BIOCHEMICAL AND MOLECULAR GENETIC STUDIES OF PATIENTS WITH ANDROGEN INSENSITIVITY SYNDROME

by

Denise D. Belsham

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For my family

To laugh is to risk appearing a fool
To weep is to risk appearing sentimental
To reach out for another is to risk involvement
To expose feelings is to risk exposing your true self
To place your ideas, your dreams before the world is to risk loss
To live is to risk dying
To hope is to risk despair
To try at all is to risk failure

But to risk we must...
Because the greatest hazard in life is to risk nothing

The person who risks nothing...
Does nothing,
Has nothing,
Is nothing.

- anonymous

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List of Abbreviations

A_{260} - absorbance at 260 nm; 1 A_{260} unit = 50 µg of DNA

ADP - adenosine-5'-diphosphate

AIS - androgen insensitivity syndrome

AR - androgen receptor

ATP - adenosine-5'-triphosphate

bis - N,N'-methylenebisacrylamide

bp - nucleotide base pairs

BSA - bovine serum albumin

CAIS - complete androgen insensitivity syndrome

cDNA - complementary deoxyribonucleic acid

CHAPS - (3-[(3-cholamidopropyl)-dimethylammoniol]1-propanesulfonate)

ddH_2O - deionized distilled water

DHT-BA - dihydrotestosterone bromoacetate

DNA - deoxyribonucleic acid

DTT - dithiothreitol

Eagle's MEM - Eagle's minimal essential medium

EDTA - (ethylenedinitrilo)tetraacetic acid

ER - estrogen receptor

GR - glucocorticoid receptor

GSF - genital skin fibroblasts

HEPES - (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid

HRE - hormone response/regulatory element

IEF - isoelectric focussing

kb - kilobase pairs

kDa - kilodaltons

LTR - long terminal repeat

MB - mibolerone

MR - mineralocorticoid receptor

mRNA - messenger ribonucleic acid

MT - methyltrienolone

PAGE - polyacrylamide gel electrophoresis

PAIS - partial androgen insensitivity syndrome

PCR - polymerase chain reaction

PEG - polyethylene glycol

pI - isoelectric point

PMSF - phenylmethylsulfonyl fluoride

RNA - ribonucleic acid

SBMA - spinal and bulbar muscular atrophy

SDS - sodium dodecyl sulfate

SSC - sodium citrate buffer solution

TAE - Tris acetate/EDTA buffer

TBE - Tris borate/EDTA buffer

TCA - trichloroacetic acid

TEMED - N,N,N',N'-tetramethylethylenediamine

Tfm - testicular feminization

Tris-HCl - tris(hydroxymethyl)aminomethane hydrochloride

2D - two dimensional

UTR - untranslated region

Abstract

Our understanding of the mechanism of androgen action in the development of normal males will be greatly facilitated by studying patients with natural mutations of the androgen receptor (AR). We have been able to identify the mutations in numerous individuals with AR defects by amplification of the eight exons of the AR gene using the polymerase chain reaction. These analyses immediately became valuable in carrier testing and genetic counselling of the extended families.

Of six kindreds with complete androgen insensitivity syndrome (AIS), the molecular basis of their disorder have been detected within the AR gene. Family 1 has a C→T mutation in exon 4 creating an *Msp*I site changing Leu676 to Pro, and Family 2 has an A→T mutation in exon 8 creating a *Mae*I site changing Lys882 to a translation termination signal. Of the other four families studied, two have a C→T mutation in exon 6 eliminating a *Kpn*I site changing Arg773 to Cys, while the other two families have a G→A mutation eliminating a *Sph*I site changing Arg773 to His. All of the mutations are within the steroid binding domain of the androgen receptor. A mutation has not yet been detected in the partial AIS Family 3, although exons 2 to 8 have all been sequenced and the size of the polyglutamine region in exon 1 of the AR gene is also within the normal range. Scatchard analysis indicates a decreased affinity of the receptor for androgen. We have also studied a kindred with spinal and bulbar muscular atrophy, a progressive, late-onset, neuromuscular disorder, thought to be caused by an AR gene defect. We have confirmed the finding that there is an increased number of CAG repeats in exon 1 of the AR gene. The affected allele in this family has 51 CAG repeats, whereas the normal allele has 23 repeats.

The 56 kDa protein with androgen binding activity, and altered expression in patients with AIS, has been further characterized. We now know that it is not the AR itself or a fragment thereof, it is not synthesized from the same gene as the AR, and the recent cloning indicates it is cytosolic aldehyde dehydrogenase 1. The role it plays in AIS is yet to be elucidated.

Summary of Contributions

Because some of the research presented in this thesis was a result of a collaborative effort between our laboratory and those of Drs. Pinsky and Faiman, I will outline my personal contributions to each section. I have attempted to accurately describe the work of others throughout the thesis body as well. The figure legends indicate if the work was done by someone else with the statement "with permission of", otherwise assume I have done the experiments.

Part III 2. Results and Discussion

A. Family 1 - All of the work presented was done in our laboratory by me.

B. Family 2 - Complete linkage analysis of the kindred with *DXS1* and *DXYS1*, specific androgen receptor binding activity, Southern analysis for a large deletion or gene alteration, and the Northern analysis were all done in our laboratory.

C. Family 3 - Specific androgen receptor binding activity, Scatchard analysis, linkage analysis with *DXS1* and *DXYS1*, Northern analysis, and mutational analysis were all done by me.

D. Other families - Southern analysis for a large deletion or gene rearrangement, Northern analysis, and confirmation/ discovery of the *Kpn*I mutation, were our contributions to this study.

E. SBMA kindred - All of the work described was done entirely by me.

Part IV 2. Results and Discussion

A. The comparison with other proteins was primarily done by me, except were indicated in the figures.

B. The confirmation of the complete deletion in the AIS patient was performed by me, while the study of the expression of the 56 kDa protein expression was done in our laboratory.

C. The additional studies, including both the analysis for the expression of the 56 kDa protein and the 56 kDa protein induction analysis, were all performed by me.

Part I

General Introduction

A. Preamble

The main reason to study a scientific problem, I am told, is the quest to uncover new knowledge. What is done with this new knowledge depends on the ingenuity of the investigator. Although science is a constantly changing field, the main purpose of research remains unchanged.

The scientist often has to refine the methods and direction taken on the quest due to limited funding. If we take a brief historical journey, we would find that often the scientists of the past have not always known the expected answers of their questions before they started their research project. A recent illustration of this type of investigation would be the unexpected finding that more than one protein may be produced from a single gene, which goes against the accepted dogma of "one gene-one protein". The research on the neurofibromatosois gene, which has three separate gene products encoded by one of the introns of the NF1 gene, is a good illustration (Roberts, 1990; Viskochil *et.al.,* 1990; Wallace *et.al.,* 1990). There are other genes with similar possibilities.

It seems a tragedy that the science of today is more often than not expected to be pre-meditated, that is, the results should have a conceived endpoint, and the actual science is merely a formality to reach this endpoint. Yet it is always reassuring to read of a discovery that has happened due to chance alone and was not part of an original plan.

The present study was originally intended to continue the study of an unusual observation made in our laboratory years ago. It is often described as a serendipitous discovery. Serendipity is the ability to make happy discoveries by accident, originating from the title of the fairy tale *The Three Princes of Serendip*, in which the heroes of the story were always making such discoveries (Heath, 1985). Although it was not considered mainstream science, the amount of knowledge gained by this particular quest, and the tangents we have followed in the process of adding to this knowledge base, have been significant.

The primary discovery, through serendipity, was that a novel 56 kDa protein was present in genital skin fibroblasts, but not in non-genital skin fibroblasts. This protein was found to have even greater significance, as it was absent in most patients with androgen insensitivity syndrome. Because the androgen receptor gene had not been cloned before this discovery was made, the complete characterization of this protein was the initial focus of our laboratory.

Our initial hypothesis was that this 56 kDa protein belonged to the same super gene family as that described as the steroid/ thyroid/ retinoic acid family of receptor proteins, due to its ability to specifically bind androgens. We did not believe that this protein was the androgen receptor itself, as was reported by another group working on what seemed to be the same protein (Kovacs, Turney, 1988). The identity of these two proteins was established and our hypothesis that our discovered protein was not the androgen receptor itself was further substantiated by acknowledging that it was too small, too abundant, but also not androgen induced due to the fact that it was found to be expressed normally in two patients with complete androgen insensitivity.

Although the other group working with this protein continued to believe that it was still the androgen receptor or a portion thereof, we still maintained that it was not. To encompass all of our findings, we then hypothesized that the 56 kDa protein was synthesized from the same gene as the androgen receptor by processes such as alternate splice sites, promoters or polyadenylation sites, even possibly by alternate strand synthesis. This hypothesis was proven false once a patient with a complete deletion of the androgen receptor gene was found to express our protein, albeit in reduced amounts.

By this time the androgen receptor had been cloned in 1988. This allowed the continuation of the studies of the three families with androgen insensitivity syndrome using the androgen receptor cDNA, as restriction fragment length polymorphism (RFLP) studies were already possible before this time utilizing centromeric X-chromosome probes for linkage analysis. The analysis of these families has led to the elucidation of the mutation

in the two families with complete androgen insensitivity. Family 1 has been found to have a point mutation in exon 4, encoding part of the hormone binding domain at a highly conserved site in the steroid receptor family. This mutation changes Leu676 to Pro via a single T to C transition mutation, creating a diagnostic *Msp*I restriction enzyme site. The second family was found to have a single point mutation (A to T) in exon 8, again coding for a part of the hormone binding domain, which changed Lys882 to an amber (UAG) translation termination signal. This mutation creates a recognition sequence for the restriction endonuclease *Mae*I, permitting the identification of carriers in this family as well.

The study of the partial androgen insensitivity family has not led to the identification of the causative mutation as of yet, but the study of this family has given some insight into the potential site of the mutation. Using Scatchard analysis, it was determined that reduced binding affinity of androgen to the receptor is present in the patients' genital skin fibroblasts. This would indicate a problem in the hormone binding domain of the androgen receptor and further studies may eventually lead to the disease causing mutation.

In a collaborative effort, it has also been possible to find a single amino acid codon which is mutated in four different families. This mutation has been proven to be the cause of androgen insensitivity in these families by expression analysis of the wild-type and mutant androgen receptor gene in transfected COS 1 cells. It is incredible that in such a short time span, the field of androgen receptor analysis has exploded so tremendously that we are at the point of actually proving the causative nature of a single base change in the androgen receptor gene.

Analysis of the androgen receptor gene itself has also been the topic of another recent finding, that being that an increase of CAG repeats in the polyglutamine region of the N-terminal of the androgen receptor gene may be the cause of an apparently unrelated disorder called X-linked spinal and bulbar muscular atrophy (La Spada *et.al.*, 1991). It has been possible to determine the carrier status and indicative disease status in females and

males, respectively, in a large kindred with this late-onset progressive disorder by analyzing this region of the androgen receptor gene. The implications of this finding are surprising, as different mutations in this single gene have now been shown to cause two completely unrelated disorders.

The last frontier that we must face in this study is the elucidation of the role of the 56 kDa protein in normal androgen action and if its absence in patients with androgen insensitivity syndrome has a distinct causative nature. While the above mentioned studies were taking place another student in our laboratory was able to clone the cDNA of the 56 kDa protein (Pereira *et.al.*, 1991). This protein was found to have complete identity with an aldehyde dehydrogenase, particularly cytosolic ALDH-1 (EC 1.2.1.3). We now must try to discover the biological function of this protein; furthermore we must discover why it has both androgen binding activity and aldehyde dehydrogenase activity and how these functions relate to the androgen insensitivity syndrome.

The study of the androgen insensitivity syndrome has been of immeasurable significance to the fields of both endocrinology and genetics due to its unusual characteristics. It is rare to find a disease in which the patients are completely changed by the effects of a single gene and yet the results are not lethal. The serendipitous discovery of the 56 kDa protein, which seems to play a role in this disorder, only makes the syndrome more fascinating to study. Combining the findings from both facets of this study has and will most likely continue to add to the ever expanding knowledge base of the androgen insensitivity syndromes.

The main focus of this thesis project consisted of biochemical and molecular genetic studies of two Manitoba families and numerous individuals with complete androgen insensitivity syndrome, and one family with partial androgen insensitivity syndrome. This has recently been expanded to include one family with X-linked spinal and bulbar muscular atrophy. The study of these patients included determining the possible functional receptor defects, the potential cause for the disease in the DNA sequence, and the

relationship or involvement of the 56 kDa protein, which was the protein discovered in our laboratory and is known to be altered in these patients. This thesis divides these components into two parts, initially the study of the androgen insensitivity syndrome, by investigating the androgen receptor gene, and then the study of the 56 kDa protein.

B. Central focus

The central focus of the thesis includes the full characterization of the families and patients with respect to two components involved in androgen insensitivity syndrome; those being the androgen receptor, mutations of which are known to be the direct cause of the disorder, and the 56 kDa protein, which seems to be secondarily involved.

Both of these components, the androgen receptor and the 56 kDa protein, will be studied at the DNA, RNA, and protein level with respect to structure and function, to further our understanding of the molecular basis of the disorder.

Part II

General Methods and Materials

(Note: the general methods section consists of routinely used methods, while supplementary methods specific to each individual results and discussion section are presented therein)

A. Patient material

Human genital skin fibroblasts from individual AIS patients were obtained from the Repository for Mutant Cell Strains, Montreal, and from the collection of Dr. L. Pinsky, Montreal, or by Drs. C.R. Greenberg and C. Faiman in Winnipeg, consisting of foreskin from normal circumcision specimens, labium majus skin, or skin from various non-genital regions donated with informed consent according to the protocols approved by the Ethics Committee of the Sir Mortimer B. Davis-Jewish General Hospital, Montreal and the Faculty of Medicine, the University of Manitoba, Winnipeg. The donors ranged in age from newborn infants to adults of 19 to 30 or 40 years.

Peripheral blood was collected from the patients and family members after informed consent was obtained. The description of the pedigrees and patients used in the studies are found in the appropriate results sections that follow.

B. Cell culture

Cells were grown to confluence in Human McCoy's 5A medium (Gibco, Grand Island, NY) supplemented with 10 % fetal calf serum and 100 μg/ml of penicillin G and streptomycin as described (Wrogemann *et.al.,* 1988). The cells were grown in 100 mm plates with medium changes twice per week or as needed. For storage of cell strains, the cells were trypsinized, washed in medium, then frozen in 1 ml medium with 8% DMSO.

C. Detection of the 56 kDa protein

(i) Protein preparation

The confluent dishes of fibroblasts were washed thrice with PBS at 37°C, then washed thrice with ice-cold 0.18 M ammonium bicarbonate (pH 7.4). The cells were harvested in 0.5 M ammonium bicarbonate with protease inhibitors (PMSF in isopropanol

1 mM, leupeptin 100 μM, pepstatin 10 μM), sonicated, then centrifuged at 100,000 x g for 20 min. The supernatants were either lyophilized or the proteins precipitated in a 10% TCA solution on ice for 30 min, spun in an Eppendorf centrifuge, then dried. The proteins were resuspended in CHAPS lysis buffer (for IEF/2D-polyacrylamide gel electrophoresis) or SDS sample buffer (for denaturing SDS-polyacrylamide gel electrophoresis). Protein estimations were performed using the Bio-rad protein assay estimation system (microassay procedure) according to the manufacturer's instructions (Richmond, CA). The reaction included 0.8 ml diluted protein sample with 0.2 ml Dye Reagent Concentrate. The absorbency was read at 595 nm spectrophotometrically versus a reagent blank. Bovine serum albumin was used as a protein standard (3-20 μg) and the reaction was linear to 20 μg of protein.

(ii) Specific labelling of the 56 kDa protein

Photoaffinity labelling of intact fibroblasts was performed using various concentrations of 17ß-hydroxy-[17α-methyl-^{3}H]estra-4,9,11-trien-3-one (methyltrienolone (MT)) (New England Nuclear, Boston, MS) and 7α-17α-dimethyl-[17α-methyl-^{3}H]19-nortestosterone (mibolerone (MB)) (Amersham, Arlington Heights, IL) to a specific activity of 85 Ci/mmol, as described (Wrogemann *et.al.,* 1988). Briefly, the plates were washed in medium buffered with 15 mM HEPES, incubated with 5-50 nM [^{3}H]androgen analog (2 ml/100 mm plate total volume) alone or with 1 μM (200 fold excess) for 2 hr at 37°C in the incubator. The medium was subsequently removed, and the plates were exposed to ultraviolet light at 295 nm for 5 min at room temperature to covalently link the bound analog. The protein sample was then processed as described above.

Affinity labelling with [^{3}H-1,2,4,5,6,7]dihydrotestosterone-17ß-bromoacetate (DHTBrAc) of intact fibroblasts was performed as described (Pereira *et.al.,* 1991). DHTBrAc was synthesized according to Kovacs and Turney (1988) and Kovacs *et.al.* (1989) based on Chang *et.al.* (1984), starting from [1,2,4,5,6,7,16,17-$^3H_8$]dihydrotestosterone (179.4 Ci/mmol, New England Nuclear, Boston, MS). The cells were incubated alone or with 1 μM (200 fold excess) of radioinert DHTBrAc for 2 hr at 37°C. The protein was then prepared as

described above.

(iii) Polyacrylamide gel electrophoresis

Denaturing SDS-gel electrophoresis was performed utilizing the method of Laemmli (1970). Isoelectric focussing (IEF) in preparation for 2D-PAGE was performed according to O'Farrell (1975) with the following modifications: (i) a mixture of 0.8% pH 5-7 ampholytes (LKB, Bromma, Sweden), 0.8% pH 6-8 ampholytes, 0.4% pH 3.5-10 ampholytes and 2% CHAPS (Perdew *et.al.,* 1983) was used in the gel; (ii) isoelectric focusing gels of 12.5 cm were formed in 17 cm long tubes (2.4 mm i.d.); (iii) about 15 or 50 μg (for silver stained or Coomasie stained gels respectively) or 80 μg (for immunoblotting) of protein were applied to each gel. IEF was carried out for 16 hr at 400 V followed by 1 hr at 800 V for a total of 7200 volt-h. IEF gels were equilibriated for 20 min in 10% glycerol, 3 mM dithiothreitol, 2.3% (wt/vol) SDS, 62.5 mM Tris-HCl pH 6.8. Gels for the second dimension were 14 x 13.5 cm and 0.75 mm thick with a gel acrylamide/bis-acrylamide concentration of 10%. The slabs were electrophoresed at constant current of 25 mA and a constant temperature of 17°C until the dye front reached the bottom of the plates. Rainbow molecular weight markers used were from Amersham (Arlington Heights, IL). The molecular weight standards included: phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa), which run at slightly higher molecular weights on gels due to the color detection additives.

(iv) Staining of the gels

Gels were routinely stained overnight with 0.05% Coomasie Brilliant blue R-250 (Bio-rad, Richmond, CA) dissolved in 50% ethanol containing 10% acetic acid and destaining in a solution of 50% methanol and 10% acetic acid. Silver staining was performed according to the method of Morrissey (1981). Briefly, the method was as follows: (i) gels were fixed in 7.5% acetic acid for 1 hr, then in 50% methanol overnight with one change; (ii) wash 3-4 x in water to remove remaining detergent before placing in reducing solution (0.5% DTT) for 30 min; (iii) add oxidizing solution (0.1% $AgNO_3$) for

30 min; (iv) wash twice with water, then add developer (3% Na_2CO_3 and 0.05% formaldehyde) until protein spots appear; (v) add 1% acetic acid to stop developing, then change to H_2O and dry onto filter paper.

(v) Fluorography

The gels were first stained with Coomasie blue as described for documentation, then destained in 50% methanol, 10% acetic acid overnight. The polyacrylamide gels were impregnated with scintillator by the method of Bonner and Laskey (1974) or by using Autofluor (National Diagnostics, Manville, NJ), dried and then exposed to preflashed Kodak XAR-5 films at -75°C. Immune blots were treated with EN^{3}HANCE spray (New England Nuclear, Boston, MS).

D. Western blotting

(i) Antiserum preparation

An antiserum to the abundant 56 kDa protein was prepared, by F. Pereira previous to the studies herein, using isolated 56 kDa protein material from two-dimensional gels. The protein spots were washed and homogenized in phosphate buffered saline and mixed to an emulsion with an equal volume of Freund's complete adjuvant (Calbiochem, San Diego, CA) to a final volume of 2 ml. Intradermal or subcutaneous inoculation were performed on a rabbit and blood collected later from an ear vein.

(ii) Immunoblotting

Samples were electrophoretically transferred to nitrocellulose paper (Micron Separations, Westboro, MT) using a transblotter (Hoeffer Scientific, San Francisco, CA) at a constant voltage of 80 V and a constant temperature of 4°C for 1 hr 15 min. The transblot buffer contained 25 mM Tris; 192 mM glycine; 0.1% SDS; 20% methanol. After air drying overnight, the filters were blocked with 5% albumin in TBST buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl and 0.05% Tween 20), then incubated with a 1:500 dilution of the antiserum in TBST buffer overnight at 4°C. Washes with TBST solution for 3X 5 min were performed between each step. Antigen bound antibodies were visualized using an

enzyme linked immunosorbent assay with alkaline phosphatase (Bio-rad, Richmond, CA) according to the manufacturer. Color development was done in 0.1 M Tris, pH 9.5; 0.1 M NaCl; 5 mM $MgCl_2$; with 0.02 mg/ml nitro blue tetrazolium and 0.01 mg/ml 5-bromo-4-chloro-3-indolyl phosphate.

E. Receptor binding assays

(i) Charcoal assay

Specific androgen binding activity in intact genital skin fibroblasts was performed essentially as described previously (Nickel *et.al.,* 1988; Belsham *et.al.,* 1989). Briefly, confluent monolayers were washed with serum-free medium and incubated for 2 hr. at 37°C in serum-free medium buffered to pH 7.4 with 15 mM Hepes containing 5 nM 7α-17α-dimethyl-[17α-methyl-^{3}H]19-nortestosterone (mibolerone) (75 Ci/mmol, Amersham) alone or together with 1 μM of the same radioinert androgen. After washing thrice with ice-cold 0.18 M ammonium bicarbonate, the cells were scraped and harvested in 0.5 M ammonium bicarbonate (with protease inhibitors), sonicated, centrifuged at 100,000 x g for 20 min., then the free counts were bound to charcoal and the binding determined (Kaufman *et.al.,* 1979). The radioactivity was assayed in 10 ml toluene solution containing Omnifluor (4 g/l, New England Nuclear, Boston, MS) and counted on a Beckman scintillation counter (Fullerton, CA).

(ii) Scatchard plot analysis

Scatchard plot analysis was performed according to the method of Kaufman *et.al.* (1990). Genital skin fibroblasts were grown to confluency in 60 mm plates with McCoy's medium supplemented with 10% fetal calf serum and 100 μg/ml of penicillin G and streptomycin. The above medium was removed and serum-free minimal essential medium (Hanks' salts) buffered to pH 7.4 with 15 mM HEPES was added to the cells 24 hr before the binding assay was performed. Replicate monolayers were incubated in the presence of 0.1 to 3.0 nM mibolerone (75 Ci/mM, Amersham) alone or with 1 μM of the same radioinert analog (to measure non-specific binding). After incubation for 2 hr at 37°C, the dishes were placed on a bed of ice, washed twice with 5 ml of 20 mM Tris-HCl (pH 7.4)

containing 0.15 M NaCl and 0.2% BSA and twice with the same buffer lacking BSA. Trypsin (0.1%) was added at room temperature for 5 min, then the cells were scraped, resuspended in Tris buffer lacking BSA and centrifuged (4°C) at 2000 g for 5 min. After washing in the same buffer, the cells were solubilized with 0.5 N NaOH (1.5 ml total volume), sampled for protein (Bio-rad protein assay system as previously described) and radioactivity, the latter in 10 ml of a toluene solution containing Omnifluor (4 g/l; New England Nuclear, Boston, MS). Specific binding (total minus non-specific) was plotted as a function of the free androgen concentration by the method of Scatchard (1949) and values of B_{max} and K_d were derived from lines fitted by linear regression.

F. Southern blot analysis

(i) DNA isolation

DNA was isolated from either peripheral blood or genital skin fibroblasts. Genomic DNA was isolated from peripheral blood according to the method of Greenberg *et.al.* (1987), which is essentially the method used at the Molecular Diagnostic Laboratory, Genetics Division (previous Director, Dr. D. Hoar; present Director, Dr. T.Garber), Alberta Children's Hospital in Calgary. Briefly, 7.5 ml of heparinized blood was incubated in 5 vol NH_4Cl:Tris at 37°C to separate white from red blood cells, then washed thrice in 37°C saline solution (0.85% NaCl). The white cells were lysed in 100 mM Tris; 40 mM EDTA; 0.2% SDS; 1M NaCl. The lysed mixture was TE saturated phenol extracted twice, then chloroform:isoamyl alcohol (24:1) extracted. The DNA was precipitated in 1/50 vol 5 M NaCl and an equal volume of isopropanol, condensed to a pellet, dried, and resuspended in low TE solution (10 mM Tris; 1 mM EDTA).

Genomic DNA was isolated from genital skin fibroblasts by the method described above with some modifications. Essentially, a total of 10 confluent 150 mm plates were washed twice with 0.85 % NaCl solution at 37 °C. The DNA was then isolated as described above after lysis directly on the plates.

(ii) DNA fractionation

Genomic DNA of approximate 20 µg aliquots to a 100 µl total volume was digested with various restriction endonucleases according to the manufacturer's guidelines. An estimation of the quantity of DNA per digest was performed by running 5 % of the sample on a 1 % agarose mini-gel in 1 % TAE buffer for 1 hr at 100 V. The gel was stained with a 0.5 µg/ml ethidium bromide solution and photographed for estimation of quantity by eye insepection and comparison to previous results for assurance of equal loading. The DNA was then precipitated with 0.1 M NaCl; 0.01% SDS; 2 vol absolute ethanol and placed at -70 °C for 20 min, spun in an Eppendorf centrifuge for 20 min, washed with 70% ethanol and vacuum centrifuge dried for 15 min. The sample was then resuspended in an appropriate amount of low TE buffer with gel-loading buffer (30% glycerol in water with 0.25% bromophenol blue), and equal amounts were loaded onto the large agarose gel (20 x 20 cm). The Southern agarose gel (1% agarose in 1% TAE buffer) was run according to the method found in Sambrook *et.al.* (1989) at 25 V for 30 min, then at 50 V for a total of 20-22 hr with circulating buffer. *Eco*RI/*Hind*III-cut lambda molecular weight markers were used (Promega, Madison, WI), which yield molecular weights of 21.226, 5.148, 4.973, 4.277, 3.530, 2.027, 1.904, 1.584, 1.330, 0.983, 0.831, and 0.125 kb. After the run, the gel was stained in 0.5 µg/ml ethidium bromide solution and photographed on Polaroid Type 57 film.

(iii) Southern blotting

The size-separated DNA was filter blotted essentially according to the original method by Southern (1975). Two types of filter paper were used for blotting: diazobenzyloxymethyl (DBM) paper prepared according to Alwine *et.al.* (1979) or Hybond-N membrane (Amersham, Arlington Heights, IL). The preparation of the gel for Hybond-N blotting entailed denaturation for 30 min (1.5 M NaCl; 0.5 N NaOH), then neutralization for 30 min (1.5 M NaCl; 1 M Tris-HCl, pH 7.2; 0.001 M Na_2EDTA). The transfer buffer was 10X SSC (1.5 M NaCl; 0.15 M Na citrate). After blotting, the DNA was covalently linked to the membrane by ultraviolet illumination, then air-dried overnight and baked for 2 hr at 80°C in a vacuum oven. The membranes were then placed in 20 ml of prehybridization solution at 37 °C overnight before hybridization with a DNA probe. Prehybridization

solution contained 50% deionized formamide; 4X SSPE; 1% SDS; 1% skim milk powder; 200 μg/ml salmon sperm DNA.

(iv) Membrane hybrydization

Probes used in the studies are described in the results section where appropriate. The probes were labelled by random priming (Feinberg, Vogelstein, 1983) to a specific activity greater than 5 x 10^8 cpm/μg. The probe was added to 9 ml of hybridization solution, which contained 47% deionized formamide; 10% dextran sulfate; 3X SSPE; 1% SDS; 0.5% skim milk powder; 200 μg/ml salmon sperm DNA, then placed with the membrane in a sealed polythene sandwich bag and incubated overnight at 37 °C. The membrane was washed with SDS/SSC solutions at the appropriate stringency (Sambrook *et.al.,* 1989), usually at 0.1% SDS; 0.1X SSC. The damp blot was then covered with a plastic cellophane "cling" wrap and exposed to Kodak X-OMAT film with a lightening-plus intensifying screen and placed at -70 °C. Developing was done in a X-OMAT X-ray film processor (National Imaging Ltd., Winnipeg, MB). The blot was stripped for subsequent usage in 0.4 N NaOH for 30 min, then neutralized in 0.1X SSC;0.1% SDS; 0.2 M Tris-HCl, pH 7.5.

G. Northern blot analysis

(i) RNA isolation

Poly (A+) RNA was isolated using the Fast Track mRNA Isolation Kit (Invitrogen Corporation, San Diego, CA) exactly according to the manufacturer's recommendations. The method allows the isolation of PolyA+ mRNA directly from cells or tissue, which are initially lysed in an SDS buffer containing RNase/Protein Degrader. The lysed mixture is applied directly to oligo (dT) cellulose for adsorption of only the mRNA. Then the DNA, the dissolved membranes, proteins and cell debris are washed off the resin with high salt buffer, the non-polyadenylated RNAs are washed off with a low salt buffer, and the mRNA is then eluted in the absense of salt. The method typically yields 10-85 μg per 1 x 10^8 cells grown in culture. 10 confluent 150 mm plates were used for each preparation.

(ii) RNA fractionation and blotting

The RNA was initially precipitated in 0.15 vol 2 M Na acetate; 2 vol absolute ethanol, then denatured in formamide/ formaldehyde solution in gel loading buffer consisting of 50% glycerol; 1 mM EDTA (pH 8.0); 0.25% bromophenol blue. Ten µg (approximately) of each sample was run on a 1 % agarose gel containing 2.2 M formaldehyde in 1X gel running buffer (5X = 0.2 M MOPS buffer; 50 mM Na acetate; 5 mM EDTA, pH 8.0) as described (Pereira *et.al.,* 1991). An estimation of the quantity of RNA for each sample was initially determined by running 5% of the RNA sample on a mini-Northern gel (a scaled down version of the Northern described above) and stained with a 0.5 µg/ml ethidium bromide solution. The amount of RNA to be loaded in each lane was estimated by eye inspection of the stained gel to be sure of equal loading. Ribosomal RNA, 28S and 18S from calf liver; 23S and 16S from E. coli MRE-600, were used as molecular weight markers (Pharmacia LKB Biotechnology, Baie d'Urfe, Quebec). The 20 cm^2 gel was then run for 30 min at 50 V and then at 35 V for 20 hr with circulating buffer. The gel was stained in a 0.5 µg/ml ethidium bromide solution and photographed with Type 57 high speed Polaroid film before transfer. The RNA was blotted onto Nitroplus 2000 hybridization membrane (Micron Separations Inc., Westboro, MA.) with a 10X SSC transfer buffer. The blot was air-dried, then dried at 80°C in a vacuum oven for 1 hr.

(iii) Hybridization

The probes were prepared by random priming exactly as described for Southern analysis. The hybridization, washing and exposure conditions were essentially the same as that for Southern blot analysis described above. The prehybridization/ hybridization solution contained 50% deionized formamide; 0.9 M NaCl; 50 mM $NaPO_4$, pH 6.5; 2mM EDTA; 2X Denhardt's solution; 200 µg/ml salmon sperm DNA; 0.1% SDS; 5% dextran sulfate. The blot, which was never allowed to dry, was placed in prehybridization solution overnight at 37°C before hybridization with the labelled probe. The full-length chicken ß-actin cDNA probe (Cleveland *et.al.,* 1980) was used as a control to estimate the equality of RNA loading in each lane.

H. Polymerase chain reaction

The PCR method was first described by Saiki (1988). The primers used to amplify the DNA are found in Table I. Each 100 µl PCR reaction contained PCR buffer (Perkin-Elmer Cetus, Norwalk, CT) containing 500 mM KCl; 100 mM Tris-HCl pH 8.3, 20 nmol of each dNTP, 100 pmol of each primer, 1 µg template DNA, and 2.5 units of Taq polymerase, covered with 100 µl of light mineral oil. The reaction was carried out cyclically with denaturation at 94 °C, annealing at 55-65°C, and extension at 72 °C, each for the times appropriate for the individual primer sets. After 25-35 cycles one final extension was done at 72°C. The PCR products were either analysed on an 8-12% polyacrylamide gel run with a Bio-rad mini-gel apparatus (Richmond, CA), 1.0-2.0% agarose gel, or subcloned and sequenced. Gels were run at 150 V for 45 min, then the gels were stained in a 0.5 µg/ml ethidium bromide solution and photographed with Type 57 high speed Polaroid film. The molecular weight markers were pGEM DNA markers (Promega, Madison, WI), prepared by digesting pGEM-3Z DNA to completion with *Hin*fI, *Rsa*I and *Sin*I, yielding a molecular range including sizes of 2.645, 1.605, 1.198, 0.676, 0.517, 0.460, 0.396, 0.350, 0.222, 0.179, 0.126, 0.075, 0.065, 0.051 and 0.036 kb. The PCR products that were to be subcloned and sequenced were first chloroform extracted, purified on a 2 % agarose gel and the isolated fragments spun through glass wool at 6000 rpm for 10 min. The product was purified by ethanol precipitation and blunt-end cloned into a Bluescript vector (Sambrook *et.al.,* 1989). Positive clones were prepared essentially according to the method by Hattori and Sakaki (1986), and double strand sequenced as described below.

I. DNA sequencing

Double strand sequencing was performed essentially according to the manufacturer's recommendations (Sequenase kit, United States Biochemical Corp.,Cleveland, OH) using the dideoxynucleotide method of Sanger *et.al.* (1977) Briefly, after a partially purified DNA template was prepared by elution through polyacrylamide gels or by the method described above, the DNA was alkaline denatured in 0.2 M NaOH for 5 min at RT, then neutralized in 1.4 M ammonium acetate, pH 7.4 and precipitated in 3

vol absolute ethanol (Hattori, Sakaki, 1986). After resuspending in low TE, the primer was annealed to the DNA and strand synthesis was initiated. The synthesis was terminated upon incorporation of a nucleotide analog which will not support continued DNA elongation (hence the name chain termination). The nucleotide analogs are 2',3'-dideoxynucleotide 5'-triphosphates (ddNTP's) lacking the 3'-OH group necessary for DNA chain elongation. The four reaction mixtures (G, A, T, C) were then run in separate lanes on a pre-run 8% denaturing polyacrylamide gel for 4-6 hr at 60 W. The sequence was prepared with [^{35}S]α-dATP. The gel was fixed with 5% acetic acid; 15% methanol 30 min before drying at 80°C under vacuum. The gel was exposed to Kodak XAR-5 film at RT overnight to 7 days.

Table I - Polymerase chain reaction primer sets for the androgen receptor gene: (A) exons 2-8, the 5' and 3' regulatory regions (from Trifiro *et.al.*, 1991a); (B) the CAG repeat region of exon 1 (from La Spada *et.al.*, 1991); and internal primers of the CAG repeat region of exon 1 (from Belsham *et.al.*, 1991 (submitted)).

	Region		Primers
A.	5'	a	5'-TGTACAGCACTGGAGCGCGTA-3'
		b	5'-GAGCCAGCTTGCTGGGAGA-3'
	EXON 2	a	5'-GTCATTTATGCCTGCAGGTT-3'
		b	5'-TCTCTCTCTGGAAGGTAAAG-3'
	EXON 3	a	5'-TCAGGTCTATCAACTCTTG-3'
		b	5'-GGAGAGAGGAAGGAGGAGGA-3'
	EXON 4	a	5'-ATTCAAGTCTCTCTTCCTTC-3'
		b	5'-GCGTTCACTAAATATGATCC-3'
	EXON 5	a	5'-GACTCAGACTTAGCTCAACC-3'
		b	5'-ATCACCACCAACCAGGTCTG-3'
	EXON 6	a	5'-CAATCAGAGACATTCCTCTGG-3'
		b	5'-AGTGGTCCTCTCTGAATCTC-3'
	EXON 7	a	5'-TGCTCCTTCGTGGGCATGCT-3'
		b	5'-TGGCTCTATCAGGCTGTTCTC-3'
	EXON 8	a	5'-ACCTCCTTGTCACCCTGT-3'
		b	5'-AAGGCACTGCAGAGGAGTA-3'
	3'	a	5'-CTATACTCTGCAGAGGTGAC-3'
		b	5'-TGTCACTGGGTTGTGGAG-3'
B.	EXON 1	a	5'-GCCTGTTGAACTCTTCTGAGC-3'
		b	5'-GCTGTGAAGGTTGCTGTTCCTC-3'
C.	EXON 1	a	5'-TGGAAGATCAGCCAAGCTC-3'
		b	5'-TTCCTCATCCAGGACCAGGT-3'

Part III

The Study of Androgen Insensitivity

1. Introduction

A. What is a normal male?

The mechanisms of androgen action in the development of the male phenotype during embryogenesis are specific (Jost, 1970); thus, normal sexual development consists of three sequential, ordered, and interrelated processes. Chromosomal sex is determined at fertilization, but the establishment of embryonic development does not begin until the eighth week of gestation, in order to determine the gonadal sex. The full mechanism for this process is still not completely understood, but it is known that the Y chromosome is responsible for the development of the indifferent gonad into a testis. Much work has been done in this area of research, which has seen the cloning of a 230 kb segment on the Y chromosome thought to contain the testis-determining gene (*TDF*) (Page *et.al.,* 1987). More recent findings have allowed this area to be further defined, and now it is thought to contain the *SRY* locus, a specific gene within the *TDF* region of the Y chromosome, known to be responsible for primary male development (Walter *et.al.,* 1991). Phenotypic sex refers to the anatomic features of the urogenital tract that characterize males and females, and is a direct consequence of the type of gonadal sex determined.

The development of the normal male phenotype depends ultimately on the endocrine secretions of the fetal testis, which will in turn convert internal and external genital anlagen into the male form, and determine the male sexual, behavioral, and functional characteristics (Griffin, Wilson, 1989). Under normal conditions the development of the sexual phenotype is as prescribed by the chromosomal sex, that is the male is the heterogametic sex (XY) and the female is the homogametic sex (XX). Alfred Jost concluded in the 1950's that phenotypic development in the absence of a gonad, either testis or ovary, is female.

In the case of male sexual development (reviewed in Griffin, Wilson, 1989), the internal genitalia result from the wolffian ducts, which give rise to the epididymides, vasa deferentia, and seminal vesicles, and the mullerian ducts disappear. The mullerian ducts,

which are formed secondarily to the wolffian ducts, give rise to the fallopian tubes, uterus, and upper vagina in the female. The external genitalia develop from common anlagen. In the male, the fusion and elongation of the genital folds cause formation of the penile urethra and shaft of the penis. The genital tubercle becomes the glans penis. The fusion of the urethral fold converts the genital swellings into the scrotum, while the urogenital sinus gives rise to the prostate and the prostatic urethra (George, Wilson, 1986).

Male development of the embryo is controlled primarily by three hormones. Mullerian-inhibiting substance causes regression of the mullerian ducts, preventing development of the female internal genitalia. Testosterone, the principal secretion of the fetal and adult testis, is responsible for the stimulation of the wolffian ducts, which develop into the male internal genitalia. Testosterone is irreversibly converted into dihydrotestosterone, the third necessary hormone for male virilization, by the enzyme 5α-reductase within the urogenital sinus and external genitalia (Siiteri, Wilson, 1974; George *et.al.,* 1987). Dihydrotestosterone is responsible for the development of the prostate and male external genitalia. The development of the male urogenital tract is complete by the end of the first trimester, but growth of the male external genitalia takes place later during gestation (Griffin, Wilson, 1987).

Testosterone not only serves as a precursor for its 5α-reduced metabolites, but may also be converted, in small quantities, to estrogens in the peripheral extraglandular tissues of both sexes, mainly in the adrenal cortex, but also in liver, fat and skin where there are low levels of the cytochrome P-450 aromatase responsible for transformation of androgens to estrogens (Figure 1). Estrogens can enhance or counteract androgen action, but also exert independent effects on cellular function. Normal androgen action in males requires an approximate 100-fold excess of androgens to estrogens in the circulation. Normal men form about 50 μg per day of estradiol and about 5 to 10 mg per day of testosterone (Wilson, 1987). Both testicular and adrenal androgens can act as precursors for estrogen formation, but testicular testosterone is the major source of dihydrotestosterone, which has plasma levels about one-tenth that of testosterone; therefore following castration or

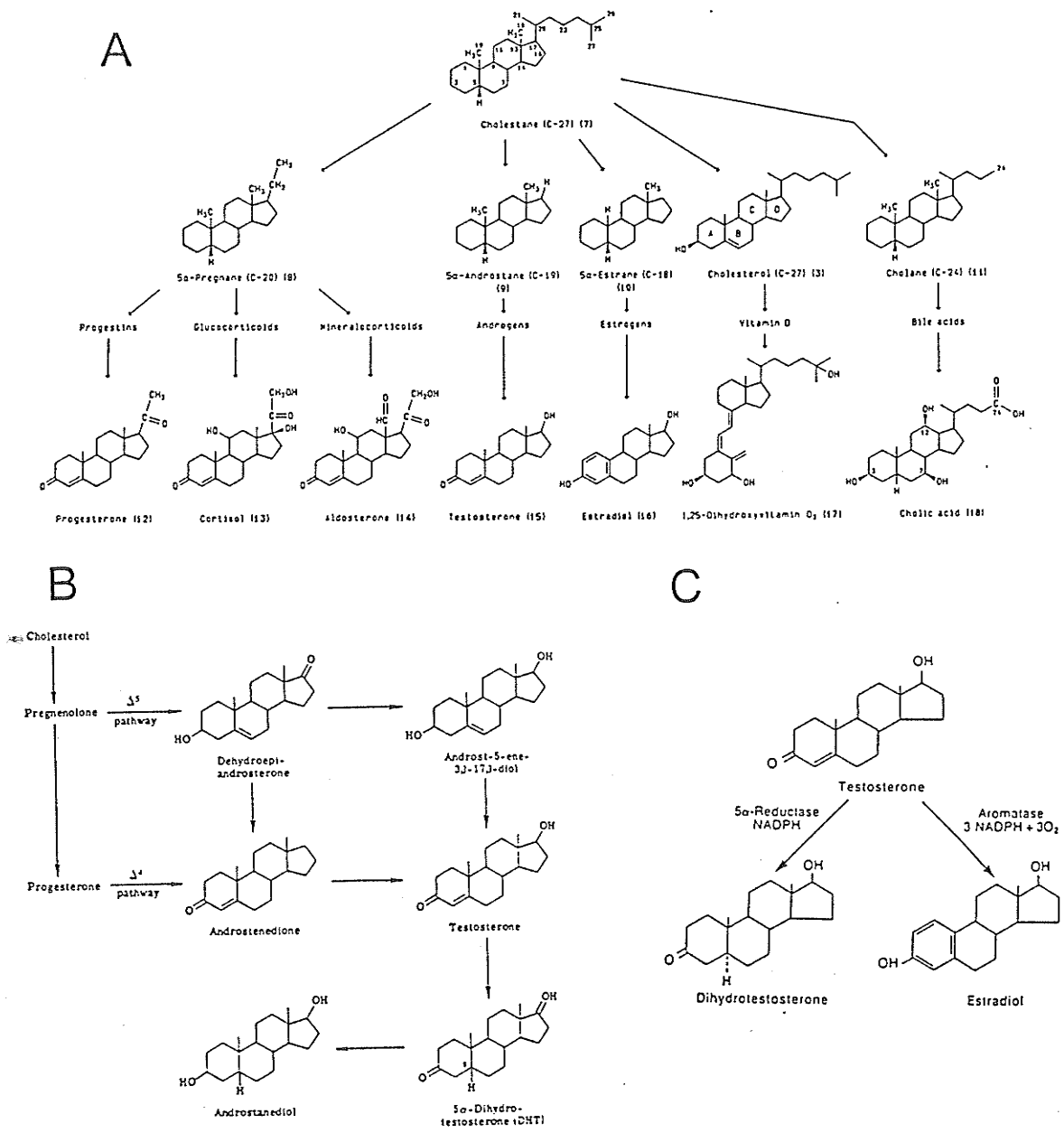


Figure 1 - (A) Family tree of seven principal classes of steroids (bottom row) which are structurally derived from the parent cholestane (top row) (from Hormones p. 54); (B) Metabolic pathway for the production of key androgenic steroids; in man the Δ^4 pathway is the major route of production of testosterone (from Hormones p. 494); (C) Principal hormones formed from testosterone by the testes and in peripheral tissues (from Griffin and Wilson, 1989 p. 1920).

testicular failure, sufficient estrogen can be produced to result in feminization, particularly gynecomastia, but due to the decreased dihydrotestosterone levels virilization is lost (Wilson *et.al.,* 1980). Thus, one notes that normal levels of these hormones are required not only to produce a normal male phenotype, but also to maintain this phenotype.

