

CHARACTERIZATION OF THE 56 kDa ANDROGEN-BINDING PROTEIN

by

Frederick A. Pereira

A thesis submitted to the Faculty of Graduate Studies
The University of Manitoba
Winnipeg, Manitoba, Canada

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry and Molecular Biology

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Dedication

*To Tracy, Korey and Graeme.
Thanks for keeping me in touch with reality.*

*Knowing the ways we can go wrong will help us to take the right way.
(From Psychology and Life, 1975, p610, by Philip G. Zimbardo,
Scott, Foresman & Co. Glenview, Illinois.)*

<i>Attribute</i>	<i>Enables us to</i>	<i>But can also lead us to</i>
Memory	Profit from past mistakes Develop and use complex concepts Relate present to past Distinguish novel events from previously experienced ones	Carry grudges, suffer from former conflicts and past traumatic events Lose spontaneity of behavior because of commitments and obligations Feel excessive remorse or sense of loss
Time sense	Develop a history and sense of a continuous self Relate present behavior to the future Distinguish between transience and permanence	Fear change, live in the past, feel guilt Dread an unknown future, become anxious Experience disappointments from unfulfilled expectations Concentrate on past or future, ignoring the present
Ability to associate elements and infer unseen events	Create, imagine events not experienced Generalize from partial data Construct theories, hypotheses	Form negative, crippling associations Misperceive self or others, develop stereotypic and delusional thinking
Perception of choice	Not be stimulus bound, be independent See ourselves as responsible agents Hope, build for future	Experience conflicts, indecision Suffer from inability to act when action is necessary
Responsibility, self-evaluation	Take pride in accomplishments Delay gratification, undertake difficult or unpopular tasks Be concerned about effects of our actions on others	Feel inadequate Feel guilt for not living up to standards or for letting someone down Feel constrained by obligations
Competence motivation	Do work well, set high standards Gain benefits of hard work Advance technologically, use resources to meet our needs	Fear failure, suffer feelings of inadequacy Be anxious about tests of our ability Work for self-aggrandizement, to be "number one," to beat others down
Concept of justice	Protect individual rights Set up fair rules binding on all Value equality of opportunity	Sacrifice individual needs to group principles or rules Torture, imprison, execute dissenters Impose our own solutions on others
Ability to use language and other symbols	Communicate with others, present and absent, for information, comfort, pleasure, planning, social control	Circulate and be prey to rumors and falsehoods; conceal true feelings Use "word magic"—curses, verbal derogation—to inflict harm Mistake the symbol for the reality
Susceptibility to social influence	Follow group standards Learn and transmit values Cooperate; establish community	Overconform, sacrifice integrity Reject innovation and stifle creativity in ourselves and others
Love	Experience tender emotions Nurture growth and independence of others Support, encourage, comfort others Feel wanted and special	Become jealous, vengeful Possessively limit another person's freedom Become depressed and suicidal from loss of love ¹

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I would like to thank Dr. Wrogemann for allowing me to learn the intricacies of basic research. Over the last 10 years, I have been fortunate to have witnessed the rigors of basic research in Canada. We experienced the exhilaration and excitement of cloning a cDNA for the first time and the melancholy of realizing it had been cloned before. However, as proper researchers would do, we were able to make the best of the situation and become innovators in the field of aldehyde dehydrogenases. Thank you for allowing me to develop at my own pace.

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List of Abbreviations

α	-	Alpha
A	-	Adenine or adenosine
A ₂₆₀	-	Absorbance at 260 nm; 1 A ₂₆₀ unit = 50 μ g of double stranded DNA 1 A ₂₆₀ unit = 40 μ g of single stranded DNA or RNA 1 A ₂₆₀ unit = 33 μ g of single stranded DNA oligomer
A ₂₆₀ /A ₂₈₀	-	Ratio of absorbancies at 260 nm and 280 nm
ADH	-	Human alcohol dehydrogenase
AHD2	-	Mouse aldehyde dehydrogenase 2
CAIS	-	Complete androgen insensitivity syndrome
ALDH1	-	Aldehyde dehydrogenase 1
ALDHs	-	Aldehyde dehydrogenases
AR	-	Androgen receptor
Arg	-	Arginine
ATP	-	Adenosine-5'-triphosphate
β	-	Beta
bis	-	N,N'-methylenebisacrylamide
BCIP	-	5-bromo-4-chloro-3-indolyl phosphate
bp	-	Nucleotide base pairs
BSA	-	Bovine serum albumin
C	-	Cytosine or cytidine
°C	-	Degrees Celsius
Ca ²⁺	-	Calcium
CaCl ₂	-	Calcium chloride
CAIS	-	Complete androgen insensitivity syndrome
cAMP	-	Cyclic 3'5'-adenosine monophosphate
cDNA	-	Complementary deoxyribonucleic acid
CHAPS	-	(3-[(3-cholamidopropyl)-dimethylammoniol]1-propanesulfonate)
Ci	-	Curie = 37 gigabecquels
cm	-	Centimeter
cpm	-	Counts per minute
CTP	-	Cytosine 5'-triphosphate
Cys	-	Cystine
dCTP	-	Deoxycytosine 5'-triphosphate
DHT	-	Dihydrotestosterone
DHTBrAc	-	Dihydrotestosterone 17 β -bromoacetate
DMSO	-	Dimethyl sulphoxide
DNA	-	Deoxyribonucleic acid
DNase I	-	Deoxyribonuclease I
dNTP	-	Mixture of deoxynucleotide triphosphates
DTT	-	Dithiothreitol
Eagle's MEM	-	Eagle's minimal essential medium
EDTA	-	Ethylenediamine tetraacetic acid
fmol	-	Femtomole
γ	-	Gamma
g	-	Gram
G	-	Guanine or guanosine
GBq	-	Gigabecquerel = 27 millicuries
GSF	-	genital skin fibroblasts
GTP	-	Guanosine 5'-triphosphate
h	-	Hour or hours

H	-	Histidine
^3H	-	Tritium
HCL	-	Hydrochloric acid
HEPES	-	(N-2-hydroxyethylpiperazine)-N'-2-ethanesulphonic acid
Hg	-	Mercury
H_2O	-	Water
H_3PO_4	-	Phosphoric acid
HRE	-	Hormone responsive/regulatory element
i.d.	-	Internal diameter
IEF	-	Isoelectric focusing
inch.	-	Inches
IPTG	-	β -D-isopropyl-thiogalactopyranoside
kb	-	Kilobase pairs
KCL	-	Potassium chloride
Kd	-	Dissociation constant
kDa	-	Kilodaltons
KH_2PO_4	-	Potassium phosphate, dibasic
K_m	-	Michelis-Menton constant
λ	-	Lambda
l	-	Liter
LTR	-	Long-terminal repeat
M	-	Molar
mA	-	Milliamperes
MB	-	Mibolerone
mCi	-	Millicurie = 37 megabecquerels
MgCl_2	-	Magnesium chloride
MgSO_4	-	Magnesium sulfate
min	-	Minute or minutes
ml	-	Milliliter
μCi	-	Microcurie = 37 kilobecquerels
μg	-	Microgram
μl	-	Microliter
μM	-	Micromolar
mm	-	Millimeters
mM	-	Millimolar
MR	-	Mineralocorticoid receptor
mRNA	-	messenger RNA
MT	-	Methyltrienolone; R1881
MW	-	Molecular weight
NaCl	-	Sodium chloride
NAD^+	-	Nicotinidine diamide
NaOH	-	Sodium hydroxide
Na_2HPO_4	-	Sodium phosphate, dibasic
NaH_2PO_4	-	Sodium phosphate, monobasic
NaPO_4	-	Sodium phosphate buffer
NBT	-	Nitro blue tetrazolium
NH_4HCO_3	-	Ammonium bicarbonate
nm	-	Nanometers
nmol	-	Nanomole
nM	-	Nanomolar
non-GSF	-	Non-genital skin fibroblasts
OD	-	Optical density
%	-	Percentage

PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
pfu	-	Plaque-forming units
pg	-	Picogram
pI	-	Isoelectric point
pmol	-	Picomole
PMSF	-	Phenylmethylsulfonyl fluoride
Pol I	-	<i>E. coli</i> DNA polymerase I
PNK	-	<i>E. coli</i> polynucleotide kinase
r-	-	Restriction minus
RAR	-	Retinoic acid receptor
RBCs	-	Red blood cells
RFLP	-	Restriction fragment length polymorphism
RNA	-	Ribonucleic acid
RNase A	-	Ribonuclease A
RNase H	-	Ribonuclease H
RT	-	Reverse transcriptase
s	-	Seconds
SA	-	Specific activity
ssDNA	-	Single-stranded DNA
SDS	-	Sodium dodecyl sulfate
SSC	-	Sodium citrate buffer solution
T	-	Thymine or thymidine
TAE	-	Tris acetate/EDTA buffer
Taq	-	DNA dependent DNA polymerase from <i>Thermus aquaticus</i>
TBE	-	Tris borate/EDTA buffer
TBq	-	Tetrabecquerel = 27 curies
TBS	-	Tris buffered saline
TBST	-	Tris buffered saline with tween-20
TCA	-	Trichloroacetic acid
TE	-	Tris-EDTA solution
TEMED	-	N,N,N',N'-tetramethylethylenediamine
Tfm	-	Testicular feminization
tRNA	-	Transfer RNA
Tris-HCL	-	Tris(hydroxymethyl)aminomethane hydrochloride
2D	-	Two-dimensional
U	-	Unit
UTR	-	Untranslated region
V	-	Volts
Vh	-	Volthours
(v/v)	-	Volume:volume ratio
(w/v)	-	Weight:volume ratio
x	-	Times
x g	-	Times gravity
X-gal	-	5-bromo-4-chloro-3-indolyl- β -D-galactoside

Summary of Contributions

Modern research is done on a collaborative nature due to the immense amount of data necessary for quick publications today. The work presented here is a culmination of research in Dr. Wrogemann's laboratory over the last 12 years. Ten years of my life have been spent on this "56 kDa protein", first as a technician and then for the last 6 years as a student working on the majority of the data presented here in this thesis. Where experiments were performed by other members of the laboratory, I have indicated from which publication the data were taken. However, all the published data presented here are taken from papers with my authorship in which I had contributed to the experimental design, protocol and/or writing of the manuscripts.

Abstract

Over the last few years, a new marker for the phenotypic heterogeneity seen within the class of X-linked complete androgen insensitivity syndrome has been characterized. The syndrome is caused by molecular defects of the androgen receptor and the marker, a 56 kDa protein, is not detected in genital skin fibroblasts of most patients with the syndrome. This finding agrees with the observation that gene alterations of the AR result in a heterogenous phenotype. The two species of the 56 kDa protein doublet are found to be identical and did not arise due to phosphorylation nor glycosylation. The 56 kDa protein is not a heat shock protein and its long half-life probably contributes to its abundance. The protein is expressed in all 23 normal genital skin fibroblast cell strains of either sex (genital skin fibroblasts are an androgen target tissue) but not found in 12 of 16 genital skin fibroblasts from patients with complete androgen insensitivity studied to date. However, the protein is not androgen induced. The protein binds androgens, and this led one group to describe it as the androgen receptor itself. Using an antiserum to the 56 kDa protein, partial and full-length clones were identified from cDNA expression libraries constructed from a GSF cell strain which has an abundant expression of the 56 kDa protein. The sequence obtained from these clones is identical to the human liver cytosolic aldehyde dehydrogenase 1 (Aldehyde:NAD⁺ oxidoreductase, EC 1.2.1.3, ALDH 1), which had previously been cloned. Aldehyde dehydrogenase activity from genital skin fibroblast cytosol is found to co-migrate with the single band labeled specifically with [³H]dihydrotestosterone bromoacetate on non-denaturing isoelectric focusing activity gels. Thus, the 56 kDa protein is an aldehyde dehydrogenase with an affinity for androgens and it is prominently expressed in normal genital skin fibroblasts, but not in non-genital skin fibroblasts. This proved that the 56 kDa protein is not synthesized from the androgen receptor nor is it a stable degradation product of the receptor protein. The 56 kDa protein is found to be under-expressed in genital skin fibroblasts from patients with complete androgen insensitivity syndrome and not missing as thought before. The exact mechanism of how a defective androgen receptor gene effects the altered expression of this ALDH 1 gene is still unknown. The delineation of the *trans*-acting factors involved in the regulation of the expression of ALDH 1 will assist in this analysis. The genomic 5'-promoter region of ALDH 1 was cloned, sequenced, and analyzed for consensus transcription factor binding site motifs. This analysis revealed that there are multiple consensus motifs present which include: multiple GATA, AP-1, and half-site GRE sites; and single CAAT, octamer, *junB*-IR, ETS and TATA motifs. Nuclear proteins, which are differentially expressed in cells which have the 56 kDa protein present and compared to those that do not, are found to bind to a portion of the 5' flanking region of ALDH 1.

A mechanism is proposed for the role of ALDH 1 in the development of the different sexual phenotypes and in the pathogenesis of complete androgen insensitivity syndrome.

I. Introduction

The 56 kDa protein was discovered in our laboratory through serendipity as a protein spot on two-dimensional gels. This protein was present on protein maps from genital skin fibroblasts, but missing on those from non-genital skin fibroblasts. It was soon learned that this protein was also missing in genital skin fibroblasts from most patients with complete androgen insensitivity syndrome and that it could bind androgens.

Complete androgen insensitivity syndrome results when the anlagen for the sex organs of a chromosomal male do not recognize and respond to androgenic hormones due to a defective androgen receptor. The process of male development is not well understood. The identification of 56 kDa androgen-binding protein as an aldehyde dehydrogenase suggested a structural or at least functional relationship to the androgen receptor. This offered us an opportunity to begin the characterization of this system.

The aim of my thesis work was to characterize the 56 kDa protein by determining its structure, regulation, and function in genital skin fibroblasts as a logical step towards understanding its role in the pathogenesis of complete androgen insensitivity syndrome.

The following review of the literature will therefore be focused on the status of the 56 kDa protein at the outset of my project, the current knowledge of complete androgen insensitivity syndrome, the androgen receptor gene and protein, and the field of aldehyde dehydrogenases in an attempt to shed light on the function of the 56 kDa protein.

A. The 56 kDa protein

1. Discovery

The 56 kDa protein was discovered in the search of mutant proteins in fibroblasts from patients with Duchenne muscular dystrophy (Rosenmann *et al.* 1982) using two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) in combination with dual-labeling autoradiography (Walton *et al.* 1980). The protein compositions of normal and Duchenne muscular dystrophy fibroblasts were compared by labeling each cell type with one of two different isotopes (^{14}C - and ^3H -leucine), mixed and then co-electrophoresed on the same two-dimensional gel. Whether protein spots come from normal, dystrophic or from both cell types was determined by dual label autoradiography which produces two superimposable X-ray films (one film shows only tritium labeled proteins and the other only ^{14}C labeled proteins). With this technique, numerous differences between normal and dystrophic fibroblast proteins were detected but only the 56 kDa protein was consistently seen to be absent in dystrophic cells. By mischance (now fortuitous), further analysis established that the 56 kDa protein was not present in skin fibroblasts from normal females, Duchenne muscular dystrophy carrier females, nor in normal male fibroblasts obtained from sources other than the original source of normal fibroblasts (Thompson *et al.* 1983). Further inquiry led to the discovery that all the normal male fibroblast cultures obtained from the first source for the initial study (Rosenmann *et al.* 1982) were from preputial skin. Whereas, dystrophic fibroblast cultures from patients, carriers, as well as from other normal individuals, were derived from non-genital skin biopsies. The conclusion was that the absence of the 56 kDa protein was not specific for Duchenne muscular dystrophy, but rather was related to the site of the skin biopsy (Thompson *et al.* 1983).

2. Identification as a phenotypic marker of CAIS

Genital skin fibroblasts are an androgen target tissue known to have an increase in steroid 5α -reductase activity, the androgen receptor and androgen induced proteins. Genital skin fibroblasts are also the tissue source of choice for the study of androgen receptor mutations, which are the primary cause of X-linked androgen insensitivity syndrome. Thus, in a collaborative study, it was determined whether the 56 kDa protein was altered in genital skin fibroblasts from patients with androgen insensitivity syndrome. The findings were subsequently reported by Nickel *et al.* (1988). The 56 kDa protein was found in each of 23 genital skin fibroblast strains (6 labium majus, 17 prepuce) but absent in 30 of 32 control non-genital skin fibroblast strains. In addition, the protein was found in the labium majus skin fibroblast strains from 2 of 12 unrelated subjects

with complete androgen resistance due to negligible androgen-receptor activity. Finally, the 56 kDa protein was absent from 12 of 14 labium majus strains from subjects with complete insensitivity to androgens, regardless of 5 α -reductase activities, which indicated that it was neither a constitutive cytotypic marker of genital skin fibroblast differentiation nor a reflection of that enzyme.

These differences were proposed to be as a result of a 3-fold greater concentration of androgen-binding activity in genital skin fibroblasts. However, no change was detected in the 'spot' size under conditions of preincubation of the fibroblasts for 48 h with 3 nM of the non-metabolizable androgen analog methyltrienolone, or in the [³⁵S]methionine pulse-labeled spot in the presence or absence of 3 nM of the analog. Whereas, under the same conditions, there would be an increase of androgen-binding activity. In addition, the protein was absent from fibroblast strains of two other unrelated patients (who had the same phenotype) despite a normal level of qualitatively abnormal androgen-receptor activity in their fibroblasts. Nickel *et al.* (1988) concluded from this that the 56 kDa protein was unlikely to be an androgen-induced protein nor did it represent a functional androgen receptor.

Nevertheless, the 56 kDa protein doublet was found to be the only specifically labeled proteins when intact prepuce fibroblasts were covalently labeled by photolysis with 50 nM [³H]methyltrienolone and separated by two-dimensional gel electrophoresis. To account for this, Nickel *et al.* (1988) postulated that the size and intensity of the 6.7 isoelectric point 56 kDa protein was causally related to the androgen receptor and that its presence or absence in genital skin fibroblast lysates represented a new marker for the phenotypic heterogeneity seen within the class of complete androgen resistance.

At about the same time, one group described a 59 kDa heat shock protein, with no steroid-binding activity, that formed complexes with several different non-transformed steroid receptor complexes (Tai *et al.* 1986). It was not known whether this protein could bind steroids with lower affinity as described by Nickel *et al.* (1988). Other proteins which could be related to the 56 kDa protein included the retinoic acid receptor (Petkovich *et al.* 1987; Giguere *et al.* 1987) and a ubiquitously expressed protein of similar size, which is inappropriately expressed in hepatocellular carcinoma cells (de The *et al.* 1987). Finally, Kovacs and Turney (1988) identified a 58 kDa protein that was covalently labeled by an electroaffinity androgen analog and they considered this protein to be the androgen receptor protein or a portion of it. This protein was very similar in characteristics to the 56 kDa protein especially the fact that it was the only protein in genital skin fibroblasts that labeled with androgen, and thus could be identical to the 56 kDa protein. This created a dilemma as Nickel

et al. (1988) concluded that the 56 kDa protein was not the androgen receptor. For these reasons, it was important to study the 56 kDa protein by trying to determine its origin and function in normal sexual development and to discover reason(s) why it was missing in genital skin fibroblasts from patients with androgen insensitivity syndrome.

B. Development of sexual phenotypes

Many proteins are involved in the determination, differentiation and development of the sexual phenotypes. The development of the sexual phenotypes is pre-determined by the chromosomal sex but in the absence of gonads (testis or ovary), a phenotypic female develops (Jost, 1970). Thus, without the effects of the hormones produced by the testes, the male phenotype is suppressed. Furthermore, resistance to the action of androgens is characterized by partial or complete absence of the usual effects of the sex hormones and normal or increased hormone production (Wilkins, 1957). The syndrome of insensitivity, resistance or unresponsiveness to androgens is a form of male pseudohermaphroditism in which genetic males with testes differentiate as phenotypic women as a result of a single-gene defect (Morris, 1953). Since androgens are important for normal male sexual development and fertility, defects in androgen action are often associated with abnormal sexual differentiation, infertility, or both (Griffin, 1992). This overview will highlight the mechanisms by which androgens promote virilization of the normal male during fetal development and prenatal life, detail some of the proteins involved in the cascade necessary for the development of sexual phenotypes and for postnatal life, and summarize the current concepts of the pathogenesis of complete androgen insensitivity syndrome .

1. Biosynthesis of steroid hormones in males

The enzymes responsible for the synthesis of sex hormones from cholesterol can be induced as a consequence of stimulation by gonadotropic hormones in sex hormone target cells (Table 1). Luteinizing hormone activates the synthesis and secretion of testosterone from Leydig cells of the testis and from the adrenals. Follicle stimulating hormone activates the synthesis of estradiol in the Sertoli cells of the testes. A critical step for steroid hormone synthesis is the cell's ability to mobilize and transport cholesterol to the mitochondria. The primary signals for transport are usually elevations of cAMP and Ca^{2+} , but inositol trisphosphate as a second messenger may be involved. An overview of steroid hormone biosynthesis and its control is presented in Figure 1. Cholesterol is the main precursor of steroid hormone biosynthesis and undergoes side chain cleavage to form Δ^5 -pregnenolone which is converted to the sex hormones (Fig. 2).

Table 1. Hormones that directly stimulate the synthesis of steroid hormones (from Biochemistry, ed. T.M. Delvin, 1992).

Steroid hormone	Steroid producing cell	Signal	Second messenger	Signal system
Cortisol	Adrenal zona fasciculata	ACTH	cAMP, PI cycle, Ca^{2+}	Hypothalamic-pituitary cascade
Aldosterone	Adrenal zona glomerulosa	Angiotensin II	PI cycle, Ca^{2+}	Renin-angiotensin system
Testosterone	Leydig cell	LH	cAMP (PI cycle?)	Hypothalamic-pituitary cascade
17 β -Estradiol	Ovarian follicle	FSH	cAMP (PI cycle?)	Hypothalamic-pituitary-ovarian cycle
Progesterone	Corpus luteum	LH	cAMP	Hypothalamic-pituitary-ovarian cycle

ACTH = adrenocorticotrophic hormone; LH = luteinizing hormone;
FSH = follicle stimulating hormone; PI = phosphatidylinositol

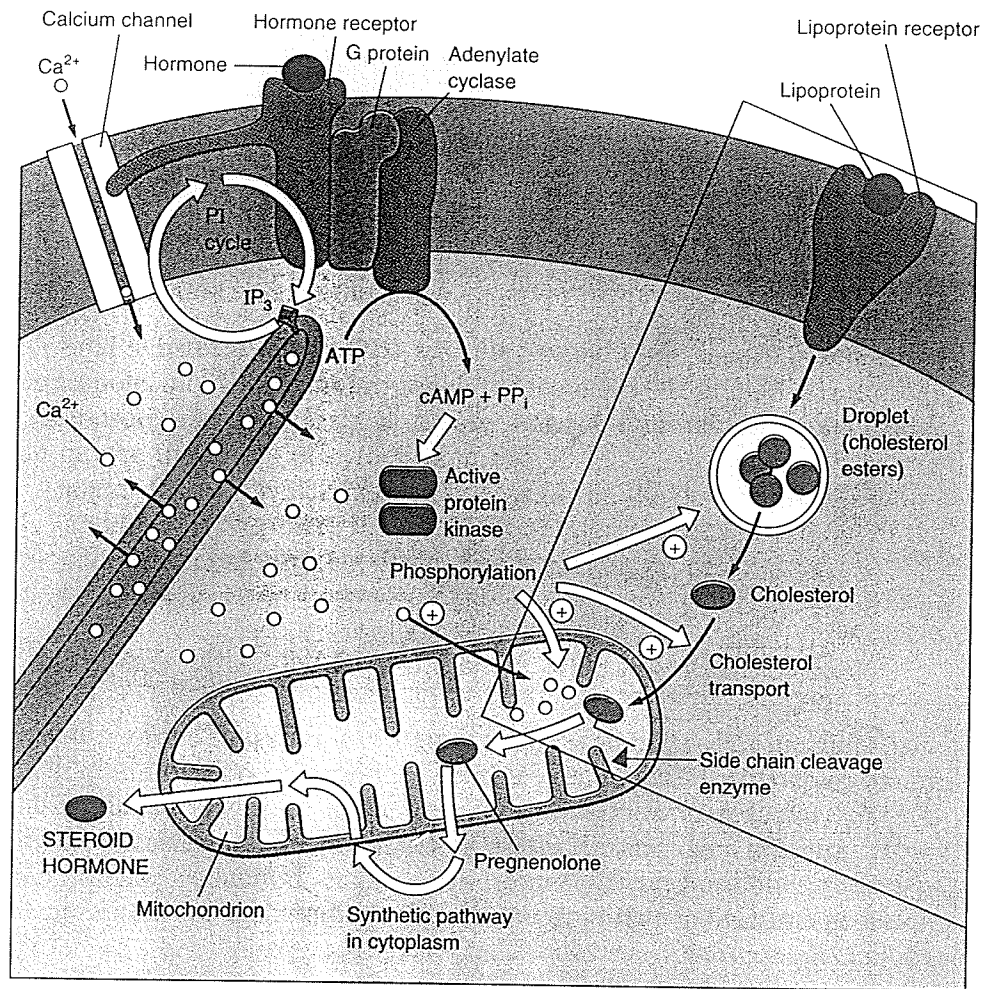


Figure 1. General overview of hormonal stimulation of steroid hormone biosynthesis. The stimulating hormone binds to cell membrane receptor and activates adenylate cyclase mediated by a stimulatory G protein. The activated receptor may directly stimulate a calcium channel or indirectly stimulate it by activating the phosphatidylinositol pathway (PI). If the PI pathway is concurrently stimulated, IP₃ could augment cytoplasmic calcium levels from the intracellular calcium stores. The increase in cAMP activates protein kinase whose phosphorylations cause increased hydrolysis of cholesteryl esters from the droplet to free cholesterol and increase cholesterol transport into the mitochondrion. The combination of elevated calcium levels and protein phosphorylation brings about induced levels of the side chain cleavage reaction. These combined reactions overcome the rate-limiting steps in steroid biosynthesis and more steroid is produced, which is secreted into the extracellular space and circulated to the target tissues in the bloodstream (from Biochemistry, ed. T.M. Delvin, 1992).

