OBs are highly stable structures, responsible for the protection of virions from damage in the environment.

Baculoviral proteins are expressed in four temporally distinct stages. During the first phase of infection, the early stage, host RNA polymerase II in association with host transcription factors transcribes immediate early proteins from promoters in the viral genome (*ie*-genes). This phase of infection is characterised by the translation of proteins involved in the transcription of genes during the delayed early stage. Consequently, the late expression factors (LEFs) required for viral DNA replication, and transcription during the late stage, are expressed. Host gene transcription shutoff correlates with progression of infection to the late stage (316). During the late and very late stages of infection, viral genes are transcribed by a virus-encoded RNA polymerase. Viral synthesis starts following the delayed early stage. Within the nucleus, viral particles are replicated by poorly understood mechanisms within the electron-dense, chromatin-like viral stroma (317). Multiple sequences, known as *hrs* regions, behave as functional origins of replication and some evidence that replication may occur by a recombinant-dependent mechanism, a rolling circle mechanism, or by a means which incorporate characteristics of both, exist (318). Between 6 and 24 hours post infection (hpi) late genes are expressed, followed by rampant expression of the very late genes starting at 18 hpi. At approximately 10 hpi the GP64 fusion protein is targeted to the cell membrane where it facilitates membrane fusion and the release of BV (319, 320). Interestingly, structural proteins and GP64 is transcribed during the late stage of infection, yet it has been reported that virions become associated with the membrane between two to four hpi. The suggestion is that a cohort of the original virus inoculum immediately translocates to the nucleus, initiates replication and prepares the membrane for budding by means of GP64, while some virions pass through the cytoplasm to infect adjacent cells (321), possibly by a mechanism involving microtubules consisting of host α- and β-tubulin (322).

Proteins involved in ODV formation are maximally expressed following the essential viral replication phase, i.e. during the very late stages of infection. Throughout this phase, two proteins, polyhedrin and p10, are expressed at extremely high amounts. Within the natural environment, the filamentous p10 protein is involved with the release of ODV from the cells by means of the controlled disintegration of the host cellular structure (323). Expression of virally encoded polyhedrin and p10 proteins from the very strong *polh* and *p10* promoters are under the control of an essential baculovirus core gene transcript, very late factor-1 (VLF-1). However, neither p10, nor polyhedrin are essential for the generation of infectious particles.

The BEVS exploits the presence of these very strong viral promoters by inserting heterologous coding sequences to downstream loci, in lieu of the non-essential native genes. Under the control of *polh*, heterologous proteins produced in BEVS can achieve up to 50% of the total cellular content (324). The large baculoviral genome, however, complicates direct manipulation of native genes or the insertion of foreign DNA. Therefore, two methods of baculovirus engineering are commonly employed in the production of recombinant infectious viral particles. The first method utilises the transferral of a gene of interest from a donor plasmid to the baculovirus genome by means of homologous recombination (307). Sequences flanking the heterologous gene cassette correspond to identical sequences up- and downstream of the site of insertion. Co-transfection of insect cells, generally the *S. frugiperda* clonal isolate Sf9, with the donor plasmid and baculovirus DNA facilitates an exchange of genetic material between the two molecules, thus introducing the foreign DNA to the baculovirus genome. This classical method of producing recombinant viruses generally resulted in the creation of recombinant viral particles at an efficiency of less than 1% which could be identified by altered plaque morphology. However, improvements to the system by Kitts and Possee (325, 326) during the 1990's significantly increased the effectivity of the process. Linearisation of the baculovirus genome and the subsequent introduction of three novel Bsu36I endonuclease recognition sites, which disrupt the essential *Orf1629* gene, increased the recovery rate to approximately 30% and ~100%, respectively.

The alternative method of combining foreign DNA with the baculovirus genome was developed in parallel with linearizable DNA by Luckow *et al.* (327). Expression of heterologous protein is achieved by the introduction of a recombinant bacmid to a suitable insect host. The F-Bacmid is a recombinant baculovirus that can replicate autonomously in *E. coli* DH10β and to which *attTn7*, the target site for bacterial Tn7, has been introduced. Foreign genes are transposed to the bacmid in *E. coli* cells when flanked by the left and right ends of Tn7. Creation of recombinant bacmids is easily verified by PCR prior to its transfection into insect cells. BV particles can be harvested from the culture media following translation of the baculoviral genes involved in replication. Viral titres are then easily increased by infection of new cells with the bacmid-derived BV. However, recombinant bacmids can be unstable and some parental bacmid background often occurs. An improved method for the creation and maintenance of recombinant bacmids by transposition was described by Airenne *et al* (328) called BVBoost. The transfer plasmid has been engineered to express the negative selection marker *sacB* from *Bacillus*

amyloliquefaciens. *E. coli* cells containing transfer plasmids that did not undergo transposition will express the product of *sacB*, levansucrase. Levan, which is converted by this enzyme (EC 2.4.1.10) from sucrose accumulates in the cytoplasm and is lethal to *E. coli* (329). Occupation of the chromosomal cryptic *att*Tn7 site of *E. coli* DH10β with an Amp resistance cassette resulted in the creation of a new *E. coli* strain (DH10 BacΔTn7) which, upon selection with sucrose, yields recombinant viral particles with negligible parental background (330).

The potential to achieve high levels of heterologously expressed protein in BEVS rivals that of *E. coli*. Since its inception, many advances have been made in BEVS. Not only has the system been optimised for rapid selection and purification of recombinant baculoviruses, several viral expression vectors have been designed for the expression of folding aides, such as calreticulin, PDI and calnexin (331). Furthermore, efficient systems of directing heterologous proteins to the cell surface have been successfully implemented. The differences in glycosylation patterns between mammals and insects have also been addressed by the creation of stably transfected insect cell lines which express mammalian glycosylation enzymes (332, 333). Owing to these advances, recent unique entries to the PDB, with less than 90% sequence identity to other entries, have been dominated by the BEVS (334).

CHAPTER 3

EXPRESSION AND PURIFICATION OF BACTERIALLY PRODUCED ANDROGEN AND ESTROGEN RECEPTOR LIGAND BINDING DOMAINS

The detrimental effects imposed on human and animal health by EDCs provide impetus for the development of methodologies capable of their rapid detection within our environment. Since EDCs can modulate the regulatory mechanisms of the endocrine system by interaction with nuclear receptors, the production of receptor proteins by heterologous gene expression will provide biological molecules with which EDCs are known to interact. If available, these proteins may be used to fashion a novel means to determine the presence of natural and synthetic endocrine-active substances within the environment by the immunohistochemical detection of conformational changes induced to the receptor LBDs upon ligand binding.

Heterologous protein production refers to the phenomena of recombining DNA from various sources with the goal to express proteins in organisms which do not naturally produce them. To this end many systems have been developed over the course of the last few decades, exploiting a variety of host organisms, each contributing a unique set of challenges and advantages (335). However, considering the broad array of cloning vectors available, along with the vast amounts of literature that have accumulated regarding prokaryotic physiology and genetics, it is easy to understand why *E. coli* is the most widely used expression host for both structural analysis and screening of protein targets (19–21) and why it is generally regarded as a first tier host for the production of heterologous proteins (336).

A challenging step in the expression of any protein or protein fragment, however, is predicting whether such a construct can be produced in large, active quantities considering many heterologous proteins express poorly or fold improperly. Small variations in the length of recombinant constructs or alterations to the amino acid sequence can alter the expression profile of proteins in host organisms. Nevertheless, despite all the data generated by various groups that focus on expression and purification of recombinant proteins for therapeutic, structural or academic research, a means of predicting which proteins will readily express and purify, based on amino acid sequence alone, is still lacking (337, 338). There are, however, many reviews that focus on possible strategies to enhance active heterologous proteins recovery in *E. coli* by discussing the host genetic milieu, fermentation conditions, lysis environments and purification strategies (259, 273, 339).

Historically, purification of nuclear receptors or their ligand binding domains to high concentration and purity have been hampered by low solubility and instability of the heterologously expressed proteins (340, 341). This chapter describes efforts to increase the solubility of a recombinant construct for the human androgen receptor ligand binding domain (ARLBD) produced in *E. coli*, with reference to media additives and lysis buffer composition. Specific mention is made of the influence of buffering, osmotic,

stabilising and solubilising additives. Attention to these parameters led to a net increase in soluble recombinant protein. Furthermore, purification of the human receptor LBDs for both androgens and estrogens, via immobilised metal affinity chromatography (IMAC) and immobilised ligand affinity chromatography, is discussed.

3.1 METHODOLOGY

3.1.1 Cell lines and vectors

The gene sequences encoding the ligand binding domains of the human androgen (ARLBD) and estrogen (ERαLBD) receptor major isoforms have been cloned previously (342). cDNA of the ERαLBD is contained within two expression vectors, pTrcHis and pET-15b. DNA encoding the ARLBD has been inserted to only the pTrcHis vector. pTrcHis/ARLBD and pTrchis/ERαLBD vectors have been transformed into the *E. coli* TOP10 strain. The pET-15b/ERαLBD vector was transformed into the BL21(DE3)pLysS strain.

Prior to all experiments, cells from cryogenic storage were routinely and aseptically streaked onto 1.5% Luria Bertani (LB)-agar plates containing 100 µg/mL ampicillin. Plates were incubated at 37°C until well defined single colonies of ± 2 mm in diameter had formed. Unless stated differently, single colonies were then inoculated in 5 mL LB media, with antibiotics, and incubated at 37°C with agitation at 235 RPM for 12 – 16 hours as initial cultures.

3.1.1.1 Extraction of plasmid DNA

Plasmid DNA was extracted using a Zyppy™ Plasmid Miniprep Kit (Zymo Research), following the classical centrifuge-based procedure according to the manufacturer's protocol. In brief, cells from 3 mL bacterial cultures, cultivated overnight at 37°C, were collected by centrifugation in a Heraeus Biofuge fresco microfuge at maximum speed. Following cell lysis and precipitation of genomic DNA, proteins and other cellular constituents, the soluble supernatants were transferred to Zymo-Spin™ IIN column, washed and eluted with 45 µL Zippy™ elution buffer.

3.1.1.2 Verification of construct identity

DNA sequencing was performed by the Central Analytical Facility (CAF) at the University of Stellenbosch. A primer that anneals to the T7 gene promoter region (5'-TAATACGACTCACTAT-3') of the pET15b-ERαLBD plasmid was used to determine the forward coding sequence of the ERαLBD insert. pTrc vectors were not sequenced as the data for these vectors were available.

3.1.2 Establishment of a bacterial growth curve

TOP10/pTrcHisARLBD initial cultures were diluted one hundredfold to 400 mL LB medium containing 50 µg/mL ampicillin. The culture was then incubated for 26 hours during which time 1.6 mL aliquots

were collected hourly, frozen in liquid nitrogen and stored at -20°C until further analysis. Subsequently, all samples were thawed and kept on ice. After homogenisation by inversion, 100 µL of each collected aliquot was pipetted in triplicate into the wells of a 96 well plate and the optical density was measured at 600 nm using a PowerWave 340 plate reader spectrophotometer (BioTek Instruments, Inc.) under the control of the software package Gen5, version 1.05.11. The OD_{600} of aliquots pertaining to the exponential phase of bacterial growth was consequently determined using a cuvette with a 1 cm light path.

3.1.3 Optimisation of parameters for ARLBD production

3.1.3.1 Cultivation media formulations

To assess the effect of media formulation on ARLBD production bacterial cells from initial cultures were diluted one hundredfold to 400 mL with either LB, LB media buffered with 30 mM sodium/potassium phosphate (bLB), or buffered LB containing 0.5% glucose (bLBg). Protein expression was induced at an OD_{600} of 0.75 by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM. Cells grown in bLBg were harvested and resuspended in bLB prior to induction. After 1 hour at 37°C the cells were collected by centrifugation at 5,000 xg for 15 minutes at 4°C in a Beckman Avanti® J-E centrifuge. After aspiration of media the cells were resuspended in chilled phosphate lysis buffer (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, 0.1% Nonidet P-40, 5 mM sodium metabisulphite (NMBS)) at 3 mL/mg wet cells. Lysis was achieved by enzymatic cell wall degradation using lysozyme at 1 mg/mL at 37°C for 1 hour, followed by ten 1 minute sonication duty cycles at 60% in an ice bath with a Sonicator™ Cell Disruptor; model W-225 (Heat Systems – Ultrasonics, Inc.). Cellular debris was then pelleted by centrifugation at 15,000 xg, 4°C, for 20 minutes. The supernatant was transferred to clean 50 mL conical tubes prior to resuspension of the cell debris in a volume equal to the supernatant.

3.1.3.2 Duration of protein expression trials

To 396 mL LB medium, containing 50 µg/mL ampicillin, 4 mL of TOP10/pTrcHisARLBD initial culture was added. At hourly intervals 1.6 mL aliquots were collected, frozen in liquid nitrogen and stored at -20°C until further analysis. Protein expression was induced by the addition of 0.3 mM IPTG to the growth media when the culture reached an OD_{600} of 0.75. Following sample collection cell aliquots were thawed and kept on ice, prior to cell harvest by centrifugation at 12,250 xg, 4°C, for 15 minutes in a Heraeus Biofuge fresco microfuge. After removal of the supernatants, cells were resuspended in 150 µL chilled phosphate lysis buffer. Cells were lysed by 25 sonication pulses using a Sonicator™ Cell disruptor, model W-10 (Heat Systems – Ultrasonics, Inc.), followed by five freeze/thaw cycles. Cellular debris was separated from the supernatant by centrifugation at 12,250 xg, 4°C, for 20 minutes. Where applicable the protein concentrations of the supernatants were adjusted to 1.9 mg/mL. Of these

supernatants 10 µL was blotted on a nitrocellulose membrane. Proteins containing histidine tags were visualised with HisProbe™-HRP (Thermo Scientific).

3.1.4 Expression of nuclear receptor LBDs

Initial cultures were used to inoculate larger volumes of bLB media in baffled shaker flasks at a ratio of 1:100. Expression was induced during the mid-exponential growth phase by the addition of 0.3 mM IPTG. Induction of ARLBD expressing cells was maintained at 37°C with agitation at 235 RPM for 1 hour. ERαLBD expressing cells were induced to express heterologous protein for 16 hours under similar conditions. Cultures were immediately placed on ice at the completion of induction, prior to cell harvest by centrifugation.

3.1.5 Determination of protein concentrations

Protein concentration determination of the lysate fractions was accomplished by means of the Pierce® BCA Protein Assay Kit (Thermo Scientific). Bovine serum albumin (BSA) standards were prepared by the standard test tube protocol and microplate procedure as described in the manufacturer's instructional document. Of all the standards and samples 10 µL were pipetted in triplicate into the wells of a 96 well microtitre plate. To each well 200 µL BCA working reagent was added using a multi-channel pipette. Plates were then incubated at 37°C for 30 minutes. After allowing the plates to cool to room temperature absorbance was measured at 540 nm with a Multiskan EX plate reader spectrophotometer (Thermo Scientific).

3.1.6 Densitometric analysis

Densitometric analysis of SDS-PAGE gels and Western blots was carried out using the software package UN-SCAN-IT gel™, version 6.1 (Silk Scientific Corporation). All images were converted to greyscale for analysis and all analyses were performed with a single region background subtraction correction factor. Optical density calculation was set to linear with saturation checking.

3.1.7 Optimisation of lysis conditions

The environment into which a recombinant protein is released upon cell lysis is an important consideration for the maintenance of protein stability and activity. Hydrophobic interactions between proteins with especially high grand average of hydropathy indexes may be markedly enhanced within the extracellular environment, as compared to the cell interior. Lysis conditions affecting the solubility of the ARLBD protein were investigated with regards to pH, salt concentration, known stabilising agents and a range of detergents and surfactants.

As before, TOP10/pTrcHisARLBD initial cultures were diluted one hundredfold to 400 mL antibiotic containing bLB and incubated at 37°C. Upon reaching mid-logarithmic growth 0.3 mM IPTG was added to induce heterologous gene expression. Induction was maintained for 1 hour after which the culture was

placed on ice. Subsequently, 3.5 mL aliquots were collected and centrifuged in a Heraeus Biofuge fresco microfuge at 2,600 xg, 4°C for 25 minutes. After aspiration the pelleted cells were frozen in liquid nitrogen and stored at -80°C until further analysis. Thawed samples were resuspended in 350 µL chilled buffer as described in Table 3.1. Following a 10 minute incubation period on ice, the resuspended cells were subjected to sonication with a Sonicator™ Cell Disruptor; model W-10 (Heat Systems – Ultrasonics, Inc.) for three 15 second bursts with 10 second cooling intervals in a wet ice bath. The cellular debris was then separated from the supernatant by centrifugation at 12,250 xg at 4°C for 20 minutes in a Heraeus Biofuge fresco microfuge. After transferral of the supernatant to clean micro centrifuge tubes the cellular debris was resuspended in 500 µL of the previously employed lysis buffers.

Samples were prepared for SDS-PAGE and 15 µL of each was loaded on to 12% SDS-PAGE gels. After transferral to Pall nitrocellulose membranes, histidine tagged proteins were visualised with HisProbe-HRP. Densitometric analyses of the resulting blots were carried out by means of the software package UN-SCAN-IT gel version 6.1. Signals were normalised against the sum of the average pixel densities obtained for the supernatant and cellular debris of individual lysates.

3.1.8 Radioligand binding studies for the ARLBD construct

Determination of whole cell ligand binding ability was assessed by means of a competitive radioligand binding assay. E. *coli* Top10/pTrcHisARLBD and untransformed *E. coli* Top10 cells were grown as before and induced to express recombinant protein. As the recombinant bacteria exhibited slower growth than their untransformed counterparts, Top10 cells were diluted to OD_{600} equivalent to that of Top10/pTrcHisARLBD post protein expression induction. The cells were harvested by centrifugation at 6,000 xg at 4°C for 20 minutes. The LB medium supernatant was subsequently aspirated and the cells were washed by resuspension in 20 mL PBS (pH 7.5), followed by centrifugation at 6,000 xg at 4°C for 20 minutes. The PBS wash buffer was subsequently aspirated and the cells were resuspended in 10 mL PBS (pH 7.5). Cell aliquots (95 µL) were incubated overnight with decreasing concentrations of ^{3}H-testosterone (range 75 nm to 1 nm). To determine non-specific binding cell aliquots were incubated with ^{3}H-testosterone with a thousand fold excess of unlabelled testosterone whilst agitating. The following morning ligand-incubated cells were harvested by centrifugation in a bench top centrifuge at 6,000 xg for 5 minutes. The resulting supernatant was transferred to 4 mL Flo-Scint™ III scintillation fluid (Perkin Elmer) and the cells were washed by resuspension in 100 µL PBS (pH 7.5). After centrifugation for 10 minutes in a bench top centrifuge at 6,000 xg, the wash buffer was transferred into 4 mL scintillation fluid. The pellet was subsequently resuspended in 100 µL PBS and then transferred into 4 mL scintillation fluid. Radioactivity was counted using a Perkin Elmer scintillation counter.

Table 3.1 **Formulations of buffers used during the optimisation of TOP10/pTrcHisARLBD to increase solubilisation of the recombinant ARLBD . pH calibration was performed at 4°C. All buffers included 250 µg/mL lysozyme and 0.1% PMSF, which was added from stock immediately prior to use.**

	Designation	pH	Buffer	Salts	Stabilisers	Detergents/Surfactants
pH Screen	**$P_{7.0}$**	7.0	50 mM Sodium phosphate	**50 mM NaCl**	**None**	**None**
	$P_{7.5}$	7.5	50 mM Sodium phosphate			
	$T_{8.0}$	8.0	50 mM Tris			
	$T_{8.5}$	8.5	50 mM Tris			
	$H_{8.0}$	8.0	50 mM HEPES			
	$H_{8.5}$	8.5	50 mM HEPES			
	$HdG_{8.0}$	8.0	30 mM HEPES + 30 mM glycylglycine			
	$HdG_{8.5}$	8.5	30 mM HEPES + 30 mM glycylglycine			
Salt Screen	**$S_{0.05}$**	**8.0**	**30 mM HEPES + 30 mM glycylglycine**	50 mM NaCl	**None**	**None**
	$S_{0.25}$			250 mM NaCl		
	$S_{0.50}$			500 mM NaCl		
	$S_{0.75}$			750 mM NaCl		
	$S_{1.00}$			1 M NaCl		
	$S_{0.05}$ $A_{0.010}$			50 mM NaCl 10 mM **$(NH_4)_2SO_4$**		
	$S_{0.05}$ $A_{0.025}$			50 mM NaCl 25 mM **$(NH_4)_2SO_4$**		
	$S_{0.05}$ $A_{0.50}$			50 mM NaCl 50 mM **$(NH_4)_2SO_4$**		

	Designation	pH	Buffer	Salts	Stabilisers	Detergents/Surfactants
Osmolyte Screen	-	**8.0**	**30 mM HEPES + 30 mM glycylglycine**	**250 mM NaCl**	No stabilisers	**None**
	5% Glycerol				5% Glycerol	
	10% Glycerol				10% Glycerol	
	Glucose				25 mM Glucose	
	Sucrose				25 mM Sucrose	
	Maltose				25 mM Maltose	
	Lactose				25 mM Lactose	
	Galactose				25 mM Galactose	
	Glucose/Glycerol				25 mM Glucose 5% Glycerol	
Detergent Screen	-	**8.0**	**30 mM HEPES + 30 mM glycylglycine**	**250 mM NaCl**	**None**	No detergents
	Propylene glycol					0.5% Propylene glycol
	Nonidet P-40					0.5% Nonidet P-40
	Triton X-100					0.5% Triton X-100
	Emulgen 913					0.5% Emulgen 913
	Poly-ethylene glycol					0.5% Poly-ethylene glycol
	Tween-20					0.5% Tween-20

3.1.9 Purification of receptor LBDs produced in bacterial expression system

3.1.9.1 Immobilised metal affinity chromatography

For purification of the ARLBD from *E. coli*, 400 mL bacterial cultures, in LB medium, was induced to express heterologous protein for 1 hour by addition of 0.3 mM IPTG when an OD_{600} of 0.75 was reached. Cells were collected by centrifugation at 5,000 xg at 4°C for 15 minutes and lysed in 35 mL chilled HDI_{20} buffer (30 mM HEPES, 30 mM glycylglycine (pH 8.0), 20 mM imidazole, 250 mM NaCl, 0.5% emulgen 913, 5 mM NMBS, 2 mM DTT, 0.25 mg/mL lysozyme, 1 mM PMSF) by ten cycles of sonication in an ice bath with 1 minute bursts using a Sonicator™ Cell Disruptor, model W-225 fitted with an H-1 probe (Heat Systems – Ultrasonics, Inc.), set to 60% duty cycle, with one minute cooling periods.

Cells expressing the ERαLBD were lysed following the procedure outlined above, following resuspension in phosphate lysis buffer containing estradiol (50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, 5 mM NMBS, 0.1% Nonidet P-40, 10 μM 17β-estradiol, 0.1 mM PMSF). After clarification by centrifugation at 15,000 xg at 4°C for 15 minutes, the supernatant was filtered through a 0.45 μm pore size syringe filter. Of the resulting ARLBD containing filtrate, 30 mL was applied to a 5 mL HiTrap Chelating HP column (GE Healthcare), of which the iminodiacetate moieties had been charged with Ni^{2+} ions. The column was previously equilibrated with a wash/equilibration buffer containing 30 mM HEPES, 20 mM imidazole, and 5 mM NMBS. Removal of proteins rich in histidine and other chelating amino acids was facilitated by raising the imidazole concentration to 92 mM (15% elution buffer). Elution of the recombinant protein from the IMAC column was achieved by increasing the concentration of imidazole to 500 mM (100% elution buffer). The fractionated eluent was collected in tubes containing, at final concentrations, 5 mM ethylene diamine tetra-acetic acid (EDTA) and 0.2% (v/v) emulgen 913 (Kao Chemical Corporation).

IMAC of ERαLBD containing samples was approached in a similar fashion as for the ARLBD, but the column was equilibrated with buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 20 mM imidazole, 5 mM NMBS, 20 mM β-mercaptoethanol and 10% glycerol.

3.1.9.2 Desalting of collected IMAC fractions

Fractions collected during IMAC containing proteins of interest were desalted by gel permeation chromatography. ARLBD containing samples were applied to a 5 mL Sephadex G-25 desalting column equilibrated with H-GPC buffer (30 mM HEPES (pH 8.0), 250 mM NaCl, 5 mM NMBS). Samples containing the ERαLBD were desalted with a 25 mL Superose-12 GPC column equilibrated with EPE buffer (10 mM sodium/potassium phosphate (pH 7.4), 135 mM NaCl, 3 mM KCl, 2% emulgen 913, and 2.5 mM EDTA).

3.1.9.3 Affinity chromatography

Affinity chromatography to purify nuclear receptor LBDs from bacterial lysates was performed using nortestosterone- or 17β-estradiol-Sepharose 6B resin in 1 mL columns. Of the filtered cell lysates 20 mL was applied to the ligand-immobilised columns which had been equilibrated with AF buffer (30 mM HEPES (pH 8.0), 250 mM NaCl, 200 mM Sucrose, 1 mM EDTA) and eluted with a linear gradient with final concentration at 100% of 500 μM testosterone (ARLBD) or 17β-estradiol (ERαLBD) using a Bio-Rad FPLC system.

3.1.10 Determination of protein identity

3.1.10.1 SDS-PAGE and Western Blot analysis

SDS-PAGE analysis was carried out at each stage of the purification process according to the procedure of Laemmli (343). Polyacrylamide gels (12%) were hand-cast and samples were electrophoresed with a Bio-Rad Mini-PROTEAN system at 4°C, 150 V. Western blot procedures were performed after overnight transferral of proteins from SDS-PAGE gels to BioTrace NT pure nitrocellulose blotting membrane (Pall Life Sciences) at 4°C, 10 V. Detection of proteins containing histidine-tags was performed using HisProbe-HRP (Thermo Scientific). Rat anti-oestrogen receptor monoclonal IgG (Thermo Scientific) was used as primary antibody for the detection of the ERαLBD, whilst peroxidase labelled goat anti-rat IgG (Kirkegaard & Perry Laboratories) served as secondary antibody. All Western blots were developed using either Pierce ECL (Thermo Scientific) or Clarity ECL (Bio-Rad) and exposed on Fuji medical X-ray film (Fuji Photo Film Co.).

3.1.10.2 Peptide analysis via mass spectrometry

In gel trypsin digestion

Protein bands were excised from SDS-PAGE gels, cut into smaller pieces and washed twice with water. The gel pieces were then incubated in 50% (v/v) acetonitrile for ten minutes. The acetonitrile was replaced with 50 mM ammonium bicarbonate and incubated for a further ten minutes. This procedure was repeated twice. After incubation in 100% acetonitrile until they turned white, the gel pieces were dried *in vacuo*.

Dried gel pieces were then treated with 10 mM DTT at 57°C for 1 hour to reduce the contained proteins. The gel pieces were then briefly washed with 50 mM ammonium bicarbonate, followed by 50% acetonitrile. Proteins were subsequently alkylated with 50 mM iodoacetamide for 1 hour in the dark. Following alkylation the gel pieces were washed with ammonium bicarbonate for ten minutes and then 50% acetonitrile for twenty minutes. The gel pieces were dried *in vacuo* and digested with 200 ng sequencing grade trypsin (Promega) at 37^0C. Following overnight incubation the gel pieces were treated twice with 70% acetonitrile in 0.1% formic acid for 30 minutes. The pooled supernatants of these washes were then dried and stored at -20^0C.