B. Mechanisms of androgen action in the normal male

The currently accepted theory for androgen action within target cells can be summarized by a reasonably brief statement. The androgen combines with its receptor protein and the receptor-hormone complex becomes anchored in the nucleus, attaches to chromatin, and promotes the transcription of genes through the formation of mRNA, ultimately resulting in increased protein synthesis (Evans, 1988). This can be illustrated (Figure 2, found later in thesis). Unfortunately (or fortunately for the researchers involved in studying this process), this is not nearly the entire story for the mechanism of androgen action. The process of androgen action, similar to all steroid hormone action, is constantly becoming more complex. A review of the most recent findings regarding steroid hormone action will be presented in a later section of this thesis. As for the action of androgen, there are differences from the other steroid hormones studied.

The two main differences between androgen action and other steroid hormone actions are: testosterone must be converted to dihydrotestosterone to fully exert its effects; and androgens also act during embryogenesis to promote differentiation of the tissues that serve as the main androgen target tissue in later life (Griffin, Wilson, 1989).

Testosterone circulates in the plasma bound to two proteins, the testosterone-binding globulin (TeBG, also called the sex-hormone-binding globulin, or SHBG) and albumin. The hormone-protein complex is in equilibrium with unbound or free hormone, comprising 1 to 3 percent of the total (Partridge, 1986). The unbound hormone is thought to enter the cell by passive diffusion, as this action is not energy-dependent and the concentration of androgen is lower in target tissues than in the plasma (Lasznitzki *et.al.,* 1974; Moore *et.al.,* 1979).

Both testosterone and dihydrotestosterone bind to the same high-affinity receptor protein, which was established by studies with mice carrying the *Tfm* mutation, synonymous to androgen insensitivity syndrome in the human (Goldstein, Wilson, 1972), and recently with human genital skin fibroblasts (Grino *et.al.,* 1990). The single gene mutation impairing the function of the receptor caused resistance to the action of both of the hormones. The receptor itself will be reviewed in a more complete form later. The receptor-androgen complex then undergoes a transformation process which allows it to bind DNA. In this DNA-binding state the complex is anchored to the nuclear compartment, where it attaches to chromatin. The testosterone-receptor complex is responsible for the regulation of the secretion of the gonadotropin luteinizing hormone (LH) and for the development of the external genitalia as discussed previously. The dihydrotestosterone-receptor complex is responsible for the development of the prostate and male external genitalia, but also mostly for the androgen-mediated events of male puberty, such as growth of facial and body hair, temporal hair recession, and maturation of the external genitalia (George, Wilson, 1986). Which of the two hormones is responsible for spermatogenesis and muscle growth at puberty has not yet been determined.

The reasons for the enhanced androgenic action of dihydrotestosterone over testosterone includes firstly the irreversible conversion by 5α-reductase, thereby no further metabolism is possible to neutralize or circumvent its androgen action; secondly testosterone binds less avidly to the receptor than does dihydrotestosterone; and thirdly the dihydrotestosterone-receptor complex is transformed more efficiently into the DNA-binding state (Wilson, 1987; Grino *et.al.,* 1990). How the various tissues recognize each complex for their different functions is not resolved, although relative concentrations of the two forms has been postulated to affect their different actions (Grino *et.al.,* 1990).

C. The disorders of sexual development: The androgen insensitivity (AIS) and related syndromes

Historically, the concept that hormone resistance at the cellular level could be due to endocrine dysfunction was first described by Albright *et.al.* (1942), when he deduced

that pseudohypoparathyroidism was due to an unresponsiveness to the parathyroid hormone. Clinical hormone insensitivity syndromes are now described for all classes of hormones except the estrogens (Verhoeven, Wilson, 1979). A form of male pseudohermaphroditism due to resistance to androgens was originally named testicular feminization syndrome because of the presence of a testis in a phenotypic female (Morris, 1953). It was also shown that true hormone resistance was indicated by the lack of virilization in an individual given exogenous androgens (Wilkins, 1957). Variants of this syndrome have been recognized, and it is now referred to as androgen insensitivity (AIS) or androgen resistance syndrome.

Disorders of sexual development can be caused by a disturbance of normal sexual differentiation at any stage of embryogenesis. These disorders can be caused by environmental insult (teratogenic drugs), nonfamilial abnormality of the sex chromosomes (such as Turner syndrome), developmental birth defect of multifactorial etiology (as in most cases of hypospadias), or a hereditary disorder resulting from a single-gene mutation (such as AIS) (Griffin, Wilson, 1989). There are at least 19 simply inherited disorders of sexual development recognized (Wilson, Goldstein, 1975). Mutations that impair androgen action appear to be quite common, and are often recognized as androgen action is essential for reproduction but not for the life of the individual. This is not the case for other hormones essential for life, such as insulin. The ascertainment bias, due to concern for reproductive capability, combined with X-linkage of the receptor, may indicate that these disorders are more common than in reality. Nevertheless, male infertility is common (about 6% (or more depending on presentation to a physician) of men in the United States), and if mutations of the androgen receptor account for a significant fraction of this disorder, then insensitivity to androgen action could prove to be one of the most common disorders of mankind (Griffin, Wilson, 1987).

The disorders of androgen insensitivity are classified as a disorder of phenotypic sex, resulting when anatomical development does not correspond to chromosomal and genetic sex, ie. the male embryo fails to virilize completely (often referred to as male

pseudohermaphroditism). Classification of these disorders may be under the persistent mullerian duct syndrome, deficiency of testosterone formation or the androgen insensitivity syndromes. Enhanced androgen action, on the other hand, does not have a clinically defined disorder, which differs from most other hormone systems in this way. A consequence of the enhancement of androgen metabolism and action in the peripheral tissues is a cause of two distinctive disorders in men, these being feminization due to increased extraglandular aromatization of androgen, and benign prostatic hyperplasia (Wilson, 1987).

Androgen insensitivity is probably responsible for most cases of male pseudohermaphroditism. Testosterone production and mullerian duct regression are normal in these individuals; thus, no female internal genitalia are present. Originally, individuals with these disorders were only recognized with defects severe enough to present as male pseudohermaphrodites with ambiguous genitalia, but since that time it has been accepted that anatomically normal men with this type of defect may present with idiopathic azoospermia or severe oligospermia, absence or severe deficiency of sperm production (Aiman, Griffin, 1982). The androgen insensitivity syndromes are now thought to encompass a wide spectrum of abnormalities ranging from completely phenotypic women with primary amenorrhea to phenotypically normal, infertile men (Griffin, Wilson, 1989; McPhaul *et.al.,* 1991b). Recently, normal, fertile men with signs of androgen insensitivity in somatic tissues, indicated by conditions such as gynecomastia and decreased axillary hair, have also been included in the androgen insensitivity group of disorders (Pinsky *et.al.,* 1990).

The molecular defects responsible for the androgen insensitivity syndromes can occur at any of the three major sites in the pathway of androgen action (Figure 2). These can be due to abnormalities in 5α-reductase, the androgen receptor, termed receptor-negative resistance, or in the subsequent phases of androgen action, termed receptor-positive resistance. Thus, there is considerable genetic heterogeneity in these disorders.

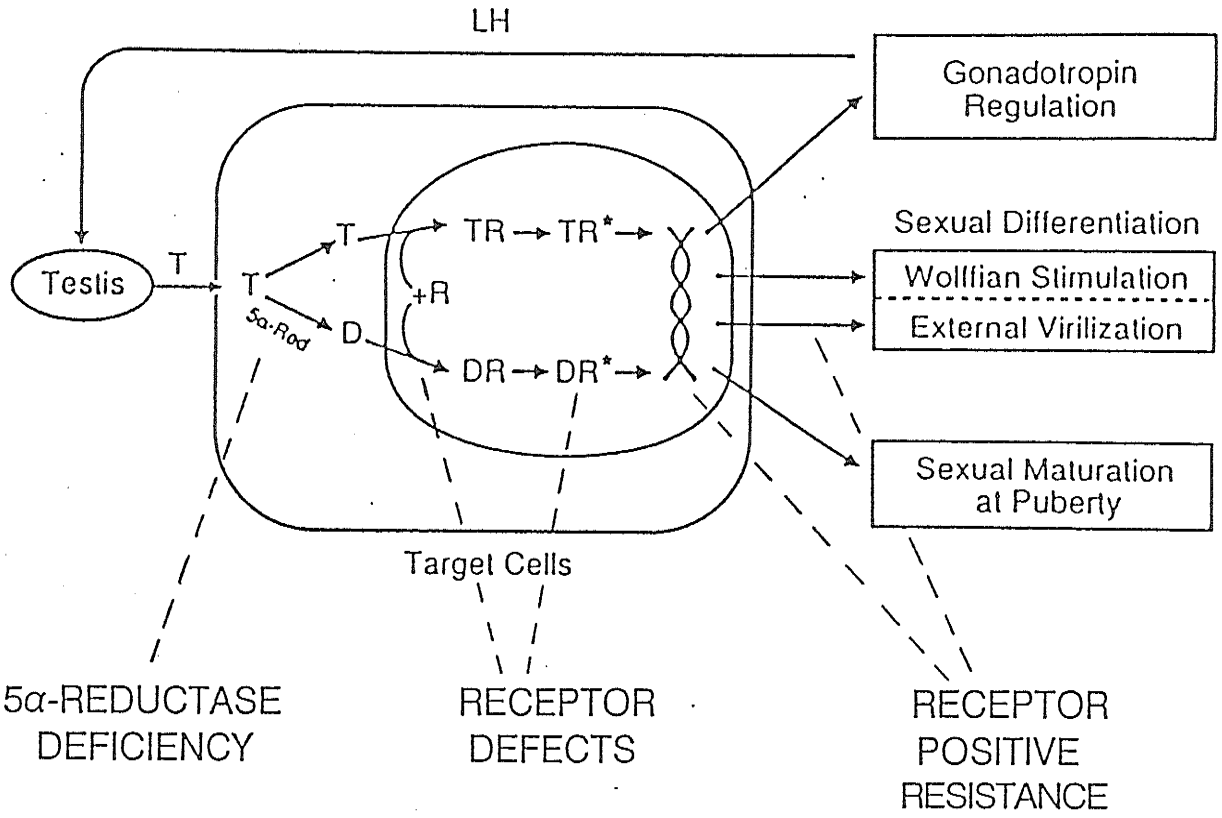


Figure 2 - Conceptual diagram for the mode of action of androgens within a target cell with the androgen receptor defects classified by the site of defect in androgen action. Major anomalies include 5α-reductase deficiency, receptor disorders, and receptor positive resistance (which may involve post-receptor events). (Modified from McPhaul *et.al.*, 1991b) T = testosterone; D = dihydrotestosterone; R = receptor; * = activated or transformed complex; LH = leutinizing hormone.

(i) 5α-reductase deficiency

The 5α-reductase deficiency syndrome, originally called pseudovaginal perineoscrotal hypospadias (Nowakowski, Lenz, 1961) and also called familial incomplete male pseudohermaphroditism type 2 (Walsh *et.al.,* 1974), is known to result from a deficient conversion of testosterone to dihydrotestosterone. The characteristic individual is a 46,XY male with normal testosterone levels and normal internal genitalia, but feminine external genitalia, so that the external phenotype is female. It is an autosomal recessive disorder. Affected individuals are usually raised as girls, but at the time of puberty often exhibit variable virilization of the external genitalia and develop normal male axillary and pubic hair; thus, a complete reversal of gender role may occur. The reason for the increased virilization at puberty compared to that in utero is unclear. Spermatogenesis is either profoundly impaired or absent. This disorder is more completely reviewed in Griffin and Wilson (1989), Imperato-McGinley *et.al.* (1974), or Imperato-McGinley *et.al.* (1979).

(ii) Receptor-positive androgen resistance

The term receptor-positive androgen resistance was first coined by Amrhein *et.al.* (1976) when describing a form of androgen resistance that did not seem to involve 5α-reductase or the androgen receptor. Subjects have been described with symptoms ranging from complete androgen insensitivity to infertile male syndrome, in other words covering the complete spectrum of abnormalities (Griffin, Wilson, 1989). The site of the molecular abnormality in these families is unclear, but in some the receptor may be qualitatively abnormal with a defect too subtle to detect with present analytical methods. In fact the family first described in the report above (Amrhein *et.al.,* 1976) was later found to have a qualitatively abnormal receptor (Brown *et.al.,* 1982); thus, had to be removed from the receptor-positive category. It is likely that the defects described in this category will be due to a heterogeneous group of molecular defects. In some, the defect may be due to events distal to the receptor in androgen action and would represent the true post-receptor disease, which would provide additional information on the action of androgens and phenotypic development.

(iii) Androgen insensitivity due to androgen receptor defects

Disorders of androgen receptor function have a wide variety of clinical manifestations (Griffin, Wilson, 1989; McPhaul *et.al.,* 1991b). These could include any of the androgen insensitivity syndromes, Reifenstein syndrome, and the infertile male syndrome. Since this thesis is mainly concerned with the complete and partial forms of androgen insensitivity due to defects of the androgen receptor, a more complete overview of these two disorders will be presented below, with a more general overview of the other two classes.

By pedigree analysis, it has been determined that the disorders of androgen receptor function indicate an X-linked recessive trait. Similar traits have been reported in the dog, cat, rat, mouse, horse, and chimpanzee (Griffin and Wilson, 1989 and references therein). In the mouse, X-linkage was established by standard mapping techniques (Lyon, Hawkes, 1970), and because there are no known instances, other than in marsupials (Sinclair *et.al.,* 1988), in which genes are X-linked in one species and autosomal in another, the inference of X-linkage was accepted. Furthermore, Meyer *et.al.* (1975) confirmed random inactivation of one X-linked allele, as would be predicted by the Lyon hypothesis (Lyon, 1972), by determining that some clones of skin fibroblasts from an obligate heterozygote had normal androgen binding, while others had deficient binding. X-linkage and localization on the X chromosome was formally established by Migeon *et.al.* (1981). Recently, the androgen receptor gene was mapped by somatic cell analysis and *in situ* hybridization to the X chromosome in both marsupial and monotreme species (Spencer *et.al.,* 1991).

It would be useful for genetic counselling if a means were available for diagnosing the obligate heterozygote before pregnancy (Griffin, Wilson, 1989). This is not easily done by phenotypic evaluation, although some heterozygous carriers have been reported to have diminished and uneven body hair (Nowakowski, Lenz, 1961), and sometimes have delayed menarche. It is theoretically possible to diagnose heterozygous carriers for this X-linked disorder by cloning skin fibroblasts, as was done by Meyer *et.al.* (1975) or more

recently by Elawady *et.al.* (1983), but such studies are rarely practical, time consuming, costly, and probably unreliable in carriers with skewed X-inactivation. It is not possible to diagnose fetuses at risk early in gestation, as the expression of the androgen receptor in normal amniotic cells is too variable (Sultan *et.al.,* 1984). However, by ultrasound, the absence of the male external genitalia in a 46,XY fetus can be determined at 20 weeks gestation (Stephens, 1984). It would therefore be beneficial to have a molecular diagnostic test to determine the carrier status of any individuals at risk for this disorder.

(a) Complete androgen insensitivity syndrome

The syndrome of complete androgen insensitivity was first described in 1937 by Pattersson and Bonnier. They deduced by family studies that the affected individuals were genetic males, the mode of inheritance was either X-linked or a sex-limited autosomal mode of transmission, and the cause was a developmental problem during embryogenesis resulting in a female phenotype. A review of 79 reported cases which fit the syndrome with an additional two new cases was published by Morris in 1953 in which he introduced the term testicular feminization.

Patients are seen by a physician because of either an apparent inguinal hernia, at prepuberty, or primary amenorrhea, postpubertal. The phenotype is that of a normal female, except axillary and pubic hair are diminished or absent. Breast development and distribution of body fat are typically feminine, and the external genitalia are unambiguously female. The internal genitalia are absent, although there are usually gonads that have histologic features of undescended testes, which are found in the abdomen, the inguinal canal, or in the labia majora (Griffin, Wilson, 1989). The cryptorchid testes of affected individuals often develop tumors (Morris, 1953; Hauser, 1963; Dewhurst *et.al.,* 1971; O'Connell *et.al.,* 1973). The vagina, if present, is blind-ending. A summary of the clinical features of this disorder is found in Table II (A).

The affected individuals are genetic males, 46,XY, and the resistance to both

Table II - Clinical features of: (A) complete androgen insensitivity syndrome; (B) partial androgen insensitivity syndrome; and (C) Reifenstein syndrome. (From Griffin and Wilson, 1989)

A

External phenotype: Female external genitalia with underdevelopment of the labia and a blind-ending vagina, female habitus and breast development, paucity of axillary and pubic hair
Urogenital tract: Testes that may be intraabdominal, along the course of the inguinal canal, or in the labia; absent Wolffian and müllerian derivatives
Karyotype: 46,XY
Inheritance: X-linked recessive
Endocrinology:
Testosterone: Normal or high male plasma levels and production rates
Estrogen: Plasma levels and production rates higher than in normal men
Gonadotropin: Elevated plasma LH levels
Pathogenesis: Complete resistance to all actions of testosterone and dihydrotestosterone

B

External phenotype: Clitoromegaly and partial fusion of the labioscrotal folds, female habitus and breast development, normal axillary and pubic hair
Urogenital tract: Testes that may or may not be cryptorchid, Wolffian duct derivatives emptying into the vagina, no müllerian duct derivatives
Karyotype: 46,XY
Inheritance: X-linked recessive
Endocrinology:
Testosterone: Normal or high male plasma levels and production rates
Estrogen: Plasma levels and production rates higher than in normal men
Gonadotropin: Elevated plasma LH level
Pathogenesis: Partial resistance to the actions of testosterone and dihydrotestosterone

C

External phenotype: Usually a male with perineoscrotal hypospadias, normal axillary and pubic hair but scant beard and body hair; breast enlargement at time of expected puberty
Urogenital tract: Testes which are often cryptorchid, Wolffian duct structures varying in the degree of male development, no müllerian duct derivatives
Karyotype: 46,XY
Inheritance: X-linked recessive
Endocrinology:
Testosterone: Normal or high male plasma levels and production rates
Estrogen: Plasma levels and production rates higher than those in normal men
Gonadotropin: Elevated plasma LH levels
Pathogenesis: Variable resistance to the action of testosterone and dihydrotestosterone

exogenous and endogenous androgens is virtually absolute in the complete form of the disorder. The only differences noticed from phenotypic women is that these subjects tend to be taller than average (Varrela *et.al.,* 1984) and their tooth size is as large as in normal men (larger than normal women) (Alvesalo, Varrela, 1980), indicating the involvement of the Y chromosome in these parameters. Intelligence is normal (Masica *et.al.,* 1969), and gender role is feminine in respect to behavior, outlook, and maternal instincts (Money *et.al.,* 1968).

Family analysis often points to other family members with similar symptoms, but about one third of subjects have negative family histories and are therefore thought to represent new mutations of this X-linked disorder, as affected individuals cannot reproduce (Griffin, Wilson, 1989). Estimates of the incidence vary from 1 in 20,000 to 1 in 64,000 male births (Hauser, 1963). It is the third most common cause of primary amenorrhea, after gonadal dysgenesis and congenital absence of the vagina (Ross, 1985).

Individuals with complete androgen insensitivity syndrome are always raised as females. Appropriate hormone treatment is prescribed and counselling is often recommended. The most serious complication of this disorder is the development of tumors in the undescended testes (Morris, 1953; Hauser, 1963; Dewhurst *et.al.,* 1971; O'Connell *et.al.,* 1973). Approximately 1 in 64 undescended testes becomes malignant; thus, it is accepted that the testes is removed. The time of surgery is disputed. Most physicians agree that removal of the tissue should take place after secondary sexual maturation is complete, due to the normal pubertal spurt in growth and feminization at this time (Griffin, Wilson, 1989). If the removal of the testes is required prior to puberty, then estrogen therapy is required at the appropriate age to ensure normal growth and breast development.

(b) Partial androgen insensitivity

Partial androgen insensitivity syndrome, or incomplete testicular feminization as termed by Prader (1957) and by Morris and Mahesh (1963), is used to describe a

syndrome which is similar to the complete form, but associated with partial virilization of the external genitalia and partial virilization, as well as feminization at puberty. The definition of the disorder is not as clearly defined as in complete androgen insensitivity and is often used to describe a wide variety of incomplete male pseudohermaphoditism manifestations. Nevertheless, it is possible to recognize a distinct phenotype among most of these subjects, which is associated with defects of the androgen receptor (Rosenfield *et.al.,* 1971). A summary of the clinical features of this disorder is found in Table II (B).

The affected individuals often have a female appearance, but are genetically male, 46,XY. The frequency of this disorder is unknown, but it is thought to be about one-tenth as common as the complete form (Griffin, Wilson, 1989). Again, as in the complete form, these patients present with primary amenorrhea or with undescended testes thought to be an inguinal hernia. The external genitalia are distinct as the labioscrotal folds are partially fused, and clitoromegaly is common. The vagina is short and ends blindly. The presence of wolffian duct structures and partial virilization differentiates this disorder from the complete form (Rosenfield *et.al.,* 1971; Madden *et.al.,* 1975). Certain features of this disorder resemble complete androgen insensitivity syndrome, some resemble 5α-reductase deficiency, while others resemble Reifenstein syndrome. Because affected individuals have varying degrees of abnormal external genitalia, a diagnosis is often made in the newborn or quite early in life. The assignment of gender is critical to the normal psychological development of the subject and should be done as early as possible. Depending on the decision, appropriate hormonal replacement or supplementation is often required and suitable counselling, either psychological or genetic, is often recommended (Griffin, Wilson, 1989).

(c) The Reifenstein syndrome

The Reifenstein syndrome is a term applied to a variety of X-linked disorders of incomplete male pseudohermaphroditism, which are generally less severe than in partial androgen insensitivity syndrome (Reifenstein, 1947; Bowen *et.al.,* 1965; Lubs *et.al.,* 1959). Each phenotypic presentation was originally thought to be due to a distinct entity, but now

it is believed that all of the syndromes represent a single gene mutation due to the extensive evaluations of familial cases from very large pedigrees in which the various members had varying degrees of expression (Ford, 1941; Walker *et.al.,* 1970). The predominant phenotype is male, often with perineoscrotal hypospadias and gynecomastia, but a wide amount of variation is often seen. A summary of the clinical characteristics is found in Table II (C). Again, for a more complete review see Griffin and Wilson (1989).

(d) The infertile male syndrome

The infertile male syndrome refers to a large number of men who are phenotypically normal, therefore not termed male pseudohermaphrodites, but are infertile. This problem has occasionally been found to be due to a defective receptor in men with negative family histories (Aiman, Griffin, 1982), but more often in men with more severely affected relatives (Aiman *et.al.,* 1979). The prevalence of this form of androgen insensitivity as a cause of infertility has not been established, but it may account for a significant fraction of male infertility associated with idiopathic azoospermia or severe oligospermia (Aiman, Griffin, 1982; Morrow *et.al.,* 1987).

D. The nature of androgen action: The androgen receptor and its family

(i) The steroid/ thyroid/ retinoic acid supergene family

(a) The hormones

Hormones have been classically placed into three categories, which include steroids, polypeptides, and amino acid and fatty acid-derived compounds, based on their chemical structures. Since that time, the hormones have been placed into three major groups based not on their structures or on the nature of their receptors but as endocrine, paracrine, and autocrine in the order of decreasing effective distances. Recently, a relatively new concept of "intracrine" regulation has also been suggested to include those hormones that are both synthesized and utilized by the same cell (Figure 3) (O'Malley, 1989). The steroids, which we are considering, are an exclusive product of the endocrine glands, where they are synthesized and stored until an appropriate signal occurs for

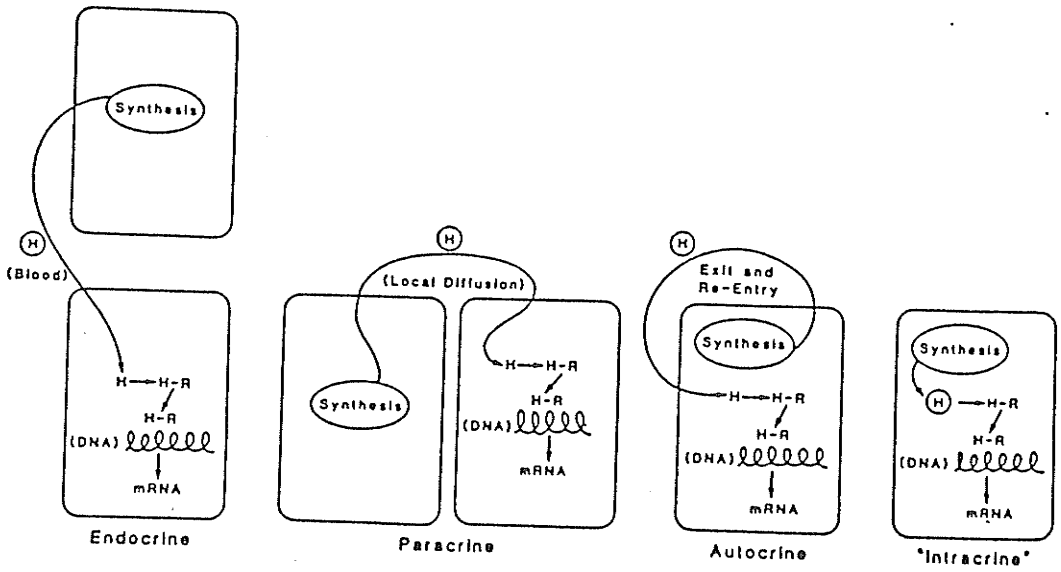


Figure 3 - System variations in hormonal regulation of endocrine target cells, including endocrine, paracrine, autocrine and intracrine regulation (from O'Malley, 1989).

release into the bloodstream. Once in the bloodstream, the steroid hormone travels to distant cellular targets to recognize the appropriate high-affinity receptors located either in the cellular nucleus or cytoplasm (Norman, Litwack, 1987).

The function of steroid hormones was studied early in medical history. Kendall was the first to crystallize the thyroid hormone, and ten years later with Reichstein completed the structural analysis of cortisol, eventually leading to the conclusion that all steroid hormones are derived from cholesterol (Figure 1) (Evans, 1988). For a complete review of the detailed chemistry, metabolism, and biosynthesis of steroids see Chapter 2 of Hormones (Norman, Litwack, 1987). Well over 225 naturally occurring steroids have been isolated and chemically characterized. The three major classes of steroid hormones are the adrenal steroids, the sex steroids, and vitamin D_3 (Evans, 1988). Each of these classes has its own diverse and complex actions, and each molecule has been shown to be important to specific areas of development and physiology.

The adrenal steroids are mainly involved in body homeostasis, including glycogen and mineral metabolism, and the stress response influencing the immune and nervous systems, as well as involvement in growth and differentiation *in vitro*. The sex steroids are involved in sex determination, fetal development, and after birth, control growth, sexual maturation and reproductive behavior, as was discussed in Part III (1 (A)) of this thesis. Vitamin D_3 is required for calcium metabolism and bone differentiation essential for normal bone development. If any of these molecules is abnormally produced, a number of clinical disorders may occur (Evans, 1988), which include Addison's disease (adrenocortical insufficiency), Cushing's disease (hypercortisolism), diabetes (insulin deficiency), goiter (thyroid hormone synthesis defect), rickets and osteomalacia (vitamin D deficiency), feminization (androgen/estrogen imbalance) among many others (Norman, Litwack, 1987).

The discovery of the mechanism of action of these small molecules came about after the preparation of radioactively labelled ligands became feasible. These are now routinely used to label receptors after receptor-hormone complex formation. The hormones

were deduced to bind to a receptor and in turn associate with high affinity binding sites on chromatin. This action resulted in a change in transcription of a limited number of genes, estimated to be between 50 and 100 (Evans, 1988).

(b) The receptor family members

The isolation and characterization of steroid hormone receptors proved to be difficult at first as these molecules are present in only trace amounts in the cell (10^3-10^4 per cell) (Carson-Jurica *et.al.,* 1990). The production of high affinity synthetic analogs of the ligands made the job much easier and first allowed the isolation of the glucocorticoid receptor (Yamamoto, 1985). Presently the receptors for the steroids: glucocorticoid, androgen, estrogen, progesterone, and mineralocorticoid, and the sterol, 1,25-dihydroxyvitamin D_3, have been identified and cloned using standard methods (Walters, 1985; Yamamoto, 1985; Ringold, 1985; de Boer *et.al.,* 1986; Haussler, 1986).

The viral oncogene erb A had significant homology to the known steroid receptor segments; and unexpectedly it was eventually found to be the gene for the cellular thyroid hormone receptor upon characterization of the gene product (Weinberger *et.al.,* 1986; Sap *et.al.,* 1986). There are two similar, but separate, thyroid hormone receptors, known as T_3R_β and T_3R_α.

Receptors for nonsteroidal ligands have since been added to the initial family of steroid receptors due to their similar structures. This led to the proposal that there is a large gene family, called the steroid/ thyroid/ retinoic acid supergene family, whose products are ligand-responsive transcription factors (Evans, 1988). These include molecules such as the E75 gene product from *Drosophilia*, postulated to be the receptor for the insect steroid ecdysone (Evans, 1988); estrogen-related receptors, ERR1 and ERR2, whose ligands are yet unknown, but were identified with the estrogen receptor DNA-binding region under low stringency hybridization conditions (Giguere *et.al.,* 1988); the receptor for the vitamin A-related metabolite retinoic acid (Petkovich *et.al.,* 1987; Giguere *et.al.,* 1987); a receptor, related to the retinoic acid receptor, thought to be involved in the

etiology of hepatocellular carcinoma, called HAP (Dejean *et.al.,* 1986; de The *et.al.,* 1987); and the chicken ovalbumin upstream promoter-transcription factor, named COUP, was also found to be a member of the family (Wang *et.al.,* 1989; Wang *et.al.,* 1987).

The evolution of the members of the supergene family has been postulated to have begun a "thousand million years ago in very primitive organisms" (O'Malley, 1989). O'Malley (1989) has suggested that primitive members of the receptor family, termed regulators, were intracellular receptors, whose ligands were environmental nutritional agents or metabolic substrates. The action of these receptors apparently took place within the cell exclusively for the survival of the organism. Eventually the genes for these regulators were duplicated within the genome, creating a family of receptors, each with its own unique functional specificity. The steroid hormone receptors can further be divided into two groups, which most likely diverged at some intermediate point in the evolutionary scheme with the development of the endocrine system. The first includes glucocorticoid, progesterone, androgen, and mineralocorticoid receptors, while the second would include receptors for estrogen, thyroid hormone, retinoic acid, and vitamin D_3 (Beato, 1989). A question that has been considered is if the more primitive forms of the receptors are still active in cells. The discovery of "orphan receptors", with more than 10 published without any known ligand (Giguere *et.al.,* 1988; O'Malley, 1990), may lead to the answer to this question. Work with the COUP-TF protein (Wang *et.al.,* 1987) indicates that it is either active constitutively, without any required ligand or that it is regulated intracrinely by a ligand produced within the cell in which it is synthesized. Because of the evolutionary postulates mentioned above, the latter explanation would seem possible. The possibility of an intracrine regulatory system is predicted to add much excitement to the future of molecular endocrinology (O'Malley, 1990; O'Malley, 1989).

(c) Steroid receptor-hormone interaction

The high affinity binding of the hormone to the receptor is necessary for the elucidation of specific gene transcriptional activation (Green, Chambon, 1986). In target cells, the steroid enters the cell by passive diffusion and travels to the nucleus where the

binding occurs. Receptors are synthesized on cytoplasmic ribosomes from single mRNAs, probably on the outer nuclear membrane allowing the translocation or transfer to the nucleus during or shortly after synthesis (Tuohimaa *et.al.,* 1988). The mechanism for this translocation may occur by diffusion or by interaction of the protein with nuclear pores, mediated by a signal sequence on the protein (Dingwall, Laskey, 1986). The exclusion limit of the pores is 67 kDa (Paine *et.al.,* 1975), but the individual shape of the receptors may still allow this type of transfer. The translocation sequences, with homology to other known nuclear translocation signals, have been found on the C-terminal side of the DNA-binding domain of all receptors (Figure 4).

The intracellular localization of the receptors is not yet fully understood, although this aspect of receptor research has been studied extensively, nevertheless it is believed that unoccupied receptors for progesterone, estrogen and androgen are found in the nucleus (Perrot-Applanat *et.al.,* 1985; King, Green, 1984; Husmann *et.al.,* 1990). The glucocorticoid receptor is an exception to this postulate and is found in the cytosol (Wilkstrom *et.al.,* 1987).

The complete understanding of the relationship of receptor binding with the action of hormones requires an accurate measurement of the kinetics of this interaction. The biological response to steroid hormones is a saturable phenomenon, since the number of receptor binding sites is finite. This is demonstrated by measuring subsequent bound and/or free steroid. Unfortunately this is more complex due to the existence of more than one class of binding sites for a given steroid. The specific ligand exhibits high affinity binding kinetics for its receptor, appropriate for the low levels of circulating hormones (Clark, Peck, 1979). This high affinity binding does not allow interference from other signals; thus, steroid specificity is achieved.

The kinetics of steroid receptor-hormone interactions can be viewed as simple monomolecular reactions defined by:

$$[H] + [R] \leftrightarrow [RH]$$

"Nuclear Location" Domains of Transactivators

SV40 T Antigen		P	K	K	K	R	K	V	
GR	^{491}R	K	T	K	K	K	I	K
MR	^{673}R	K	S	K	K	L	G	K
AR	^{628}R	K	L	K	K	L	G	N
PR	^{637}R	K	F	K	K	F	N	K
ER	^{256}R	K	D	R	R	G	G	R
T3Rβ	^{179}K	R	L	A	K	R	K	L
RAR	^{162}R	K	A	H	Q	E	T	F
VDR	^{102}R	K	R	E	M	I	L	L

Figure 4 - SV 40 T antigen nuclear transfer signal sequence and homologous sequences from human steroid receptors (from Carson-Jurica *et.al.*, 1990). Numbers indicate the amino acid position in the receptor sequence.

where H is the free hormone, R is the unoccupied receptor, and RH is the receptor-hormone complex (Norman, Litwack, 1987). Assuming simple Michaelis-Menten kinetics, the equilibrium can be expressed as

$$K_a = [RH]/[H][R] = k_{+1}/k_{-1} = 1/K_d$$

where K_a refers to the equilibrium constant of the reaction components and the second expression is the ratio of the forward and reverse rate constants, k_{+1} is the on-rate and k_{-1} is the off-rate. The receptor should be saturated by 20-fold the K_d (dissociation constant), the concentration at which 50% of the receptors are occupied. To measure specific binding of the receptor to the ligand, one must calculate total binding minus nonspecific binding.

The parameters defined by equilibrium constant equation are best determined by Scatchard plot analysis (Scatchard, 1949). The results are obtained by plotting bound hormone (x axis) versus bound/free hormone (y axis). The Scatchard plot conforms to the equation:

$$[RH]/[H] = -K_d[RH] + nK_d$$

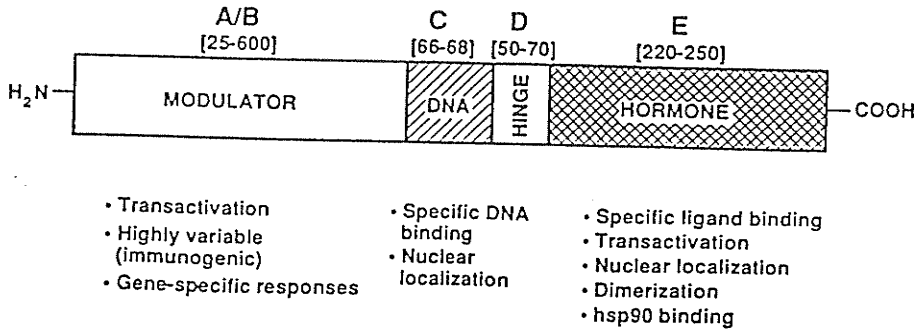
$$(y = m x + b)$$

which is a straight line equation for a single class of binding sites. The X-intercept represents the number of receptor binding sites (n), where the slope is $-1/K_d$. From this analysis one can determine differences in binding affinity or the total number of binding sites available. Although the physical parameters of hormone-receptor binding can become more complex than this brief description, a more complete overview will not be attempted here and for a more complete review see Chapter 1 of Hormones (Norman, Litwack, 1987) or Laboratory Methods Manual for Hormone Action and Molecular Endocrinology (Clark *et.al.,* 1988).

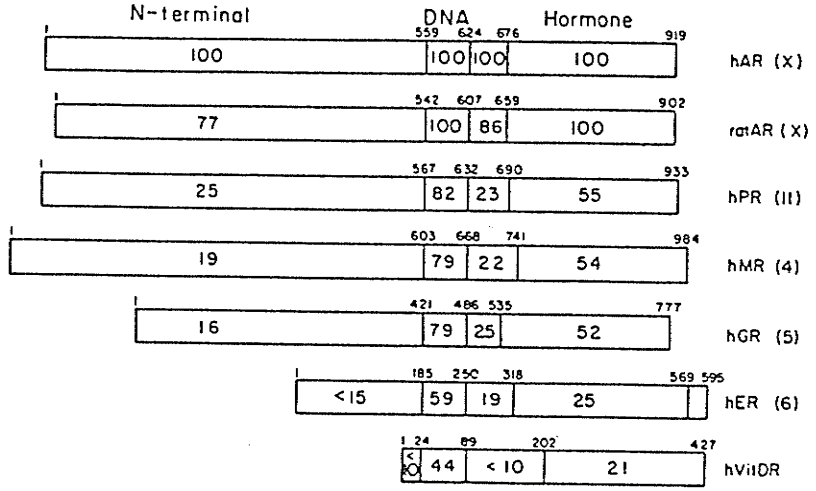
(d) Steroid receptor structure

The most striking feature of this family is the similarity of the structural organization of the receptor molecules (Figure 5(A)), although the action of each of these molecules is distinct and extremely diverse. The similarities are based on the amino acid sequence,

A



B



C

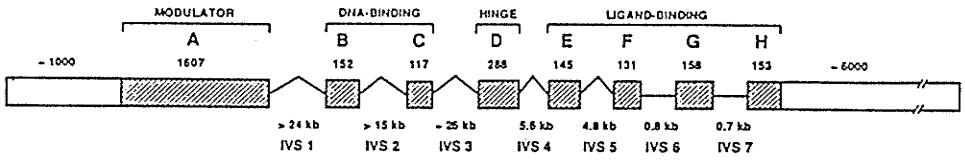


Figure 5 - (A) Structure of a hormone receptor protein, including the proposed function of each domain (from Janne and Shan, 1991); (B) Amino acid homology comparisons among nuclear receptor proteins (from Lubahn *et.al.*, 1988a). Indicated are the percent sequence homologies with the hAR. Note the sequence differs from Chang *et.al.*, 1988a by 2 amino acids due to the poly(amino acid) region repeat differences. (C) The genomic structure of the androgen receptor gene. Exons numbered as A-H, also referred to as 1-8 by other authors. The exon sizes are presented as base pairs, while the intervening sequences (IVS) are kilobase pairs. (From Janne and Shan, 1991)

which reveals three conserved regions. The DNA-binding domain is the most highly conserved, followed by the hormone-binding domain. The N-terminal domain is a hypervariable region that varies in both length and amino acid composition.

The first most highly conserved region is part of the DNA binding domain of the receptors, including a 66 amino acid sequence. In particular, there are nine cysteine residues of which eight are thought to form two zinc fingers (Figure 6), which are responsible for the physical binding of the receptors to DNA (Freedman *et.al.,* 1988). Each finger binds a zinc ion that is tetrahedrally coordinated by the cysteine residues. The steroid receptor family is distinguished from other transcription factors by the presence of the "two cysteine:two cysteine" subtype of zinc fingers, where each zinc ion forms a tetrahedral coordination complex with cysteine residues only. The other factors, such as the amphibian transcription factor TF IIIA, have the "two cysteine:two histidine" subtype for the coordination of the zinc ion (Green, Chambon, 1988; Evans, Hollenberg, 1988).

The first finger, consisting of four cysteines and several hydrophobic amino acids determines hormone responsive element (HRE) specificity. The significance of the HRE's will be further discussed later. The second finger is thought to be involved in protein-protein interactions, such as receptor dimerization (Evans, 1988; Green, Chambon, 1988). It has five cysteine residues along with several basic residues. With more complex mutational analysis, the number and type of amino acid residues found in the fingers and the number located between the two fingers has also been found to be important for the gene activation specificity of the receptor molecules (Berg, 1989; Evans, 1988; Umesono, Evans, 1989). Replacing the DNA-binding region of one receptor, such as the hER, with another such as the hGR, has resulted in a hybrid receptor molecule with the predicted switch in DNA-binding specificity (Green, Chambon, 1987). This technology, called the finger swap, has been exploited to characterize novel hormone receptors, such as that of the retinoic acid receptor (Giguere *et.al.,* 1987).

The next most highly conserved region of the receptors is the hormone-binding

The Zinc Finger Regions of Steroid Receptors

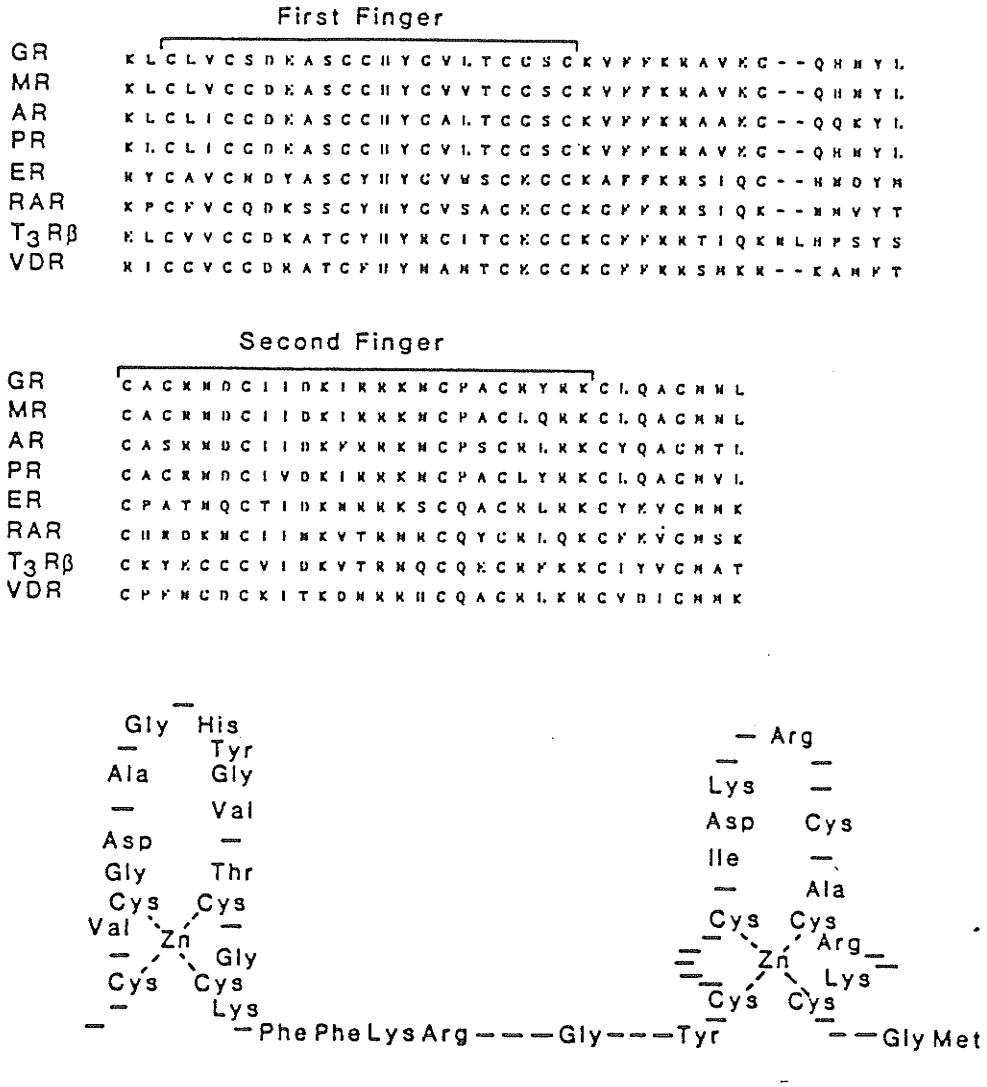


Figure 6 - Amino acid sequence of the C1 region of several steroid receptors and proposed finger structure. The proposed zinc finger region structure indicates the most highly conserved amino acids; dashes indicate variable amino acids. (From Carson-Jurica *et.al.*, 1990).

domain. There are two separate stretches of amino acid residues in this region, consisting of approximately 42 and 22 amino acids respectively, that are homologous among the different receptors (Figure 5(B)). These regions are known to be hydrophobic and are likely involved directly in hormone-binding, protein-protein interactions or transcriptional activation, although the specific mode of action is unclear. Mutational analysis of this region indicates that the entire region must remain intact for high affinity ligand binding, indicating that most mutations in this region would result in a loss of specific binding activity (Carson-Jurica *et.al.,* 1990).