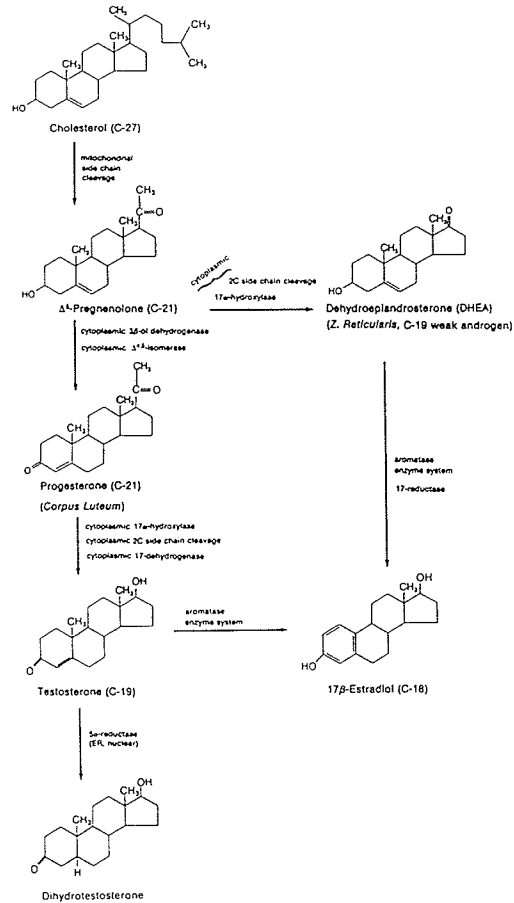


Figure 2. Conversion of cholesterol to sex hormones. Cholesterol is converted to the sex hormones by one or more complexes of the cytochrome P₄₅₀ side chain cleavage enzyme complex to form Δ^5 -pregnenolone releasing a C₆ aldehyde, isocaproic aldehyde (not shown). Δ^5 -pregnenolone is mandatory in the synthesis of all steroid hormones. Progesterone can be formed directly from Δ^5 -pregnenolone with the help of two cytoplasmic enzymes, 3 β -ol dehydrogenase and $\Delta^{4,5}$ -isomerase. Progesterone is further converted to testosterone by the action of the cytoplasmic enzymes. The conversion of testosterone to dihydrotestosterone in androgen target cells requires the activity of 5 α -reductase located in the endoplasmic reticulum and nuclear fractions. Pregnenolone can enter an alternative pathway to form dehydroepiandrosterone with the action of two other cytoplasmic enzymes, 17 α -hydroxylase and 2C side-chain cleavage, respectively. This compound can be converted to 17 β -estradiol via the aromatase enzyme system and the action of 17-reductase. Additionally, estradiol can be formed from testosterone through the action of the aromatase system (from Biochemistry, ed. T.M. Delvin, 1992).

2. Metabolic inactivation of steroid hormones in males

Inactivation of steroid hormones involves reduction of the very stable ring system of the steroids. A more active form of androgen is produced when testosterone is reduced, by 5α -reductase in androgen target cells, to form dihydrotestosterone. However, further reduction results in inactivation. The inactivation reactions predominate in liver and generally render the steroids more water soluble, as marked by subsequent conjugation with glucuronides or sulfates that are readily excreted in the urine. Table 2 summarizes reactions leading to inactivation and excretory forms of the steroid hormones.

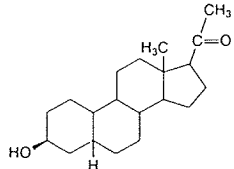
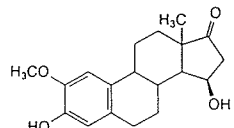
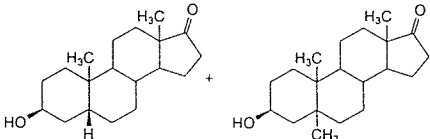
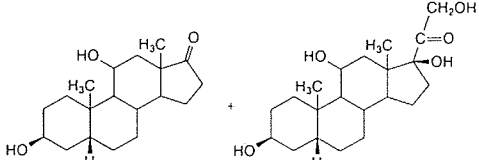
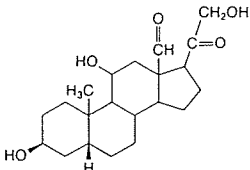
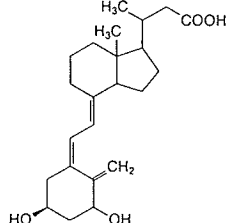
3. Dynamics of androgen and estrogen metabolism

The principle androgen secreted by the testes and present in male plasma is testosterone. Testosterone also serves as the precursor for two other active hormones — dihydrotestosterone and estradiol. The ratio of the production rate of testosterone to that of estradiol in normal men is about 100 to 1 (MacDonald *et al.* 1979). About 85 % of the estradiol in normal men is formed by the extraglandular aromatization of circulating testosterone, and the remainder is secreted directly by the testes (MacDonald *et al.* 1979). Whereas adipose tissue is a major site of extraglandular estrogen production, the testes may secrete significant amounts of estrogen directly into the circulation when gonadotropin concentrations are elevated or when plasma luteinizing hormone activity is elevated in pathologic states (Griffin, Wilson, 1980). As a consequence, estradiol serves a major role in determining the final phenotype in several disorders of androgen action. Feminization results when the normal 100-fold excess of androgens to estrogens is disturbed either by an increase in estrogen production or by a decrease in testosterone formation (or action) under circumstances in which estrogen production remains appreciable (Wilson *et al.* 1980). Estradiol exerts its function by interaction with its own receptor, either to potentiate the action of androgens, as in the prostate (Moore *et al.* 1979), or to oppose their effects, as in the male breast (Wilson *et al.* 1980). Therefore, excess estrogen — either relative or absolute — causes feminization in men, particularly enlargement of the breasts or gynecomastia (Wilson *et al.* 1980).

4. Normal androgen physiology

It is heuristically important to recognize that the androgen-response begins with the delivery of sufficient androgen to cells that are targets of androgen action, and culminates with an appropriate increase or decrease in the expression of the products of androgen-regulated genes (Pinsky *et al.* 1992). Testosterone is the principle androgen secreted by the testes and the principle circulating

Table 2. Excretion Pathways for the sex hormones (from Biochemistry, ed. T.M. Delvin, 1992).

Steroid class	Starting steroid	Inactivation steps	A : B ring junction	Steroid structure representations of excreted product	Principal conjugate present ^a
Progestins	Progesterone	1. Reduction of C-20 2a. Reduction of 4-ene-3-one or 2b. 3 β -steroid dehydrogenase	(cis)	 Pregnanodiol (5β-pregnane-3α,20-diol)	G ^a
Estrogens	Estradiol	1. Oxidation of 17 β -OH 2. Hydroxylation at C-2 with subsequent methylation 3. Further hydroxylation or ketone formation at a variety of positions, e.g., C-6, C-7, C-14, C-15, C-16, C-18		 One of many possible compounds	G
Androgens	Testosterone	1. Reduction of 4-ene-3-one 2. Oxidation of C-17 oxo	(cis and trans)	 Androsterone Etiocholanolone	G, S ^a
Glucocorticoids	Cortisol	1. Reduction of 4-ene-3-one 2. Reduction of 20-oxo group 3. Side chain cleavage	(trans)	 11β-OH-androsterone Allo tetrahydrocortisone	G
Mineralocorticoids	Aldosterone	1. Reduction of 4-ene-3-one	(trans)	 3α,11β,21-(OH)₃-20-oxo-5β-pregnane-18-al	G
Vitamin D metabolites	1,25(OH) ₂ D ₃	1. Side chain cleavage between C-23 and C-24	—	 Calcitroic acid	?

^a G = Glucuronide; S = sulfate.
Reproduced from A. W. Norman and G. Litwack, *Hormones*. Orlando, FL: Academic, 1987.

androgen. In the circulation, it is bound to two proteins, namely, testosterone-binding globulin (also termed sex-hormone-binding globulin) and albumin (Pardridge, 1986). About 1 to 3 % of the circulating testosterone is in the free form which may enter cells by passive diffusion (Lasznitzki, 1974). Inside the cell it either combines directly with the high-affinity androgen receptor in the nucleus, is converted to dihydrotestosterone by 5 α -reductase before binding to the same high-affinity androgen receptor or is aromatized to estradiol (Fig. 3). The receptor-androgen complexes then undergo a transformation reaction. The transformed complex promotes more efficient DNA-binding in chromatin which effectively promotes or suppresses transcription of mRNA. The major actions of androgens are summarized in Figure 3 (Griffin, 1992). Although dihydrotestosterone and testosterone bind to the same receptor protein, the two hormones perform different roles in androgen physiology. The receptor-testosterone complex is responsible for the negative feedback regulation of the secretion of the gonadotropin luteinizing hormone in the hypothalamic-pituitary system, and for the virilization of the Wolffian ducts during male phenotypic sex differentiation (Wilson, 1975; George, Wilson, 1986). The receptor-dihydrotestosterone complex, in contrast, is responsible for the development of the male external genitalia and prostate during embryogenesis and for most of the androgen-mediated events of sexual maturation at the time of male puberty (growth of facial and body hair, temporal hair recession, maturation of external genitalia) (Wilson, 1975; George, Wilson, 1986). It is not clear which of the androgens mediates stimulation of spermatogenesis (Wilkins, 1957). Dihydrotestosterone may be a more potent androgen than testosterone because it binds more tightly to the androgen receptor (Wilbert *et al.* 1983), and promotes the transformation reaction (Kovacs *et al.* 1984). Thus, the irreversible synthesis of dihydrotestosterone results in a major amplification of the androgenic signal. Furthermore, androgen action differs from other steroid hormones because many of its effects are mediated by the sum of the effects of testosterone and its major metabolites, dihydrotestosterone and estradiol, and because androgens promote the differentiation during embryogenesis of those tissues that will be major sites of action in postnatal life.

5. Normal male gonadal sex determination

Normal sexual development during embryogenesis consists of three sequential, ordered, and interrelated processes (Jost, 1970) involving establishment of chromosomal sex which is translated to gonadal sex and ultimately to phenotypic sex. Chromosomal sex is determined at conception (fertilization) where in mammals, the heterogametic sex (XY) is male and the homogametic sex (XX) is female. The product of the sex-determining region Y (SRY) gene, which is found on the Y chromosome, enacts the transition of chromosomal sex to gonadal sex by promoting the differentiation of the indifferent gonads into testes in males (Koopman *et al.* 1991). The

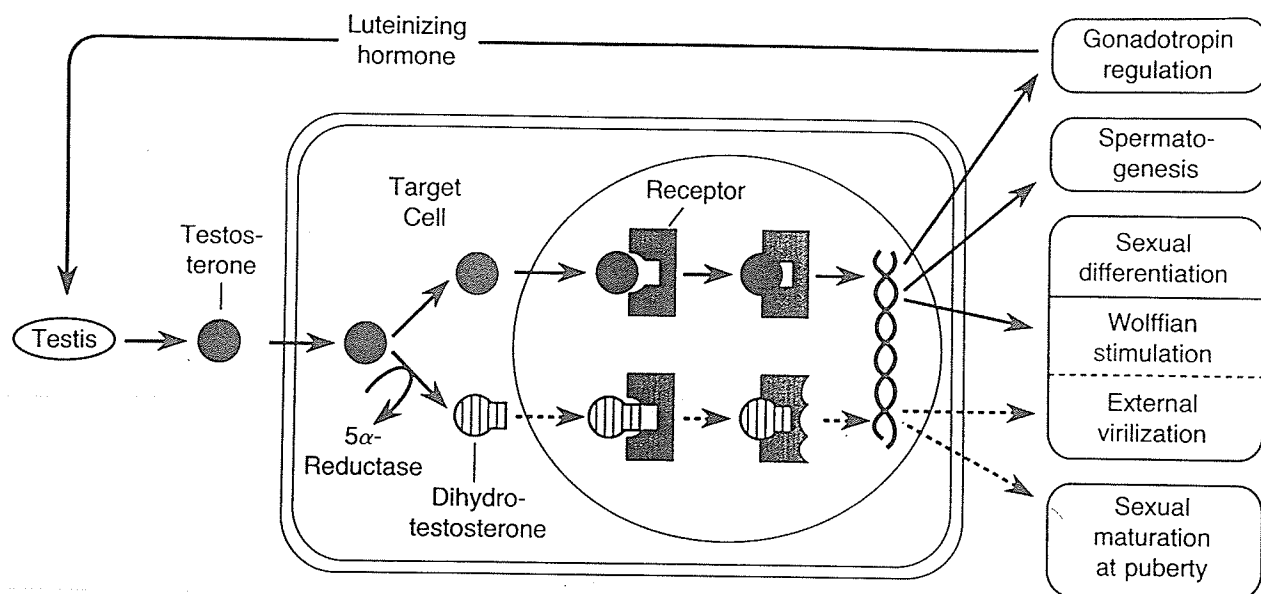


Figure 3. Normal androgen physiology. Testosterone, secreted by the testis, binds to the androgen receptor in a target cell, either directly or after conversion to dihydrotestosterone. Dihydrotestosterone binds more tightly than testosterone, and the complex of receptor-dihydrotestosterone can bind more efficiently to the chromatin. The major actions of androgens, shown on the right, are mediated by testosterone (solid lines) or by dihydrotestosterone (broken lines) (from Griffin, 1992).

mechanisms involved are unknown except that the human SRY gene has been cloned and is found to be an intronless gene encoding a 120 amino acid protein. This protein contains a region homologous to a DNA-binding motif (high-mobility group box) present in non-histone chromosomal proteins and transcription factors (Su, Lau, 1993). Thus, the SRY may directly interact with and promote activation of genes responsible for the differentiation of the male phenotype. However, several mutations in the SRY gene have recently been described in males (Hawkins *et al.* 1992). This contradicts the original concept of the hypothetical "testis-determining gene," TDF, thought at one time to produce the testes-determining factor." An alternate working hypothesis (Mittwoch, 1973, 1992) based on the interaction of a cascade of multiple factors has been proposed to aid in the understanding of the role played by SRY and other genes in the process of sexual development (Mittwoch, 1993). Many other protein factors responsible for this cascade resulting in the different sexual phenotypes are yet to be discovered.

6. Hormonal control of male development

Development of the male sexual phenotype during embryogenesis is controlled primarily by three hormones (George, Wilson, 1986; Goldstein, Wilson, 1975) in the indifferent anlagen. Müllerian-inhibiting substance causes regression of the müllerian ducts, probably acting in concert with testosterone, preventing development of the female internal genitalia. Testosterone acts directly to stimulate the Wolffian ducts to differentiate into the male internal genitalia. Differentiation of the Wolffian ducts begins just prior to the eighth week of development and is completed at about 13 weeks, before the capacity to form dihydrotestosterone, the third hormone, is acquired by these tissues (Siiteri, Wilson, 1970). Furthermore, testosterone is irreversibly reduced to dihydrotestosterone which is necessary for virilization of the urogenital sinus and external genitalia. However, in the absence of testes as in the normal female, or in male animal embryos castrated prior to the onset of phenotypic differentiation, the phenotypic female sex develops. Thus, both the Wolffian ducts and the external genitalia virilize in the characteristic male fashion when female embryos are exposed to androgens at the appropriate time in embryonic development (Schultz, Wilson, 1979). This implies that the receptor-androgen mechanism is fundamentally the same in male and female embryos. Thus, the hormones produced by the fetal testes at the critical period of embryonic development and not differences in the receptors for the hormones result in the different sexual phenotypes.

7. Development of phenotypic sex during embryogenesis

Differentiation of the gonadal sex into phenotypic sex is the direct consequence of the type of gonad formed and the resulting endocrine secretions of the fetal testis. In the male, the Wolffian ducts give rise to the epididymides, vasa deferentia, and seminal vesicles, and the müllerian ducts disappear (Fig. 4). In the absence of testes, as in the female, the müllerian ducts give rise to the fallopian tubes, uterus, and upper vagina, and the Wolffian ducts either disappear or persist in vestigial form as Gartner's ducts. In contrast to the internal genital development, the external genitalia and urethra of both sexes develop from common anlagen, the genital tubercle, genital folds, and genital swellings. In the male, fusion and elongation of the genital folds cause formation of the urethra and shaft of the penis and ultimately bring the urethral orifice to the genital tubercle (glans penis). Additionally, the fused genital swellings become the scrotum, and the prostate forms in the wall of the urogenital swellings (George, Wilson, 1986).

8. Disorders of phenotypic sex development

Male pseudohermaphroditism is a disorder of phenotypic sex in which chromosomal and gonadal males do not develop as normal men. There are three general categories of disorders: the persistent müllerian duct syndrome; deficiency of testosterone formation; and the androgen insensitivity syndromes (Griffin, Wilson, 1986). This section will be confined to the third category because androgen insensitivity is thought to be responsible for most cases of male pseudohermaphroditism (Savage *et al.* 1978). The androgen insensitivity syndromes are single-gene disorders of phenotypic sexual development in persons with a karyotype 46,XY who have bilateral testes, normal regression of the müllerian ducts, and quantitatively normal secretion of testosterone (Griffin, 1992). The known molecular defects responsible for the syndromes involve either the 5 α -reductase enzyme or the androgen receptor. The clinical features of the different syndromes are given in Table 3 (Griffin, 1992).

9. Complete androgen insensitivity

Disorders of androgen-receptor function result in a spectrum of phenotypic abnormalities ranging from those in phenotypic women with complete testicular feminization to those in minimally affected, phenotypically normal men (Table 3). Although the various syndromes differ in their clinical manifestations and management, they have similar X-linked inheritance and hormonal profiles. Subjects with complete insensitivity usually come to medical attention because of an inguinal hernia before puberty or primary amenorrhea after puberty. There are no reliable estimates of the frequency of androgen insensitivity in any one or all of its clinical forms. The figure usually quoted for the complete form (1:50,000), based on the finding of testes in inguinal

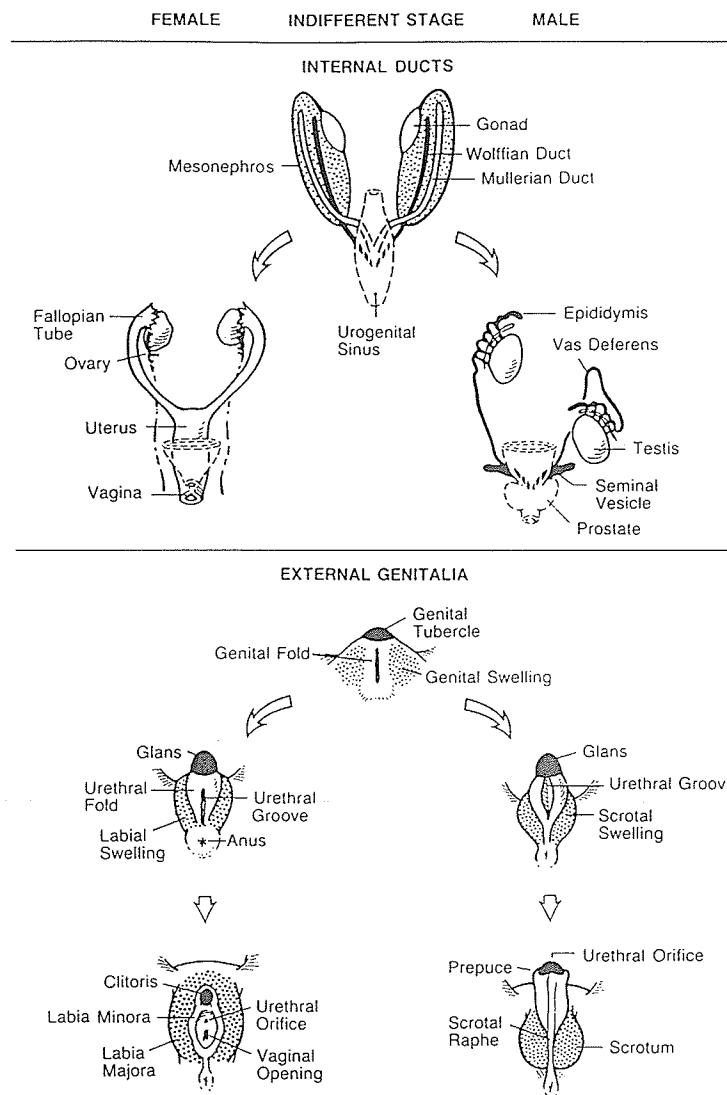


Figure 4. Phenotypic differentiation of internal ducts and external genitalia in male and female embryos (from Metabolic Basis of Inherited Disease, Eds. C.R. Scriver, A.L. Beaudet, W.S. Sly and D. Valle, 1989).

Table 3. The clinical features of the different androgen insensitivity syndromes (from Griffin, 1992).

FEATURE	5 α -REDUCTASE DEFICIENCY	ANDROGEN RECEPTOR DISORDERS				
		COMPLETE TESTICULAR FEMINIZATION	INCOMPLETE TESTICULAR FEMINIZATION	REIFENSTEIN'S SYNDROME	INFERTILE MALE SYNDROME	UNDERVIRILIZED MALE SYNDROME
Inheritance	Autosomal recessive	X-linked recessive	X-linked recessive	X-linked recessive	X-linked recessive	X-linked recessive
Hormonal profile	Normal male androgen and estrogen production	Increased androgen and estrogen (usually)	Increased androgen and estrogen (usually)	Increased androgen and estrogen (usually)	Increased androgen and estrogen (usually)	Increased androgen and estrogen (usually)
Phenotypic features						
Spermatogenesis	Decreased	Absent	Absent	Absent	Absent or decreased	Normal or decreased
Müllerian derivatives	Absent	Absent	Absent	Absent	Absent	Absent
Wolffian derivatives (epididymis, vas deferens, seminal vesicle)	Male	Absent	Male	Male	Male	Male
Urogenital sinus derivatives (prostate and urethra)	Female	Female	Female	Underdeveloped male	Male	Male
External genitalia (penis and scrotum)	Female (may virilize at puberty)	Female	Posterior fusion and clitoromegaly	Perineoscrotal hypospadias	Male	Male
Breasts	Male	Female	Female	Gynecomastia	Gynecomastia in some	Gynecomastia

hernias of affected girls, is likely to be an underestimate (Pinsky *et al.* 1992). The phenotype is female with normal external genitalia, except that axillary and pubic hair are diminished or absent (Morris, 1953). Actually, sparse central pubic hair is often present even when axillary hair is absent, since pubertal feminization depends upon peripheral aromatization of testosterone to estradiol, whose action is unopposed because of the insensitivity to androgens. The vagina is short and blind or absent. Internal genital structures are absent except for the testes which may be located in the abdomen, along the course of the inguinal canal, or in the labia majora. Spermatogenesis is incomplete or absent, and Leydig cells are normal. There is usually a family history of the disorder, but about one third of the subjects represent new mutations. Estrogen therapy is necessary to initiate feminization in patients who undergo gonadectomy before puberty and to maintain it in those who are allowed to feminize spontaneously before castration.

Biochemically, complete insensitivity to androgens is defined by a quantitative and qualitative analysis of specific androgen-binding in genital skin fibroblasts from the labium majus (Pinsky *et al.* 1992). About 50 % of subjects have negligible specific androgen-binding activity (receptor negative with binding activity of ≤ 5 fmol/mg protein with dihydrotestosterone as ligand). This implies that the androgen receptor is absent, non-functional, or present in a form unrecognizable by the assay. About 25 % of the time, the specific androgen-binding activity is quantitatively normal (receptor positive with binding activity of 15 - 40 fmol/mg protein) but qualitatively abnormal (increased rate of dissociation of the dihydrotestosterone-receptor complexes). There are many other intracellular and extracellular indices of a qualitative androgen receptor defect which may include: thermolability of the androgen-receptor complex (Griffin, 1979); failure of up-regulation of the androgen-receptor binding activity during prolonged incubation of the cells with a synthetic, non-metabolizable androgenic ligand (Kaufman *et al.* 1986); failure of the androgen-receptor complexes to localize in the nuclei (Eil, 1983); resistance of the androgen-receptor complexes to the stabilizing effects of molybdate during prolonged ultracentrifugation through sucrose gradients (Griffin, Durrant, 1982); and lability of the dihydrotestosterone-receptor complexes under conditions that transform normal complexes to the DNA-binding state (Kovacs *et al.* 1984). Occasionally, the specific androgen-binding is borderline-normal or clearly less than normal, but easily measurable (receptor deficient). This phenotype is usually produced due to an aggregate of qualitative defects of the androgen receptor.