Liquid chromatography/mass spectrometry

The dried peptides, dissolved in 5% acetonitrile in 0.1% formic acid, were analysed on a Thermo Scientific EASY-nLC II liquid chromatograph connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electrospray source. Injections of 10 µL were made.

Liquid chromatography of pET15b-derived ERαLBD samples was performed, at a flow rate of 300 nL/min, on an EASY-Column (2 cm, 100 µm ID, 5 µm, C18) pre-column, followed by an EASY-column (10 cm, 75 µm ID, 3 µm, C18). For analysis of ARLBD samples an XBridge BEH130 NanoEase column (15 cm, ID 75 µm, 3.5 µm, C18) was used. Peptides were eluted with a gradient of 5 – 40% solvent B (100% acetonitrile in 0.1% formic acid) in 20 minutes, 40 – 80 % solvent B over 5 minutes, and then isocratic for 10 minutes. Solvent A was water containing 0.1% formic acid.

Mass spectrometry was performed at 1.5 kV, capillary temperature of 200°C, with no sheath and auxiliary flow, and the ion selection threshold set to 500 counts for MS/MS. An activation Q-value of 0.25 and activation time of 10 milliseconds were also applied for MS/MS.

Automated database searching (Mascot, Matrix Science, London, UK and Sequest) of all tandem mass spectra against the Swissprot 57.15 and UNIPROT human databases was performed by using Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany) to identify proteins. Carbamidomethyl cysteine was set as fixed modification, while oxidized methionine, N-acetylation and deamidation (NQ) was used as variable modifications. The precursor mass tolerance was set to 10 ppm, and fragment mass tolerance set to 0.8 Da. Two missed tryptic cleavages were allowed. Proteins were considered positively identified when they were identified with at least 2 tryptic peptides per proteins with a Mascot score of more than $p < 0.05$, as determined by Proteome Discoverer 1.3.

3.2 RESULTS AND DISCUSSION

3.2.1 Cell lines and vectors for the expression of nuclear receptor LBD

The expression cassette of the pTrcHis–TOPO cloning vector is indicated in Figure 3.1. Transcription of heterologous protein coding sequences to mRNA is under the control of the strong *E. coli* trc promoter. This hybrid between the -35 region of the trpB and the -10 region from the *lacUV5* (344, 345) promoters is located upstream of a *lac* operator which is normally bound by the lac repressor protein (LRP). LRP is the product of the *lacI*^{q} gene. The repressor protein suppresses transcription by inhibiting host RNA polymerase from binding to the *trc* promoter. Upon availability of allolactose, or allolactose analogues such as IPTG, repression is lifted and recombinant protein expression is induced. The plasmid also contains a pBR322 derived origin of replication and the ampicillin resistance gene, *bla*, which encodes for the expression of β-lactamase as a selective marker. The T1 transcription terminator from the *E. coli* rrnB gene occurs 16 bp downstream of the inserted gene. Upstream from the encoded hexahistidine tag the ribosomal binding site of the transcribed mRNA includes the Shine Dalgarno sequence (TAAGGAGG) and the canonical ATG translation initiation codon.

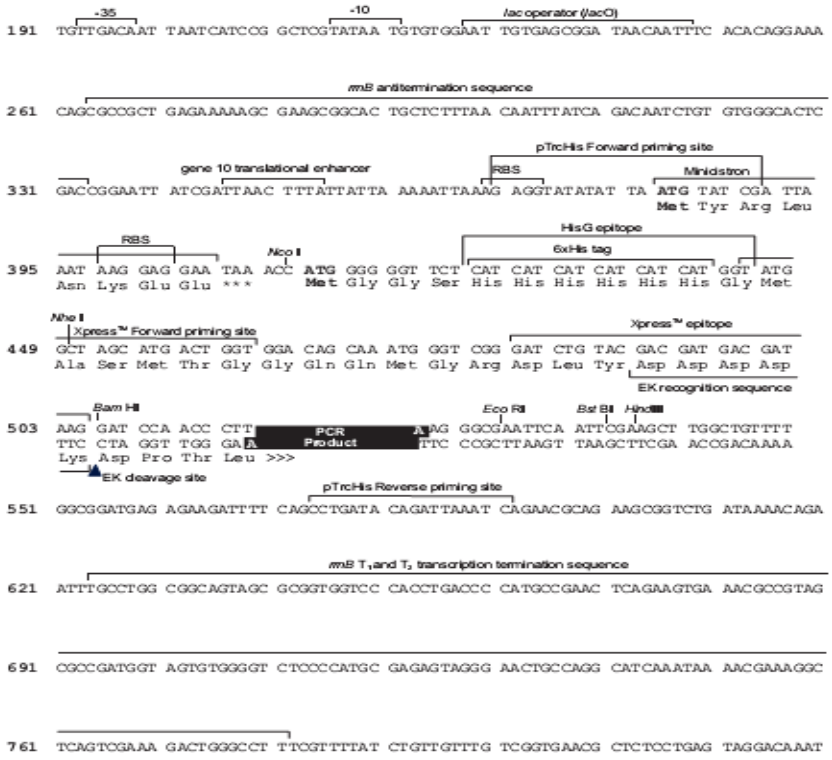

Figure 3.1 **Multiple cloning site, downstream termination region and 5' regulatory map of the pTrcHis-TOPO cloning vector. Heterologous genes are placed under the control of the pTrc promotor and expressed as fusion proteins with an N-terminal hexahistidine tag. The DNA coding for such recombinant proteins are amplified with polymerases that adds adenylates the 3' of PCR products, which are then ligated to the linearised cloning site, downstream of an enterokinase digestion site.**

As with the pTrcHis system described above, the pET-15b vector expresses proteins with an N-terminal 6xHis tag which confers metal chelating capability to the molecule, such proteins may therefore be purified by means of IMAC. Also, the *lac* operator is present in the region 5' of the multiple cloning site (Figure 3.2) and in the absence of lactose analogues is bound by the repressor, inhibiting the formation of transcription complexes at the upstream promoter region. Expression of these heterologous proteins, however, occurs under the control of the T7 promoter, which is not recognised by the host RNA polymerase.

Bacterial hosts such as the BL21(DE3)pLysS strain of *E. coli* contain a chromosomal copy of the T7 RNA polymerase from the λ-lysogen DE3 phage. The expression of this bacteriophage polymerase is controlled by the *E. coli lacUV5* promoter and its expression can therefore be controlled by the addition of IPTG. Also, derivatives of the BL21 strain lack the lon and OmpT proteases which increases the stability of heterologous proteins expressed in these cell lines (273). Furthermore, the BL21(DE3)pLysS cells express T7 lysozyme which serves to inhibit T7 RNA polymerase prior to induction of protein expression. The presence of this protein enhances cell lysis by increasing cell wall degradation under milder conditions (337).

As with the pTrcHis vectors, this plasmid contains a pBR322 origin of replication which ensures high maintenance of plasmid stability and a *bla* coding sequence, which imparts selectivity by antibiotic resistance.

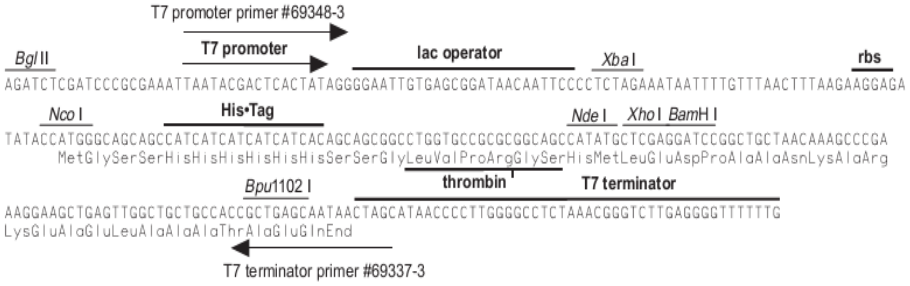

Figure 3.2 pET-15b cloning/expression region. Recombinant proteins are transcribed from the T7 promotor in the presence of the inducer molecule, IPTG. Presence of IPTG activates the expression of bacteriophage T7 RNA polymerase and relieves the repression of heterologous gene transcription.

3.2.2 Heterologous expression and recovery of proteins in *E. coli*

3.2.2.1 Initial expression analysis

The *E. coli* system is well known and widely employed as a means of heterologous protein synthesis. Cases of recombinant proteins, directed at expression in the cytoplasm, which encompass up to 30% of the biomass in this host have been reported (20, 339). Yet the converse is also true, several proteins have proven problematic when expressed in prokaryotic systems (346–348). Initial small scale induction experiments indicated that the heterologous hexahistidine fused receptor LBDs are expressed by the bacterial host cells. However, for both the androgen and estrogen constructs the majority of the expressed protein was sequestered to the cellular debris (Figure 3.3 and Figure 3.4).

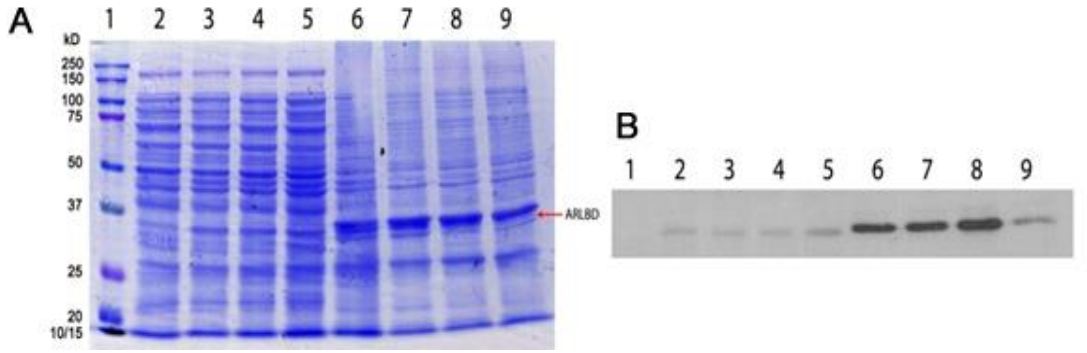

Figure 3.3 **Image of a 10% SDS-PAGE gel indicating the expression a recombinant hexahistidine-tagged ARLBD protein produced in *E. coli* TOP10/ARLBD. Panel A: lane one contains 7.5 µg Kaleidoscope protein standard. Each of lanes two to four were loaded with 20 µg of the supernatants of cultures induced with IPTG to express heterologous protein for four hours. In lane 5 uninduced culture supernatant was loaded (20 µg). Lanes six to eight contains the cellular debris of triplicate cultures (11.5 µg, 8.3 µg and 8.4 µg, respectively). Lane nine contains 9.2 µg of the cellular debris of an uninduced culture. Panel B: an image of a duplicate western blot indicating the presence of a his-tag fusion protein.**

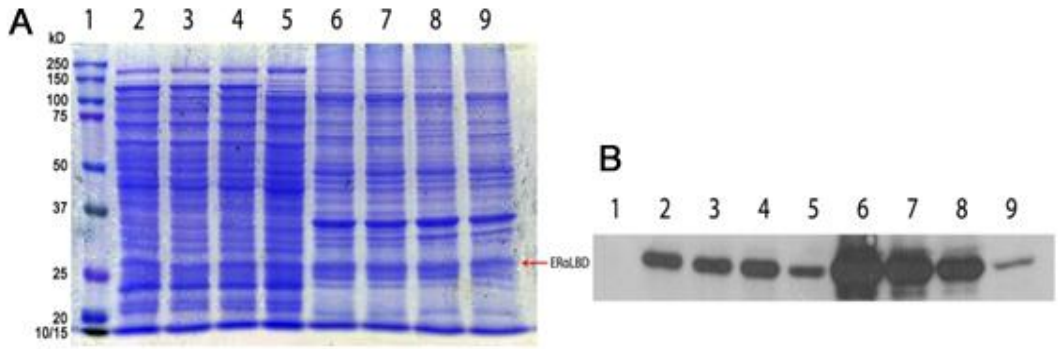

Figure 3.4 **Samples from a four hour induction of *E. coli* BL21(DE3)/ERαLBD cells with IPTG resolved by SDS-PAGE (10%). Panel A: lane one contains 7.5 µg Kaleidoscope molecular marker. Lanes two to four each contain 20 µg of proteins from the soluble fraction of induced cultures. The supernatant from an uninduced culture was loaded in lane five (20 µg). The insoluble fractions of induced cultures were loaded to lanes six to eight (9.6 µg, 9.6 µg and 9.4 µg, respectively). In lane nine the cellular debris of from an uninduced culture was loaded (9.5 µg). Panel B: an image of a duplicate western blot indicating the presence of a his-tag fusion protein.**

The images above indicate that a significant amount of the heterologous proteins, expressed by the bacterial cells, are not liberated into the supernatant during cell lysis, but is mostly confined to the insoluble fraction. On average, about 90% of the total chemiluminescent signal observed for the ARLBD was confined to the insoluble component ($p < 0.001$). The ERαLBD, however, contained substantially ($p < 0.05$) more recombinant protein in the supernatant; averaging at approximately 40%.

Several heterologous proteins overexpressed in *E. coli* often accumulate in accumulated, insoluble structures known as inclusion bodies. The formation of these dense aggregates can be a significant hindrance in obtaining soluble, active protein in some situations. It has been postulated that the proteins in inclusion bodies have native-like secondary structures (349) and are formed from intermediates of the folding pathway (350). Therefore, a possible strategy is the solubilisation of inclusion bodies by denaturing conditions, but subsequent refolding of proteins into active proteins may prove difficult (349,

351–355). Many strategies have been developed to address the problem of inclusion body formation at a genetic level (21), yet inhibition of the formation of these aggregates must virtually always, to some extent, be addressed by optimisation of growth and lysis conditions. Induction temperature, addition of various small molecules, co-expression with chaperones and concentration of inducer are all parameters that may be modulated to optimise protein production. Furthermore, the maximum obtainable yields of heterologous proteins may also be influenced by the time and duration of expression induction (356). Long induction times may lead to exhaustion of available nutrients in the culture.

Marked expression of the receptor LBDs, in the absence of induction by IPTG, can be detected for both the ARLBD and ERαLBD constructs. Interestingly, the signal observed in the soluble phase of ERαLBD containing lysate was three times as much as for the cellular debris (Figure 3.4, insert lane 5). Many promoters suffer from basal level activation, even in the absence of inducer due to incomplete repression. Promoters based on the lac promoter, such as *trc* and *lacUV5*, suffer from higher basal transcription levels in the absence of glucose due to elevated levels of cyclic-AMP (cAMP) (357). A general approach to reducing incomplete repression is by the inclusion of glucose to the pre-induction cultivation medium as *E. coli* preferentially uses this sugar as a carbon source until it is exhausted. This strategy decreases intracellular levels of cAMP. If present, cAMP induces a conformational change in catabolite activator protein (CAP), thus allowing it's binding to regulatory elements upstream of *lac*-derived promoters, leading to the initiation of transcription (356). Control over heterologous gene expression in *E. coli*, prior to induction, is important since the overexpression of such genes impose a high metabolic burden on the host cells (21, 358). The energy requirements of cells expressing recombinant protein, even at basal level, may therefore impact negatively on the growth rate, since heterologous protein synthesis and specific growth rate is inversely correlated (358).

Many protocols that focus on the expression of recombinant proteins in *E. coli* recommend that induction should be initiated during the exponential growth phase (359–362). Figure 3.5 shows the growth characteristics of the pTrcHis-ARLBD transformed bacterial strain indicating that the logarithmic growth phase for these cells under culture conditions occurs roughly 2 – 6 hours after inoculation. During this phase of fast bacterial growth cells are actively replicating and increasing the culture biomass. Transcription of the four ribosomal RNAs is pronounced (363), with much of the cytoplasm being occupied with ribosomes to facilitate elevated levels of protein synthesis.

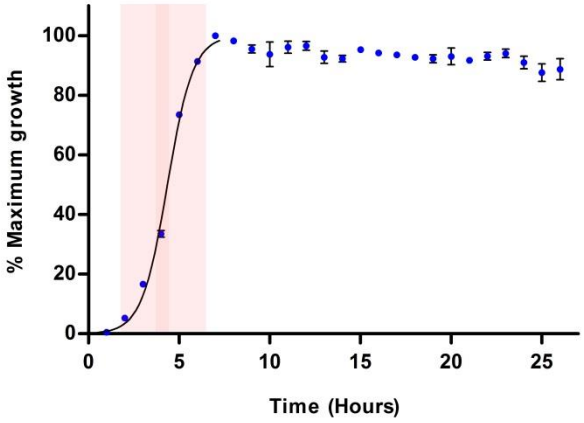

Figure 3.5 **Growth curve for the *E. coli* TOP10/pTrcHisARLBD strain cultivated in LB media at 37°C in baffled shaker flasks. Active bacterial gowth occurs between two and six hours post inoculation with mid-exponential growth at approximately four and a half hours under culture conditions. The OD_{600} in a 1 cm cuvette at the midpoint of growth is ± 0.75.**

There is interplay between the synthesis and degradation of recombinant proteins *in vivo* depending on the rate of mRNA translation by the ribosomes, sequestration to insoluble inclusion bodies and degradation by bacterial proteolytic enzymes. Hourly detection of recombinant protein after induction of bacterial cells to express ARLBD indicated that the optimal duration of expression is one hour post-induction (Figure 3.6, A6). A gradual decrease in signal is noticeable in aliquots taken during the sequential hours.

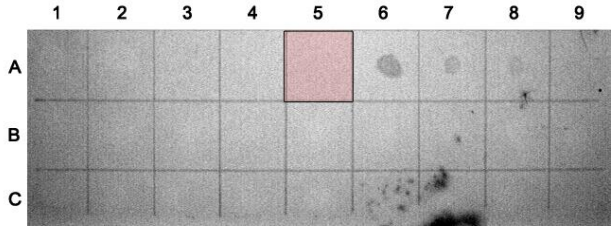

Figure 3.6 **Dotblot of proteins extracted from *E. coli* TOP10/pTrcHisARLBD cells. Induction of protein expression occurred 4 hours after inoculation of the growth media with initial cultures (A5, red overlay). Hourly aliquots were blotted and developed with HisProbe HRP.**

For the ERαLBD protein, however, a steady increase in signal strength can be observed on a Western blot (Figure 3.7) of aliquots taken at hourly intervals after induction of a BL21(DE3)pLysS/ERαLBD culture with 0.3 mM IPTG. Overnight induction markedly increases the intensity of the protein band, compared to induction periods of five hours or less.

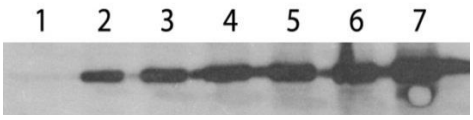

Figure 3.7 **Western blot of of induced BL21(DE3)/ERαLBD cells at time of induction (lane one), hourly intervals post induction (lanes two to six) and at sixteen hours post induction (lane seven) developed with rabbit anti-estrogen receptor alpha monoclonal IgG.**

3.2.2.2 Effects of buffering and glucose inclusion in growth media

Due to its ability to support fast growth and high cellular yields LB is the most widely used rich medium for the cultivation of a variety of prokaryotes. Even though growth in LB is not as good as in more nutrient rich formulations such as terrific broth, high cell densities are often achieved. However, high cell density could theoretically promote a variety of problems, including plasmid loss, limited oxygenation of the environment and decreases in pH due to elevated cellular metabolism (364), all of which could potentially reduce the maximal achievable amount of recombinant protein within the system. According to Strandberg and Enfors (365) the total and relative amounts of inclusion bodies formed during fermentation increases with a reduction in pH. Indeed, a decrease of 0.2 pH units as recorded for cultures grown in LB medium without the addition of a buffering system, whilst no such change was recorded for cultures to which 30 mM sodium/potassium phosphate was added. Control of the internal pH is important for optimal growth of *E. coli*. Cytoplasmic pH in its natural habitat, the human gastrointestinal tract, is maintained between 7.2 and 7.8 (366) due to active and passive mechanisms, such as ATPase transporter system and the buffering effect provided by the cell's metabolic pool. In a paper discussing the physiology of *E. coli* cultivated in LB, Sezonov *et al.* (367) reports that the pH of the medium at growth arrest is routinely near nine. Cells inoculated into spent media did not grow. However, upon the addition of glucose such media could support growth in a dose dependant manner, revealing that depletion of essential carbon sources affecting cellular metabolism, rather than toxification of the environment, results in arrest of growth. Inclusion of glucose in growth media therefore tends to lead to higher cell densities of *E. coli*, which often results in higher levels of expressed soluble protein (368, 369). Furthermore, as discussed in the previous section, the presence of glucose in the cultivation medium of cells expressing protein under *lac* derived promoters, prior to induction, alleviates the metabolic burdens associated with protein overexpression.

Indeed, the addition of a buffer to the growth medium led to a significant increase ($p < 0.001$) in the amount of protein that was released into the soluble fraction of bacterial lysates. Moreover, the addition of 0.5% glucose to the medium led to further significant ($p < 0.001$) total protein concentrations within lysate supernatants (Figure 3.8).

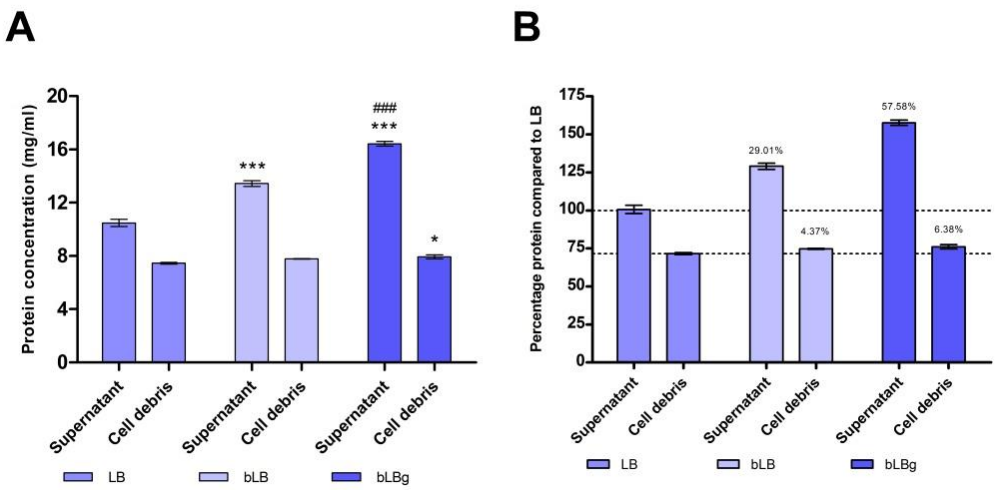

Figure 3.8 **Effect of media formulation on total protein content produced by TOP10/pTrcHisARLBD cells induced to express heterologous protein. Total protein concentrations contained within each fraction of lysates from cells incubated in growth medium with or without supplementation with a phosphate buffering system or glucose is presented in panel A. These concentrations are normalised against the concentration of the supernatant from cells grown in unmodified LB medium in panel B. Percentage shift from LB is indicated above the bars.**

The results of SDS-PAGE electrophoreses of cell cultures induced to express recombinant ARLBD are presented in Figure 3.9. Interestingly, alterations to media composition, to withstand pH fluctuations, had no significant impact on the production of heterologous ARLBD in *E. coli* in this trial (Figure 3.10), as determined by two-way ANOVA. Moreover, the substantial increase in soluble protein observed upon the addition of glucose to the medium did not result in increases in soluble ARLBD. In fact, in relation to unmodified LB media, significantly more recombinant protein was obtained in the insoluble fraction of cell lysates from cultures grown in bLBg.

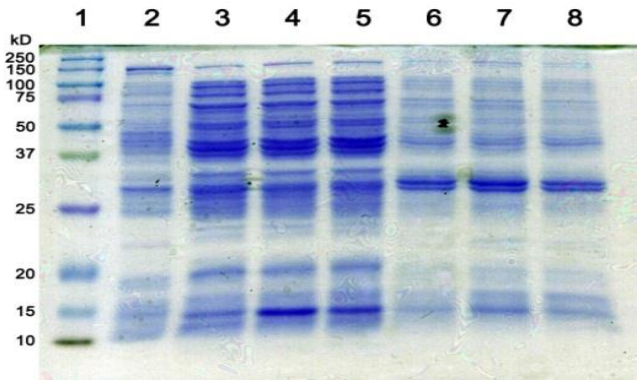

Figure 3.9 **SDS-PAGE gel image of TOP10/pTrcHisARLBD bacterial lysates produced under different growth media conditions. In lane one, 6 µg Kaleidoscope protein marker (Bio-Rad) was loaded. *E. coli* negative lysate control (Pierce) was loaded in lane two (30 µL). To lanes three to five, 20 µL of prepared LB supernatant, bLB supernatant and bLBg supernatant was respectively loaded. Of the prepared cellular debris 20 µL was loaded into lanes six to eight.**

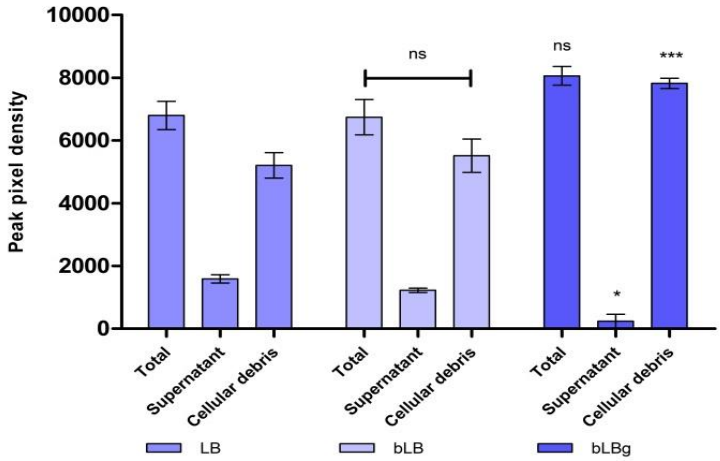

Figure 3.10 Relative distribution of *E. coli* produced ARLBD between the supernatant and cell debris of cell lysates is indicated in the graph to the left. A comparison between the SDS-PAGE band densities for the fractionated protein, obtained with differing media formulations, indicates no significant change in the signal provided by the recombinant protein between LB and bLB. There is, however a highly significant increase ($p < 0.001$) in the insoluble ARLBD produced with media containing glucose. Also, there is a significant decrease in the amount of soluble ARLBD obtained.

It is implausible that catabolite repression would reduce the metabolic burden and therefore lead to increased levels of proteins seen in Figure 3.8. Arguably, the reason for higher levels of protein is increased cell density due to higher metabolic turnover provided by a more nutritious environment. Increased rates of protein synthesis may therefore lead to enhanced sequestration of misfolded recombinant protein intermediates to the bacterial inclusion bodies due to high local concentrations within the cytosol.

3.2.2.3 Preparation and optimization of bacterial cell lysates

According to Leibly *et al*. (278) a mere 35% of recombinant proteins produced in *E. coli* are adequately soluble and properly folded to be purified in acceptable quantities for procedures such as crystallisation. Cell lysis is the first step in the fractionation of cellular constituents. Within the cell interior molecules are protected from degradation by harsh extracellular conditions. Lysis of cells disrupts a carefully controlled environment, which allows the unregulated release of endogenous proteases and phosphatases. The potential for a protein of interest to be degraded or modified therefore increases. When the goal of cell lysis is to purify or test the function of a particular protein, special attention must be given to the effects of the lysis reagents on the stability and function of the protein of interest. This is especially true for proteins that are inherently hydrophobic, since the stability provided by cellular compartmentalisation is removed upon lysis. It is, however, also important to consider any downstream procedures, such as protein purification, when making allowances for alterations in the parameters of the buffering systems employed. In the current study, the lysing conditions pertaining to pH, salt, stabilisers and detergents were optimised.