Using artificial expression systems, it is possible to study structure-function relationships. This type of research has been gaining popularity recently and several studies using transient and stable transfection into cultured cells have allowed definition of the functional domains of numerous receptor molecules (Carson-Jurica *et.al.,* 1990). Expression of receptor fragments in *Escherichia coli* has allowed a detailed structural analysis (Carson-Jurica *et.al.,* 1990; Dahlman *et.al.,* 1989; Lin *et.al.,* 1990; Power *et.al.,* 1990; Tsai *et.al.,* 1989; De Vos *et.al.,* 1991; Young *et.al.,* 1990b), which will eventually lead to insights into receptor higher order structure and the interaction with other proteins and DNA.

(e) Steroid receptor function

In the past, the mechanism of action of the steroid hormones had been loosely postulated to occur as a consequence of the association of an appropriate hormonal ligand with its receptor, resulting in a biological response. These hormones bind to their receptors and in turn control gene networks that exert their effects on growth, development and cell function. The function of steroid receptors has been elucidated and the classical model of steroid hormone action is best summarized by Evans (1988) where it is proposed that binding of the ligand to the receptor induces an allosteric change allowing the complex to bind to its DNA responsive element in the promoter region of a target gene. This is the most basic understanding of receptor function and further research indicates that the process is much more complex than first postulated.

Steroid receptors only exert their function after receiving a signal upon binding of the hormone. DNA-binding and transcriptional activation do not occur if transformation of the receptor by either hormone-binding or artificial activation is absent. However, if the C-terminal portion of the receptor is deleted constitutive expression of target genes occurs, which indicates that the hormone-binding domain acts as a repressor of gene activation without the presence of hormone (Godowski *et.al.,* 1987). The mechanism by which a receptor is then activated is through a structural conformation change. There are also proteins known to be complexed with the inactive receptors, such as heat shock protein 90 (hsp90) (Bresnick *et.al.,* 1989; Picard *et.al.,* 1988; Binart *et.al.,* 1989; Joab *et.al.,* 1984), which are thought to play a role in maintaining the inactive receptor in a conformation suitable for hormone binding.

Although the hormones are specific for their individual receptors, there is crossreactivity between the various steroids with receptors. The specificity is dictated by the affinity of the receptor for the hormone and in some cases by tissue specificity of receptor expression or intracellular enzymatic conversion of the hormone to a more responsive form, such as for the androgen receptor. The actual requirement of the hormone beyond the initial activation event is unclear, although it seems to prevent reassociation of the receptor with hsp90 or any other proteins found in the inactive complex (Groyer *et.al.,* 1987; Tai *et.al.,* 1986; Koutsilieris *et.al.,* 1988). Another proposal for the function of the hormone is involvement in receptor dimerization (Kumar, Chambon, 1988).

The DNA-binding domain, particularly the zinc fingers interact with specific gene sequences in the promoter region, called the hormone responsive or regulatory elements (HRE) (Green, Chambon, 1988). The HREs are cis-acting and enhancer-like, as they function in an orientation and position-independent manner (Chandler *et.al.,* 1983). The HREs are palindromic, binding receptor dimers, and are 13 to 15 bp in length, however as little as two base changes in an HRE can change a glucocorticoid responsive gene to an estrogen responsive gene (Jensen *et.al.,* 1968), and altering single bases can alter

receptor binding and destroy the response to hormone (Gorski *et.al.,* 1968). The consensus HRE 5'-AGGN$_X$CAN$_{(0-3)}$TGN$_Y$CCT-3' matches most of those described (Martinez, Wahli, 1991). There are two subfamilies of HREs depending upon the nucleotide sequence. For instance, if N_X of the consensus sequence above is T and N_Y is A, then the HRE belongs to the estrogen responsive element subfamily. Meanwhile if N_X and N_Y are A and T, respectively, then the HRE belongs to the glucocorticoid/progesterone responsive element family (Klock *et.al.,* 1987). The third class of HREs are those that bind the thyroid receptor, which are identical to the estrogen HRE except the three bp at the centre of the dyad axis of symmetry are lacking (Glass *et.al.,* 1988). A more complete review of the mutagenesis experiments and the analysis of the importance of each nucleotide in the HRE consensus sequence can be found in Umesono and Evans (1989), Wahli and Martini (1991), Beato (1989), and Carson-Jurica *et.al.* (1990).

The genes that contain these HRE sequences in their regulatory regions have also been studied in some detail and some are reviewed (Beato, 1989; Carson-Jurica *et.al.,* 1990; Beato *et.al.,* 1989), especially in relation to the glucocorticoid receptor, the first to be studied extensively. The technology used to study the function of the HREs includes protein-DNA binding studies and gene transfer experiments of mutated HREs linked to reporter genes.

The genes can be positively regulated, negatively regulated, or repressed by steroid hormones. Genes found to be positively regulated include the mouse mammary tumor virus (Ringold, 1979; Scheidereit *et.al.,* 1983; Payvar *et.al.,* 1983; Chalepakis *et.al.,* 1988), the human metallothionein II_A gene (Karin *et.al.,* 1984), the chicken lysozyme gene (Moen, Palmiter, 1980; Renkawitz *et.al.,* 1984), the vitellogenin genes (Burch *et.al.,* 1988; Martinez *et.al.,* 1987; Scheidereit *et.al.,* 1985), the growth hormone genes (Slater *et.al.,* 1985; Moore *et.al.,* 1985), the Moloney murine sarcoma virus (Miksicek *et.al.,* 1986), the rabbit uteroglobin gene (Bailly *et.al.,* 1983), the rat tyrosine aminotransferase gene (Jantzen *et.al.,* 1987), the rat tryptophan oxygenase gene (Danesch *et.al.,* 1987), and the rat acidic glycoprotein gene (Klein *et.al.,* 1987). Of course this is only a relatively small

representative group of genes regulated by steroid hormones, and the number will undoubtedly increase as the mechanisms of gene regulation are elucidated.

Steroid receptors not only induce gene expression, but are also known to repress the expression of certain genes. Negatively regulated genes include the gene for the α-subunit of the glycoprotein hormones (Jameson *et.al.,* 1987; Akerblom *et.al.,* 1988), the rat prolactin gene (Sakai *et.al.,* 1988), and the proopiomelanocortin gene (Drouin *et.al.,* 1987). There are other genes also found to be negatively regulated by steroids, but the sequences involved in the regulation have not yet been defined. The consensus sequence for negative regulation versus positive regulation has not yet been clarified, but the differences in the nucleotide sequence may be responsible for the opposite effect seen in the various genes mentioned above.

The N-terminal region of the steroid receptors is the least conserved and is likely another necessary component of each receptor's specific functionality. The total length is highly variable. The epitopes of most antibodies to the receptors have been prepared against this hypervariable region (Carson-Jurica *et.al.,* 1990). The exact function of this region is not known, but a few speculations have been proposed. It is known to be necessary for function as deletion analysis indicates reduced activity of the receptor (Hollenberg *et.al.,* 1987). Another speculated functional activity includes involvement in transactivation of the receptor, particularly transcriptional modulation through DNA-binding and thus, ultimately target gene activation (Beato, 1989; Evans, 1988). The transcriptional modulation may occur through aggregate charge and higher order structure. The recent finding of the importance of the length of the CAG repeat region in the N-terminal of the androgen receptor may lead to more concise clues to the function of this domain (La Spada *et.al.,* 1991).

There are still a few questions that must be considered when studying hormone receptors. The role of chromatin organization should be considered in the future, as the experiments to date have mainly been done *in vitro* with naked DNA. The preliminary

studies indicate that the precise nucleosomal organization is essential for hormonal regulation (Beato, 1989; Richard-Foy, Hager, 1987; Beato, 1987; Beato *et.al.,* 1989). It is suspected that the information required for nucleosome phasing may be found in the HRE region, and that perhaps the nucleosome acts as a "repressor" of transcription by preventing other transcription factors from binding to the promoter region (Beato, 1989). Furthermore, post-translational modification of hormone receptors should be important in the functionality of the molecules. In particular, phosphorylation of the receptors is thought to be necessary for steroid binding, but may also play a role in activation, chromatin binding and recycling of the molecules (Auricchio, 1989; Beato, 1989). The regulation of the expression of the receptor genes and the precise mechanism(s) determining specificity of function of the numerous members of the family are other expanding areas of research bound to contribute to this ever expanding knowledge base of steroid hormone action.

(ii) The androgen receptor

(a) Preliminary findings

One of the members of the steroid receptor supergene family that had eluded cloning and sequencing of its cDNA was the androgen receptor. Before 1988, the study of this receptor molecule depended mainly on the knowledge that it was located on the X chromosome and RFLP studies were possible. Initially it was reported that the androgen receptor was X-linked and was homologous to the *tfm* locus in the mouse (Meyer *et.al.,* 1975). Since that time, Migeon *et.al.* (1981) narrowed the localization of the androgen receptor locus on the X chromosome to the pericentromeric region between Xq13 and Xp11, proximal to the locus for phosphoglycerate kinase (PGK). Linkage analysis with RFLPs in families with AIS was possible with the three cloned DNA sequences from the centromeric region and proximal long arm of the X chromosome, p8, pDP34 and S9 which define the chromosomal segments *DXS1*, *DXYS1* and *DXS17* respectively (Wieacker *et.al.,* 1987) or with *PGK*1 (Imperato-McGinley *et.al.,* 1990). The genetic linkage analysis with these markers has added to the lod scores known for androgen insensitivity syndrome. Lod scores represent the log 10 of the ratio of the likelihood of linkage to the androgen receptor gene, at various recombination fractions (ϕ), relative to the likelihood of non-

linkage (ϕ = 0.50). In general, a lod score of +3 indicates the odds of linkage are 1000:1, predicting linkage, whereas a score of -2 is 1:100 and may exclude linkage. Published maximum lod scores for *DXS1* are 3.5 (ϕ = 0) (Wieacker *et.al.,* 1987) and 3.2 (ϕ = 0.06) (Imperato-McGinley *et.al.,* 1990), while the maximum lod score for *DXYS1* is 0.76 (ϕ = 0) (Wieacker *et.al.,* 1987). Because the gene was not yet identified, the families could not yet be analyzed for molecular defects at the gene level; thus, this was the most convenient method for carrier testing. Recently, an 18-locus linkage map of the pericentromeric region of the human X chromosome has been reported which presents the order of the loci around the androgen receptor gene (Figure 7) (Mahtani *et.al.,* 1991).

Many biochemical studies involving the androgen receptor were reported in the past and a few significant features should be mentioned at this point. The androgen receptor had resisted purification to homogeneity longer than any other steroid receptor, probably due to the high proteolytic activity in androgen responsive tissues. The molecular weight of the androgen receptor was initially estimated to be 60,000 when it was isolated from steer seminal vesicle (Chang *et.al.,* 1982). This may have represented the 56 kDa protein discovered and characterized in our laboratory, which will be discussed in detail later in this thesis. Since that time, the androgen receptor was also isolated and characterized in rat ventral prostate with a molecular mass of 86,000 kDa (Chang *et.al.,* 1983; Tindall *et.al.,* 1984b), rat uterine cytosol with a molecular mass of 98,000 kDa (Chang, Tindall, 1983) and 120,000 kDa in hyperplastic human prostate (Murthy *et.al.,* 1984). A compilation of all the estimated molecular weights, which include those mentioned above and many others ranging from 25,000 to 167,000 kDa, are found in Johnson *et.al.* (1987), where the common size was estimated to be approximately 110,000 to 120,000 kDa. Finally, the androgen receptor was purified to near homogeneity recently from calf uterus and human genital skin fibroblasts, and was reported to be a 110 kDa protein (van Loon *et.al.,* 1988; van Laar *et.al.,* 1989). Another recent report suggested the size could be 60-65 kDa (McEwan *et.al.,* 1989), but since the cloning of the androgen receptor cDNA, this would seem to be an underestimation of the predicted size.

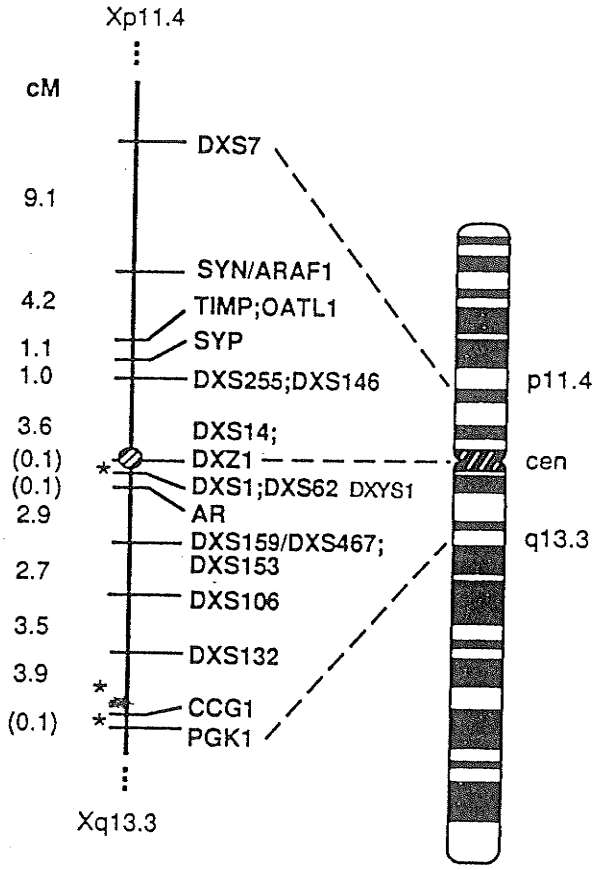


Figure 7 - Localization of the androgen receptor gene (AR, formerly named *DHTR*) on the X chromosome, including some of the flanking markers to this locus (from Mahtani *et.al.*, 1991).

The androgen receptor was detected in genital skin fibroblasts upon labelling with numerous tritiated androgen analogs, including those mentioned in the general methods section, methyltrienolone, mibolerone, and 5α-dihydrotestosterone. The binding kinetics described earlier for steroid receptors in general also apply to the androgen receptor. Specific androgen receptor binding analysis has been performed by numerous groups and the details of such can be found in some representative reports (Janne, Bardin, 1984; Tindall *et.al.,* 1984a; Kovacs, Turney, 1988; Mainwaring, Randall, 1984; Gronemeyer, Govindan, 1986). Before the cloning of the androgen receptor cDNA, diagnosis of androgen insensitivity syndrome was routinely confirmed by specific androgen receptor binding assays and Scatchard analysis in the patient's genital skin fibroblasts. Many of the androgen receptor defects (reviewed in detail below) have been determined in this fashion before the advent of the androgen receptor cDNA and molecular diagnosis at the DNA level, and may still be invaluable to provide clues as to the cause of the disorder in patients where the molecular defect is yet to be determined.

Antibodies to the androgen receptor have been generated by numerous groups. The antibodies to the androgen receptor were generated from high titre autoantibodies found in some patients with prostate cancer (Liao, Witte, 1985), as purification of the androgen receptor was difficult. More recently, monoclonal antibodies against the androgen receptor have been produced (Young *et.al.,* 1988), but the cells were apparently unstable and lost antibody producing capability. Since the cloning of the androgen receptor, it has been possible to produce large quantities of specific androgen receptor peptides to use as antigens in raising specific poly- and monoclonal antibodies (Chang *et.al.,* 1989; Zegers *et.al.,* 1991; Janne, Shan, 1991). Another group has also raised antipeptide antibodies against two distinct regions of the androgen receptor (Husmann *et.al.,* 1990).

Using a mouse monoclonal antibody against a fragment of the N-terminal domain of the androgen receptor, the expression of the androgen receptor in human tissues has been determined immunohistochemically (Ruizeveld de Winter *et.al.,* 1991). The results

were mostly consistent with previous assignments by biochemical ligand binding and autoradiographic data. The androgen receptor is present in all reproductive organs of the male, while variable expression was noticed in female reproductive tissues. Non-genital skin revealed no detectable staining, although sebaceous glands, sweat glands and ducts were immunopositive. The digestive, respiratory and urinary tracts were all immunonegative, which is in contrast to previous reports. This may be explained in part by species differences, but the very low androgen receptor content in these tissues and high background due to nonspecific labeling with the previous methodology is a more probable explanation (Ruizeveld de Winter *et.al.,* 1991). There was comparatively weak reactivity found in the nuclei of hepatocytes. The androgen receptor protein was not detectable in skeletal muscle, smooth muscle of the bronchi, intestines and bladder, furthermore there was low intensity staining in the myocardial specimens of two male subjects, while no staining was detected in the same tissue of female subjects. Lymph nodes, thymus, thyroid gland, parathyroid gland and adrenal gland did not reveal any immunoreactivity; similarly peripheral neural tissue, including ganglion cells, had negative results (Ruizeveld de Winter *et.al.,* 1991).

(b) Cloning of the androgen receptor

Three independent research groups cloned the androgen receptor cDNA in 1988 (Figure 8). Chang *et.al.* (1988b) and Lubahn *et.al.* (1988b) both published their reports in the same issue of *Science*, while Trapman *et.al.* (1988) published their report one month later. Another report followed these initial three publications a half year later (Tilley *et.al.,* 1989). All of these groups used the highly homologous DNA-binding region from other members of the steroid receptor gene family to prepare oligonucleotides to screen cDNA libraries at low stringency as their strategy to isolate positive clones of the androgen receptor gene.

The libraries used, in the order of the studies mentioned above, were human testis, human epididymis and human foreskin fibroblasts, human breast cancer T47D cells, and human prostatic hyperplasia, respectively. These libraries were chosen due to the known

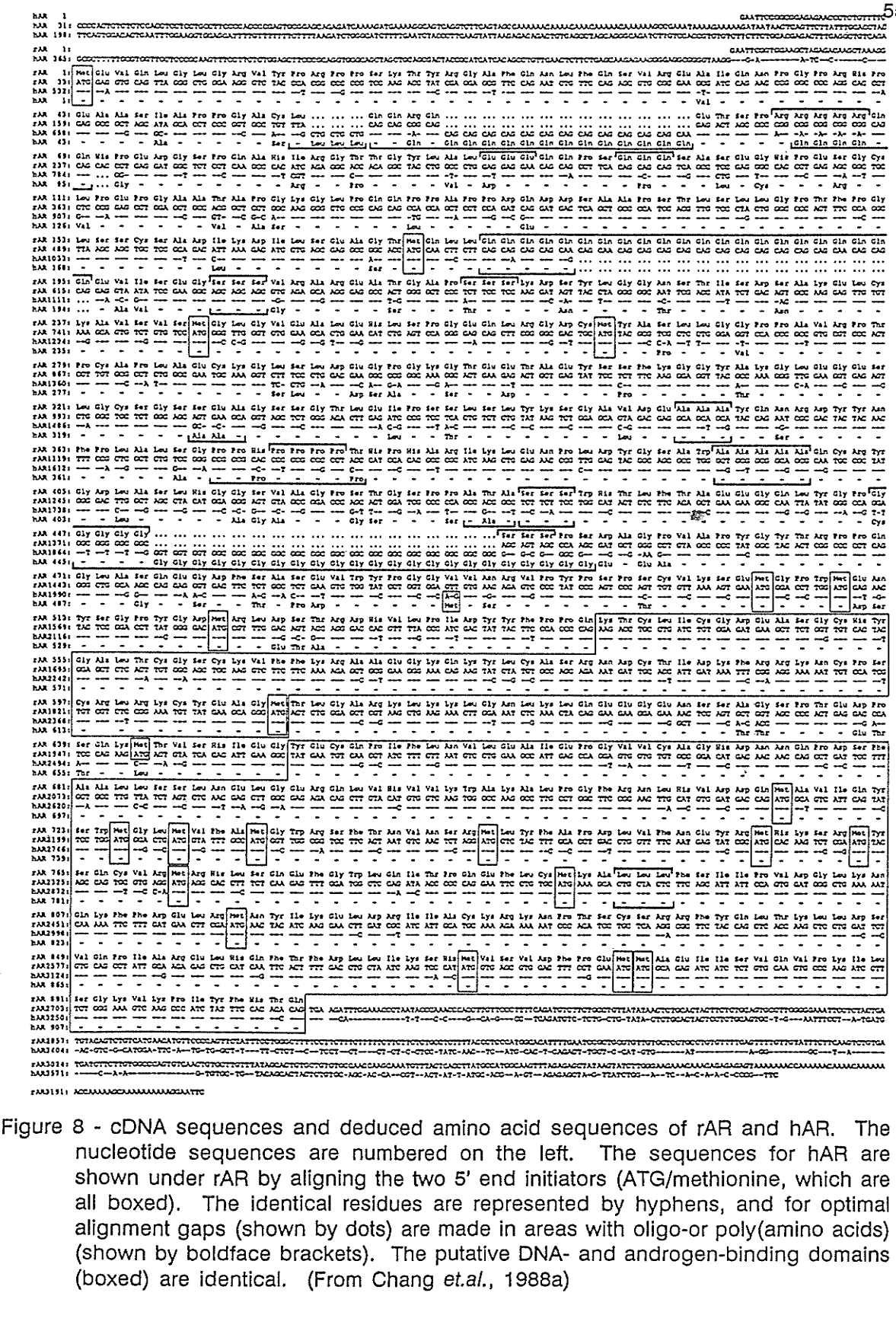


Figure 8 - cDNA sequences and deduced amino acid sequences of rAR and hAR. The nucleotide sequences are numbered on the left. The sequences for hAR are shown under rAR by aligning the two 5' end initiators (ATG/methionine, which are all boxed). The identical residues are represented by hyphens, and for optimal alignment gaps (shown by dots) are made in areas with oligo-or poly(amino acids) (shown by boldface brackets). The putative DNA- and androgen-binding domains (boxed) are identical. (From Chang *et.al.*, 1988a)

presence of the androgen receptor and the lack of large quantities of other steroid receptors. Two groups also initially screened an X chromosome library in order to isolate potential cDNAs to subsequently screen the above mentioned libraries, as the androgen receptor is the only known steroid receptor to be encoded by this chromosome (Lubahn *et.al.,* 1988b; Tilley *et.al.,* 1989). The initial cloning analysis indicated that the androgen receptor was also a member of the steroid receptor super gene family, as was expected. The cDNA consisted of a highly homologous DNA-binding domain, a homologous hormone-binding domain and a large N-terminal domain containing two homopolymeric regions, those being a polyglutamine and polyglycine repeat region. The total number of polyglutamines in the polyglutamine region varied, including 17 (Chang *et.al.,* 1988a), 21 and 25 (Lubahn *et.al.,* 1988a), 20 (Tilley *et.al.,* 1989), and 21 (Marcelli *et.al.,* 1990b) from the various reported sequences. The significance of this region will be further discussed later in this thesis. The polyglycine region had similar differences with numbers including 27 (Chang *et.al.,* 1988a), 24 (Lubahn *et.al.,* 1988a), and 23 (Tilley *et.al.,* 1989).

Proof that the isolated cDNAs were from the androgen receptor gene included expression of the cDNA in mammalian cells producing a protein with the binding characteristics, immunoprecipitable with androgen receptor antibodies, and size as was expected for the androgen receptor. Dosage analysis of Southern blots of human cell lines containing multiple X chromosomes was also used to identify positive clones by two groups (Lubahn *et.al.,* 1988b; Tilley *et.al.,* 1989). Extensive analysis of the cDNA has lead to very similar results, which include a translated receptor protein of approximate molecular mass 98 kDa, with an open reading frame of between 917 and 919 amino acids. The first reported mRNA species were 11 and 8.5 (major species), and 4.7 (minor species) kb (Trapman *et.al.,* 1988). These numbers have been modified since that time with the accepted mRNA sizes being 10 and 7 kb, with the 7 kb species probably resulting from differential processing of a precursor mRNA (Lubahn *et.al.,* 1988a; Faber *et.al.,* 1991). The 4.7 kb species reported above was also seen in other analyses and was thought to result from cross-hybridization with ribosomal RNA (Lubahn *et.al.,* 1988a). The most accurate size of the androgen receptor mRNA from the analysis of the complete size of the cDNA

is 10.6 kb (Faber *et.al.,* 1991).

The rat androgen receptor has also been cloned and analyzed (Chang *et.al.,* 1988b; Chang *et.al.,* 1988a; Tan *et.al.,* 1988). The rat cDNA was 100% homologous to the human androgen receptor cDNA at the DNA- and hormone-binding domains, and showed very few differences at the N-terminus (Figure 5) (Chang *et.al.,* 1988a; Tan *et.al.,* 1988). In particular, the rat cDNA encodes a protein of 902 amino acids with a very similar molecular weight. The mRNA species is 10 kb. The poly(amino acid) motif, on the other hand, is a polyglutamine region further downstream from the specific polyglutamine region found in the human cDNA, which is absent in the human cDNA at that position. The mouse androgen receptor is 97% homologous to the rat androgen receptor and 83% homologous with the human androgen receptor (Charest *et.al.,* 1991). Since the time of the original cloning, another group has utilized a polymerase chain reaction technique to obtain cDNAs of the androgen receptor from divergent species, including mouse, dog, guinea pig and clawed frog (He *et.al.,* 1990a).

The most recent mapping of the human androgen receptor gene locus, using the androgen receptor cDNA, has been to the Xq11 to Xq12 region on the human X chromosome (Figure 7) using somatic cell hybrid panels segregating portions of the X chromosome (Brown *et.al.,* 1989). Using the androgen receptor cDNA, a *Hind*III (6.7/3.5 kb) polymorphism, with a frequency of 0.1 in the general population, was also described (Brown *et.al.,* 1989), which has been utilized in a subsequent study of a family with partial androgen insensitivity syndrome (Lobaccaro *et.al.,* 1991).

(c) Genomic organization of the androgen receptor

The complete coding region of the human androgen receptor gene has been isolated from a genomic library (Kuiper *et.al.,* 1989; Brinkmann *et.al.,* 1989; Lubahn *et.al.,* 1989). Analysis of this genomic clone reported in these papers indicates that the receptor gene consists of eight exons and is longer than 90 kb (Figure 5(C)). The exons are either numbered 1 to 8 (Kuiper *et.al.,* 1989) or A to H (Lubahn *et.al.,* 1989). Exon 1 begins with

the ATG translation start site and encodes the complete N-terminus of the androgen receptor. The DNA-binding region is encoded by exons 2 and 3, with each zinc finger produced by a separate exon. The hormone binding domain is encoded by the last 5 exons, 4 to 8. The coding sizes of exons 1 to 8 are 1586, 152, 117, 288, 145, 131, 158, and 153 bp, respectively. The total size of the first exon is over 3 kb due to the large 5'-noncoding sequence. Exon 8 also has the large 3'-noncoding region consisting of over 6 kb. The introns between the coding regions are >24, >15, 26, 5.6, 4.8, 0.8, 0.7 kb, respectively. The group also confirmed that the gene is found in a single copy in the human genome using Southern analysis (Kuiper *et.al.,* 1989), as was initially reported by Lubahn *et.al.* (1988b).

Little is known about the regulation of androgen receptor expression, except for tissue specificity (Mooradian *et.al.,* 1987) and autologous down-regulation of androgen receptor mRNA by androgen (Quarmby *et.al.,* 1990; Shan *et.al.,* 1990; Janne, Shan, 1991). It has also been found that androgens, like the epidermal growth factor (androgens enhance synthesis of the EGF receptor), stimulate cell growth; thus, androgens effect tumor cell growth. This has been noted in studies with a human prostate tumor cell line, LNCaP, where autocrine and paracrine mechanisms effectively make cells sensitive to growth factor mediated stimuli (Mulder *et.al.,* 1989). A complete analysis of the androgen receptor regulatory regions has recently been reported (Faber *et.al.,* 1991), and these studies will add tremendously to the elucidation of the control of expression of this gene.

The characterization of the human androgen receptor transcription unit has uncovered some surprising findings. The untranslated regions (UTR) are unusually long (5'-UTR is 1.1 kb and 3'-UTR is 6.8 kb). In fact, the 5'-UTR is likely the longest published for any gene so far, while the 3'-UTR is the longest for any of the steroid hormone receptor mRNAs, although all are known to have relatively long 3'-UTRs (Faber *et.al.,* 1991). The promoter region of the human androgen receptor contains two major initiation sites, being one consensus Sp1 binding site and a homopurine stretch of 60 bp containing several GGGGA and GGGA sequence motifs. The initiation sites are not like

those reported for the human estrogen receptor and the chicken progesterone receptors (Huckaby *et.al.,* 1987; Jeltsch *et.al.,* 1990; Green *et.al.,* 1986), as there are no TATA or CCAAT box elements nor a large (G+C)-rich region (Faber *et.al.,* 1991), which indicates that the promoter region of the various members of the steroid receptor family must have diverged during evolution (if the same primordial gene hypothesis is correct) to adapt to their differing expression and functional capabilities. There are also no similarities to the "initiator" sequence (Smale, Baltimore, 1989) nor the "HIP1" sequence (Means, Farnham, 1990), both found at the transcription initiation sites of several (G+C)-rich promoters in order to position the transcriptional machinery. Two functional polyadenylation signals have been found at the 3'-UTR, ATTAAA at +10377 and CATAAA at +10604, directing poly(A) addition to the mRNA (Faber *et.al.,* 1991).

The rat androgen receptor gene regulatory region has also been characterized (Baarends *et.al.,* 1990). Like the human gene promoter, the rat promoter region lacks typical TATA and CCAAT box elements, but does have one Sp1 site located about 60 bp upstream from the major transcription initiation site (Baarends *et.al.,* 1990). Again a homopurine-rich region is found containing a total of 8 GGGGA elements, very similar to that of the human androgen receptor promoter region.

(d) Gene regulation by androgens

The knowledge required to fully understand the regulation by androgens has not come easily (Berger, Watson, 1989), especially in comparison to other steroids such as glucocorticoid gene regulation. Genes known to be regulated by androgens have been reported, in particular, the mouse mammary tumor virus (MMTV) has been studied in the androgen receptor containing T47D cell line (Darre *et.al.,* 1986; Parker *et.al.,* 1987; Cato *et.al.,* 1987; Ham *et.al.,* 1988; Gowland, Buetti, 1989). Androgen response elements (AREs) have been found to overlap with glucocorticoid/progesterone response elements (GRE/PRE) in the MMTV LTR (Ham *et.al.,* 1988; Gowland, Buetti, 1989). In a more recent study using both monkey CV1 cells and human HeLa cells, neither containing an endogenous functional androgen receptor, CAT activity was increased using the MMTV

LTR as a source of AREs when analyzing the effects of rat ventral prostate androgen receptor cDNA (Rundlett *et.al.,* 1990). This type of study may be useful to investigate the deleterious effects of conceivably mutant androgen receptors and the failure of a response from specific genes after androgen therapy in malignant cells, especially in prostate cancer.

Other genes known to be regulated by androgens include ß-glucuronidase (GUS), ornithine decarboxylase (ODC), the RP2 gene, the kidney androgen-regulated protein (KAP), and alcohol dehydrogenase (ADH), all of which have been mainly studied in the mouse kidney (Berger, Watson, 1989). The kallikrein gene of human prostatic tissue has been found to be regulated by androgen (Young *et.al.,* 1990a). The 20 kDa glycoprotein (Ho *et.al.,* 1989; Ho *et.al.,* 1991), the prolactin-inducible protein (Murphy *et.al.,* 1987), and probasin (Spence *et.al.,* 1989; McQueen *et.al.,* 1991), from the rat prostate, and the rat seminal vesicle secretory protein IV (Kistler, Kistler, 1991) are other characterized androgen-regulated genes. Androgens are therefore thought to be modulators of transcription initiation. The reported post-transcriptional effects of androgens as stabilizing factors of the mRNA (Berger *et.al.,* 1986; Page, Parker, 1982) would be considered a secondary response to their role as transcriptional activators. There has also been some speculation as to the requirement for other cell-specific factors in addition to the androgen receptor for transcription initiation (Parker *et.al.,* 1987; Allison *et.al.,* 1989), predicting that androgen receptor regulation may be more complex than that postulated for other steroid receptors.

The studies of androgen regulation of gene expression is still in its infancy and will, no doubt, explode in the very near future, as has the whole field of androgen research. Many of the abstracts published from the most recent Endocrine Society Meeting (73rd, 1991) have been related to the genes regulated by androgens and the response elements necessary for this regulation. Many unique questions must be answered before a true understanding of this process is unveiled. Does the androgen receptor have its own response element, as has been suggested by some groups (Rundlett *et.al.,* 1990; McQueen *et.al.,* 1991), (B. Matusik, personal communication), or is the ARE the same as

the GRE/PRE (Marschke *et.al.,* 1991; Ho *et.al.,* 1991; Kistler, Kistler, 1991; Riegman *et.al.,* 1991)? What are the unique features of androgen regulated genes which dictate the specific reaction to androgens? Are other factors required for androgen action, such as specific transcription factors (Beato, 1989)? Does phosphorylation play a part in the functionality of the androgen receptor (Kemppainen *et.al.,* 1991), as has been suggested for other steroid receptors (Beato, 1989)? All of these questions will soon be answered and will add to the steroid receptor research field perhaps as no other steroid research has thus far.

E. Androgen receptor defects

(i) Causes of AIS before molecular studies

Before the advent of the cDNA of the androgen receptor, the study of androgen receptor defects was based mainly on qualitative or quantitative investigations using primarily biochemical strategies. These studies were based on the identification of apparent specific androgen binding to an as yet physically unidentified receptor molecule. The binding assays were done in homogeneous or partially enriched fractions containing potentially many molecules with some affinity for androgens. Characterization of the androgen receptor molecule depended upon inference of binding characteristics, such as Scatchard analysis.

From this analysis, androgen insensitivity was thought to be caused by abnormalities of the androgen receptor. Measurement of the specific androgen receptor binding activity in skin fibroblasts (Keenan *et.al.,* 1974; Keenan *et.al.,* 1975; Brown, Migeon, 1981), and then, more appropriately in the genital skin fibroblasts of patients allowed the pathogenesis of the disorder to be slowly unravelled (Griffin *et.al.,* 1976). The genital skin fibroblasts of some patients with complete androgen insensitivity syndrome have absent binding activity (Keenan *et.al.,* 1975; Griffin *et.al.,* 1976; Kaufman *et.al.,* 1976), which would explain the profound consequences of complete resistance to androgen action. At that time, it was not known whether this absence of binding was due to the absence of the receptor protein or the presence of a mutant receptor protein.

The cause of AIS in other patients has been found to be due to qualitatively abnormal receptor molecules (Table III). The initial study of this sort detected a thermolabile receptor (Griffin, 1979), which was active at 37°C and 26°C, but had binding less than one-fifth of normal activity at 42°C. The presence of thermolabile androgen receptors has also been reported by other investigators (Brown *et.al.,* 1982; Evans *et.al.,* 1984; Schweikert *et.al.,* 1987; Smallridge *et.al.,* 1984).

Other functional assays, comparing the physiochemical characteristics of the wild-type androgen receptor to those of the patient, have been performed which also indicate a structural abnormality of the receptor protein. Examination of the effect of molybdate, a compound with a profound stabilizing effect on the normal unbound 8-S androgen receptor, but not the receptor of androgen insensitive patients, has been studied (Smallridge *et.al.,* 1984; Schweikert *et.al.,* 1987; Griffin, Durrant, 1982). Molybdate has been found to stabilize aggregated, non-transformed receptors and allows increased receptor binding activity in cytosol preparations, which is exhibited by significantly higher total binding over the same preparation without molybdate (Dahmer *et.al.,* 1984; Rowley *et.al.,* 1984; Carroll *et.al.,* 1984). The finding of decreased affinity of ligand binding to the receptor, determined by Scatchard analysis, has been reported in numerous studies (Pinsky *et.al.,* 1991; Brown *et.al.,* 1982; Kaufman *et.al.,* 1984; Pinsky *et.al.,* 1985; Jukier *et.al.,* 1984).

Other qualitative defects could include: increased dissociation of the hormone-receptor complex (Pinsky *et.al.,* 1991; Brown *et.al.,* 1982; Evans *et.al.,* 1984; Kaufman *et.al.,* 1984; Pinsky *et.al.,* 1985; Jukier *et.al.,* 1984; Kaufman *et.al.,* 1982; Pinsky *et.al.,* 1981); impaired nuclear retention of the androgen receptor molecule (Eil, 1983); defective transformation (Kovacs *et.al.,* 1984), whereas normal transformation is the change of the receptor from its inactive to active form, required before subsequent androgen receptor-dependent events may occur; and defective up-regulation of the androgen receptor (Kaufman *et.al.,* 1981; Pinsky *et.al.,* 1991; Kaufman *et.al.,* 1984; Pinsky *et.al.,* 1985; Kaufman *et.al.,* 1983; Kaufman *et.al.,* 1986; Evans, Hughes, 1985). The amount of specific

Table III - Qualitative abnormalities of the androgen receptor protein. (From Griffin and Wilson, 1989)

Marker	References
1. Thermolability in monolayers	Smallridge *et.al.*, 1984
	Griffin, 1979
	Schweikert *et.al.*, 1987
	Brown *et.al.*, 1982
	Evans *et.al.*, 1984
2. Instability of cytosol receptor	Smallridge *et.al.*, 1984
	Schweikert *et.al.*, 1987
	Griffin and Durrant, 1982
3. Decreased ligand binding affinity	Pinsky *et.al.*, 1984
	Brown *et.al.*, 1982
	Kaufman *et.al.*, 1984
	Pinsky *et.al.*, 1985
	Jukier *et.al.*, 1984
4. Impaired nuclear retention	Eil, 1983
5. Failure of up-regulation of the androgen receptor by androgens	Pinsky *et.al.*, 1984
	Kaufman *et.al.*, 1984
	Pinsky *et.al.*, 1985
	Kaufman *et.al.*, 1981
	Kaufman *et.al.*, 1983
	Kaufman *et.al.*, 1986
6. Increased rate of dissociation of ligand from receptor	Pinsky *et.al.*, 1984
	Brown *et.al.*, 1982
	Evans *et.al.*, 1984
	Kaufman *et.al.*, 1984
	Pinsky *et.al.*, 1985
	Jukier *et.al.*, 1984
	Kaufman *et.al.*, 1982
	Pinsky *et.al.*, 1981
7. Lability of the receptor under transforming conditions	Kovacs *et.al.*, 1984

receptor binding activity in normal genital skin fibroblasts, over basal activity in unexposed replicates, is roughly doubled after a 20 hr preincubation with androgen. This phenomenon is called up-regulation and is believed to be due to androgen-induced receptor synthesis (Kaufman *et.al.,* 1981). The importance of up-regulation in the androgen response system is generally accepted, as this may confer an advantage on target cells that are able to increase their androgen receptor activity in response to an increase in androgen levels; conversely without the ability to up-regulate androgen receptor levels, deleterious developmental effects may occur, such as the androgen insensitivity in target cells.

All of the above mentioned qualitative, and most likely the quantitative, abnormalities can be traced to a defect of the androgen receptor protein. The mutational analysis of the androgen receptor gene itself was not possible until the molecular cloning and sequencing of the cDNA. There was much speculation as to the underlying cause of AIS, whether it was due to a single mutation or perhaps to many different types of mutations at the same gene locus was yet to be determined.

(ii) After the cloning of the androgen receptor

The elucidation of the molecular structure of the androgen receptor gene has led to an explosion of molecular studies involving patients with AIS. More than 40 mutations have been reported and more have been found which have not yet been published (Table IV).

Most of the mutations found to date are unique to each individual family and the location of the mutation often reflects the receptor abnormality predicted from the specific androgen binding activities or the other qualitative abnormalities deduced from other biochemical methods, although this is not always the case (McPhaul *et.al.,* 1991b). To make this statement more evident to the reader, a good example of this phenomenon would be a mutation in the androgen binding domain being responsible for the production of a qualitatively abnormal receptor molecule which has known decreased androgen

Table IV - Mutations of the androgen receptor gene.

Amino acid change	Base change	Exon	Reference
A. Point mutations			
Cys 557 → Tyr	G → A	2	Zoppi *et.al.*, 1991
Gly 568 → Val	GG → TT	2	Chang *et.al.*, 1991
Cys 574 → Arg	T → C	2	Zoppi *et.al.*, 1991
Cys 576 → Phe	G → T	2	Chang *et.al.*, 1991
Lys 588 → Ter	A → T	3	Marcelli *et.al.*, 1990b
Arg 615 → Pro	G → C	3	Marcelli *et.al.*, 1991
Leu 616 → Arg	T → G	3	De Bellis *et.al.*, 1991
Leu 676 → Pro	T → C	4	Belsham *et.al.*, 1991a
Asp 686 → Asn	G → A	4	Ris-Stalpers *et.al.*, 1991
Asp 686 → His	G → C	4	Ris-Stalpers *et.al.*, 1991
Trp 717 → Ter	G → A	4	Sai *et.al.*, 1990
Arg 724 → His	G → A	5	Marcelli *et.al.*, 1990a
Ser 759 → Phe	C → T	5	De Bellis et.al., 1991
Tyr 761 → Cys	A → G	5	McPhaul *et.al.*, 1991a
Arg 772 → Cys	C → T	6	Marcelli *et.al.*, 1991
Arg 773 → Cys	C → T	6	Trifiro *et.al.*, 1989 Prior *et.al.*, 1991 Mebarki *et.al.*, 1990
Arg 773 → His	G → A	6	Prior *et.al.*, 1991 De Bellis *et.al.*, 1991
Arg 774 → Cys	C → T	6	Brown *et.al.*, 1990
Arg 775 → Cys	C → T	6	Tilley *et.al.*, 1990
Trp 794 → Ter	G → A	6	Marcelli *et.al.*, 1990c
Ser 813 → Asn	G → A	6	Trifiro *et.al.*, 1990
Arg 831 → Ter	C → T	7	De Bellis *et.al.*, 1991
Arg 831 → Gln	G → A	7	Brown *et.al.*, 1991
Arg 840 → His	G → A	7	De Bellis *et.al.*, 1991
Arg 855 → His	G → A	7	Chang *et.al.*, 1991
Arg 855 → Cys	C → T	7	De Bellis *et.al.*, 1991
Asp 864 → Gly	A → G	7	De Bellis *et.al.*, 1991
Val 866 → Met	G → A	7	Lubahn et.al., 1989
Lys 882 → Ter	A → T	8	Trifiro *et.al.*, 1991b
B. Deletions			
A.A. 69-76		1	Marcelli *et.al.*, 1990
Second zinc finger		3	Quigley *et.al.*, 1990
Steroid-binding domain			Brown *et.al.*, 1988
Complete androgen receptor gene			Trifiro *et.al.*, 1991a Quigley *et.al.*, 1991
C. Others			
Aberrant splicing of mRNA			Ris-Stalpers *et.al.*, 1990
CAG repeat region - decreased number		1	McPhaul *et.al.*, 1991a
increased number		1	La Spada *et.al.*, 1991 Belsham *et.al.*, 1991b (sub.)

binding activity (Lubahn *et.al.,* 1989; Pinsky *et.al.,* 1990).