10. The mouse model for androgen insensitivity syndrome, the *Tfm* mouse

Equivalents to complete androgen insensitivity syndrome due to androgen receptor defects have been observed in several animal species, including mouse, rat, cat, chimpanzee and Finnish

raccoon dog (Meyers-Wallen *et al.* 1989). Recently, the underlying mutation in the *tfn* mouse has been identified as a single base deletion in the androgen receptor, leading to a reading-frame shift and a premature stop codon at amino acid 412 (Charest *et al.* 1991; He *et al.* 1991; Gaspar *et al.* 1991). This predicts that a non-functional receptor without DNA and hormone binding domains is synthesized. However, a smaller receptor with high-affinity androgen binding of less than 10% wild-type activity is observed in *Tfn* mice (Young *et al.* 1989). This protein appears to result from an internal translation initiation site downstream of the mutation (He *et al.* 1991; Gaspar *et al.* 1991).

11. Androgen receptor gene structure

By the fall of 1988, several groups had succeeded in cloning the cDNA for the androgen receptor (Brinkmann *et al.* 1989; Chang *et al.* 1988; Lubahn *et al.* 1988; Tilly *et al.* 1989) allowing direct comparisons to be made with a cDNA for the 56 kDa protein as described in the results section of this thesis.

The human androgen receptor gene is located in band q12 of the X chromosome (Brown *et al.* 1989). The eight exons of the gene encompass more than 90 kb (Fig. 5 C) (Kuiper *et al.* 1989; Lubahn *et al.* 1989) encoding two major mRNA species, 7 - 8 kb and 10 - 11 kb, differing in length of the 3' untranslated region because of alternate splicing (Faber *et al.* 1991). The 5' untranslated region encoded by exon 1 is 1.1 kb long and contains one major and one minor site for initiation of transcription in a 13 bp region. The promoter lacks TATA and CCAAT-like DNA binding elements, but has a putative SP-1 binding motif in a GC-rich region -50 to -60, and an AG-rich region at -185 to -230 (Tilly *et al.* 1990; Faber *et al.* 1991). The 3' untranslated region encoded by exon 8 is 6.8 kb long. The mRNA codes for a protein of 910 amino acid with a calculated molecular mass of 98 - 99 kDa (Brinkmann *et al.* 1989; Chang *et al.* 1988; Lubahn *et al.* 1988; Tilly *et al.* 1989)(Fig. 5 B). The protein is a prototypical member of the subfamily of the superfamily of steroid/thyroid/retinoic acid receptors, which include the glucocorticoid, progesterone and mineralocorticoid receptors. The DNA binding domain has the highest homology between members of the subgroup, followed by the hormone-binding domain. The N-terminal domain is the hypervariable region which varies both in length and amino acid composition. The proposed functions of the different domains of the androgen receptor are listed in Figure 5A.

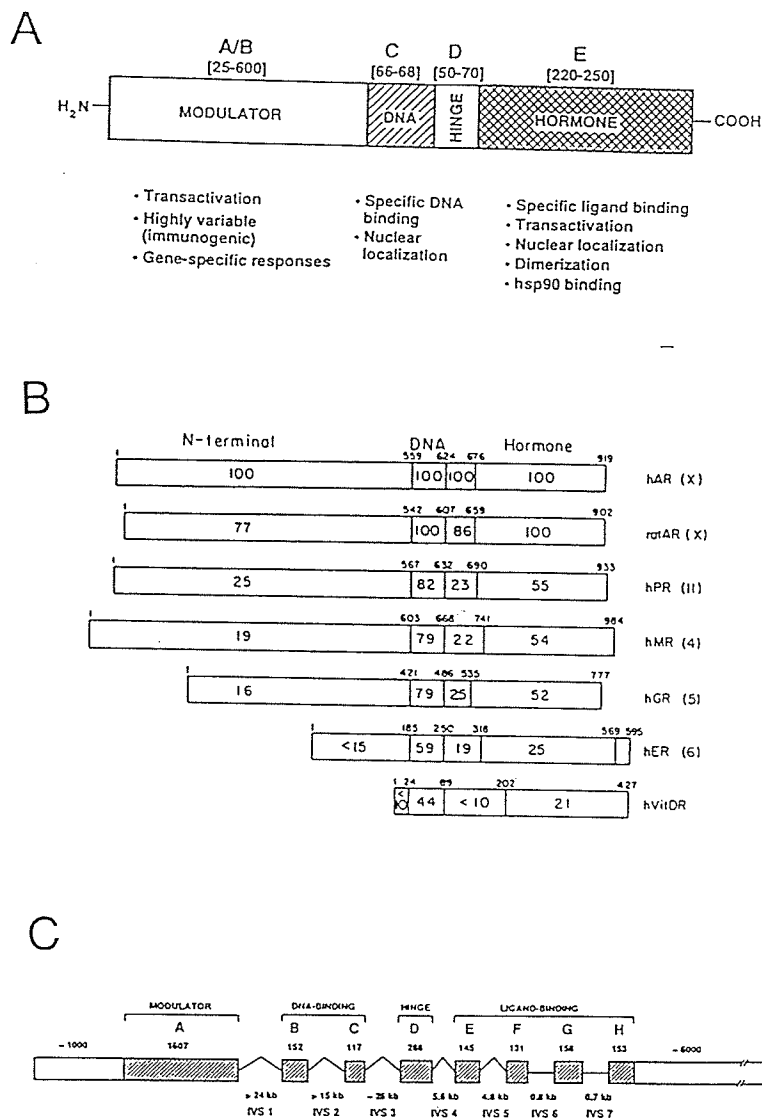


Figure 5. The general structure of a steroid hormone receptor protein; amino acid homology comparisons among receptor proteins; and the genomic structure of the androgen receptor. A) Structure of a hormone receptor protein, including the proposed functions of each domain (from Janne, Shan, 1991); B) Amino acid homology comparisons among nuclear receptor proteins (from Lubahn et.al. 1988). Indicated are the percent of sequence homologies with the human androgen receptor. Note: the sequence differs from Chang et.al., (1988) 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. The exon sizes are presented as base pairs while the intervening sequences (IVS) are in kilobase pairs (from Janne, Shan, 1991).

12. Genes known to be regulated by androgens

Some of the genes known to be regulated by androgens include the mouse mammary tumor virus long terminal repeat studied in the breast cancer cell line T47D (Darre *et al.* 1986), β -glucuronidase, ornithine decarboxylase, the RP2 gene, the kidney androgen-regulated protein, and alcohol dehydrogenase, all of which have been studied in the mouse (Berger, Watson, 1989). The kallikrein gene of human prostatic tissue (Young *et al.* 1990a), the 20 kDa glycoprotein (Ho *et al.* 1989; Ho *et al.* 1991), the prolactin-inducible protein (Murphy *et al.* 1987), the rat prostate probasin gene (Spence *et al.* 1989; McQueen *et al.* 1991), the prostatic steroid-binding protein C3 gene (Parker *et al.* 1988; Rennie *et al.* 1989), the prostate-specific antigen (Reigman *et al.* 1992), and the rat seminal vesicle secretory protein IV (Kistler, Kistler, 1991) have all been shown to be regulated by androgens.

Steroid receptors are believed to regulate gene activities through binding to specific DNA sequences, referred to as hormone-responsive elements. These elements can be grouped into two categories of inverted repeat consensus sequences: the TGACC motif that mediates estrogen, retinoic acid, and thyroid hormone responses (Klein-Hitpass *et al.* 1986; Umesco *et al.* 1988); and the TGTTCT sequence that confers regulation by glucocorticoids, progestins, and androgens (Scheidereit *et al.* 1986; Strahle *et al.* 1987; Ham *et al.* 1988). The inclusion of the androgen-responsive element in this latter group is based largely on the observation that the androgen receptor can induce transcription through glucocorticoid-responsive elements of the mouse mammary tumor virus DNA and the tyrosine aminotransferase gene (Ham *et al.* 1988; Denison *et al.* 1989). A consensus DNA-binding site for the androgen receptor has been identified by a site selection assay and found to be conserved with the glucocorticoid-responsive element (Roche *et al.* 1992).

A typical androgen regulated gene is the C3 gene which is regulated by androgens and glucocorticoids *in vivo* (Parker *et al.* 1988; Rennie *et al.* 1989) and contains a glucocorticoid-responsive element motif that functions equally well with androgens, glucocorticoids, and progesterone receptors under *in vitro* conditions (Claessens *et al.* 1989; De Vos *et al.* 1991). Only the rat prostatic probasin gene whose androgen-responsive element, which only contains a half-site of the glucocorticoid-responsive element, has been shown to be more effectively induced by androgens than by glucocorticoids or progestins *in vitro* (Rennie *et al.* 1993a) and in transgenic mice (Sheppard *et al.* 1993, Rennie *et al.* 1993b).

More androgen-responsive elements, identified by functional assays to be specifically responsive to androgens, will have to be studied before any certainty can be reached as to whether they are similar to the glucocorticoid-responsive element.

13. Androgen receptor mutations

Analysis of the mutations identified to date reveal that a wide variety of androgen receptor gene mutations underlie the heterogeneity in phenotypic expression of androgen insensitivity. Complete or partial gene deletions are an uncommon cause of the syndrome, accounting for up to 10 % of cases (Trifiro *et al.* 1991; Quigley *et al.* 1992a; Quigley *et al.* 1992b; Brown *et al.* 1988). Single base mutations in the coding region can result in altered amino acid sequence (Brown *et al.* 1990; McPhaul *et al.* 1991; Marcelli *et al.* 1991; Ris-Stalpers *et al.* 1991), introduction of a premature termination codon (Marcelli *et al.* 1990; Sai *et al.* 1990; Marcelli *et al.* 1990b), or altered mRNA splicing (Ris-Stalpers *et al.* 1990). The majority of these substitutions are localized to the DNA-binding domain and to two segments of the androgen-binding domain (McPhaul *et al.* 1993) (Fig. 6).

Studies of the molecular defects indicate that there is no clear-cut relationship when the phenotypes of patients with classic forms of androgen insensitivity are compared to the type of receptor defect as assayed by tests of quantitative or qualitative androgen-binding (Griffin, 1992, McPhaul *et al.* 1993). Although, mutations resulting in premature termination codons are a common cause of absent binding, point mutations leading to amino acid substitutions in the hormone-binding domain may also lead to either absent binding or qualitatively abnormal binding.

Other factors must affect the observed phenotype in some subjects. For example, subjects have been detected who have identical mutations in the androgen receptor gene but result in different phenotypes. This type of result suggests that factors other than the coding sequence of the androgen receptor itself can modify patient phenotype (Zoppi *et al.* 1993). This clearly points towards the importance of determining the function(s) of any protein(s) found to be altered in relation to the androgen insensitivity syndrome.

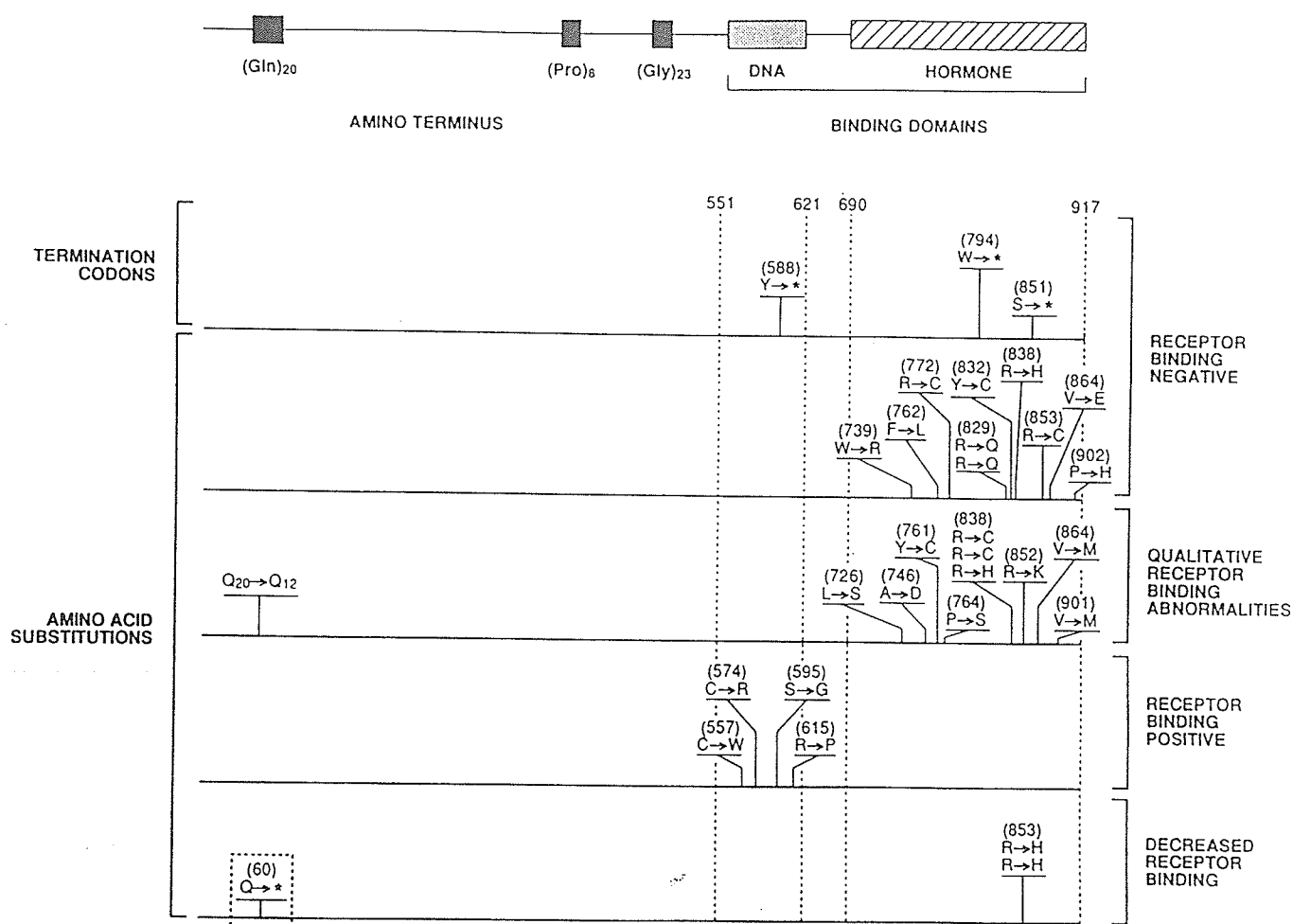


Figure 6. Distribution of mutations in the androgen receptor gene (below) and a schematic structure of the androgen receptor is shown above. The relative positions of the homopolymeric domains, DNA-binding, and hormone binding domains are indicated. The mutations identified in 30 patients with androgen resistance are shown. Amino acid substitutions are shown using the single amino acid code, and termination codons are indicated by an asterisk. The amino acid residue number is indicated for each mutation in parenthesis, using the numbering coordinates of Tilley et.al., (1989). The individual patient categories determined by ligand-binding assays are indicated to the right. The type of genetic mutation identified (termination codons, amino acid substitutions) is shown to the left (from McPhaul *et al.* 1993).

C. Aldehyde dehydrogenases

1. Identification of the 56 kDa protein as an aldehyde dehydrogenase

The following is an overview of the field of aldehyde dehydrogenases with specific concentration on the class 1 aldehyde dehydrogenases, of which ALDH 1 is a member and will be described in the results section.

Aldehyde dehydrogenases (ALDHs) are a family of enzymes with common structural and functional features which catalyze the conversion of aldehydes to their corresponding acids by means of an NAD⁺-dependent, irreversible reaction. A broad range of substrates can be recognized, including a spectrum of aliphatic and aromatic aldehydes. Based on primary sequence analysis, three major classes of mammalian ALDHs — 1, 2, and 3 — have been identified. Classes 1 and 3 contain both constitutively expressed and inducible cytosolic forms. Class 2 consists of constitutive mitochondrial enzymes. Each class appears to oxidize a variety of substrates that may be derived either from endogenous sources such as amino acids, biogenic amines, or lipid metabolism or from exogenous sources, including aldehydes derived from xenobiotic metabolism (Lindahl, 1992). Lindahl provides an excellent review of the aldehyde dehydrogenase field in general and their role in carcinogenesis.

2. Biochemistry of ALDH enzyme system

a. Nomenclature of mammalian aldehyde dehydrogenases

Based on primary sequence: class 1 represent the cytosolic forms; class 2, the mitochondrial forms; and class 3, the tumor forms (Table 4 A). In total, 13 complete or partial mammalian ALDH sequences are known. Based on overall alignments and positional identities, all can be placed into one of the three existing classes (Table 4 B). The human and horse cytosolic ALDHs, the mouse cytosolic ALDH, the rat phenobarbital-inducible ALDH, and the mouse retinal ALDH all share greater than 85 % nucleotide and 65 % amino acid identity, and are, therefore, class 1 enzymes (Lindahl, 1992). The rat formyl-tetrahydrofolate dehydrogenase also shows considerable identity to class 1 ALDHs.

Table 4. Nomenclature and known primary sequences of mammalian aldehyde dehydrogenases (from Lindahl, 1992).

Nomenclature of Mammalian ALDHs

Class 1 Aldehyde dehydrogenase (ALDH1)

Members of the class 1 ALDHs are the constitutive and inducible cytoplasmic ALDHs *human*, *horse*, *beef*, *rat*, *mouse*, *sheep*, and *dog* liver and the ALDH *inducible by PB*

Class 2 Aldehyde dehydrogenase (ALDH2)

Members of the class 2 ALDHs are the mitochondrial ALDHs isolated from *human*, *horse*, *rat*, *beef*, *mouse*, *sheep*, and *dog* liver

Class 3 Aldehyde dehydrogenase (ALDH3)

Members of the class 3 ALDHs are the rat liver *tumor-specific/TCDD-inducible/3-MC-inducible* cytoplasmic ALDHs. Also included in this class are the major constitutive or inducible ALDHs from rat liver microsomes and mammalian cornea, stomach, lung, and urinary bladder

Note: Sources identified in italics are considered the prototype enzyme for the class, based on primary structure determination. Other representatives are based on kinetic, physical, and immunological properties or subcellular distribution. See Reference 36 for details of the assignments of a particular form to a class.

Known Mammalian ALDH Primary Sequences

Enzyme	Source
Class 1 constitutive	Human liver cytosol Horse liver cytosol Mouse liver cytosol
Class 1 inducible	Rat liver cytosol Mouse retina
Class 2 constitutive	Human liver mitochondria Horse liver mitochondria Rat liver mitochondria Beef liver mitochondria
Class 3 constitutive	Rat liver microsomes
Class 3 inducible	Rat liver Beef cornea

b. Isozyme Distribution

The isozymes can further be differentiated by electrophoretic mobility, kinetic properties, subcellular localization, tissue distribution, disulfiram sensitivity, and primary molecular sequences (Harada *et al.* 1980). The distribution of the three classes of ALDHs amongst mammalian tissues is complex. All tissues likely possess mitochondrial ALDHs. However, depending upon the tissue and species, they may also possess a constitutive cytosolic ALDH. In addition, some tissue cytosolic ALDHs can also be induced under certain conditions. A typical distribution is found in human liver which contains multiple ALDHs distributed approximately equally between mitochondria and cytosol. Both high- and low- K_m (for short-chain aliphatic aldehydes) forms are known. The low- K_m mitochondrial and the high- K_m cytosolic forms account for the majority of the acetaldehyde-oxidizing capacity of human liver. A human liver microsomal ALDH has been identified. Tissues like cornea, lung, stomach, and urinary bladder, have a different subcellular distribution and activity profile from those of liver. Over 90 % of the total ALDH activity is cytosolic in cornea, but only 50 % in stomach and urinary bladder. Furthermore, mouse and human hearts appear to lack the constitutive cytosolic ALDH, which is the only form found in human erythrocytes (Inoue *et al.* 1979).

c. Substrate specificities

The various ALDHs have different substrate preferences. Only a few have had their putative physiological substrates identified. In fact, it is believed that each ALDH may be able to oxidize many aldehyde substrates, with the exact substrate varying with the tissue and/or physiological situation. With respect to coenzyme specificity, all ALDHs have K_m s for NAD^+ in the micromolar range. Generally, class 1 ALDHs have K_m s of 1 to 10 μM , whereas class 2 forms have K_m s in the 20 to 50 μM range and class 3, the most variable class, having K_m s from 5 μM for the corneal enzyme to near 100 μM for the microsomal form. There exists a wide spectrum of endogenous and exogenous aldehydes which are potential substrates for ALDHs (Table 5 and 6). Small aliphatic aldehyde substrates (e.g. acetaldehyde and propionaldehyde) are excellent substrates for both class 1 and 2 constitutive enzymes. However, large aldehyde-containing molecules (e.g. some corticosteroid aldehydes and aldophosphamide derived from cyclophosphamide) and biogenic aldehydes (derived from neurotransmitter metabolism, 4-hydroxyalkenals from lipid peroxidation) may be substrates for certain class 1 and/or 2 forms. Class 1 enzymes are the only aldehyde dehydrogenases capable of oxidizing retinal to retinoic acid in man (Dockham *et al.* 1992) with an extremely high affinity ($K_m = 0.06 \mu M$) for retinal (Yoshida *et al.* 1993).

Table 5. Example of endogenously produced aldehydes (from Lindahl, 1992).

Aldehyde	Source
Acetaldehyde	Threonine catabolism
γ -Aminobutyraldehyde	Putrescine catabolism
Betaine aldehyde	Choline catabolism
21-Dehydrocorticosteroids	Corticosteroid catabolism
3,4-Dihydroxyphenylacetaldehyde	Dopamine catabolism
Glutamic- γ -semialdehyde	Proline biosynthesis
Hexanal	Lipid peroxidation
5-Hydroxyindoleacetaldehyde	Serotonin catabolism
4-Hydroxynonenal	Lipid peroxidation
Malondialdehyde	Lipid peroxidation
Retinal	Vitamin A metabolism
Succinic semialdehyde	GABA shunt

Table 6. Examples of aldehydes found in foods and from exogenous sources (from Lindahl, 1992).

Examples of Aldehydes Found in Foods

Formaldehyde	Benzaldehyde	Malondialdehyde
Acetaldehyde	Phenylacetaldehyde	Acrolein
Propanal	4-Hydroxynonenal	Glyoxal
Hexanal	Crotonaldehyde	Methylglyoxal
Hexenal	Citral	
Octanal	C ₂₄₋₃₀ aldehydes	
Decanal		

Examples of Exogenous Sources of Aldehydes

Source	Aldehyde
Combustion	Formaldehyde, acetaldehyde, acrolein
Ethanol	Acetaldehyde
2-Butoxyethanol	Butoxyaldehyde
Diethylnitrosamine	Acetaldehyde
<i>N</i> -Butyl- <i>N</i> -(4-hydroxybutyl)nitrosamine	<i>N</i> -Butyl- <i>N</i> -(propyl-3-aldehyde)nitrosamine
Succinylcholine	Betaine aldehyde
Benzene	<i>trans, trans</i> -Muconaldehyde
Toluene	Benzaldehyde
Xylene	Tolualdehyde
Laetrile	Benzaldehyde
Codeine	Formaldehyde
Phenacetin	Acetaldehyde
Cyclophosphamide	Aldophosphamide
Nicotine	γ -3-Pyridyl- γ -methyaminobutyraldehyde

3. Genetics of ALDH isozymes

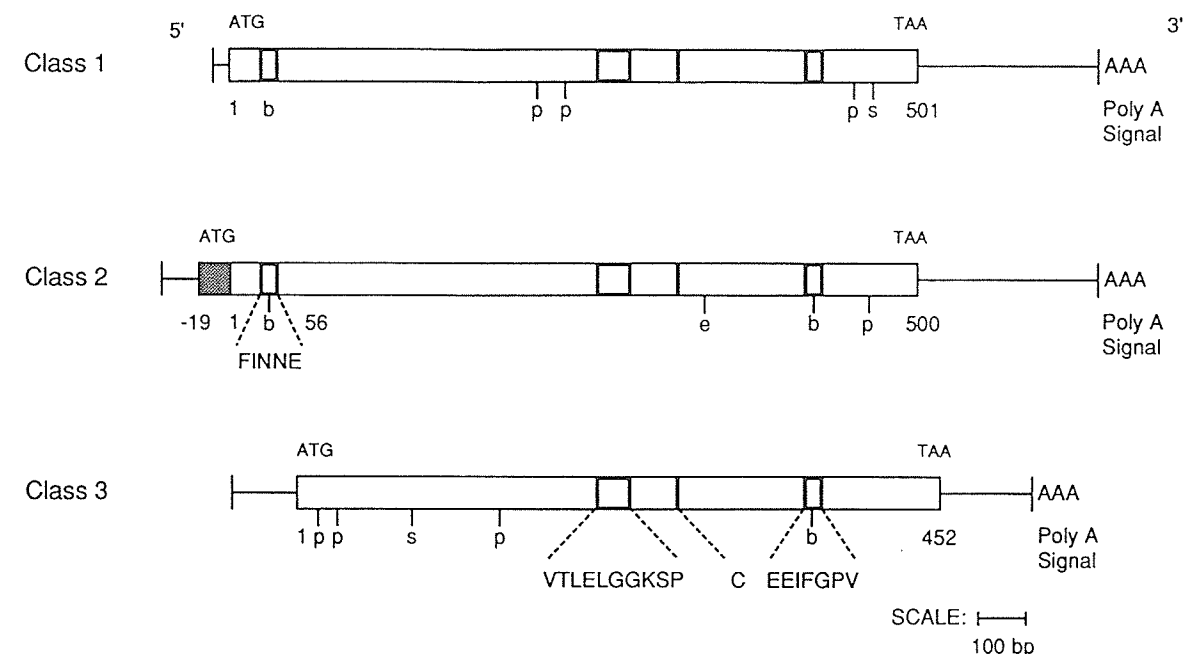
d. Gene structures

The human class 1 and class 2 ALDH genes have been cloned and the intron-exon organizations in the two genes are very similar (Fig. 7 B), as compared to class 3 genes. Both enzymes are encoded by 13 exons with nine of the 12 introns interrupting the coding sequences at homologous positions, and one intron (intron 9) being displaced by only one nucleotide from its counterpart (Hsu *et al.* 1988; Hsu *et al.* 1989). The ALDH 1 gene encodes 501 amino acid residues together with 53 bp at the 5'-non-coding region and 538 bp at the 3'-non-coding region. The promoter region has an ATA box and a CCAAT box, which are located 32 and 74 bp upstream, respectively, from the start of transcription initiation. The class 1 gene (ALDH 1) is mapped to chromosome 9 at q12 (Hsu *et al.* 1989), whereas its mouse counterpart (*Ahd 2*) is at q12 on chromosome 19 (Algar *et al.* 1986). The human mitochondrial class 2 gene is mapped to chromosome 12 (Braun *et al.* 1986; Hsu *et al.* 1988). None of the other genes have been mapped.