It has been well documented by several authors (370, 371) that proteins become insoluble at their respective pI's, even though the protein may be soluble in the native conformation. At these pH values the interaction between proteins increases because the electrostatic forces of the molecules are at a minimum. As a consequence less water interacts with protein molecules, resulting in favourable conditions for protein aggregation and subsequent precipitation. Conversely, proteins have net negative or positive charges above and below the pI. The net charged state of the protein leads to increases in charge repulsion between proteins, diminishing protein-protein interaction. Furthermore, more water can interact with charged, solvent accessible residues, thus contributing to greater protein solubility. The phenomenon of isoelectric precipitation has been successfully exploited in isolation of particular target proteins from complex mixtures (372–374). However, it is also known that a decrease in solubility leading to aggregation negatively impacts on protein functionality (375, 376) especially when subsequent dissolution of such aggregates and refolding of proteins into the native form is inadequate. It is therefore essential to control the pH of the lysis environment, since it is generally assumed that over-expression of heterologous proteins, especially proteins with a high degree of hydrophobicity, is more prone to lead to the accumulation of inclusion bodies in *E. coli* (349).

The ARLBD, as with the other nuclear receptor LBDs, consists mainly of hydrophobic residues arranged in twelve helices folded into a three-layered, antiparallel helical sandwich (133). The calculated theoretical isoelectric point for the current ARLBD protein, according to the SIB ExPASy Bioformatics Resources Portal, is 6.73 (377). In agreement with the argument above, solubility should therefore be negatively impacted at this pH. The relative solubility of the recombinant protein was therefore assessed over a range of pHs between 7.0 and 8.5, at 0.5 increments (Figure 3.11). Densitometric analysis indicated an optimal pH of 8.0 utilizing a HEPES/glycylglycine buffering system.

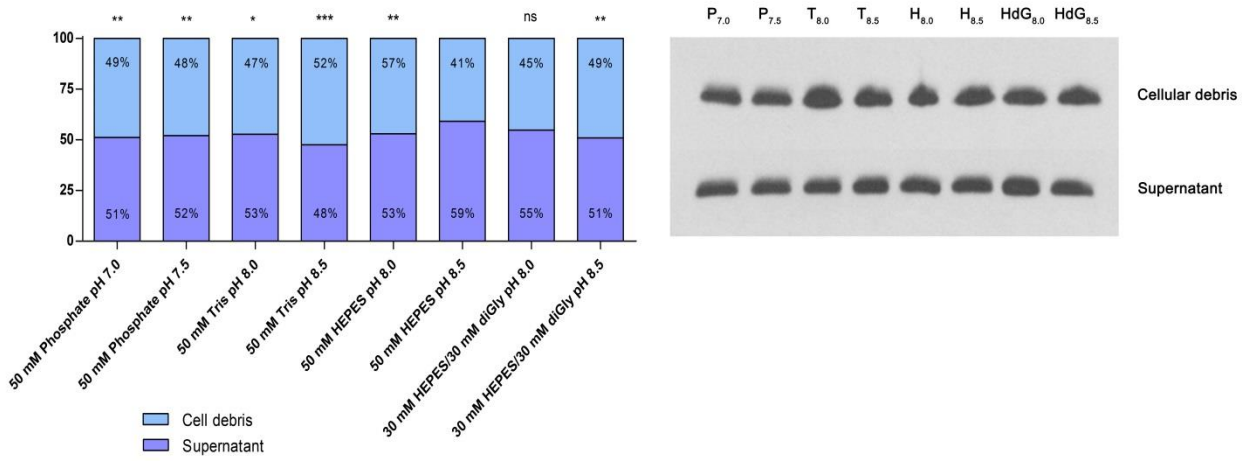

Figure 3.11 Comparison between the relative distribution of recombinant ARLBD between the soluble and insoluble phases of *E. coli* lysates obtained from lysis in buffers consisting of either sodium phosphate, Tris, HEPES or HEPES and glycylglycine at a pH range of 7.0 to 8.5. Responses that indicate significantly less soluble protein in the supernatants were analysed against 50 mM HEPES (pH 8.5), which gave the highest response. The visualised protein blots were digitised and the relative pixel obtained were expressed as percentage distribution between the cell debris (top) and supernatant (bottom) of the bar graphs.

Control of pH is a prominent factor to consider in the solubility of proteins. However, of equal importance is the ionic strength of the solution to which proteins are released upon cell lysis and should not be ignored. The processes of salting in and salting out are common practice in many biological laboratories and are useful techniques in the isolation and purification of a variety of proteins. Salt can have different effects on proteins in solution, depending on the concentration added. Low ionic strength tends to lead to increases in solubility. However, depending on the nature of the salt employed, as the concentration is increased past a certain point, solubility may be affected differentially by either stabilisation or denaturation of the proteins in question (378). The general theory explaining the phenomenon of salting out is that the free ions in solution compete with proteins for hydration by water. If the concentration of salt reaches a certain threshold, proteins become unable to compete and are forced from the solvent (379). Salting in, on the other hand, may be explained by the Debye-Hückel theory. Ions in electrically neutral solutions are not uniformly distributed but are surrounded by higher local concentrations of counter ions. This ionic atmosphere shields the ion from similarly charged ions. In this manner the electrostatic free energy of proteins, surrounded by counter ions, are concealed, thus increasing the activity of the solvent; and by extension leads to an increase in solubility (380).

The relative distribution of the ARLBD in cell lysates was not significantly increased towards the soluble fraction by increasing the concentration of NaCl (Figure 3.12), nor did the addition of low levels of ammonium sulphate. Increasing NaCl concentrations to 750 mM and above, however, significantly affected the solubility of the recombinant protein and skewed the distribution towards the cellular debris.

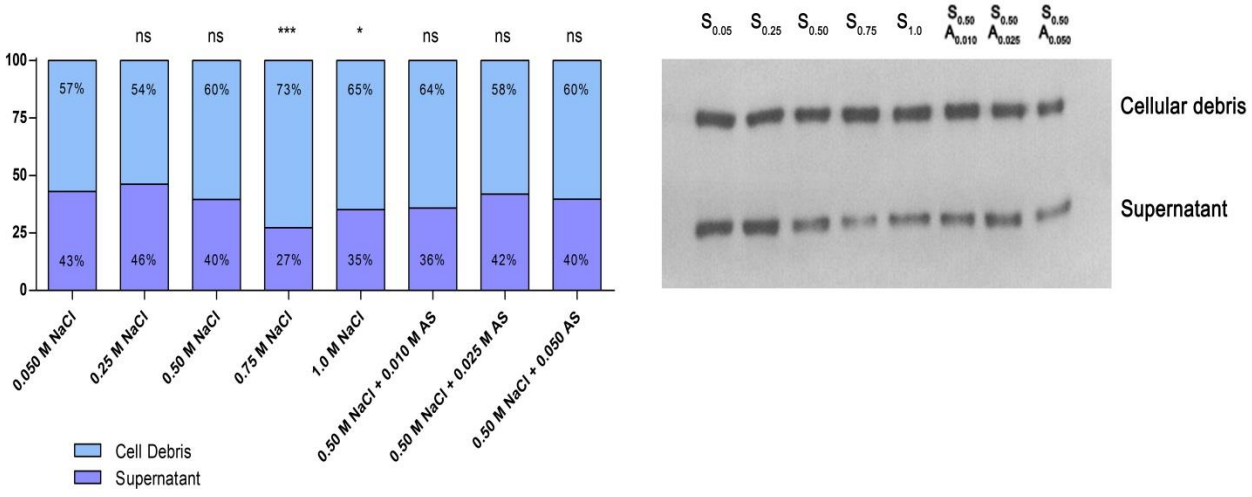

Figure 3.12 Relative distribution of recombinant ARLBD between the soluble and insoluble fractions of *E. coli* lysates containing varying concentrations of NaCl and ammonium sulphate. Reductions of solubility at NaCl concentrations above 750 mM, as expressed by the digitised products of the obtained Western blots (bar graphs), were seen to be significant.

The inclusion of certain monosaccharides, amino acids or polyols has been shown to affect solubilisation of proteins in *E. coli* by stabilisation (278, 376). High concentrations of macromolecules within the cytosol are rapidly diluted upon cell lysis and may lead to protein aggregation due to hydrophobic interactions. Osmolytes, such as sugars and polyhydric alcohols, have greater interaction with the polar residues of proteins than water (381), leading to the displacement of water from these hydrophilic sites whilst simultaneously repelling nonpolar substances and interacting favourably with water. As a result, water and osmolytes are redistributed around the hydrophobic moieties of proteins in such a manner as to minimise unfavourable contacts. This solvophobic effect leads to an exchange reaction at hydrophobic loci on the surface of proteins which is favourable to water. Therefore, osmotic stabilisers exert effects by destabilising aggregates and enhancing native protein stability. The inclusion of such stabilisers in lysis conditions, however, did not significantly impact on the solubilisation of the protein of interest in the current investigation (Figure 3.13).

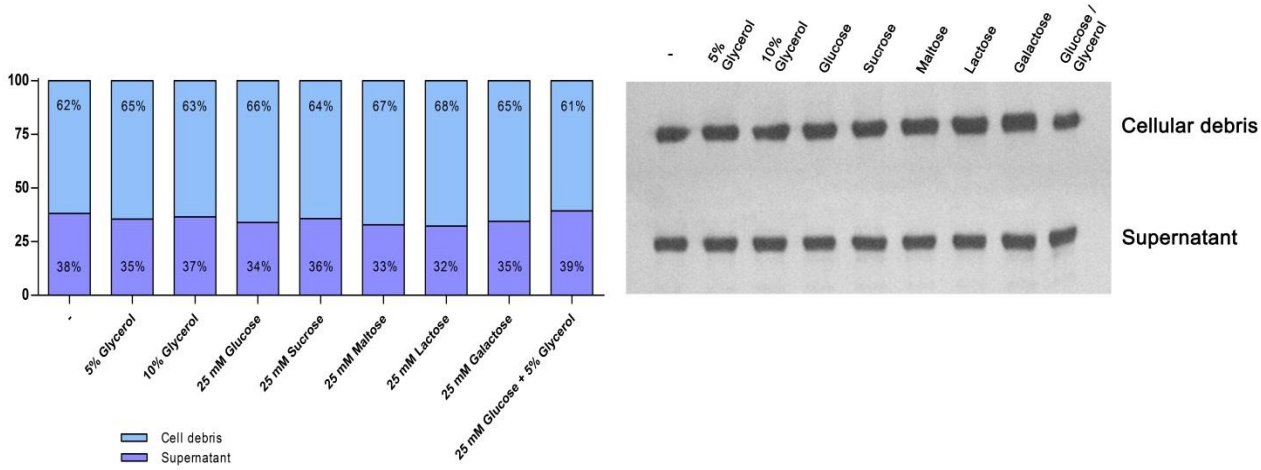

Figure 3.13 Effects of osmolyte addition to lysis buffers on the solubility of bacterially expressed ARLBD. No significant difference could be observed between lysates obtained from buffers, containing different osmolyte additions, following digitisation of Western blots (bar graphs) and analyses of relative pixel densities.

The addition of surfactants or detergents to homogenisation buffers enhances cellular lysis by disruption of membranes and has been used in the refolding of proteins from isolated and denatured bacterial inclusion bodies (354). Moreover, the amphipathic properties of detergent molecules have been extensively exploited in the isolation of membrane proteins which generally exhibit low solubility (382). The hydrophilic moieties of detergent molecules interact with the polar solvent, forming hydrogen bonds, while the hydrophobic tails form highly organised globular structures called micelles. In this manner, the cellular membranes are mimicked by the detergent, facilitating solubilisation of the protein of interest. The high degree of hydrophobicity exhibited by nuclear receptors, especially the ligand binding domain, require that such proteins be shielded from the aqueous environment to diminish possible hydrophobic effects which would result in aggregation. The hydrophobic tails of detergents associate with hydrophobic amino acid residues located on the protein exterior, while the polar heads interact with the dipoles of water molecules.

The extraction of recombinant protein into cell lysates prepared with buffers to which detergents or surfactants were added are presented in Figure 3.14. Addition of the non-ionic detergent Nonidet P-40 or the surfactant Triton X-100 significantly ($p < 0.01$) raised the amount of recombinant protein that could be liberated to the soluble supernatant of cell lysates. Very significant increases ($p < 0.001$) in the distribution of the ARLBD to the soluble fraction of cell lysates were, however, obtained by inclusion of the non-ionic polyoxyethylene nonyl phenol ether detergent emulgen 913 and the polysorbate surfactant Tween-20.

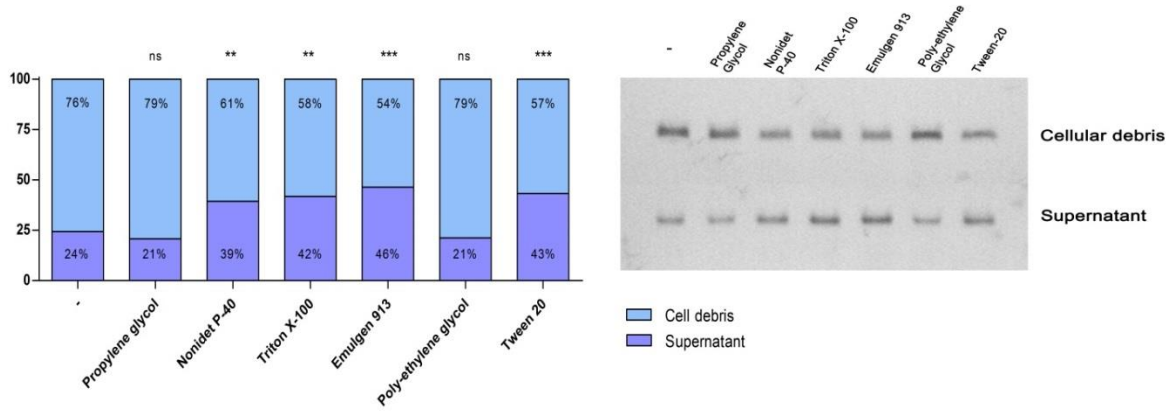

Figure 3.14 Densitometric comparison of the relative distibution of heterologously expressed ARLBD between the soluble and insoluble phases of *E. coli* lysates. As shown in the bar graphs, the product of the digitisation of the accompanying Western blots, significant increases in solubility of the recombinant protein could be achieved upon cell lysis by the addition of Nonidet P-40, Triton X-100, Emulgen 913 and Tween-20.

3.2.2.4 Inclusion of glycylglycine in growth and induction media

The use of small molecules such as L-arginine (383, 384), sorbitol (384, 385), glycylglycine (386), among others, have been reported to enhance the solubility of proteins during protein expression or refolding experiments. In this study glycylglycine was added to cultivation media, with subsequent protein expression induction when cultures reached the mid-logarithmic growth stages. Noticeable reduction in doubling times could be observed for cultures grown in media to which 250 mM glycylglycine had been added (Figure 3.15).

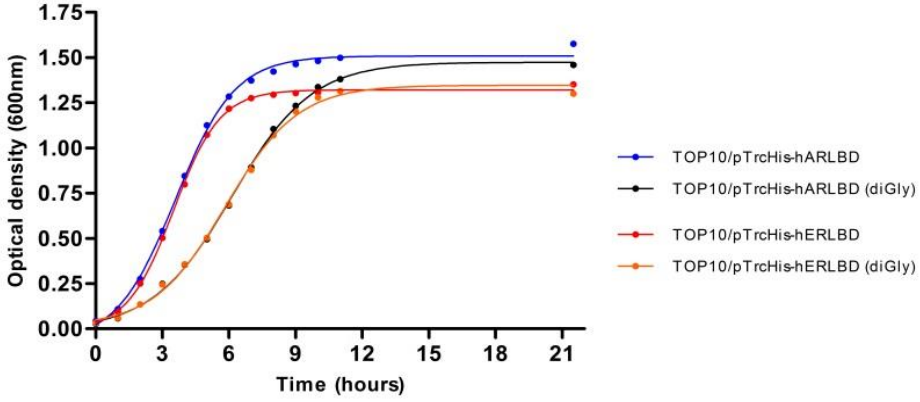

Figure 3.15 Growth curves of TOP10 cell lines containing pTrcHis vectors expressing the ARLBD and ERαLBD constructs. The addition of glycylglycine markedly decreased doubling time of the bacterial cells, but did not affect the maximum cell density reached.

Addition of the dipeptide at this concentration did not appear to enhance the expression of the sex steroid ligand binding domains in the bacterial cells. In fact, the results (Figure 3.16) indicate that supplementation of the media with the dipeptide impacted negatively on the solubility of the proteins.

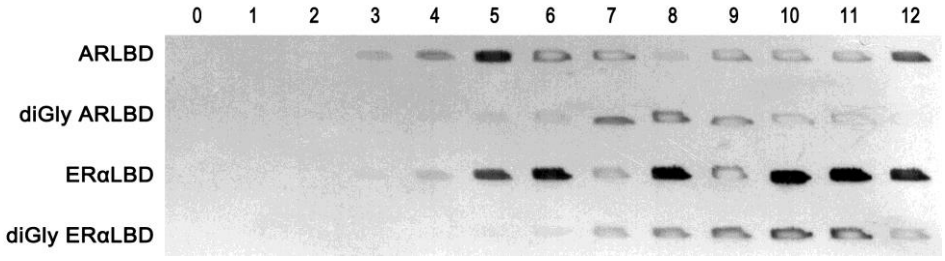

Figure 3.16 Dot blot of cell lysates from *E. coli* cells induced to express heterologous nuclear receptor ligand binding domains. Induction of ARLBD and ERαLBD expression in the absense of glycylglycine was initiated at 4 hours post inoculation. Cultures to which glycylglycine had been added were induced to express the recombinant protein at 6 hours post induction during the mid-exponential growth phase.

It is notable that, in comparison to the initial expression duration trial depicted in Figure 3.6, expression of the ARLBD could be detected for several hours after induction. Optimisation of lysis conditions may explain why increased levels of the recombinant protein were extracted to the soluble supernatant fraction of the lysate, enabling visualisation of the protein via immunodetection. However, the strongest signal for the ARLBD was again detected at one hour post induction. The signal obtained for the ERαLBD, also, was weaker in blots of aliquots taken hourly from cultures induced to express protein in the presence of glycylglycine. A degree of basal heterologous protein expression could be detected prior to induction of transcription from the *trc* promoter by IPTG for both constructs under all conditions. No glucose was added to the cultivation media prior to induction, thus incomplete repression of the promoter region allowed transcription to occur in the absence of the inducer molecule.

3.2.3 Purification

Metal affinity chromatography was first introduced in the 1970's and has remained the form of affinity chromatography with the broadest application in protein biochemistry. The incorporation of histidine-tags to the C- or N-terminus of proteins has become an increasingly popular method of protein purification due to the dative covalent bonds which form between metal chelating amino acid residue configurations and divalent metal ions, such as those of nickel, cobalt, copper and iron. The recombinant proteins transcribed and translated from the vectors encoding the ARLBD and ERαLBD have been engineered to contain an N-terminal hexahistidine tag. Hence, purification of the bacterially produced sex steroid receptor LBDs from the filtered lysate supernatant was approached via a two-step chromatographic process. First, IMAC was used to isolate heterologous protein from bacterial lysates and the obtained eluents were then desalted via gel permeation chromatography.

3.2.3.1 Isolation of the ARLBD

Immobilised metal affinity chromatography

Initial attempts to purify the ARLBD, however, did not give positive results on chromatograms obtained during IMAC separations. None of the 20 amino acids found in proteins absorb light within the visible range. Three aromatic ring containing residues, however, significantly absorb in the ultraviolet. Phenylalanine weakly absorbs light at this wavelength, with a major peak at 257.5 nm. Tryptophan, however, absorbs strongly at 280 nm due to its absorbance maximum at 278 nm. Tyrosine, which naturally occurs at a higher frequency in proteins than tryptophan, the residue of least abundance, absorbs less strongly at 280 nm, with an absorbance maximum at 274.25 nm. As a result proteins are amenable to detection when absorbance at 280 nm is measured. However, proteins which contain few of these amino acids are therefore expected to have little absorbance at 280 nm.

The recombinant ARLBD protein contains four tryptophan (1.3%), nine tyrosine (2.9%) and seventeen (5.5%) phenylalanine residues. The theoretical extinction coefficient of the ARLBD, as calculated by a modified Edelhoch method which utilises the coefficients of Trp and Tyr determined by Pace *et al.* (387), is 35410 M^{-1} cm^{-1}. Consequently a 1 mg/mL solution should have an absorbance of 0.997 in water at standard 1 cm light pathlength, assuming all cysteine residues are reduced. The initial purification conditions included emulgen 913 in the chromatographic buffers as a means to maintain solubility of the ARLBD during isolation. However, it was found that this detergent absorbs light at 280 nm, leading to high background and diminished resolution after subtraction. Moreover, imidazole at the elution concentration of 500 mM has an A_{280} of 0.5. The chromatographic mobile phase system therefore markedly contributed to the inability to resolve the presence of the recombinant protein in the eluent via UV absorption (Figure 3.17).

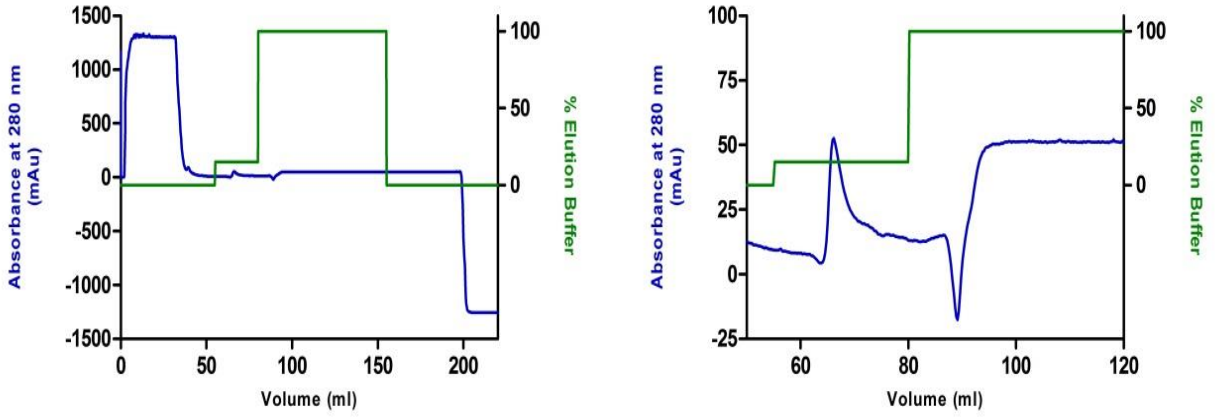

Figure 3.17 Chromatograms obtained during the initial ARLBD purification conditions. The left panel indicates the full chromatographic run from sample application to completion. Note the drop in absorption upon removal of the mobile phase. The panel to the right zooms in on the expected elution volume of the ARLBD, a peak indicating the elution of the target protein could not be obtained due to high background interference from the chromatographic buffers.

Nevertheless, collection of the fractions surrounding the expected elution volume, with subsequent blotting of these fractions onto nitrocellulose membrane and visualisation with antibodies against the HisG epitope (Figure 3.18, B4), revealed that the protein was indeed being retained on the column.

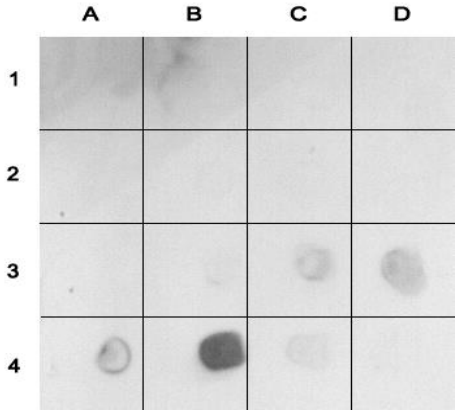

Figure 3.18 **Dot blots of selected fractions from IMAC developed with HisProbe HRP. The response in block B4 indicates that the column does retain the hexahistidine tagged protein.**

Removal of interfering UV absorptive substances markedly increased the resolution obtained on the chromatograms during IMAC purification of the ARLBD (Figure 3.19). A peak at 90.22 mL, near to the expected elution volume of the target protein was collected and analysed by SDS-PAGE (Figure 3.20). Some contaminating *E. coli* proteins co-eluted with the protein of interest and can be seen as bands occurring at higher and lower molecular masses on the gel image than the ARLBD.

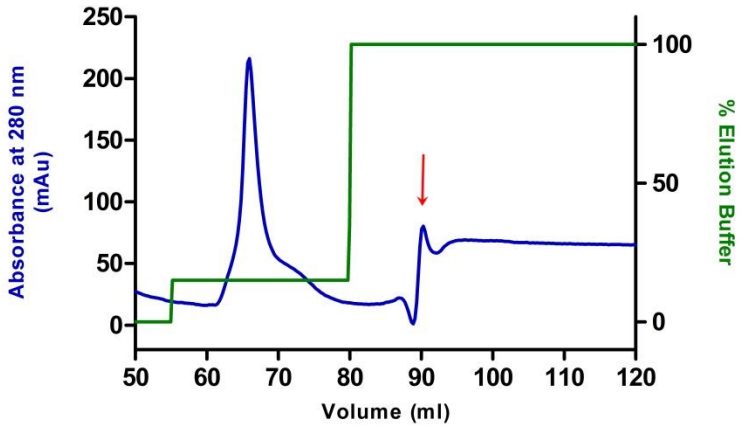

Figure 3.19 **IMAC chromatogram of indicating elution of metal chelating proteins from a 1 mL HisTrap chelating column. The major peak at 65.89 mL is the result of proteins rich in amino acid residues such as histidine, cysteine and tryptophan. The peak at 90.22 mL, indicated with a red arrow, contains the hexahistidine tagged ARLBD protein.**

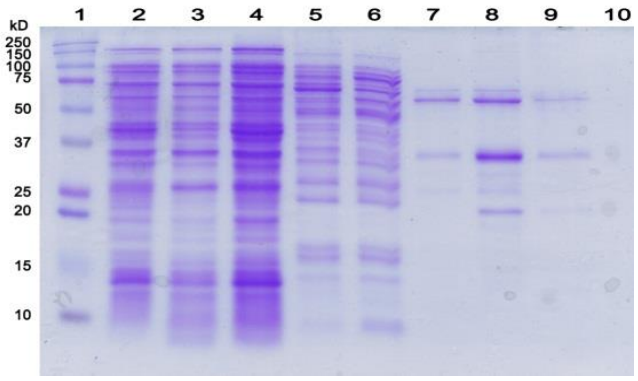

Figure 3.20 **Image of a 12% SDS-PAGE gel containing resolved fractions collected during IMAC seperation of *E. coli* TOP10/pTrcHisARLBD lysate supernatant. Lane one contains Kaleidoscope marker (Bio-Rad). Of the filtered supernatant a volume containing 20 µg was loaded in lane two. Lanes three to six contains fractions collected during the elution of proteins rich in histidine and other metal chelating amino acid residues. In lanes seven to ten the fractions collected during the elution of the protein of interest was loaded. Protein concentrations of collected fractions could not be determined due to the high concentration of imidazole which interferes with the BCA protein assay.**

The results could successfully be reproduced during multiple purifications utilising similar conditions (Figure 3.21). Attempts were made to diminish the co-elution of *E. coli* proteins from the column by increasing the duration of isocratic elution at 15% elution buffer, and by incorporation of a gradient elution step from 15% to 100% elution buffer as shown in run number two and three in Figure 3.21, respectively. These alterations did not markedly affect the separation of the recombinant protein from the contaminating proteins, nor did raising the imidazole concentration in the wash and equilibration buffers to 40 mM (Figure 3.21, chromatogram 4).