Most of the mutations reported are caused by single base pair changes in the DNA sequence in the coding regions of the androgen receptor gene, although this does not rule out the possibility of finding deleterious mutations in other regions such as the intronic regions or in the regulatory regions of the gene. A list of the mutations found in the hormone-binding domain can be found in Table IV (Belsham *et.al.,* 1991a; Ris-Stalpers *et.al.,* 1991; Marcelli *et.al.,* 1990a; DeBellis *et.al.,* 1991; McPhaul *et.al.,* 1991a; Trifiro *et.al.,* 1990; Brown *et.al.,* 1990; Chang *et.al.,* 1991; Lubahn *et.al.,* 1989; McPhaul *et.al.,* 1991b). Other reported mutations include those with single base pair changes leading to a truncated receptor molecule due to replacement of the normal amino acid residue with a stop codon (Sai *et.al.,* 1990; Marcelli *et.al.,* 1990b; Marcelli *et.al.,* 1990c; DeBellis *et.al.,* 1991; Trifiro *et.al.,* 1991b). This type of mutation was also found in one of our families and will be thoroughly discussed in the results section (Part III.2.B.) of this thesis. There appears to be one mutational "hot-spot" at Arg773, also called Arg772, Arg774 or Arg775 depending on the primary androgen receptor cDNA sequence used for analysis, as the numbers differ due to the homopolymeric regions, as several groups have reported a single mutation at this amino acid residue (Trifiro *et.al.,* 1989; Prior *et.al.,* 1991; Mebarki *et.al.,* 1990; Tilley *et.al.,* 1990; DeBellis *et.al.,* 1991; Marcelli *et.al.,* 1991; McPhaul *et.al.,* 1991b) (Table IV). This particular mutation will be further discussed in the results and discussion section (Part III.2.D.) in this thesis. Finally a complete deletion of the androgen-binding domain has also been reported (Brown *et.al.,* 1988).

Some mutations of the androgen receptor may alter the primary structure of the androgen receptor mRNA. In particular, mutations creating a termination signal; thus, a truncated receptor molecule, often have a decreased amount of mRNA (Marcelli *et.al.,* 1990b; He *et.al.,* 1991; Charest *et.al.,* 1991). Aberrant splicing of the androgen receptor mRNA may produce a nonfunctional receptor (Ris-Stalpers *et.al.,* 1990). It has also been suggested that both single point mutations causing an altered protein structure and a depressed level of androgen receptor mRNA are necessary for receptor-negative complete

androgen insensitivity syndrome (Marcelli *et.al.,* 1991; McPhaul *et.al.,* 1991b).

Mutations that involve specific regions responsible for androgen receptor function other than hormone binding have also been found (Table IV). A mutation of the second zinc finger has been reported (Quigley *et.al.,* 1990), as have other mutations in the DNA-binding domain, which have been found to be the frequent cause of receptor-positive androgen insensitivity discussed previously (Zoppi *et.al.,* 1991; Chang *et.al.,* 1991; DeBellis *et.al.,* 1991). One patient was found to have no detectable mRNA (Tilley *et.al.,* 1990). One of our patients with a complete deletion of the androgen receptor is the subject of one of the results sections (Part IV.2.B.) in this thesis (Trifiro *et.al.,* 1991a), and since the time of the report of our patient another subject with a complete deletion of the androgen receptor has now been reported (Quigley *et.al.,* 1991).

Mutations of the homopolymeric regions of the androgen receptor, particularly the glutamine repeat region at the N-terminus of exon 1 thought to be involved in transcriptional regulation, have also been detected. One group has found that a decreased number of glutamine repeats in exon 1, in cooperation with a single point mutation changing Tyr761 to Cys, amplifies the impairment of receptor function caused by the latter point mutation in one patient (McPhaul *et.al.,* 1991a; McPhaul *et.al.,* 1991b). Furthermore, an increased number of glutamine repeats has been postulated to be the cause of another seemingly unrelated disorder, spinal and bulbar muscular atrophy (La Spada *et.al.,* 1991), which will be discussed further.

The field has progressed at such a rate that it is now necessary to prove the causative nature of the detected mutation in mammalian expression systems before the mutation can be published. The experimental procedures used for this type of analysis are discussed in the results section of this thesis. A few reports have been published using this technology (Marcelli *et.al.,* 1990b; Marcelli *et.al.,* 1990c; Prior *et.al.,* 1991; Marcelli *et.al.,* 1991; McPhaul *et.al.,* 1991a), and it is likely that this will become the normal procedure for the analysis of androgen receptor defects in the future.

One particularly interesting study mentioned above, which reported two separate mutations in the androgen receptor gene of the patient, proved that the effects of the two mutations were additive (McPhaul *et.al.,* 1991a). When analyzing the mutations in an androgen receptor construct, individually and in combination, the ability of the receptor to stimulate a reporter gene was strikingly diminished only when both mutations were present. This study emphasizes the potential problems that can be encountered when analyzing single point mutations, and also that the full analysis of the mutation by expression studies is probably wise. It is also possible that these types of study will advance even further, perhaps utilizing transgenic mice as experimental vehicles.

Along with the studies of human AIS patients, other significant mutations in the androgen receptor gene have also been uncovered. The *Tfm* mouse was initially reported to lack androgen receptor mRNA in the liver (Lubahn *et.al.,* 1988b; Gaspar *et.al.,* 1990) and to have a shortened androgen receptor due to a single point mutation (He *et.al.,* 1990b; Young *et.al.,* 1989). This finding was eventually confirmed to be due to a frameshift mutation at a stretch of 6 cysteines approximately two-thirds into the N-terminal domain of the mouse androgen receptor gene, creating a premature termination codon at amino acid 412, resulting in an unstable androgen receptor mRNA (Charest *et.al.,* 1991; He *et.al.,* 1991; Gaspar *et.al.,* 1991). This is the first frameshift mutation reported in the steroid supergene family; furthermore three internal initiation codons have been postulated to result in the reinitiation of translation from the mRNA creating shortened, but partially active androgen receptor peptides, which would account for the decreased amount of binding activity found in the *Tfm* mouse (He *et.al.,* 1991; Gaspar *et.al.,* 1991). The *Tfm* rat mutation has been found to be single point mutation changing Arg735 to Glu in the steroid binding domain (Yarbrough *et.al.,* 1989; Yarbrough *et.al.,* 1990). The prostate tumor cell line, LNCaP, known to overexpress the androgen receptor protein resulting in highly increased levels of specific androgen binding activity, has also been studied for the presence of a specific mutation. It has been found that the androgen receptor gene in this cell line has a single point mutation at Thr868 changing the amino acid residue to Ala (Veldscholte *et.al.,* 1990). The expression of this mutated androgen receptor gene was

found to result in an androgen receptor molecule capable of binding not only androgens, but also progestagens, estrogens and anti-androgens, activating an androgen regulated reporter gene construct in COS or HeLa cells (Veldscholte *et.al.,* 1990).

The study of specific mutations in patients with AIS will eventually lead to a clear picture of the mechanisms of androgen action in the normal human. By mutational analysis, it is possible to elucidate the exact consequence of each specific mutation in a mammalian system and the deleterious results when the normal coding sequence is not present. Of course, one must keep the experimental results in perspective, as the normal physiological system is bound to have many extragenic factors involved in the processes, but nevertheless it is a good beginning to uncovering the mysteries of sexual development and differentiation.

F. X-linked spinal and bulbar muscular atrophy:
Another androgen receptor gene mutation

(i) Overview of the disorder

X-linked spinal and bulbar muscular atrophy (SBMA) was first described by Kennedy et al in 1968 in his report of two families in which 11 members, all male, were affected by an unusual, slowly progressive spinal and bulbar muscular atrophy affecting the anterior horn cells. The disease often presented in the fourth or fifth decades and initially involves weakening of the proximal muscles, due to degeneration of the anterior horn cells. The patient life span is normal. The proximal muscle weakness gave a clinical picture similar to muscular dystrophy in some patients. Since the initial description, six cases were described (Barkhaus *et.al.,* 1982) followed by an additional 10 cases (Harding *et.al.,* 1982). The list includes more than 20 families reported over the past 25 years or so.

The chronic spinal muscular atrophies developing in the adult life are a clinically and genetically heterogeneous group of disorders (Harding *et.al.,* 1982). The proximal

chronic spinal muscular atrophies can be caused either by autosomal dominant or autosomal recessive genes, both of which have been described, and although pathologically similar, they are known to be clinically separate entities from X-linked recessive SBMA. The disorder has been difficult to diagnose in males without a positive family history, although this does not necessarily indicate the occurrence of a new mutation.

The clinical features of X-linked SBMA often include gynecomastia, often the earliest manifestation of the disease, and when thoroughly examined some patients have also been noted to have testicular atrophy and azoospermia (Harding *et.al.,* 1982; Arbizu *et.al.,* 1983). Recently, a patient with SBMA was also shown to have decreased specific androgen-binding affinity (Warner *et.al.,* 1991). These characteristics are similar to those often found in patients with disorders of androgen action. Fischbeck *et.al.* (1986) described linkage to *DXYS1* on the proximal long arm of the X-chromosome, and loose linkage or nonlinkage to markers elsewhere. They concluded that the gene defect for X-linked SBMA is localized to this area on the X-chromosome, near *DXYS1*. This investigation provided a marker to be used for genetic counselling in families with this disorder. Interestingly enough, positive linkage analysis has been shown between the androgen receptor gene locus and *DXYS1* (Wieacker *et.al.,* 1987).

(ii) Relationship to the androgen receptor

These preliminary findings indicated that the gene for the androgen receptor may be near the gene for SBMA, which was also thought to be near the gene for X-linked neuropathy due to its linkage to *DXYS1* (Fischbeck *et.al.,* 1986). The disorder could then be hypothesized to be due to a contiguous gene syndrome because of the overlap of clinical characteristics (Schmickel, 1986).

Recently, a report indicated that an increased number of CAG repeats in exon 1 at the N-terminus of the androgen receptor gene could be the cause of X-linked SBMA (La Spada *et.al.,* 1991). The amplified repeats, roughly double the number over normal

controls (average 21), were absolutely associated with the disease. The study included 35 unrelated SBMA patients and 75 control subjects. The CAG or polyglutamine repeats segregated with the disease in 15 families, with no recombination in 61 meioses, giving a maximum log likelihood ratio (lod score) of 13.2 at a recombination rate of 0 (La Spada *et.al.,* 1991).

Because of this finding, it is now possible to use these methods to predict the carrier status in females and also the possible occurrence of this late onset progressive disorder in male members of families with X-linked SBMA.

2. Results and Discussion

A. Family 1 with complete AIS: Detection of a novel *Msp*I RFLP for direct mutation detection/ carrier testing in a Hutterite kindred.

(from Belsham *et.al.*, 1991a, and 1991b, Amer.J.Hum.Genet. (submitted))

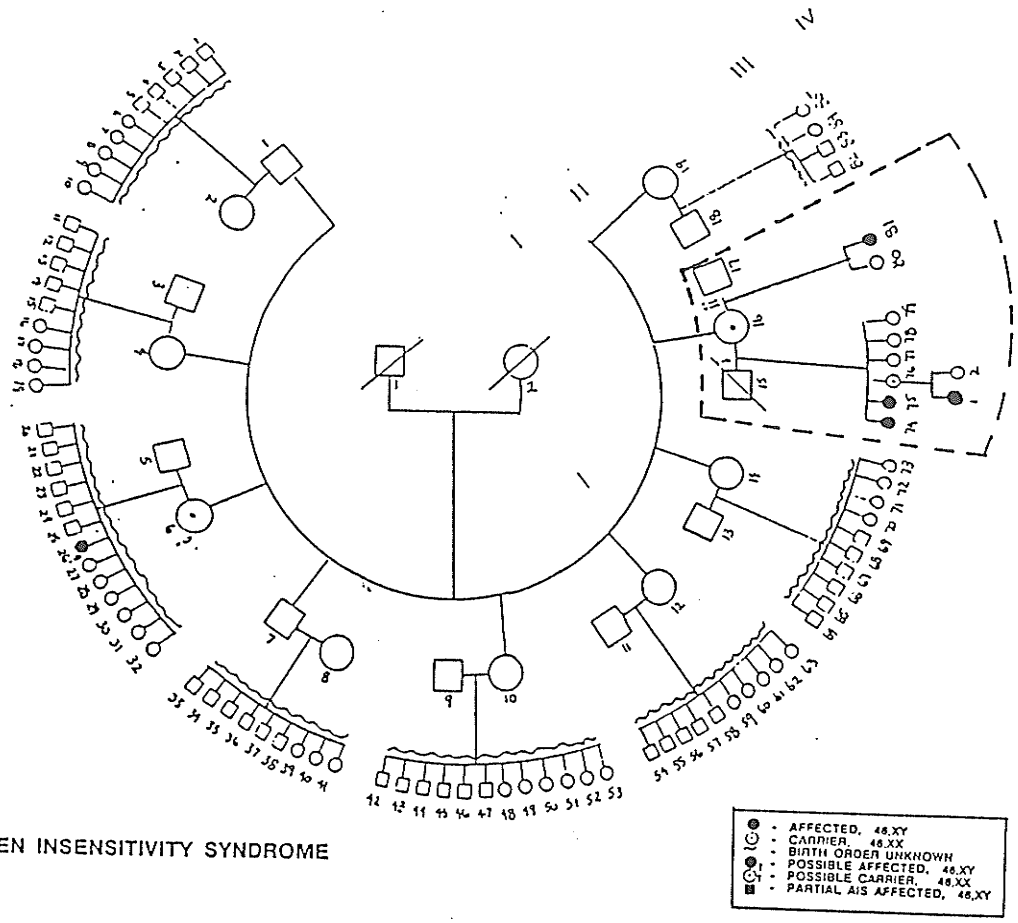
(i) Abstract

Herein we describe a Manitoba Hutterite kindred with AIS. Carrier testing in this family was requested but not feasible as the biochemical assay for androgen receptor binding activity requires a genital skin biopsy from all family members and there is still a considerable overlap of androgen binding activity values between carriers and non-carriers. Cloning skin fibroblasts to determine the expression of a defective androgen receptor in approximately half the cells is theoretically possible, this is not practical (Griffin, Wilson, 1989); thus, linkage analysis was attempted with the anonymous DNA sequences from *DXS1* and *DXYS1*, which are known to be very closely linked to the AR locus (Wieacker *et.al.,* 1987). As this analysis did not prove completely informative for the kindred, we continued this study and are now able to report a novel *Msp*I restriction fragment length polymorphism (RFLP) pattern, created by a single base pair substitution, which probably is the cause of the disease in this kindred.

(ii) The subject and family

The proband (III-1 in Figure 9(B)) presented at age 5 with an inguinal hernia, which was removed and found to contain testicular tissue. A genital skin biopsy was taken at that time. A chromosome analysis revealed an XY karyotype and negligible specific androgen binding activity in her genital skin fibroblasts confirmed the complete androgen insensitivity syndrome diagnosis. A genital skin biopsy was obtained. A complete family history was taken (Figure 9(B)) and the proband was found to have three maternal aunts with complete AIS (II-1, II-2, and II-9). All three aunts were phenotypic females, with 46, XY karyotypes and had undergone gonadectomies. Testicular tissue was confirmed histopathologically. The maternal grandmother (I-2), an obligate carrier, has 6 fertile sisters, 2 normal brothers, 34 healthy nephews and 33 healthy nieces (Figure 9(A)). There

A



ANDROGEN INSENSITIVITY SYNDROME

FAMILY 1

B

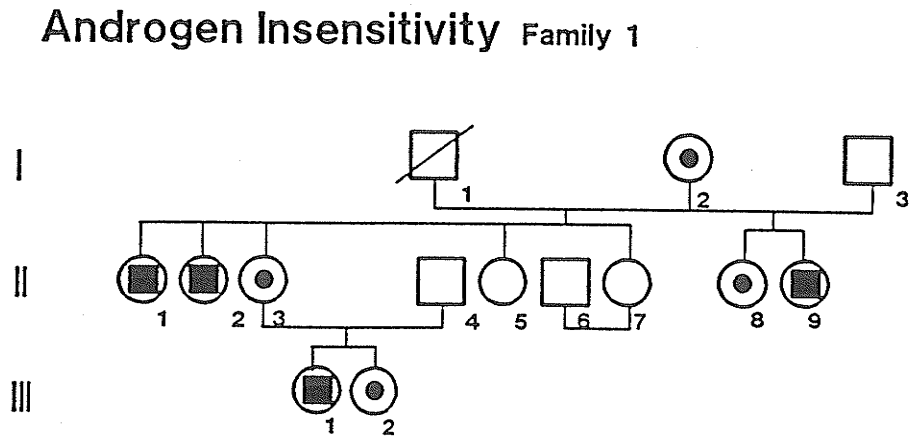


Figure 9 - The pedigree of the large extended Hutterite kindred of Family 1 (top), with the studied members indicated in the smaller pedigree (bottom). Circle with filled square, CAIS.

is no known history of androgen insensitivity in the large extended pedigree, with the exception of one questionable AIS in a distant cousin without formal diagnosis (Figure 9(A)). After informed consent was obtained, peripheral blood was collected from 13 members of the Hutterite kindred (Figures 9(B)).

(iii) Supplementary methods

Polymorphism analysis

To detect potential RFLPs in this family with the androgen receptor cDNA, genomic DNA from the obligate carrier mother (I-2 in Figures 9, 11, and 12) and one of her affected daughters was digested with the following restriction endonucleases; *Taq*I, *Bam*HI, *Eco*RI, *Hind*III, *Msp*I, *Rsa*I, *Pst*I, *Hpa*II, *Sau*3A, *Xba*I, *Sac*I, *Alu*I, *Bcl*I, *Bgl*II, *Bst*XI, *Hae*III, *Xmn*I, *Stu*I, and *Eco*RV. A polymorphism was detected only with *Msp*I using the 0.7 kb fragment of the androgen receptor cDNA (2223(HindIII) to 2935(EcoRI) according to the sequence by Chang *et.al.* (1988b). The probes used were various human androgen receptor cDNAs (obtained from Drs. Chang and Liao, University of Chicago) that recognized its DNA- and androgen-binding regions (Figure 10), and p8 (Aldridge *et.al.,* 1984) and pDP34 (Page *et.al.,* 1982), that recognize the anonymous loci *DXS1* and *DXYS1*, respectively.

PCR Amplification and DNA Sequencing of Exons 4, 5, and 6

The PCR method was initially described by Saiki *et.al.* (1988). Amplification of exons 2 to 8 and the polyglutamine region were performed using flanking sets of primers for these three exons of the androgen receptor from published sequences (Table I). Each 100 µl PCR reaction contained PCR buffer (Perkin Elmer Cetus, Norwalk, CT) containing 500 mM KCl; 200 mM Tris-HCl pH 8.3, 20 nmol of each dNTP, 100 pmol of each primer, 1 µg DNA, and 2.5 units of Taq polymerase, covered with 100 µl of mineral oil. The reaction was carried out cyclically with denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. After 25 cycles, a final extension was performed for another 7 min at 72°C. The PCR products were chloroform extracted, purified on a 2% agarose gel, and the fragments isolated by centrifugation of the gel band through glass wool at 6000 rpm for 10 min. The products were purified by ethanol

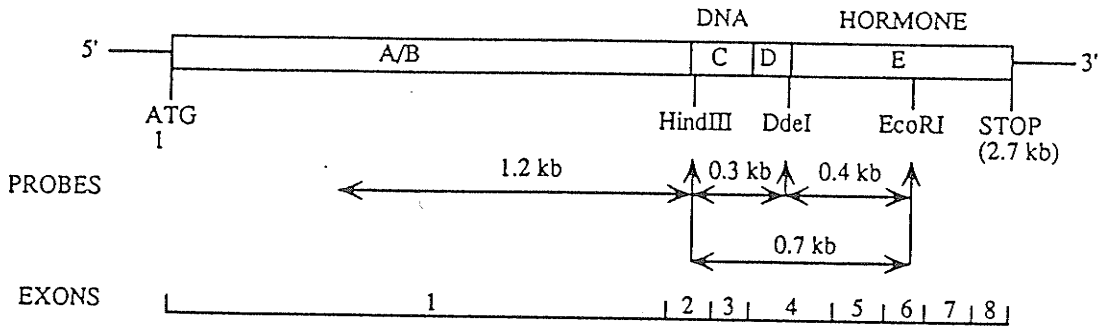


Figure 10 - A cDNA map of the androgen receptor gene with probes used in the studies presented in this thesis. The location of the exon/intron boundaries are indicated below the diagram. Note: hAR-1 is the 0.7 kb segment; hAR-2 is the 0.3 kb fragment from the *Eco*RI site to the 3' end of the cDNA clone; hAR-1.2 is the 1.2 kb segment; and hAR-2.1 is the fragment from the 5' end of the cDNA clone to the *Hin*dIII site.

precipitation and blunt-end ligated into a Bluescript vector (Sambrook *et.al.,* 1989). Positive clones were prepared essentially according to the method of Hattori and Sakaki (1986), and double-strand-sequenced according to the manufacturer's recommendations (Sequenase kit, United States Biochemical Corp., Cleveland, OH) using the method of Sanger *et.al.* (1977).

(iv) Results

Genital skin fibroblasts of the proband (III-1 in Figure 9(B)) had negligible specific androgen receptor binding activity, (Table V), which agrees with the complete AIS diagnosis. The control cell lines used in this experiment included a normal genital skin fibroblast strain, MCH6 and a lymph node carcinoma of the prostate cell line (LNCaP), which is known to overexpress the androgen receptor (van Laar *et.al.,* 1990). The binding activities measured were 24.4 fmol/mg protein and 481.0 fmol/mg protein, respectively, for these controls.

Because carrier testing was requested for 4 potential carriers in the kindred, linkage analysis with the X-chromosome probes p8 and pDP34, representing the anonymous DNA sequences of *DXS1* and *DXYS1* was attempted. These probes have previously been described as very closely linked to AIS (Wieacker *et.al.,* 1987; Imperato-McGinley *et.al.,* 1990). Family member III-2, the sister of the proband in the subfamily of the kindred including II-3, II-4, III-1 and III-2, was determined to be a carrier with the available information. In Figure 11, it is apparent that the mutant phenotype segregates with the 15 kb allele using probe p8 (11(A)) and the 12 kb allele using probe pDP34 (11(B)). The linkage analysis did not prove helpful for the majority of potential carriers in this family, as the obligate carrier grandmother I-2 was uninformative. Since I-2 is homozygous for both X-chromosome alleles with both probes, we cannot determine the carrier status of the other 3 potential carriers in this family (II-5, II-7 and II-8). From the linkage analysis, we are able to add positive information to the previously reported lod scores for both *DXS1* and *DXYS1* (Wieacker *et.al.,* 1987; Imperato-McGinley *et.al.,* 1990), as Family 1 had one informative meiosis, phase unknown. We therefore add 0.301 to both of the previously

Table V - Specific androgen receptor binding activity of the probands of: Family 1 (KJH), Family 2 (DB), Family 3 (JMR); the complete androgen receptor deletion patient (8812); control GSF cell strains, MCH6 and MCH49; control non-GSF, WP09: prostatic carcinoma cell line which overexpresses the AR, LNCaP; and HeLa cells. (S.E., standard error; brackets indicate number of separate trials; each experiment done with 5 nM [^{3}H]androgen analog, either MT or MB)

Cell strains/lines	No. trials	Activity (fmol/mg protein) $\pm$ S.E.
KJH	(5)	2.22 $\pm$ 0.70
DB	(3)	1.20 $\pm$ 0.51
JMR	(5)	3.64 $\pm$ 0.48
8812	(3)	0.83 $\pm$ 0.39
MCH6	(9)	24.40 $\pm$ 1.92
MCH49	(2)	24.45 $\pm$ 4.45
WP09	(5)	4.30 $\pm$ 1.93
LNCaP	(4)	481.03 $\pm$ 46.31
HeLa	(8)	0.25 $\pm$ 0.17

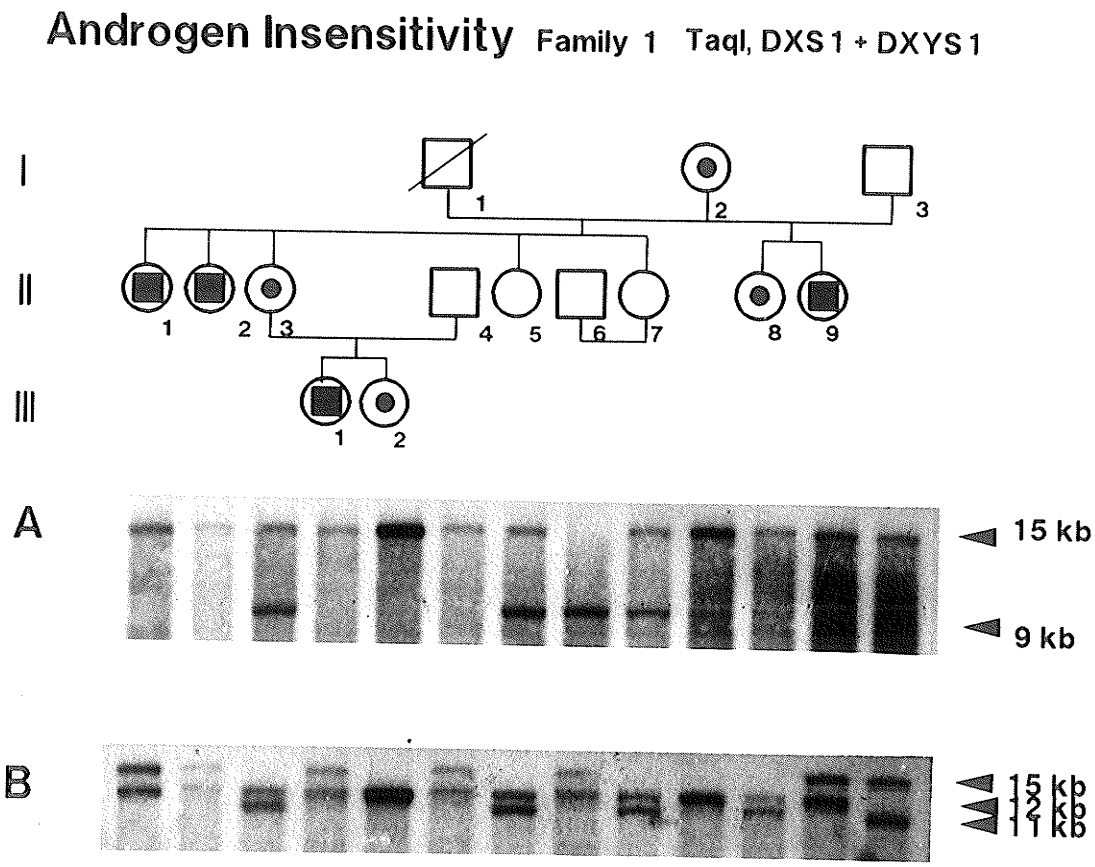


Figure 11 - Full pedigree of the studied kindred (top) in which the proband is III-1. Southern blot of *TaqI* digested genomic DNA of the kindred members probed with the anonymous DNA sequences (A) *DXS1* and (B) *DXYS1*. Polymorphic band distribution of the 15 and 9 kb X-chromosome alleles at the *DXS1* locus. At the *DXYS1* locus, the 15 kb allele is a constant Y-chromosome specific band, while the polymorphic X-chromosome alleles are represented at 12 and 11 kb.

Table VI - Linkage analysis with loci, *DXS1:AR* and *DXYS1:AR*, of Family 1 and Family 2; the results represent hand-calculated lod scores.

		DXS1:AR	ϕ	*DXYS1:AR*	ϕ
Family 1	1 phase known meiosis 1 NR: 0 R	0.301	0.0	0.301	0.0
Family 2	3 phase unknown meioses 3 NR: 0 R	0.602	0.0	0.602	0.0
Previous published lod scores		6.7	0.06	0.76	0.0
Lod score total		7.603	0.06	1.663	0.0

Note: For both markers, the 95% confidence limits have not been calculated;
ϕ = recombination frequency; NR = non-recombinant; R = recombinant

reported lod scores for the markers (Table VI).

In order to resolve the problem of determining the carrier status in the three potential carriers, we decided to screen for other possible RFLPs using a spectrum of restriction enzymes and the 0.7 kb fragment of the androgen receptor cDNA as a probe (see Figure 10) until we found one which rendered the obligate carrier grandmother (I-2) heterozygous or informative. We found an RFLP with the enzyme *Msp*I. A Southern blot which included the entire kindred was prepared. Figure 12(A) shows the autoradiogram of the blot probed with the 0.7 kb AR cDNA. Two RFLP patterns are apparent on this blot (and were also present on repetitions of three other Southern blots with separate *Msp*I digests). The fragment sizes are approximately 4.7(A1), 4.55(A2), 3.7(A3), and 3.55(A4) kb. The pattern indicated that the A2 and A4 bands were segregating with the disease (4.55 and 3.55 kb), and on that basis we could determine the carrier status of the three remaining daughters in this family. II-5 and II-7 are not predicted to be carriers and II-8 is predicted to be a carrier of the mutant gene using this analysis.

To determine the frequency of the *Msp*I RFLP in a control population sample, Southern blots were prepared from control persons containing 79 unrelated X-chromosomes. Of these, 22 were from Hutterite controls, which may not be considered totally unrelated as the Hutterites have a very small founder population (Lewis *et.al.,* 1985). The control population was random, with both sexes equally represented. The polymorphism was found to be unique to our kindred, as it was not seen in our control population.

In order to further localize this RFLP, we cut the 0.7 kb AR cDNA into two fragments (0.3 and 0.4 kb) with the restriction enzyme *Dde*I (Figure 10). This allowed us to localize the RFLP pattern, with the downstream 0.4 kb probe, to an area including the three exons 4, 5 and 6, which encode part of the hormone binding domain of the androgen receptor. We then amplified these three exons from two affected patients (II-

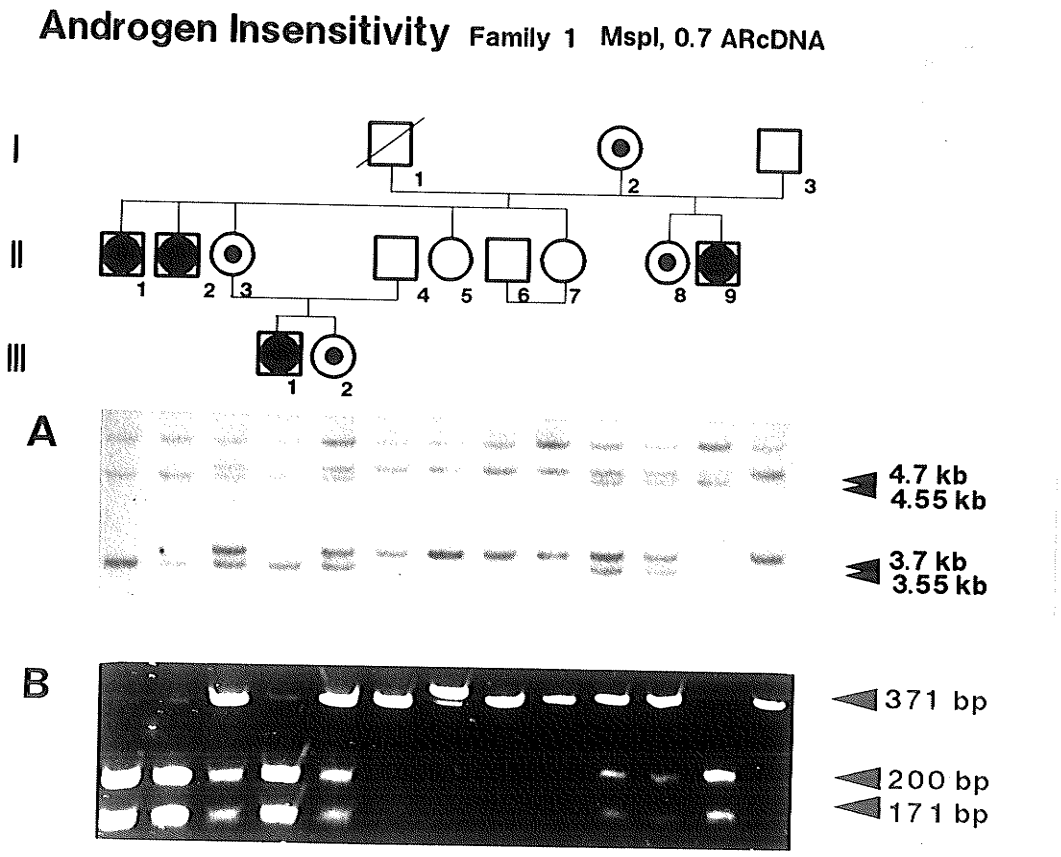


Figure 12 - *Msp*I polymorphic pattern. (A) Southern blot of *Msp*I digested genomic DNA probed with 0.7 kb h-AR cDNA (Figure 10). (B) Polyacrylamide gel of *Msp*I digested PCR amplified exon 4 of the kindred members. Gel stained with ethidium bromide.

2 and II-9) using PCR. Upon sequencing, a point mutation was detected in exon 4 of both patients, while the other two exons were the same as published for the wild-type androgen receptor (Chang *et.al.,* 1988b). The mutation was a single base substitution (T->C) at nucleotide 2558, which results in the creation of a new *Msp*I site (Figure 13). The appearance of four separate alleles from a single mutation will be further discussed below. This transition mutation, which has not been previously described to our knowledge, replaces Leu 676 with Pro at a site which is conserved in numerous members of the steroid receptor supergene family (Evans, 1988), including receptors for androgen, progesterone, glucocorticoid, mineralocorticoid, and estrogen (Figure 14, sequence from Chang *et.al.*, 1988).

The PCR products from exon 4 were then amplified for all members of this kindred, digested with *Msp*I, and run on an 8% mini-polyacrylamide gel in order to confirm the carrier status of the daughters. The pattern seen in Figure 12(B) confirms our previous carrier assignments on the basis of the Southern blot analysis.

(v) Discussion

A large Hutterite kindred with complete androgen insensitivity syndrome was ascertained after the proband (III-1) presented with an inguinal hernia, found to contain testicular tissue. Chromosomal analysis confirmed an XY karyotype. Androgen binding studies on the proband's genital skin fibroblasts revealed no androgen binding activity.

RFLP linkage analysis was performed with the anonymous DNA sequences from *DXS1* and *DXYS1*, which allowed carrier detection in one informative branch of the family, but was uninformative for a majority of potential carriers. The proband's sister (III-2) was tentatively assigned as a carrier of the mutant gene. Analysis of the proband's DNA by Southern blot analysis with the complete androgen receptor cDNA probe did not reveal any major deletion or gene rearrangements. Furthermore, the mRNA of the proband was normal by Northern analysis (Figure 15).

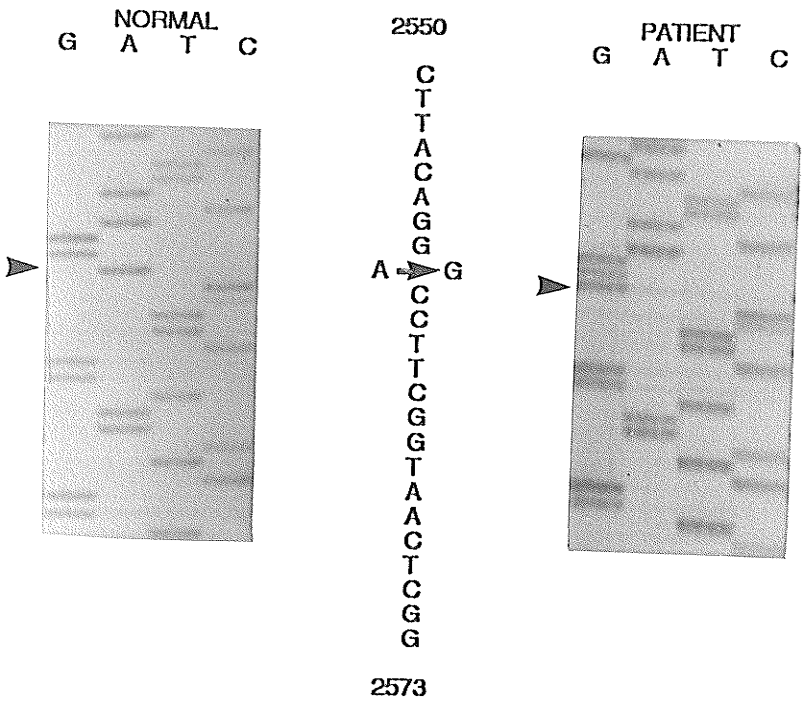


Figure 13 - The complementary dideoxynucleotide sequence of 2550 to 2573 bp from normal control DNA compared to that of the patient. Arrows mark the A(T) → G(C) transition mutation.

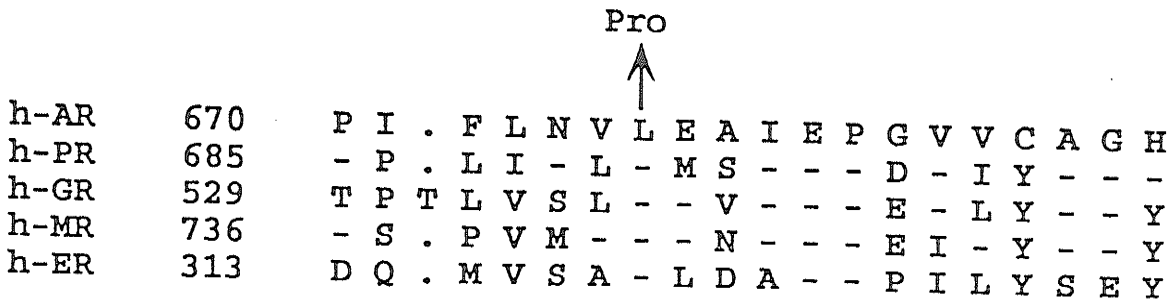


Figure 14 - The conserved amino acid sequences of members of the steroid receptor family in the hormone binding regions. The receptors are androgen (AR), progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR), and estrogen (ER). Position 676 of the h-AR (Leu 676 → Pro is our mutation) is conserved in all the receptors shown.

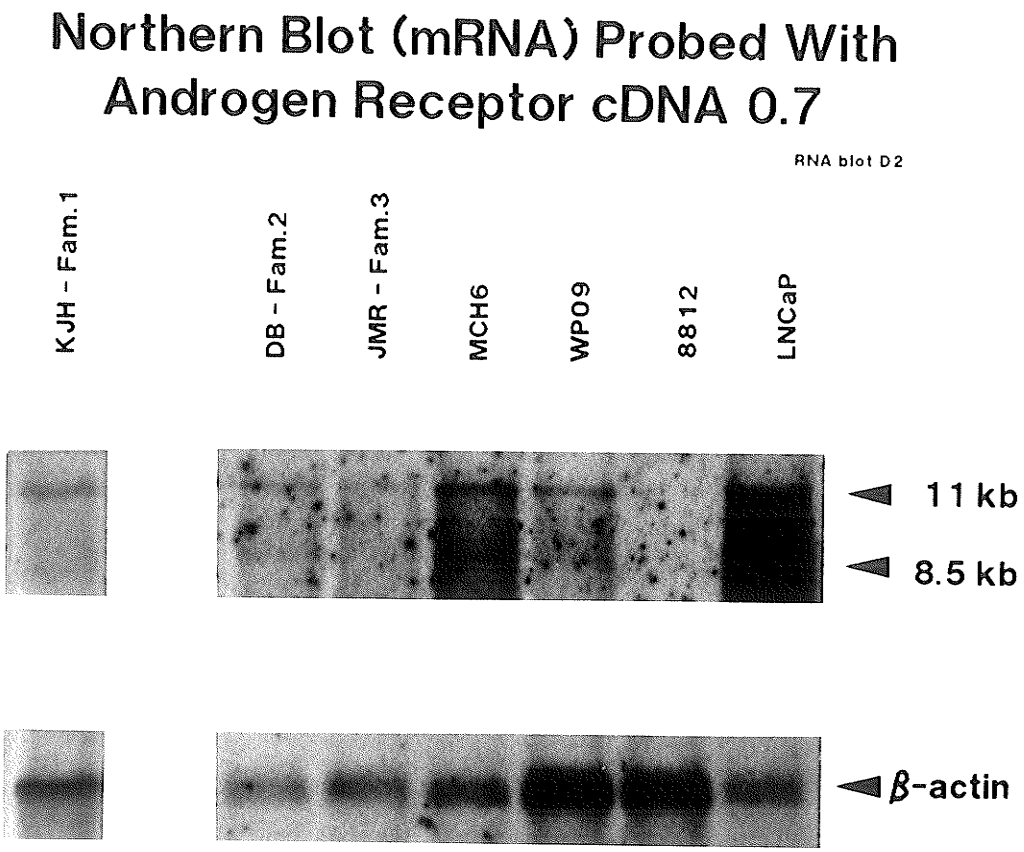


Figure 15 - Northern analysis of the androgen receptor mRNA extracted from the genital skin fibroblasts of the two CAIS probands (KJH (Family 1) and DB (Family 2)), the proband JMR of the PAIS Family 3 and a normal individual (MCH6); from the non-genital skin fibroblasts of a normal individual (WP09); and from LNCaP (a human cell line that overproduces the AR). The AR mRNA was detected with the h-AR 0.7 kb used as a probe (Figure 10). Actin (2 kb) was measured with a full-length chicken ß-actin cDNA probe.

Because carrier assignments in this kindred were requested for the other three potential carriers, all in the child-bearing age group, a search for RFLPs using the androgen receptor cDNA as a probe was undertaken. This screening, which included 19 restriction enzymes, revealed a novel *Msp*I polymorphism pattern that cosegregated with the disease, and confirmed the previous carrier assignments. The approximate band sizes are 4.7/A1, 4.55/A2, 3.7/A3, and 3.55/A4 (Figure 12(A)). There is one additional daughter in this kindred living in another city that has the option of knowing her carrier status in the future if she so chooses.

The four polymorphic bands on the Southern blots were hybridizing inconsistently and the size differences between A1/A2 and A3/A4, respectively, were very close, suggesting they might be the result of the same mutation. To investigate that this polymorphic pattern may not be a true RFLP, but rather due to an incomplete digestion, we did a time course digestion experiment.

In order to determine which side of exon 4 was responsible for this pattern, the exon 4 PCR amplified product was first digested with *Msp*I and the two fragments (171 and 200 bp) were used as probes to determine if the polymorphism could be seen with both probes. Figure 16(A) demonstrates the fragment sizes used and the results. The 171 bp fragment only detected the 4.7/A1 and 3.7/A3 bands in the normal individuals and carriers, as expected, but the 200 bp fragment detected all four of the bands as was seen in Figure 12(A) (data not shown), indicating that the problem was upstream of exon 4.