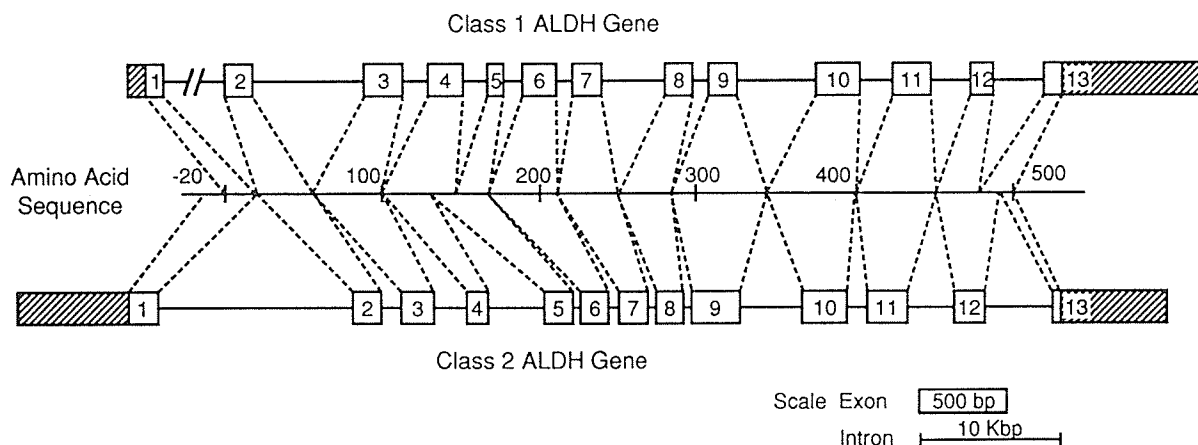
e. Genetic variations and polymorphisms at the ALDH 1 locus

Racial differences have been found between Orientals and Caucasians in the mitochondrial ALDH 2 locus of individuals sensitive to alcohol (Wolff, 1972; Goedde *et al.* 1979). Approximately 50 % of Orientals lack mitochondrial ALDH 2 activity in their livers, but no Caucasians have been found to exhibit this deficiency. Orientals who lack the ALDH 2 isozyme activity exhibit an alcohol-flushing characteristic, proposed to be due to the accumulation of toxic acetaldehyde (Goedde *et al.* 1979; Mitzoi *et al.* 1979).

Several variants with biochemical, electrophoretic and immunological differences of the ALDH 1 isozymes have been identified in individuals sensitive to alcohol. In contrast to the high frequency of the mitochondrial variants observed in the Orientals, the ALDH 1 variants are much less common. A variant with severely diminished catalytic activity but strong immunological cross-reactivity with an antiserum towards ALDH 1 was found in one of 10 (Yoshida, 1983) and again in two of 60 livers from individuals of Japanese descent (Kanayama *et al.* 1984). Another variant with different electrophoretic mobility was found in two Oriental subjects (Eckey *et al.* 1986). Two kinetic variants, ALDH 1-Columbo and ALDH 1-Harrow, were found in Caucasian alcohol-flushers (Yoshida *et al.* 1989). The former had 60 % of the normal activity and a lower rate of utilization of deamino-NAD and ethanoamide-NAD, while the latter had only 20 % of the normal activity. Both variants exhibited altered electrophoretic mobility. It was proposed that since no



A



B

Figure 7. Mammalian aldehyde dehydrogenase cDNA and genomic organization of human class 1 and 2 aldehyde dehydrogenases. (A) Mammalian ALDH cDNAs. The alignment is based on the human, rat, and mouse class 1 sequences; the human, rat, and bovine class 2 sequences; and the rat tumor-specific and TCDD-inducible sequences. Arabic numbers represent positions of key amino acid residues. The bold areas and corresponding one-letter sequences are regions of complete primary sequence identity between all known ALDHs. Lowercase letters represent restriction enzyme sites in rat cDNAs: b — Bgl II; e — Eco RI; p — Pst I; s — Sac I. Hatched area at the 5' end of the class 2 cDNA encodes the mitochondrial signal peptide. (B) Genomic organization of human class 1 and class 2 ALDHs. Exons are boxed and dotted lines indicate alignment of amino acid sequences encoded by exons. 5'- and 3'-untranslated regions are indicated by shaded boxes (from Lindahl, 1992).

Caucasians have an Oriental-type mitochondrial ALDH 2 variant (responsible for alcohol-flushing in 50 % of Orientals), these ALDH 1 variants may be directly responsible for the alcohol-flushing found in 5 - 10 % of Caucasians.

The mutations responsible for the ALDH 1 variants have not been elucidated to date. However, a Msp I restriction fragment length polymorphism has been identified for the ALDH 1 locus (Yoshida, Chen, 1989). Two alleles were identified, allele 1 is a 20 kb band and allele 2 is a 7 kb band, and were polymorphic in both Caucasians (with genotype frequencies 1-1 = 0.3, 1-2 = 0.7 and allele frequencies of 0.65 for the homozygous state and 0.35 for the heterozygous state) and Orientals (genotype frequencies 1-1 = 0.6, 1-2 = 0.2, and 2-2 = 0.2). The homozygous genotype 2-2 has not been identified in Caucasians. The Msp I alleles were found to be compatible with Mendelian codominant inheritance in one family.

f. ALDH gene regulation

Although some of the human class 1 and 2 genes have been isolated, no data are yet available on the regulatory regions involved in ALDH gene expression (Lindahl, 1992). However, Lindahl (1992) has speculated on the presence of certain DNA response elements as compared to the regulation of expression of class 3 genes. He proposes that a CACACA repeat element be present in the 5' promoters of this class of genes. For the cytochrome genes, the length of the CACACA repeat is inversely related to the basal level of expression of the gene (Suwa *et al.* 1985). Furthermore, genetic evidence suggests the existence of a gene encoding a protein that specifically activates the class 1 ALDH gene in response to phenobarbital, predicting a binding site of a positive trans-acting factor. Because class 1 genes are both constitutively expressed and inducible, he also proposes that negative cis-acting and/or positive tissue-specific cis-acting elements be present. None of these elements have been proven or even identified to exist. Furthermore, the class 1 and class 2 ALDHs are developmentally expressed in human fetal and infant liver samples (Santisteban *et al.* 1985; Yoshida *et al.* 1991) as determined by immunodetection and by starch gel activity staining.

Thus, the normal control of ALDH1 gene expression has not been analysed to date. Furthermore, the transcriptional control elements responsible for aberrant ALDH1 gene expression have not previously been examined. Lindahl suggests that certain cis-acting elements present in other members of the genes for aldehyde dehydrogenases should be present in ALDH1. However, in our analysis of the reported sequence of the 5' promoter region (Hsu *et al.* 1989) we were unable

to identify any elements proposed by him. Thus, an attempt will be made to define the transcriptional control elements involved in normal and aberrant expression of the ALDH1 gene, and determine whether there is any component of androgen regulation of the ALDH1 gene.

D. Research Aims

The overall objective of my research was to examine the characteristics of the 56 kDa protein in normal and complete androgen insensitivity syndrome genital skin fibroblasts. These studies provide compelling evidence that there is a relationship between the androgen receptor and the 56 kDa protein. The identification of the 56 kDa protein as ALDH 1, during the course of this work, provided the basis for the initiation of studies of ALDH 1 regulation. This also provided the basis to establish a hypothesis of the relationship of the 56 kDa protein to the androgen receptor.

II. Material and Methods

A. Patient material

Surgical acquisition of foreskin from normal circumcision specimens, labium majus skin, or skin from various non-genital regions were obtained from Dr. C. R. Greenberg, Winnipeg. Other cell strains and lines were obtained from three sources: the Repository for Mutant Human Cell strains, Montreal; from the collection of Dr. L. Pinsky, Montreal; and from the American Type Culture Collection (Rockville, Maryland). Peripheral blood was collected into Lavender Vacutainer® tubes (containing 4 mM EDTA, Becton-Dickinson, Franklin Lakes, New Jersey). Specimens from patients with androgen insensitivity syndrome or from normal individuals were donated with informed consent according to the protocol approved by the Ethics Committee for use of Human Subjects of the Sir Mortimer B. Davis, Jewish General Hospital, Montreal and of the Faculty of Medicine, University of Manitoba, Winnipeg.

B. Tissue culturing

1. Primary skin cultures

Patient biopsy specimens for primary cultures were collected in sterile Human McCoy's 5A medium supplemented with 15 % fetal calf serum (CanSera, Rexdale, Ontario). The specimens were cut into small pieces with sharp curved scissors. Four to five explants were transferred to each of six to eight 60 mm dishes. Excess medium was aspirated and explants were covered with a single round sterile coverslip. Three to four milliliters of medium were added, making sure not to disturb the coverslip and explants. Dishes were placed in the incubator and checked after one week when the medium was changed. When there was sufficient growth around the explants, the coverslip was transferred to another 60 mm dish and placed such that the cells were facing up and overlaid with sufficient medium as to cover the cells. When it was judged to have sufficient cells to harvest, the medium was changed 24 h before harvesting by standard trypsinization.

2. Cell culturing

In general, GSF were cultured in Human McCoy's 5A medium supplemented with 10% fetal calf serum, 100 µg/ml each of penicillin G and of streptomycin, whereas other cells were cultured in the appropriate media and supplements according to the suppliers. Cells were cultured under standard conditions of 5 % carbon dioxide, 95 % air in a 37°C humidified incubator (Hotpack, Waterloo, Ontario). Cells were cultured in tissue culture dishes of different sizes as needed and the medium was changed every 3 - 5 days as needed in a laminar flow hood (Canadian Cabinets,

Montreal, Quebec). Cells were passed by trypsinization in 1 - 3 ml of 2.5 mg/ml trypsin and 0.1 mg/ml EDTA for 5 - 10 min or until the cells were seen to round-up and detach from the dish. Sharp agitation was used to help in the detachment. The cells were aspirated into a sterile Pasteur pipette and the solution used to wash the rest of the cells off the dish by strong expulsion against the dish. The cells - trypsin mixture was immediately diluted into the supplemented medium to inactivate and dilute the trypsin. Appropriate aliquots were dispensed to fresh tissue culture dishes with the desired dilution (e.g., MCH 6 required a 1:3 to 1:5 dilution, whereas, HepG2 required a minimum of 1:5 to 1:8 dilution).

3. Storage of cells

Cells were trypsinized as described above and diluted to 10 ml in supplemented medium. The solution was mixed and the cells were pelleted by centrifugation at 500 x g for 5 min at room temperature. The medium was discarded by aspiration in the flow hood. The cells were then resuspended into 1 ml of freezing medium (8 % DMSO in supplemented medium or 8 % DMSO in serum). The mixture was rapidly transferred to sterile 2 ml cryovials (Nalgene, Rochester, New York) and placed at -20°C for 24 h and subsequently transferred to -70°C or to liquid nitrogen for long-term storage.

C. Analysis of proteins

1. Preparation of tissue culture cells for fractionation of proteins

The medium was removed from the dishes and the cells washed twice with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄ and 1.5 mM KH₂PO₄, pH 7.4), and the cells lysed directly into the appropriate buffer.

For two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis, the cells were lysed with CHAPS lysis buffer (9.5 M urea, 3 % CHAPS, 30 mM DTT and 2 % ampholyte mixture containing 0.8 % pH 5-7 Ampholine® (Pharmacia LKB Biotechnology, Bromma, Sweden), 0.8 % pH 6-8 Ampholine®, 0.4 % pH 3.5-10 Ampholine®). The cells were scraped with a plastic spatula and the homogenate collected into a microcentrifuge tube. The homogenate was sonicated briefly to ensure complete lysis of the cells. The sample was then stored at -20°C if used within a week or at -80°C for use within 1 month.

For SDS-PAGE analysis, cells were lysed and scraped-off with SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 20 % glycerol, 2 % SDS, 4 % 2-mercaptoethanol and 0.002 % Bromophenol Blue). The homogenate was immediately boiled for 5 min. Samples were again stored at -20°C if used within a week or at -80°C for use within 1 month with boiling before loading.

Alternatively, cells were washed another two times with ice cold 0.18 M ammonium bicarbonate and scraped in an appropriate volume of 0.18 M NH₄HCO₃ (pH 7.4) containing a protease

inhibitor mix (1 mM PMSF, 10 μ M pepstatin A and 100 μ M leupeptin). The mixture was sonicated on ice, centrifuged at 100,000 x g for 20 min at 4°C, and the supernatant was lyophilized. The proteins were resuspended into the appropriate buffer for gel electrophoresis.

2. Protein quantitation

Protein concentration was determined by the Coomassie Blue protein binding assay of Bradford (1976). Essentially, 200 μ l of dye-reagent concentrate (BioRad, Richmond, Virginia) was added to aliquots of protein extracts containing ammonium bicarbonate or phosphate buffer, which had previously been diluted with water to a final volume of 800 μ l. Albumin was used as a protein standard. The increase in absorption, over a sample with no protein added, of the resultant mixture at 595 nm was monitored. The absorption values for the standard protein were plotted against the known concentration of protein aliquots generating a standard curve. The concentrations of the protein extracts were interpolated from this curve. However, when the protein concentration of extracts solubilized by CHAPS lysis or SDS sample buffer needed to be determined, aliquots were fractionated by SDS-PAGE along with protein extracts of known concentrations. Samples in CHAPS lysis buffer were diluted with an equal volume of 2 x SDS sample buffer and boiled prior to loading. The gels were stained by Coomassie Blue which allowed a visual approximation of the concentration of proteins in each mixture. This procedure was necessary because reagents in the two buffer systems interfered with the Bradford assay (Bradford, 1976).

3. Denaturing two-dimensional PAGE

Denaturing 2-D PAGE was performed according to O'Farrell (1975) with the following modifications: (i) 2 % ampholyte mixture and 2 % CHAPS were used in the isoelectric focusing (IEF) gel; (ii) IEF gels of 12.5 cm were formed in 17 cm long tubes (2.4 mm i.d.); (iii) approximately 15 μ g (for gels to be stained by silver) or 80-150 μ g (for gels to be immunoblotted or for purification of the 56 kDa protein) were applied to each gel. The IEF gel contained 600 μ l of solution (9.1 M urea, 4 % acrylamide-bisacrylamide (28.4:1.6), 2 % CHAPS, 2 % ampholytes, 0.01 % ammonium persulphate and 0.07 % TEMED). Prior to addition of the ammonium persulphate and TEMED the solution was degassed for 10 min with a vacuum pressure of 29 inch. of Hg. The gel solution was overlaid with 20 μ l of 8 M urea and allowed to polymerize for 1 h. The urea solution and unpolymerized acrylamide was removed and the samples loaded. 30 μ l of sample overlay solution (8 M urea, 2 % ampholytes) was added. 0.02 N NaOH was used as the anodic solution and 0.01 M phosphoric acid was used at the cathode. Initially, IEF was run under constant current of 0.25 mA/gel until the Voltage reached 400 and then at constant Voltage of 400 for 16 h and finally at 800 V for 1 h for a total of 7200 Vh. Gels were extruded from the glass

tube by air pressure which was applied using a syringe connected to the tube with a short piece of tubing. Gels were equilibrated for 30 min in O'Farrell's buffer C (10 % glycerol, 3 mM DTT, 2.3 % (w/v) SDS, 62.5 mM Tris-HCl pH 6.8). Gels for the second dimension SDS-PAGE were 14 x 13.5 cm and 0.75 mm thick with a gel acrylamide/bisacrylamide concentration of 10 %. The IEF tube gel was connected to the second dimension slab with a small volume of 1 % agarose, 0.002 % Bromophenol Blue in O'Farrell's buffer C. The slabs were electrophoresed at a constant current of 20 mA and a constant temperature of 17°C until the Bromophenol blue dye was 1 cm from the bottom of the glass plates. Rainbow molecular weight markers (Amersham, Arlington Heights, Illinois) were used to determine the approximate molecular weights of the proteins on the two-dimensional protein maps.

4. Denaturing SDS-polyacrylamide gel electrophoresis

Samples dissolved in SDS sample buffer were electrophoresed through a 10 % acrylamide/bisacrylamide gel according to Laemmli (1970) using a vertical slab gel electrophoresis system (Hoefer Scientific Instruments, San Francisco, California). Routinely, the discontinuous gel system consisted of a stacking gel and separating gel. The gel solutions were diluted from a 30 % acrylamide stock (29.2:0.8, acrylamide:bisacrylamide). The separating gel contained 16 ml of solution (10 % acrylamide-bis, 0.375 M Tris-HCl pH 8.8 and 0.1 % SDS). The solution was degassed and 9.3 μ l of a 10 % solution of ammonium persulphate and 9.3 μ l of TEMED were gently mixed in. The solution was poured into a glass sandwich with 0.75 mm spacers. The gel solution was overlaid with 5 ml of water saturated 2-butanol and allowed to polymerize 1h to overnight. After complete polymerization, the butanol was removed and the gel washed with water and stacking gel buffer (without acrylamide). The stacking gel which contained 5 ml of solution (4.5 % acrylamide-bis, 0.125 M Tris-HCl pH 6.8, 0.1 % SDS), was degassed and 14.5 μ l of 10 % ammonium persulfate and 4.2 μ l of TEMED were mixed in. The solution was poured, a gel comb inserted and the gel allowed to polymerize at least 45 min before washing the gel surface with water. The wells were washed with electrode buffer (25 mM Tris, 192 mM glycine and 0.1 % SDS) prior to loading of samples. Samples previously denatured in SDS sample buffer were boiled for 5 min and loaded into the wells. The gels were run under constant current of 25 mA/gel at 17°C until the Bromophenol Blue dye front reached the bottom.

5. Visualization of proteins

a. Coomassie Blue staining

Coomassie Brilliant Blue R-250 (BioRad, Richmond, Virginia) was dissolved to a concentration of 0.4 % in 95 % ethanol and filtered through Whatmann no. 1 filter paper (Whatmann International, Maidstone, England). Gels were stained in a buffer containing 0.05-0.2 %

Coomassie Blue (depending on the protein concentration loaded on the gel) and 10 % acetic acid for 2 h to overnight. Gels were then destained in 250 ml of 50 % methanol and 10 % acetic acid with many changes until the desired intensity of staining was reached.

b. Silver staining

Silver staining was performed according to Morrissey *et al.* (1981) with modifications. Essentially, gels were fixed in 7.5 % acetic acid for 2 h to overnight, soaked in 50 % methanol for 1 h to overnight. The fixed gel was washed thrice in double distilled water to remove any remaining detergent and soaked for 30 min in 100 ml of reducing agent (5 μ g/ml DTT) and subsequently in 100 ml of an oxidizing agent (0.1 % silver nitrate). The gels were washed twice with double distilled water to remove excess silver nitrate and briefly rinsed twice with the developing solution (3 % sodium bicarbonate and 0.05 % of formaldehyde). Finally, the gels were developed in 100 ml of the developing solution until the desired intensity of stain was achieved. Further development of the stain was stopped by washing the gels in 1-7 % acetic acid for 30 min. Finally, the gels were neutralized by soaking for 30 min in water before they were dried on to 3 MM filter paper (Whatmann International, Maidstone, England). An approximate 100-fold increase in sensitivity was achieved over Coomassie Blue staining.

c. Metabolic labeling of proteins

The use of L-[35 S]-methionine (185.0 MBq/mmol, New England Nuclear, Mississauga, Ontario) or 3 H-Leucine and 3 H-cysteine as metabolic labels provided a much more sensitive method of visualizing proteins from tissue culture cells. Essentially, the cells were incubated with 50 μ Ci/100 mm dish of radioactive amino acid for 36 h. The cells were harvested as described before, SDS or 2D-PAGE analysis performed and the gel treated for fluorography and exposed to film.

d. Fluorography

Gels of proteins labeled with 3 H, 14 C or sometimes 35 S were visualized by fluorography according to Bonner and Lasky (1974). Briefly, the gels were stained with Coomassie Blue as described and a photocopy or a Polaroid picture taken for documentation. The gels were destained and impregnated with an aqueous based scintillant (Autofluor, National Diagnostics, Manville, New Jersey) prior to drying on to 3 MM paper. Fluorography was performed by exposing the dried gel to preflashed Kodak XAR-5 X-ray film at -80°C . Western blots of radioactive proteins were treated with a scintillant spray (EN 3 HANCE, New England Nuclear, Boston, Massachusetts) prior to exposure to X-ray film for fluorography.

D. Physico-chemical characterization of the 56 kDa protein

1. Extraction of 56 kDa proteins from GSF

GSF cells were extracted either with low or high salt buffers to determine whether the 56 kDa proteins were differentially extracted. Cells were washed 4 x in PBS and 4 x in 0.18 M ammonium bicarbonate. Cells were then harvested in 1 ml of buffer (0.18 M or 0.5 M ammonium bicarbonate with or without 1 mM PMSF and 3 mM DTT). The cells were homogenized and centrifuged at 100,000 x g for 20 min at 4°C. An aliquot of the supernatant was taken for protein estimation with the remainder lyophilized and the protein residue resuspended to 5 µg/ml in CHAPS lysis buffer for 2D-PAGE.

2. *In vivo* phosphorylation in GSF

Phosphorylation of GSF was performed according to Erickson and Shealy (1981). Cells from 4 confluent 100 mm dishes of MCH 6 were trypsinized and washed in 10 ml of medium. The cells were resuspended in medium containing 1 % FCS and ³²P-orthophosphate (10 mCi(370 MBq)/ml, Amersham, Oakville, Ontario). The cells were incubated at 37°C for 2 h and subsequently pelleted. The supernatant was removed and the cells washed thrice in PBS, thrice with 0.18 M ammonium bicarbonate. The cells were homogenized for 10 s using a Kinematica homogenizer in 1 ml of 0.5 M ammonium bicarbonate with 1 mM PMSF. The homogenate was centrifuged at 100,000 x g for 20 min and the supernatant was extracted with 3.75 ml of chloroform:methanol (1:2) per milliliter of supernatant. The precipitated material was pelleted and washed in 2.4 ml of chloroform:methanol:water (1:2:0.8). The precipitate was again pelleted and the chloroform and methanol evaporated under a stream of nitrogen. The remaining aqueous layer was frozen and lyophilized. The residue was resuspended into 50 µl of CHAPS lysis buffer and 2D-PAGE performed.

3. Immunological detection of lectin binding proteins in GSF

GSF cells from MCH 6 were separated by SDS-PAGE and immunoblotted to nitrocellulose. The lanes of protein were cut into strips and used to adsorb to lectins. Lectins used were from a kit (Vector Laboratories, Burlingame, California) and were biotinylated. Avidin conjugated to horse radish peroxidase enzyme was used as a reporter of the bound biotin-lectin. Any lectin found to bind glycoproteins within the 50 - 60 kDa size range were noted. These lectins were then used in the analysis of 2D-PAGE immunoblots to determine if the 56 kDa proteins were glycoproteins.

4. Heat shock response

Cells were grown at 37°C or at 41°C in a humidified incubator for 2 - 4 h in the presence of [¹⁴C]glycine and [¹⁴C]leucine. The cells were harvested and 2D-PAGE analysis performed and the gels treated for fluorography.