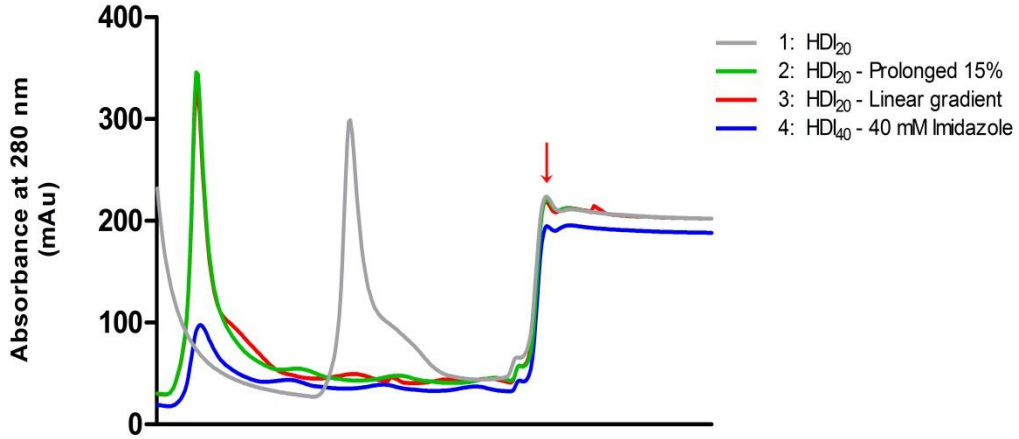

Figure 3.21 **Overlay of IMAC chromatograms obtained during the purification of the ARLBDb protein from *E. coli* lysate. The retention time of purification number 1 was adjusted to the elution volume for the heterologous protein peak of the subsequent purifications (red arrow).**

Desalting of IMAC samples by gel permeation chromatography

Determination of the concentration of proteins that occur in fractions obtained during the elution phase of immobilised metal affinity chromatography was not possible without prior removal of the high concentration of imidazole. Various options, such as dialysis, protein precipitation and buffer exchange, may be employed for the removal of excess salts from proteinaceous mixtures.

Gel permeation chromatography was used during the current study to remove excess imidazole from protein samples. The chromatogram in Figure 3.22 was obtained after desalting of collected ARLBD containing IMAC fractions, with a Sephadex G-25 column. Elution of the protein from the column yielded a large peak of nearly 1,000 mAu, indicating successful purification of the recombinant protein. The removal of excess imidazole from these fractions can be seen by the increase in conductivity on the chromatogram.

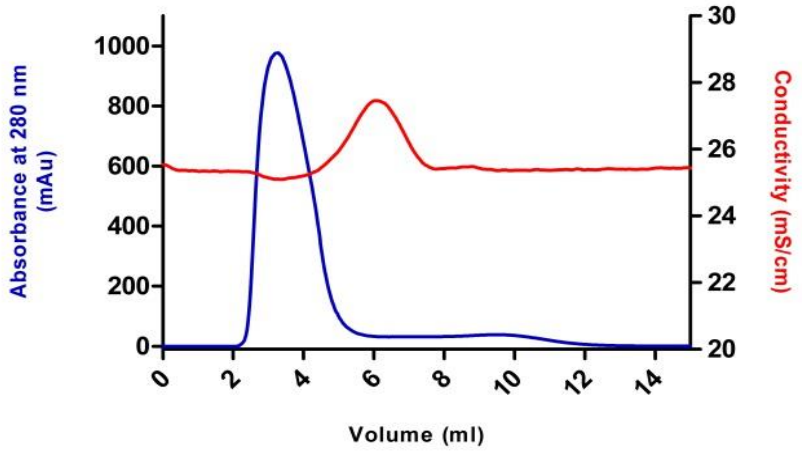

Figure 3.22 Chromatogram indicating desalting of IMAC fraction containing the ARLBD. The rise in conductivity indicates elution of imidazole from the Sephadex G-25 column.

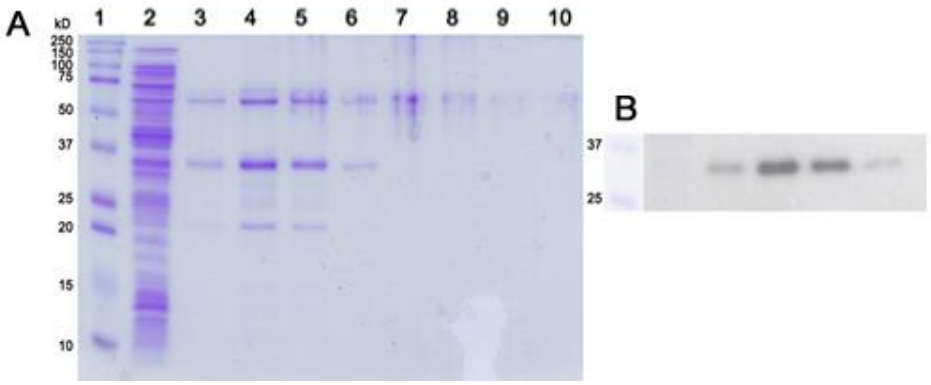

Figure 3.23 Image of an SDS-PAGE gel of fractions collected after desalting of collected IMAC fraction. Panel A: lanes one contains 6 µg of Kaleidoscope marker. In lane two 20 µg of TOP10/pTrcHis-ARLBDb lysate (7.12 mg/mL) was loaded. In lanes three to ten 25 µL (with 2x sample buffer) of GPC fractions 1 – 8 were loaded. Panel B: Image of a duplicate SDS-PAGE developed as a Western blot with anti-AR antibodies following transferral to a nitrocellulose membrane.

Removal of excess imidazole from the protein containing IMAC samples allowed the determination of protein concentrations by the BCA method. A total of 2.68 mg protein was recovered from the gel permeation column. Densitometric analysis of the fraction loaded in lane four (Figure 3.23, A), which yielded the highest visual response representing the ARLBD, indicated that 57% of the protein complement in this fraction is the recombinant receptor. Inferring from densitometry (Table 3.2), a total of 1.71 mg ARLBD was produced in the *E. coli* expression system.

Table 3.2 **Densitometric analysis of the SDS-PAGE gel presented in Figure 3.23 (lanes 3 – 6). Highlighted values pertain to the bands on the gel image that are associated with the ARLBD protein.**

Elution Volume mL	Fraction Concentration µg mL^{-1}	Calculated Molecular Mass Da	Segment	Pixel Total	Percentage of Pixel Total	Estimated Concentration µg mL^{-1}
3.0	862.08	58399.87	3·1	792	0.89	7.67
		53887.63	3·2	34101	38.37	330.78
		33121.83	3·3	46225	52.01	**448.37**
		20618.34	3·4	7764	8.74	75.35
4.0	948.75	58399.87	4·1	2651	1.03	9.77
		53433.42	4·2	71164	27.68	262.61
		32842.65	4·3	147409	57.34	**544.01**
		20444.55	4·4	35847	13.94	132.26
5.0	872.08	57419.54	5·1	2857	1.72	15.00
		53660.05	5·2	39053	23.48	204.77
		32842.65	5·3	101339	60.92	**531.27**
		20444.55	5·4	23103	13.89	121.13
6.0	552.08	53660.05	6·1	37258	59.98	331.14
		32703.95	6·2	21494	34.60	**191.02**
		20186.61	6·3	3369	5.42	29.92

3.2.3.2 Isolation of the ERαLBD

Immobilised metal affinity chromatography

Co-purification of bacterial proteins during IMAC is not an uncommon occurrence and is especially problematic when contaminating metal chelating *E. coli* proteins are of similar molecular size to target proteins, or when such proteins are expressed at a high level (388, 389). Bolanos-Garcia and Davies (390) have proposed a classification of the most commonly co-purified protein, concluding that the majority of these contaminants are stress-responsive in nature. Proteins that co-elute with 6xHis tagged heterologous proteins either possess native metal binding capabilities as a function of their cellular machinery, or surface accessible histidine clusters are present which confer metal binding capabilities. Moreover, hydrophobic proteins may interact with the scaffold matrix by hydrophobic interactions.

These contaminating proteins were also observed to co-elute with the protein of interest during purification of the ERαLBD from BL21(DE3)pLysS/ERαLBD lysates (Figure 3.26). Nevertheless, comparatively, the elution of recombinant histidine-tagged protein from the IMAC column with free imidazole at 500 mM could be detected (Figure 3.24) considerably easier than for the AR counterpart.

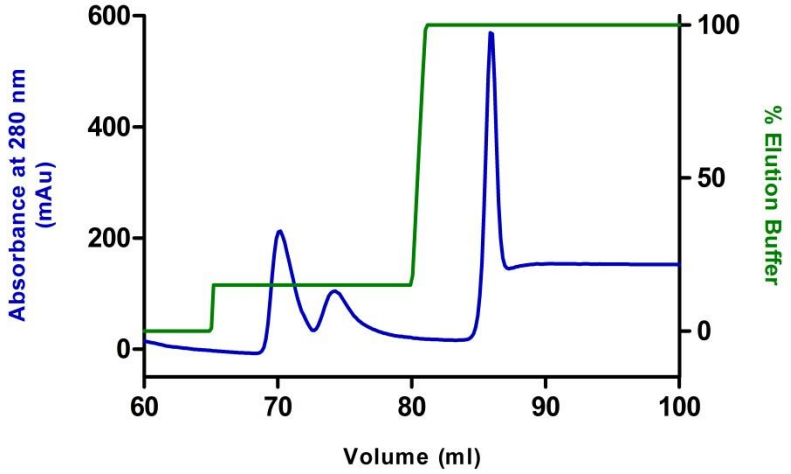

Figure 3.24 **Chromatogram indicating the purification of the bacterially produced ERαLBD protein. Elution of the protein of interest occurred at 85.93 mL, with *E. coli* proteins rich in metal chelating residues eluting with 92 mM imidazole at 70.17 mL and 74.18 mL.**

Desalting and clean-up of ERαLBD by gel permeation chromatography

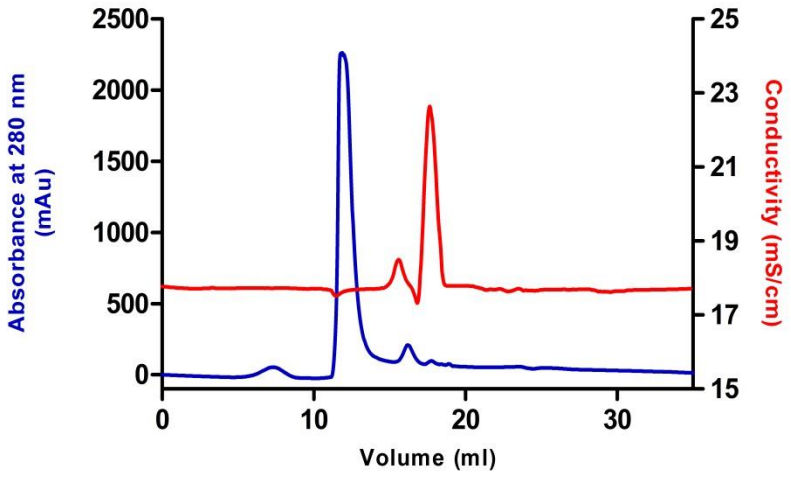

Figure 3.25 **Chromatogram of desalting of a fraction obtained from IMAC of bacterial lysates containing recombinant ERαLBD. Elution of the target protein from the Sepharose-12 column occurred at 11.87 mL. Removal of excess imidazole is evident as a rise in conductivity following elution of the ERαLBD.**

The removal of salts from the IMAC purified fractions could again be seen in a sharp raise of conductivity of the eluent following elution of a major absorbance peak at 11.87 mL. Smaller peaks earlier and later on the chromatogram indicate elution of the higher and lower molecular weight *E. coli* contaminating proteins from the column. With this method 832 µg of the ERαLBD protein could be obtained at high homogeneity (Figure 3.26, lane 8).

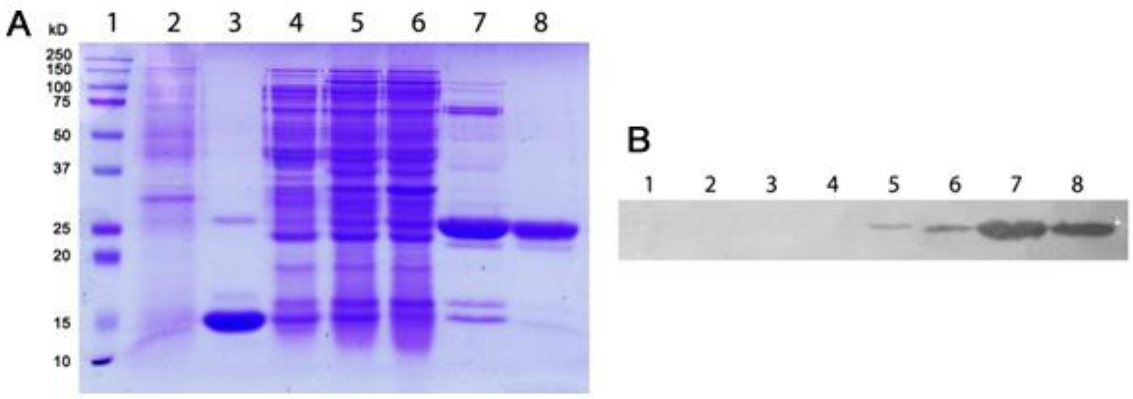

Figure 3.26 **Panel A: SDS-PAGE image indicating the purification of the ERαLBD from *E. coli*. Lane one contains 6 µg Kaleidoscope molecular marker. *E. coli* negative control lysate (25 µL) and lysozyme (20 µg) was loaded to lanes two and three, respectively. To lanes four and five 20 µg of crude lysates were added, respectively from uninduced and IPTG induced cultures. In lane six 20 µg of the resuspended cell debris from an induced culture was added. The fraction of eluent from IMAC which contains the recombinant ERαLBD was loaded in lane seven (12.5 µL). In lane six 4.1 µg of purified bacterially produced ERαLBD was loaded. Panel B: Western blot of a duplicate gel developed with rat anti-ERα IgG.**

3.2.3.3 Protein identification

Bands excised from SDS-PAGE could be successfully identified by tryptic digest LC-MS/MS as derivatives of the human androgen receptor, isoform 2. Three unique peptides were detected (accession P10275-2) with 28.35% coverage and a Mascot/Sequest score of 535.41. A protein derived from the human estrogen receptor, alpha isoform, could also be identified with six unique peptides, coverage of 13.95% and a score of 1449.76.

3.2.3.4 Affinity chromatography

For the purification of both the ARLBD and ERαLBD a high degree of homogeneity could be achieved by the application of metal affinity chromatography. To obtain a near pure protein sample, however, requires at least one step. Contaminants could be removed from ERαLBD containing IMAC fractions by gel permeation chromatography. The ARLBD proved more challenging in this regard due to the comparatively lower levels of stability in solution. The imidazole in both LBD preparations could be removed, which was important for downstream analyses and applications.

Although IMAC is an effective affinity isolation technique, further processing to eliminate contaminants from fractionated ARLBD containing lysates generally resulted in the loss of the protein in dilution. An alternative approach for one-step purification, therefore, is to employ another form of affinity chromatography to purify the receptor LBD's. Ligands to the receptors, immobilised to a stationary matrix, is a highly specific form of affinity chromatography that depends upon protein-ligand interaction. Figure 3.27, A and B, shows dot blots of fractions obtained after affinity chromatography performed on *E. coli* lysates containing, respectively, recombinant ARLBD and ERαLBD, with Sepharose 6B resin, coupled to either nortestosterone or 17β-estradiol.

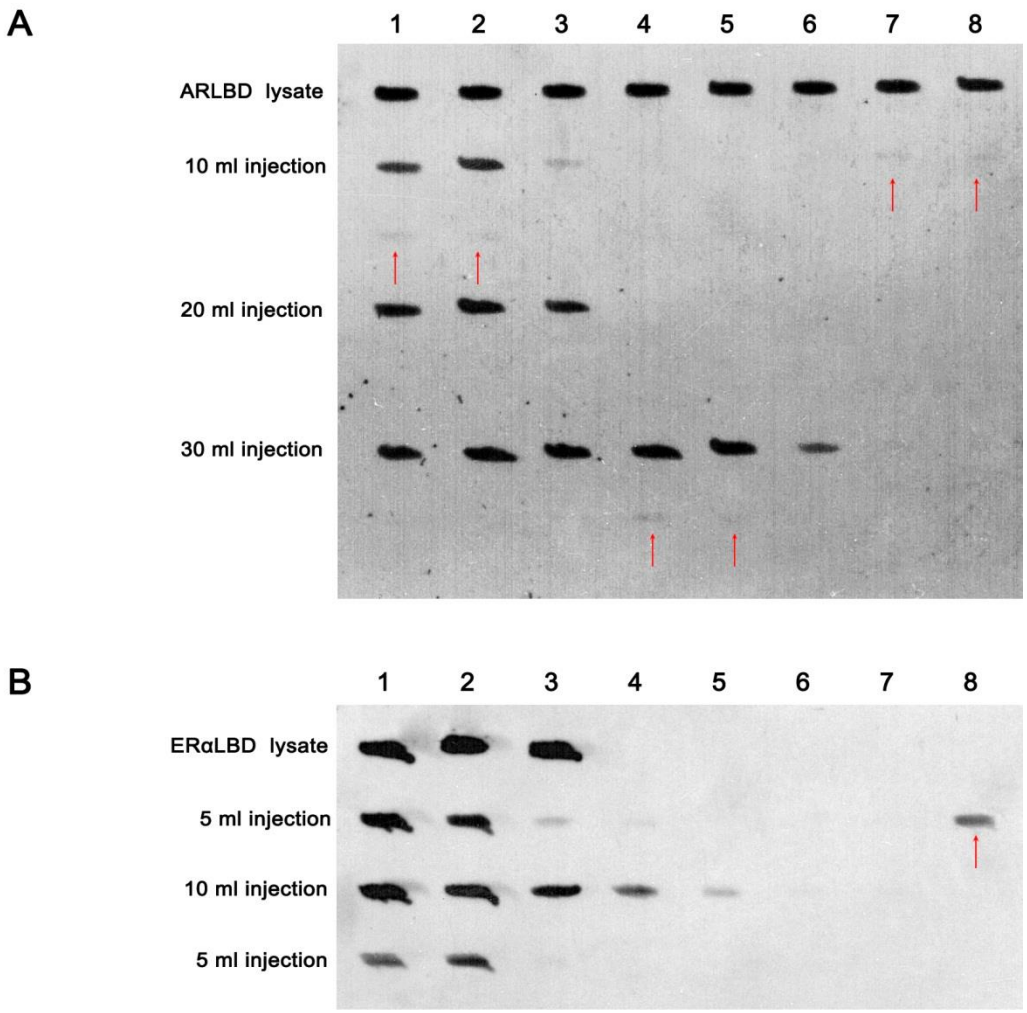

Figure 3.27 Dot blot of fractions collected during multiple injections of TOP10/pTrcHis-ARLBD (panel A) and TOP10/pTrcHis-ERαLBD (panel B) lysate supernatants to 1 mL nortestosterone-Sepharose 6B and 1 mL 17β-estradiol-Sepharose 6B affinity columns. The majority of the proteins did not bind to the column and eluted during the application and wash steps of the chromatographic runs. Minor binding of the bacterially produced receptor LBD's occurred and was eluted by the addition of free testosterone or 17β-estradiol (red arrows). The top rows contains 2 µL spots of the lysate as control.

Several attempts were made at isolation of the recombinant receptor LBDs with these systems. However, no peaks indicating the elution of protein from the columns could be resolved during treatment with free ligand. Apart from a single detection of the ERαLBD and minute amounts for the ARLBD in two runs, indicating poor retention of the LBDs on the columns (Figure 3.27, red arrows), the sex steroid receptor ligand binding domains could not be purified by affinity chromatography. The majority of the recombinant proteins did not interact with the resin matrix, but eluted in the wash.

3.2.4 Binding studies

The inability of ligand-immobilised affinity columns to retain the target proteins cast doubt on the ligand binding capabilities of the bacterially expressed human sex steroid receptor LBD's. Radioligand saturation binding is a sensitive means to assess the activity of receptor proteins in *in vivo* and *in vitro* systems by means of scintillation counting. Poor retention of the recombinant LBD's to immobilised ligand chromatography matrixes provided impetus for the investigation of ligand binding capabilities via radioligand assays.

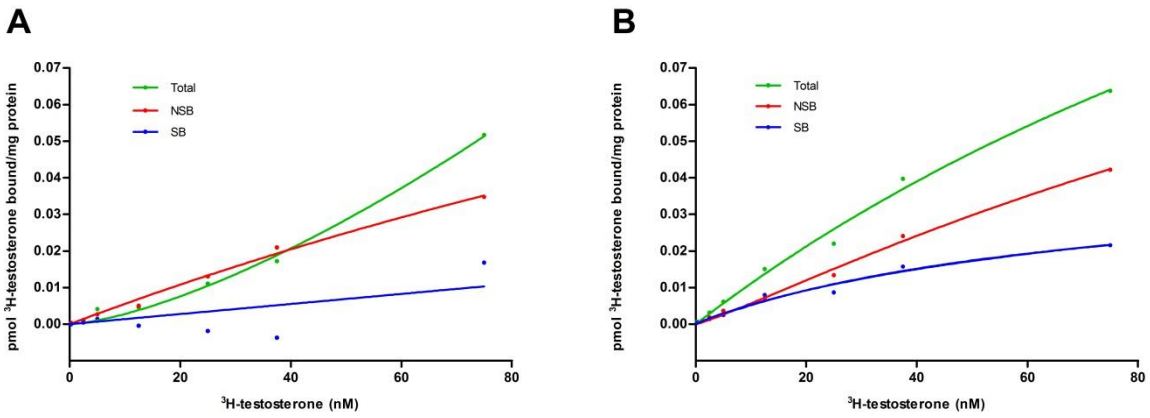

Figure 3.28 Saturation binding plots of whole cell radioligand binding experiments performed with ^{3}H-testosterone (green) and excess non-radioactive ligand (red). Results of the TOP10/pTrcHisARLBD experiment is presented in panel A, with results of the control, untrasformed TOP10 cells in panel B.

Previous radioactive binding experiments (342) of the ARLBD and ERαLBD expressed as fusion proteins with maltose binding protein (MBP) indicated a measure of biospecific capture of radioligands by immobilised receptors produced in *E. coli*. To a large degree, however, non-specific binding was observed during these studies.

The lipophilic natures of steroids allow these molecules to cross cellular membranes by simple diffusion. Therefore, in the current study a whole cell saturation binding assay was used to evaluate the binding capabilities of recombinant ARLBLD. Intact cells are easily pelleted by centrifugation and therefore permit facile separation of bound and free ligand. Furthermore, the use of intact cells to assess binding, prior to purification, allows an assessment of the maximal concentration of functionally expressed receptors produced that bind ^{3}H-testosterone as a function of the total protein complement. No distinction could, however, be made between the total binding of tritiated ligand to expressed receptors and binding that occurs non-specifically to other surfaces available during the assay ($p = 0.8707$). Results in Figure 3.28 indicate that the functionality of the majority of receptors produced in the bacterial system may be impaired.

3.3 CONCLUSION

6xHis-tagged proteins containing the correct primary structures of the human sex steroid receptor LBD's were purified to near homogeneity by means of IMAC and GPC. However, these recombinant proteins could not be isolated from bacterial lysates to quantifiable levels by immobilised ligand affinity chromatography. Nevertheless, a small population of the receptor LBD produced in this system may be correctly folded and active if one considers the results obtained during affinity chromatography since faint chemiluminescent signals were observed during elution with free ligand. The majority of the recombinant protein, however, eluted during application and washing. Furthermore, whole cell radioligand saturation assays pertaining to the ARLBD corroborated that the heterologously expressed protein exhibits insignificant binding activity towards a natural ligand of the androgen receptor.

However, nuclear receptors and their respective ligand binding domains from various species have been successfully cloned and expressed in bacterial systems with a variety of fusion tags (391–394), many structural works with regards to these proteins have been carried out in *E. coli* (395). Nonetheless, as is well known, there are many parameters that must be considered when attempting to produce eukaryotic proteins in a prokaryotic expression system. For one, not all strains of *E. coli* are equally suited to express certain proteins. For instance, the level of connate amino-acylated tRNAs available in the cytoplasm reflects the usage of codons by *E. coli* (21). Certain codons occur infrequently in the genetic material of bacterial host cells, these minor codons are generally distributed to genes that are expressed at low levels. However, preferences for such codons are often abundant in heterologous genes from eukaryotes, archaea and other prokaryotes distant from *E. coli* (396). The presence of such rare codons in highly transcribed mRNA could lead to translational errors such as amino acid substitutions, frameshifting events, or premature translational termination (397) due to ribosomal stalling when minor codon tRNAs carrying the required amino acid is absent (397–399). Kane *et al*. (400) reported on a single disfavoured AGA codon which caused a two codon shift during *E. coli* synthesis of bovine placental lactogen. The expression of heterologous proteins of seemingly the correct length may therefore be ambiguous due to poor translational integrity. Problems such as these may be addressed by site directed mutagenesis, thus abolishing aberrant protein variants, yet this approach is only viable if the rare codons in question do not affect protein activity or mRNA stability. Furthermore, alterations to all rare codon positions may become a lengthy process, depending on their frequency within the specific gene. Alternative approaches include the co-transformation of bacterial cells with plasmids encoding the necessary rare tRNAs or utilisation of strains of *E. coli*, such as Rosetta-gami (EMD-Novagen) which contain increased pools of rare tRNAs and, as with the Origami™ strain, possesses a greatly enhanced ability at disulphide bond formation due to *trxB* and *gor* gene mutations (273). The ARLBD contains a total eighteen rare codons, nine arginine (AGG, AGA, CGA), seven proline (CCC) and two leucine (CTA). Within the coding sequence of the ERαLBD fifteen rare codons are present, six of which codes for arginine, four for proline, four for leucine and one for isoleucine (ATA).

The choice of promoter may also influence the quality of protein produced within the bacterial system. mRNA of proteins expressed under control of the strong *trc* promoter contain the Shine-Dalgarno sequence which facilitates optimal translation initiation. Moreover, the *trc* promoter binds the host transcription machinery more efficiently (344) than promoters such as the 2.5-fold promoter-up mutant, *lacUV5* (401), resulting in high levels of mRNA from recombinant origin. Although high-level expression of heterologous nuclear receptor LBD can be obtained, optimising the yield of properly folded protein might require a reduction in the induction level. Over synthesis of mRNA may lead to the accumulation of misfolded protein in the cytosol, increasing molecular crowding, thus driving protein aggregation due to elevated macromolecular association constants (402).