This result could be explained by an *Msp*I site in intron IV which does not cut as readily as the other sites, which is called *Msp*I "partial" in Figure 16(A), since the sequenced exon 4 did not have any other MspI sites. In order to investigate the possibility of an MspI partial digest, a time course digestion with MspI was undertaken. The results indicate that after long digestion times (72 hr) with excess amounts of restriction enzyme (100 units/μg total) the larger polymorphic pair disappears and the 3.7/3.55 kb pair remains (Figure 16(B)), indicating that there is a site about 1000 bp

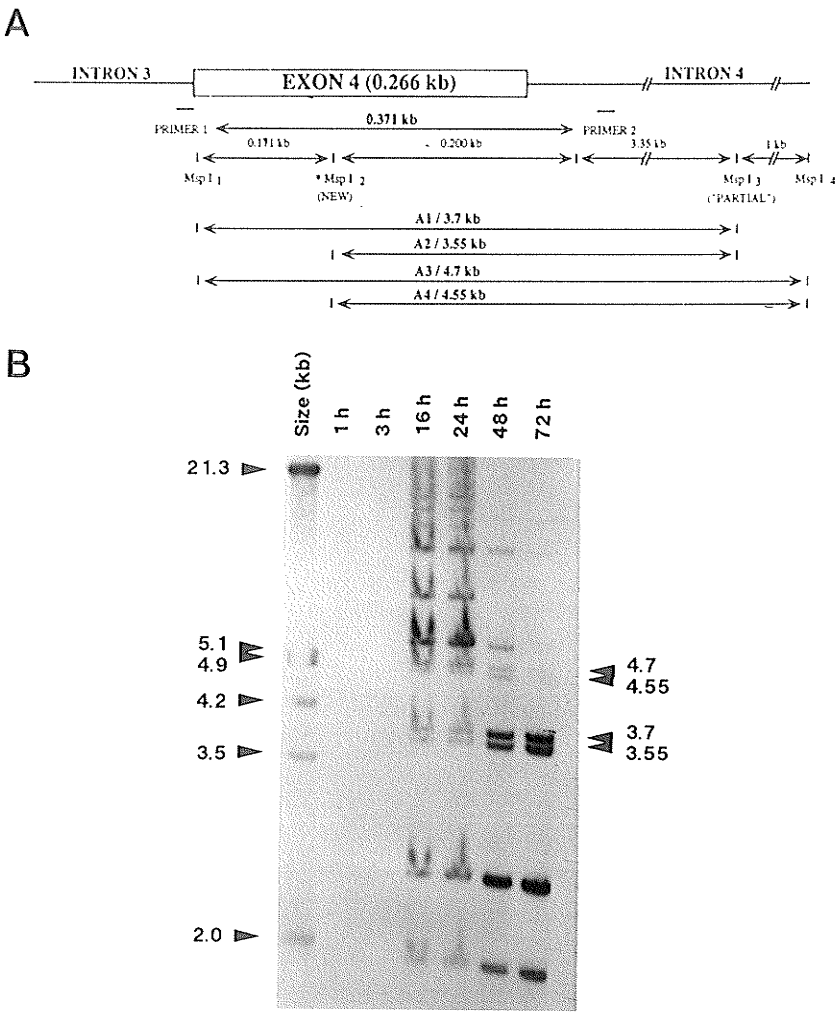


Figure 16 - (A) Diagrammatic representation of the assumed *MspI* sites in the genomic DNA of the patients, which explains the polymorphic pattern. If the "partial" *MspI* site does not completely digest the DNA, then all four bands are present ie. 4.7/A1 kb, 4.55/A2 kb, 3.7/A3 kb, and 3.55/A4 kb. (B) Southern blot of *MspI* digested carrier DNA probed with the 0.7 kb AR cDNA. Upon longer digestion times and increased amounts of enzyme the upper bands, 4.7/A1 and 4.55/A2 kb, disappear and appear as the lower bands 3.7/A3 and 3.55/A4 kb only.

upstream in the intron which does not digest properly (see Figure 16(A)) and mimics a true RFLP. Partial digestion is notorious for *Msp*I (Camerino *et.al.,* 1985). This phenomenon might explain other similar *Msp*I RFLP patterns documented in other publications such as Greenberg *et.al.* (1990), but detailed analysis of the pattern and probable cause were not presented.

The *Msp*I polymorphism was not detected in 79 unrelated X-chromosomes of which 22 were from Hutterite controls. The Hutterite control group may not all be totally unrelated as the founder population consisted of an estimated maximum of 124 ancestral genomes (Lewis *et.al.,* 1985). The Manitoba Hutterites are *Schmiedeleut*, which is a branch of the Hutterite Bretheren, a religious, socioeconomic, communal isolate (Lewis *et.al.,* 1985). There is a higher occurrence of many genetic disorders in this community, some of which have been published (Bowen, 1985; Hostetler, 1985), and androgen insensitivity is not included in the published list of prevalent disorders found in this inbred population. To date, this RFLP pattern and mutation has not been described in other Hutterite communities (Bowen, 1985), but this may be due to the relatively recent cloning of the androgen receptor cDNA in 1988. The maternal grandmother (I-2) in our kindred therefore probably represents a new mutation, as her extended family history is negative. Unfortunately, additional screening of her extended family is not possible.

In order to determine the molecular lesion in this family, we were able to localize this polymorphism to exons 4, 5, and 6 using a partial androgen receptor cDNA described previously. Part of the hormone binding domain of the androgen receptor is encoded by these three exons. Upon amplification of these exons using PCR, followed by sequencing, a mutation was detected in exon 4. Exons 2, 3 and 5 to 8 were sequenced and no difference from the published sequence was detected.

A single base substitution replacing a thymidine with a cysteine (T->C) was the novel mutation, which creates a new *Msp*I site at position 2558 in the cDNA. This transition mutation, which has not been previously described, replaces Leu 676 with Pro

at a site that is conserved throughout the evolution of the various steroid receptors (Figure 14). The high conservation of the DNA binding and hormone binding domains in the members of the steroid/thyroid/retinoic acid supergene family has been described by numerous investigators (Chang *et.al.*, 1988a; Evans, 1988). The receptors for androgen, progesterone, glucocorticoid, mineralocorticoid, and estrogen, all have this Leu 676 at the conserved position, which implies an important role of this amino acid in the normal function of the receptor. If this amino acid is mutated, as in these patients, a defective protein is present, which is likely the cause of the androgen insensitivity in this kindred. The deleterious consequences of a change of a Leu or any other amino acid to a Pro has also been described (MacArthur, Thornton, 1991), as proline is known to be a "helix-breaker" because of its imino acid structure.

In order to determine if the base change in exon 4 is the disease-causing mutation in this kindred, expression of the mutant construct will be performed in a transfected mammalian cell system. If the specific androgen binding activity dissappears when this single mutation is introduced into the normal androgen receptor construct (Brinkmann *et.al.*, 1989), this will be considered proof that the base change is responsible for the complete androgen insensitive phenotype in the kindred. Complete sequencing of all the eight exons of the androgen receptor gene, along with the 5' and 3' regulatory regions, will be undertaken if there is no change in specific androgen binding in an attempt to find another mutation.

The carrier assignments in the kindred are reliable whether the mutation is disease-causing or not. If the mutation is the direct cause of androgen insensitivity, then the carrier assignments are definite. Another mutation in the AR gene causing the phenotype would not affect the carrier assignments unless recombination had occurred within the androgen receptor gene itself. Because the gene is only 90 kb, the probability of a cross-over within this region is negligible, but not impossible. Thus, if we consider linkage analysis 95% reliable with markers located much further away, then one should have no problem accepting the carrier assignments based on an RFLP within the gene itself.

The results presented here demonstrate the use of a novel *Msp*I RFLP pattern to predict the carrier status in a Manitoba Hutterite AIS kindred. In the process of carrier testing, we were able to localize the pattern to a region which included part of the hormone binding domain and subsequently found a mutation in exon 4 creating a new *Msp*I restriction enzyme site. To explain the complementary pairs of alleles in the RFLP pattern, we demonstrated the problem of incomplete digestion that may occur when using the *Msp*I enzyme and longer digestion times with increased enzyme concentration are recommended. This mutation is likely the cause of the disorder in this kindred. Although this is not conclusive, the evidence of a deleterious amino acid change at a highly conserved residue predicts such a consequence.

B. Family 2 with complete AIS: Detection of an amber (termination) mutation creating a diagnostic *Mae*I site.

(from Trifiro *et.al.*, 1991b)

(i) Abstract

Herein we describe a family with complete AIS with affected individuals in two generations. Early studies on this family indicated cosegregation of the mutant phenotype and the RFLPs at the loci *DXS1* and *DXYS1*. Androgen binding activity in the proband's genital skin fibroblasts was negligible, although normal levels of androgen receptor mRNA were detected. With the collaboration of the laboratory of Dr. L. Pinsky, a single nucleotide substitution in the X-linked androgen receptor gene was discovered. The mutation is an adenine-to-thymine transversion in exon 8 that changes the sense of codon 882 from lysine (AAG) to amber (UAG) translation termination signal, predicting a truncated receptor lacking 36 amino acids at the carboxy terminus of its 252-amino acid androgen-binding domain. Thus, the mutation is probably the cause of complete AIS in this family.

(ii) The subject and family

The family was ascertained thrice by the laboratory of Dr. L. Pinsky. Firstly when the proband (III-2 in Figure 17) appeared as a 12-year-old with a lump in the left groin after a history of left inguinal herniorrhaphy in infancy, then as an adult when she was

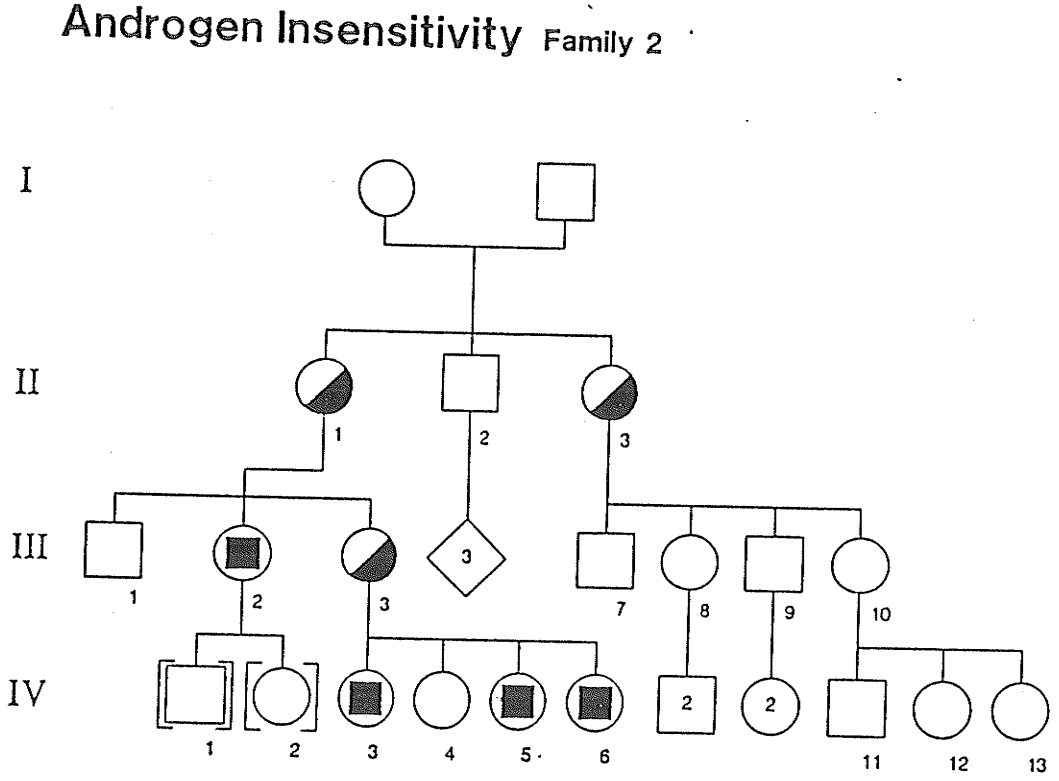


Figure 17 - Full pedigree of Family 2 in which the proband is III-2 (from Trifiro *et.al.*, 1991b). Circle with filled square, CAIS.

contacted for further studies after her first cousin (III-10) was referred for genetic counselling, and finally through three affected maternal nieces (IV-3, IV-5, IV-6). Negligible specific androgen-binding activity in her nongenital skin fibroblasts was discovered, following a report by Keenan *et.al.* (1974), but because of lost contact, this finding could not be confirmed, until more recently, in her genital skin fibroblasts. Using the 20-year-old nongenital skin fibroblast line from her deceased mother (II-1), we can demonstrate heterozygousity at the androgen receptor locus by molecular genetic techniques.

(iii) Supplementary methods

PCR amplification of genomic exons 2-8

(done in the lab of Dr. L. Pinsky, McGill University, Montreal)

This was performed essentially as described by Saiki *et.al.* (1988) using sets of intronic primers (Table I) whose composition was given to us before publication by J. Trapman and A.O. Brinkmann, Erasmus University, Rotterdam, The Netherlands. Those that bracket exon 8 are shown in Figure 21. Each 100 µl reaction mixture contained PCR buffer, 0.01% gelatin, 20 nmol each dNTP, 100 pmol of each primer, 1 µg DNA, and 2.5 units of *Taq* polymerase, and was covered with 100 µl of mineral oil. The reaction was carried out cyclically with denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. At the end of the 35th cycle, the reaction was incubated at 72°C for 7 min. The PCR products were extracted with chloroform and then used for direct sequence analysis or to demonstrate *Mae*I sensitivity of mutant exon 8 by analysis of the digestion products in an 8% polyacrylamide gel.

Direct DNA sequencing

(done in the laboratory of Dr. L. Pinsky, McGill University, Montreal)

PCR-amplified DNA was purified by electrophoresis on low melt LMP agarose (BRL, Gaitherburg, MD). The primary product was excised and stored at 4°C, or melted before removal of a 4 µl sample. One or another of the primers (2 pmol) used in the PCR was ^{32}P-labelled and added to the sample. The mixture was heated to 95°C for 5 min then allowed to cool to 50°C. Sequencing reactions using Sequenase (United States

Biochemical Corp., Cleveland, OH) were initiated exactly as described (Higuchi *et.al.,* 1988), except that the incubation temperature was 50°C, and Mn^{2+} (final concentration, 2 μM) was added to the sequencing buffer (Tabor, Richardson, 1989).

(iv) Results

Genital skin fibroblasts of the proband had negligible specific androgen-binding activity in accord with the clinical diagnosis of complete androgen insensitivity (Table V). In the same assay, a normal genital skin fibroblast strain had a B_{max} of 40 fmol/mg protein (normal, 20-45 fmol/mg protein) with K_d of 0.1 nM (normal, 0.1-0.25 nM), and one subject with partial androgen insensitivity had 25 fmol/mg protein with a K_d of 0.7 nM.

Figure 18 shows a partial pedigree, wherein the proband is III-2, and the results of Southern analysis with the probes p8 and pDP34 on genomic DNA digested with *Taq*I. It is apparent that the mutant phenotype segregates with the 9-kb allele as detected by probe p8 at the *DXS1* locus (Figure 18(A)). Yet II-3, the maternal aunt of the proband, could not be assigned carrier status even though she is heterozygous for the 9-kb and 15-kb alleles at the locus, as we cannot predict the origin of her X chromosomes from the members studied. On the other hand, the mutant phenotype segregates with the 11-kb allele at the *DXYS1* locus as detected by pDP34 (Figure 18(B)), and II-3 is homozygous for that allele. This result increased the suspicion that she was a carrier. In contrast, both sets of results indicated that IV-4 was not a carrier. Again, as for the linkage analysis for Family 1, we are able to add positive information to the previously published lod scores for both *DXS1* and *DXYS1* (Wieacker *et.al.,* 1987; Imperato-McGinley *et.al.,* 1990). In Family 2, there are three informative meioses, phase unknown; thus, we add 0.601 to the previously published lod scores for both of the markers (Table VI).

We were able to analyze the androgen receptor gene of the proband in a number of ways, particularly using Southern analysis. Using cDNA probes recognizing the DNA- and androgen-binding domains, we utilized Southern analysis to show that genomic DNA extracted from the proband's genital skin fibroblasts yielded normal patterns with the

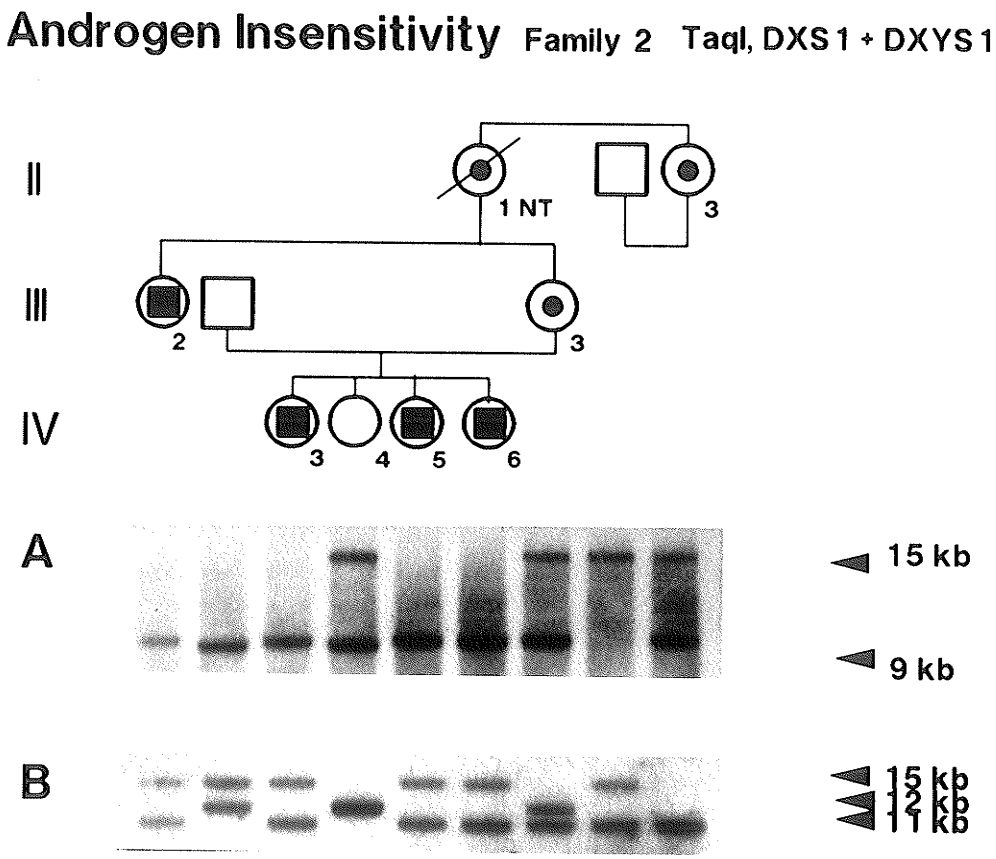


Figure 18 - A partial pedigree in which all relevant individuals are identified numerically exactly as in Figure 16 (top). The distribution of the 9 and 15 kb alleles at the *DXS1* locus (middle). The distribution of the 11, 12 (X-chromosome) and 15 kb (Y-chromosome) alleles at the *DXYS1* locus. Individual II-1 was not tested. (From Trifiro *et.al.*, 1991b)

restriction enzymes *Eco*RI and *Taq*I (Figure 19). She had the common 7-kb allele of the *Hind*III dimorphism (Brown *et.al.,* 1989) (Figure 19(A)); and the results of the concurrent studies made it unnecessary to further analyze other family members for this RFLP.

Using Northern analysis (Figures 15 and 20), we found a normal amount of 10-kb androgen receptor mRNA in the proband's genital skin fibroblasts (DB). This demonstrated that the gene itself, and its transcription were intact, although subtle differences can not be detected in this manner.

In the laboratory of Dr. L. Pinsky, direct sequencing of the PCR-amplified genomic exons 2-8 revealed a unique sequence alteration in exon 8 (Figure 21), which represented an adenine-to-thymine transversion at codon 882 that changes its sense from lysine to a translation termination signal of the amber type.

The nucleotide substitution predicted the creation of a recognition sequence for the restriction enzyme *Mae*I. To test the prediction by PCR analysis and to determine whether the maternal aunt of the proband was heterozygous, the PCR-amplified exon 8 from the proband, her sister, her mother and her maternal aunt was subjected to *Mae*I digestion and subsequently analyzed by PAGE in Dr. Pinsky's laboratory. The normal exon 8 is *Mae*I-resistant, whereas exon 8 from the proband is totally cleaved into the two expected fragments, and that her mother, aunt, and sister have both the normal and mutant types of exon 8. Using this analysis, III-8 and III-10 have not been found heterozygous for the androgen receptor gene mutation; thus, the previous RFLP analysis (Figure 18) implying that IV-4 is also not a carrier was confirmed.

(v) Discussion

Using standard molecular-genetic technology, we have been able to define the molecular cause of complete androgen insensitivity in two generations of a Canadian family, originating in Manitoba. The presence of normal androgen receptor mRNA and the absence of specific androgen-binding activity in the genital skin fibroblasts of the proband

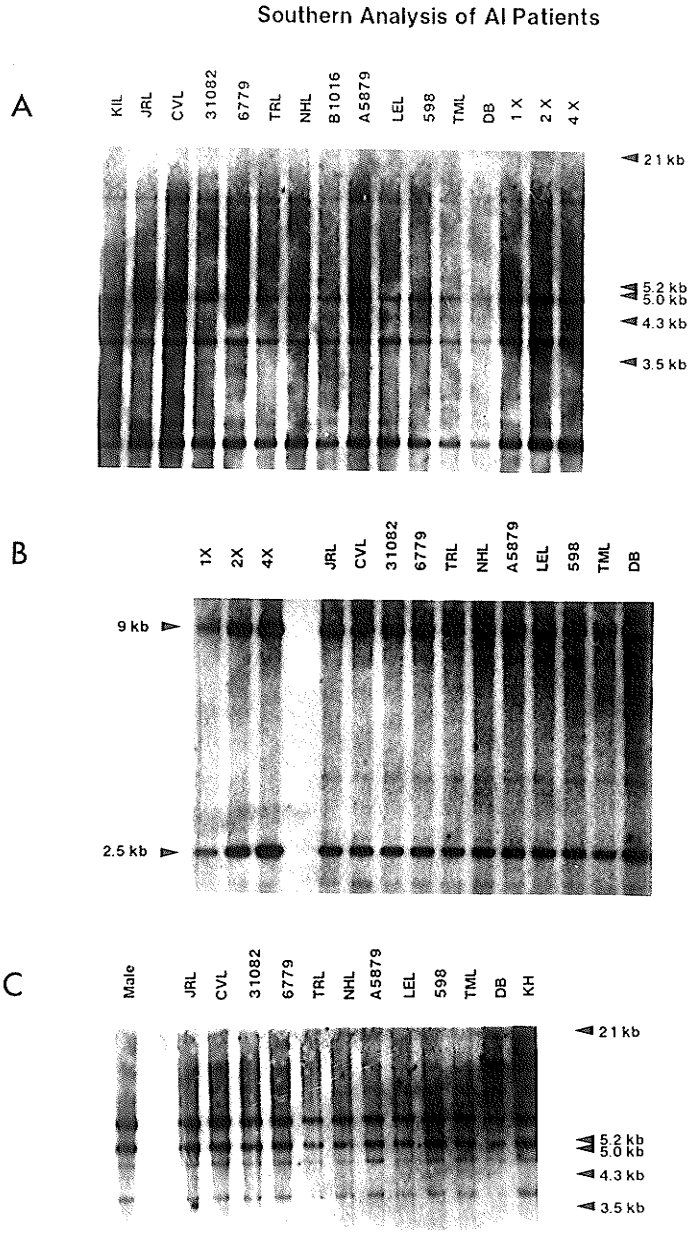


Figure 19 - Southern analysis of genomic DNA from a number of CAIS patients with the restriction enzymes (A) *Taq*I; (B) *Eco*RI; and (C) *Bam*HI. The proband III-2 (DB) of Family 2 and TML are discussed further in this thesis. No large deletion or gene rearrangement was detected. Note that with *Bam*HI, the genomic DNA of DB was not completely digested and upon redigestion followed by Southern analysis, the extra band disappeared. Note: molecular weight markers indicated in (A) and (C). ((A) and (B) with permission of T. Nylen)

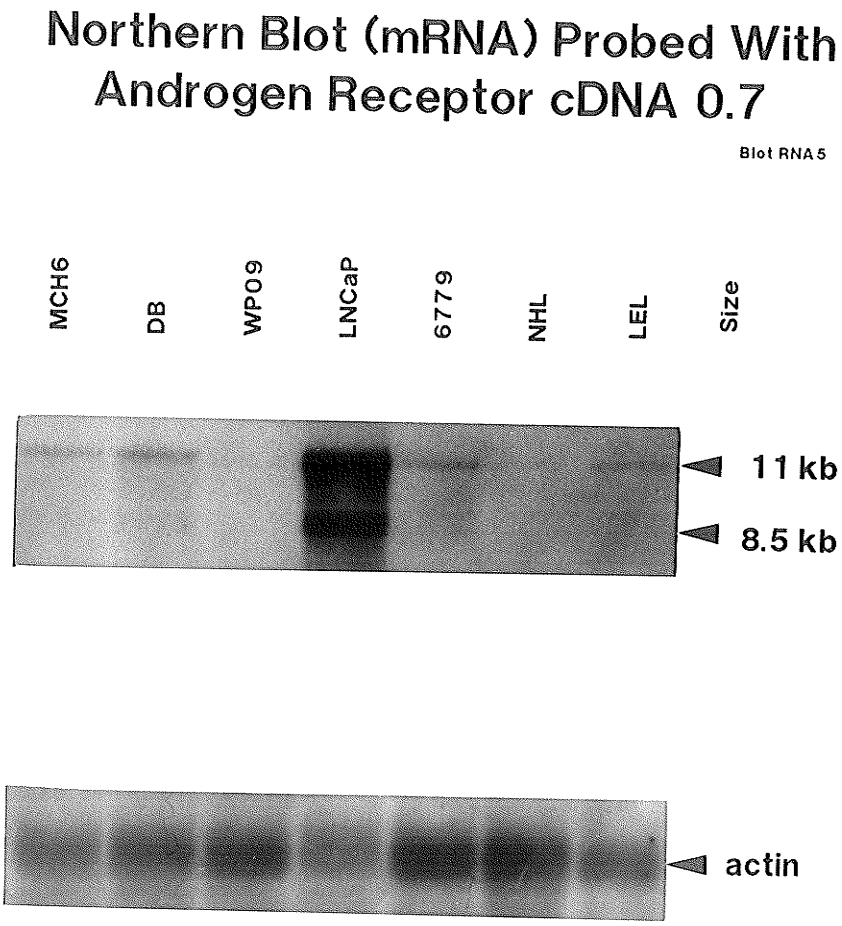


Figure 20 - Northern analysis of the androgen receptor mRNA from genital skin fibroblasts of a normal control (MCH6), two studied CAIS individuals, LEL and 6779, the proband DB of Family 2, and NHL. WP09 is a control non-genital skin fibroblast line that expresses the AR weakly, LNCaP overexpresses the AR. AR mRNA was detected with the h-AR 0.7 kb used as a probe (Figure 10). Actin (2 kb) was measured with a full-length chicken ß-actin cDNA probe. (With permission of T. Nylen from Prior *et.al.*, 1991 (submitted))

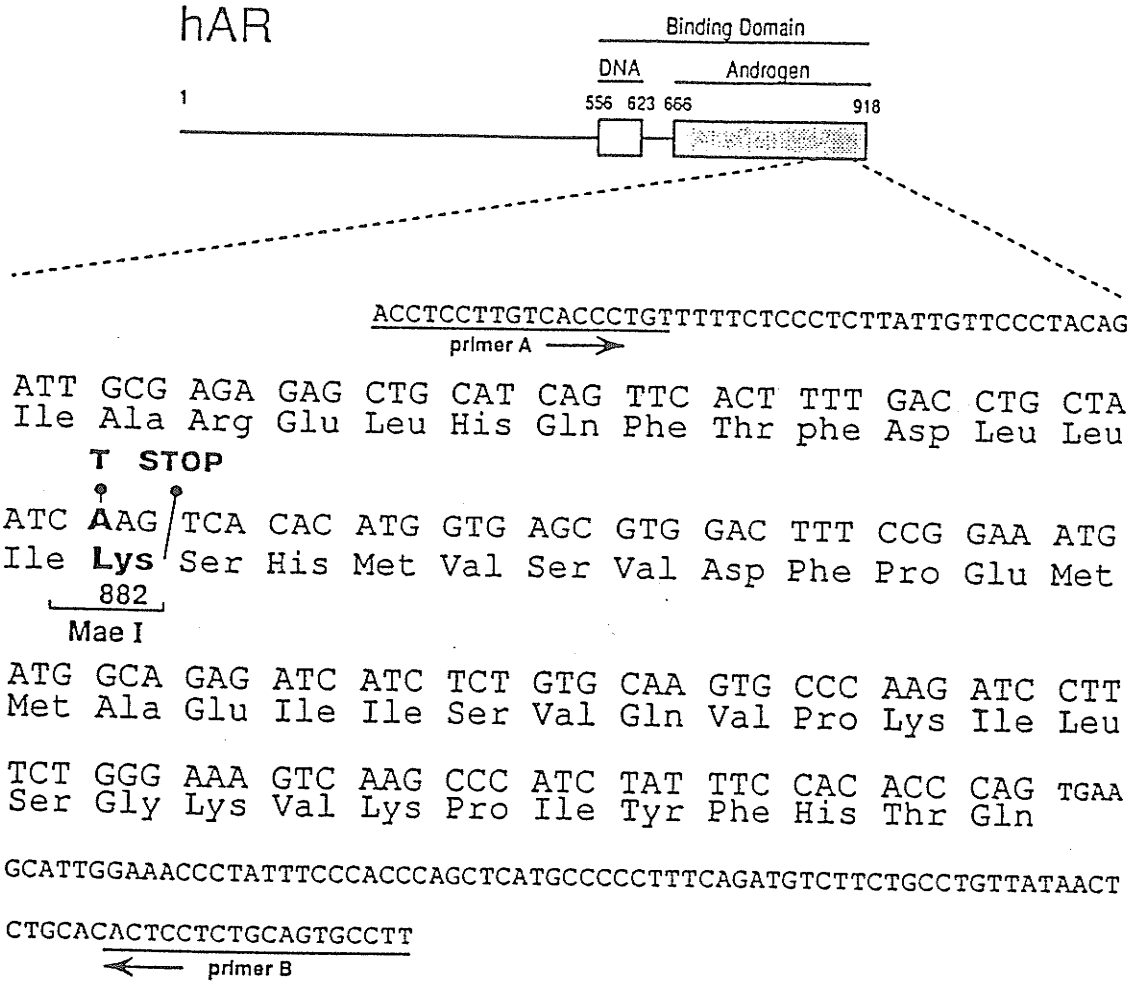


Figure 21 - Location and context of the Lys882 stop mutation in the AR, and in the nucleotide and amino acid sequences of exon 8 of the AR gene. The flanking primers used for PCR are indicated by underlines and arrows. The A→T transversion and the Lys→ter substitution at codon 882 are indicated in bold face type. The *MaeI* site created by the mutation (CTAG) is identified by the bracket connecting codons 881 and 882. (From Trifiro *et.al.*, 1991b)

suggested a missense or nonsense mutation in the coding portion of the androgen receptor gene. By sequencing exons 2-8, the exons encoding the DNA-binding and androgen-binding domains of the androgen receptor, Dr. Pinsky's laboratory was able to find a single nucleotide substitution that is the putative pathogenic mutation of the androgen receptor gene, that being an A->T transversion at codon 882 that changes its sense from lysine to an amber translation stop signal.

We can not rule out the small chance of another stop mutation or any other deleterious mutation in exon 1, the exon encoding the relatively long N-terminal domain of the androgen receptor (Faber *et.al.,* 1989), but it is worth noting that there is considerable evidence that all parts of the steroid binding domain contribute to normal steroid binding by a steroid receptor (Carson-Jurica *et.al.,* 1990). For example, C-terminal truncations removing as little as 5 or 14 amino acids from the glucocorticoid receptor seriously impair its hormone-binding affinity (Rusconi, Yamamoto, 1987). Furthermore, removing 29 amino acids from the C-terminal leaves barely detectable glucocorticoid-binding activity (Rusconi, Yamamoto, 1987). The Lys882 amber mutation in the present family seems to be similar to the latter. Likewise, a missense mutation at codon 546, close to the N-terminus of the glucocorticoid-binding domain, of the mouse glucocorticoid receptor abolishes its hormone-binding activity (Danielson *et.al.,* 1986). Pinsky's group has also shown such a mutation at the N-terminus of the androgen-binding domain in the DNA of a subject with complete AIS whose genital skin fibroblasts have negligible specific androgen-binding activity (Pinsky *et.al.,* 1990).

The androgen-binding domain of the androgen receptor shares at least 50% amino acid sequence homology with the hormone-binding domains of the receptors for progesterone (P), glucocorticoid (G), and mineralocorticoid (M) (Chang *et.al.,* 1988a). In contrast, the codons homologous to Lys882 in the androgen receptor are represented by glutamine, glutamic acid, and aspartic acid in the PR, GR, and MR, respectively. This complies with the expectation that nonsense mutations will be more likely than missense mutations to be found at positions that are evolutionarily unconserved.

The creation of a *Mae*I restriction site by this mutation has been valuable for confirming, identifying or ruling out heterozygosity for the mutant allele at the androgen receptor locus in the present family.

Previous research from the laboratory of Dr. Pinsky has determined 4 translation stop mutations among 21 different coding sequence alterations in the AR genes of 26 unrelated families with various degrees of androgen insensitivity (Pinsky *et.al.,* 1990). One, at codon 717, has been published previously (Sai *et.al.,* 1990). Other stop mutations at two different positions in the coding region of the androgen receptor gene of two unrelated patients with complete AIS have also been reported (Marcelli *et.al.,* 1990b; Marcelli *et.al.,* 1990c). The first was associated with a decreased concentration of genital skin fibroblast androgen receptor mRNA (Marcelli *et.al.,* 1990c); the other, in exon 6, was not (Marcelli *et.al.,* 1990b). The premature termination mutation reported by our group appears to mimic the latter.

C. Family 3 with partial AIS: Characterization of a subject with a less severe AIS phenotype.

(from Keely *et.al.*, 1991, J.Clin.Endocrinol.Metab. (submitted) and subsequent studies)

(i) Abstract

Herein we report a family with partial androgen insensitivity syndrome, characterized by androgen receptor abnormalities in 46, XY individuals. The failure of plasma levels of sex hormone-binding globulin (SHBG) to decline in response to exogenous androgens has been proposed recently as an in-vivo test for androgen resistance. We describe a 21-year-old male with partial androgen insensitivity, with decreased specific androgen receptor-binding activity, who was given long-term high dose exogenous androgens as a therapeutic trial for sexual dysfunction. Despite the absence of a clinical response, there was a significant reduction in both SHBG and gonadotropins suggesting both hepatic and pituitary androgen recognition, indicating that the response to androgen may vary among tissues and that the SHBG response may not predict the genital tissue response. To analyze the cause of the partial androgen insensitivity, molecular genetic studies were

undertaken. The preliminary results indicate that the underlying mutation is not in exons 4-6, encoding part of the hormone binding domain. Other molecular analyses will also be presented.

(ii) The subject and family

A 21-year-old Phillipino was referred to the Reproductive Endocrinology Clinic at the Health Sciences Centre, Winnipeg, because of a presentation of micropenis. He was initially assigned a female gender, but was subsequently raised as a male. He had not received any prior medical attention. He was married. His family history revealed multiple affected kin, most of whom were raised as males, although there was an affected phenotypic female, his sister, in Canada (Figure 22).

On physical examination, he had normal height and weight. He had scant sexual hair, bilateral gynecomastia and a decreased penile length with hypospadias. He had a short blind-ending vagina and bilateral inguinal masses. He subsequently underwent bilateral subcutaneous mastectomies and hypospadias repair. A genital skin biopsy was taken at that time. Subsequently, peripheral blood samples were obtained from the proband, his mother, his unaffected brother, two unaffected sisters, and his affected sister for DNA studies.

Initial laboratory investigations revealed a high normal total serum testosterone of 33.5 nmol/l (normal, 8-35 nmol/l), elevated serum luteinizing hormone (LH) 37 IU/l (normal, 2.4-8.0 IU/l), elevated serum follicle-stimulating hormone (FSH) >25 IU/l (normal, 1.3-5.0 IU/l) and a serum estradiol <40 pmol/l (normal, <180 pmol/l). He had a 46,XY karyotype.

(iii) Supplementary methods

Exogenous androgen treatment

(done in Dr. Faiman's laboratory, University of Manitoba, Winnipeg)

Due to his strong desire for improved sexual function and previous reports of beneficial effects of supraphysiological levels of androgens in such patients, two non-

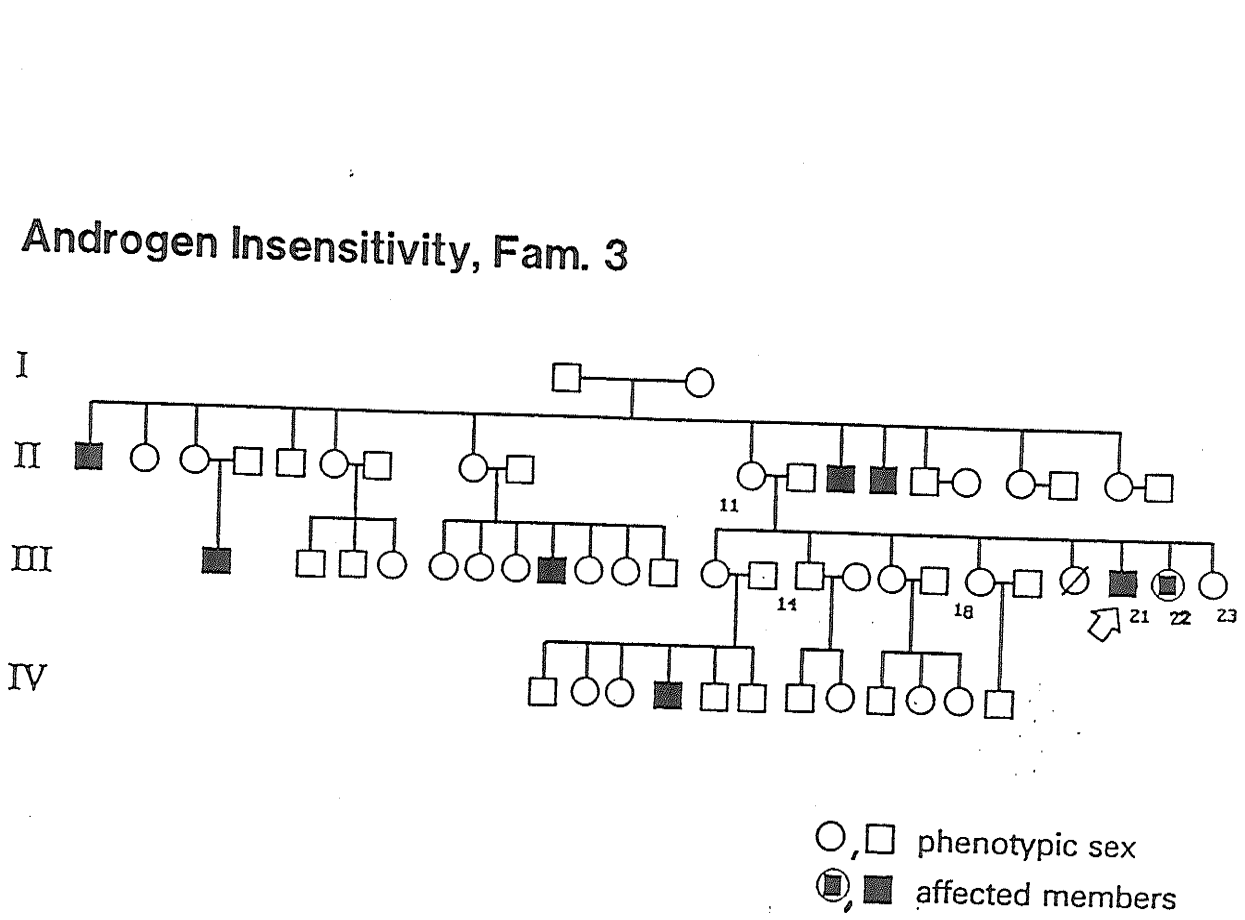


Figure 22 - Full pedigree of Family 3 with multiple affected individuals, all but one raised as males. The proband is III-21. Filled square, PAIS; circle with filled square, CAIS.

aromatizable exogenous androgens were administered (Price *et.al.,* 1984; Grino *et.al.,* 1989). Under the care of the Endocrinology Clinic, he received fluoxymesterone 10 mg orally four times daily for two weeks followed by dihydrotestosterone propionate (DHT) 100 mg im daily for four weeks after a two-month washout period. LH, FSH and SHBG serum levels were measured by commercially available solid phase fluoroimmunoassays (DELFIA Wallac Oy, Turku, Finland) before each treatment and at weekly intervals (13 samples over two hours by indwelling catheter) during treatment. All samples were stored at -20°C and run in a single assay. Approximately one year later he received fluoxymesterone 10 mg four times daily for three months with monthly measurements of LH, FSH and SHBG (single samples only). Multiple analysis of variance and Duncan's multiple range test were used where more than one value were available (i.e. first two treatments only) to detect treatment induced changes in LH, FSH and SHBG levels.

(iv) Results

At no time during androgen administration was there any change in penile length, sexual performance or signs of virilization. He did develop breast tenderness with no appreciable change in size.

Initial androgen receptor binding studies using 5 nM MT indicated a reduced binding activity in the patient's genital skin fibroblasts compared to normal genital skin fibroblasts consistent with a diagnosis of partial androgen insensitivity. In order to determine if the problem was due to a decreased binding affinity or a decreased number of binding sites, Scatchard analysis was undertaken. Figure 23 demonstrates the saturation curves for the patient (23(A)) and normal (23(B)) genital skin fibroblasts. The Scatchard analysis of these values are seen in Figure 23(C). The androgen receptor binding affinity is decreased in the patient. The patient K_d is 0.65 compared to the control K_d of 0.11. The number of binding sites available are within the normal range.

The response of gonadotropins and SHBG to exogenous androgens are shown in Figure 24. There was a significant decrease in each of these hormones following both

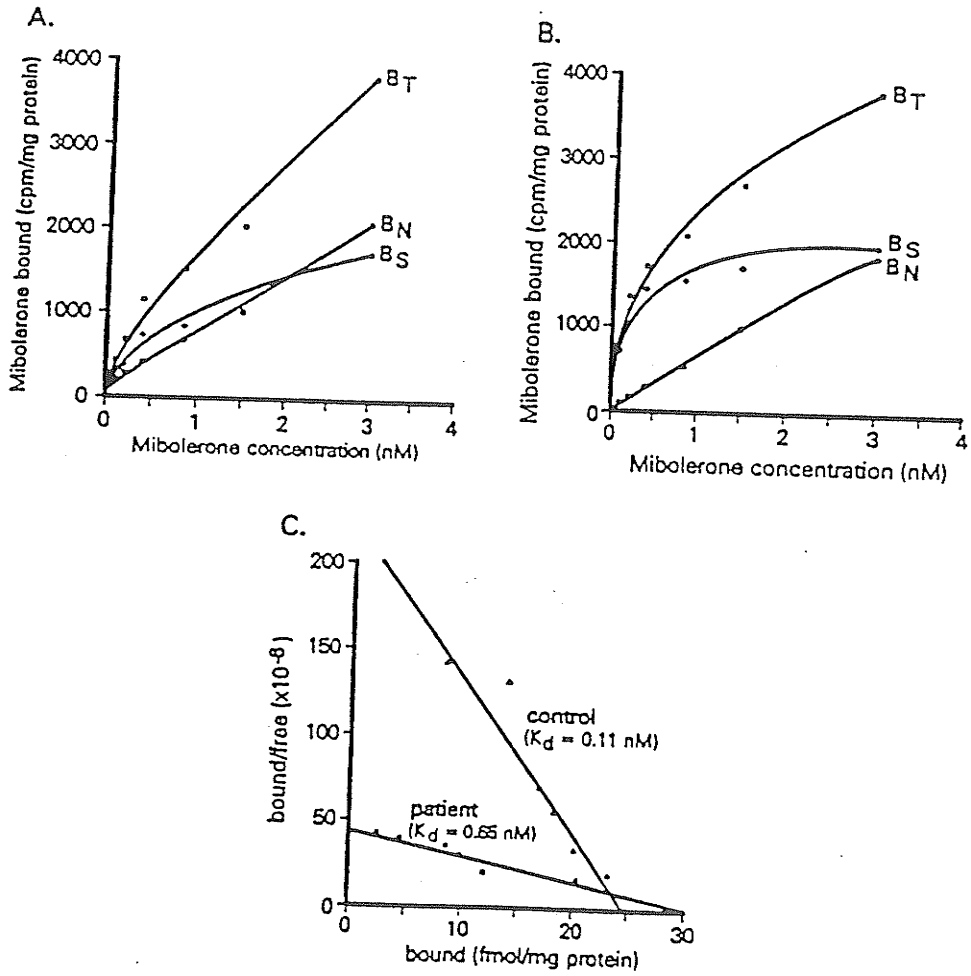


Figure 23 - Saturation analysis of [^{3}H]MB binding in the patient (A) and normal (B) genital skin fibroblasts, as represented in Michaelis-Menten plots. B_T, total bound; B_N, nonspecific binding; and B_S, specifically or saturable bound ($B_S = B_T - B_N$). (C) Scatchard plot of saturable bound [^{3}H]MB, same primary data as in (A) and (B). Each point is the average of four determinations. (From Keely *et.al.*, 1991 (submitted))

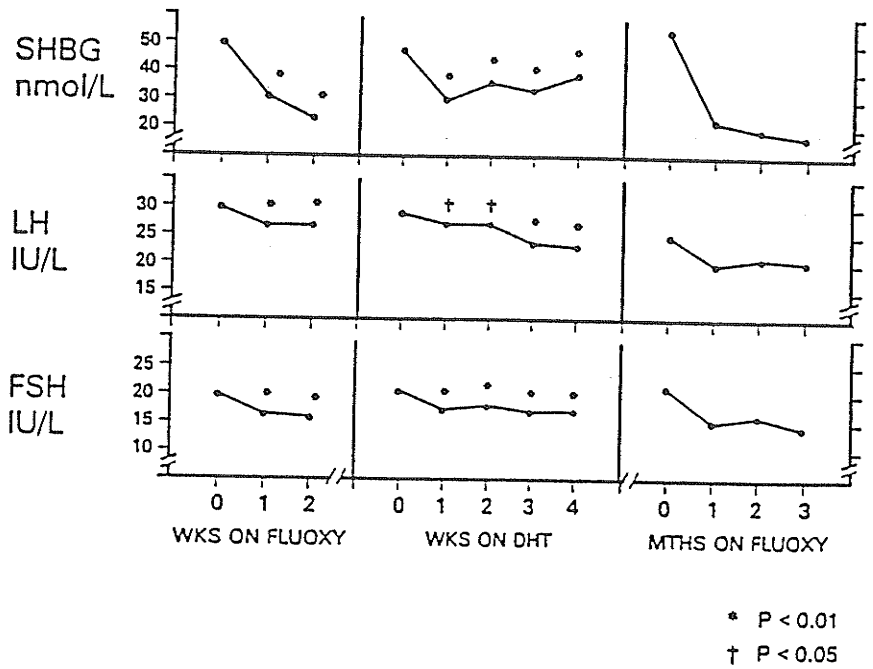


Figure 24 - Sex hormone binding globulin (SHBG) and gonadotropin (LH anf FSH) responses to fluoxymesterone (fluoxy), 10 mg orally four times daily, and dihydrotestosterone (DHT), 100 mg I.M. daily, for the duration indicated. Intervals between studies (l. to r.) - two months and approx. one year. Where sufficient data collected for statistical analysis, differences vs. mean pretreatment levels are shown (see text for details). (With permission of C. Faiman from Keely *et.al.*, 1991 (submitted))

forms of androgen administration.