5. Identity of 56 kDa doublet proteins

a. Protease digestion of isolated 56 kDa protein

S. aureus V8 protease (endoproteinase Arg-C, Miles Laboratories, USA) and chymotrypsin (Chymotrypsin A4 from bovine pancreas, Bohringer Mannheim) were used according to the method of Cleveland *et al.* (1977) to determine whether there was any homology between the two 56 kDa doublet proteins of pI 6.5 and 6.7. Eight spots of pI 6.5 and 4 spots of pI 6.7 were excised from 2D-PAGE gels stained with Coomassie Blue. The spots were soaked in 10 ml buffer (0.125 M Tris-HCl pH 6.8, 0.1 % SDS and 1 mM EDTA) and then pushed to the bottom of sample wells which had been filled with the same buffer. Spaces around the gel pieces were filled with 10 µl of the same buffer containing 20 % glycerol. Finally, 10 µl of this buffer containing 10 % glycerol and a given amount of protease was overlaid into each well and electrophoresis performed. The gel was a 17.5 % SDS-polyacrylamide gel with 1.5 mm spacers and 1 mM EDTA within the 5 cm long stacking gel. BSA and other non-specific spots were used as digestion standards for comparison. Several concentrations of the two proteases were loaded over the spots in the different lanes and the gel was electrophoresed at 20 mA until the Bromophenol Blue dye front reached the bottom of the stacking gel. The current was turned off and the gel left in the chamber for 30 min to allow for digestion of the proteins. The gel was subsequently electrophoresed at 20 mA as usual. The gel was stained first with Coomassie Blue and then with silver to visualize the oligo-peptides.

b. Densitometric analysis of peptide bands

The peptide banding pattern was compared by eye and after densitometric scanning of the stained gels. Scanning was done on a single beam scanning densitometer (model 1650 transmittance/reflectance, BioRad, Richmond, Virginia) using the reflectance mode. The reflectance observed for a single pass of the beam over the center of the stained band was recorded by a chart recorder. The pattern and intensity of the peaks identified were compared between the pI 6.5 and pI 6.7 56 kDa protein doublet peptide patterns to determine their identity.

6. Radioactive labeling of androgen binding proteins

a. Photoaffinity labeling of intact fibroblast cells

Confluent 35, 60 or 100 mm dishes of cells were rinsed once with 3-5 ml of sterile PBS and once with HEPES-MEM buffer (15 mM HEPES pH 7.4 in Eagle's MEM). The dishes were placed in a

37°C humidified incubator for 2 h with various concentrations of the following androgen analogs in HEPES-MEM buffer: 17 β -hydroxy-[17 α -methyl-³H]estra-4,9,11-trien-3-one (methyltrienolone, MT, or R1881, 70-87 Ci(2.59-3.22 TBq)/mmol, New England Nuclear, Mississauga, Ontario) or with 7 α ,17 α -dimethyl-[17 α -methyl-³H]19-nortestosterone (mibolerone, MB, 70-85 Ci(2.6-3.1 TBq)/mmol, Amersham, Arlington Heights, Illinois). Duplicate dishes were incubated with a 200-fold molar excess of radioinert ligand in HEPES-MEM buffer. The incubation medium was removed and placed on ice. For MT photolysis, the dishes were placed inverted on a UV transilluminator, Chromato-Vue model C-63B (Ultraviolet Products, San Gabriel, California). Dishes were irradiated for 10 min each to crosslink the photoligand to the proteins. For MB photolysis, the dishes were kept on ice and irradiated for 2 min using a hand-held short wave UV lamp (Spectroline® model EF-140C, Westbury, New York) by placing the lamp onto the rim of the dish. The cells were harvested by scraping in a small volume (60 μ l for 35 mm, 100 μ l for 60 mm and 200 μ l for 100 mm dishes) of the appropriate buffer (CHAPS lysis, SDS or NH₄HCO₃) and treated as described above for fractionation of proteins.

b. In vitro photoaffinity labeling of tissue extracts

Tissues from mouse or rat were excised and washed in ice cold PBS. Tissues were cut into small pieces and homogenized on ice for 30 s using a Kinematica homogenizer (Brinkmann Instruments, Westbury, New York) in 6 x (w/v) of buffer (10 % glycerol, 1.5 mM EDTA, 50 mM NaPO₄ pH 6.0, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A and 1 mM PMSF). The homogenate was centrifuged at 100,000 x g for 20 min at 4°C and the supernatant stored on ice. The solvent of the required amount of [³H]Mibolerone was evaporated under a stream of nitrogen and residue resuspended into the same volume of ethanol. 150 μ l aliquots of extract were then placed into microcentrifuge tubes and the aliquot of [³H]mibolerone added and incubated for 3 h at 4°C. Each aliquot of supernatant was spotted onto a sheet of polyfilm which was placed on a sheet of glass sitting on crushed ice. The puddles of homogenate were irradiated for 5 min with the hand-held short wave UV lamp from a distance of 2 cm. The samples were recovered and 60 μ l aliquots were immediately mixed with an equal volume of 2 x SDS sample buffer and boiled for 5 min. Five microliter aliquots were also taken from the homogenate and diluted 5 fold in water and the protein concentrations determined by the Bradford method (Bradford *et al.*, 1976). Eighty microgram aliquots of homogenate were mixed with 20 μ l of CHAPS lysis buffer and the proteins fractionated by 2D-PAGE as described.

c. Affinity labeling of intact fibroblasts

³H-dihydrotestosterone bromoacetate (DHTBrAc) was synthesized according to Kovacs and Turney (1988) and Kovacs *et al.* (1989) based on the method of Chang *et al.* (1984). Synthesis

began from [1,2,4,5,6,7,16,17-³H(N)]-(5 α -Androstan-17 β -ol-3-one) (dihydrotestosterone, 170-210 Ci(6,29-7.77 TBq)/mmol, New England Nuclear, Mississauga, Ontario). The cells were incubated with ligand in HEPES-MEM buffer alone or with a 200-fold molar excess of radioinert steroid for 2 h in a 37°C humidified incubator. Dishes were rinsed once with PBS, harvested into the appropriate buffer and treated as described above.

7. Steroid receptor binding assays

a. Charcoal binding assay

Specific androgen binding activity in intact GSF were performed as described by Nickel *et al.* (1988). Briefly, confluent monolayers were washed with serum-free medium (HEPES-MEM). Cells were incubated for 2 h at 37°C with HEPES-MEM containing 5 nM MB $\pm$ 200 fold molar excess of radioinert steroid. The cells were washed thrice in 0.18 M ammonium bicarbonate buffer and harvested in 0.5 M ammonium bicarbonate buffer with 1 mM PMSF, sonicated, and centrifuged at 100,000 x g for 20 min at 4 °C. The supernatant was incubated with dextran coated charcoal for 5 min on ice prior to pelleting the charcoal for 1 min at 12,000 x g. Binding activity was determined according to Kaufman *et al.* (1979). Aliquots of the initial supernatant prior to binding with charcoal and of the charcoal bound supernatant were added to 10 ml of toluene containing omnifluor (4g/l, New England Nuclear, Boston, Massachusetts) and counted in a Beckman scintillation counter (Beckman, Fullerton, California).

b. Scatchard plot analysis

Scatchard plot analyses were performed according to Kaufman *et al.* (1990). Confluent 60 mm dishes of GSF were placed on ice. The medium was aspirated and replaced with HEPES-MEM 24 h prior to performing the assay. Duplicate dishes were incubated for 2 h at 37°C in the presence of 0.1 - 0.3 nM MB $\pm$ 200 fold molar excess of steroid. The dishes were placed on a bed of ice and washed twice with 5 ml of buffer (20 mM Tris-HCl pH 7.4, 0.15 M NaCl and 0.2 % BSA), and twice with the same buffer without BSA. The cells were trypsinized for 5 min at room temperature and the cells scraped and resuspended in buffer lacking BSA. The cells were pelleted at 500 x g for 5 min at room temperature and washed once in the same buffer. The cells were solubilized with 1.5 ml of 0.5 N NaOH and aliquots were taken for protein and radioactivity determinations. Specific binding (total binding less the non-specific binding in the presence of 200 fold molar excess radioinert ligand) was plotted as a function of the free concentration according to Scatchard (1949) and the values of B_{max} and K_d were derived from the curve.

E. Immunological characterization of the 56 kDa protein

8. Antibody production

Several hundred 2D-PAGE fractionations were performed on extracts from a GSF strain, MCH 6. 56 kDa protein spots (right spot, pI 6.7) were excised from Coomassie Blue stained gels. However, gels stained for the preparative isolation of 56 kDa protein spots were by the method of Hunkerpillar *et al.* (1983). This was because it was believed that a high amount of N-termini were susceptible to acetylation in the presence of 10 % acetic acid in the staining and destaining solutions normally used. This modification blocked N-terminal sequencing procedures and may alter specificities of antibodies and was avoided. Thus, gels were stained in a solution containing 0.5 % Coomassie Blue, 1 % acetic acid, 30 % 2-propanol. Gels were stained only long enough to visualize protein spots (approximately 15 min). The background was destained (in 250 ml of 5 % acetic acid and 16.5 % methanol) for a short period of time (5 - 30 min) until the individual spots were detectable for excision. Gel pieces were stored at -80°C until sufficient numbers were collected.

a. Antiserum preparation

The protein spots were washed in a small volume of PBS and subsequently homogenized in the same buffer. An equal volume of Freund's complete adjuvant (Calbiochem, San Deigo, California) was added to a final volume of 2 ml and the mixture homogenized to an emulsion. This homogenate was injected intradermally in 0.1 ml aliquots into many positions on the shaven lower-back of a 6 month old white New Zealand rabbit. The primary inoculation was performed using 100 gel spots whereas the two subsequent boosts given intraperitoneally two weeks apart. Each boost utilized 50 spots which were homogenized with incomplete Freund's adjuvant. Blood was collected by bleeding from the ear vein after application of a small amount of xylene and Vaseline to help in the collection. The blood was allowed to clot and the coagulated material was pelleted at 3000 x g. The serum was collected and stored at -80°C.

9. Immunochemical detection of 56 kDa protein

a. Determination of antibody titer

Serial dilutions were made and tested for reactivity against serial dilutions of MCH 6 protein extracts immobilized on nitrocellulose using a dot blot apparatus (BioRad, Richmond, Virginia). An enzyme-linked immunosorbent assay (ELISA) was used to visualize the primary antibodies from the antiserum immobilized by bound antigen. MCH 6 protein extracts were serially diluted to a total volume of 100 μ l with TBS buffer (10 mM Tris pH 8, 150 mM NaCl). This volume was allowed to passively absorb to nitrocellulose membrane by flowing through the wells of a dot blot apparatus. Serial dilutions of the antiserum in 1 % BSA, 0.1 % Thimerosal in TBST (TBS with 0.05 % Tween 20 (polyoxyethylene-sorbitan monolaurate)) were then added to the wells and

allowed to bind for 1 h without any flow through. The addition of the BSA had the added effect of blocking the rest of the surface of the nitrocellulose from adsorbing anymore protein. The solution was vacuumed through and the wells washed with 5 volumes of TBST. The nitrocellulose was removed from the apparatus and incubated in 5 % BSA and 0.01 % Thimerosal for 30 min to block the rest of the surface. The membrane was then washed once with TBST. A 1:1000 dilution of the horse radish peroxidase (HRP) conjugated or a 1:3000 dilution of the alkaline phosphatase conjugated goat anti-rabbit IgG was made in TBST. The blot was incubated with the second antibody solution for 1 h and washed twice with TBST and once with TBS. HRP development was done in the dark for up to 40 min with 30 ml of a solution containing 15 mg of 4-chloro-1-naphthol (BioRad, Richmond, California) dissolved in 5 ml methanol at -20°C and mixed with 25 ml TBS and 15 µl hydrogen peroxide. Alternatively, alkaline phosphatase color development was done in 100 ml carbonate buffer (0.1 M sodium bicarbonate pH 9.8, 1 mM MgCl₂ containing 30 mg nitro blue tetrazolium (NBT, Sigma, St Louis, Missouri) in 1 ml 70 % dimethyl formamide and 15 mg BCIP (toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate, Sigma, St Louis, Missouri) in 1 ml dimethyl formamide. Color development was allowed to proceed from 1 h to overnight to yield the desired intensity of stain.

b. Partial purification of antiserum

The polyclonal antiserum was successively immunoabsorbed to protein extracts from a cell strain, WP009, that had no expression of the 56 kDa protein as determined by silver and Coomassie staining, or by photolytic and affinity labeling and Western immunoblot analysis of proteins fractionated by SDS-PAGE. This was accomplished by diluting the antiserum 1:500 in 1 % BSA in TBST and adsorbing it to Western blots of WP009 for 1 - 3 h at 4°C. The partially purified antiserum was then stored at 4°C.

c. Western immunoblotting

After size fractionation by PAGE, proteins were electrophoretically transferred to Nitro Plus nitrocellulose paper (Micron Separations, Wesboro, Montana) using a Transphor system (Hoefer Scientific Instruments, San Francisco, California), a Lauda circulating bath (Brinkmann Instruments, Rexdale, Ontario) and a buffer system developed by Towbin *et al.* (1979). Following PAGE separation, the gel was equilibrated for 30 min in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol, and 0.1 % (w/v) SDS) before electroblotting. The nitrocellulose membrane was cut to a size slightly larger than the gel, hydrated in water for 30 min and placed in transfer buffer for 30 min prior to transfer. A sandwich consisting of two sheets of 3 MM blotting paper, the nitrocellulose paper, the gel, and two more sheets of blotting paper were clamped together and placed into the blotting chamber. Excessive bubbles trapped in the sandwich were

removed by firmly knocking the submerged sandwich against the chamber bottom. Transfers were performed at 100 V for 1.5 h with cooling to 5°C. Following transfer, the nitrocellulose was removed, blotted between filter papers and air dried overnight at room temperature. The blots were placed between two filter papers, wrapped in foil and the proteins fixed to the membrane by heating at 65°C for 30 min

d. India ink stain of proteins on nitrocellulose membranes

Nitrocellulose blots were washed four times for 10 min each in TBS - 0.03 % (v/v) Tween 20 at 37°C. Subsequently, the blots were rinsed with water and stained in a solution of 1 μ l India drawing ink (Pelikan, Hannover, Germany) per milliliter of TBS - 0.03 % Tween 20 and using a minimum of 0.6 ml cm⁻² nitrocellulose. The blots were stained overnight with gentle shaking at room temperature. Excessive background stain was removed by destaining with water for 5 min and then air dried. Filters were stored dry in the dark to avoid fading or alternatively, a Polaroid picture was taken (f-stop of 22 and 1/60 shutter speed).

e. Immunochemical staining on Western blots

The alkaline phosphatase color development procedure for Western blot analyses were different from that of the dot blot methods. The dried Western blots were placed, protein side down, in 50 ml of 5 % non-fat dry milk in TBST for 30 min to saturate the nitrocellulose with non-specific protein. The membranes were then placed into quick seal plastic bags and rinsed with TBST. The antiserum was diluted appropriately (for anti-56 kDa sera in general, a 1:500 dilution was sufficient) in a total volume of 10 ml of TBST and placed into the bags. The bags were heat sealed and incubated overnight at 4°C with gentle rocking on a shaking platform. The bags were cut open, the antibody solution removed and stored, and the membrane removed and rinsed thrice for 15 min each with 25 - 50 ml of TBST. The membranes were rinsed briefly with 5 % non-fat dry milk in TBST and then with TBST. The membranes were placed into new bags and the second antibody solution added in a volume of 10 ml in TBST. The bags were incubated as before for 1 h with gentle shaking at 4°C. The blots were removed from the bags and rinsed thrice for 15 min each with TBST. The blots were developed for color in 50 ml of buffer (9 volumes of an alkaline phosphatase buffer containing 100 mM Tris pH 9.5, 100 mM NaCl and 5 mM MgCl₂; 1 volume of a 2 mg/ml NBT in 100 mM Tris pH 9.5; and 0.1 volumes of 10 mg/ml of BCIP in dimethylformimide). Color development was allowed to proceed for 1 - 3 h after which fresh buffer was used when longer development was necessary.

10. Immunological analysis of *in vitro* translated proteins from GSF

a. Isolation of total RNA from cell cultures

Total RNA was isolated from thirty 150 mm dishes of GSF MCH 6 by the guanidinium isothiocyanate method (Chirgwin *et al.* 1979). The medium was aspirated and the monolayer of cells washed twice with PBS. The cells were lysed with 2.5 ml of guanidinium solution/dish (5 M guanidinium isothiocyanate, 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.7 M β -mercaptoethanol, and 2 % N-lauroylsarcosine (Sarkosyl) and filter sterilized). Lysis of the cells was immediate and the viscous solutions pooled and triturated by being drawn up into a syringe attached with a 18 gauge needle. 0.1 g of CsCl was added for each milliliter of solution and layered over 9 ml of 5.7 M CsCl in a Quick Seal polyallomer tube (Beckmann, Westbury, New York). The tube was sealed and centrifuged at 40,000 x g in a Ti 70 rotor at 20°C for 16 h. The tube was sliced open near the top and the solution carefully aspirated until 3 cm above the bottom of the tube. The tube was inverted to discard the rest of the solution, the tube was cut 2 cm from the bottom and placed inverted on the benchtop. The inside wall of the tube around the pellet was carefully wiped and the RNA pellet dissolved in 3 ml of buffer (5 mM EDTA, 0.5 % Sarkosyl, and 5 % β -mercaptoethanol). The solution was extracted sequentially with phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol and precipitated with 0.3 M sodium acetate pH 5.2 and 2.5 volumes of ethanol. The pellet was washed in 70 % ethanol and dried. The pellet was dissolved by adding 1 ml of water and heating at 65°C for up to 30 min. Ten microliters of sample was quantified by determining the absorbance at 260 nm and comparing it to that at 280 nm. The value of 40 μ g/ml RNA for each unit of absorbance at 260 nm was used to determine the concentration of RNA and a value of 1.7 - 1.9 for $A_{260/280}$ ratio was considered pure enough.

b. Isolation of mRNA

Messenger RNA was purified from this total RNA by passing over an oligo-d(T) cellulose (Pharmacia, Uppsala, Sweden) column according to Maniatis *et al.* (1982). The mRNA was precipitated, dried and resuspended into 20 μ l of sterile water.

c. In vitro translation

In vitro translation was performed using a kit (New England Nuclear, Mississauga, Ontario). Essentially, 1 μ l of mRNA was translated for 1 h at 37°C. The 25 μ l translation mix contained 1 μ l mRNA, 10 μ l rabbit reticulocyte lysate, 5 μ l L-[35 S]-methionine (800 Ci(30 TBq)/mmol, Amersham, Arlington Heights, Illinois), 5.5 μ l translation cocktail, 2 μ l of 1 M KCl, 0.5 μ l of 32.5 mM magnesium acetate and 1 μ l of sterile distilled water. TCA precipitable counts were determined for 0.1 μ l aliquots.

d. Immunoprecipitation and analysis of translated protein

The rest of the translation mixture was diluted to 500 μ l with washing buffer (0.05 M Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM EDTA, 1 % SDS and 0.02 % sodium azide). This yielded a solution of 1×10^6 cpm/ml TCA precipitable counts. 10 - 15 μ l of specific antiserum or pre-immune serum were added and the mixture placed on an orbital rotator at 4°C for 1 h. Subsequently, 50 μ l of a 5 % solution of protein-A Sepharose (Pharmacia, Uppsala, Sweden) were added and returned to the rotator for 16 h at 4°C. The Sepharose beads were pelleted and washed 9 times with 1 ml each of washing buffer. The immune complexes bound to the protein-A Sepharose were released by addition of 50 μ l of 1 M acetic acid. The beads were pelleted and the supernatant was lyophilized. The residue was resuspended into CHAPS lysis buffer for analysis by 2D-PAGE. The gels were prepared for fluorography and exposed to X-ray film to reveal the immunoprecipitated translation products.

11. Immunocytochemical analysis of 56 kDa protein

MCH 6 cells were seeded onto 35 mm dishes and grown for 24 h. The cells were washed thrice with PBS and fixed to the dishes with 2 ml of Bouins reagent (4 % paraformaldehyde in PBS) for 15 min at room temperature. Dishes were washed thrice with TBST for 10 min each and incubated with 5 % BSA for 30 min to block non-specific binding of antibodies. Dishes were washed once with TBST and incubated overnight at 4°C with a 1:750 dilution of antiserum to the 56 kDa protein or with non-immune serum. The next day, the dishes were washed thrice with TBST at room temperature and incubated for 1 h at room temperature with a second antibody (goat anti-rabbit IgG alkaline phosphatase conjugate). The dishes were washed thrice with TBST and color development for alkaline phosphatase performed. Dishes were washed in water and saturated with 50 % glycerol. Phase contrast microscopy was used to view the specific staining and photographs were taken.

F. Molecular characterization of the cDNA for the 56 kDa protein

1. cDNA library construction

The first cDNA library was constructed in a λ gt11 expression vector (Pharmacia, Uppsala, Sweden) with random primed poly A⁺ RNA from GSF strain MCH 6 isolated using the guanidinium isothiocyanate method of Chirgwin *et al.* (1979). The second library was primed with oligo(dT) (Pharmacia, Uppsala, Sweden) using cloned Moloney-murine leukemia virus (M-MLV, BRL, Bethesda, Maryland) reverse transcriptase. The third cDNA library was constructed in Lambda ZAP[®] II (Stratagene, La Jolla, California) using the cloned M-MLV RT and poly A⁺ RNA from MCH 6, isolated by the Fast-Track mRNA kit (Invitrogen, San Deigo, California) and primed with oligo (dT₁₈). Only the third library construction will be described in detail.

a. Messenger RNA isolation

Poly A⁺ RNA was isolated from cell strains using oligo-dT cellulose supplied with a Fast Tract mRNA isolation kit (Invitrogen Corporation, La Jolla, California) based on the method by Bradley *et al.* (1988). This method allowed the isolation of Poly A⁺ RNA directly from cells or tissues. Tissue culture cells were lysed directly on the dish with 15 ml of lysis buffer (0.2 M NaCl, 0.2 M Tris-HCl pH 7.5, 1.5 mM MgCl₂, 2 % SDS, 200 µg/ml proteinase K) and the solution passed from dish to dish. Alternately, up to 1 g of animal tissue was excised, washed in PBS and cut into small pieces, subjected to 30 s of homogenization with a Kinematica homogenizer (Brinkmann, Hannover, Germany). The homogenates were incubated at 45°C for 30 min with gently shaking for the digestion of proteins. The homogenates were also triturated through plastic syringe fitted with a 18 gauge needle with 4 up and 4 down strokes. Any insoluble material was pelleted by centrifugation at 4000 x g for 5 min at room temperature. The lysate was adjusted to 0.5 M with 5 M NaCl stock solution and mixed thoroughly and any insoluble nucleic acid precipitate was sheared by trituration using a 21 gauge needle. 50 - 100 mg of oligo(dT) cellulose beads for each 10⁹ cells were added to each tube and rocked gently at room temperature for 30 min. The beads were pelleted at 4000 x g at room temperature, washed in 20 ml of binding buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.5), pelleted and washed again in 10 ml binding buffer and pelleted. The pellet was resuspended into 10 ml of low salt buffer (0.2 M NaCl, 10 mM Tris-HCl pH 7.5) and pelleted. This wash was repeated until the wash buffer had an OD₂₈₀ below 0.05 units. The beads were transferred to a spin-column and washed in low salt buffer once. The mRNA was eluted from the beads by mixing 200 µl of elution buffer (10 mM Tris-HCl pH 7.5) into the beads with a plastic pipette tip. The solution was spun through and a second volume of elution buffer was added and spun through. The mRNA was precipitated with 2.5 volumes of ethanol and 0.3 M sodium acetate. The mRNA pellet was resuspended in 50 µl of elution buffer and stored at -80°C.

b. Reverse transcription of mRNA

The first strand reverse transcription was done in a 50 µl volume. Five micrograms of mRNA from MCH 6 was diluted to 32.5 µl and heat denatured at 65°C for 3 min and quickly placed on ice for 5 min. 10 µl of 5 x M-MLV RT buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl₂ and 50 mM DTT), 2.5 µl of 10 mM dNTP mix, 2.5 µl (2.5 µg) of oligo(dT)₁₂₋₁₈ and 2.5 µl (500 U) of M-MLV reverse transcriptase were added and the mixture incubated at 37°C for 1h. The reaction was stored on ice.

c. Efficiency of first strand synthesis

Ten microliters of the initial reaction volume was removed and added to 1 μl of [α - ^{32}P]dCTP (3000 Ci(TBq)/mmol, Amersham, Arlington Heights, New York). The reaction was incubated at 37°C for 1 h after which 1 μl of 0.25 M EDTA pH 8.0 was added to stop the reaction. It was then stored on ice.

d. Second strand synthesis

The following was added to the 40 μl of reverse transcribed reaction mix to a total volume of 320 μl : 32 μl 10 x second strand buffer (188 mM Tris-HCl pH 8.3, 906 mM KCl, 46 mM MgCl_2 and 37.5 mM DTT), 6 μl 10 mM dNTP mix, 1 μl [α - ^{32}P]dCTP, 231.6 μl water, 8 μl (80 U) DNA Pol I and 1.4 μl (2.7 U) RNase H. The reaction was incubated for 2 h at 16°C and then stopped by adding 25 μl of 0.25 M EDTA pH 7.5.

e. TCA precipitable counts

One microliter of the first strand synthesis efficiency reaction and 5 μl of the second strand reaction were diluted to 10 μl with water. 5 μl aliquots were spotted onto two nitrocellulose filters and air dried. One of each duplicate filters were washed thrice for 5 min each in 10 ml of ice cold 10 % TCA and 20 mM sodium phosphate buffer. The filters were then washed in 10 ml of ethanol for 5 min and air dried. The washed and unwashed filters were then counted according to Cherenkov.