Several experimental approaches may also be followed to reduce the formation of aggregates *in vivo*. Lowering of the temperature at which expression is induced (346, 403, 404), expression in the late (405) or early (403) exponential phase, increasing the intracellular concentration of molecular and chemical chaperones (347), lowering of inducer molecule concentration (406) and the use of cold inducible expression systems (360, 407, 408) have all been shown to affect protein solubility and activity. Along with the alterations to growth and induction parameters, countless variables influence the stability and solubility of heterologous proteins during cell lysis. Many of these variables may be manipulated to improve recombinant protein recovery, over and above the approaches took in this work. However, such approaches are mostly cumbersome, difficult to interpret and subject to a large degree of experimental error and bias. High-throughput techniques should therefore not only be based on representative sample sizes, but must also be sensitive enough to distinguish between results.

E. coli is an attractive expression host for a variety of reasons (273) and many strategies exist to maximise the production of heterologous proteins in prokaryotes at minimal cost (337, 409). The diversity of genetically engineered strains, expression vectors, induction strategies, broad cultivation parameters and general ease of use all contribute to the popularity of prokaryotic systems. Unfortunately, not all proteins are amenable to heterologous expression in *E. coli*, either due to toxicity to the organism or by the nature of the protein itself. As a consequence there will be circumstances when the choice of expression host will not be obvious and therefore several alternative expression hosts must be evaluated.

Owing to high degrees of intrinsic hydrophobicity, resulting in instability and subsequent precipitation from solution upon purification, the human nuclear receptor LBDs present such a circumstance. The inability of *E. coli* to correctly fold the human-derived proteins to the correct conformations, along with low production yields of functional protein achieved with this system require that substitutions to this form of heterologous gene expression be considered. In the following section an alternative means to produce nuclear receptor LBDs by utilisation of an insect cell/baculovirus expression system is described.

CHAPTER 4

CLONING, EXPRESSION AND PURIFICATION OF THE HUMAN ANDROGEN AND ESTROGEN RECEPTOR LIGAND BINDING DOMAINS WITH THE BACULOVIRUS EXPRESSION VECTOR SYSTEM

The difficulties associated with the purification of active forms of bacterially produced human sex steroid receptor LBDs, along with the inability of these proteins to specifically bind natural ligands to these receptors necessitated an investigation into an alternative heterologous expression system.

Members of the Baculoviridae are the most prominent entomopathogenic viruses. However, individual baculovirus isolates normally infect only closely related species of arthropods. Particularly, species of the orders Lepidoptera, Hymenoptera, Diptera, Neuroptera, Coleoptera, Trichoptera, Thysanura, Siphonoptera and some Crustaceae have been known to serve as hosts for the rod shaped, double stranded DNA viruses (410). Many of the insects in these orders cause devastation to fruit, vegetable and crop fields during their reproductive cycles. As a result, baculoviruses have been studied for use as biological pest controlling agents and have successfully been employed for the protection of apples, soybean and cotton from, respectively, the codling moth (*Cydia pomonella*), the velvet bean caterpillar (*Anticarsia gemmatalis*) and the cotton bollworm (*Helicoverpa armigera*) (411). Growing public concerns over genetically modified crops, along with known detrimental effects of synthetic pesticides on humans and animals, provides impetus for the production of safe and economical alternative means of crop protection against insect pests. Nevertheless, most wild-type baculoviruses are not sufficient to replace chemical insecticides due to latent infectious cycles that may take between five and ten days to kill the host, with decimation of crops during this period due to continued insect feeding. Recombinant protein technology has, however, provided a mechanism of reducing the time between virus application, feeding cessation and death. Transgenic baculoviruses expressing toxic genes from, among other organisms, spiders (412), scorpions (413), bees, bacteria (414) and fungi (415) have successfully improved insecticidal activity. Thus, in addition to the use of these viruses as possible insecticides, baculovirus expression vectors are used extensively to produce a variety of recombinant proteins *in vitro*. Modern methods of DNA manipulation have massively increased the ease of use associated with transgenic baculovirus generation. In this study, one of the methods used to transfer genetic materials to the viral genome is presented. A double homologous recombination event between a donor plasmid and linearised viral DNA is utilised to incorporate two human genes into the polyhedrin locus of an AcMNPV-derived baculovirus genome. This resulted in the creation of three recombinant viruses. One expresses the human ARLBD, the other two viruses express derivatives of the human alpha estrogen receptor LBD, hERαLBD, which encodes for the E domain of the receptor, and hERαLBD-f, a protein containing both the E and F domains of the hERα.

4.1 METHODOLOGY

4.1.1 The pSV.ARo and pSG5ERα mammalian expression vectors

The DNA encoding isoform 2 of the human androgen receptor (hAR, NCBI accession: M20132.1) was obtained from the 2751 nucleotide cDNA of the full length protein contained in the mammalian expression vector pSV.ARo (416). *E. coli* DH5α cells, transformed with the mammalian expression vector pSG5ERα (417), was donated by the Louw/Africander laboratory (Department of Biochemistry, Stellenbosch University). The plasmid contains the full cDNA sequence encoding the alpha isoform of the human estrogen receptor (hERα, NCBI accession: NM_000125.3) with a molecular mass of 66 kDa.

4.1.2 Transformation of *E. coli* JM109 with pSV.ARo

4.1.2.1 Production of chemically competent cells with a method involving $CaCl_2$

E. coli JM109, regarded as an all-purpose cloning strain suited to the generation of high quality plasmid DNA (418), was transformed with the pSV.ARo plasmid. Cells from cryogenic storage were streaked out on non-selective LB plates and incubated at 37°C for 36 hours. A single bacterial colony was selected from these plates, inoculated into 5 mL LB and incubated at 37°C with agitation at 235 RPM. After sixteen hours, 1 mL of this culture was diluted to 100 mL LB media in a 500 mL baffled Erlenmeyer flask. Incubation continued until an OD_{600} of 0.4 was reached, at which point the culture was placed on ice for 10 minutes. Cells were then collected by centrifugation at 2,800 xg, 4°C, for 10 minutes. After aspiration of the supernatant the tubes were kept inverted for 1 minute after which cells were gently resuspended in 10 mL chilled 75 mM $CaCl_2$. Following 30 minutes on ice the cells were collected as before and resuspended in 2 mL chilled 75 mM $CaCl_2$. The resultant chemically competent cells were immediately transferred to ice cold 15 mL conical tubes as 200 µL aliquots.

4.1.2.2 Transformation *E. coli* JM109 with of pSV.ARo

To 200 µL chemically competent *E. coli* JM109 cells 36.4 ng of pSV.ARo was added. Following 30 minute incubation on ice the cells were heat shocked at 42°C for exactly 90 seconds. Subsequently, the tubes were placed on ice for 90 seconds after which 800 µL SOC medium (2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added to each. Cell suspensions were then incubated for one hour at 37°C with shaking at 235 RPM. Subsequently, 100 µL of the transformation reactions were spread out on LB agar plates and incubated at 37°C. Single colonies from these transformation plates were inoculated into 5 mL LB, containing 100 µg/µL ampicillin, and cultured overnight. Plasmid DNA extraction was performed on all transformation cultures, followed by agarose gel electrophoreses to establish the presence of the pSV.ARo pDNA.

4.1.3 Extraction of DNA from bacterial cells

Zyppy™ Plasmid Miniprep Kits (Zymo Research) was used in the isolation of plasmid DNA from bacterial cells. Plasmid-containing bacteria were cultivated overnight in LB medium, containing the relevant antibiotic for selection, at 37°C with agitation at 235 RPM. Of these cultures 3 mL was centrifuged in a Heraeus Biofuge fresco microfuge at maximum speed for one minute. Following aspiration of the growth media the cells were lysed and treated as per the manufacturer's instructions to obtain the purified pDNA.

4.1.4 Agarose gel electrophoreses

Agarose gel electrophoreses of PCR fragments and extracted plasmids were performed with either 0.8% or 1% molecular grade agarose D1 LE (Whitehead Scientific, Brackenfell, RSA) in TAE buffer. DNA samples were mixed in a ratio of 5:4 with Ficoll Orange loading dye (0.1% Orange G, 20% Ficoll, 10 mM EDTA). No more than 450 ng of DNA was loaded to the wells of agarose gels. Electrophoreses was carried out at 75 V after entrance of DNA samples to the gel matrix at 110 V. In all cases the 1 kb DNA ladder by Promega was employed as a molecular size marker. Following electrophoreses, agarose gels were stained with 0.01% GR Green (Excellgen, Inc., Rockville, MD, USA) in TAE buffer for 25 minutes, visualised with a UV transilluminator and analysed with E-Capt, version 12.9 for Windows (Vilber Lourmat, Marne-la-Vallée, France).

4.1.5 Cloning of cDNA to the pAB6xHis baculovirus transfer plasmid

The baculovirus transfer plasmid, pAB6xHis, was obtained from AB Vector (San Diego, CA, USA). The plasmid allows for the transferral of heterologous DNA to the baculovirus genome by double homologous recombination. The multiple cloning site is located downstream of a polyhedrin promoter region and sequences expressing a 6xHis affinity tag and a thrombin recognition site.

4.1.5.1 Primer design

Primers for the amplification of the human AR and ER ligand binding domains (hARLBD and hERαLBD) were obtained from Inqaba Biotec (Muckleneuk, Pretoria, RSA) (Table 4.1). The mammalian expression vector, pSV.ARo, was used as template for the amplification of the hARLBD coding region corresponding to bases 2319 to 3136 of the human androgen receptor CDS. Recognition sequences for the restriction enzymes XbaI and PstI were introduced at the 5'-ends of the hARLBD left primer and right primer, respectively, facilitating insertion of the DNA into the polylinker of pAB6xHis at these restriction sites. DNA encoding the hERαLBD, and the hERαLBD including the C-terminal F-domain was amplified from the mammalian expression vector pSG5ERα. Two pairs of primers were designed for the amplification of the hERαLBD; bases 1266 to 2006 were amplified by the primer pair hERαLBD LP and hERαLBD RP, and bases 1266 to 2156 were amplified by the hERαLBD RP and hERαLBD-f RP. To the 5'-end of the shared hERαLBD left primer a recognition sites for the restriction

enzyme Cfr9I was added. The hERαLBD RP introduces a stop codon to the hERαLBD protein. Both of the right primers introduce the recognition site for the restriction enzyme EcoRI.

Table 4.1 **Primers for the amplification of the human sex steroid receptors' LBDs into the pAB-6xHis vector. Required stop codons are highlighted in blue. Sequences in red refers to additional nucleotides needed to encode for restriction endonuclease recognition sites (↓ denotes a cleavage position). Nucleotides indicated in black 5' of endonuclease recognition sites were added to facilitate restriction enzyme docking following PCR amplification.**

Primer	Sequence	Endonuclease	Overhang type
hARLBD LP	5'-GCT↓CTAGAGGAGACAACCCAGAAGCTGACAG-3'	XbaI	5'
hARLBD RP	5'-AACTGCA↓GGTTTCCAATGCTTCACTGGGTGTG-3'	PstI	3'
hERαLBD LP	5'-TCTAC↓CCGGGAAGAAGAACAGCCTGGCCTTGTC-3'	Cfr9I	5'
hERαLBD RP	5'-AAG↓AATTCTCATAGGCGGTGGGCGTCCAG-3'	EcoRI	5'
hERαLBD-f RP	5'-AAG↓AATTCGAGCTCTCAGACCGTGGCAGG-3'	EcoRI	5'

4.1.5.2 Amplification of hNRLBD coding regions by polymerase chain reaction

Amplification of LBD coding regions was carried out with a Thermo Hybaid PCR Express Thermal Cycler (Thermo Scientific, Waltham, MA, USA) using Pfu DNA polymerase. To each PCR reaction tube 4.0 mM $MgCl_2$, 10 mM deoxyribonucleotide triphosphates mix, 1.25 U Pfu polymerase and either 76.56 ng pSV.ARo or 19.14 ng pSG5ERα was added in 1x Pfu PCR buffer. All reactions were initiated by denaturation of the template DNA at 95°C for 120 seconds, followed by four cycles of denaturation at 95°C for 30 seconds, primer annealing at 58°C for 30 seconds and string elongation at 72°C for 60 seconds. Following these four cycles the annealing temperature for the pSV.ARo and pSG5ERα reactions was raised to 62°C and 64°C, respectively, for 35 cycles. The temperature was maintained at 72°C for 15 minutes to ensure final elongation of amplified PCR products. Following successful amplification, PCR products were purified using the Wizard SV Gel and PCR Clean Up System.

4.1.5.3 Dephosphorylation of host plasmids

Dephosphorylation of host plasmids was performed with rAPid Alkaline Phosphatase (Roche). Linearised pAB 6xHis plasmid DNA, prepared for the insertion of the hARLBD PCR, contained recessive 5' ends due to the activity of PstI. Subsequently, dephosphorylation reactions pertaining to these plasmids were carried out by incubation with the enzyme at 37°C for 30 minutes (419). pAB-6xHis plasmids, to which the hERαLBD or hERαLBD-f PCR fragments were ligated, were dephosphorylated at 37°C for 10 minutes. Following incubation, the reactions were arrested by transferral of tubes to a water bath set to 75°C for three minutes.

4.1.5.4 Ligation of digested PCR fragments and dephosphorylated host plasmids

Prepared PCR fragments were ligated to 50 ng dephosphorylated pAB-6xHis plasmids by incubation at ambient temperature for 5 minutes in the presence of T4 DNA ligase. Incubations were carried out with an insert to vector ratio of either 5:1 or 3:1. Subsequently, ligated plasmids were stored at -20°C until transformation into *E. coli* cells.

4.1.6 DNA Sequencing of PCR fragments and transfer plasmids

The three purified amplicons of the genes of interest were sent to the Central Analytical Facility, Stellenbosch University, for sequencing. All fragments were diluted to a concentration of 10 ng/µL, whilst PCR primers used for amplification were provided to CAF at 1.1 pmol/µL.

Recombinant baculovirus transfer vectors, extracted from *E. coli* propagation hosts following transformation, were diluted to a concentration of 100 ng/µL and sequenced with the phF (5'-AGACGCACAAACTAATATCACAAACTGGA-3') and mR (5'-CGTGTCGGGTTTAACATTACGGATT-3') primers (Inqaba Biotec). All sequencing reactions were performed on a 3730XL DNA Analyzer (Applied Biosystems).

4.1.7 Transformation of *E. coli* cells with baculovirus transfer plasmids.

4.1.7.1 Preparation of chemically competent cells

Chemically competent *E. coli* cells were prepared according to the protocol by Inoue et al. (420). Briefly, *E. coli* DH5α and *E. coli* JM109 colonies were cultured on non-selective LB-agar plates at 37°C. Twelve colonies (2 mm to 3 mm in diameter) were subsequently inoculated to 250 mL SOB media (2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$). Filter-sterilised Mg^{2+} constituents were added to the media after sterilization by autoclaving. Cells were incubated at 18°C in a 2 litre baffled shaker flask with constant agitation at 235 RPM. Upon reaching an OD_{600} of 0.6 the flask was removed and incubated on ice for 10 minutes. Cells were then harvested by centrifugation at 2,500 xg, 4°C, for 10 minutes. After aspiration of growth media the cells were gently resuspended in 80 mL cold TB buffer (10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$). $MnCl_2$ was added to the buffer only after the pH was set to 6.7. Resuspended cells were subsequently incubated in an ice bath for 10 minutes prior to centrifugation at 2,500 xg, 4°C, for 10 minutes. Following aspiration of the supernatant the pelleted cells were gently resuspended in 20 mL cold TB buffer. Dimethyl sulphoxide (DMSO) was subsequently added to a final concentration of 7% while gently swirling the resuspended cells. After incubation in an ice bath the cells were frozen with liquid nitrogen in 1 mL aliquots prior to cryogenic storage at -80°C.

4.1.7.2 Transformation of cells with recombinant plasmids

Frozen competent cells were thawed at room temperature and immediately placed on ice. Cells were then dispensed as 200 µL aliquots into chilled 15 mL conical tubes using chilled pipette tips. To these cells 2 µL of the ligated plasmids were added and the mixture was incubated for 30 minutes in an ice bath. After incubation in the ice bath, the cells were heat shocked at 42°C for exactly 30 seconds after which the tubes were immediately transferred to an ice bath for 2 minutes. To each tube 800 µL SOC media (SOB with 20 mM glucose) was added prior to incubation at 37°C with vigorous shaking. Subsequently, 50 µL, 100 µL and 200 µL of the reactions were added to 3 mL 0.65% soft LB-agar, containing 75 µg/mL

ampicillin, which had been pre-incubated at 47°C. The mixture was then overlaid on LB-agar containing 60 µg/mL ampicillin. Transformation plates were incubated inverted at 37°C.

4.1.8 Restriction endonuclease digest

Prior to the amplification of target gene sequences by PCR the integrity of the template DNA was assessed by restriction enzyme digest. All reactions were performed with five units of the respective restriction endonucleases in 2x Tango™ buffer at 37°C for 2 hours. pSV.ARo (484 ng) was digested with BamHI and HindIII, whilst pSG5ERα (638 ng) was digested with HindIII and SalI. For insertion of the obtained PCR fragments into the polylinker of the pAB-6xHis host vector 1 µg of pDNA and 1 µg of the respective inserts were prepared by restriction enzyme digest. For the ARLBD the amplicon and baculovirus transfer plasmid was digested with XbaI (10 U) and PstI (20 U) in 1x Tango buffer. hERαLBD and hERαLBD-f PCR fragments and host vectors were prepared for cloning with Cfr9I (10 U) and EcoRI (5 U) in 1x Cfr9I buffer. All reactions were incubated at 37°C for 3 hours.

Of the pAB-6xHis/hARLBD and pAB-6xHis/ERαLBD baculovirus transfer plasmids extracted from potential transformants 1 µg was analysed with HindIII in 1x buffer R. The pAB-6xHis/ERαLBD-f plasmids were digested with 5 U SalI in 1x SuRE/Cut Buffer H.

4.1.9 Culturing of insect host cell lines

4.1.9.1 Monolayer cultures

Spodoptera frugiperda (Sf9) and *Trichoplusia ni* BTI-TN-5B1-4 (*T. ni*) cells were obtained from Allele Biotechnology and Pharmaceuticals/Orbigen (San Diego, CA, USA). These cells were initially grown as monolayers in a non-humidified, ambient air-regulated incubator set to 27°C in complete TNM-FH medium consisting of Grace's insect cell culture medium (Gibco®, Life Technologies) supplemented with yeastolate (3.330 g/L), lactalbumin hydrolysate (3.303 g/L), sodium bicarbonate (0.35 g/L), 10% foetal bovine serum (FBS), 100 U penicillin, 100 µg/mL streptomycin, 10 µg/mL gentamycin and 2.5 mg/mL amphotericin B. Cells were counted by means of a haemocytometer to assess cell density and viability was assessed by the Trypan Blue exclusion method (421). Passages of monolayers were performed at 90 – 95% confluency; cells were subsequently plated at seeding densities of 5×10^4 viable cells/cm^2.

4.1.9.2 Suspension cultures

Sf9 cells were transferred to suspension culture once regular exponential growth was obtained at a sustained cell viability of greater than 90%. Cells were suspended in 100 mL complete TNM-FH in sterile 250 mL polycarbonate Erlenmeyer flasks at constant agitation of 100 RPM at 27°C. Pluronic F-68 was added at a final concentration of 0.1% to reduce cell shearing. Cells were routinely passaged to a cell density of 2×10^5 viable cells per millilitre when the culture reached a density of $> 2 \times 10^6$ viable cells per millilitre.

4.1.9.3 Adaptation of *T. ni* cells to suspension cultures

T. ni cells require adaptation to suspension culture conditions upon initial transferral from monolayers. Cells from two confluent 175 cm^2 flasks of adherent cells were collected and used to inoculate 100 mL complete TNM-FH at a seeding density of 2.25 x 10^6 viable cells/mL in polycarbonate Erlenmeyer flasks. To reduce aggregation of cells in suspension, 10 U heparin per mL culture volume was added during the initial phases of transferral of cells from monolayers to suspension. Cells were considered adapted to suspension growth conditions when viability exceeded 98%, doubling times of 18 – 24 hours were reached and clumping was reduced to less than ten cells per aggregate.

4.1.10 Co-transfections and viral rescue by homologous recombination

4.1.10.1 Preparation of Sf9 cells for co-transfection

Sf9 monolayers were prepared by seeding 9.6 cm^2 polypropylene dishes with 2.5 mL complete TNM-FH medium containing 1 x 10^5 viable cells/cm^2. Cells were left to adhere at 27°C for 1 hour. The adhered cells were then washed twice with sterile 1x PBS to remove antibiotics and FBS. Subsequently, 2 mL incomplete TNM-FH (no FBS), containing no antibiotics, was added to each well.

4.1.10.2 Generation of recombinant baculovirus

Recombinant baculoviruses were generated by co-transfection of Sf9 cells in 9.6 cm^2 dishes with linearized baculovirus DNA and the pAB-6xHis transfer plasmids containing human sex steroid receptor LBD cDNA in the polyhedrin locus. In brief, 10% ProFectin™ (AB Vector) transfection reagent was added dropwise to a mixture of 100 ng of the respective pAB-6xHis/hNRLBD transfer pDNA and 5 µL linearized ProFold™-C1 baculovirus DNA (AB Vector) and incubated for 15 minutes at ambient temperature. The DNA-ProFectin emulsion was then added dropwise to Sf9 monolayers and incubated at 27°C. Three days after co-transfection the virus containing media was collected and centrifuged at 1,500 xg for 5 minutes. The supernatant, containing P0 virus stock, was transferred to sterile polypropylene tubes and stored at 4°C, protected from light.

4.1.10.3 Confirmation of protein production

Following the generation of P0 virus stocks small scale infections of Sf9 cells were carried out to assess recombinant protein production. Triplicate wells of 9.6 cm^2 dishes were seeded with Sf9 cells at 90% confluency and allowed to settle for one hour at 27°C. These cells were then infected with 200 µL P0 ProFold™-C1/hARLBD, ProFold™-C1/hERαLBD or ProFold™-C1/hERαLBD-f virus stocks. Cells from these infections were collected by centrifugation at 48 hpi, 60 hpi and 72 hpi (hours post induction) and resuspended in 100 µL TNN buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40). Lysis was accomplished by osmotic shock and shear force by agitation with a bench vortex. The cell debris was separated from the supernatant and resuspended in 100 µL TNN buffer.

After treatment with 2x sample buffer (343) at 95°C for 10 minutes the supernatant and cell debris samples were analysed by SDS-PAGE and Western blot.

4.1.11 Expansion of virus stocks and titration

4.1.11.1 Virus propagation – increase of titer

The initial P0 virus stocks are of a titer which is too low for protein expression studies and must therefore be amplified. Towards this end, 500 µL of each recombinant virus was added directly to 75 cm^2 Sf9 monolayers at 25% confluency in complete TNM-FH. Four days post infection (dpi) ProFold™-C1/hNRLBD infected cells were divided into two 75 cm^2 flasks with an equal volume of fresh complete TNM-FH. At 7 dpi all infected cultures exhibited clear signs of enlargement and non-adherence (Figure 4.1). At this time the media supernatant of these cultures, containing P1 virus stock, was collected by centrifugation at 1,500 RPM for 5 minutes and stored, protected from light at 4°C.

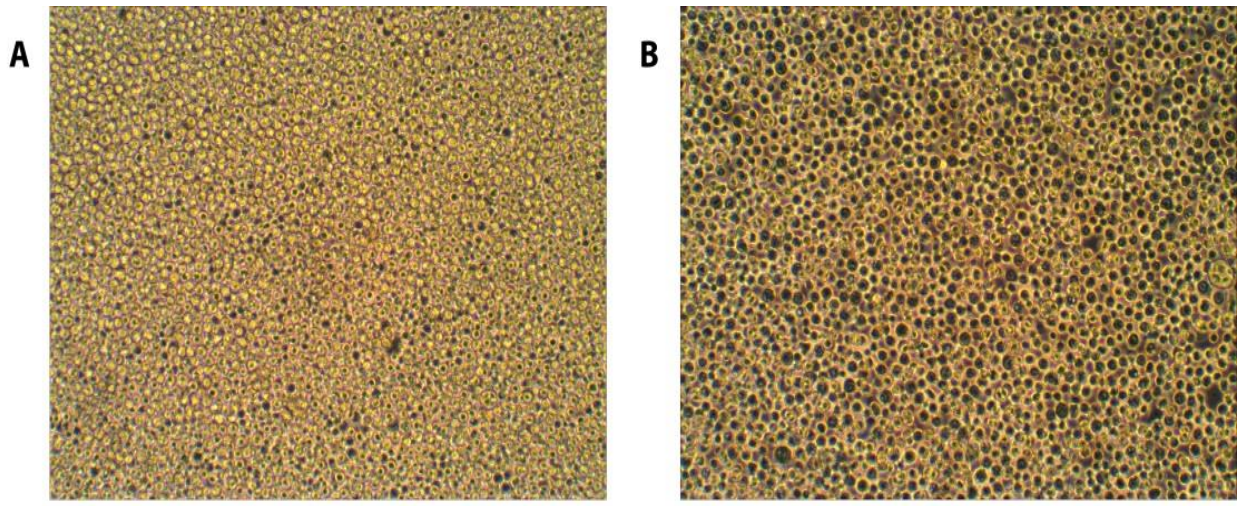

Figure 4.1 Photographs (100x magnification) of Sf9 cells infected with P0 ProFold™/hERαLBD P0 cells showing clear signs of infection (panel B) as compared to an non-infected control culture. Infected cells appear larger and are less prone to adherence. At later stages of infection cell debris becomes higly evident as cells rapidly deteriorate.

For the generation of larger volumes of recombinant P2 viral stock 100 mL Sf9 suspension cell cultures, at a density of 4 x 10^5 cells/mL, were infected with 100 µL of the respective P1 virus stocks. After seven days' incubation at 27°C with constant shaking at 100 RPM, cultures were centrifuged at 1,500 xg for 5 minutes. The supernatant of these cultures was stored under the same conditions as for the P1 stocks.

4.1.11.2 Determination of viral titer with Green Control

Green Control, a titrated baculovirus stock (10^8 pfu/mL) was obtained from AB Vector. Titration of virus stocks was accomplished by terminal green dilution. Duplicate serial dilutions (1:1 – 1:32) of recombinant viral stocks and Green Control were made in black low auto-fluorescence 96-well plates. The virus dilutions were then subjected to fluorometry at an excitation wavelength of 395 nm, with detection of emission at 509 nm. Obtained emission values were plotted against the dilution factor. Virus titres were approximated as the mean of the fold decrease observed for 1:1 and 1:2 dilutions of the recombinant against Green Control.

4.1.12 Expression of heterologous proteins

4.1.12.2 Comparison of protein expression in Sf-9 and *T. ni* cells

To determine the relative levels of recombinant protein production in *T. ni*, as compared to Sf9 cell cultures, triplicate 9.6 cm^2 wells were seeded at ± 95% confluency with these cell lines in 2.5 mL complete TNM-FH. The cells were allowed to settle in the medium and adhere to the bottom of the wells prior to infection with 200 µL of the three P2 recombinant virus stocks. Following incubation at 27°C for 60 hours, the infected cells were harvested by centrifugation at 1,500 xg for 3 minutes at 4°C and resuspended in 100 µL TNNB buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM NMBS, 0.5% Nonidet P-40, 5 mM β-mercaptoethanol, 0.1 mM PMSF). Cells were then lysed by osmotic shock and shear force, by agitation with a bench vortex. The cellular debris was subsequently separated from the soluble fraction of the lysate by centrifugation in a Hereaus Biofuge fresco microfuge at 12,250 xg for 30 minutes at 4°C. The fractions were resolved on SDS-PAGE gels and analysed by Western blot.