Because there are two potential carrier sisters in this family, we then attempted linkage analysis with two pericentromeric DNA markers at the *DXS1* and *DXYS1* loci. The results of Southern analysis with the closely linked probes, p8 and pDP34 at loci *DXS1* and *DXYS1*, respectively, on genomic DNA digested with *Taq*I revealed that the Phillipino family did not possess the polymorphic pattern required for RFLP linkage analysis (Figure 25(A) and (B)). Probe p8 recognized only the 12 kb allele, not the 11 kb allele. Probe pDP34 recognized the appropriate Y specific fragment at 15 kb, but only recognized the 12 kb X chromosome-specific allele. This does not necessarily indicate that the RFLP pattern is not present in any members of this kindred, but simply that the two markers were uninformative for the individuals studied.

Northern analysis (Figure 15) revealed a normal amount of 11-kb androgen receptor mRNA in his genital skin fibroblasts. This demonstrated that the gene itself, and its transcription were grossly intact.

Because specific androgen-binding activity was decreased and upon Scatchard analysis, the patient was found to have decreased androgen receptor binding affinity, the cDNA encoding the androgen-binding domain of the androgen receptor protein would be the best place to begin looking for a mutation, which could be responsible for the syndrome. Exons 2 to 8 were amplified using the polymerase chain reaction (PCR), and upon sequencing of these regions from the two affected patients (III-21, III-22 in Figure 22), a different mutation was detected in exon 4 from each sibling. Because this did not seem probable, subsequent sequencing of this region revealed that both of these mutations were most likely due to misincorporation of a nucleotide at the PCR level. Exons 2, 3 and 5 to 8 were normal from both affected family members.

(v) Discussion

Androgen insensitivity either complete or partial, results from a variety of qualitative

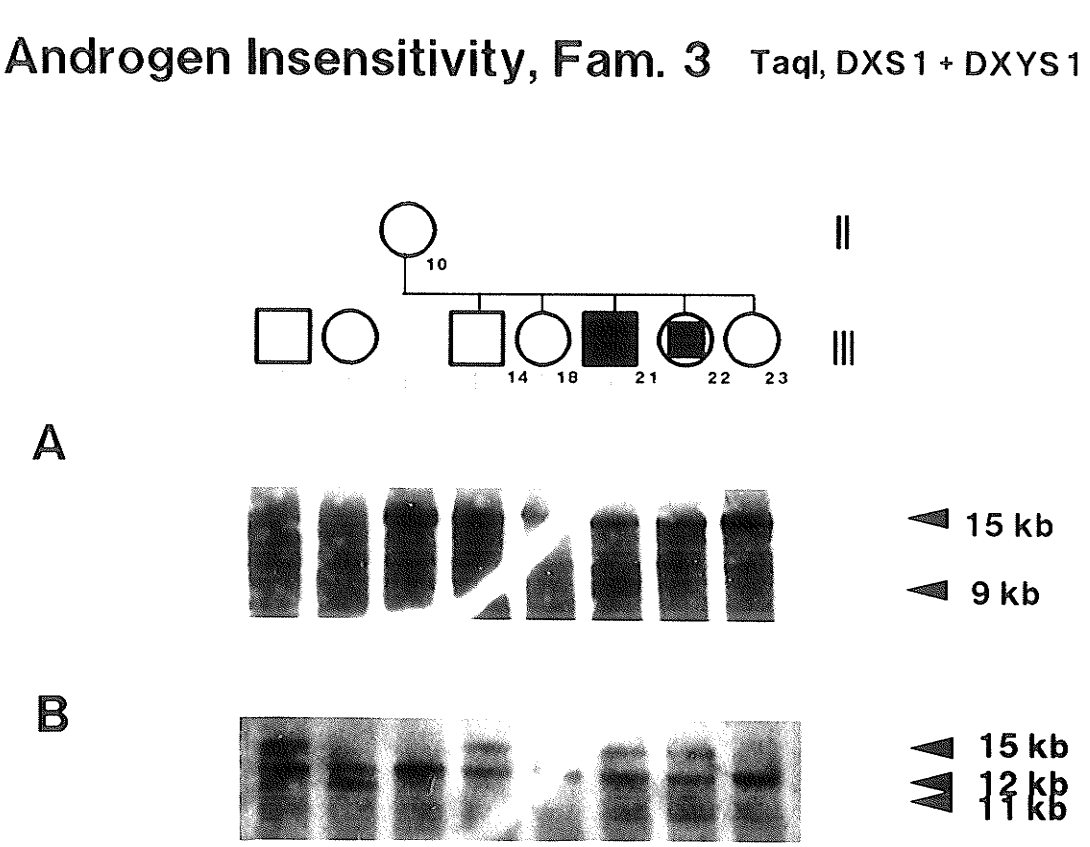


Figure 25 - The pedigree of Family 3 with the studied individuals (top). Southern blot of *TaqI* digested genomic DNA of family members probed with the anonymous DNA sequences (A) *DXS1* and (B) *DXYS1*. No polymorphic band pattern was found with either marker; thus the kindred was uninformative at these two loci.

or quantitative abnormalities of the androgen receptor which appear to be unique to each family studied.

The syndrome itself is associated with high levels of LH and sometimes FSH, with high normal or elevated testosterone levels indicating diminished recognition of the hypothalamic-pituitary axis to androgen negative feedback (Faiman, Winter, 1974). Others have failed to show a significant reduction in LH and FSH following administration of non-aromatizable androgens to subjects with complete or partial AIS (Faiman, Winter, 1974; Boyar *et.al.,* 1978; Lacroix *et.al.,* 1979). Our patient showed a significant, albeit partial, reduction of both gonadotropins in response to fluoxymesterone and DHT. This gonadotropin decline may be due to a direct effect of the non-aromatizable androgens. Alternatively, it may result from displacement of endogenous testosterone from SHBG and a negative feedback action on hypothalamic-pituitary receptors, itself, or through aromatization to estradiol (Vigersky *et.al.,* 1976).

Several recent studies investigated the usefulness of the normal lowering of SHBG plasma levels following exogenous androgens (Selby, 1990) as an "androgen insensitivity test" to aid with gender assignment in male pseudohermaphroditism (Sinnecker, Kohler, 1989; Ciacco *et.al.,* 1989; Forest *et.al.,* 1986). The data suggest a good correlation between androgen receptor responsiveness and SHBG response to androgens. However, most of the patients studied were prepubertal and evidence for such a relationship in postpubertal patients with AIS is lacking.

The presence of androgen receptors in hepatocytes has been demonstrated by immunohistochemical methods (Takeda *et.al.,* 1990) and has been characterized as a single, high affinity receptor. Although hepatic androgen receptors appear to be similar to prostate and genital androgen receptors, some unexpected discrepancies have been found, which suggest differences in the hepatic androgen receptor binding or response to antiandrogens compared to classical genital receptors (Bannister *et.al.,* 1988; Winneker *et.al.,* 1989).

Steroid hormone receptor binding results in a complicated process of target gene activation, translation and protein synthesis which is unique to individual tissues (Carson-Jurica *et.al.,* 1990). The tissue specific factors controlling gene activation are as yet unknown. Alterations in receptor binding, as seen in AIS, may therefore have a different degree of effect on individual tissue androgen responses. It is known that different androgen receptor mutations lead to a wide spectrum of clinical phenotypes of the AIS affecting different tissues to different degrees (Pinsky *et.al.,* 1990). Androgen receptor mutations in the N-terminal domain (the A/B region) have recently been implicated in X-linked spinal and bulbar muscular atrophy (La Spada *et.al.,* 1991), further supporting the view that different mutations of the single androgen receptor gene can have widely differing effects in different tissues. The significant reduction in SHBG and gonadotropins, despite the absence of clinical improvement may be secondary to varying degrees of "androgen resistance" between the liver, pituitary gland and genitalia.

The clinical response to androgen therapy in the postpubertal patient can not be predicted accurately based upon biochemical markers (SHBG and gonadotropins). Nor are there any *in vitro* tests available at present capable of predicting a clinical response. Notwithstanding the possibility that the response may be limited by age/maturation events in the penis, as there is some evidence in the rat that expression of the androgen receptor gene mRNA in penile tissue and cells decreases with sexual maturation (Gonzalez-Cadavid *et.al.,* 1991), a therapeutic trial should be recommended in such cases.

The direct sequencing of exons 2 to 8, 4 to 8 encoding part of the hormone-binding domain, did not provide a disease-causing mutation in either of the two family members. Although we would predict a mutation in the androgen-binding domain, this does not necessarily rule out the possibility of a mutation in any other region of the androgen receptor gene, which may include parts of exon 1 not yet analyzed or the 3'and 5' regulatory regions. Further studies are indicated with this family, particularly if predictive carrier testing is a priority. The initial results of the different mutations in each of the affected brother and sister would allow us to stress the importance of multiple sequencing

attempts, following separate PCR reactions. It is possible to have different mutations on each of the X-chromosome alleles originating in the mother. Since the mother is obviously not affected (she has had eight children), this could be an example of allelic complementarity, where the one mutation compliments the other and in effect produces a normal phenotype. Although one would expect this phenomenon to be extremely rare, as it is difficult to cite a disorder with this occurrence, one could not rule out its plausibility, that is until the mutations were found to be due to PCR artifacts.

D. Studies of other complete AIS individuals: Detection of a mutational "hot-spot" at Arg773 in four individuals.

(from Prior *et.al.*, 1991, Amer.J.Hum.Genet. (submitted))

(i) Abstract

Herein we describe two different point mutations in a single codon of the androgen receptor gene in four unrelated Canadian families. Two of these families have a mutation involving a cytosine-to-thymine transition at a CpG sequence in exon 6 that changes the sense of codon 773 from arginine to cysteine, eliminating a *Kpn*I site at the intron-exon boundary. The patient's genital skin fibroblasts have decreased specific androgen-binding activity. The other two families have a guanine-to-adenine transition at the same codon that changes its sense to histidine and eliminates a *Sph*I site. These patients have normal androgen-binding capacity (receptor-positive), but a several-fold lower than normal affinity for androgens. Furthermore the receptor is known to be thermolabile in the presence of androgens. The Arg773Cys mutation yielded barely detectable androgen-binding activity as determined by transient transfection of COS cells with an expression vector containing the appropriate mutation, while the Arg773His mutation had abundant activity with abnormal properties. The conservation of this Arg773 in the progesterone and glucocorticoid receptors, with a conservatively replaced Lys in the mineralocorticoid receptor, demonstrates the importance of this region to normal steroid-binding activity of steroid receptors and their post-binding stability.

(ii) The subjects and their families

The proband of family 1 (coded LEL) is one of two sisters diagnosed as having complete AIS. Both have the classical clinical picture and a 46,XY karyotype. The genital skin fibroblasts of LEL were previously reported to have less than 5 fmol/mg protein specific androgen-binding activity (Pinsky *et.al.,* 1977).

The proband of family 2 is TML (Kaufman *et.al.,* 1976) (Figure 26, IV-3), who also has two affected maternal aunts. This family originated in Manitoba. Again, the subject has the expected clinical picture and negligible specific androgen-binding activity.

Family 3 was more fully described in Gottlieb *et.al.* (1987). The subject's genital skin fibroblasts have been found to have a normal capacity for androgen-binding, but the receptor binds androgens with decreased affinity, and is unstable in their presence. The patient, although not completely described clinically, has the complete AIS characteristics.

The subject from family 4, coded 6779, again has classical complete AIS. The androgen-binding activity in her genital skin fibroblasts were in the normal range, although previous analysis of her condition was receptor-negative when the androgen receptor was extracted from whole cells. Upon whole cell analysis, done for this paper by Dr. Pinsky's laboratory, a normal binding activity was found, but also that various androgen-receptor complexes dissociated in her cells much faster than normal and failed to up-regulate their specific androgen-binding activity during prolonged incubation with non-metabolizable androgens, MB or MT, as was the case in 30285 of family 3. Normal cells double their activity within 24 hours (Gottlieb *et.al.,* 1987).

(iii) Supplementary methods

(All of the following methods were done in the laboratory of Dr. L. Pinsky, McGill University, Montreal)

Construction of AR cDNA expression vector with Arg773Cys

A 147 bp *Eco*RI-*Hinc*II fragment containing codon 773 from pSK.hARLEL, and a *Kpn*I-*Hinc*II fragment (760 bp) from pGEM-3Z.hAR$_0$ were ligated into a *Kpn*I-partial *Eco*RI-

Complete Androgen Insensitivity Syndrome - TML Pedigree

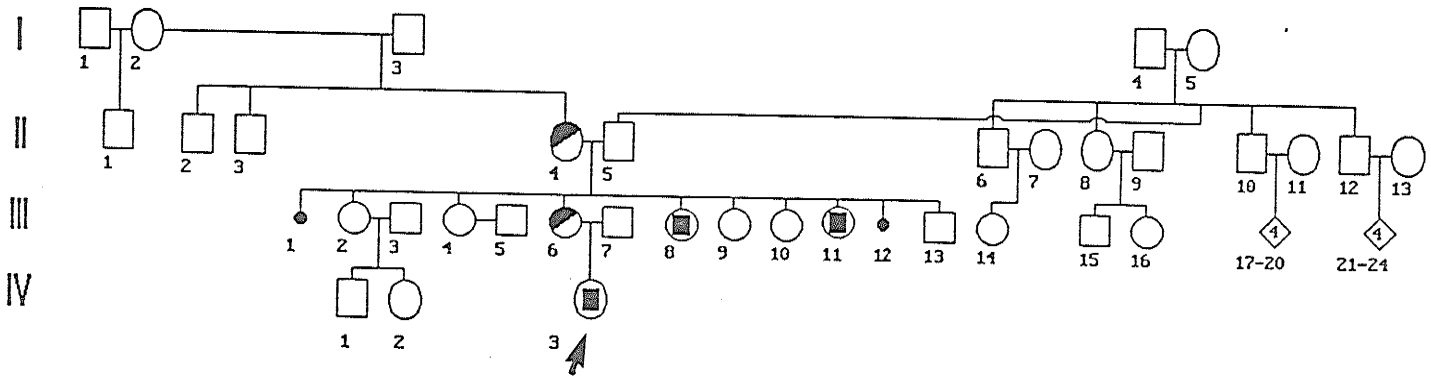


Figure 26 - Full pedigree of the kindred with one of the studied probands (TML or IV-3) from Prior *et.al.*, 1991 (submitted). Circle with filled square, CAIS.

digested fragment of pGEM-3Z.hAR$_0$ to form pGEM-3Z.hARLEL.

pSV.ARo is a pBR328-based, full-length hAR cDNA expression vector containing the SV40 early promoter, and the rabbit ß-globin poly-A signal (Brinkmann *et.al.,* 1989). A 1011 bp *Kpn*I-*Pvu*I fragment of it was removed, and replaced by a homologous insert from *Kpn*I-*Pvu*I-treated pGEM-3Z.hARLEL containing codon 773. The presence of the C->T substitution (in subject LEL) was ascertained by dideoxy sequencing. The normal and mutant plasmid DNA were purified twice on CsCl gradients, and ethanol precipitated before transfection into COS-1 cells.

Construction of AR cDNA expression vector with Arg773His

Two opposite sense 20 bp oligonucleotides were synthesized to contain the appropriate single nucleotide substitution. (Sense: 5'AATGAGTACCACATGCACAA 3'; 5'TTGTGCATGTGGTACTCATT 3'). Using normal hAR cDNA as a template, separate PCRs were conducted: the sense oligonucleotide with the downstream anti-sense primer; the anti-sense oligonucleotide with an upstream sense primer. The two products, containing upstream or downstream sequences, overlapped at the mutation site. To minimize the generation of spurious sequence alterations, a relatively high amount of template (1 µg) was used, with a relatively low number of cycles (24), and Vent (New England Biolabs, Beverly, MA), rather than *Taq* polymerase, because the former's exonuclease activity permits error correction. After purification on a low-melt agarose gel, the two overlapping products of the first PCR, and the same up- and downstream primers, were used in a second PCR. The single product was cleaved with *Sca*I and *Bst*BI to yield a 276 bp fragment containing the mutation, that was ligated into the *Sca*I-*Bst*Bi-treated expression vector, pBSV.hAR$_0$, a form of pSV.hAR$_0$ modified to contain a unique *Bst*BI site. The relatively small size of the fragment transferred further minimized the chance of including adventitious substitutions. Recombinant clones were tested for the mutation by resistance to *Sph*I digestion, sequenced for confirmation and to exclude unwanted errors, then used for transfection studies.

Construction of the MMTV.GH reporter plasmid

pMMTV.CAT (Miesfeld *et.al.,* 1986) was obtained from J. Drouin (Montreal). This was digested with *Nhe*I and *Hind*III to yield a 1447 bp fragment containing its long terminal repeat (LTR). pϕGH, a pUC12 vector (Nichols Institute, Los Angeles) containing the human growth hormone (hGH) gene was digested with *Xba*I and *Hind*III. The LTR fragment was ligated cohesively into pϕGH because *Nhe*I and *Xba*I ends are sticky. The resulting reporter construct puts the hGH gene under the regulatory influence of four glucocorticoid/ progesterone/ androgen response elements from the LTR of the MMTV (Ham *et.al.,* 1988).

Transfection of COS-1 cells by electroporation

COS-1 cells were washed and resuspended in ice-cold PBS to a concentration of 20 million per ml. 0.5 ml of the cell suspension were transferred to a sterile, cold cuvette (electrode gap 0.4 cm, Bio-rad, Richmond, CA), 10-20 µg of plasmid DNA were added, and the mixture was incubated on ice for 5 min. The shock parameters were: 250 V, 960 µF, 35 msec. After 10 min, post-shock incubation on ice, the cells in each cuvette were resuspended in 1.5 ml of medium (if more than one cuvette was used, the suspensions were fused), and 0.5 million cells were placed in each 35 mm petri dish.

At various specified times after transfection, various concentrations of androgen were added to the medium, the specific androgen-binding activity in the cells was characterized and, when applicable, the concentration of growth hormone in the medium was measured radioimmunometrically as recommended by the manufacturer of the assay kit (Nichols Institute, Los Angeles).

SDS-Page and Western analysis of the AR in transfected COS-1 cells

Confluent monolayers (about 5 million cells) in 60 mm petri dishes, washed twice with PBS, were lysed with 80 µl of 50 mM Tris-Cl, pH 6.8, containing 10 % glycerol, 2 % SDS, 100 mM DTT and 100 µg/ml of PMSF. Thereafter, preparation of cell extracts, SDS-PAGE, Western transfer by electroblotting, and blocking were performed as described by Sambrook *et.al.* (1989). The nitrocellulose filters were incubated overnight at 4°C with a

monoclonal antibody (F39.4.1) to a peptide (SP61) corresponding to amino acids 301-320 in the N-terminal portion of the hAR (Zegers *et.al.,* 1991). The antibody was diluted 1/400 in 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, (Tris-buffered saline, TBS). After 3 washes in TBS, the filters were incubated with a 1/1000 dilution of horseradish peroxidase-rabbit anti-mouse immunoglobulin G for 1 hr at room temperature. After 3 more washes each in TBS with and without 0.5 % Tween-20, the blots were developed with 4-chloro-1-naphthanol in the presence of H_2O_2 for 15 min.

(iv) Results

Because this paper is a collaboration of four laboratories, it is most appropriate to identify the work performed in our laboratory. To complete this section of the thesis, the results obtained in the other laboratories will also be described, but only briefly. For a complete overview of the work, one should refer to the above mentioned paper when it becomes available.

A major gene disruption was initially ruled out by examining the genomic DNA of LEL and 6779 by Southern analysis after digestion with *Bam*HI, *Eco*RI and *Hin*dIII (Figure 18), using the cDNA probe hAR-1 (Figure 10). Northern analysis revealed normal amounts of an approx. 10 kb AR mRNA in the fibroblasts of LEL and 6779 (Figure 20).

The Arg773Cys mutation in LEL was first discovered in Dr. Pinsky's laboratory upon sequencing the complete coding portion of the androgen-binding domain (exons 4-8) after PCR amplification of her cDNA. It was the only mutation found by amplifying and sequencing the remaining exons 2-8. The Arg773Cys mutation predicted the elimination of a *Kpn*I restriction enzyme site at the intron 5-exon 6 boundary. Our laboratory was able to confirm this by Southern analysis of *Kpn*I-digested genomic DNA, while screening for the same alteration among a set of eleven unrelated subjects with complete AIS. This screening uncovered another positive subject, TML, who had a new band of approx. 15 kb in place of one each at approx. 10 kb and 5 kb, as in LEL (Figure 27).

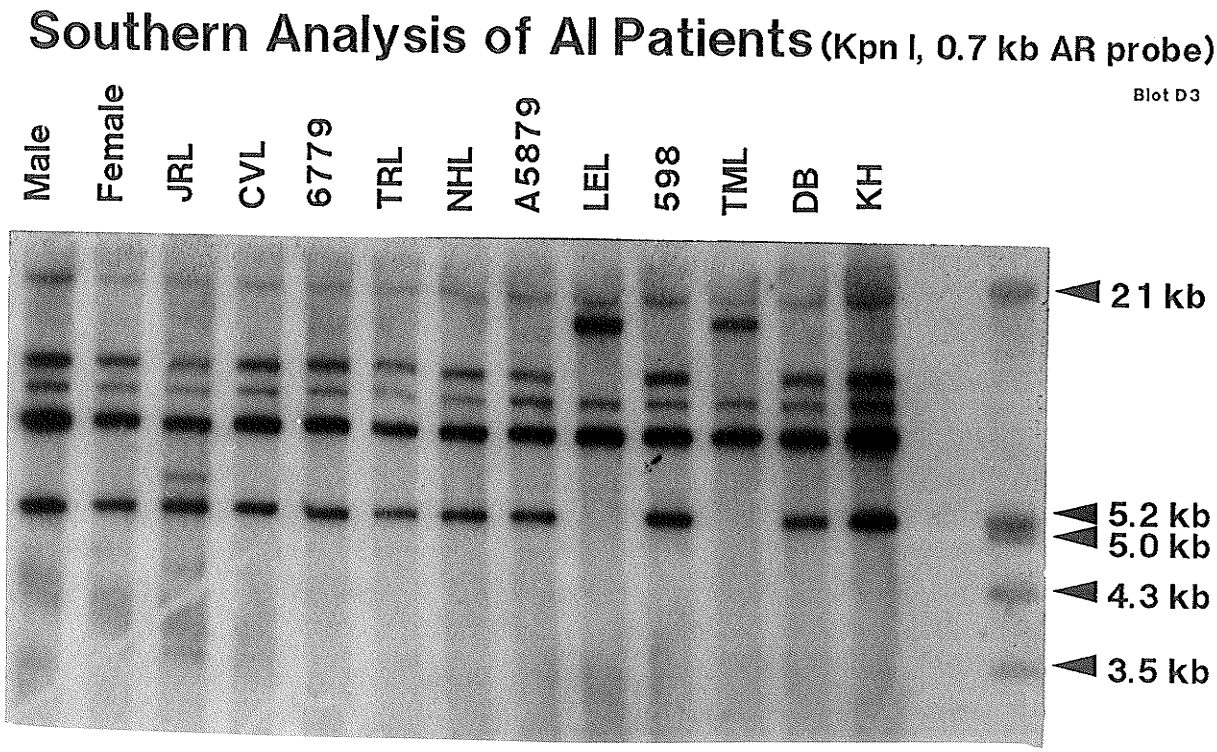


Figure 27 - Southern analysis of *KpnI* digested GSF genomic DNA with the h-AR 0.7 kb cDNA probe (Figure 10). LEL and TML share the Arg773Cys mutation. 6779 has the Arg773His mutation. The remainder are unrelated individuals with CAIS. DB is the proband of Family 2.

Dr. Pinsky's lab continued this analysis by studying the relatives of the two affected patients. The affected sister of LEL had only *Kpn*I resistant exon 6, which implied the Arg773Cys mutation (Figure 28) was linked to the mutant phenotype. The mother of LEL, who was expected to yield a hybrid pattern due to her obligate heterozygousity, instead was found to have PCR-amplified exon 6 that was completely *Kpn*I sensitive. This indicated that she is a somatic-germ cell mosaic. TML's mother, on the other hand, did have the additional two fragments representing *Kpn*I sensitivity of the normal exon 6 allele, as expected for an obligate carrier mother.

The Arg773His mutation (Figure 28) was discovered in both families in the laboratory of Dr. L. Pinsky using similar PCR technology as mentioned above.

The expression of mutant androgen receptor cDNAs and transactivational activity of mutant ARs was performed completely in the laboratory of Dr. Pinsky. The constructs were first tested for the normal production of AR protein by immunoblotting. The LEL construct had negligible specific androgen-binding activity, and when cotransfected with pMMTV.GH in the presence of androgen, insignificant amounts of GH were produced, as expected.

The 30285 construct, containing the Arg773His mutation, makes an AR with a lower than normal affinity for androgen as demonstrated by Scatchard analysis and by dissociation rates of the complexes with three different androgen analogs, those being MT, MB and DHT. When cotransfected with pMMTV.GH, they required 3.2 nM MB to reach maximal GH production, compared to < 1 nM with a normal AR cDNA expression vector.

The above results suggest that the AR with the Arg773His mutation is less competent than normal as a transactivator of the androgen response elements in the long terminal repeat of the MMTV.GH. A comparison with a normal AR cDNA construct was prepared to prove that despite an equivalent transfection efficiency, the androgen-binding activity in cells transfected with either would not be equal after prolonged incubation with

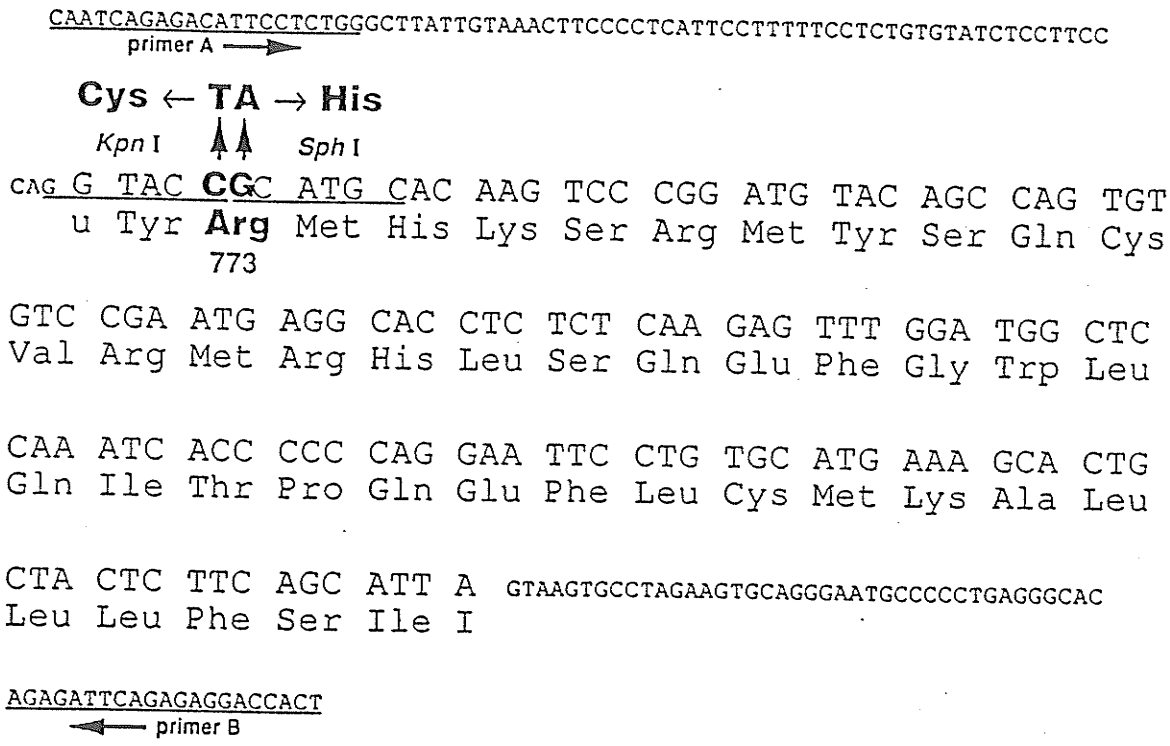


Figure 28 - The amino acid and nucleotide sequence of exon 6 of the AR with flanking intronic sequences. The primers used for PCR amplification are shown. The C-T and G-A transitions and their resulting amino acid substitutions are indicated in bold type. The respective abolished restriction sites are underlined. (From Prior *et.al.*, 1991 (submitted))

MB, that being 72 hr after transfection in order to determine up-regulation compared to incubation for only 2 hr between 70 and 72 hr after transfection.

This expectation was realized as the transfection efficiency was about equal as judged by the fact that COS-1 cells have about 2000 fmol/mg protein of "basal" androgen-binding activity at 6.4 nM MB between 70 and 72 hours after transfection with either expression vector, and secondly the normal androgen-binding went up, while the mutant activity went down with prolonged MB incubation. The comparison suggested that a sufficiently high concentration of Arg773His mutant AR is fully competent to transactivate the AREs in the LTR of the MMTV, but at concentrations between 250 and 500 fmol/mg protein, it is 3-fold less competent than normal.

(v) Discussion

Using standard molecular biology, we were able to identify, in two pairs of unrelated families with complete AIS, two transition mutations (C->T and G->A) in codon 773 (CGC) of exon 6 (or exon G) that changes its sense from Arg to Cys (TGC) or His (CAC), respectively (Figure 28).

Three types of circumstantial evidence pointed to the pathogenicity or causative nature of these sequence alterations. Neither mutation was found in 10 normal X-chromosomes, or in 18 other known putative disease causing mutations in 18 other complete AIS families or 10 partial AIS families studied in Dr. Pinsky's laboratory. The Arg773 of the AR is conserved as Arg or Lys in three other receptors of the AR subfamily. Finally the mutation is in a region of the androgen-binding domain that shares 85% amino acid identity with the homologous region in the steroid-binding domains in the AR subfamily of steroid receptors.

To prove their pathogenicity, Dr. Pinsky's laboratory undertook the job of creating expression vectors containing the mutations, followed by transfection into COS-1 cells. The results obtained were as expected from the evaluation of the patients' genital skin

fibroblasts, that is the Arg773Cys had barely measurable androgen-binding activity, while Arg773His had an abundant amount of abnormal form of the AR. The results suggest that Arg773 contributes not only to the androgen-binding affinity of the normal AR, but also to the transregulatory capacity, as judged by the AREs in the LTR of the MMTV. It would also be of interest to study the response of the AREs in other naturally androgen regulated genes to investigate the possibility of a similar response.

The different phenotypes produced by two amino acid substitutions at codon 773 (Figure 28) will help to discover the stereochemistry responsible for the high affinity with which the AR binds androgen. In this case, it is possible that the replacement by Cys disrupts androgen binding more completely than by His due to the potential intramolecular disulfide bond formation (Wilson *et.al.,* 1986).

The fact that both codon 773 mutations described involved G:C to A:T transitions correlates with the fact that cytosines at CpG dinucleotides are often methylated and form thymines by spontaneous deamination (Barker *et.al.,* 1984). That such dinucleotides are mutational "hot spots" is supported by the identification of this mutation in a second Canadian family of different ethnic origin among the first 19 families with complete AIS studied in Dr. Pinsky's lab. This Arg773Cys mutation was one of the first described in two different individuals in the USA (Brown *et.al.,* 1990; Tilley *et.al.,* 1990) and in one each from France (Mebarki *et.al.,* 1990) and Canada (Trifiro *et.al.,* 1990). The Arg773His has also been included in a preliminary report from another laboratory (DeBellis *et.al.,* 1991). Thus, one can see that Arg773 is a relatively common target of AR gene mutation in families with complete AIS. One would assume that Arg773 is required by the AR not only to bind androgen, but most likely also to form AR-hormone complexes that are stable and are capable of normal transcriptional activation.

E. Family 4 with X-linked spinal and bulbar muscular atrophy: Analysis of the CAG repeat region of the androgen receptor gene allowing predictive carrier testing/ disease diagnosis.

(from Belsham *et.al.*, 1992, J.Neurol.Sci. (submitted))

(i) Abstract

Herein we describe a family with X-linked spinal and bulbar muscular atrophy (SBMA or Kennedy's Disease) which is a disease of adult onset characterized by slow progression, predominant proximal and bulbar muscle involvement and absence of sensory or pyramidal tract signs. One characteristic, which conforms with the disorders of androgen action, is the appearance of gynecomastia. This disorder was also previously shown to be linked to the marker *DXYS1* on the proximal X-chromosome long arm. Recently, a report implicated a mutation at the N-terminus of the androgen receptor gene involving a gene amplification of CAG repeats as the cause of X-linked SBMA (La Spada *et.al.,* 1991). We amplified this region in a number of individuals from a kindred with this disorder by PCR followed by Southern analysis in order to predict the carrier status and possible occurrence of the disease. Upon sequencing, the mutated allele was found to have an increased number of CAG repeats, while the normal allele was within the expected range of repeats. Control studies with other unaffected individuals and one individual with AIS revealed repeat numbers within the normal range, while another X-linked SBMA patient had an enlarged CAG repeat region.

(ii) The subject and family

The proband (IV-14 in Figure 29) was examined by Dr. W.C. Yee when he presented with muscle weakness in his 20's. His symptoms continued to progress with increasing muscle twitching, muscle cramps and weakness with dysphagia. He does not have any signs of gynecomastia. He was variously diagnosed as having peripheral neuropathy and a muscular dystrophy, before a diagnosis of X-linked spinal and bulbar muscular atrophy was accepted. His brother (IV-16 of Figure 29) had complained of similar, yet milder symptoms of this disorder such as muscle weakness after exercise, but a formal examination has not been done. Two of the proband's three sisters have

Spinal and Bulbar Muscular Atrophy Family

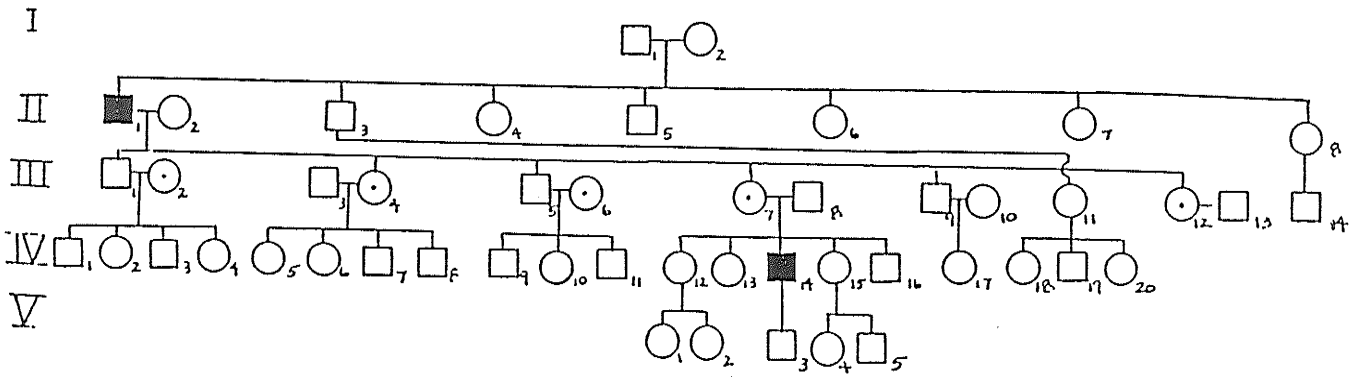


Figure 29 - The full pedigree of the spinal and bulbar muscular atrophy (SBMA) kindred, or Family 4. The proband is IV-14.

been examined and show no signs of the disorder. His maternal aunt (III-3 in Figure 29) may be a manifesting carrier, as she also complains of muscle aching with exertion and has signs of very mild chronic denervation. Peripheral blood samples were obtained from 14 family members (Figure 30). Seven members of this family were interested in their carrier status, while two male members of this family, particularly the brother of the proband, wanted to know their status with respect to developing this late onset, progressive disorder.

(iii) Supplementary methods

PCR amplification of the CAG repeat region in exon 1 of the AR

The PCR method was essentially the same as that described by Saiki *et.al.* (1988) and amplification of the CAG repeat region was performed using the primer set described (La Spada *et.al.,* 1991) and a set synthesized previously for our lab (Table I). Each 100 µl PCR reaction contained PCR buffer (Perkin Elmer Cetus, Norwalk, CT), 20 nmol of each dNTP, 75 pmol of each primer, 1 µg DNA, 0.01% gelatin and 2.5 units of *Taq* polymerase covered with 100 µl of mineral oil. The reaction was carried out cyclically with denaturation at 95°C for 1 min, annealing at 67°C for 2 min, and extension at 72°C for 1.5 min. After 35 cycles, extension continued for another 8.5 min at 72°C. The PCR products were chloroform extracted, purified on either a 12% PAGE gel or a 1.2% agarose gel, and then the DNA was eluted as described (Sambrook *et.al.,* 1989).

Direct sequencing of the PCR amplified CAG repeat region

The purified DNA was precipitated in 2.5 vol of ethanol, then resuspended in low TE. The double-stranded DNA was sequenced with the dsDNA Cycle Sequencing Kit (BRL, Gaithersburg, MD) according to the manufacturer's recommendations using the primary method of Sanger *et.al.* (1977). 7-deaza-dGTP was already used in the kit to avoid unnecessary secondary structure formation, particularly hydrogen bond formation, which causes band compressions during the sequencing run. One of the primers used to initially amplify the fragment (Table I) was end-labeled with [^{32}P] -dATP and used in the cycle sequencing reaction. The method utilizes native dsDNA which is introduced into a

set of dideoxy sequencing reactions, and is then subjected to a repetitive series of temperature changes (i.e. thermal cycling). The DNA is heat denatured, primers are annealed, and then the primers are extended as template-complementary oligonucleotides to dideoxy terminations. One positive feature of the cycle sequencing system is that less DNA is required than for a typical non-cycling reaction.

(iv) Results

The amplification of the CAG repeats or polyglutamine region located in exon 1 at the N-terminus of the androgen receptor gene was performed for all 14 kindred members. The size of the fragment representing the normal allele from this region is predicted to be about 400-450 bp, while the affected allele, with its amplified region is expected to yield a fragment of about 500 bp or more. The analysis of the PCR reaction products was done on a 1.2% agarose gel stained with a 0.5 μg/ml ethidium bromide solution. The two sets of bands could be easily detected (Figure 30(A)). The predicted size of the amplified fragment was approximately 400 bp, and the normal fragment was approximately 500 bp.

To be sure that the two bands were indeed from the androgen receptor gene, and were not unspecific products, the agarose gel was Southern blotted onto a filter and probed with a region of the androgen receptor gene representing this area. The results are seen in Figure 30(B). From this analysis, we can see that III-3, III-4, III-7, and III-12 are confirmed obligate carriers, while IV-12, IV-13, IV- 15 are also predicted to be heterozygous for the two alleles from this CAG region. III-11, IV-6, and IV-20 are not carriers upon CAG repeat analysis. The proband (IV-14) has only the upper mutated fragment, while his brother (IV-16) has only the lower normal fragment, predicting that he is unaffected and will not develop the disorder. Other males, III-4, and III-14, are also found to be unaffected.