f. Calculation of yields of transcribed cDNA

The first strand yield was calculated as follows:

$$\begin{aligned}\text{pmol dCTP}/\mu\text{l} &= (25,000 \text{ pmol dCTP}/50 \mu\text{l reaction volume}) \times (10 \mu\text{l aliquot}/11 \mu\text{l total}) \times 1 \mu\text{l} \\ &\hspace{25em} \text{aliquot} \\ &= 454.5 \text{ pmol dCTP}/\mu\text{l}\end{aligned}$$

$$\text{SA (cpm/pmol dCTP)} = (\text{total cpm}/\mu\text{l}) / 454.5 \text{ pmol}/\mu\text{l} = \text{SA cpm/pmol dCTP}$$

$$\begin{aligned}\text{Yield } (\mu\text{g DNA}): &= \frac{(\text{TCA ppt. cpm}) \times (11 \mu\text{l}/1 \mu\text{l aliquot spotted}) \times (4 \text{ pmol dNTP}/\text{pmol dCTP})}{(\text{SA cpm/pmol dCTP}/\mu\text{l}) \times (3030 \text{ pmol dNTP}/\mu\text{g DNA})} \\ &= \mu\text{g DNA (first strand) in } 10 \mu\text{l reaction} \\ &= \mu\text{g DNA} \times 40 \mu\text{l total}/10 \mu\text{l reaction}\end{aligned}$$

The second strand yield was calculated as follows:

$$\text{pmol dCTP}/5 \mu\text{l} = (80,000 \text{ pmol}/345 \mu\text{l}) \times 5 = 1159 \text{ pmol dCTP}/5 \mu\text{l}$$

$$\text{SA (cpm/pmol dCTP)} = (\text{cpm}/5 \mu\text{l}) / 1159 \text{ pmol dCTP}/5 \mu\text{l}$$

performed in a 32 μ l volume (20 μ l cDNA, 3.2 μ l 10 x ligase (300 mM Tris-HCl pH 7.8, 100 mM MgCl₂, 100 mM DTT and 10 mM ATP), 3.2 μ l 1 mg/ml BSA, 1.5 μ l (15 pmol) EcoRI adaptors and 3.7 μ l (7.5 Weiss U) T4 DNA ligase). The reaction was mixed gently and incubated overnight at 15°C. The reaction was stopped by heating at 70°C for 10 min

k. Kinasing of adaptor 5'-overhangs

Directly after the ligation reaction, the tube was cooled on ice and the following was added to a total reaction volume of 40 μ l: 4 μ l 10 x kinase (10 mM Tris-HCl pH 8.0), 2 μ l of 0.1 mM ATP, 3 μ l [γ -³²P]ATP, 1 μ l (10 U) T4 PNK. The reaction was incubated at 37°C for 30 min and boosted with 0.5 μ l of 10 x kinase buffer, 4.5 μ l 10 mM ATP and 1 μ l T4 PNK to ensure that all ends were phosphorylated. The reaction was phenol:chloroform:isoamyl alcohol extracted and precipitated.

l. Purification of adaptored cDNA from free adaptors

Then the free adaptors were removed from the adaptored/kinased cDNA by passing over a Sephadex G-50 spin column. The volume was collected and 1 μ l was counted. The cDNA precipitated with 0.25 M ammonium acetate and 2.5 volumes of ethanol. The cDNA was collected by centrifugation and the pellet washed, dried and resuspended in 2.5 μ l of water.

m. Ligation of adaptored/kinased cDNA to EcoRI digested/phosphatased Lambda ZAP II

The cDNA was ligated to 1 μ g of EcoRI digested and phosphatased lambda ZAP II arms in a volume of 5 μ l (2.5 μ l cDNA, 1 μ l ZAP II, 0.5 μ l 10 x T4 DNA ligase buffer, 0.5 μ l 10 mM ATP and 0.5 μ l (10) T4 DNA ligase). The reaction was incubated overnight at 4°C.

n. Packaging of libraries

Packaging of the ligated phage was performed using a kit (Gigapack II gold packaging extract, Stratagene, La Jolla, California). One microliter of ligated phage was added to 15 μ l of a quickly thawed extract (red tube) and immediately 15 μ l of Sonic extract was added and mixed. The reaction was spun briefly and then incubated at room temperature for 2 h. 500 μ l of SM buffer (100 mM NaCl, 10 mM MgSO₄, 50 mM Tris-HCl pH 7.5 and 0.01 % gelatin) and 20 μ l of chloroform was added and mixed. The reaction was spun briefly to sediment debris and the supernatant removed and stored at 4°C.

o. Determination of library titers

Preparation of plating bacteria

A streak of XL1-Blue cells were inoculated from glycerol stocks onto NYZ plates (5g NaCl, 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g yeast extract, 10 g NZ amine (casein hydrolysate), 15 g agar per liter) containing 12.5 $\mu\text{g/ml}$ tetracycline. The plate was incubated overnight at 37°C. A single colony was picked and inoculated into 50 ml of TB medium (5 g NaCl, 10 g bacto tryptone pH to 7.4 with NaOH per liter and autoclaved, add MgSO_4 to 10 mM and maltose to 0.2 %). The cells were grown at 37°C with vigorous shaking until an $\text{OD}_{600} = 1.0/\text{ml}$. The cells were pelleted at 700 x g for 10 min and resuspended to $\text{OD}_{600} = 0.5$ with sterile 10 mM MgSO_4 . These cells were stored at 4°C for up to 5 days.

Infection of bacteria with phage particles

The packaged library was serially diluted and 10 μl aliquots were adsorbed to 200 μl of plating cells for 15 min at 37°C. 3 ml of melted top agar (NYZ broth with 0.7 % agarose) cooled to 50°C and supplemented with 50 μl of 0.5 M IPTG and 50 μl of 125 mg/ml X-GAL was added to the cell mixture. The tube was quickly capped and inverted to mix and poured on to prewarmed NYZ agar plates. The plates were incubated at 37°C for 16 h and the plaques (white recombinant and blue non-recombinant) counted.

p. Amplification of cDNA libraries

Libraries were amplified prior to screening by infecting 500 μl of plating cells with 20,000 pfu for 15 min at 37°C. The mixture was plated onto NYZ agar plates with the addition of 2.5 ml of top agar. The plates were incubated at 37°C for 16 h after which 5 ml of SM buffer were added and the dishes incubated overnight at 4°C on a shaking platform with gentle rocking. The solution was removed and chloroform added to 5 %. The cellular debris was removed by centrifugation at 7,000 x g for 30 min and the supernatant was recovered and chloroform added to 0.3 %. The titer of this amplified library was checked.

q. Storage of libraries

The amplified libraries were stored in SM buffer with 0.3 % chloroform at 4°C for several months. For long term storage, DMSO was added to 7 % and aliquots stored at -70°C.

2. Screening of cDNA libraries

a. Expression screening

The cDNA libraries were immunoscreened using the rabbit antiserum against the 56 kDa protein. Essentially, 5,000 pfu were plated onto each 100 mm LB agar plate and incubated for 3.5 h at 43°C to permit plaque formation. Three hours after the plates had been in the incubator, nitrocellulose filter disks (S & S NC™ BA 85, Schleicher & Schuell, Keene, New Hampshire)

were numbered with a soft pencil. Then they were impregnated with IPTG by dunking the filter disks into a 10 mM IPTG solution and then placing them onto a sheet of 3 MM paper for air drying in the dark. This process was repeated until the desired number of disks were impregnated (usually no more than 20). The filter disks were overlaid onto the plates and incubated for 4 h at 37°C to induce controlled expression of recombinant peptides fused to β -galactosidase. The filters were marked with three non-symmetrical stabs (with a hypodermic needle dipped in India ink) of the filter and agar to allow for identification of the positive clones. The filter was gently blotted between two 3 MM filters to remove any adhering top agarose and washed in TBS buffer for 10 min. The filters were then placed into a solution of 2 % BSA for 1 h - overnight at room temperature to block non-specific binding of antiserum. Two filters were placed back-to-back and placed in a plastic petri dish which had 10 ml of partially purified antiserum solution (1:500 dilution in TBS). The dishes were placed on a slowly shaking platform at 4°C and incubated overnight. The next day, the antiserum solution was saved and stored at 4°C. The filters were washed twice with 10 ml TBST and incubated with 5 ml of a 1:3000 dilution of affinity purified goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate human IgG adsorbed (BioRad, Richmond, California). The dishes were incubated at room temperature for 1 h with gentle shaking. The antibody solution was discarded and filters washed three times with TBST. Color development was exactly as describe for Western immunoblotting. Positive plaques were identified and excised from the original plate by aligning the orientation marks on the filter with those in the agar. Each plug of agar was added to 1 ml of SM buffer, 20 μ l of chloroform, and incubated overnight at 4°C. The next day a dilution of each selected clone solution was made (assuming approximately 1×10^{11} pfu/plug/ml or 10^6 /plaque/ml) and rescreened until all plaques were positive.

b. Hybridization screening

Filter-Lifts

Five thousand - 10,000 pfu were used to infect Y1090r- plating cells and plated onto LB agar using top agarose. The plates were incubated at 37°C for 8 - 16 h to allow plaque growth. The plates were removed from the incubator and cooled to 4°C to harden the top agarose (in cold room at least 1 h). Each plate overlaid with a numbered dry, sterile nitrocellulose filter and left for 30 s (a second duplicate filter-lift was made by adsorbing for 60 s). Orientation marks were made and the filter removed and placed plaque side upwards for 3 min on a sheet of 3 MM filter paper soaked in denaturing solution (1.5 M NaCl in 0.5 N NaOH). Prolonged exposure to this solution was avoided (only a total of 10 disks were treated in each cycle). The filters were then transferred to a second 3 MM filter soaked in neutralization solution (1.5 M NaCl in 0.5 M Tris-HCl pH 8.0)

and incubated for 3 min. The filters were then rinsed briefly in 2 x SSC and placed on 3 MM paper. The filters were exposed to UV to crosslink the DNA to the filter disk in the Stratalinker as before.

Pre-hybridization

Filters were washed at room temperature three times each for 15 min in 3 x SSC, 0.1 % SDS (500 ml per 40 - 45 filters). Filters were washed in 1 l of the same buffer at 65°C for 3 h and then at 50°C for 1 h. The filters were prehybridized overnight at 43°C in 100 ml of buffer (6 x SSC, 5 x Denhardt's solution, 0.05 % sodium pyrophosphate, 0.5 % SDS and 100 µg/ml denatured salmon sperm DNA).

Oligonucleotide labeling

75 pmol of ALDH 1 P2 (5' TAGCCGCATCCAGGATT 3') oligonucleotide, corresponding to the 5' most of the first ALDH 1 clone, was end labeled with T4 polynucleotide kinase in a 75 µl reaction mix (200 µCi of γ -³²P ATP (6000 Ci(222 TBq)/mmol, New England Nuclear, Markham, Ontario), 7.5 µl One-Phor-all buffer, 46.5 µl water, 50 U T4 PNK). The reaction mixture was incubated at 37°C for 30 min and then at 68°C for 30 min to inactivate the enzyme. 30 µg of yeast tRNA was added and the reaction phenol:chloroform:isoamyl alcohol extracted. The organic phase was back-extracted with 25 µl of TE and the aqueous phases pooled. The oligonucleotide DNA was precipitated with 0.25 M ammonium acetate and 2.5 volumes of ethanol. The pellet was washed with 70 % ethanol and dried. The pellet was resuspended into 200 µl of TE and 1 µl aliquot was counted in a scintillation counter and the specific activity determined.

Hybridization

The oligonucleotide was diluted to 1 x 10⁶ cpm(1.85 ng)/ml hybridization buffer (6 x SSC, 1 x Denhardt's, 0.05 % NaPO₄, 100 µg/ml yeast tRNA). The filters were hybridized at 43°C for 24 h with gentle mixing. Filters were washed in 6 x SSC and 0.5 % NaPO₄ at room temperature. Then 3 times for 30 min each at 50 °C. Filters were checked for hot-spots of label with a Geiger counter at 0.1 x setting. Filters were placed on to a used sheet of X-ray film and covered with polyfilm wrap. The filters were exposed to X-ray film with an intensifying screen. Positive clones identified were picked from the original plates and rescreened with a random primed 0.7 kbp HincII-PstI fragment of ALDH 1 cDNA.

3. Subcloning from positive clones

a. λgt11 clones

Inserts from the λgt11 clones were subcloned by double digestion with KpnI - SstI releasing fragments from the vector. These fragments were purified from a 1 % agarose gel and cloned into double digested pUC 18/19 and M13 18/19. Subsequently, a double digest of the inserts with

HindII - XbaI was subcloned again and finally a HincII - PstI digest of this insert yielded a 0.7 kb fragment.

b. Ligation of DNA fragments to plasmids

Cloning of DNA fragments generated by restriction endonucleases

Fragments with overhanging nucleotides generated by endonuclease digestions were gel purified before cloning into plasmid vectors (usually containing the pUC/M13 multiple cloning site). The vectors were first digested with the same restriction enzyme and treated with calf intestinal alkaline phosphatase to remove the 5' phosphate. Thus, the vector was unable to self-ligate. Aliquots of the two DNAs (3:1 molar ratio of insert:vector) were mixed and precipitated. The pellet was resuspended in 20 μ l of ligase buffer on ice (50 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, and 5 % (w/v) polyethylene glycol-8000) with 0.1 U of T4 DNA ligase (BRL, Gaithersburg, Maryland). The reaction was allowed to proceed at 4°C overnight. An aliquot of the ligation was checked on an agarose gel prior to precipitation of the reaction. The pellet was resuspended to 5 μ l and 1 μ l was electroporated into competent bacteria for amplification and selection of the modified vector.

Digestion of DNA

Plasmid DNA, gel purified PCR amplified fragments, or DNA fragments isolated from plasmid and gel purified were routinely digested in a reaction volume of 20 μ l in the appropriate buffer for the specific restriction enzyme. In general, the buffer system of BRL was used preferentially as it gave the most consistent complete digestions. However, digestion of PCR amplified products were also performed in the PCR buffer. Enzyme was added to an aliquot of the reaction and incubated at the appropriate temperature. The extent of digestions were followed by fractionation of an aliquot on a 1 % mini-agarose gel.

c. Lambda ZAP II clones

The Lambda ZAP II vector had been designed to allow *in vivo* excision and recircularization of any cloned insert contained within the lambda vector to form a phagemid (pBluescript SK⁻) containing the cloned insert (Stratagene). This "subcloning" process required the co-infection of a helper phage capable of producing a set of f1 phage proteins which Lambda ZAP II cannot. This would allow the phagemid to be excised replicated and packaged. The co-infection required 200 μ l of XL1-Blue cells, combined with 200 μ l of Lambda ZAP II phage clone stock (assuming $>1 \times 10^7$ phage particles) and 1 μ l of R408 helper phage (no more than 1×10^7 pfu/ml). This mixture was incubated at 37°C for 15 min and 5 ml of 2 x YT medium (10 g NaCl, 10 g yeast extract and 16 g bacto-tryptone per liter) were added and the reaction placed in a 37°C shaking incubator for 3 h.

The reaction was then heated at 70°C for 20 min to kill the bacteria, and centrifuged at 4000 x g to remove the precipitated proteins. The supernatant must be decanted immediately to a new tube to remove the possibility that some living bacteria be used for the helper phage to replicate thus contaminating the preparation. This stock contained the pBluescript phagemid clone packaged as filamentous phage particles and could be stored at 4°C for 1 - 2 months. The phagemid was rescued by infecting 200 µl of XL1-Blue plating cells with 50 µl of the phage stock and incubating at 37 °C for 15 min before plating 50 µl onto LB agar plates containing 50 µg/ml Ampicillin. Colonies appearing on the plate contained plasmid clones whereas the helper phage would not grow since it lacked Ampicillin resistance.

d. Storage of clones

To maintain plasmid clones, single colonies were streaked on to a new LB/Ampicillin plate and grown overnight at 37°C and the plate sealed with parafilm for storage at 4°C for 1 - 3 months. For long term storage (up to 1 year at -20 °C or -70°C), single colonies were picked and inoculated into 2 ml of LB/ampicillin medium and grown overnight. 0.5 ml of this saturation culture was mixed with an equal volume of bacterial freezing medium (2 x Hogness buffer, 71 mM K₂HPO₄, 3 mM sodium citrate, 0.7 mM MgSO₄·7H₂O, 14 mM ammonium sulfate, 26 mM KH₂PO₄, 8.8 % (w/v) glycerol).

4. Purification of plasmid DNA

Most of the plasmids used were based on the pUC series (containing pMB1 replicon) which grow at a copy number high enough (>20 copies/cell) to routinely yield 1 to 5 mg of plasmid DNA from a 500 ml culture grown to saturation in LB (10 g bacto- tryptone, 5 g yeast extract, 5 g NaCl and 1 ml of 1 N NaOH) medium. Thus, chloramphenicol amplification (Norranders *et al.*, 1983) of plasmid DNA was seldom used.

a. Mini-preps of plasmid DNA

Plasmids were routinely isolated from bacteria by mini-preparations using a modification of the alkaline lysis method (Birnboim, Doly, 1979, Birnboim, 1983). Essentially, 1 ml of an overnight saturation culture of bacteria was centrifuged for 1 min to pellet the bacteria. The supernatant was discarded and the bacterial pellet was completely resuspended by mixing in the presence of 100 µl of glucose buffer (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl pH 8.0). The mixture was incubated at room temperature for 5 min after which 200 µl of lysis buffer (0.2 N NaOH, 1 % (w/v) SDS) was injected, using a pipette, into the mixture to thoroughly mix and lyse the bacteria. The tube was tapped to completely mix the contents and the tube incubated on ice for another 5 min. The solution was neutralized with the addition of 150 µl of potassium acetate solution (made by

titrating 29.5 ml of glacial acetic acid to pH 4.8 by addition of KOH pellets and making the volume to 100 ml), mixing for 2 s and incubating on ice for 5 min. The cell debris and chromosomal DNA was pelleted by centrifugation at 12,000 x g at 4°C for 5 min. The supernatants were transferred to fresh tubes, phenol and chloroform/isoamyl alcohol extracted and precipitated by mixing with 0.8 ml of absolute ethanol. The mixed solution was allowed to sit at room temperature for 5 min before pelleting the plasmid DNA by centrifugation at 12,000 x g at room temperature for 5 min. The pellet was washed with 70 % ethanol and dried. The plasmid DNA was dissolved into 50 μ l of RNase A in TE (100 μ g/ml DNase free RNase A in TE). This amount of RNase A quickly dissolved the pellet which mainly consisted of RNA. The solution was stored at -20°C indefinitely. This method of preparation allowed the plasmids to be sequenced or digested by restriction endonucleases.

b. Large scale preparation of plasmid DNA

Large-scale preparation of plasmids also employed the alkaline lysis method with modifications. Routinely, 2 ml from a 10 ml saturated overnight cultures were inoculated into 800 ml of LB medium. This culture was allowed to proceed overnight with vigorous agitation at 37°C. The bacteria were pelleted at 6000 x g in a 250 ml centrifuge bottle at 4°C for 5 min. The pellet from the entire 800 ml culture was resuspended into 4 ml of glucose buffer by vigorous mixing. 1 ml of lysozyme buffer (freshly made 25 mg/ml hen egg white lysozyme in glucose buffer) was added and the mixture placed at room temperature for 10 min. 10 ml of lysis buffer was added and mixed gently until homogenous and placed on ice for 10 min. The mixture was neutralized by the addition of 7.5 ml of potassium acetate solution and sharp agitation of the covered bottle until a white flocculant precipitate appeared. The suspension was incubated on ice for a further 10 min prior to centrifugation at 20,000 x g at 4°C for 10 min. The supernatant was carefully removed to a fresh tube without transferring any precipitate, made to 200 μ g/ml RNase A, and incubated at room temperature for 30 min. The plasmid DNA was precipitated with the addition of 0.6 volumes of isopropanol and mixing by inversion then pelleting at 15,000 x g for 1 min at room temperature. The pellet was washed and dried as before. The pellet was then dissolved in 1 ml of 200 μ g/ml RNase A buffer and incubated at 37°C for 15 min prior to 3 extractions with phenol and 1 extraction with phenol:chloroform/isoamyl alcohol. The DNA was precipitated a second time with the addition of 2.5 volumes of ethanol and 1/50 volume of NaCl. The plasmid DNA was pelleted, washed, dried, resuspended into 0.5 ml of TE and stored at 4 °C. The plasmid DNA isolated by this method had greater than 90 % superhelical DNA and was digested, sequenced or transformed into bacteria without problems.

c. Purification of plasmid DNA for transfection into mammalian cells

Plasmid DNA isolated by the large scale alkaline lysis procedure was purified using a QIAGEN-Tip 500 (QIAGEN Inc., Chatsworth, California). 500 μg of plasmid was diluted in 10 ml of buffer PI (100 $\mu\text{g}/\text{ml}$ RNase A, 50 mM Tris-HCl pH 8.0, 10 mM EDTA), diluted with 10 ml of buffer P2 (200 mM NaOH, 1 % SDS) and incubated at room temperature for 5 min. 10 ml of chilled buffer P3 (3 M potassium acetate pH 5.5) was mixed in and incubated on ice for 20 min. One QIAGEN-Tip 500 column was equilibrated with 10 ml of QBT buffer (750 mM NaCl, 50 mM MOPS pH 7.0, 15 % ethanol and 0.15 % Triton X-100) and the chilled plasmid DNA solution applied to the column. The column was washed twice with 30 ml each of buffer QC (1 M NaCl, 50 mM MOPS pH 7.0 and 15 % ethanol) and the DNA eluted with 15 ml of QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5 and 15 % ethanol). The DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 15,000 $\times$ g at 4°C for 30 min. The DNA pellet was washed with cold 70 % ethanol, air dried for 5 min, and redissolved in 0.5 ml of TE. The plasmid DNA was stored at 4°C indefinitely.

5. Introduction of plasmid DNA into bacteria

a. Transformation using calcium chloride

Preparation of competent cells

250 μl of an overnight saturation culture of MV1193 or DH5 α bacteria were transferred to 25 ml of LB incubated at 37°C with vigorous agitation for 2 h. The culture was chilled on ice for 10 min and the cells pelleted at 4,000 $\times$ g for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in 12.5 ml of ice cold sterile CaCl_2 buffer (50 mM CaCl_2 and 10 mM Tris-HCl pH 8.0) and incubated on ice for 15 min. The cells were pelleted and the supernatant discarded and the pellet resuspended in 1.7 ml of ice cold sterile CaCl_2 buffer and 200 μl aliquots were stored at 4°C for 4 h to overnight.

Transformation of competent bacteria

One - 5 μg of plasmid DNA was added to one 200 μl aliquot of competent bacteria with gentle mixing. The mixture was stored on ice for 30 min, heat shocked at 42°C for 2 min then diluted with 1 ml of LB at 37°C and incubated at 37°C water bath for 45 min. Aliquots of 20 μl , 200 μl and 400 μl were spread onto LB agar plates containing 100 $\mu\text{g}/\text{ml}$ ampicillin. Plates were left at room temperature for 20 min to allow absorption of the volume. The plates were incubated inverted in a 37°C incubator for 16 h. Individual colonies were picked and 2 ml overnight saturation cultures were grown and the plasmids isolated and digested to determine presence of correct clones.

b. Transformation by electroporation:

Preparation of bacteria

This method (Dower *et al.* 1988) had become the main procedure used for transformation of bacteria. 2.5 ml of an overnight saturation culture of *E. coli* cells were used to inoculate 500 ml of LB in a 2-liter flask and grown with vigorous agitation until the culture reached a logarithmic phase (OD₆₀₀ reached 0.5 - 0.6). The culture was chilled in an ice-water bath for 10 - 15 min, the cells transferred to prechilled 250 ml centrifuge tubes and centrifuged for 20 min at 4,000 x g at 2°C. The supernatant was discarded and the pellet resuspended into 5 ml of ice-cold water and diluted with 500 ml of ice-cold water. Cells were pelleted, supernatant was poured off immediately and the pellet resuspended by swirling in the remaining liquid. The cell suspension was mixed well with a second 500 ml volume of ice-cold water and pelleted again. The supernatant was poured off as before and the pellet resuspended in the remaining liquid. An equal volume of 10 % glycerol was added and 50 µl aliquots were dispensed into prechilled microcentrifuge tubes and frozen on dry ice. The samples were stored at -80°C.

Electroporation of plasmid DNA

The electroporation apparatus (BioRad, Richmond, California) was set to 2.5 kV and 25 µF and the pulse controller set to 400 ohms. Routinely, 5 pg of pure plasmid DNA or 1 µl of a ligation reaction (containing up to 50 pg of DNA) were added to a fresh tube on ice. One vial of competent cells was rapidly thawed (by holding between fingers) and 25 µl aliquots was added to each vial to be electroporated. The cell suspension was mixed with a pipette tip and transferred to a prechilled sterile (UV irradiated 10 min and chilled on ice 5 min) 1 mm electroporation cuvette. The outside of the cuvette was wiped of excess moisture and slightly tapped on the bench-top to settle the cells to the bottom. The cuvette was immediately placed into the sample chamber and the pulse discharged. The cuvette was removed and 1 ml of SOC (0.5 % yeast extract, 2 % tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) medium was immediately used to resuspend the cell suspension. The volume was transferred to a 10 ml culture tube and incubated with agitation at 37°C for 1 h. Three aliquots (2 µl, 20 µl and 200 µl) of total volume 200 µl were spread onto LB agar plates supplemented with 100 µg/ml of ampicillin. Plates were allowed to dry, were inverted and incubated overnight in a 37°C incubator. Transformation efficiencies were typically 1 x 10⁷ - 1x 10⁹/µg plasmid DNA which was far more than was required for simple cloning purposes.