4.1.12.1 Determination of optimal protein expression duration

To analyse the optimal expression period for the production of recombinant nuclear receptor LBD's produced with the BEVS, an expression time course was carried out. Nine 9.6 cm^2 wells were seeded at ± 95% confluency with *T. ni* cells and allowed to adhere as before. To triplicate wells, 200 µL of recombinant ProFold™-C1/hARLBD, ProFold™-C1/hERαLBD or ProFold™-C1/hERαLBD-f was added. Cells were then incubated at 27°C. At 48 hpi, 60 hpi and 72 hpi cells were washed from the wells with the growth media, transferred to 2 mL microcentrifuge tubes and harvested. After aspiration of the media, cells were resuspended in 100 µL TNNB buffer, lysed and analysed by Western blot as before.

4.1.13 Purification of recombinant protein produced in insect cells via immobilised metal affinity chromatography

4.1.13.1 Initial protein purification studies

For initial protein purification studies *T. ni* cells were grown in 75 cm^2 culture flasks and infected at ± 85% confluency with ProFold™-C1/hERαLBD at a multiplicity of infection (MOI)[1] of > 5 and > 10. These cells were then incubated for 60 hours at 27°C. Subsequently, the infected cells were collected by washing with the growth media. The flask was rinsed with 2 mL PBS which was added to the collected cells. After centrifugation at 1,500 xg for 10 minutes the supernatant was aspirated and cells were resuspended in 4 mL TNNI buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM NMBS, 0.5% NP40, 10 mM imidazole, 5 mM β-mercaptoethanol, 0.1 mM PMSF). Cells were lysed by osmotic shock and sonication for 5 seconds with a Sonicator™ Cell disruptor (model W-225R, Heat Systems – Ultrasonics, Inc.), fitted with a H-1 probe, after which cellular debris was separated from the soluble fraction by centrifugation at 3,500 xg for 25 minutes. The supernatant was then filtered with a 0.45 µm pore size, low protein binding Sartorius syringe filter. Of this filtrate, 2 mL was injected onto a 1 mL HisTrap column (GE Healthcare), connected to an ÄKTAprime purification system. The column had been equilibrated with NP-IMAC buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM NMBS, 0.1% NP40, 20 mM imidazole, 5 mM β-ME and 0.1 mM PMSF). Histidine-tagged proteins were eluted from the column by raising the concentration of free imidazole in the buffer to 500 mM.

4.1.13.2 Protein purification

Following initial studies, the cell lysis buffer was adapted by inclusion of 25 mM sucrose and 5% glycerol as stabilizing agents (TNNI+ buffer). Furthermore, 0.6 µg/mL pepstatin A, 6.5 µg/mL aprotinin, 6.5 µg/mL leupeptin and 6.5 µg/mL antipain was added to the lysis buffer as additional protease inhibitors. For larger scale protein purification 200 mL suspension cultures of *T. ni* cells, exhibiting viabilities of over 99%, were infected with recombinant baculovirus at an MOI higher than 10. After 60 hours incubation at 27°C infected cells were collected by centrifugation at 15,000 xg, 4°C, for

[1] Multiplicity of infection is an important concept in the BEVS production process. The MOI is defined as the number of infective particles (pfu) added to a culture per insect cell. At low MOI (<< 1 pfu/cell) there are not enough infective particles present to allow every cell to take up at least one viral particle, therefore only a small population of the cells in culture will be infected. However, infected cells cease to divide and instead become factories for the generation of additional virus. Uninfected cells, on the other hand, continue to grow until enough progeny virus has budded from infected cells to be able to infect the rest of the non-infected population. Current strategies for the amplification of baculovirus vectors therefore rely on infecting low cell densities with low MOI (502). This approach is preferred because it is presumed that beyond the first round of infection the remaining cells in culture will be infected synchronously via the budded progeny virus. As a result, the occurrence of defective interfering particles (DIPs) is diminished. These defective viral particles may contain several mutations or deletions in their genome, ultimately increasing their rate of replication over that of the intact recombinant virus. DIP formation reduces the quality of recombinant baculovirus stock and leads to a reduction in recombinant protein production (503). Adequate DIP replication, however, requires the help of the complete virus. Asynchronous infection at a low MOI is therefore advantageous, since the probability of a DIP and an intact virus infecting the same cell is very low.

20 minutes. Following aspiration of the media, the obtained cell pellets were respectively resuspended in 33 mL chilled TNNI+ buffer. Cells were lysed by osmotic shock and brief sonication with 20 pulses at duty cycle 50, adjustment 7 using a Sonicator™ Cell disruptor, model W-225R (Heat Systems – Ultrasonics, Inc.) fitted with an H-1 probe. The cell lysate was then cleared by centrifugation at 16,000 xg, 4°C for 15 minutes. Collected lysate supernatants were subsequently stored at -80°C after flash freezing in liquid nitrogen.

The buffering system utilised during IMAC was altered by the substitution of NP40 with 0.15% CHAPS (C-IMAC buffer). Lysate supernatants were thawed, filtered with 0.45 µm pore size Sartorius syringe filters and kept on ice prior to chromatography. IMAC was carried out with an ÄKTAprime protein purification system by injection of protein samples on to 1 mL HisTrap Chelating columns equilibrated with C-IMAC buffer. During purification of the hARLBD, 20 mL of the filtrate was applied undiluted (9.62 mg/mL) to the column. The protein concentration of lysates containing the hERαLBD (11.24 mg/mL) and hERαLBD-f (10.47 mg/mL), however, were adjusted to approximately 5.5 mg/mL and then filtered with 0.45 µm pore size Sartorius syringe filters prior to commencement of chromatography. Following application of protein samples the column was washed with C-IMAC buffer. The imidazole concentration was then increased to 92 mM to remove contaminating metal chelating proteins. Elution of the target proteins from the column was achieved by raising the concentration of imidazole to 500 mM.

4.1.13.3 Dialysis of IMAC purified protein

Following IMAC, fractions containing the protein of interest were dialysed overnight at 4°C against CD buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM NMBS, 0.15% CHAPS, 7.5 mM DTT and 1 mM PMSF) in a ratio of no less than 1:200.

4.1.14 Identification of proteins

4.1.14.1 SDS-PAGE and Western Blot

Hand-cast 12% SDS-PAGE gels were routinely used to separate proteins according to size. Protein samples were diluted in 2x SDS-PAGE sample buffer according to Laemmli (125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.001% bromophenol blue) (343). Electrophoresis was performed with a Bio-Rad Mini-PROTEAN system at 4°C, 150 V. After electrophoresis, proteins were visualised by gel staining with Coomassie Brilliant Blue R-250, prior to fixing and destaining with glacial acetic acid and methanol.

To determine the optimal duration of expression and as a comparison between Sf9 and *T. ni* cells an equal volume of lysate supernatant was mixed with 2x sample buffer and heated at 95°C for 10 minutes. Of each prepared sample, 15 µL was then loaded into the wells of SDS-PAGE gels and electrophoresed at 150 V until the bromophenol blue front reached the bottom of the matrix. The resolved proteins were then transferred to BioTrace NT pure nitrocellulose blotting membrane (Pall Life Sciences) at a constant

amperage of 400 mA for one and a half hours. The membranes were subsequently blocked overnight in casein buffer (10 mM Tris (pH 7.6), 0.15 M NaCl, 0.5% Casein) at 4°C with gentle shaking. Afterwards the membranes were incubated at 37°C in a working solution of rabbit anti-hAR (Santa Cruz Biotecnology, Inc., Santa Cruz, CA, USA) or rat anti-ERα IgG (Thermo Scientific) in casein buffer (1:1,000). Following washing with TBST, the membranes were incubated at 37°C in a working solution of HRP conjugated goat anti-rabbit (Sigma) or goat anti-rat (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) IgG (1:10,000) in casein buffer. The membranes were again washed with TBST prior to incubation at room temperature for 5 minutes in Clarity ECL substrate (Bio-Rad). The blot was then exposed on Fuji medical X-ray film (Fuji Photo Film Co.) and developed with AXIM X-ray developer and fixer (Africa X-ray Industrial & Medical (Pty) Ltd.).

4.1.14.2 Mass spectrometry

SDS-PAGE gels containing bands corresponding to positive identification of the target proteins by Western blotting were submitted to the Stellenbosch University Central Analytical Facility at Tygerberg Medical Campus, Cape Town, for analysis by mass spectrometry. All reagents used in analysis were of analytical grade or equivalent.

In-gel trypsin digest

Identified protein bands were excised from SDS-PAGE gels and transferred to 1.5 mL microfuge tubes and destained with 200 mM NH_4HCO_3:Acetonitrile 50:50 (Sigma) until clear. Samples were then dehydrated with desiccant prior to reduction with 2 mM triscarboxyethyl phosphine (TCEP, Fluka) in 25 mM NH_4HCO_3 for 15 minutes at room temperature with agitation. Following removal of excess TCEP, the gel pieces were again dehydrated. Subsequently, 20 mM iodoacetamide (Sigma) in 25 mM NH_4HCO_3 were added and the gel pieces were incubated in the dark for 30 minutes at room temperature to carbamidomethylate cysteine residues. The gel pieces were then dried and washed with 25 mM NH_4HCO_3 followed by another dehydration step. Proteins were digested overnight at 37°C by addition of sequencing grade trypsin (Promega) solution (20 ng/µL). Following tryptic digest, the gel pieces were treated once with 50 µL water and once with 50% acetonitrile to extract peptides from the matrix. The pooled supernatants of these washes were then dried and the resulting peptides were resuspended in 30 µL 2% acetonitrile in water containing 0.05% trifluoroacetic acid (TFA).

Residual reagents from tryptic digests were removed with C_{18} stage tips (CAF, Tygerberg, RSA). These tips were activated with 30 µL methanol (Sigma) and equilibrated with 30 µL 2% acetonitrile in water containing 0.05% TFA. Peptides were loaded onto the tips and then washed with 30 µL 2% acetonitrile in water containing 0.05% TFA before elution with 30 µL 50% acetonitrile in water containing 0.05% TFA. The eluate was then dried by evaporation and the dried peptides were reconstituted in 2% acetonitrile in water containing 0.1% formic acid for LC-MS analysis.

Liquid chromatography

Liquid chromatography was performed on a Thermo Scientific Dionex UltiMate 3000 Rapid Separation Liquid Chromatography system equipped with a 2 cm x 100 μm C_{18} trap column and a 25 cm x 75 μm Pepmap C_{18} analytical column. Solvent A consisted of 2% acetonitrile in water containing 0.1% formic acid and solvent B consisted of 100% acetonitrile containing 0.1% formic acid. Samples were loaded from a temperature controlled autosampler, set to 7°C, onto the trap column with solvent A at a flow rate of 5 μL/min for 10 minutes. Samples were then eluted to the analytical column with a gradient at 250 nL/min consisting of 2.5% solvent A for 10 minutes, then 2.5% to 30% solvent B for 30 minutes, followed by 30% to 50% solvent B for 15 minutes (using Chromeleon non-linear gradient 6), after which the gradient was raised from 50% to 80% solvent B. Solvent B was then kept constant at 80% for 5 minutes. Re-equilibration of the column was performed for 10 minutes between chromatographic runs. Chromatography was performed at 50°C and the outflow delivered to the mass spectrometer through a stainless steel nano-bore emitter.

Mass spectrometry

A Thermo Scientific Fusion mass spectrometer was used for all mass spectrometric analyses. Data was acquired in positive mode using a Nanospray Flex (Thermo Scientific) nano-ESI source with spray voltage set to 1700 V and ion transfer tube temperature set to 275°C. MS1 scans were recorded in the Orbitrap mass analyser set to 120,000 resolution over the scan range m/z = 350 – 1,650 with a fill time of 100 ms or until adaptive gain control (AGC) target of $2e^5$ were reached. All spectra were internally calibrated using the polysiloxane ion at m/z = 371.10024. Ion filter criteria were set to mono-isotopic precursors only with charge state 2 – 7 and dynamic exclusion of 1 over 45 s with mass tolerance of 10 ppm. Precursor selection was performed in Top Speed data dependent mode with the most intense precursor selected first with a cut off intensity higher than 50,000.

Precursor selection was performed using the quadrupole mass analyser, with an isolation window of m/z = 2, prior to higher-energy collisional dissociation (HCD) fragmentation. HCD collision energy was set to 30%. Detection was performed in the orbitrap mass analyser with an ion injection time of 35 ms and resolution 30,000 or until an AGC target of $5e^4$ was reached.

Data Analysis

The RAW files generated by the mass spectrometer were imported into Proteome Discoverer, version 1.3 (Thermo Scientific) and processed using the Mascot 2.3.1 (Matrix Science) and Sequest (Thermo Scientific) algorithms. Database interrogation was performed against IPI human database with trypsin cleavage allowing for 2 missed cleavages. Precursor mass tolerance was set to 10 ppm and fragment mass tolerance set to 0.8 Da. Protein N-terminal acetylation, deamidation (NQ) and oxidation (M) was allowed as dynamic modifications and carbamidomethylation of cysteine residues as static modification. Peptide validation was performed using the peptide validator node set to search against a decoy database with strict false discovery rate at 1%.

4.1.15 Radioligand binding assay

To assess the biofunctionality of the recombinant nuclear receptor LBDs, radioligand binding assays were conducted using 1β,2β-3H(N)-testosterone (^{3}H-T; Du Pont: NEN® Products, Boston, MA) and 2,4,6,7-^{3}H(N)-estradiol (^{3}H-E_2; Perkin Elmer: NEN® Radiochemicals, Boston, MA). The wells of a 48-well plate (Costar®, Cambridge, MA) were coated with 1.5 mL blocking buffer (1.5% BSA in TBST (pH 7.6); 33 mM HEPES, 100 mM NaCl, 6.5% imidazole) for 1 hour at 4°C. Following coating, the wells were washed three times with TBST, after which 400 µL equilibration buffer (100 mM HEPES (pH 8.0), 300 mM NaCl, 20 mM imidazole) was added to each well. To each well, 100 µL of a 10 mg/mL SiMAG-IDA/Nickel magnetic particle suspension (Chemicell GmbH, Germany) was added and the beads were washed thrice with 1 mL equilibration buffer. The magnetic nanoparticles were then suspended in 480 µL binding buffer (100 mM HEPES (pH 8.0), 300 mM NaCl, 20 mM imidazole, 0.2% Tween-20). To triplicate wells, 20 µL of purified 6xHis-LBD or 6xHis-β-galactosidase were added to saturate the Ni^{2+}-binding sites of the particles with hexahistidine-tagged protein. The plate was then incubated for 30 minutes at ambient temperature with constant mixing following which the protein-immobilised particles were washed twice with 500 µL binding buffer. The beads were again resuspended in 490 µL binding buffer and to each well 10 µL of ^{3}H-T or ^{3}H-E_2, containing approximately 100,000 CPM, was added, with subsequent incubation for 30 minutes at ambient temperature. Subsequently, the supernatant was aspirated and the beads were washed twice with 500 µL binding buffer. Protein was then removed from the beads by incubation for two periods of 5 minutes at ambient temperature in 500 µL elution buffer (100 mM HEPES (pH 8.0), 300 mM NaCl, 500 mM imidazole, 0.2% Tween-20). The 500 µL steroid supernatants, binding wash buffers and eluted proteins were transferred to 4 mL scintillation fluid in individual vials. Radioactivity of each sample was then measured in triplicate with a Tri-Carb® 2810 TR Liquid Scintillation Analyzer (Perkin Elmer) as counts per minute (CPM) over periods of 5 minutes.

4.1.16 Statistical analysis

Statistical differences between different treatment groups were determined by ANOVA and Bonferroni post-tests to compare replicate means. The data were presented as mean standard deviation for at least three separate determinations for each treatment. All analyses were performed by the software package Prism 5, version 5.00 for Window (GraphPad Software, Inc).

4.2 RESULTS AND DISCUSSION

4.2.1 Confirmation of template cDNA identity and integrity

Prior to commencement of a cloning strategy for the amplification of the hARLBD and hERαLBD(-f) coding regions, the integrity of the template DNA was assessed by digestion with restriction endonucleases, followed by agarose gel electrophoresis (Figure 4.2). The pSV.ARo expression vector is a 7219 bp DNA molecule containing two recognition sites for HindIII and one for BamHI. pSG5ERα, on the other hand, is 5864 bp in length and contains one recognition site for HindIII and two for SalI.

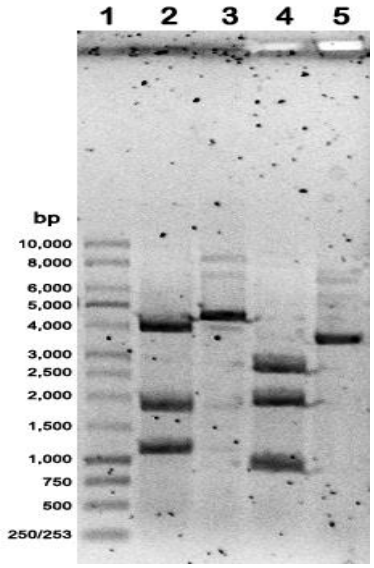

Figure 4.2 **Image of a 0.8% agarose gel following electrophoresis of the mammalian expression vectors pSV.ARo and pSG5ERα. Lane one contains a 1 kb DNA ladder (Promega). In lane two 204 ng pSV.ARo, digested with HindIII and BamHI, was loaded with 351 ng of the undigested plasmid in lane three. The pSG5ERα, digested with SalI and HindIII, was loaded to lane four (263 ng), with 452 ng of undigested pSG5ERα in lane five.**

Results observed following agarose gel electrophoreses correlated well with the expected fragments from endonuclease restriction. A comparison between the expected and the obtained fragment sizes is presented in Table 4.2.

Table 4.2 **Comparative results of expected fragment sizes to experimentally obtained bands on agarose gel following electrophoresis of uncut and endonuclease digested mammalian expression vectors, pSV.ARo and pSG5ERα.**

DNA molecule	Expected fragment size	Gel fragment size
pSV.ARo	4182 bp	4053 bp
	1687 bp	1856 bp
	1350 bp	1159 bp
pSG5ERα	2874 bp	2673 bp
	2063 bp	1938 bp
	927 bp	939 bp

4.2.2 Cloning of nuclear receptor ligand binding domains

4.2.2.1 Amplification of target cDNA and the generation of transfer plasmids

Direct manipulation of AcMNPV DNA (NCBI accession: NC_001623) is a complex endeavour due to its 134 kb size and frequency of restriction endonuclease sensitive sites (325). The insertion of foreign genes into the baculoviral genome, in contrast to bacterial plasmids, is therefore difficult to accomplish. Prior to the production of any recombinant baculovirus, the gene of interest must be cloned into a plasmid designed for the transferral of genetic elements into the baculovirus genome. The construction of such a transfer vector is performed by routine techniques involving the design of primers for the amplification of a gene of interest along with the introduction of endonuclease recognition sites to the 5' and 3' ends of resulting PCR fragment, the restriction of this fragment and a host plasmid with the relevant enzymes, followed by ligation of the two molecules.

Depending on the transfer vector employed recombinant genes may be placed under a variety of temporally distinct promoters in the BEVS (422). The pAB-6xHis™ baculovirus transfer vector (Figure 4.3) is designed for expression of proteins with N-terminal 6xHis tags under the control of the strong polyhedrin promoter. The polyhedrin promoter provides for transcription of the maximum level of RNA encoding the protein of interest during the very late stages of infection. Downstream of this promoter the pAB-6xHis polylinker contains six unique endonuclease restriction sites (SmaI, XbaI, EcoRI, NotI, PstI, BglII) for insertion of a PCR fragment encoding a gene of interest. A sequence encoding a thrombin (LVPRGS) site occurs directly upstream of the polylinker and, as such, the 6xHis tag can be cleaved from the expressed protein after purification by IMAC, if so desired. The vector contains the *bla* gene which expresses β-lactamase and confers resistance to ampicillin, carbenicillin and related antibiotics. The high-copy-number pBR322 bacterial origin of replication is also present for propagation of the plasmid in *E. coli*.

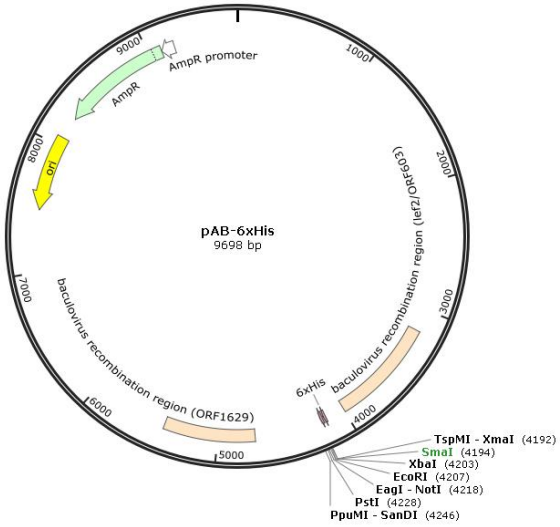

Figure 4.3 Map of the baculovirus transfer vector, pAB-6xHis™ indicating the multiple cloning site downstream of a sequence encoding a 6xHis metal affinity tag. The baculovirus recombination regions ORF 1629 and lef2/ORF603 mediate transferral of the inserted gene of interest to the AcMNPV genome.

The target sequences of the hAR and hERα LBDs were successfully amplified by PCR. Furthermore, restriction endonuclease recognition sites were introduced at each end of the molecules and the reverse primer to the hERαLBD sequence inserted the translation stop codon 'TGA' to the amplified region. Additional nucleotides to the 5'-ends of the primers were added to increase the docking platform for endonuclease interaction with the DNA. The expected outcome for the amplification of the hARLBD gene sequence, along with the extra nucleotides needed for insertion to the baculovirus transfer plasmid, is an 830 bp PCR fragment. For the amplification of the two ERαLBD genetic sequences, the expected fragment sizes are 765 bp and 909 bp, respectively for the E-domain and E/F-domain constructs. The successful amplification of these coding regions were confirmed by agarose gel electrophoreses (Figure 4.4) and direct DNA sequencing.

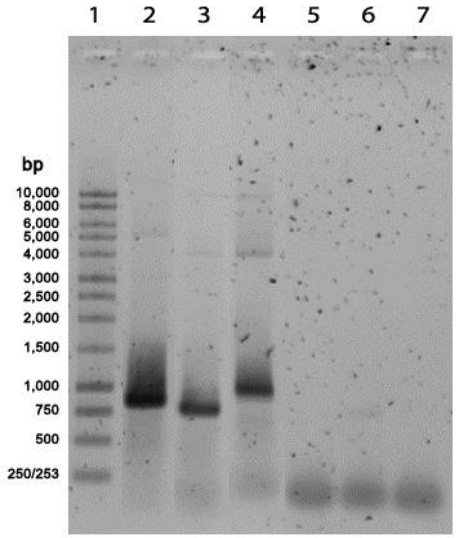

Figure 4.4 **Image of a 1% agarose gel confirming the successful amplification of the gene sequences encoding the hARLBD in lane two, the hERαLBD in lane two and the hERαLBD-f in lane three. Negative controls to these PCR amplifications containing no template DNA were loaded to lanes five to seven. A 1 kb DNA ladder (Promega) was loaded in lane one.**

After dephosphorylation of the linearized host plasmid, ligation of endonuclease treated pAB-6xHis™ with similarly prepared amplicons resulted in the generation of the pAB-6xHis/hARLBD, pAB-6xHis/hERαLBD pAB-6xHis/hERαLBD-f baculovirus transfer plasmids. These new vectors, which facilitate the transferral of genetic material to the baculovirus genome, were successfully transformed to *E. coli* propagation hosts DH5α and JM109. Subsequent to bacterial replication, the plasmids were extracted and assessed by restriction enzyme digest with the endonucleases HindIII or SalI. Electrophoreses of the digested plasmids and subsequent visualisation affirmed insertion of the nuclear receptor PCR fragments to the host plasmids (Figure 4.5). Sequencing of the new transfer vectors with the phF and mR primers, which anneal to DNA sequences flanking the inserts, confirmed the correct nucleotide sequences within the polyhedrin loci.

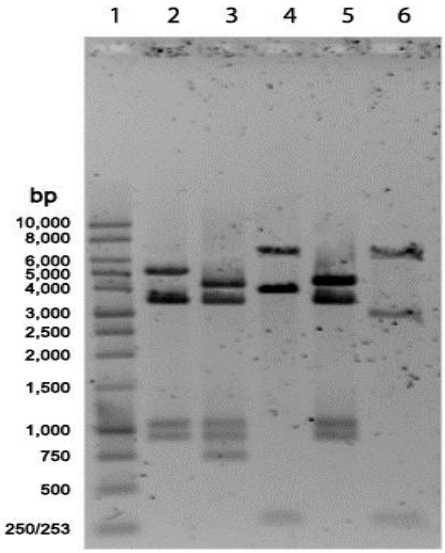

Figure 4.5 **Image of results obtained from 1% agarose gel electrophoresis of restriction enzyme digested baculovirus transfer plasmids extracted from *E. coli* JM109 cells. Lane one contains the 1 kb DNA ladder. To lane two, HindIII digested pAB-6xHis/hARLBD was loaded, with HindIII digested pAB-6xHis/ERαLBD and SalI digested pAB-6xHis/ERαLBD-f in lanes three and four, respectively. Lanes five and six contains the parent plasmid, pAB-6xHis, digested with HindIII and SalI, respectively.**

4.2.3 Generation of recombinant baculoviruses by homologous recombination

Following mapping of the polyhedrin gene (423) and demonstration that this protein was not essential for the production of infectious extracellular virus (424), the first report of the insertion of a foreign gene into the polyhedrin locus of the AcMNPV by homologous recombination was presented in 1983 by Smith *et al.* (307). Wild-type AcMNPV infections are characterised by the formation of a crystalline matrix within the cell nucleus which encapsulates viral particles known as occlusion bodies (OB). Polyhedrin deficient phenotypes may be readily distinguished from wild-type infections by altered plaque morphology due to the absence of OB in cells infected with the recombinant. This initial method of heterologous gene introduction via homologous recombination was, however, inefficient due to the low frequency (< 0.1%) of DNA exchange between circular wild-type virus and transfer vector. Linearization of the viral genome at the polyhedrin locus decreased infectivity, as compared to the circular form, by 15- to 150-fold (325), the virus must be in its circular form to be infectious. Co-transfection of the linear virus with appropriate transfer vectors increased the frequency of recombination by up to 30% due to re-circularisation of the virus. The relatively high background (~70%) observed after linearisation led to the insertion of novel Bsu36I (CC$^{\downarrow}$TNAGG) restriction sites in genes upstream and downstream of the polyhedrin locus (326). Digestion of parental virus with this enzyme therefore removes sections of these genes (Figure 4.6). The upstream gene, *ORF603*, is non-essential for viral replication, however, *ORF1629*, downstream of the *polh* gene, encodes for a WASP (Wiscott-Aldrich syndrome protein)-like protein, p78/83 (321), which is involved in nuclear actin filament formation and must be present in order for the formation of infectious virions.