Sequencing of this area was performed after purifying the DNA fragments from 1.5% agarose gels. The amplified region in the proband was found to contain 51 CAG repeats (Figure 31(A)), which is fairly close to the predicted number by analysis of agarose

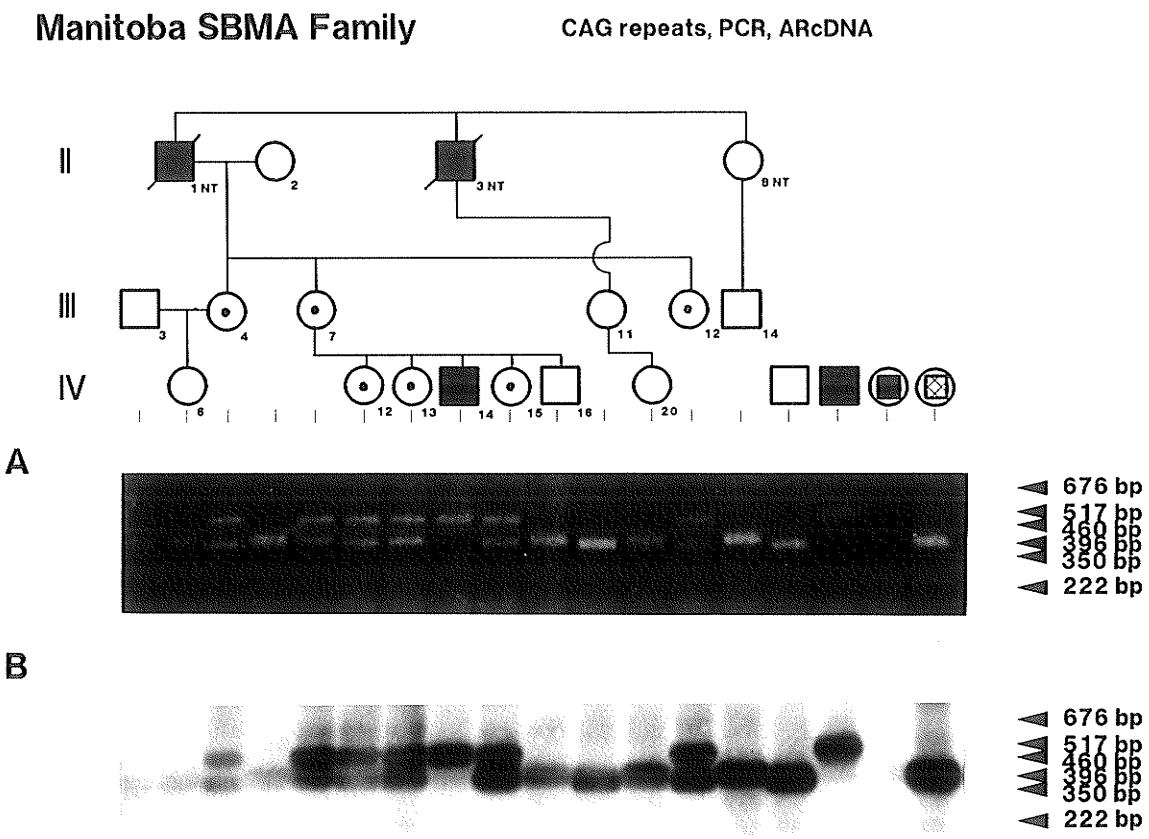
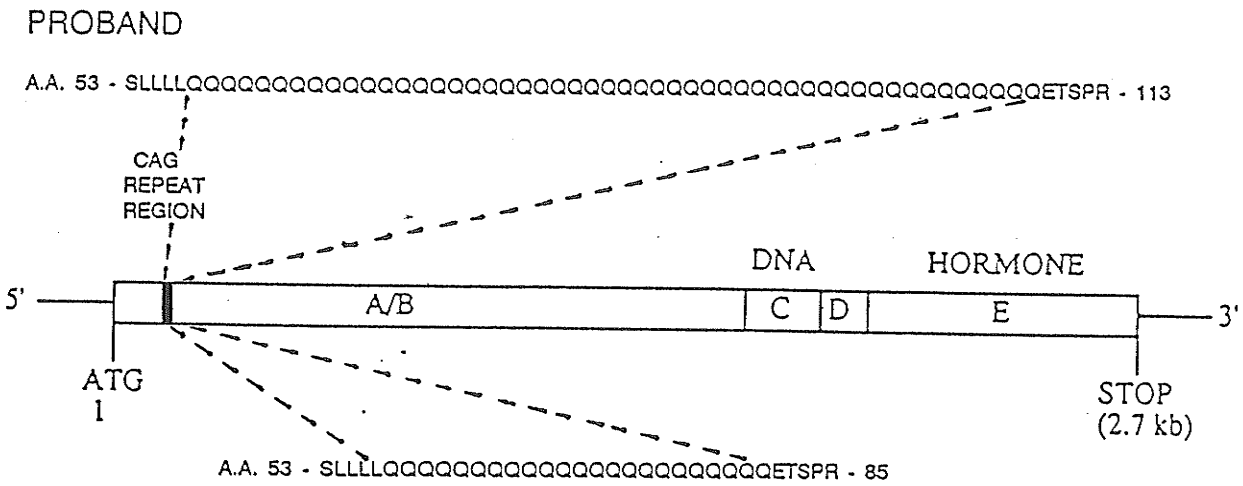


Figure 30 - (A) Agarose gel electrophoresis (1.2%) of PCR products obtained by amplification of the AR CAG repeat in the studied pedigree. Gel is stained with ethidium bromide. (B) Southern analysis of the gel in (A) probed with the h-AR cDNA defined by *Sma*I 641 → *Stu*I 1153 (sequence according to Figure 7) containing the CAG repeat region. Controls are lane 11, unrelated SBMA patient; lane 12, control male; lane 13, CAIS patient; and lane 14, PAIS patient. NT = not tested.



S = SERINE; L = LEUCINE; Q = GLUTAMINE; E = GLUTAMIC ACID; T = THREONINE;
P = PROLINE; R = ARGININE

Figure 31 - Deduced amino acid number from the sequencing of the androgen receptor gene of the proband (IV-14) and his normal brother at the CAG repeat region. The sequencing results indicate that the proband has nearly twice as many glutamine repeats as his normal brother (51 and 23 repeats respectively).

and PAGE gels. The normal fragment has 23 CAG repeats (Figure 31(B)). These numbers are within the range predicted by La Spada *et.al.* (1991).

(v) Discussion

A kindred with X-linked spinal and bulbar muscular atrophy was ascertained after the proband was diagnosed to have this adult onset, progressive disorder. The diagnosis of this disorder is often difficult, and is often misdiagnosed as another form of spinal muscular atrophy or any of the other similar degenerative diseases of the lower motor neuron (Kennedy *et.al.,* 1968). Once diagnosed, other family members decided to participate in a genetic study in order to determine potential carrier status or possible onset of the disease.

Using the methods reported (La Spada *et.al.,* 1991), we were able to determine the carrier status of seven potential carriers and to determine the potential disease diagnosis in the two male members of this kindred. Since there is a large extended kindred, any members have the chance to have predictive testing done if they so choose.

The diverse number of polyglutamine repeats or CAG repeats in the coding region in exon 1 at the N-terminal of the androgen receptor has been reported to range from 17 to 26 with an average of 21 in normal individuals (La Spada *et.al.,* 1991). The differences noted in this area were first described upon sequencing of the androgen receptor cDNA in separate laboratories (Lubahn *et.al.,* 1988a; Tilley *et.al.,* 1989). There is another region further downstream from this polyglutamine region in the androgen receptor gene also known to contain varying numbers of GGT/GGC repeats, called the polyglycine region. It had been thought that perhaps patients with differing degrees of androgen insensitivity would have predictive numbers of these repeats (Lubahn *et.al.,* 1988a). Upon analysis of a few patients with complete and partial AIS the number of repeats fall into the normal range. Nevertheless, using PAGE it should be possible to detect subtle differences in CAG repeat number, which may be useful for RFLP analysis upon detection of the different X chromosome alleles due to differing number of CAG repeats. Perhaps this analysis

would be useful for genetic counselling in families, where other linkage analysis and mutation detection has failed, to predict carrier status not only in SBMA but also in AIS.

Patients with AIS are feminized but do not show any signs of muscle weakness, which is very different from patients with SBMA patients with normal fetal sexual development, despite later signs of feminization. Specific androgen receptor binding studies in patients with SBMA indicate that more often than not the binding activity is normal, although two patients have been found to have decreased binding activity (A.R. La Spada, International Congress of Human Genetics, Washington, DC, October, 1991). The insertional mutation probably does not affect the DNA- or androgen-binding characteristics of the AR in most patients, but possibly alters the function of the AR in motorneurons. Androgen receptors have been found to be concentrated in spinal and bulbar motorneurons, the cells that degenerate in SBMA (Sar, Stumpf, 1977). In the rat, androgens are known to play an important role in normal spinal motorneuron growth, development and response to injury (Yu, 1989).

It is known that the number of CAG repeats does not seem to correlate with the severity or onset of the disease (A.R. La Spada, International Congress of Human Genetics, Washington, DC, October, 1991). In our study, the proband does not have gynecomastia, but the control SBMA patient is known to present with this characteristic condition of SBMA. The number of CAG repeats is significantly smaller in our proband than in the control patient; thus, it would be interesting to search for a correlation between the number of CAG repeats and the presentation of gynecomastia in other SBMA patients to determine if this finding has any significance.

The significance of this finding has yet to be elucidated, but the increased number of CAG repeats has been implicated to be the cause of X-linked SBMA (La Spada *et.al.*, 1991). Yet, how this occurs is difficult to imagine. One hypothesis is that the N-terminus of the androgen receptor is required for an operational task associated with motorneuron function. The regulation of genes by the AR could be predicted to be altered in patients

with SBMA (La Spada *et.al.,* 1991), as repeats with this structure have been implicated in both developmental regulation and transcriptional regulation in other species (Wharton *et.al.,* 1985; Duboule *et.al.,* 1987; Mitchell, Tijan, 1989; Kao *et.al.,* 1990). Since this region of the androgen receptor is cloaked in mystery, one would believe that any study of its functionality would help to uncover the significance of the N-terminal region of not only the androgen receptor, but perhaps other members of the steroid/ thyroid/ retinoic acid supergene family (Evans, 1988; Carson-Jurica *et.al.,* 1990) due to the structural similarities of these proteins.

Part IV

The Study of the Novel 56 kDa Protein With Androgen Binding Activity

1. Introduction

A. Historical perspectives on the discovery of the "missing spot"

The study of genetic disorders has often relied on the determination of the mutant protein involved in the disorder before any knowledge of biochemical or molecular cause of the disease could be determined, as was done for the androgen insensitivity syndromes due to defects of the androgen receptor. Before the cloning of the androgen receptor gene, much research was directed towards characterizing the androgen receptor protein itself. Our laboratory decided in 1978 to use this method to look for the mutant protein involved in certain genetic disorders, particularly Duchenne muscular dystrophy.

Using a newly developed dual labeling technique our laboratory was able to find a protein that appeared to be missing in the fibroblasts of patients with Duchenne muscular dystrophy (Rosenmann *et.al.,* 1982). Essentially the method involved differential labeling of normal and mutant cells with [^{3}H]leucine and [^{14}C]leucine. After the labeling, the cell preparations were mixed together and ran as a single sample by 2D-PAGE in order to avoid the problem of difficult reproduction of single gels. The gels were dried and subsequently exposed using the appropriate system, autoradiography for [^{14}C] and fluorography for [^{3}H]. The negative of the autoradiogram (to give white spots) was superimposed on the fluorogram (black spots). White spots (normal) would be covered by black spots (dystrophic) if the protein were present in both cell strains, but if a protein was missing in the mutant cell strain only the white spot would be detected. In this way we were able to find the "missing spots".

This incredible discovery of the missing protein in Duchenne muscular dystrophy was published in 1982 (Rosenmann *et.al.,* 1982), but unfortunately the initial findings of the initial publication had to be corrected (Thompson *et.al.,* 1983), for indeed a virtually unavoidable oversight occurred. The final conclusions of this study were that the biopsy site was responsible for the presence or absence of this protein, and there was no relationship to Duchenne muscular dystrophy. The protein was present in normal genital

skin fibroblasts, but absent in normal non-genital skin fibroblasts. Unfortunately the biopsy site was not recorded in either the cell repository catalogues or the specification sheets, which led to the unintentional comparison of normal genital skin fibroblasts with non-genital skin fibroblasts. This discovery, although with results which were rather disappointing at first, was indeed serendipitous.

B. Initial studies of the 56 kDa protein

The discovered protein was called the 56 kDa protein after its molecular weight. The fact that this protein is only present in normal genital skin fibroblasts and not in non-genital fibroblasts could arise causally or coincidentally. Genital skin fibroblasts differ from other fibroblasts in that they are known to be an androgen target tissue. Therefore they are enriched in the androgen receptor, 5α-reductase, and other components required for androgen dependent gene expression, which was reviewed in the introduction to androgen insensitivity syndrome section of this thesis. Since the protein was present in an androgen target tissue, it became apparent that it could be involved in the androgen insensitivity syndrome or might be the androgen receptor itself, since patients with this disorder are deficient in proteins involved in androgen action, such as the androgen receptor.

A preliminary blind study (the code was broken after 8 cell strains were tested), involving a mixture of androgen insensitive patients and normal controls, was undertaken by our laboratory. The cell lines were donated by Dr. L. Pinsky from Montreal. The initial study investigated the differences between normal control cell lines and androgen insensitive patients with regards to the newly discovered missing protein. In each patient, the 56 kDa protein was found to be missing by 2D-PAGE followed by silver staining. A more complete study was published soon after (Nickel *et.al.,* 1988). The results may be found in Table VII. The protein was found to be present in 23 normal control genital skin fibroblasts, absent in 30 of 32 normal control non-genital skin fibroblasts and absent in 12 of 14 labium majus skin fibroblast strains. The above study allowed us to conclude that the presence or absence of the 56 kDa protein in GSF was a novel marker of genetic heterogeneity within the class of complete AIS.

C. Characteristics of the 56 kDa protein

Since that time, the characterization of the 56 kDa protein has continued. This protein can be detected as a cytosolic protein by 2D-PAGE as two spots with isoelectric points of 6.7 and 6.5 (Figure 32). The 56 kDa protein was estimated, on the basis of silver or Coomassie Blue staining, to comprise approximately 0.1% of total GSF cellular protein (Nickel *et.al.,* 1988). The two spots can be covalently labelled with [^{3}H]MT (Figure 33) and [^{3}H]MB (Figure 34). Using concentrations ranging from 0.5 to 50 nM, the fibroblasts are labelled for 2 to 48 hr followed by photolysis, 2D-PAGE and fluorography. Two spots are specifically labelled with the androgen analogs, as labeling of the spots is suppressed by the presence of a 200-fold excess of radioinert androgen analog, although the apparent affinity of this protein for the synthetic analog is lower than that estimated for the authentic androgen receptor as competition with excess cold analog did not completely suppress labeling (Figure 33) (Wrogemann *et.al.,* 1988). The fluorogram of the covalently labelled spots can be perfectly superimposed onto the two 56 kDa spots stained with Coomassie Blue (Wrogemann *et.al.,* 1988). The relationship of the two spots was determined by a comparison of the peptide patterns after partial hydrolysis with *Staphylococcus aureus* V_8 protease and with chymotrypsin. The patterns are virtually identical, suggesting that they represent the same protein (Wrogemann *et.al.,* 1988).

D. Comparisons to the androgen receptor

Because this protein can be labelled with androgen analogs, one could postulatethat this protein is the androgen receptor, but evidence argues against this postulate. The size of the androgen receptor monomer in various species is thought to have a common molecular size of approximately 90-110 kDa (Johnson *et.al.,* 1987; Brinkmann *et.al.,* 1988; Gyorki *et.al.,* 1988; van Loon *et.al.,* 1988; Mulder *et.al.,* 1989). The cloning and sequencing of the androgen receptor cDNA indicates the intact androgen receptor monomer should be a 98 kDa protein of 917-919 amino acid residues (Chang *et.al.,* 1988b; Trapman *et.al.,* 1988; Lubahn *et.al.,* 1988b; Faber *et.al.,* 1989), the inconsistencies in size resulting from differences in the homopolymeric regions. Labeling of the androgen receptor with androgen analogs for 48 hr results in an increase in

Table VII - Specific androgen binding activities and the 56 kDa protein status in the genital skin fibroblasts of numerous patients with androgen insensitivity syndrome, including normal genital skin fibroblasts from control MCH6 and MCH49, non-genital skin fibroblasts WP09, and LNCaP, which overexpresses the AR (modified from Wrogemann *et.al.*, 1988).

Cell strain	AR binding	56 kDa protein*	MT or DHT# labeling
TML	1	-	-
6779	1	-	-
598	5	-	-
30284	17	-	-
KIL	11	-	-
TRL	1	+	+
NHL	1	+	+
JRL	0	-	-
A5879	2	-	-
LEL	1	-	-
CVL	0	-	-
31082	2	-	-
2379	5	-	-
8481	1	-	-
DB	1	+/-	+#
KJH	2	+/-	+#
JMR	4	+/-	+#
8812	1	+/-	+#
Normal controls:			
MCH6	24	+	+
MCH49	24	+	+
WP09	4	-	-
LNCaP	481	-	-

*Based on silver or Coomasie stained protein maps of 2D-gels.
+/- variable on gels depending upon protein amount and upon staining/destaining
#Determined subsequent to use of [^{3}H]DHT-BA in the labeling method, which is more efficient and specific than the previous labeling with MT or MB, for the 56 kDa protein.

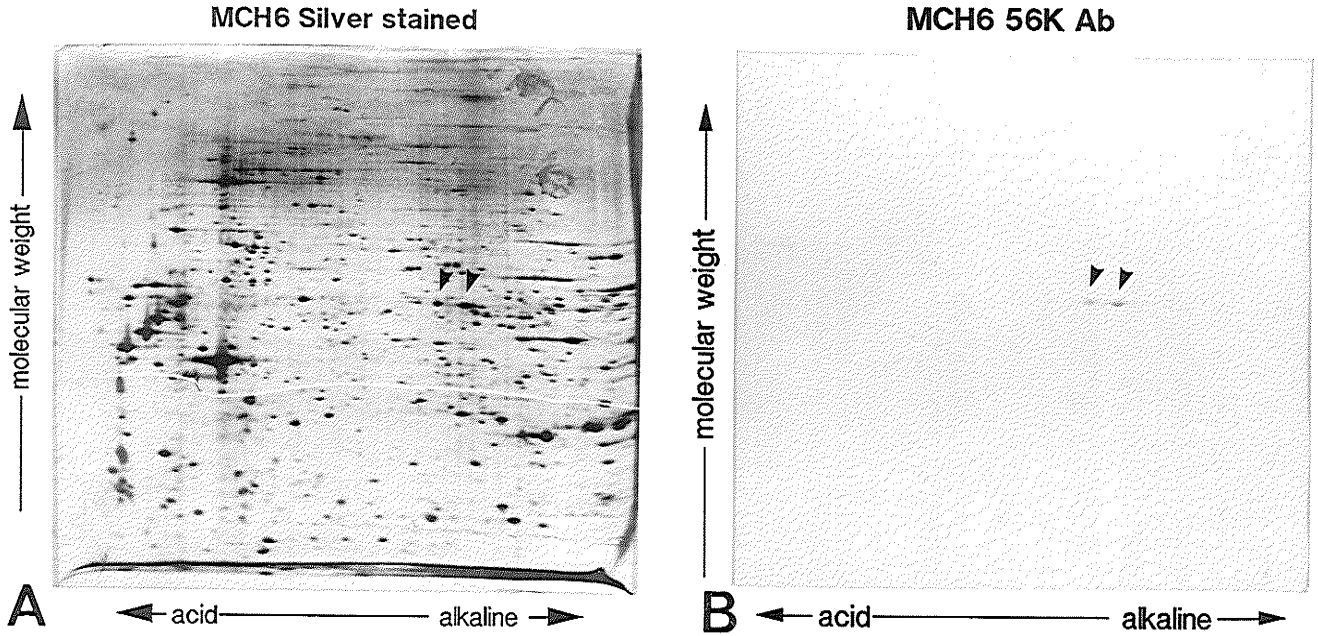


Figure 32 - Protein map of genital skin fibroblasts, strain MCH6 and Western blot analysis with the antiserum against the 56 kDa protein. (A) Silver-stained gel of 100,000xg supernatant proteins; and (B) Immunoblot of an MCH6 lysate reaction with the anti-56 kDa rabbit antiserum. The 56 kDa protein doublet is marked with arrows. (From Pereira *et.al.*, 1990)

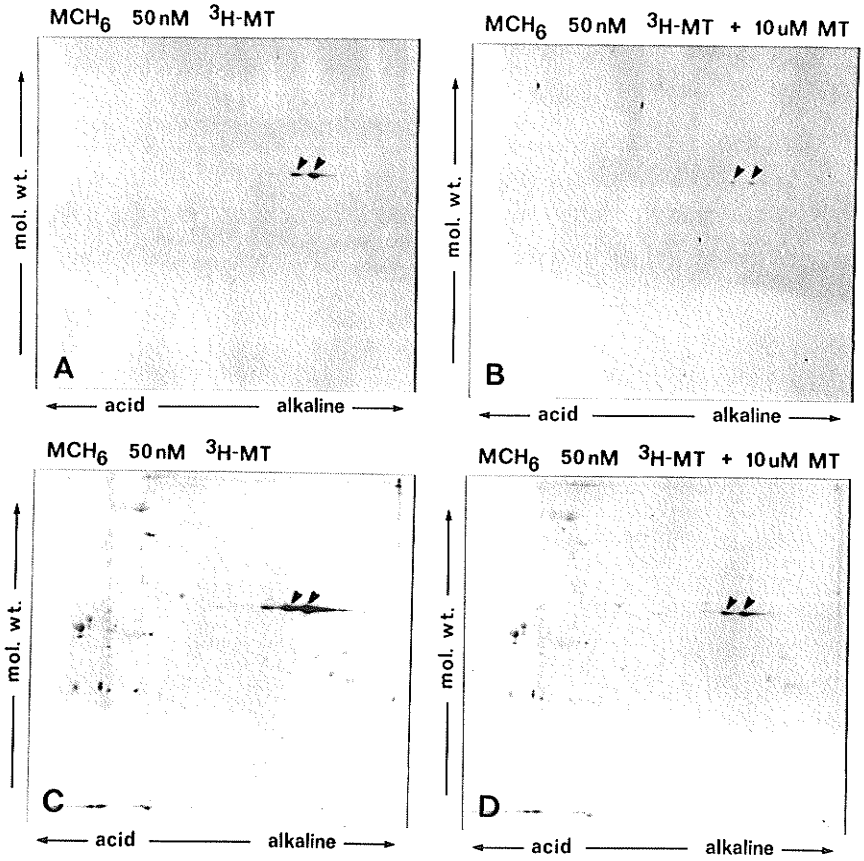


Figure 33 - Specific and non-specific labeling of proteins by photolysis of GSF strain MCH6 with 50 nM [^{3}H]MT (A) and (C); and 50 nM [^{3}H]MT + 200-fold excess radioinert MT (B) and (D). Exposure: 33 days in (A) and (B); 201 days in (C) and (D). Separation by 2D-PAGE and overexposure of fluorograms allow clear distinction between specifically labelled proteins (competed by excess cold ligand, arrows) and non-specifically labelled proteins (unsaturable with ligand, all other spots in (C) and (D)). (With permission of E. Rosenmann from Wrogemann *et.al.*, 1988)

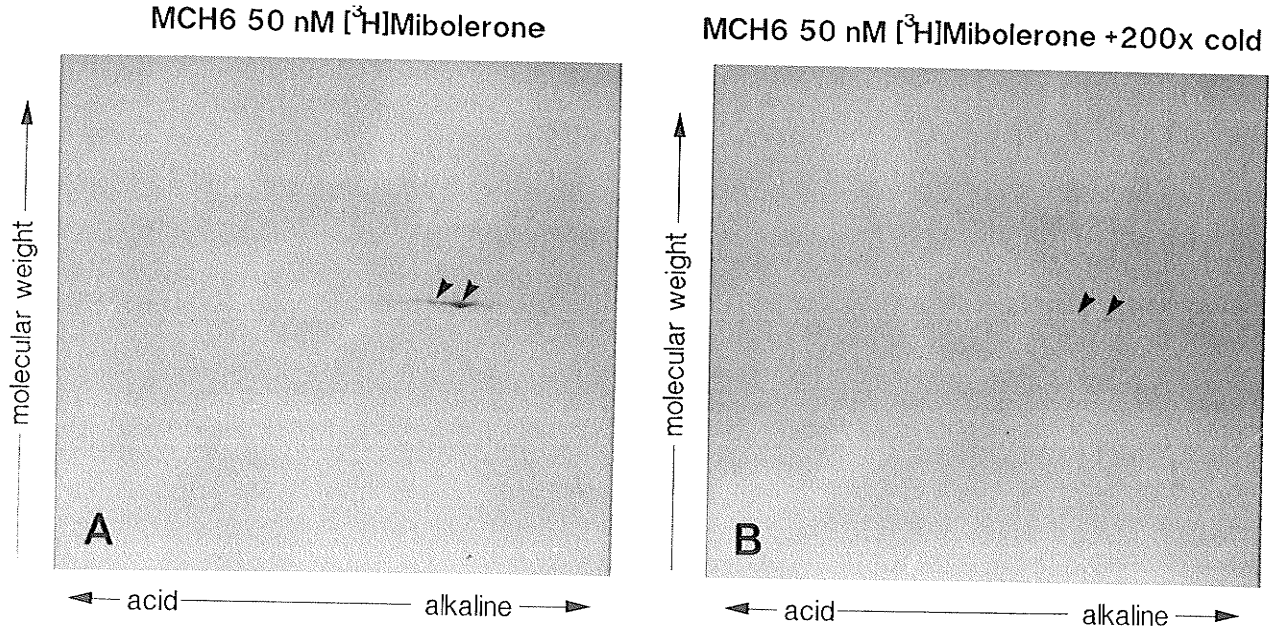


Figure 34 - Photoaffinity labeling with [^{3}H]MB of GSF strain MCH6. (A) Cells labelled with 50 nM [^{3}H]MB separated by 2D-PAGE and exposed by fluorography and (B) with 200-fold excess radioinert MB. Exposure: 44 hr. Arrows point to the position of the 56 kDa protein doublet. (From Pereira *et.al.*, 1990)

androgen receptor binding (approximately double), which is termed "upregulation" (Kaufman *et.al.,* 1981). A comparison study was undertaken with the 56 kDa protein in which the cells were labelled with [^{3}H]MT for two hours, which should show no upregulation, to 48 hr, which should produce maximal upregulation. The results indicate that the 56 kDa protein is not "upregulated" and that subjecting cells to only 2 hr of incubation with the ligand results in maximal labeling (Wrogemann *et.al.,* 1988). The affinity of the androgen receptor for its natural ligand, androgen, is high. At 2 nM of MB, which would saturate the androgen receptor (Gottlieb *et.al.,* 1987), labeling of the 56 kDa protein is observed, but competition with a 200-fold excess of radioinert MB is not detected. Photolysis experiments with increasing MT or MB concentrations (2 to 30 nM) suggest that the apparent affinity of the 56 kDa protein for androgen is low, as half maximal binding required almost 1 μM MT or 4 μM MB, although this is probably not completely accurate due to the fact that the labeling is not performed in equilibrium conditions, required to measure the parameters of hormone binding, due to the covalent binding of the ligand to the receptor. An accurate determination of the affinity of the 56 kDa protein for androgen has not yet been accomplished.

Competition experiments with other steroids reveal a hierarchy similar to that reported for the androgen receptor (Keenan *et.al.,* 1984). Dihydrotestosterone was shown to be the most potent competitor and hydrocortisone the least (Table VIII). The labeling efficiency of the 56 kDa protein has been determined to occur at approximately 0.125% with [^{3}H]MT (Wrogemann *et.al.,* 1988), if one assumes that one molecule of ligand can bind per molecule of the 56 kDa protein.

Although this protein shares many characteristics in common with the androgen receptor, it is doubtful that it is the androgen receptor itself, but up to this point in the story, one cannot yet rule out the possibility that this protein could be a part of the androgen receptor, perhaps a product of its degradation as has been suggested previously (Nickel *et.al.,* 1988; Kovacs *et.al.,* 1989). Evidence for a biosynthetic precursor of the progesterone receptor has been reported (Mullick, Katzenellenbogen, 1986), but there is

Table VIII - Hierarchy of competition by other steroids when photolytically labeling the 56 kDa protein with MT (with permission of E. Rosenmann from Wrogemann *et.al.*, 1988)

Steroid	Peak Height (mm)	% Density
MT (control)	153	100
Dihydrotestosterone	9	6
Progesterone	18	11
ß-estradiol	24	15
Hydrocortisone	49	32

* Cells of strain MCH6 were incubated for 48 hr with 50 nM [^{3}H]MT in the absence (control) or presence of a 500-fold excess of radioinert competing steroids. The cells were photolysed, fractionated by 2D-PAGE, exposed fluorographically and the peak label intensity over the 56 kDa protein quantitated by densitometric scanning of the X-ray film.

little information on storage forms or cycling of steroid receptors. Furthermore, this protein could be one of the smaller proteins known to be produced by the in vitro transcription and translation of the androgen receptor cDNA (Chang *et.al.,* 1988b). An antiserum to this protein was prepared to test this hypothesis by in vitro translation from mRNA of GSF. To see whether the 56 kDa protein was synthesized as a larger precursor, its synthesis was tested by in vitro translation. 2D-PAGE maps of total translation products clearly reveal the 56 kDa protein doublet of pI 6.5 and 6.7. This result was confirmed by immunoprecipitating the translation product with the anti-56 kDa antiserum and separating the precipitate on 2D-PAGE. In this way, the 56 kDa protein was shown not to be synthesized as a larger precursor (Pereira *et.al.,* 1990).

The antiserum described above has had much use in our laboratory. The antiserum was originally prepared by excising the righthand, pI 6.7, 56 kDa protein spots from 2D-PAGE gels and inoculating the prepared emulsions into rabbits. The polyclonal antiserum produced recognizes both of the 56 kDa spots on preparative 2D-PAGE gels which were subsequently Western blotted (Figure 32 (B)). The Western blot reveals that the antiserum is specific for the 56 kDa protein, and detects it reliably at 1:500 dilutions (Pereira *et.al.,* 1990). The immunoanalysis also confirms the postulate that the two spots are structurally related (Wrogemann *et.al.,* 1988), as the antiserum prepared only against right-hand spots also detects the left-hand spot equally well.

To add further evidence to our case that the 56 kDa protein is not the androgen receptor, we have also described two exceptional AIS patients which express this protein normally, although their GSF have negligible androgen receptor binding activity (Nickel *et.al.,* 1988). The presence of the protein has been tested by both immunoanalysis and covalent labeling with MT and MB (Wrogemann *et.al.,* 1988; Pereira *et.al.,* 1990). If the 56 kDa protein was a degradation product of the androgen receptor, one might expect the 56 kDa protein in these two mutant strains to be structurally altered, but this has been shown not to be the case. The observation of the presence of the 56 kDa protein in these two exceptional patients has allowed us to infer that although the 56 kDa protein

is quantitatively and causally related to the androgen receptor protein, it is not always dependent on the latter's functional capability or more simply it does not appear to be androgen-dependent.

E. Other proteins reported with similar characteristics

In a separate report, another group had described a 58 kDa protein, which they called the androgen receptor, that could be labelled with DHT-BA (Kovacs, Turney, 1988). DHT-BA has previously been reported to covalently bind to androgen receptors in several species (Chang *et.al.,* 1982; Chang *et.al.,* 1984). The radioactive ligand covalently labelled a single protein of estimated molecular weight 58,000 that was absent from normal non-GSF or GSF from patients with "receptor-negative" AIS. Under non-denaturing conditions, the protein covalently labelled by DHT-BA has been described to be indistinguishable from the authentic [^{3}H]DHT labelled androgen receptor on the basis of Stokes' radius, sedimentation coefficient, isoelectric point (by chromatofocusing) or hydrophobicity (Kovacs *et.al.,* 1989). The possibility that the above described protein could be identical to our 56 kDa protein had to be investigated.

The description of the 56 kDa protein has instigated a study that has led to many unanswered questions. Could this protein be the androgen receptor itself? If it is not the intact androgen receptor, could it be a proteolytic fragment of the androgen receptor? It has also been postulated to be a natural relative of the androgen receptor, such as a biosynthetic precursor, a potential storage form, or a version that represents its post-ligated state, as part of a potential recycling process (Nickel *et.al.,* 1988). Another thought was that this protein could be part of a heterooligomeric untransformed androgen receptor complex, as is the 90 kDa heat shock protein, known to be a component of most untransformed steroid receptor complexes (Binart *et.al.,* 1989; Joab *et.al.,* 1984; Bresnick *et.al.,* 1989); thus, we considered whether our protein could be the 59 kDa protein recognized to be a component of untransformed androgen, estrogen, progestin, and glucocorticoid receptor complexes in rabbit uterus and liver (Tai *et.al.,* 1986). Although this 59 kDa protein was not considered a steroid binding protein, it has not been tested

whether it possibly has a lower affinity for steroid as does the 56 kDa protein. Any one of the above or even a combination of the above explanations was possible and we found that it was necessary to further characterize the 56 kDa protein in order to resolve the unanswered questions.

2. Results and Discussion

A. Comparison study of proteins with similar characteristics

(from Belsham *et.al.*, 1989)

(i) Abstract

Herein we describe the comparison of a 56 kDa protein with other steroid receptor associated or related proteins of seemingly similar characteristics. The 56 kDa protein from genital skin fibroblasts is visualized as a protein doublet by 2D-PAGE with isoelectric points of 6.7 and 6.5. The protein is absent in the GSF of patients with complete androgen insensitivity syndrome. It also specifically binds androgens. A protein of 58 kDa from GSF has been found to be covalently radiolabelled by the affinity ligand dihydrotestosterone 17ß-bromoacetate. The two proteins have been found to be indistinguishable by 1- and 2-D PAGE. Antibodies raised against the 56 kDa protein also recognized the protein covalently labelled with DHT-BA. A third protein of 59 kDa has been found to be associated with several steroid hormone receptor complexes, but has no ligand binding activity. This protein was found to be clearly separate from the 56 kDa protein upon 2D-PAGE analysis and by immunoanalysis with the 56 kDa antiserum.

(ii) Supplementary methods

Affinity labeling with DHT-BA

(done in the lab of Dr. Kovacs, Vanderbilt University, Nashville)

DHT-BA was synthesized by the method of Chang *et.al.* (1984) and the product analyzed as previously described (Kovacs, Turney, 1988). Human GSF cytosol was incubated for 30 min at 4°C with 5 nM [^{3}H]DHT-BA and the sample chromatographed on a column of Bio Gel A 0.5 M (180 ml bed volume, 1.6 x 90 cm). The radiolabelled peak corresponding to a Stokes' radius of 4.4 nm was recovered and used for subsequent one- and two-dimensional electrophoresis.

59 kDa monoclonal antibody preparation

(done in the lab of Dr. Faber, Medical College of Ohio, Toledo)

Anti 59 kDa immunoglobulin G_1 (IgG_1), derived from pooled ascites produced by cell line KN382/EC1 (Nakao *et.al.,* 1985) was purified by DEAE-Affi-Gel blue chromatography (Bio-rad, Richmond, CA) (Brunk *et.al.,* 1982). Protein peaks were located with the Bradford protein assay (Bradford, 1976) and the immunoglobulins were concentrated by ammonium sulfate precipitation. Concentration of IgG_1 was determined by radial immunodiffusion (Hudson, Hay, 1980).

(iii) Results

The analysis of human GSF lysates by 2D-PAGE reveals a distinct, relatively abundant 56 kDa protein doublet on the alkaline side of the map at pI 6.7 and 6.5 (Figure 32). This protein can be specifically and covalently labelled with the androgen analog [^{3}H]MB (Figure 35(A), lanes 3-6). A preparation of the "58 kDa" protein covalently labelled with [^{3}H]DHT-BA migrates identically on one-dimensional SDS gels with the "56 kDa" protein labelled with [^{3}H]MB, suggesting that the two proteins might be identical (Figure 35(B)).

The identity of the two proteins was further studied using 2D-PAGE and the antiserum generated against the 56 kDa protein. This antiserum recognizes the 56 kDa protein doublet on Western blots and does not readily react with other proteins in GSF (Figure 32(B)). Occasionally, a faint 110 kDa species is also detected by this antiserum, but these results can not always be confirmed (data not shown). The antiserum recognizes, on 2D-Western blots of the 58 kDa protein preparation labelled with [^{3}H]DHT-BA, a protein doublet of the same size and isoelectric points as the 56 kDa protein (Figure 36(A) and (B)). Moreover, a fluorogram of the Western blot shows the label is also bound to a protein doublet which is perfectly superimposable on the species recognized by the antiserum (Figure 36(C) and (D)). Therefore, we can conclude that the "58 kDa" and the "56 kDa" proteins are clearly identical.

A monoclonal antibody directed against the previously described 59 kDa protein (Tai *et.al.,* 1986; Koutsilieris *et.al.,* 1988) recognizes a protein with approximate pI 5.4 in

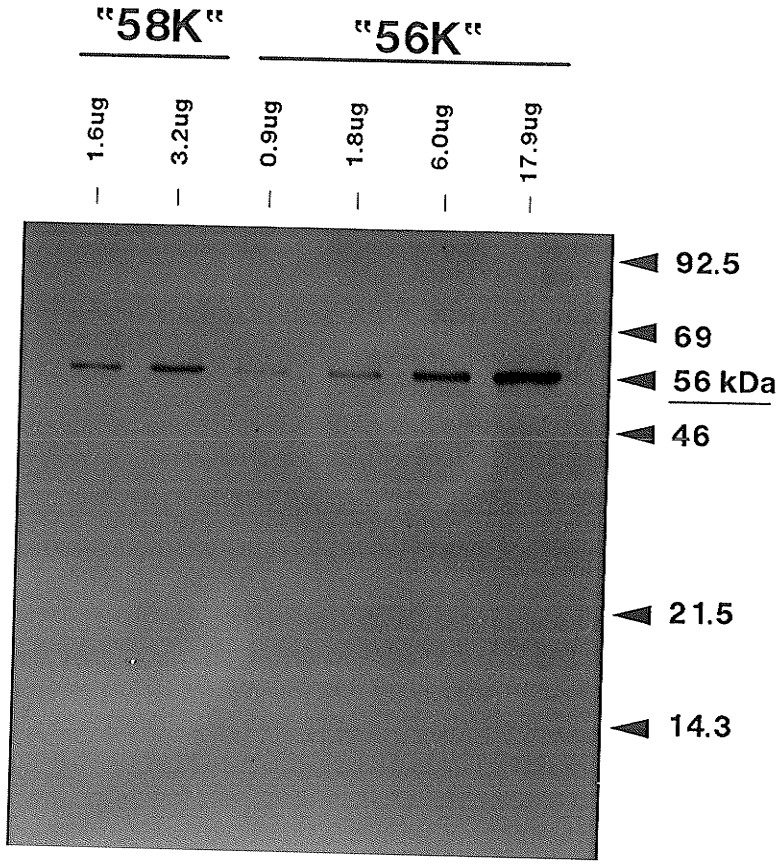


Figure 35 - Fluorogram of SDS-gel electrophoresis of a normal GSF preparation labelled with 5 nM [^{3}H]DHT-BA (lanes 1-2, marked "58 K") and of a normal GSF cell lysate labelled with 50 nM [^{3}H]MB (lanes 3-6, marked "56 K"). The 56 kDa band is marked. (With permission of E. Rosenmann from Belsham *et.al.*, 1989)

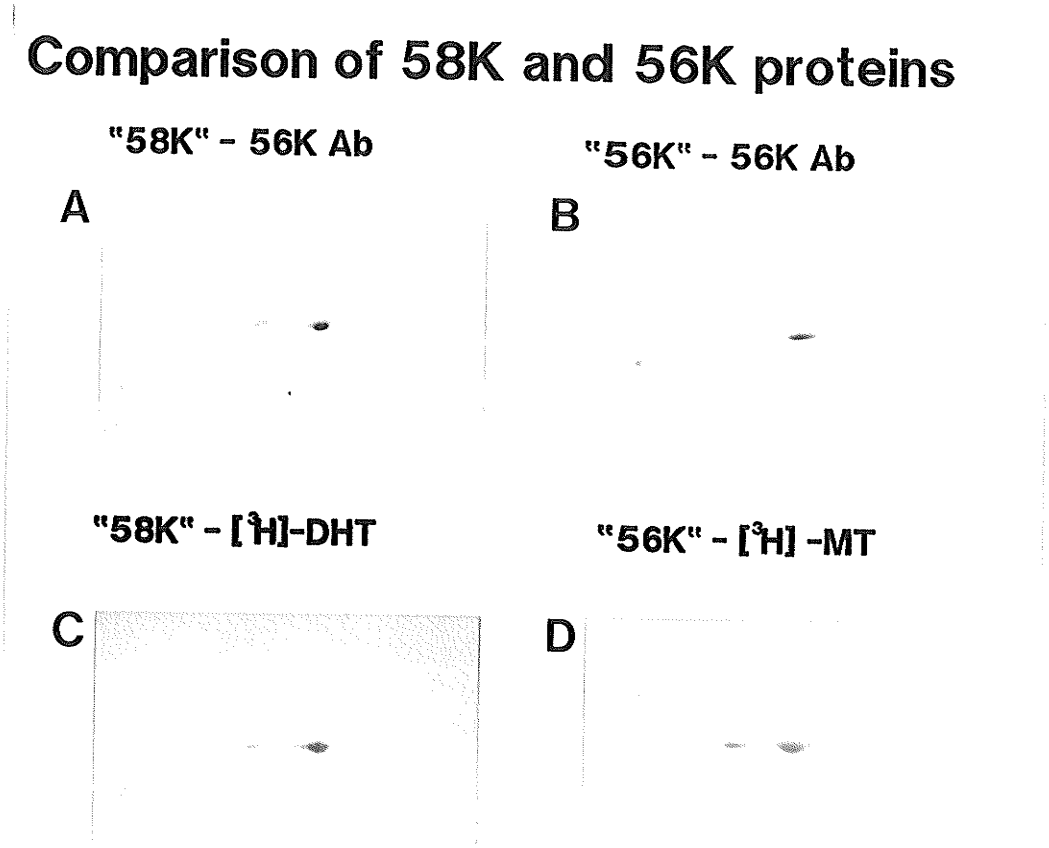


Figure 36 - 2D-PAGE, immunoblots and fluorography of [^{3}H]DHT-BA labelled or [^{3}H]MT labelled human GSF proteins. (A) Immunoblot of "58 kDa" [^{3}H]DHT-BA labelled cell extract reacted with anti 56 kDa antiserum. (B) Immunoblot of strain MCH6 GSF reacted with anti 56 kDa antiserum. (C) Fluorogram of the immunoblot shown in (A). (D) Fluorogram of the immunoblot in (B) labeled with [^{3}H]MT. (From Belsham *et.al.*, 1989)

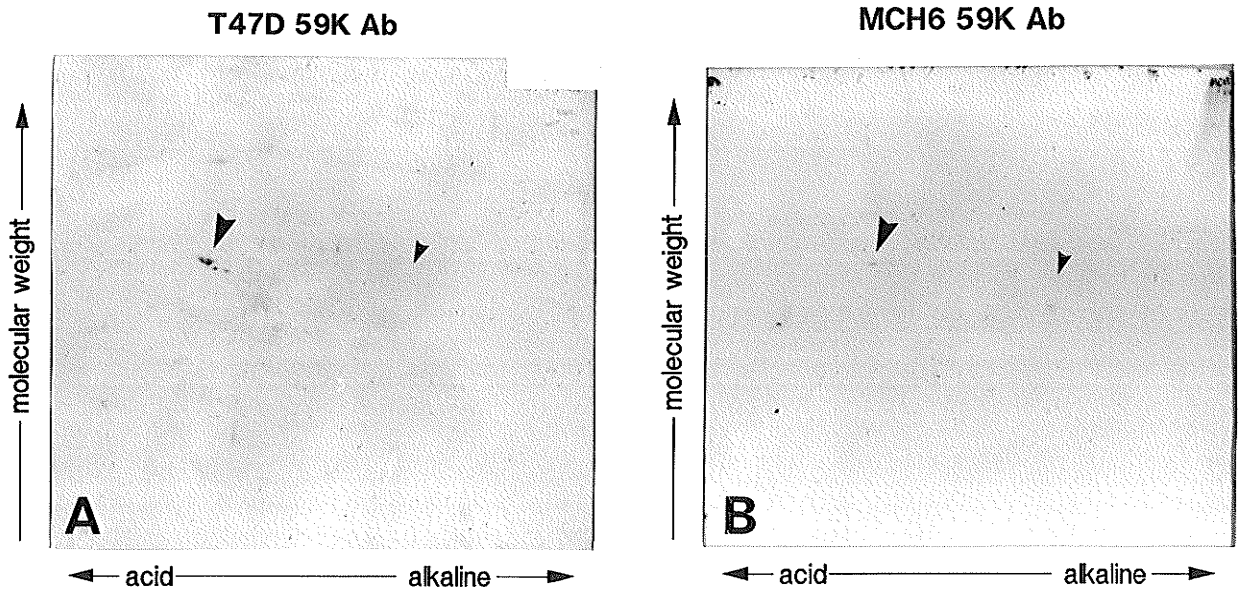


Figure 37 - Immunoblots of T47D cell lysates (A) and of MCH6 cell lysates (B) reacted with the anti 59 kDa monoclonal antibody. The large arrow points to the 59 kDa protein, the smaller arrows point to the position where the 56 kDa protein would be. (From Belsham *et.al.*, 1989)

T47D cells (Figure 37(A)) and, although much weaker, also in normal GSF (Figure 37(B)). The 59 kDa protein is thus clearly distinct from the 56/58 kDa species described above. The anti-59 kDa antibody does not recognize the 56 kDa protein in either cell type (Figure 37). Furthermore, the 56 kDa antiserum does not detect the 56 kDa protein in T47D cells, nor does it crossreact with the 59 kDa protein in either T47D or MCH6 GSF cells (data not shown).

(iv) Discussion

The above study demonstrated unambiguously that the 56 kDa protein first identified as an abundant protein in GSF (Rosenmann *et.al.,* 1982; Thompson *et.al.,* 1983; Nickel *et.al.,* 1988; Wrogemann *et.al.,* 1988) and the 58 kDa protein covalently labelled by DHT-BA (Kovacs, Turney, 1988; Kovacs *et.al.,* 1989) are identical.