6. Confirmation of selected clones

c. Southern blot hybridization of ALDH 1 cDNA to clones

Positive clones were digested with EcoRI to excise the cloned insert and the reaction products fractionated on a 1.5 % agarose gel. The gel was stained with ethidium bromide and a Polaroid picture taken.

Capillary Blotting

The size fractionated digested DNA was blotted to nitrocellulose by capillary action according to Southern (1975). Two types of membrane were utilized for blotting: activated diazobenzylloxymethyl (DBM) paper prepared according to Alwine *et al.* (1979) and Hybond-N nitrocellulose membrane (Amersham, Arlington Heights, Illinois). The activation of NBM paper for binding of DNA to DBM paper was essentially as described for Hybrid Selection. Preparation of the gel for blotting to Hybond-N required denaturation in 1.5 M NaCl in 0.5 N NaOH for 30 min and then neutralization in 1.5 M NaCl, 1 mM EDTA in 0.5 M Tris-HCl pH 7.2. 10 x SSC (1.5 M NaCl in 0.15 M sodium citrate) was used as the transfer buffer. The gel was placed upside-down on the blotting assembly and the membrane placed over it. Three sheets of 3 MM paper were wet in transfer buffer and placed onto the membrane, subsequently, 4 inches of paper hand towels (cut to the size of the blot) were placed onto the 3 MM paper. A weight of 500 g was placed onto a glass plate on top of this tower of paper and leveled. Blotting was allowed to proceed for 8 - 16 h when the blot was gently rinsed in 3 x SSC and blotted with 3 MM paper. The DNA was covalently linked to the damp Hybond-N paper by ultraviolet illumination in a Stratalinker (Stratagene, La Jolla, California) set to auto-crosslink at 1200 kilojoules. Alternately, the membranes were baked at 80°C for 2 h in a vacuum oven or gel dryer. The blots were kept wet in a small volume of TE and stored indefinitely at 4°C in sealed plastic bags.

Random primed labeling

The 0.7 kb HincII-PstI ALDH 1 cDNA fragment was used to confirm individual clones. Probes were labeled by random priming (Feinberg, Vogelstein, 1983) to a specific activity greater than 5×10^8 cpm/ μ g. Routinely, 25 - 100 ng of gel purified (using GeneClean kit, BIO101, La Jolla, California) fragment were incubated in the presence of 200 mM HEPES, 50 mM Tris-HCl, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, 0.39 mg/ml BSA, 5.4 OD260 units/ml oligodeoxyribonucleotide primers (hexamer fraction), at pH 6.8, and 20 μ M each of dGTP and dTTP, 50 μ Ci each of [³²P]dCTP and [³²P]dATP, 3000 Ci/mmol (Amersham, Arlington Heights, Illinois), and 3U of Klenow in a total volume of 50 μ l. The reaction was allowed to proceed at room temperature for 1 -3 h and then stopped with 3 μ l of 0.2 M Na₂EDTA, pH 7.5. The free label was removed from the reaction by gel filtration through a G-25 (Quick Spin®) Sephadex® column (Boehringer Mannheim, Indianapolis, Indiana). The column was drained by gravity, centrifuged at 1000 x g for 2 min in a swinging bucket centrifuge and the eluted buffer discarded. The 50 μ l of reaction mix was applied directly to the center cone of Sephadex and the column centrifuged again for 4 min. The eluate was collected and diluted to 1 ml. A 1 μ l aliquot was

counted in a scintillation counter (Beckman 7500, Beckmann Instruments, Fullerton, California) and the specific activity determined.

Membrane Hybridization

Blots were pre-hybridized at 42°C for 16 h to block the membrane of non-specific binding sites that would sequester the small amount of free labeled probe. Twenty milliliters of pre-hybridization solution for each 20 x 20 cm Hybond-N membrane contained 50 % deionized formamide, 4x SSPE (0.72 M NaCl, 4 mM EDTA and 40 mM sodium phosphate pH 7.7), 1 % SDS, 1 % skim milk powder and 200 µg/ml denatured salmon sperm DNA. After pre-hybridization, the solution was discarded. The radiolabeled probe was mixed with 14 ml of hybridization solution, which contained 47 % deionized formamide, 10 % dextran sulfate, 3 x SSPE, 1 % SDS, 0.5 % skim milk powder, 200 µg/ml salmon sperm DNA. Hybridization was performed at 42°C for another 16 h with gentle rotation on a orbital rotator. The membrane was washed as follows: 2 times 10 min at room temperature with 0.1 x SSC, 0.1 % (w/v) SDS and 2 times 20 min each at 65°C with 0.1 x SSC, 0.1 x SDS. The damp membrane was placed onto a 21.6 x 22.9 cm PVC plate, covered with plastic cellophane, a Kodak X-OMAT AR-5 X-ray film was placed on to the plastic, a lightening-plus intensifying screen was placed onto the film, and finally a second PVC plate covered the assembly and was clamped tightly. The assembly was placed into black plastic bags and placed at -80°C for an overnight exposure. Films were developed in an automatic film processor (National Imaging Ltd., Winnipeg, Manitoba). The blot was stripped for subsequent usage in 0.4 N NaOH for 30 min, then neutralized in 0.1 x SSC, 0.1 % SDS in 0.2 M Tris-HCl pH 7.5. The blots were stored in sealed plastic bags with a small volume of TE.

d. DNA sequencing

The Sanger dideoxy chain termination method (Sanger *et al.* 1977) of sequencing DNA was employed preferentially due to its ease of use and availability of standardized kits. Positive clones from λgt11 libraries were plaque purified and subcloned into pUC 19 for further amplification and insert isolation for probing blots, or for M13 for dideoxy chain termination DNA sequencing. Whereas, positive clones from lambda ZAP II libraries were subcloned by *in vivo* excision to generate recombinant pBluescript phagemids and were sequenced directly.

Sequenase®

Sequenase® Version 2.0 (United States Biochemical, Cleveland, Ohio) is a genetic variant of bacteriophage T7 DNA polymerase which has a complete lack of the 3' - 5' exonuclease activity. This version also includes high processivity, high rate of incorporation (>300 dNTP/s) and the

ability to use nucleotide analogs used for sequencing (ddNTPs, alpha-thio dNTPs, dITP, 7-deaza dGTP etc.). Single or double stranded DNA can be sequenced using the dideoxy chain termination method. Three - 5 μ g of plasmid DNA (isolated using mini-prep method) or other dsDNA (isolated from gels) were denatured for 30 min at 37°C in 0.2 N NaOH and 0.2 mM EDTA. The solution was neutralized by addition of 0.1 volumes of 3 M sodium acetate (pH 4.5 - 5.5) and the DNA precipitated with 3 volumes of ethanol. The DNA was pelleted and washed with 70 % ethanol, dried and resuspended in 7 μ l of distilled water, 2 μ l of Sequenase reaction buffer (5 x concentrate contained 200 mM Tris-HCl pH 7.5, 100 mM MgCl₂ and 250 mM NaCl) and 1 μ l of primer (1 pmol). Annealing of the primer to the ssDNA templates were done by heating the mixture in a 65°C water bath for 2 min and then allowing the reaction to cool to 37°C over a period of 30 min. The annealed primer was extended by addition of deoxy nucleotides and T7 polymerase in a 15.5 μ l reaction (10 μ l primer-template mix, 1 μ l of 0.1 M DTT, 2 μ l of diluted labeling mix (5 x concentrate contained 7.5 μ M each of dGTP, dCTP and dTTP), 0.5 μ l of [α -35S]dATP (800 Ci/mmol, Amersham, Arlington Heights, Illinois), and 2 μ l of diluted Sequenase (1:8 in 10 mM Tris-HCl pH 7.5, 5 mM DTT and 0.5 mg/ml BSA). The reaction was mixed thoroughly by tapping the side of the tube and incubated at 18°C for 5 min. The extension reaction was terminated by transferring 3.5 μ l aliquots of the reaction and mixing with 2.5 μ l of 37°C pre-warmed dideoxy NTP termination mixes. These reactions were incubated at 37°C for 5 - 10 min when the reactions were terminated by the addition of 4 μ l of stop solution (95 % formamide, 20 mM EDTA, 0.05 % Bromophenol Blue and 0.05 % Xylene Cyanol FF). The samples were stored at -20°C and heated to 80°C for 2 min prior to loading on a sequencing gel.

Taq polymerase

Taq polymerase was used to sequence the λ gt11 clones directly from the lambda templates using primers for the multiple cloning region and a kit (TaqTrack sequencing, Promega, Unionville, Ontario). Essentially, 10 pmol of λ gt11 forward or reverse primers were end-labeled in a 10 μ l reaction (10 pmol primer, 0.5 μ l (10 pmol) [γ -32P]ATP (6,000 Ci/mmol), 1 μ l 10 x PNK buffer (500 mM Tris-HCl pH 7.5, 100 mM MgCl₂, 50 mM DTT, 1 mM spermidine), 5 μ l (U) T4 PNK). The reaction was incubated at 37°C for 10 min and then the kinase was inactivated at 90°C for 2 min. The alkali denatured and precipitated lambda template was annealed to the primer in a reaction volume of 25 μ l (0.4 pmol (10 μ g) lambda template, 5 μ l Taq DNA Pol 5 x buffer (250 mM Tris-HCl pH 9.0 at 25°C) 50 mM MgCl₂), and 1 pmol of primer and water to 25 μ l). The reaction was incubated at 37°C for 10 min. 1.6 μ l Taq was added to the annealed template mix and 6 μ l of this mix was transferred to each of 4 tubes containing the ddNTP mixes (1 μ l of each individual ddNTP per tube). The tube was incubated at 70°C for 15 min after which 4 μ l of stop solution (10

mM NaOH, 95 % formamide, 0.05 % Bromophenol Blue and 0.05 % Xylene Cyanol) were added. The reactions were heated to 70°C for 5 min prior to loading on a sequencing gel.

PCR based cycle sequencing

Cycle sequencing permits direct sequencing of femtomole quantities of dsDNA using PCR technology. A dsDNA cycle sequencing kit was used (BRL, Burlington, Ontario). The primer was end labeled in a 5 μ l reaction (1 μ l 5 x kinase buffer (300 mM Tris-HCl pH 7.8, 50 mM MgCl₂, 1 M KCl), 1 μ l [γ -³²P]ATP (6,000 Ci/mmol), 1 μ l (1U) T4 PNK and 2 μ l (1 pmol) primer). The reaction was incubated at 37°C for 30 min before termination by heating at 55°C for 5 min. The reaction mixture consisted of a 36 μ l volume (4.5 μ l of 10 x Taq sequencing buffer (300 mM Tris-HCl pH 9.0, 50 mM MgCl₂, 300 mM KCl and 0.5 % (w/v) W-1), 26 μ l of template DNA (~50 fmol), and 0.5 μ l (2.5U) Taq DNA Pol). The reagents were mixed and the tube briefly centrifuged to collect the volume at the bottom of the tube. 8 μ l of this reaction were distributed to each of 4 tubes each containing 2 μ l of individual termination mixes (e.g., for the ddATP termination mix, 2 mM ddATP and 50 μ M each dATP, dCTP, 7-deaza-dGTP and dTTP). Each 10 μ l reaction was overlaid with 10 μ l of mineral oil, the tubes briefly centrifuged and placed into a thermal cycler. Temperature cycling was for 95°C for 5 min to denature the template, then a quick cycle of 10 s at 95°C and 10 s at 65°C was used. 5 μ l of stop buffer was added and samples stored at -20°C for no more than 1 week before running on a sequencing gel.

Sequencing gels

Sequencing gels of acrylamide concentrations 5 - 7 % were generally used to separate the denatured radioactive fragments. Sixty centimeter long plates with wedge spacers (thicker near the bottom) were routinely used. The wedge spacers altered the voltage gradient in the gel such that smaller bands were slowed down resulting in more sequence information being evenly spaced and retained on the gel. A 6 % gel required 100 ml of solution (5.7 g acrylamide, 0.3 g bis-acrylamide, 48 g urea, 10 ml 10 x modified TBE (1.3 M Tris, 0.5 M Borate, and 30 mM EDTA), 40 ml water, 666 μ l of 10 % ammonium persulphate). The solution was made to 100 ml and vacuum filtered through 0.45 μ filter (Millipore, Bedford, Massachusetts), 44 μ l of TEMED was added and the solution poured into the glass sandwich using a 60 ml syringe with a blunted 18 gauge needle. The glass sandwich was reclined to a 45° angle and a sharktooth comb inserted length-wise (with the flat-side) 1 cm into the solution. The gel was allowed to polymerize for 1 h before washing the top with water. The gel was connected to the apparatus holder and pre-run at 60 watts for 30 min with 1 x mTBE buffer. The samples were heated and stored on ice. Prior to loading, the sharktooth comb was inserted onto the gel to create the wells. The wells were washed out with running buffer, samples loaded and run at 60 watts (~55°C) until the

Bromophenol Blue dye front had run off the gel for 15 min. The gel was placed into 1500 ml of fixer (15 % methanol and 5 % acetic acid) for 30 min before drying onto 3 MM paper. The gel was exposed to X-ray film for autoradiography at room temperature or with an intensifying screen at -80°C.

e. Northern Blot Analysis:

Northern gel electrophoresis

Two to 10 µg of each sample were denatured with 50% (v/v) deionized formamide, 2.2 M formaldehyde and 20 mM MOPS pH 7.0 in a total volume of 40 µl. The sample was denatured at 65°C for 15 min and cooled on ice for 10 min. 4 µl of 10 x RNA loading buffer (50 % (v/v) glycerol, and 0.1 mg/ml Bromophenol blue) was added and the samples dry loaded into the wells of a 1% (w/v) agarose gel containing 2.2 M formaldehyde in 1 x MOPS buffer (10 x consisted of 0.2 M MOPS pH 7.0, 50 mM sodium acetate, 10 mM EDTA) and 0.5 µg/ml ethidium bromide. The gel was electrophoresed at 120 V for 25 min to run the RNA into the gel after which the gel was submerged with 1 x MOPS buffer and the voltage was decreased to 35 V for 20 h. The gel was photographed and washed twice in 10 x SSC for 20 min each. The gel was blotted onto a nitrocellulose filter (Hybond-N, Amersham, Arlington Heights, Illinois) as described above for Southern blotting. The filter was baked at 80°C for 2h in a vacuum oven and prehybridized overnight in 20 ml of buffer (50% (v/v) formamide, 4 x SSPE, 250 µg/ml denatured salmon sperm DNA, 1 % skim milk powder and 1 % SDS). Hybridizations in 10 ml of buffer (47 % deionized formamide, 10 % dextran sulfate, 3 x SSPE, 1 % SDS, 1 % skin milk powder and 250 µg/ml salmon sperm DNA) for 16h at 42°C. The filter was washed twice for 15 min each at room temperature and twice at 65°C in a buffer (0.1x SSC and 0.1% SDS). Filters were exposed to X-ray film with a Lightning Plus (New England Nuclear) intensifying screen at -80°C.

f. Hybrid-select *in vitro* translation

Hybrid Selection

Fifty micrograms of clone PUMB1 or carrier plasmid pUC 19 were denatured and bound to nitrocellulose filters (Huynh *et al.* 1985). The filters were used to hybridize against 40 µg of poly A⁺ RNA from strain MCH 6 in 300 µl of hybridization buffer (50% deionized formamide, 0.4 M NaCl, 0.2% SDS, 30 mM PIPES, pH 6.5, and 50 µg/ml yeast tRNA) at 50°C for 3h. The filters were washed 10 times in a Tris buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 150 mM NaCl and 0.5% SDS) at 65°C and the messages released into 300 µl of water containing 2 µl of 10 mg/ml yeast tRNA by boiling and quick-freezing in a dry ice-ethanol bath. The solution was allowed to thaw at room temperature and the filters removed. The mRNA was phenol and chloroform extracted, then precipitated with two volumes of ethanol and 0.1M NaCl.

***In vitro* Translation**

The precipitated messages were translated by a rabbit reticulocyte lysate *in vitro* translation system (Promega). Essentially, the messages were incubated in a total volume of 50 μ l containing 35 μ l of lysate, 1 μ l methionine mix, 5 μ Ci of L-³⁵S-methionine (1070 Ci/mmol, New England Nuclear) for 1h at 30°C.

TCA precipitable counts

TCA precipitable counts were determined as follows: 1 μ l of the reaction was mixed with 19 μ l of 1 % Triton X-100, 20 mM methionine in PBS. 10 μ l each was spotted onto many sections of a Whatmann 3 MM filter disk and allowed to dry. One disk was placed into an ice cold solution containing 10 % TCA and 20 mM methionine for 10 minutes. The filter was subsequently boiled in 500 ml of 5 % TCA and 20 mM methionine for 15 min, washed once in the same solution, twice in 100 % ethanol and finally once in ether and allowed to dry. Both the unwashed and TCA washed filters were placed in 10 ml Aquasol (New England Nuclear) and counted.

Immunoprecipitation with anti-56 kDa antiserum

The radioactive translation products were incubated with anti-56 kDa antiserum for 1h in immunoprecipitation buffer (10 mM Tris-HCl pH 7.4, 2 mM EDTA, 150 mM NaCl and 10% Nonidet P-40) and precipitated with Sepharose protein-A. The bound proteins and antibodies were released by boiling in the presence of SDS sample buffer. The supernatants were fractionated by SDS-PAGE and the gels treated for fluorography as above.

7. Comigration of aldehyde dehydrogenase activity and affinity label

a. Non-denaturing slab isoelectric focusing

For non-denaturing isoelectric focusing slab gels, cells were scraped into a phosphate buffer (10 mM phosphate pH 7.5, 10% glycerol, 3 mM EDTA, 1 mM PMSF, 10 μ M pepstatin A and 100 μ M leupeptin), triturated through a 10 μ l Hamilton syringe and centrifuged for 2 minutes in a microcentrifuge at 4°C. The supernatant was used for the protein fractionation. Non-denaturing slab isoelectric focusing of protein supernatants in phosphate buffer were carried out as follows: 20 μ g of cytosol labeled with ³H-DHTBrAc $\pm$ 200-fold excess cold ligand and 80 μ g of radioinert protein was applied to the same sample pad on a prefocused acrylamide slab gel (5% acrylamide, 3% bis-acrylamide, 10% glycerol, and 2% Pharmalyte pH 3-10). The gel was focused for 3h, for a total of 1800 Vh, at 4°C using 1 M H₃PO₄ and 1 M NaOH as the electrolytes.

b. Aldehyde dehydrogenase activity staining

The activity staining was essentially as described (Kurys *et al.* 1989). A 2 % agarose gel in 100 mM Tris pH 8.5, cooled to 50°C, was mixed with 0.33 mg/ml NAD⁺, 0.33 mg/ml NBT, 1 mg/30 ml phenazine methosulphate and 10 mM benzaldehyde and 10 mM acetaldehyde as substrates. The activity was allowed to proceed at 37°C in a humidified dark chamber until the stain was strongly visible. The reaction was stopped in 7.5% acetic acid and fixed (50% methanol, 10% acetic acid) before the gel was photographed.

c. Fractionation of non-denaturing slab gel for scintillation counting

The appropriate lanes were rinsed in water and sliced into 3 mm pieces, digested in 0.5 ml NCS (9:1 in water, Amersham) tissue solubilizer for 1h, neutralized with 13 µl of glacial acetic acid and counted with 15 ml of 0.4% Omnifluor (Amersham) in toluene in a liquid scintillation counter. Two separate lanes were also sliced prior to activity staining and soaked in 0.5 ml of water and the pH of each slice determined to produce a pH profile of the focused slab gel.

8. Southern blot analysis of genomic DNA:

a. DNA isolation

Seven to 9 ml of peripheral blood were collected in Lavender Vacutainer® tubes and stored at 4°C for up to one week at 4°C without decrease of recovery of genomic DNA. Isolation of genomic DNA from white blood cells was by the procedure developed by Dr. D. Hoar (Director of the Molecular Diagnostic Laboratory, Division of Genetics, Alberta Children's Hospital, Calgary) and subsequently modified by Greenberg *et al.* (1987). The red blood cells were lysed by dilution of the peripheral blood with 5 volumes of hypotonic buffer (140 mM NH₄Cl and 17 mM Tris pH 7.65) and incubating at 37°C for 5 min. The nucleated cells were pelleted by centrifugation at 500 x g for 10 min. The supernatant was aspirated being very careful not to disturb the loose reddish-white cell pellet by leaving 4 -5 ml of the supernatant above the pellet. The pellet was resuspended with 10 ml of 37°C saline (0.85 % NaCl) to wash the nucleated cells and pelleted. This process was repeated with aspiration down to the pellet. The cells were then resuspended into high TE buffer (100 mM Tris-HCl pH 8.0 and 40 mM EDTA). The cells were immediately lysed by injection of 2 ml of lysis buffer (100 mM Tris-HCl pH 8.0, 40 mM EDTA and 0.2 % SDS) using a 3 ml syringe and an 18 gauge needle. If the lysis was deemed incomplete, the mixture was gently aspirated into the syringe and re-injected into the tube and repeated if necessary. At this stage the lysate was stable for several weeks and could be stored at 4°C or shipped by mail. The genomic DNA was subsequently extracted by addition of an equal volume of TE (10 mM Tris pH 8.0 and 1 mM EDTA) saturated phenol. The two phases were mixed to a milky emulsion by end-over-end rotation for 10 min. The phases were separated by centrifugation at 1000 x g for 5 min. Using a large bore disposable plastic pipette, the upper aqueous phase was

gently aspirated and removed to a new tube without disturbing the interphase. The interphase was removed to a different tube and mixed with 2 ml of high TE and reextracted with phenol. The two aqueous phases were pooled and reextracted with phenol. Finally, this aqueous phase was extracted with an equal volume of chloroform:isoamyl alcohol (24:1). The volume of the aqueous phase was measured and 1/50 volume of 5 M NaCl was added to bring the concentration of salt to 0.1 M. An equal volume of absolute ethanol was added and the solution gently inverted end-over-end to mix and allow the genomic DNA to precipitate. After complete mixing of the phases, the partially condensed DNA material was allowed to settle to the bottom of the tube and most of the solution discarded. 5 ml of 70 % ethanol was added to allow the DNA to condense more tightly into a blob. The solution was discarded and 100 % ethanol was added to the pellet. The tightly condensed genomic DNA pellet was dried and resuspended into 5 ml of TE by gentle end-over-end rotation at 4°C. This solution was stored at 4°C indefinitely. Approximately 50 μ g of genomic DNA is usually recovered per ml of whole blood using this procedure.

Genomic DNA from tissue culture cells was isolated by a modification of the above protocol. Essentially, the medium was aspirated from 10 confluent 150 mm plates and rinsed twice with ice cold PBS and trypsinized as described above. The cells were scraped, the volumes pooled and pelleted at 500 x g. The cells were resuspended in 2 ml of high TE, lysed and the DNA isolated as above.

b. Restriction endonuclease digestions of genomic DNA

Approximately 20 μ g aliquots of genomic DNA was digested with various restriction endonucleases in a total volume of 100 μ l with the appropriate buffer as supplied by the manufacturer. Digestions were done for 16 h with a 2 fold excess of enzyme (40 U) at the appropriate temperature and boosted with 1 fold of enzyme. The reaction was checked for completion of digestion after 2 h by fractionating 5 % of the total volume on a mini-agarose gel according to Maniatis *et al.* (1982). The DNA was stained in 0.5 μ g/ml ethidium bromide in 1 x TAE. The gel was placed on a ultraviolet transilluminator (Fotodyne, New Berlin, Wisconsin) and a Polaroid picture was taken (f-stop set at 8 and 2 - 4 s exposure using a red filter and Polaroid type 57 or 667 film). The intensities of the fluorescence of the ethidium bromide bound to the DNA was used as a measure of the concentration of DNA. Furthermore, the intensity of the banding (representative of repetitive DNA) or smear of the DNA was a measure of the completeness of the digestion.

c. Precipitation of digested genomic DNA

If the digestion was deemed complete, an appropriate volume of the total reaction is removed as to normalize the concentration of DNA between the different samples. The fragmented DNA was

precipitated by bringing the solution to 0.1 M NaCl and 0.01 % SDS, and adding 2 volumes of absolute ethanol. The solution was mixed and placed at -70°C for 20 min. The samples were momentarily thawed and the precipitated DNA pelleted by centrifugation at 12,000 x g for 20 min at 4°C. The supernatant was discarded and the pellet washed with 1 ml of 70 % ethanol. As much of the 70 % ethanol was removed as possible and the pellet was vacuum desiccated. Subsequently, the pellets were dissolved in 40 µl of TE with 1 x DNA gel loading buffer (30 % glycerol in water with 0.25 % Bromophenol blue).

d. Agarose gel electrophoresis

A 'definitive' agarose gel electrophoresis was routinely performed using a horizontal gel electrophoresis system (model H4, BRL, Gaithersburg, Maryland) with 250 ml of gel (1 % agarose, electrophoretic grade with low electroendosmosis, in 1 x TAE (40 mM Tris, 1 mM EDTA and 19 mM acetic acid)). The agarose was dissolved by heating in a 500 W microwave (Sanyo) for 5 min. The solution was swirled to mix the agarose and heated for another min. The gel solution was placed in a 65°C water bath until the temperature stabilized and subsequently, was placed on the bench-top to cool to approximately 50°C and the gel poured. Wells were formed using a 1 mm x 1.2 cm 14 well comb. The gel was submerged in 1 x TAE in the buffer chamber and the samples loaded. One lane of the gel was loaded with 200 ng of Lambda DNA double digested with Hind III/EcoRI as a standard. The gel was run at 25 V for 30 min, then at 50 V for a total of 20 - 22 h with circulating of the running buffer from the anode to the cathode. After the run, the gel was stained with ethidium bromide and a picture was taken as described.