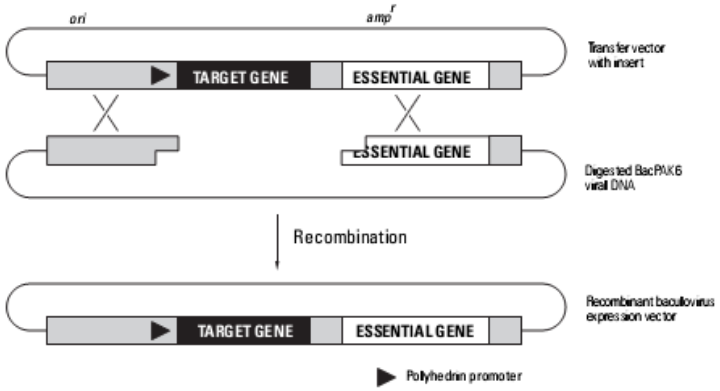

Figure 4.6 **A diagram of the AcMNPV genome based on the BakPAK6 virus DNA contain Bsu36I recognition sites within the *ORF603* and *ORF1629* genes, respectively located upstream and downstream of the polyhedrin locus. Bsu36I restricted viral particles are linear and non-infective. Re-ligation of the viral DNA in the absence of a functional *ORF1629* gene does not restore replicative prowess, since this gene is essential to viral structure. Recombination with a transfer vector rescues the virus by restoration of the truncated genes, while simultaneously introducing a heterologous gene to the polyhedrin locus of the viral genome. Image: Clontech.**

The Bsu36I alterations to the baculoviral genome described above have been incorporated into the commercially available insect expression vector ProFold™-C1 (Figure 4.7). The DNA molecule has also been engineered to express two human chaperone proteins, Heat-Shock Cognate 70 (Hsc70) and a molecular partner, Heat-Shock Protein 40 (Hsp40/ERdj2), which cooperate in protein folding in the cytoplasm (425). Up to 30% of eukaryotic proteins (426) require the availability of molecular chaperones to fold into the proper three dimensional configurations necessary for biological activity. DnaJ-like proteins, such as Hsp40, stimulate the ATPase activity of Hsc70, allowing stable interaction between the chaperone protein and a polypeptide substrate (427). Upon binding to unfolded substrates, Hsp40 can recruit Hsc70 to act upon these nascent polypeptides, thus preventing improper folding by stabilisation of the amino acid chain during translation until sufficient protein has been synthesised to obtain the correct conformation.

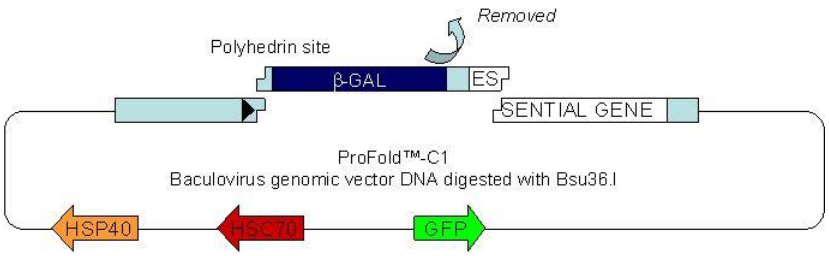

Figure 4.7 **Schematic representation of the genetic organisation of AcMNPV derived ProFold™-C1 baculovirus DNA. The vius has been linearised with Bsu36I and is therefore non infectious until rescue by recombination with a transfer vector. The genetic sequences for the human chaperones Hsp40 and Hsc70, along with the reporter protein GFP, has been inserted in positions distal to the polyhedrin locus. Image: AB Vector.**

Insect cells can synthesize these molecular helper proteins, indeed Hsp70, the stress induced connate of Hsc70, is up-regulated during baculovirus infection (428, 429). Moreover, AcMNPV replication is assisted by heat shock proteins during infection of Sf9 cells (430, 431). It has also been reported that

Hsc70 and Hsp70 associate with the VP1, VP2 and VP3 capsid proteins of polyomaviruses in the cytosol with consequent nuclear translocation, thus assisting in viral assembly (432). Upon expression of VP1 and VP2 in Sf9 cells with baculovirus vectors, co-immunoprecipitation with hsp70-like proteins was also observed. Such results suggest that molecular chaperones are involved in the prevention of premature cytosolic capsid assembly and, or alternatively, the transport of unassembled capsid proteins to the nucleus. Nobiron *et al.* (433) demonstrated by a differential display approach that Sf9 host mRNA encoded by *hsc70* was transiently up-regulated during AcMNPV infection, with expression reaching a peak at 6 hpi and complete down-regulation occurring at 24 hpi. Down-regulation of the expression of these proteins from 12 hpi onwards implies that recombinant eukaryotic constructs, expressed during the late stages of infection under the control of the polyhedrin or p10 promoters, do not have access to these molecular chaperones. Heterologous proteins which require the assistance of heat shock chaperones are thus often misfolded, which may lead to aggregation in structures reminiscent of bacterial inclusion bodies.

DNA inserted into the multiple cloning site of the pAB-6xHis plasmid, in lieu of polyhedrin, is flanked on either side by the sequences *lef2/ORF603* and *ORF1629*, respectively. As explained in the preceding discussion, these baculovirus derived gene sequences are the sites of homologous recombination with similar, yet truncated, sequences present at the ends of the linearised ProFold™-C1 genome. Co-transfection of Sf9 cells with ProFold™-C1 and the recombinant pAB-6xHis plasmids led to successful re-circularisation and rescue of the virus, along with the introduction of the heterologous human sex steroid LBD's to the baculoviral genome. Successful co-transfections were easily identified by morphological changes to the treated cultures, as compared to untransfected controls, and by visualisation of green fluorescence using simple fluorescence microscopy (434).

4.2.3.1 Viral titration and amplification

Baculoviral particles produced during the initial double homologous event are of low titer and therefore unsuitable for the large-scale production of recombinant proteins. Various methods of baculovirus titration have been developed since the original inception of traditional plaque assays (435, 436). Such methods include end-point dilutions (437), cytopathic effects based on cell size (438), flow cytometry (439), qPCR (440) and methods based on expressed reporter genes such as β-galactosidase (441) or colorimetric indicators such as 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) or AlamarBlue™ (442). Titration of recombinant baculovirus stock is important for numerous reasons. Amplification of virus at excessive MOI is not only wasteful of valuable stocks, but may lead to qualitative decreases in subsequently produced infectious particles due to the occurrence of DIPs. Furthermore, knowledge of the titer of viral stock allows for quality assurance in terms of the reproducibility of protein production runs.

Emission and excitation scanning of the recombinant baculovirus infected insect cells gave an emission maximum of 509 nm, a major excitation maximum of 395 nm, with a minor peak at 470 nm (Figure 4.8). The spectral properties are the result of expression of the 238 amino acid green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (443, 444). GFP production by ProFold-C1™ coincides with transcription from very late promoters, such as *polh*. This highly stable protein is not easily degraded by proteases, heat, chemical denaturants or pH fluctuations and therefore serves as a marker molecule for viral production.

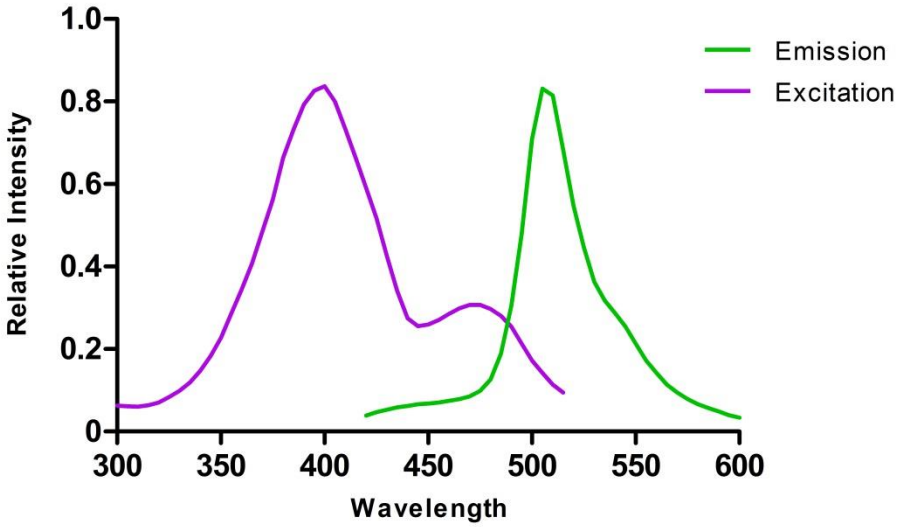

Figure 4.8 **Excitation spectrum of GFP from *Aequorea victoria* has two maxima, a major peak at 395 nm and a minor peak at 470 nm. The fluorescence emission spectrum has a peak at 509 nm and a shoulder at 540 nm. GFP is expressed by the parent baculovirus, ProFold™-C1, at the very late stages of infection. The spectra were generated by fluorescence scanning of media collected at 60 hpi from Sf9 cells infected with recombinant ProFold-C1™ virus.**

Quantitation of the emitted fluorescence of recombinant virus stock against that of the titrated reference Green Control (10^8 pfu/mL) allow for fast approximation of viral titre (445). The initial virus stock was amplified with MOI's less than one from low starting titres to 7.75 x 10^7 pfu/mL, 4.00 x 10^7 pfu/mL and 4.08 x 10^7 pfu/mL for ProFold-C1/hARLBD, ProFold-C1/hERαLBD and ProFold-C1/hERαLBD-f, respectively, as determined by terminal green dilution (Figure 4.9).

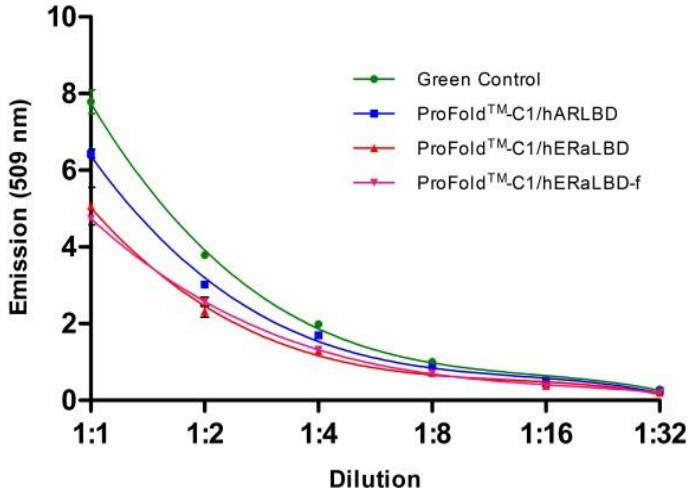

Figure 4.9 **GFP titration curves of recombinant baculoviruses against a reference stock of known titer (10^8 pfu/mL). Viral titer is approximated as the mean of the fold decrease observed for 1:1 and 1:2 dilutions of the recombinant against Green Control.**

4.2.4 Protein expression

Conversely to the low MOI commonly used in the amplification of viral stock, synchronicity of infection is required for the expression of heterologous protein in the BEVS. Asynchronous infection leads to suboptimal yields of recombinant protein due to premature cell lysis and delayed protein production.

4.2.4.1 Sf9 v *T. ni*

The Sf9 cell line, a clonal isolate of IPLB-Sf21-AE (Sf21) (446), is well suited to the production and amplification of recombinant baculovirus due to uniform monolayer formation and consistent cell morphology. However, multiple workers have expressed recombinant proteins in cells derived from different insect hosts. Davis *et al.* (447) evaluated the expression of secreted alkaline phosphatase (SEAP) by a recombinant AcMNPV baculovirus in eight insect cell lines derived from *Spodoptera frugiperda, Trichoplusia ni, Mamestra brassicae* and *Estigmene acrea*. McKenna *et al.* (448) established and compared thirty one cell lines from *T. ni* for viral and protein production. Hink *et al.* (449) examined expression levels between 23 different insect cell lines. All these studies found insect cell lines that were capable of producing greater amounts of recombinant protein than either Sf9 or Sf21.

BTI-TN-5B1-4 differs markedly from Sf9 cells in terms of its metabolism and requirements for growth. In contrast to Sf9, which, in the presence of ammonium ions, can grow in the absence of glutamine, glutamate and aspartate (450), *T. ni* cells have strict demands for glutamine and asparagine to support their growth (451). Furthermore, the production of ammonium and lactate is low in Sf9 cells (452), yet these molecules are produced at high concentrations in *T. ni* cells and could have possible inhibitory effects on cellular growth (453) translating into altered expression profiles of recombinant protein within different cell lines.

A comparison between the expression levels obtained in Sf-9 cells and the *T. ni* derived cell line BTI-TN-5B1-4 is presented in the Western blot image in Figure 4.10. Densitometric analysis (Figure 4.11) indicates that maximal expression of the hERαLBD and hERαLBD-f proteins may be achieved by selecting the *T. ni* cell line as an expression host instead of Sf9 ($p < 0.05$ and $p < 0.001$, respectively). No significant difference was observed in the responses obtained from *T. ni* cells infected with ProFold-C1/hARLBD, compared to Sf9.

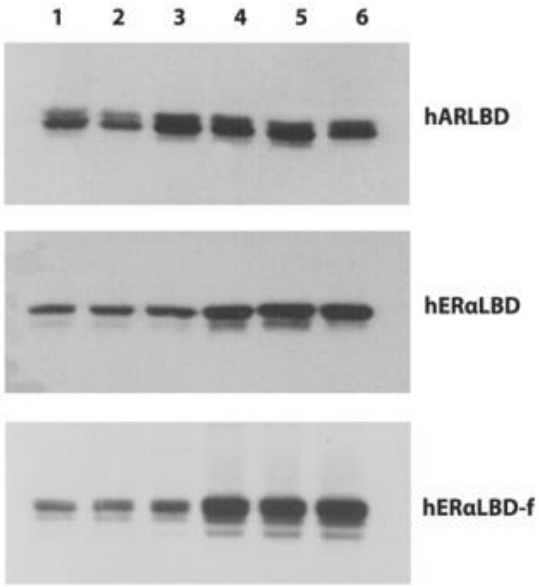

Figure 4.10 Western blots of triplicate Sf9 and *T. ni* cultures harvested at 60 hpi following infection with recombinant baculoviruses expressing the hARLBD, hERαLBD and hERαLBD-f proteins.

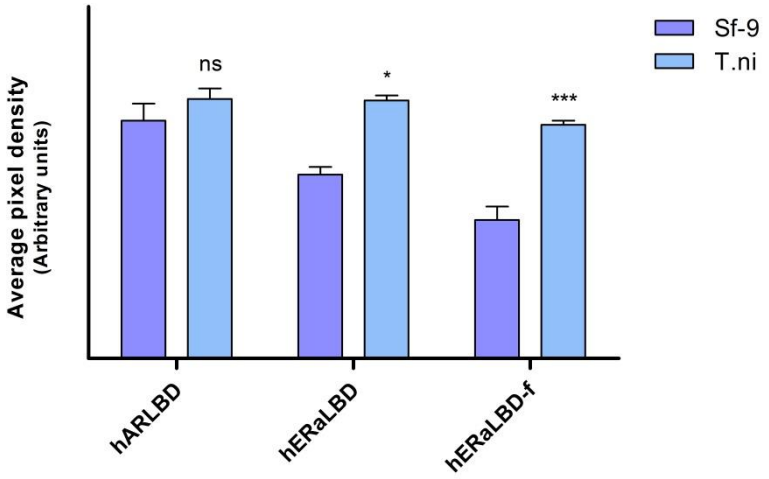

Figure 4.11 Comparitive production of sex steroid receptors LBD's in two insect cell lines, Sf9 and *T. ni*. No significant difference in the relative expression of the hARLBD could be detected between the two cell lines.

4.2.4.2 Determination of optimal infection period for LBD production

Transcription from the strong *polh* promoter is initiated during the very late stages of infection. Virally expressed very late factor 1, in combination with a virally-induced RNA polymerase leads to a burst of high-level transcriptional activity from this promoter (454). In AcMNPV-infected cells the very late genes are activated between 18 and 72 hpi (316). During wild type baculovirus infection this stage of infection is characterised by the appearance of a crystalline matrix of occluded virus (OV), with a concomitant start of cell lysis. Considering the lytic nature of the BEVS, the optimal time to harvest infected cells expressing heterologous proteins in the cytosol is an important consideration. Proteolysis is a critical issue and many techniques have been proposed to monitor the state of an infected culture in order to predict the correct time to harvest cells, thus maximising recombinant protein yield (455–457). Many of these techniques, such as the measurement of oxygen uptake rates or intracellular ATP concentrations, are impractical for routine monitoring of peak protein production due to the requirements for specialised equipment. Automated systems are available for the continual assessment of cell viability, density and size, yet Sander and Harrysson (458) concluded that the only reliable predictor of the point of peak protein production is a peak in the average cell diameter. The authors, having based their observations on the production of enhanced GFP (EGFP), caveats their findings by pointing out that proteins other than the very stable EGFP may be more sensitive to proteolysis, thus necessitating continual assessment of all parameters over multiple infections. Alternatives to strict monitoring strategies have included the addition of protease inhibitors in infected cultures, or the deletion of genes encoding specific proteases from baculovirus vectors. Other methods, such as the utilisation of pre-lytic promoters for heterologous protein expression have also been investigated (459–462).

In the absence of sophisticated equipment for continual assessment of protein production, the optimal duration of infection within the BEVS must be determined empirically. Western blots of infected *T. ni* cell lysate supernatants (Figure 4.12 and Figure 4.13) indicated maximal responses for recombinant hERαLBD after 60 hpi ($p < 0.01$ and $p < 0.05$ for the hERαLBD and hERαLBD constructs, respectively). The visual increase observed for the hARLBD at 60 hpi and 72 hpi, as compared to 48 hpi were shown to be non-significant.

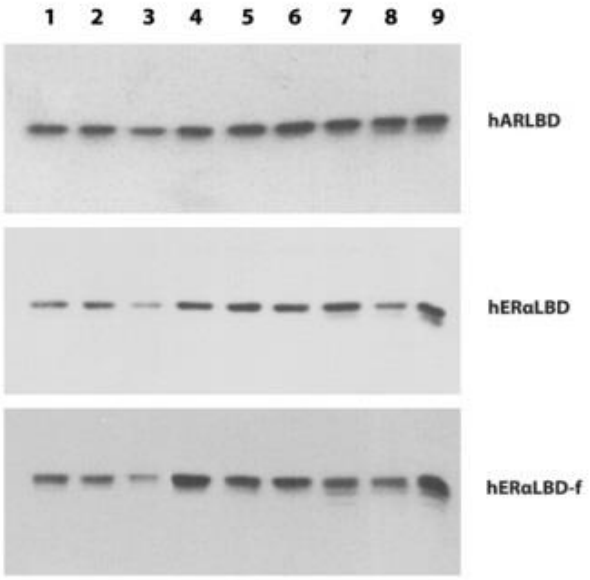

Figure 4.12 Determination of optimal expression duration for the production of sex steroid receptor LBDs in *T. ni* cells. Triplicate infections were collected at 48, 60 and 72 hours after infection with recombinant baculoviruses at a MOI > 10.

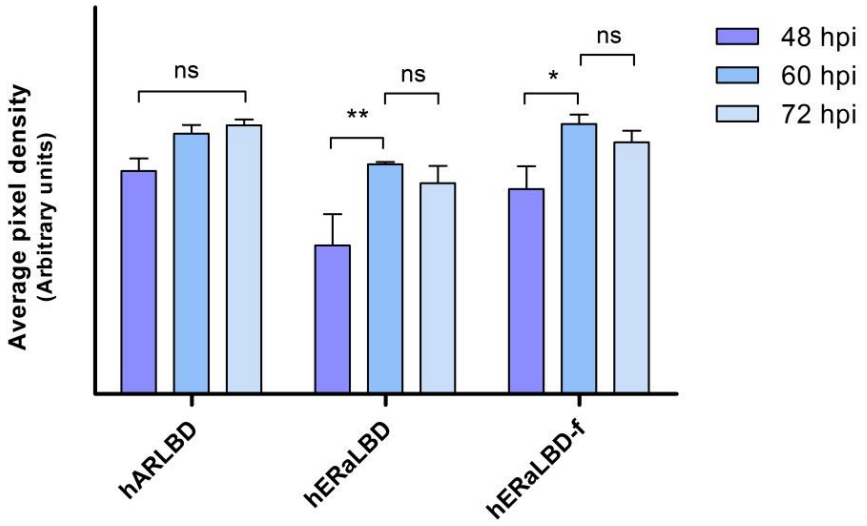

Figure 4.13 Relative chemiluminescent responses obtained from Western blots of human sex steroid receptor ligand binding domains following expression by *T. ni* cells infected with recombinant baculoviruses.

4.2.5 Purification of recombinant protein

4.2.5.1 Initial studies

Heterologous proteins expressed from the ProFold™-C1 polydedrin locus, following rescue of the virus by homologous recombination with pAB-6xHis plasmids, are fused to 6xHis metal affinity tags. The inclusion of this molecular marker enables purification of the target proteins by IMAC via the formation of coordinated bonds between the imidazole rings of consecutive histidine residues and metal ions bound to a stationary matrix. It is well documented that the ligand binding domains of nuclear receptors are highly hydrophobic (391, 463–465); major concerns in the heterologous production of such proteins always include at least some reference to issues pertaining to solubility (341, 394). Primary investigation into the purification of BEVS produced hERαLBD protein therefore included the detergent Nonidet P-40 in the chromatographic buffer as an agent to inhibit protein aggregation and precipitation due to hydrophobic interaction. The inclusion of this non-ionic polyoxyethylene detergent led to substantial background interference at the detection wavelength of 280 nm. Slight repression of peaks was evident as background absorption amounted to approximately 545 mAu. Nevertheless, an elution peak for the recombinant hERαLBD was resolved on the chromatogram at 27.59 mL (Figure 4.14).

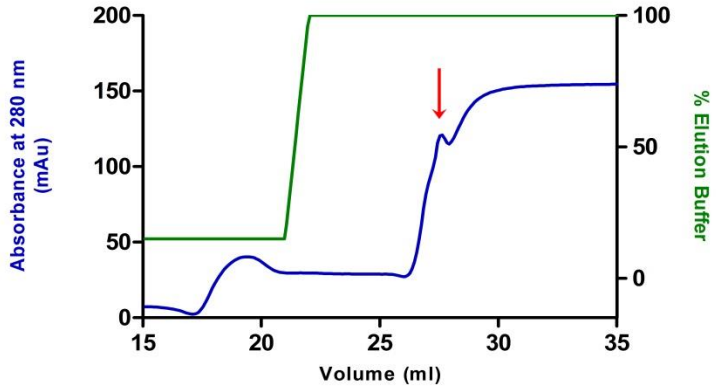

Figure 4.14 Chromatogram of the first attempt at purification of the hERαLBD construct from *T. ni* lysate supernatant via IMAC. The slight peak at 27.59 mL (indicated with a red arrow) is the elution volume for the recombinant protein of interest.

Analyses of the eluent fractions immediately surrounding the obtained peak at 27.59 mL (Figure 4.14) by SDS-PAGE and Western blot with anti-hERα antibodies (Figure 4.15) revealed that the hERαLBD could be purified to a high degree of homogeneity from insect cell lysates as nearly no contaminating proteins are observable in lanes four to six. Moreover, cultures infected at MOI greater than 10 yielded more recombinant protein, compared to an MOI > 5, as indicated by the depth of the chemiluminescent response obtained during Western blot. However, a comparison of the purified protein to the original lysate supernatants revealed that there is a degree of proteolytic activity prior to IMAC purification, resulting in removal of the polyhistidine affinity tag from the recombinant protein, thus decreasing the amount of protein that can be purified by this form of affinity chromatography under these conditions.

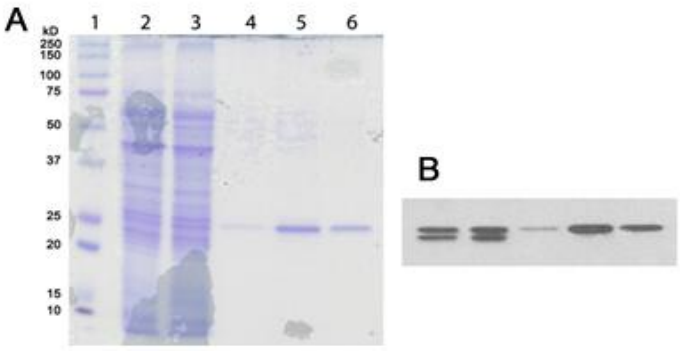

Figure 4.15 **SDS-PAGE (panel A) of hERαLBD indicate a high degree of homogeneity following single step purification by IMAC. Lanes four to six were loaded with 25 µL of 2x sample buffer prepared fractions surrounding the elution peak of the target protein (Figure 4.14). Purification of the hERαLBD from insect cell lysates indicate some proteolytic activity prior to purification as is evident from the doublet lines occuring in lanes two and three of the Western blot insert (panel B). These lanes contain lysate from cultures infected with MOIs of >5 and >10, respectively.**

4.2.5.2 Purification of heterologously expressed nuclear receptor LBDs produced in the baculovirus expression vector system

Exchanging NP-40 for the zwitterionic detergent, CHAPS (3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate), alleviated the high background absorption of the chromatographic system. CHAPS, a mild detergent that does not lead to the denaturation of proteins, has been employed by numerous groups (466, 467) for the solubilisation of generally hydrophobic proteins, such as those which are normally membrane bound. According to Stoscheck (468) up to 10% CHAPS is tolerable in protein assays that utilize absorption at 280 nm. Background absorption by NP-40 amounted to 547.5 mA, whilst CHAPS contributed less than 7 mAu to the UV absorption spectra.

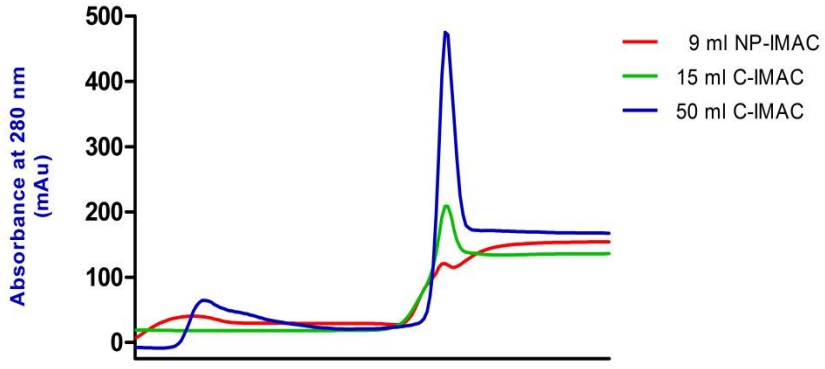

Figure 4.16 **Chromatograms of IMAC purifications of the hERαLBD from insect cell lysate. The inclusion of Nonidet P-40 (NP-IMAC buffer) in the chromatographic buffers resulted in substantial background absorbance at 280 nm, yet an elution peak for the target protein was still visible. Exchange of this detergent to CHAPS (C-IMAC buffer) markedly removed background absorbance by the buffering system.**

The hERαLBD could be detected by UV absorption during IMAC in a reproducible and injection volume dependant manner (Figure 4.16). Reproducible elution profiles for the hERαLBD-f (Figure 4.17) protein were also observed. Interestingly, larger absolute absorbance values were obtained for peaks indicating elution of the hERαLBD-f protein as compared to the hERαLBD when applying the same amount of protein from insect cell lysates to a HisTrap Chelating column. In addition to the seven phenylalanine residues, three tryptophan residues and five tyrosine residues contained within the hERαLBD protein, hERαLBD contains an additional phenylalanine residue and two tyrosine residues. It is doubtful that the increase in absorptive capacity is due to the presence of these additional aromatic residues. Most probably, the increased peak area of hERαLBD-f separations, as compared to that of hERαLBD, can be explained by the presence of additional contaminating proteins within the dialysate obtained after IMAC purification (Figure 4.18, lanes 6 and 7).