The 59 kDa protein identified as a component of several steroid receptors (Tai *et.al.,* 1986; Koutsilieris *et.al.,* 1988) has a clearly distinct pI and does not share steroid binding capacity or immunologic determinants with these proteins. It is thus unrelated to the former protein. Although no clear biological function for the 59 kDa protein has been demonstrated, we know that it is a component of many non-transformed steroid receptors in different tissues and this study describes its presence in GSF.

The 56/58 kDa protein (hereafter described as the 56 kDa protein) has many properties considered characteristic of the human androgen receptor. The protein is expressed in GSF, but not in non-GSF (Thompson *et.al.,* 1983; Nickel *et.al.,* 1988; Kovacs, Turney, 1988), it can be specifically labelled with androgens (Nickel *et.al.,* 1988; Wrogemann *et.al.,* 1988; Kovacs, Turney, 1988), and represents the most prominent protein in GSF with such characteristics (Wrogemann *et.al.,* 1988; Kovacs, Turney, 1988). Most patients with complete AIS lack this protein (Wrogemann *et.al.,* 1988; Kovacs, Turney, 1988). Furthermore, under native conditions the DHT-BA radiolabelled protein is indistinguishable from the authentic receptor complex radiolabelled with [^{3}H]DHT (Kovacs *et.al.,* 1989).

Several characteristics of the 56 kDa protein are not those expected for a classical androgen receptor. First, the protein is more abundant than published estimates of the androgen receptor content of target tissues determined by noncovalent ligand binding assays (Nickel *et.al.,* 1988). On the basis of protein staining, the 56 kDa protein spot was estimated to comprise 0.1% of soluble GSF protein (Nickel *et.al.,* 1988). Quantitation of [^{3}H]DHT-BA binding has indicated a 10-30-fold greater abundance of DHT-BA binding relative to DHT binding (Kovacs *et.al.,* 1989) and an estimated abundance of 0.005% of total soluble protein. Second, the 56 kDa protein has been found to retain its ability to be photolytically labelled with MT in cells from two subjects with complete AIS with no detectable androgen binding activity by noncovalent binding assays (Nickel *et.al.,* 1988; Wrogemann *et.al.,* 1988). Third, the apparent androgen binding affinity of the 56 kDa protein has been assessed by our laboratory to be lower than that of the androgen receptor (Wrogemann *et.al.,* 1988), although results obtained in the DHT-BA binding studies indicated an affinity characteristic of an androgen receptor (Kovacs, Turney, 1988). The reasons for these differing results are not clear. Finally, 56,000 is a lower molecular weight than would be expected for the intact androgen receptor monomer (Johnson *et.al.,* 1987). Data from the molecular cloning of the androgen receptor cDNA (Chang *et.al.,* 1988b; Lubahn *et.al.,* 1988b; Trapman *et.al.,* 1988) predict a protein molecular weight of approximately 99 kDa for the complete coding sequence (Chang *et.al.,* 1988a). However, in vitro translation of androgen receptor mRNA transcribed from full-length cDNA generates a number of receptor-related products, including one of 55 kDa (Chang *et.al.,* 1988a). Some of these (of 94, 76, 70, and 46 kDa) are the result of translation from alternate start sites. The 55 kDa fragment (as well as proteins of 32 and 30 kDa) cannot be accounted for by such a mechanism.

From this study, one can see that the exact relationship of the 56 kDa protein to the androgen receptor is, therefore, not yet fully clarified. It could still be a stable degradation product of the full length receptor monomer (Kovacs *et.al.,* 1989) or it may be synthesized as a separate protein from the androgen receptor gene by processes involving different promoters, differential splicing, alternate strand synthesis, and/or different

polyadenylation sites or a combination of these (Wrogemann *et.al.,* 1988). It may be yet another member of the steroid/thyroid supergene family (Wrogemann *et.al.,* 1988; Evans, 1988). In order to study these questions, it would be best to clone the cDNA of this protein or perhaps find an exceptional patient that produces the 56 kDa protein, but could not possibly produce a functional androgen receptor due to a genetic defect, such as a complete deletion or even a deletion of the hormone binding domain.

B. 56 kDa protein produced by a patient with a complete deletion of the androgen receptor

(from Trifiro *et.al.*, 1991a)

(i) Abstract

Herein we describe a patient with complete androgen insensitivity which has been found to have a complete deletion of the androgen receptor gene. The genital skin fibroblasts of this patient were studied for the presence of the relatively abundant 56 kDa protein, which is known to bind androgens and has been thought to be the androgen receptor or a fragment thereof. The 56 kDa protein was found to be expressed in the GSF of this patient, albeit at much reduced amounts. We therefore must conclude that the similar characteristics between this 56 kDa protein and the androgen receptor cannot arise because they share the same gene, nor can we postulate that the 56 kDa protein is produced from the same gene by processes involving different promoters, differential splicing, synthesis from the other strand and/or different polyadenylation sites or a combination of these.

(ii) The subject

The subject, coded 8812, was found to have an inguinal hernia at one year. She is mentally retarded with her full scale intellectual quotient varying from 30 to 60. She has a 46, XY karyotype and a classical complete androgen insensitivity syndrome phenotype. A GSF biopsy was taken and used to determine specific androgen-binding activity, which was found to be negligible (less than 5 fmol/mg protein); thus, she is classified receptor-negative. Her family history does not present with any other cases of AIS and upon

physical examination, her mother and two adult sisters did not appear to be carriers, as they did not have delayed menarche or sparse, asymmetric or delayed development of pubic or axillary hair, which can be taken as indications of heterozygousity.

(iii) Results

In the course of screening 27 families with complete AIS by a variety of molecular genetic techniques, the group from Dr. L. Pinsky's laboratory in Montreal discovered one who had a complete deletion of the coding portion of the androgen receptor gene (Pinsky *et.al.,* 1989). In order to further characterize the deletion, Dr. Pinsky's laboratory conducted other experiments to investigate the size and apparent termini of the subject's deletion. They used androgen receptor cDNA fragments and also the 5' and 3' regulatory regions of the gene as probes for this purpose.

When using the 5' AR and hAR-1 as probes, no restriction fragments could be detected (Trifiro *et.al.,* 1991a). With the hAR-2.1 or hAR-1.2, a 7 kb restriction fragment was detected as in the control individuals. This fragment is thought to have another origin (as will be discussed) as there is no 7 kb EcoRI-EcoRI interval in the portion of the androgen receptor gene recognized by this probe (Kuiper *et.al.,* 1989).

With the hAR-2, containing exons 7, 8 and part of the 3' region, no DNA fragments were detected from the patient, although in the preliminary report the deletion was thought to stop short of the 3' region. Our laboratory confirmed that the deletion continued into the 3' region by preparing a Southern blot with the patient's genomic DNA, with a male and female control, cut with the restriction endonucleases *Hind*III, *Eco*RI, and *Taq*I. The Southern blot was probed with the 0.5 kb hAR-2 probe (Figure 10) and also with the centromeric X-chromosome probe *DXS1* used as a control. The hAR-2 cDNA fragment did not hybridize to any bands in the patient, but hybridized to the appropriate fragments in the controls (Figure 38(A)). The *DXS1* probe detected the same bands in both the patient and controls (Figure 38(B)). The results established that the proband had a complete

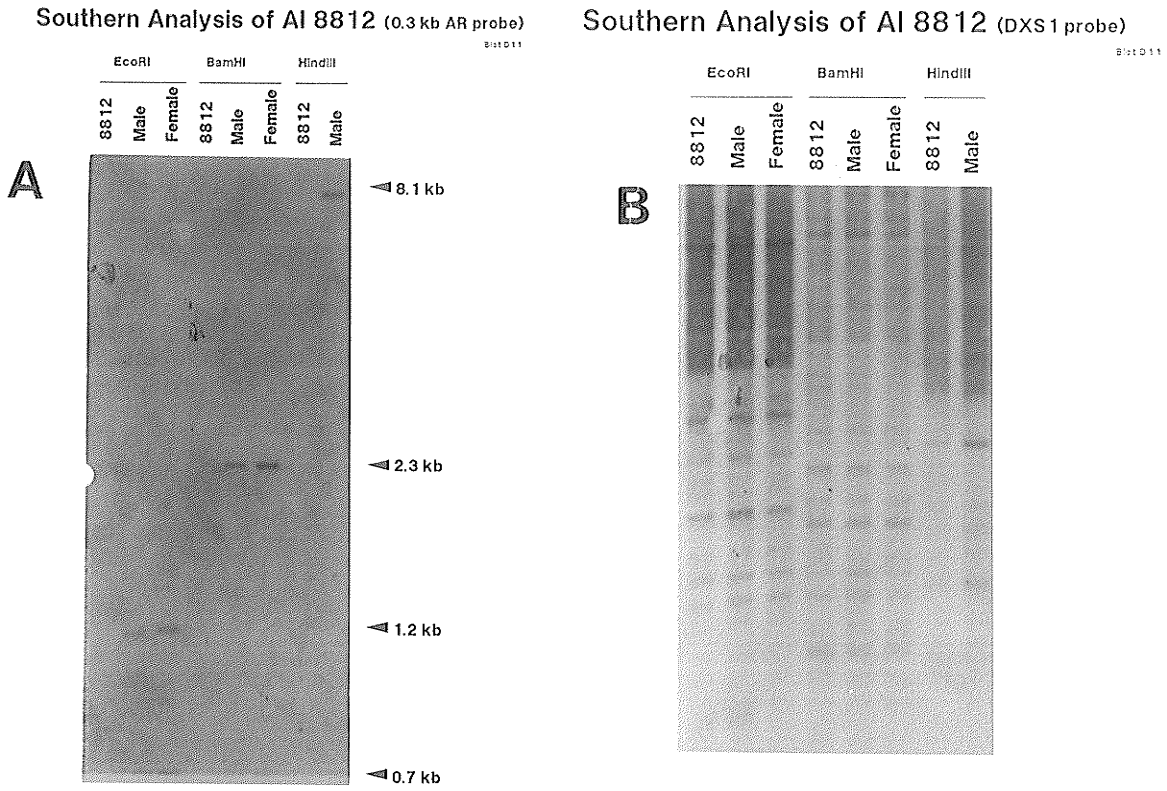


Figure 38 - Southern blot of genomic DNA from the patient 8812 and two normal control subjects from either sex. The DNA was digested with *Hin*dIII, *Eco*RI, and *Bam*HI. The probe used was the 0.7 h-AR cDNA (Figure 11) in (A) and the anonymous DNA sequence *DXS1* in (B).

deletion of the coding region of the androgen receptor gene (Trifiro *et.al.,* 1991a).

Further analysis was done to prove the full extent of the subject's gene deletion by subjecting her DNA, using conditions necessary for the polymerase chain reaction, to intronic primers capable of amplifying exons 2-8, as well as to primers flanking portions of the untranslated regions in exons 1 and 8. The DNA of the subject produced no products whether examined by ethidium bromide staining or by Southern blot hybridization (Trifiro *et.al.,* 1991a). Since the time of publication, our laboratory has been successful in amplifying DNA from exon 1, which was not tested in the proband, containing the CAG repeat region using primers described (La Spada *et.al.,* 1991). The subject's DNA did not produce any product from this region (Figure 30) and it is therefore likely that the deletion is complete, as this region is found in the center of exon 1, and it would be unusual to find a split mutation leaving only a small part of the gene intact.

Our laboratory was quick to examine the GSF from this patient to look for the presence or absence of the 56 kDa protein, once a complete deletion was determined to be the cause of the AIS in this subject. A silver stained gel shows that her GSF make the 56 kDa protein, in both of its pI forms, albeit at a lower level than do the control cells (Figure 39(A)) and this finding is also confirmed by Coomassie Blue staining (data not shown). Using [^{3}H]DHT-BA, we found that the 56 kDa protein was specifically labelled, whether analysed by 1D- (data not shown) or 2D-PAGE (Figure 39(B) and (C)).

(iv) Discussion

In the previous study (Belsham *et.al.,* 1989), we described the 56 kDa protein as being identical to the 58 kDa protein thought to be the androgen receptor due to its similar characteristics. We concluded that this protein could not be the androgen receptor itself due to its differences in molecular size, in apparent concentration within GSF, and in apparent binding affinity.

To account for the differences, we considered a few possibilities, which included

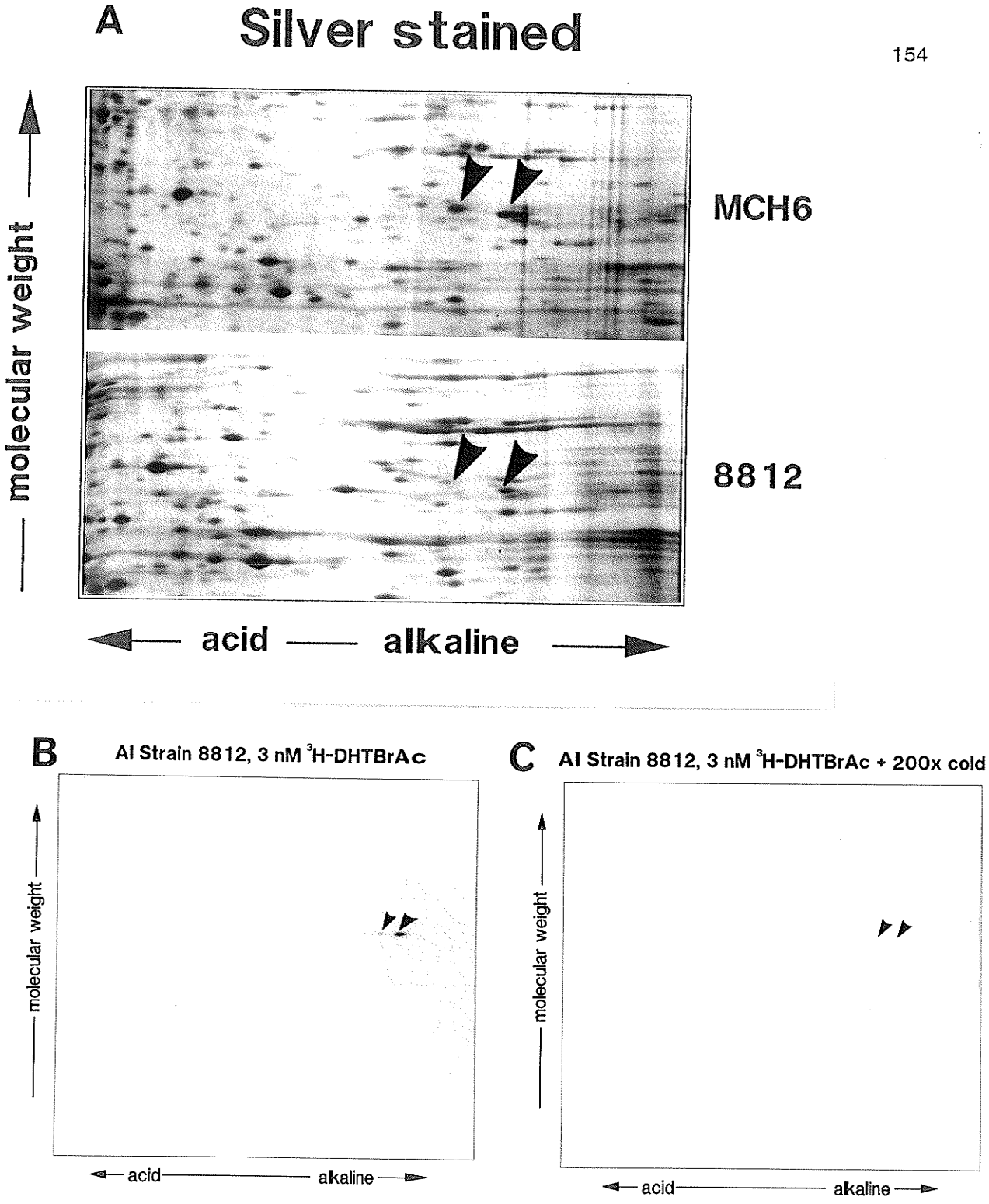


Figure 39 - (A) The 56 kDa protein doublet (p*I* 6.7, 6.5) in GSF detected by silver staining after 2D-PAGE. 8812 is the subject and MCH6 is a control. (B) The 56 kDa androgen-binding protein in its doublet form (p*I* 6.7, 6.5) after 2D-PAGE of the subject's GSF covalently labelled with 3 nM [^{3}H]DHT-BA. (C) The same analysis with 1 mM radioinert DHT-BA added to the cells. (With permission of E. Rosenmann from Trifiro *et.al.*, 1991 a)

the protein being a stable degradation product of the androgen receptor, the protein could be synthesized from the androgen receptor gene by use of different promoters, differential splicing and/or polyadenylation sites, synthesis from the opposite strand or different translation start sites. It could also be a distinct "orphan" member of the steroid/ thyroid/ retinoic acid receptor superfamily. This 56 kDa protein could therefore be a ligand-activated, DNA-binding, transcription-regulating protein of which the authentic ligand(s) have yet to be identified (Wrogemann *et.al.,* 1988; O'Malley, 1990). The results above clearly indicate that the 56 kDa protein must be encoded by a separate gene from the classic androgen receptor gene, because it is made by the GSF of a subject with complete AIS whose androgen receptor gene deletion has eliminated at least all of its coding portion.

The extent of the deletion was somewhat different than the original report (Pinsky *et.al.,* 1989). The 7 kb *Eco*RI-*Eco*RI fragment, detected by the hAR-2.1 and 1.2 cDNA fragments (described previously in the results section), does not exist in the portion of the androgen receptor gene recognized by these two probes. To explain this finding, one must know that both of these probes recognize at least one of the two poly-GC sequences that occur in exon 1 of the androgen receptor gene (Kuiper *et.al.,* 1989; Lubahn *et.al.,* 1989); thus, it is possible that they hybridize to a 7 kb fragment containing a poly-GC sequence, which does not belong to the androgen receptor gene (Trifiro *et.al.,* 1991a). Another possibility is that they hybridize to a 7 kb fragment that contains the highly conserved 5' exon for the DNA-binding domain of another gene in the steroid receptor superfamily (Trifiro *et.al.,* 1991a).

Separate genetic origins of the 56 kDa protein and the androgen receptor help us to explain the GSF strains from the complete AIS patients in which the 56 kDa is expressed normally (Nickel *et.al.,* 1988). The absence of the 56 kDa protein in the GSF of most patients with complete AIS can only be explained by a type of dependency of this protein on the androgen receptor at any of numerous molecular levels. This dependency can not be easily explained, but one speculation is a functional relation with the androgen

receptor protein that promotes relative accumulation of the 56 kDa protein. If this is the case one might imagine that exceptional mutations of the androgen receptor gene, such as in the 5' end where the functional value is relatively unknown, there may be incoordinate expression of the two proteins. To test this hypothesis, one would like to know in which region of the androgen receptor gene the mutations will be found in the two patients with complete AIS that were first shown to express the 56 kDa protein in relatively normal amounts. Communication with Dr. Pinsky indicates that this N-terminal region may contain the causative mutations, as sequencing of the rest of the androgen receptor gene has not uncovered any differences in the cDNA sequence. Again, as in the spinal bulbar muscular atrophy story (La Spada *et.al.,* 1991), the N-terminal of the androgen receptor gene may have functions of greater importance than has been realized or postulated to this point.

C. Further studies of the 56 kDa protein

(i) Abstract

Herein we describe further studies of the 56 kDa protein in order to more fully understand its involvement in the androgen insensitivity syndromes and likely relationship with the androgen receptor. This relationship can not be easily elucidated, as has been proven by the previous studies described, but with the complete characterization of the protein, we will be closer to understanding the problem and obtaining a clear answer to this problem. To confirm the initial findings that this protein is absent in the GSF of AIS patients, it would be beneficial to examine the new patients from Family 1, 2, and 3 described in the first part of this thesis for the expression of this protein using the most sensitive test found for this purpose, that is covalent radiolabelling with [^{3}H]DHT-BA. Using this method, we find that the protein is not absent, but instead is expressed at different levels in these individuals. In order to confirm our hypothesis that the 56 kDa protein is not androgen induced, a separate study, which again refutes androgen induction was also undertaken. The ultimate answer to our question of the significance of the 56 kDa protein lies in the molecular cloning of its cDNA, of which studies have been performed and will be described in the discussion.

(ii) Results

Patient studies

The 56 kDa protein is now known to be present in not only the two original exceptional AIS patients (Nickel *et.al.,* 1988), but also in the AIS patient with a complete deletion of the androgen receptor (Trifiro *et.al.,* 1991a). With the increased sensitivity of detection of this protein using DHT-BA (Kovacs, Turney, 1988; Kovacs *et.al.,* 1989), one can also detect the 56 kDa protein in the GSF of the two complete AIS patients in the kindreds studied in this thesis (Figure 40(A) and (B)). The protein is expressed but at much reduced levels from normal control GSF (Figure 40(D)). The partial AIS patient also expresses the protein in his GSF (Figure 40(C)), at a relatively higher amount than that of the complete AIS patients.

Androgen induction analysis

The hypothesis that the expression of the 56 kDa protein is not androgen dependent has always been maintained throughout our studies. In order to confirm this finding, a study was performed in which MCH6 cells, grown in hormone-stripped medium, were incubated both in the presence and absence of the androgens analog DHT-BA to determine the effect that androgens have on the expression of the 56 kDa protein. The cells were labelled with [^{35}S]methionine. The results indicate that the expression of the 56 kDa protein is not noticeably altered, both upon silver staining and after autoradiography (Figure 41(A)-(D)).

(iii) Discussion

The initial experiments studying the 56 kDa protein came to the conclusion that the protein doublet was "absent" in the GSF of patients with AIS (Nickel *et.al.,* 1988). In light of more recent studies, this conclusion may not necessarily be true, as stated. The protein was present in the original two exceptional patients, and the relative amounts of the protein appeared to be the same as the normal control GSF cell lines (Nickel *et.al.,* 1988). The detection system used in these studies was 2D-PAGE followed by silver-stain and the amount of 56 kDa protein estimated and scored by eye.

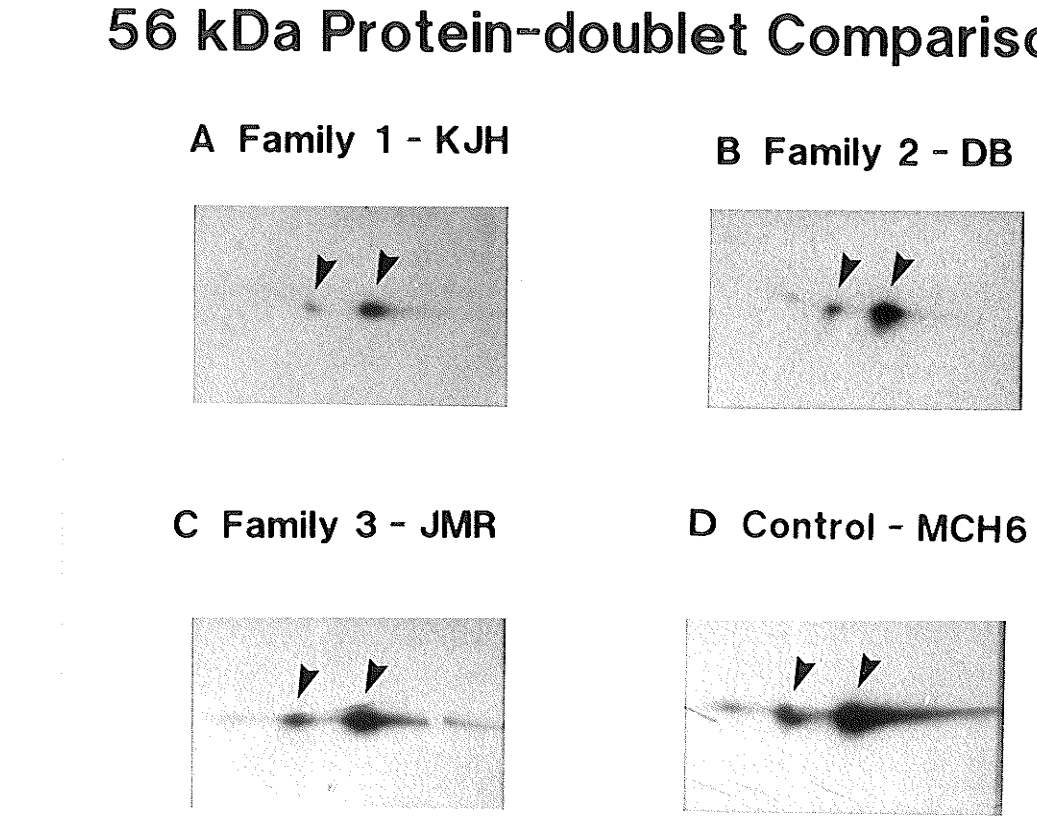


Figure 40 - Covalent labeling of GSF with 5 nM [^{3}H]DHT-BA in (A) CAIS subject KJH from Family 1; (B) CAIS subject DB from Family 2; (C) PAIS subject JMR from Family 3; and (D) control GSF strain MCH6. Exposure: 21 days. Arrows indicate 56 kDa protein doublet.

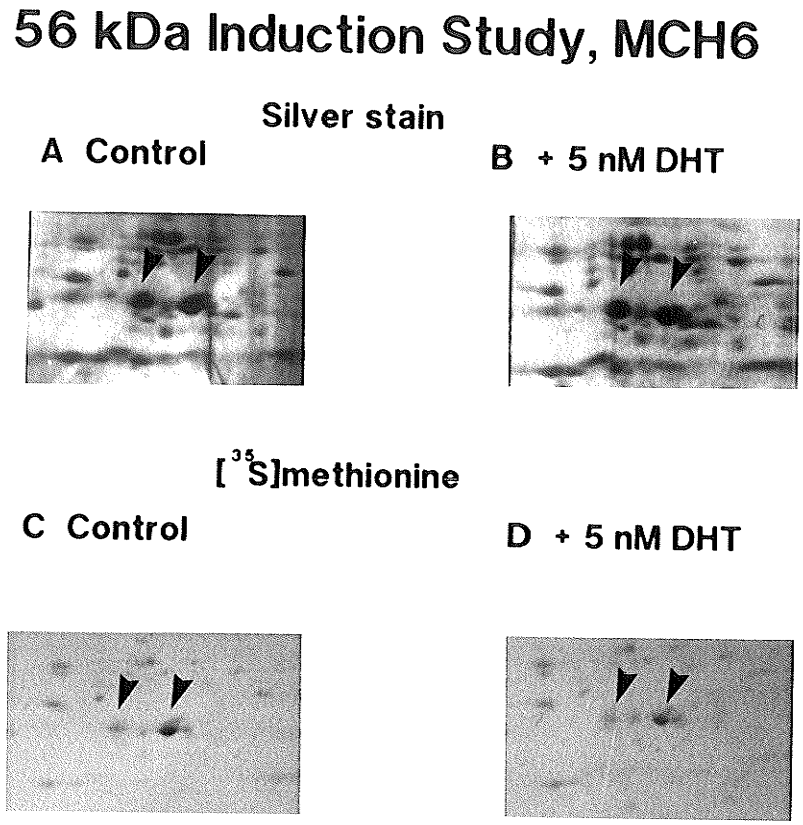


Figure 41 - Expression analysis of the 56 kDa protein with and without androgen. (A) Silver-stained 2D-PAGE protein map of [^{35}S]methionine labelled GSF strain MCH6 after a previous two day incubation in charcoal-stripped medium (free from steroid); and (B) as in (A), but with 5 nM MB. (C) Autoradiograph of the 2D-gel in (A); and (D) the 2D-gel in (B). Exposure: 7 days. Arrows point to the 56 kDa protein doublet.

Since that time, our laboratory has used tritiated androgen analogs to detect the presence of the protein on fluorograms. The most sensitive method to date has been the use of [^{3}H]DHT-BA. With this method one is able to detect the protein in patients with complete AIS, such as the patient with a complete deletion of the androgen receptor, and also in the two probands of the families studied and reported in this thesis. The relative amounts of this protein are lower than that of the control cell line, MCH6, but nevertheless it is detectable, and not "absent" as was stated in the original papers (Rosenmann *et.al.,* 1982; Thompson *et.al.,* 1983; Nickel *et.al.,* 1988). It is likely that instead there is a gradient of expression, depending upon the severity of the androgen receptor defect, as the complete AIS patient expresses the protein less than the partial AIS, which in turn is still less than the expression in normal GSF. Of course, it is difficult to draw conclusions from these four individuals and to be statistically sound, one should test the original and newly acquired AIS patients (now numbering more than 32) along with the appropriate control subjects for the expression of the 56 kDa protein by the more sensitive method, that being covalent labeling with [^{3}H]DHT-BA.

The presence of the 56 kDa protein in these patients with AIS indicates that it is a separate protein from the androgen receptor, but that there is some form of cooperative cellular effort between the two proteins. This regulatory effect may occur at the gene level. In order to determine if this is possible, it would be best to determine if the 56 kDa protein is androgen induced. The experiments to test this possibility were done previously, but there were some oversights in the experimental procedures, which have since been improved. In particular, the cells were not incubated with hormone-stripped medium before introduction of the test androgen. Although these previous results indicated that the protein was not androgen induced, it was deemed valuable to repeat this analysis with improved methodology. To reconfirm this conclusion, the relative amount of the 56 kDa protein in the presence or absence of androgens was evaluated both by Silver-stain analysis and by autoradiography. The results indicate that there is no androgen induction involved in the regulation of the expression of the 56 kDa protein upon addition of androgen (Figure 41(A)-(D)). There are perhaps more sensitive methods that should also

be attempted before completely rejecting any possibility of androgen induction. One method may include the evaluation of the GSF mRNA levels on Northern blots once the cDNA for the 56 kDa protein becomes available, at different time points, in the presence or absence of androgen .

In order to ultimately determine the origin of the 56 kDa protein, one must clone the cDNA of the protein. Another student in our laboratory, Mr. Fred Pereira, accepted this challenge and was finally successful in 1990 (Pereira *et.al.,* 1991). It was determined, after cloning the cDNA from an expression library of genital skin fibroblasts using the 56 kDa antiserum produced in our laboratory, that the 56 kDa protein is an aldehyde dehydrogenase, particularly human aldehyde dehydrogenase 1 (ALDH1), the cytosolic isoform. This 56 kDa androgen binding protein also has aldehyde dehydrogenase activity (Figure 42).

The conclusion that the 56 kDa protein was indeed an aldehyde dehydrogenase came only after a number of confirmational tests of the selected cDNA clone. The complete findings of this study can be summarized as by Pereira *et.al.* (1991): (i) the clone detects a message that reflects quantitatively the 56 kDa protein, using the various cell lines available to us, (ii) the clone selects a message which translates into a 56 kDa protein recognized by the 56 kDa antiserum, (iii) the partial sequence, now full sequence, shows 100% homology to ALDH1 (Hsu *et.al.,* 1985), (iv) The subunit size, isoelectric point on non-denaturing gels and message size for the 56 kDa protein are in close agreement for that described for the enzyme (Kurys *et.al.,* 1989; Hsu *et.al.,* 1989; Agarwal, Goedde, 1989); (v) GSF show ALDH activity, and this activity comigrates with a band that contains the 56 kDa protein specifically labelled with androgen (Figure 42); (vi) the data agree with the observation that ALDH1 is not expressed in skin fibroblasts (Anthony *et.al.,* 1989), specifically non-GSF.

The aldehyde dehydrogenases are thought to be important for the rapid detoxification of various potentially cytotoxic aldehydes (Sladek *et.al.,* 1989).

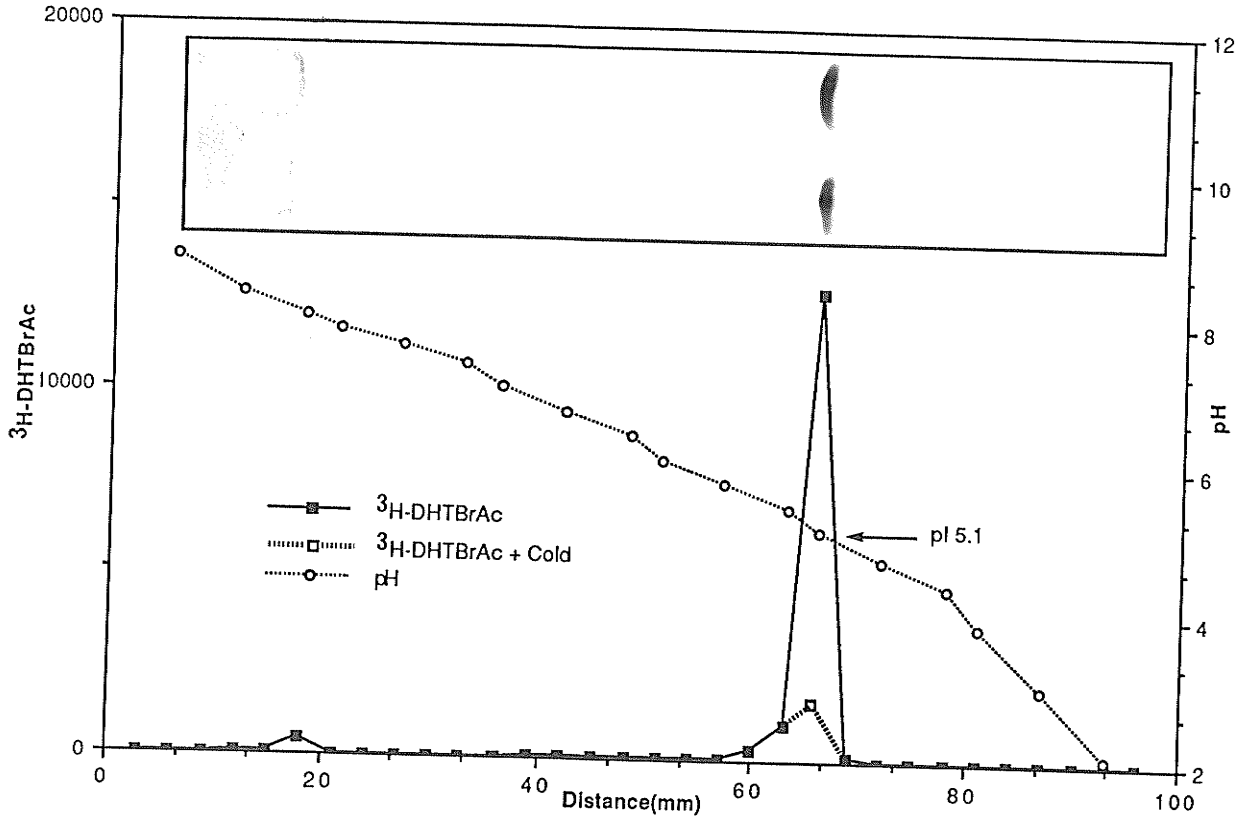


Figure 42 - Aldehyde dehydrogenase activity associated with [^{3}H]DHT-BA labelled GSF cytosol from the normal individual MCH6. Activity gel is shown as an insert. Lane A, cells labelled with 5 nM [^{3}H]DHT-BA; lane B, as in A, but with the inclusion of 200-fold cold DHT-BA. The pH profile and counts per minute are plotted against the length of the non-denaturing isoelectric focusing gel. The enzyme activity and radioactivity bands both focused at *pI* 5.1 (arrow). (With permission of E. Rosenmann from *Pereira* et.al., 1991)

The enzymes catalyze the irreversible oxidation of these highly reactive aldehydes to the corresponding acids in a NAD-dependent reaction (Kurys *et.al.,* 1989). There have been numerous isoforms of these enzymes described. ALDH-2, the mitochondrial isoform, is responsible for acetaldehyde oxidation. A mutation of this enzyme is thought to be responsible for alcohol sensitivity, which is often noticed in Orientals as "facial flushing" (Yoshida *et.al.,* 1983; Jornvall *et.al.,* 1987). The specific biological roles of the various isoforms have not yet been fully elucidated.

This finding proves that although a protein has androgen binding characteristics, one can not classify it as a receptor or a member of the steroid binding protein family (Pereira *et.al.,* 1991). The exact correlation of the expression of this protein (ALDH1) with the presence of a functionally normal androgen receptor must now be determined. The preferential expression in genital skin fibroblasts must also be examined. The aldehyde dehydrogenases have been extensively studied and perhaps by using the known characteristics of this enzyme, a more coherent story can be pieced together as to the significance of this protein in the androgen insensitivity syndromes.

Part V

Concluding Remarks and Future Implications

To truly understand the processes of normal sexual development, one must first examine the endocrine systems involved in this process. In particular, the androgen receptor plays a major role in the course of development of the normal male phenotype. The mechanism of action of the androgen receptor is currently being elucidated. Androgen is thought to bind to its receptor protein with high affinity and in turn launches an expression cascade of gene networks or a genetic program in the target cells. To unravel the complex components of these gene networks, one must begin with the most basic concepts of this process.

The androgen insensitivity syndrome is the ideal vehicle to begin this type of study and has allowed the complex processes of androgen action to be separated into essentially singular constituents, as almost every subject has been found to have a different mutation, each adding another piece of knowledge to the puzzle. Normal androgen action is necessary for reproductive fitness, but not essential for life; thus, the androgen insensitivity syndromes allow us to determine the events required for normal sexual development and to elucidate the sites where this process may go awry.

This study was established to characterize three Manitoba families and numerous individuals with androgen insensitivity syndrome with respect to two separate proteins known to be involved in the disorder. The two proteins were the androgen receptor, defects of which are known to be the direct cause of androgen insensitivity, and the 56 kDa protein, thought to be secondarily involved.

Preliminary studies indicated that the 56 kDa protein was involved in the syndrome, as this androgen binding protein was found to be absent in the genital skin fibroblasts of patients with androgen insensitivity syndrome. The protein was absent in non-genital skin fibroblasts and expressed normally in normal genital skin fibroblasts. The initial focus of the project was to further characterize the 56 kDa protein and its involvement in the syndrome.

Upon comparison to other reported proteins with similar characteristics to our 56 kDa protein, it was found that another group was indeed studying the same protein, which they claimed to be the androgen receptor itself or a proteolytic fragment thereof. We maintained throughout our studies that our 56 kDa protein was not the androgen receptor itself, as it was too small, too abundant, but also not androgen induced due to the fact that it was previously found to be expressed in two patients with complete androgen insensitivity syndrome.

To encompass all of our findings, we then hypothesized that the 56 kDa protein was synthesized from the same gene as the androgen receptor by mechanisms that could include alternate splice sites, promoters or polyadenylation sites, or possibly synthesis from the opposite strand. This hypothesis was proven to be false as a patient with a complete deletion of the androgen receptor gene was found to express our protein, albeit in reduced amounts.

Further characterization of the 56 kDa protein indicates that it may be expressed in patients with androgen insensitivity, albeit in reduced amounts, and not "absent" as initially reported. Preliminary studies indicate that there may be increasing levels of expression as the androgen insensitivity syndrome phenotype due to androgen receptor defects becomes less severe. The more sensitive method of covalent labeling with [^{3}H]DHT-BA may eventually aid in detecting the expression of the protein, most likely at low levels, in all patients, both previously tested and newly acquired.

The cloning of the cDNA of the 56 kDa protein by another member of our laboratory has revealed that it is an aldehyde dehydrogenase, precisely cytosolic ALDH-1 (EC 1.2.1.3). The next frontier of the work with the 56 kDa protein will be to determine whether and/or how both androgen binding activity and aldehyde dehydrogenase activity are involved in normal androgen action. Furthermore, why is the expression of this protein significantly decreased in patients with androgen insensitivity syndrome? The involvement of cytosolic aldehyde dehydrogenase in normal androgen action has not yet been

postulated, and with the further characterization of the 56 kDa protein with respect to these patients, including the regulation of the protein and function in androgen target tissues, an answer to these questions will eventually be determined.

Because we were already characterizing individuals with androgen insensitivity syndrome with respect to the 56 kDa protein, we subsequently decided to provide carrier testing analysis to two families requiring these services. The androgen receptor was not cloned until 1988; thus, we initially attempted linkage analysis with pericentromeric markers thought to be linked to the androgen receptor gene. Although this type of analysis was successful in a few individuals, we still could not determine the carrier status in numerous others, and decided to look for other polymorphic markers with the androgen receptor cDNA once it became available to us.

In the process of searching for RFLPs with the androgen receptor cDNA, we were able to find a novel *Msp*I RFLP in Family 1 which we could not detect in a sample of the general population. Localization of this RFLP pointed to the steroid-binding domain of the androgen receptor gene. Eventually we found a unique mutation in exon 4 at a highly conserved site in the steroid receptor supergene family. This mutation changes Leu676 to Pro via a single T to C transition mutation, creating a diagnostic *Msp*I restriction enzyme site. This finding has allowed us to determine the carrier status in all potential carriers in Family 1, which has been of considerable value to the members involved in the study.

This mutation is likely the cause of the disorder in this family, but in order to prove its causative nature we must first attempt to determine if the mutant androgen receptor gene produces a protein with a decreased amount of specific androgen receptor binding activity, determined by tests with a mutant androgen receptor construct expressed in a mammalian cell system. If a decreased amount of androgen receptor binding is not detected, then it would be best to continue to analyze the exons and regulatory regions of the androgen receptor gene not previously analyzed for another potential causative mutation. Nevertheless, the RFLP analysis and carrier predictions should not be affected

by such analysis, as the RFLP is found within the androgen receptor gene itself and the chance for recombination to occur within this region is likely slim.

Our second family with complete androgen insensitivity syndrome was also analyzed at the gene level and a mutation was found by Dr. Pinsky's laboratory. The mutation was a single point mutation in exon 8 (A to T) changing Lys882 to an amber translation termination signal. Again, a restriction enzyme site, *Mae*I, was created by this mutation and allowed the confirmation of the previous carrier detection by linkage analysis with the X chromosome markers.

A potential disease-causing mutation has not yet been found in Family 3 with partial androgen insensitivity syndrome, but the search continues. We have been able to characterize this family with respect to androgen binding activity by Scatchard analysis and the decreased affinity for androgens indicates that the mutation may lie in the steroid binding domain. Linkage analysis has not been possible and the ideal method to determine potential carriers in this family would be through direct mutation detection.

In a collaborative effort, we have also assisted in the elucidation of a mutational "hot-spot" at Arg773 in four individuals with complete androgen insensitivity. By expression analysis of the wild-type versus mutant androgen receptor gene constructs in transfected COS cells, this mutation was found to yield the deleterious consequences assumed to be responsible for the production of the androgen insensitive phenotype.

During the course of this study, it was determined that another defect of the androgen receptor gene may also be responsible for the production of a seemingly unrelated disorder, that being X-linked spinal and bulbar muscular atrophy. An increase in the number of CAG repeats in exon 1 of the androgen receptor gene has been shown to be absolutely associated with the disorder. Using this knowledge, we were able to determine the carrier status and potential disease status in a family with this late-onset progressive disorder. We have been able to determine that the affected proband in Family

4 has approximately twice the number of glutamine repeats than his unaffected brother. The other potential carriers or affected members of this family now have the option to have carrier analysis or disease diagnosis using this method.

The study of the families with defects of the androgen receptor has also contributed to the ever growing knowledge base on the mechanisms of androgen action and defects thereof. By analyzing each mutation, we become ever closer to understanding the role of each domain, or more precisely each nucleotide, in the development of an individual with a normal male phenotype.

Furthermore, by analyzing the defects in the androgen receptor gene, we should also get a more clear understanding of the role of the 56 kDa protein in this process. Presently, we have more than 30 androgen insensitive patients, in which the androgen receptor gene mutations are rapidly being elucidated. It should therefore be possible to correlate the expression of the 56 kDa protein at both the protein and mRNA levels with the specific androgen receptor gene mutations. This type of analysis may add some insight into the nature of the relationship of these two distinct proteins.

The study of the androgen insensitivity syndrome has been of immeasurable significance to the fields of both endocrinology and genetics due to its unusual characteristics. It is rare to find a disease in which the patients are completely changed by the effects of a single gene and yet the results are not lethal. The serendipitous discovery of the 56 kDa protein, which seems to play a role in this disorder, only makes the syndrome more fascinating to study. Combining the findings from all facets of this study has and will most likely continue to add to the ever expanding knowledge base of the androgen insensitivity syndromes and the deviations from normal during sexual development in general.

Part VI

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