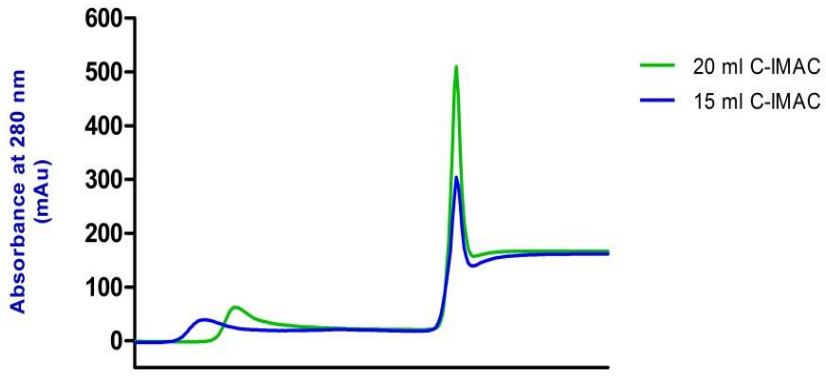

Figure 4.17 Chromatograms of IMAC purification of the hERαLBD-f from insect cell lysate. The 20 mL injection contained approximately 110 mg protein resulting in protein recovery of 8.37 mg within the elution fraction of the target protein. Aproximately 82.5 mg protein was applied to the column during the injection of 15 mL lysate, with an approximate recovery of 6.93 mg protein during target peak elution.

Positive development of Western blots using anti-hERα antibodies successfully identified the major protein bands in lanes six to nine of Figure 4.18 as being derived from this hormone receptor. Analyses of the lanes six to nine of Figure 4.19 indicates that the hERαLBD-f protein comprises 75.6% of the total protein in lanes six and seven (1.92% std.dev.), while the hERαLBD accounts for 92.9% of the protein in lanes eight and nine (1.65% std.dev.). Protein concentrations for the hERαLBD and hERαLBD-f containing fractions were respectively determined as 6.42 mg/mL (1.75 mL) and 5.58 mg/mL (1.50 mL). Therefore, the quantities of recombinant hERαLBD and hERαLBD-f that could be produced successfully in the BEVS equate to approximately 10.44 mg and 6.33 mg, respectively, from cell lysate injections of 20 mL. Considering that the initial lysate protein concentration was 5.50 mg/mL, percentage recovery of recombinant hERαLBD and hERαLBD-f was approximately 9.49% and 5.75% of the total protein complement.

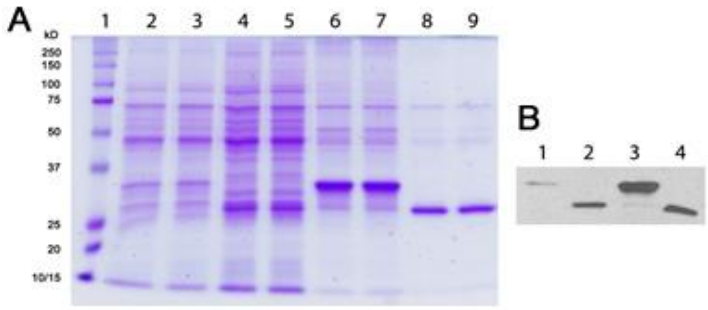

Figure 4.18 **Image of SDS-PAGE gel (panel A) indicating the purification of the hERαLBD and hERαLBD-f proteins via IMAC, with insert of Western blot of these proteins using primary monoclonal antibodies raised against the human estrogen receptor. In lane one, 7.5 µg of the Bio-Rad Kaleidoscope Precision Plus molecular marker was loaded. Lanes two and three contains lysate of *T. ni* cells infected with ProFold-C1/hERαLBD-f, with ProFold-C1/hERαLBD infected cell lysate in lanes four and five. The IMAC purified hERαLBD-f peak fraction was loaded in lanes six and seven, with IMAC purified hERαLBD fraction in lanes eight and nine. The Western blot insert (panel B) indicates the presence of the hERαLBD-f and hERαLBD proteins, at the expected sizes, in lanes one and two, respectively. The purified forms of these proteins are visible in lanes three and four, respectively.**

Figure 4.18 also gives evidence that the inclusion of additional protease inhibitors in the lysis buffer successfully inhibited cleavage of the recombinant proteins at the thrombin site, located at the carboxy-end of the polyhistidine affinity tag, as singular bands can be observed in Western blots of the cell lysates (lanes one and two).

UV absorption by proteins during elution of IMAC columns (Figure 4.19), to which *T. ni* lysates from ProFold-C1/hARLBD infections had been applied, is markedly lower for the hARLBD compared to the hERαLBD or hERαLBD proteins. However, a noticeably difference was observed in peak height with increasing injection volumes. Nevertheless, only 54.8% of the purified protein resolved by SDS-PAGE (Figure 4.20), according to densitometry, gave a positive result when subjected to Western blot utilising anti-hAR antibodies.

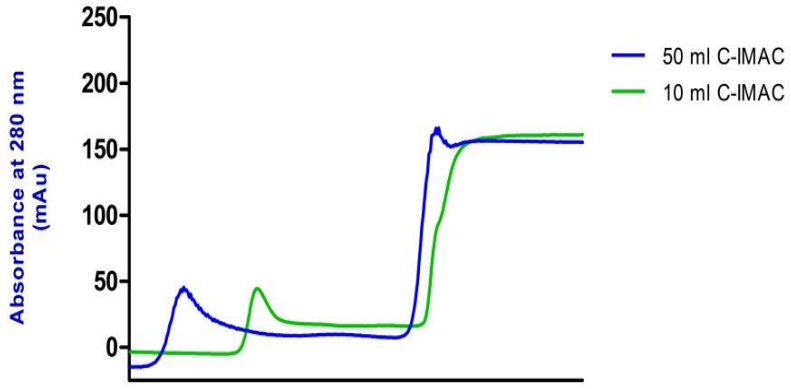

Figure 4.19 **Chromatograms of the separation of baculovirus/*T. ni* produced hARLBD. Injection of 10 mL insect cell lysate resulted in a barely detectable elution peak for the target protein. An increase of the injection volume to 50 mL markedly increased the peak height. Compared to a 50 mL injection of hERαLBD containing lysate at approximately half the concentration the elution peak height is relatively low.**

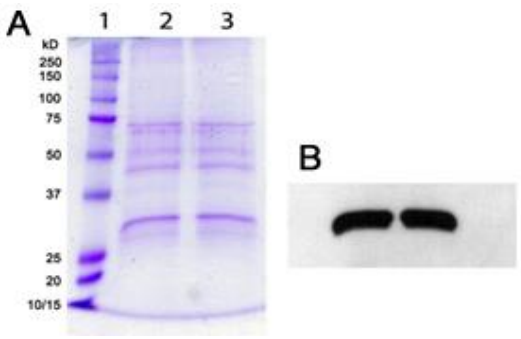

Figure 4.20 **Image of an SDS-PAGE gel (panel A) showing resolved proteins collected during elution of the major peak of the 50 mL injection of BEVS expressed hARLBD in Figure 4.19. Lane one contains the Kaleidoscope molecular protein marker, while duplicate samples of the collected IMAC fraction was loaded to lanes two and three. The major bands in the second and third lanes correspond to the target protein, as confirmed by Western blot with anti-hAR antibodies (panel B).**

Protein determinations of the initial insect cell lysate and the collected IMAC fraction, following dialysis, indicated values of 9.62 mg/mL and 1.03 mg/mL, respectively. Considering a total amount of 20 mL containing 192.4 mg protein was injected onto the column during purification, and the collected peak was 3 mL, protein recovery within this fraction amounted to 1.60% of the total protein complement. Consequently, 1.69 mg, or a mere 0.88% of the total protein content can be ascribed to the hARLBD.

4.2.6 Protein identification

4.2.6.1 Mass spectrometry

Examination of MS results against the IPI human database successfully identified fragmented peptides from SDS-PAGE gel excised protein bands as analogous to the major isoforms of the human androgen and estrogen receptors. Proteins with 2 or more peptides were detected with confidence greater than 95% (Table 4.3).

Table 4.3 **Mass spectrometric analysis of proteins excised from SDS-PAGE gels. Proteins were detected with high confidence as analogues of the human sex steroid receptors.**

Sample	Accession	Description	Coverage	Unique Peptides	PSMs	Sequest Score	Mascot Score
ARLBD	IPI00020070.4	Androgen receptor, Isoform 2	27.06%	5	155	91.01	314.83
ERαLBD	IPI00294982.1	Estrogen receptor, α Isoform	18.99%	5	89	99.58	
ERαLBD-f	IPI00294982.1	Estrogen receptor, α Isoform	40.50%	8	398	332.28	701.9

4.2.7 Radioligand binding studies

The biospecific capture of ^{3}H-T and ^{3}H-E_2 to magnetic particle immobilised recombinant steroid receptor LBDs is presented in Figure 4.21. No significant difference ($p > 0.05$) could be observed between β-galactosidase and hARLBD immobilised to Ni^{2+}-functionalised magnetic nanoparticles when treated with tritiated testosterone. However, compared to the control, significant ($p < 0.05$) amounts of radioactive estradiol could be bound by the recombinant hERαLBD and hERαLBD-f proteins. For both these constructs, three fold more ^{3}H-E_2 was retained following elution from the magnetic particles, compared to β-galactosidase treated in the same manner indicating substantial binding activity of the recombinant derivatives of the alpha isoform of the human estrogen receptor LBD.

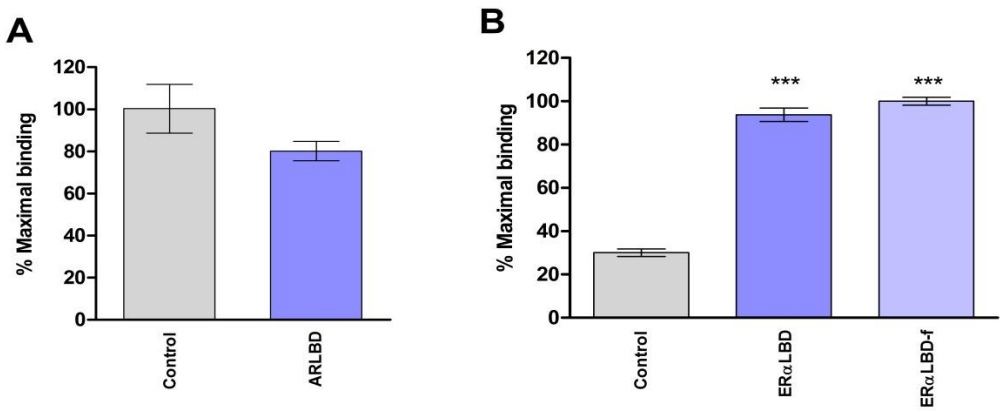

Figure 4.21 Radioligand binding to proteins immobilised on Ni^{2+}-derivitised magnetic nanoparticles. Radioactive counts per minute obtained were normalised against the maximal response observed. Panel A indicates the results of BEVS produced ARLBD (80 CPM) compared to β-galactosidase (Control) (100 CPM). Panel B compares the hERαLBD (752 CPM) and hERαLBD-f (802 CPM) produced in the baculovirus insect cell system against the control (241 CPM).

According to the manufacturer of SiMAG-IDA/Nickel, approximately 30 µg of histidine tagged protein can be immobilised to the surfaces of 1 mg particles. Assuming that the distribution of protein immobilised to the nanoparticles is the same as was determined by densitometric analysis of the SDS-PAGE separation of the collected recombinant containing fractions, and assuming that a total of 30 µg protein was sequestered to the particles, it follows that these radioligand assays were executed with 27.87 µg hERαLBD and 22.68 µg hERαLBD-f, respectively. With a tritium counting efficiency of 61.17%, the observed counts equate to 198.89 fmol ^{3}H-E_2 and 260.62 fmol ^{3}H-E_2 that could be bound, respectively, by 1 mg of the hERαLBD and hERαLBD-f forms of the human estrogen receptor derived proteins.

4.3 CONCLUSION

During this study the genome of a commercially available AcMNPV-derived insect pathogen was successfully altered, leading to the generation of three recombinant baculoviruses. The classical method of homologous DNA exchange between the baculovirus genome and a donor plasmid placed the genes encoding the histidine tagged LBDs of human nuclear receptors under the control of the very strong viral *polh* promoter. Good expression of the products from these genes were readily identified following initial creation of these viruses, with a majority complement of recombinant protein present within the soluble fractions of cell lysates. Similarly to what has been reported in literature for some constructs, the *T. ni* BTI-TN-5B1-4 cell line performed well in the expression of the hER-derived LBDs (469). Considering the yield obtained for the recombinant hERαLBD and hERαLBD-f constructs, 9.49% and 5.75% of the total protein complement, the purification of active LBD to the androgen receptor with the BEVS, as with the *E. coli* expression system, has to date proven difficult. Table 4.4 gives an indication of the theoretical amounts of recombinant human sex steroid LBDs that can be produced according to the protocols and conditions that have been employed during this study.

Table 4.4 **Comparison between the expression of histidine tag containing protein constructs of the human receptor LBDs towards androgenic and estrogenic hormones. Purification of the heterologous proteins was carried out by means of IMAC.**

	BEVS			Bacterial expression	
	hERαLBD	**hERαLBD-f**	**hARLBD**	**ARLBD**	**ERαLBD**
Total protein in 400 mL culture (mg)	741.84[†]	691.2[†]	636.24[†]	249.2	384.46
Protein applied to column (mg)	110.0	110.0	192.4	249.2	384.46
Concentration after purification (mg/mL)	6.42[℗]	5.58[℗]	1.03[℗]	0.75[‡]	0.559[‡]
Elution volume (mL)	1.75	1.50	3.00	4.00	1.50
Densitometric percentage	92.9	75.6	54.8	57.0	99.2
Total protein (mg)	10.44	6.33	1.69	1.71	0.832
Percentage of total protein complement	9.49	5.75	0.88	0.69	0.22
Theoretical quantity of recombinant protein obtainable from 400 mL culture (mg)	70.4	39.8	5.59	1.71	0.83

[†] Protein quantities inferred from 200 mL cultures infected with recombinant baculovirus at MOI > 10

[℗] Concentration of dialysate

[‡] Concentrations calculated as the average concentration of all fractions collected during GPC

For both the androgen and estrogen receptor constructs the BEVS yield considerably more recombinant protein per volume of culture. The production of human LBDs of the alpha estrogen receptor was particularly successful, for both the sole E domain, as well as the E/F construct. Although the radioligand retained by these forms of the receptor was less than anticipated, there was significant capture of ^{3}H-E_2 by the ERαLBD-derived proteins over that exhibited by the control. However, the inability of the ARLBD to sequester the tritiated ligand is a concern. Still further investigation into the binding capabilities of the heterologously produced LBDs is required. Nuclear receptors, especially the domains involved in ligand binding, are highly hydrophobic molecules. As with the bacterially produced receptor LBDs, solubility, especially during long term storage involving freezing and thawing cycles, becomes a

primary concern. Precipitation from solution is a frequent and problematic event which hampers analyses and biological applications following purification. Faster turn-around from purification to immobilisation onto solid surfaces for activity testing could possibly improve the results obtained thus far by inhibition of destabilising aggregation.

Comparatively, apart from a need for the development of sterile technique, the BEVS, as utilised in this project is easily implemented and does not require a level of skill far exceeding that needed for use of the bacterial expression system. Homologous recombination between linearised baculovirus DNA lacking the downstream essential *ORF1629* gene and a donor plasmid, carrying the missing sequence in addition to the transgene, can generate recombinant virions at nearly 100% efficiency. However, according to Kollewe and Vilcinskas (249) the partial deletion of *ORF1629* by restriction endonuclease inevitably leaves a small amount of undigested parental molecules behind. Such possible contamination should be addressed by plaque assay to assure that no contamination of viral stocks by intact parental viral particles has occurred.

Nevertheless, the purification of two variants of the human ERαLBD that could successfully bind a natural ligand to this receptor, while attached to a solid support via an incorporated affinity-tag, was achieved. Therefore, there is a possibility that these heterologously produced human-like proteins may be used to sequester molecules exhibiting estrogenic activity within aqueous solutions. The binding of such ligands to receptors may be investigated by a variety of means, including radioactive assays, SPR and immunohistochemical techniques. Consequently, availability of these proteins may be used for environmental remediation via the development of technologies capable of the facile detection or removal EDCs from aqueous solutions.

CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

The occurrence of synthetic chemicals in the aquatic, marine and terrestrial environments is of major concern in terms of global health. Current projections on population growth indicate that during the first decade and a half of the twenty first century more than one billion people will have been added to the total population. Increased access to primary health care, disease eradication programs and improved vaccines and pharmaceuticals will result in a global population of 7.1 billion people by 2015 (470). More people are surviving to childbearing age and beyond, thus raising the proportion of adolescent and elderly, nonworking people. Primarily this growth has been, and continues to be, in low- and middle income countries. The rapid addition of more people to a country can make it difficult to provide a concomitant rise in health and living standards whilst simultaneously emphasising increased environmental protection. According to the United Nations Population Fund "the eradication of poverty and the assurance of environmental sustainability are today's greatest challenges and are intrinsically linked to population dynamics" (471).

In essence, the more people there are on earth the greater the need for the provision of health care, housing, jobs, energy, tillable land, food and access to clean water. Resultantly, greater pressure is exerted on natural resources, ultimately leading to environmental deterioration. For example, farmable land is essential to sustain high rates of human population growth. Thus, in many parts of the world, especially in developing countries, deforestation and subsequent desertification may occur as more land is required for intense food producing agricultural practices. Deforestation leads to diminishment of the amount of evapotranspiration of water from soil and plants leading to a reduction in humidity, altering rainfall patterns and increasing water runoff to rivers and oceans (472), thereby promoting land demineralisation and erosion (473). Consequently, less soil nutrients and water are available in such areas for sustained agriculture. Water quality and availability is intrinsic to public health and greatly impacts on the sustainable development and economic progress of a country. Between 1990 and 2012, more than 2.3 billion people gained access to improved sources of drinking water (474). Nevertheless, within the developing world, where economic progress is limited, a variety of water related issues threatens the global gains formulated under the United Nations Millennium Development Goals (475) for the improvement of access to safer drinking water (476). Water quality assurance programs in third world countries are often circumscribed by a lack of scientific skill and knowledge, poor monitoring capabilities and inadequate regulation, resulting in the contamination of water supplies by industrial chemicals, pesticides and biological waste. Moreover, many persistent chemicals capable of disrupting the biochemical processes of the endocrine system are imported to developing countries as agricultural aids or agents for pathogen control (11).

The occurrence of EDCs and PPCPs within the environment is believed to be detrimental to public health and within industrialised countries trace level detection of synthetic biologically active chemicals have received much attention. Conversely, due to poor waste disposal practices and a lack of infrastructure to effectively test and treat contaminated water, people in developing countries are increasingly being exposed to pesticides and other EDCs. In light of the deleterious effects that EDCs may elicit, continual assessment of the environmental impact of chemical and pharmaceutical discharge at specific locations such as water and sewage treatment facilities, industrial zones and rural water access points, which may affect the lives of people and animals, is a necessity. Still, any action plan aimed at the successful management of EDCs or the formulation and implementation of strategies for environmental remediation (477–479) would ultimately require methods capable of rapid and cost-effective detection of problematic compounds. In contrast to the developing world, industrialised countries have access to the infrastructure and knowledge base needed to implement various analytical techniques for the monitoring of specific EDCs (41, 115, 116, 480). However, internationally agreed and validated testing methods capture only a limited range of the known spectrum of EDCs (102). There is therefore a growing need for the development of a rapid and economical EDC detection system that may be used *in situ* with a low demand for technical capabilities. Such a system must negate any necessity for specialised equipment and must be able to detect a range of different molecules without the need for complicated end-points or additional steps such as chemical derivitisation or the maintenance of test animals. Ideally, therefore, EDCs must be identified by their biological mode of action and not by specific molecular structures.

The first requirement in the design of such a novel system, capable of the simultaneous detection of a variety of chemicals which occur at low concentrations, is the ability to selectively sequester and concentrate molecules with analogous biological effects. Following the inception of IMAC in 1975 by Porath *et al.* (481) the availability of increasingly improved chelating agents, such as nitrilotriacetic acid (NTA), iminodiacetic acid (IDA) and carboxymethyl aspartate (CM-Asp) has, in conjunction with recombinant DNA technology, led to this technique becoming one of the most widely used forms of affinity chromatography available for the purification of proteins in research and industrial environments (482). However, the principles of IMAC also provide an easy yet effective mechanism for the facile immobilisation of recombinant proteins to a variety of stationary surfaces. Examples of surfaces to which recombinant oligohistidine-tagged proteins have been stably and functionally immobilised include, among others, glass coated with a NTA modified poly(ethylene glycol) polymer (483), paper (484) and mesoporous silica (485, 486) functionalised with surface-oxidised nickel nanoparticles, chloro-silane-functionalised magnetic nanoparticles (487), and nanoporous alumina membranes silanised with aminopropylsilane and IDA (488). The functional immobilisation of 6xHis-tagged nuclear receptor LBDs to a stationary contactor membrane surface could therefore present a means to selectively concentrate environmental EDCs, with affinity for the specific receptors, to readily detectable levels.

On the other hand, immunohistochemistry presents a possible means to detect EDCs captured by membrane-immobilised receptor LBDs. Antibodies are highly specific in their interaction with cognate

antigens. Following the development of the hybridoma methodology (489) in 1975 by Cézar Milstein and George J. F. Köhler, monoclonal antibodies (mAb's) have been extensively utilised in a variety of applications in the health, academic and commercial sectors. Vertebrates make billions of different antibodies, each with a binding site that recognises a specific region of a macromolecule. The hybridoma technique allows for the isolation and immortalisation of particular, highly specific antibodies. Via coordinated bonds to a defined epitope, mAb's are capable of detecting small changes in the molecular structures of proteins. For instance, mAb's have been used for the detection of single amino acid substitutions in the tyrosine kinase domain of the epidermal growth factor receptor which promote the proliferation of adenocarcinomas of the lung (22, 23 and references included within). Moreover, post-translational modifications of proteins have also been probed by means of phosphorylation-state (492) and acetylation-state specific (493) mAb's. Importantly, mAb's have been effectively used in the detection of conformational changes in the maize auxin-binding protein (494) and the progesterone receptor (495).

The ligand-induced conformational changes that occur within the LBDs of nuclear receptors have been well documented in multiple publications and have been discussed in chapter two of this study. By utilising mAb's that can distinguish between the ligand-bound and apo conformational states of receptor LBDs, an EDC detection kit may be assembled to detect a broad range of molecules based on receptor interaction (Figure 5.1). This system would be able to selectively concentrate EDCs with affinity to a specific class of membrane-immobilised receptor LBDs, such as the recombinant hARLBD or hERαLBD proteins. Binding of these conformation-indicative mAb's to liganded receptor LBDs may then be detected by secondary, enzyme-coupled antibodies via visualisation with standard colorimetric techniques.

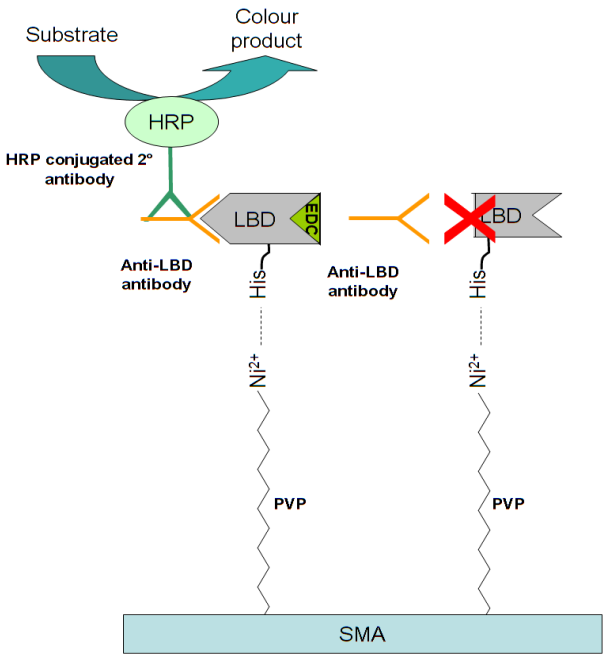

Figure 5.1 **Schematic representation of an affinity membrane based biosensor for the selective capturing, concentration and detection of EDCs using immobilized 6xHis-LBDs of steroid receptors and antibodies specific for the ligand bound form of the protein** (496).

Furthermore, the conformational changes that occur within the nuclear receptor LBD upon ligand binding may be exploited to detect specific classes of EDCs. For example, agonist binding to the estrogen receptor leads to the formation of the AF-2 groove on the surface of the LBD. As with nuclear box containing protein cofactors within the cell, monoclonal antibodies that have been raised to detect the resulting amino acid arrangement would only be able to coordinate with the LBD if the AF-2 is present. Conversely, antagonists would not be able to induce this conformation change, thus this system would selectively identify ER agonists. Weigel *et al.* (495) prepared a mAb (C-262) to a synthetic peptide containing 14 amino acids from the carboxy-terminal of the progesterone receptor (PR). This mAb was able to detect the PR when bound to the antagonist RU486. However, when bound to the agonists progesterone or R5020, C-262 was unable to recognise the PR, suggesting that the epitope present at the carboxy-terminus is unavailable to mAb interaction when in the agonist-induced conformation. Alternatively, other epitopes may be identified on LBD surfaces that only become accessible upon activation of the receptor, regardless of the nature of the ligand. Using surface plasmon resonance Kong *et al.* (497) describe the interaction of a biotinylated αII peptide (SGSGLTSRDFGSWYA) (498) with an ERαLBD construct. This peptide, while immobilised to a solid support, was able to interact with the ERαLBD when bound to both agonists (E_2, DES, genistein) and an AF-2 antagonist (OHT). Importantly, this study indicated that AF-2 was not required for αII binding, since a mutant LBD lacking H12 (ERαΔH12) retained binding capabilities to αII. Therefore, it may reasonably be argued that mAb's capable of identifying an epitope analogous to that recognised by αII would be able to non-selectively identify liganded ERαLBD in the presence of either agonists or antagonists. Moreover, several other such protein-protein interaction surfaces have also been identified (498–501).

The recombinant baculoviruses produced during this study, along with methods developed for the solubilisation of proteins of a hydrophobic nature, allow for high-level expression and purification of truncated forms of human nuclear receptors. Moreover, the incorporation of 6xHis-tags to the recombinant proteins not only facilitates directed purification of the receptor LBDs following synthesis in heterologous expression systems but will also be used for the facile immobilisation of these proteins to synthetic contactor surfaces by means of IMAC principles. Consequently, the immobilised LBDs will form the basis of a novel BBA based on the principles described above that is capable of the sequestration, concentration and indiscriminate detection of hormonal mimicking EDCs within environmental milieus. Due to the low skills requirement and economy of the proposed BBA, such EDC detection kits may provide beneficial insight into the health and safety aspects of drinking water in urban and rural areas alike, especially in parts of the developing world where scientific infrastructure is lacking.

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