G. Regulation of ALDH 1 expression

1. Cloning of the 5' promoter region of ALDH 1

Polymerase chain reaction

Mullis and Faloona (1987) were the first to take advantage of the thermostable DNA dependent DNA polymerase of *Thermus aquaticus* found in some hot springs. They used this polymerase in the *in vitro* amplification of DNA segments through a succession of incubation steps at different temperatures. Typically, the dsDNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at a low temperature and then extended at an intermediate temperature (Perkin-Elmer Cetus, Norwalk, Connecticut). A cycling of these events results in the exponential amplification of the target DNA segment. The major advantage of this polymerase was its ability to polymerase nucleotides to the 3'-hydroxyl end of an oligonucleotide primer at high temperatures with an optimum at 72°C. The major disadvantage was its moderate processivity and high rate of misincorporation (300 errors/10⁶ nucleotides incorporated).

However, modifications to the cloned enzyme have improved on these. Essentially, the polymerase chain reaction (PCR) was performed with up to 1 μ g of genomic DNA and 25 pmol each of two specific primers in a 100 μ l of reaction mix (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl₂, 200 μ M of each dNTP, 0.1 % gelatin and 2.5 U of Taq polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut, or from Gibco BRL, Life Technologies, Inc., Burlington, Ontario)). The reaction mixture was overlaid with 75 μ l of light mineral oil and placed into a Perkin-Elmer Cetus thermocycler model 480 and run for 25 - 35 cycles. The specific cycling conditions for amplification of the 5' ALDH 1 region were as follows: denaturation at 94°C for 1 min; 30 s cool to 57°C and 1 min anneal at the same temperature; 30 s heating to 72°C and extension for 2 min; cycle for 30 rounds; followed by one 8 min extension period before cooling to 4°C. Reactions were analyzed by 2 - 3 % agarose or 5 - 12 % polyacrylamide gels.

PCR fragment cloning

Prokaryote and eukaryotic DNA polymerases catalyze the non-template addition of deoxynucleotides to the 3'-hydroxyl end of blunt-ended DNA (Clark, 1988). Deoxy ATP is preferentially incorporated during the PCR amplification process, thus, the cloning of these fragments has been facilitated by the production of plasmid vectors with single 3'T-overhangs at the insertion site. Using the TA cloning system (Invitrogen corporation, San Deigo, California), 25 ng of the plasmid pCRII vector was ligated with approximately 10 ng of amplified product in a 11 μ l reaction mixture (7 μ l water, 1 μ l of 10 x ligation buffer, 1 μ l of pCRII, 1 μ l of diluted PCR product and 1 μ l of T4 DNA ligase). The mixture was incubated overnight at 4°C and then 1 μ l aliquots were transformed into DH5 α bacteria by electroporation.

2. DNA fragment purification

a. Purification of oligonucleotide fragments

The 739 bp 5' promoter region was digested with restriction endonucleases to generate a set of contiguous fragments small enough for gel shift and *in vitro* footprint analyses. 5 μ g of 739 bp fragment were digested with each enzyme and the fragments separated on a 5 % non-denaturing acrylamide gel. The ethidium stained bands were excised and soaked in 500 μ l of 0.5 M ammonium acetate and 1 mM EDTA at 37°C overnight. The gel pieces were pelleted and the supernatant transferred to a new tube and precipitated with 2.5 volumes of ethanol. The pellet washed and resuspended in 20 μ l of water. Aliquots of the fragments were separated on a gel and the DNA in the bands quantified by comparing to a standard amount of DNA run on the same gel. however, for *in vitro* footprint analysis, the 739 bp fragment was end-labeled prior to digestion with the endonuclease and the fragments separated on a 1.5 % agarose TBE gel. The separation of the DNA bands was followed during electrophoresis using a hand-held UV lamp. When the band

of interest was separated from the rest, a trough was cut out of the agarose directly in front of the band. The trough was filled with 4 x TBE and electrophoresis continued at 200 V. The band promptly migrated into the TBE in the trough and the solution was removed. The solution was phenol:chloroform:isoamyl alcohol extracted, chloroform:isoamyl alcohol extracted and precipitated with 0.3 M sodium acetate and 2.5 volumes of ethanol. The pellet was washed with 70 % ethanol, dried and resuspended into 50 μ l of water. An aliquot was counted to determine the number of counts per minute.

b. End-labeling of fragments

Fragments were end labeled by filling-in of the 5'-overhanging ssDNA. Essentially, labeling of the EcoRI 5'-overhang with [α - 32 P]dATP entailed the following reaction mix: 5 μ l (25 ng) fragment; 5 μ l 10 x Klenow buffer (0.5 M Tris-HCl pH 7.5, 0.1 M MgCl₂, 10 mM DTT and 0.5 mg/ml BSA); 20 μ M dTTP; 5 μ l of [α - 32 P]dATP (3000 Ci(111 TBq)/mmol, New England Nuclear, Markham, Ontario) 23 μ l water; and 2 μ l (6U) Klenow. The reaction was incubated at room temperature for 30 min before extracting with phenol:chloroform:isoamyl alcohol and precipitation with 0.3 M sodium acetate pH 5.5. The pellet was washed with 70 % ethanol and partially dried and then resuspended into 50 μ l of water. One microliter aliquots were counted to determine specific counts. Alternatively, if the fragment needed to be digested further, after digestion the fragments were gel purified before use.

3. Preparation of nuclear protein extracts

The method developed in Dr. Phil Sharp's laboratory has become the standard to isolate nuclei for analysis of DNA-protein interactions (Dignam *et al.* 1983). Briefly, tissue culture cells were collected by scraping into 2.5 ml PBS with a flat plastic spatula after rinsing the dish twice with PBS. The cells were transferred to 50 ml polypropylene conical centrifuge tubes on ice. All cells were pooled and pelleted at 500 x g at 4°C after which the pellets were pooled and gently resuspended in PBS and pelleted. The cells from seventy 150 mm tissue culture plates of GSF strain MCH 6 were collected and stored at -70°C until needed. The cell pellet was allowed to thaw completely on ice. The most important step of the procedure was then to resuspend the cells gently in 5 volumes of buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl and 0.5 mM DTT). This was done by aspirating the cells into a 10 ml graduated pipette and expelling the suspension back into the tube with the tip of the pipette resting at the bottom of the tube. The cells were incubated in this hypotonic buffer for 10 min on ice to swell the cells. The cells were pelleted at 500 x g for 10 min at 4°C. The supernatant was discarded and 2 packed cell volumes (of the original cell pellet) were added to the pellet. The pellet was dislodged from the tube aspirated with a wide bore plastic Pasteur pipette and transferred to a 15 ml Dounce homogenizer. The cells were

broken with 10 strokes (up and down) using the loose fitting pestle B for GSF cells. The homogenate was transferred to a centrifuge tube and the nuclei pelleted at 1000 x g for 15 min at 4°C. The supernatant was collected and stored on ice. The crude nuclear pellet was further centrifuged at 25,000 x g for 20 min at 4°C and the supernatant pooled with the first. This pellet was resuspended in 1 - 1.5 ml (in general, Dignam *et al.* (1983) called for 3 ml buffer C/10⁹ cells but this was impractical with GSF) of buffer C (20 mM HEPES pH 7.9, 25 % (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM PMSF and 1 mM DTT). The pellet was transferred to a cleaned Dounce homogenizer and homogenized with 10 strokes using pestle B. The nuclear suspension was transferred to 6 ml ultracentrifuge tubes (Oakridge type) and placed on an orbital rotator at 4°C for 30 min to mix and extract the nuclei. The broken nuclei were pelleted at 100,000 x g for 20 min at 4°C. This nuclear extract and the first cytosolic supernatant were then centrifuged at 100,000 x g for 30 min at 4°C to pellet any insoluble material. The supernatants were transferred to dialysis tubing (cleaned by boiling successively in 10 mM EDTA then 10 mM NH₄HCO₃, then washing in copious amounts of water). They were dialyzed at 4°C against 50 volumes of buffer D (20 mM HEPES pH 7.9, 20 % (v/v) glycerol, 0.1 M KCl, 0.2 mM EDTA, 1 mM PMSF and 1 mM DTT) with changes each 30 min for a total of 3 changes and a 2 h dialysis. The dialysates were aliquoted in 50 µl volumes to prechilled microcentrifuge tubes and stored at -70°C. Samples were used with up to 3 freeze-thaw cycles.

4. DNA-protein electrophoretic mobility shift assay

The radioactive 5' end-labeled fragments were used in mobility shift assays (Baldwin, 1990). Binding reactions of fragments with nuclear proteins were performed in a 15 µl volume (10,000 cpm (0.1 ng) fragment, 3 µl of 5 x binding (50 mM HEPES-NaOH pH 7.9, 250 mM NaCl, 2.5 mM EDTA, 50 % glycerol, 5 mM DTT with no MgCl₂ added), 2 µg poly(dI-dC) and the protein extract added last). The reactions incubated at room temperature for 15 - 30 min. A 4 % non-denaturing polyacrylamide gel (with a 40:1 acrylamide:bisacrylamide ratio) with 1.5 mm spacers was used to fractionate the protein-DNA mixture. Forty milliliters of gel solution (5.33 ml of 30 % acrylamide, 2 ml of 2 % bisacrylamide, 2 ml of 50 % glycerol, 2 ml of 10 x modified TBE (1.3 M Tris base, 0.5 M Boric acid and 30 mM EDTA), 300 µl of 10 % ammonium persulfate, 40 µl of TEMED and water to 40 ml) was required for two gels. The gel was pre-run at 35 mA constant current for 1 h before the samples were loaded into the wells. Gels were run until the dye front was at the bottom of the gel. The gels were removed from the glass sandwich and dried onto 3 MM filter paper. The gels were then exposed to X-ray film for autoradiography.

5. *In vitro* footprint analysis

DNase I protection was first described by Galas and Schmitz (1978) as a method to study sequence-specific DNA binding of the lac repressor to the lac operator. This method is essentially the same as a mobility shift assay however, the DNA bound to protein was digested with DNase I and the fragments were separated on a sequencing gel. Essentially, aliquots of nuclear protein extracts were incubated for 15 min on ice with 20,000 cpm of an end-labeled DNA fragment (only labeled on one end) in the presence of 2 μ g of non-specific competitor, poly (dI-dC), in a volume of 20 μ l in buffer D. The reaction was removed from the ice and kept at room temperature for 2 min. DNase I (RQ-DNase I, Promega, Unionville, Ontario) was added at various concentrations (1:200 for naked DNA and 1:20 for protein:DNA reactions) in buffer D with 2.5 mM CaCl_2 and incubated for 2 min for each reaction. The reaction was stopped with 160 μ l of stop mix (100 μ l 2 x PK buffer (0.2 M Tris-HCl pH 7.5, 25 mM EDTA, 0.3 M NaCl and 2 % SDS), 2 μ l of 10 mg/ml tRNA, 10 μ l of 10 mg/ml proteinase K and 48 μ l water) and incubated at 37°C for 25 min. The reaction was phenol:chloroform:isoamyl alcohol extracted and ethanol precipitated with 0.2 M NaCl. The dried pellet was resuspended into 4 μ l of formamide loading buffer (80 % formamide, 1 mM EDTA and 0.1 % dyes (Bromophenol Blue and Xylene Cyanol). The reactions were heated to 90°C for 2 min and cooled on ice prior to loading onto a 7 % sequencing gel.

III. Results and Discussion

A. The 56 kDa protein

Since the discovery of the 56 kDa protein in 1982, the methods and reagents available for the isolation and analysis of proteins by electrophoresis have improved greatly. This prompted an optimization of the methods for the characterization of the 56 kDa protein. This was especially important as one of the primary goals of my thesis was the isolation of the cDNA for the 56 kDa protein. This either required an antiserum towards the protein or generation of a degenerate set of oligonucleotides from a partial sequence of the protein. Thus, a better characterization of the 56 kDa protein doublet was primary to a partial purification of the protein.

1. Identification of the 56 kDa protein spot

The 56 kDa protein was identified by its coordinates on a 2-dimensional gel protein map of a soluble protein extract (100,000 x g for 1 h) from genital skin fibroblasts. The 56 kDa protein was readily stained by Coomassie Blue (Fig. 8) and appeared as a doublet with isoelectric points of 6.5 and 6.7. The fact that a low sensitivity protein stain like Coomassie Blue (50 ng/mm², Guilain *et al.*, 1983) was able to stain the doublet as large spots (1 of 13 large spots in the map) implied that the 56 kDa protein belonged to a class of abundantly expressed proteins in GSF. However, the 56 kDa protein was also less abundantly expressed in normal GSF as seen in a comparison of other normal GSF strains (Fig. 9). The four normal GSF strains have varying levels of detectable 56 kDa protein doublet and had been given arbitrary units with a grading system to define their level of expression. The 56 kDa protein was detected in MCH 6 as a large spot (4+) or detected in FXF as a much smaller spot (1+). A non-GSF strain (WP009) with no detectable 56 kDa protein (0) is shown for comparison.

2. Comparison of protein stains on 2D-PAGE of GSF

The idea that the 56 kDa protein was an abundantly expressed protein in GSF was reinforced by sensitivities of the different protein stains. Comparison of the number of spots between the Coomassie Blue (Fig. 8) and silver stained (Fig. 10) protein maps revealed many more proteins in GSF which were expressed at a level not detectable by the Coomassie Blue stain. The 56 kDa protein was also radioactively labeled with [³⁵S]methionine which implied that the protein was a methionine containing protein (Fig. 11).

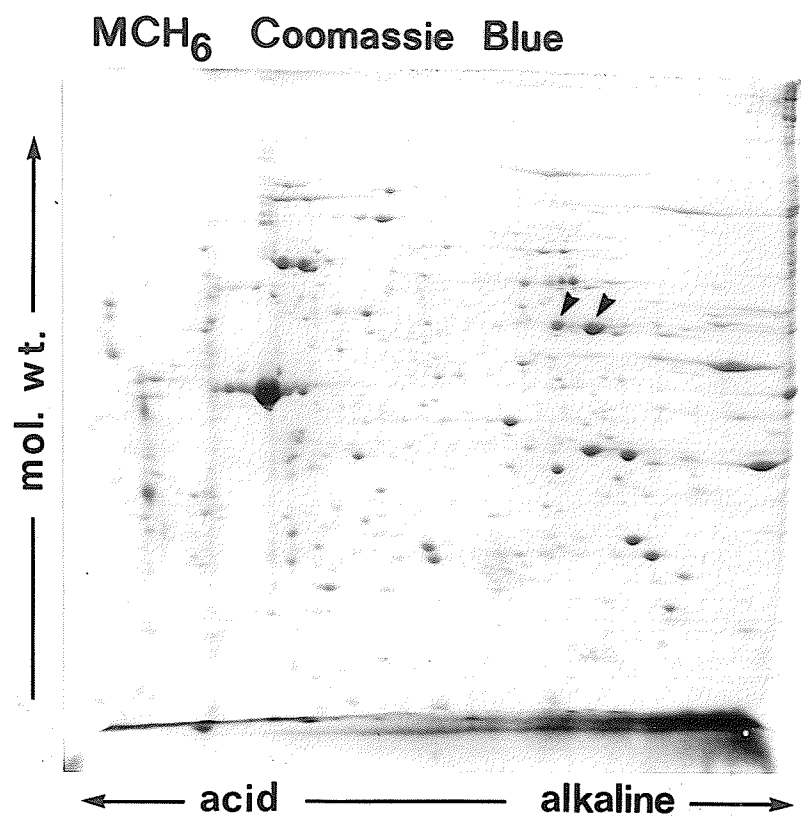


Figure 8. The 56 kDa protein detected in genital skin fibroblasts by Coomassie Blue staining after 2D-PAGE analysis of 100,000 x g supernatant from strain MCH 6. The 56kDa protein doublet is marked by arrows.

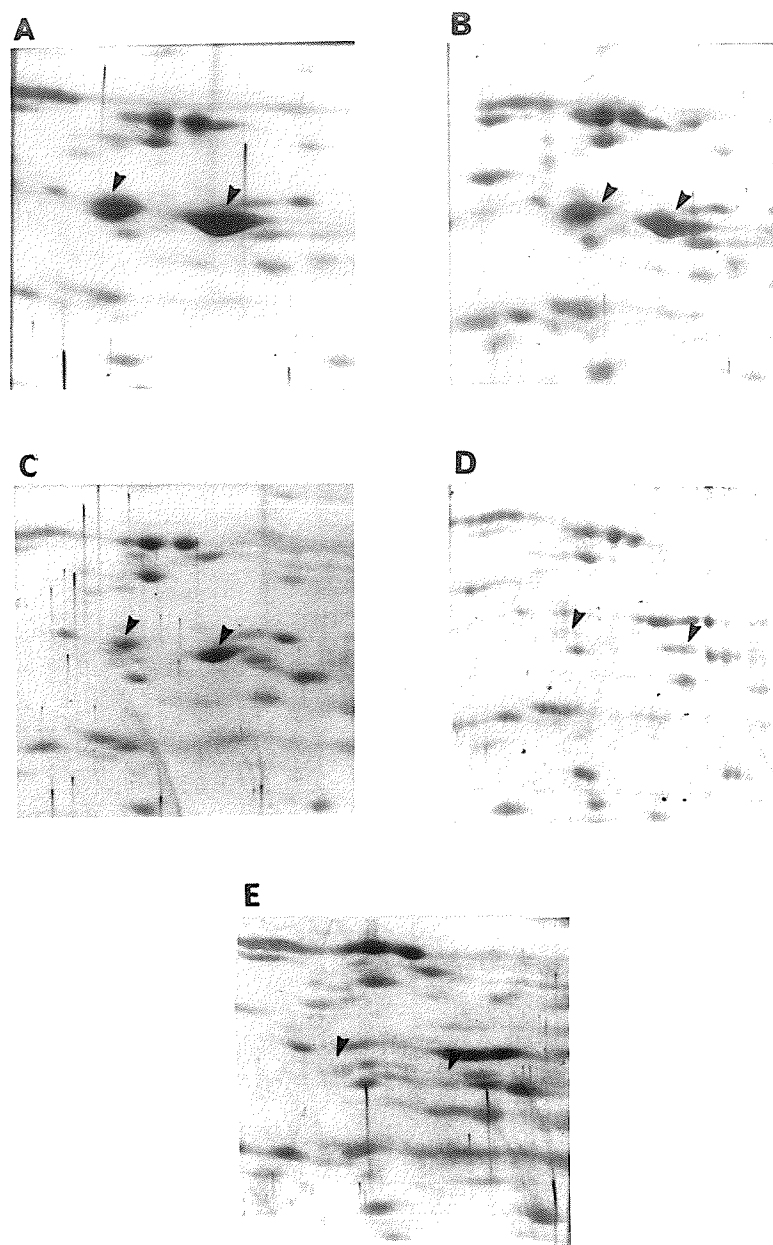


Figure 9. Expression of the 56 kDa protein in normal cells. Grading for the expression of the 56 kDa protein spot is designated according to the size and intensity as follows: A. genital skin fibroblasts (GSF) strain MCH 6 (4+); B. GSF strain 2030 (3+); C. GSF strain 2036 (2+); D. GSF strain FXF (1+); and E. non-genital stain WP009 (0).

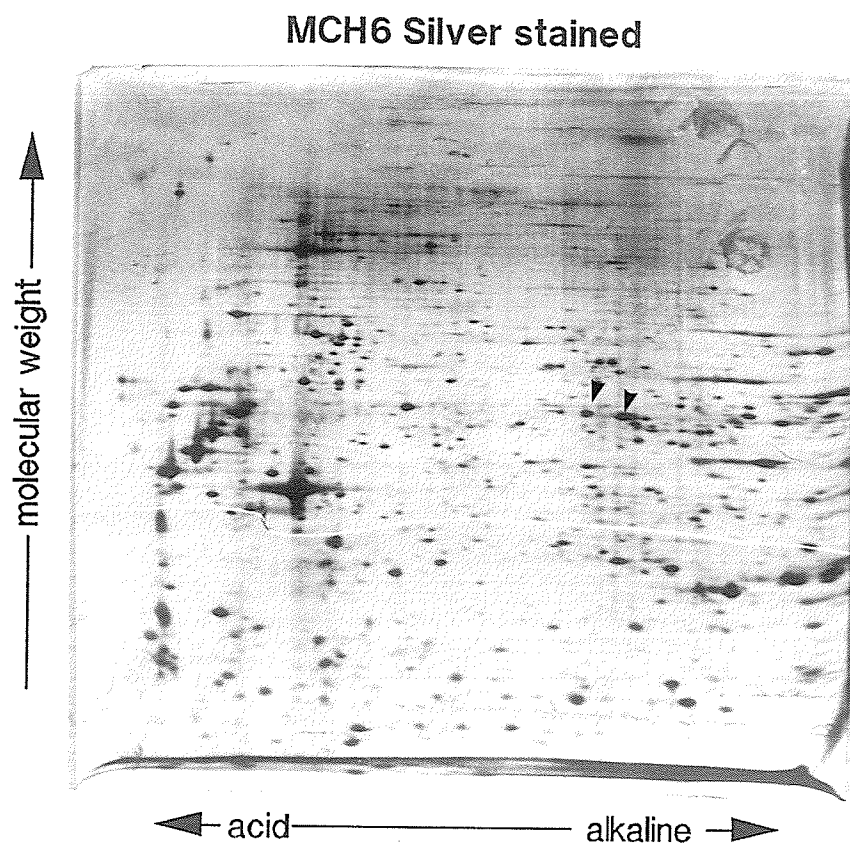


Figure 10. Two dimensional gel electrophoresis of soluble proteins from strain MCH 6 human genital skin fibroblasts. Fifteen micrograms from a 100,000 x g supernatant were applied to the gel and stained by the silver method. The 56 kDa protein doublet is marked by arrows.

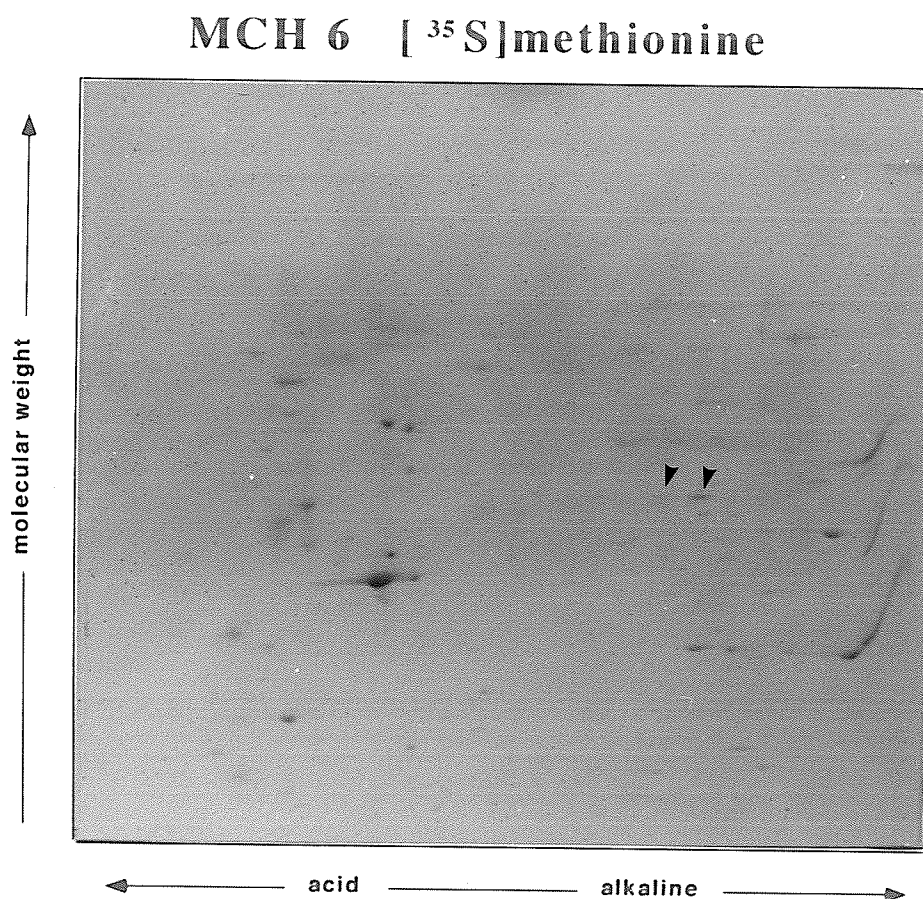


Figure 11. Human genital skin fibroblasts strain MCH 6 radiolabeled with 100 μ Ci of [³⁵S]methionine and the 100,000 x g supernatant separated by 2D-PAGE. The gel was treated for fluorography and the fluorogram is shown after 36 h of exposure.

3. Effect of salt in extraction of the 56 kDa protein from GSF

In order to optimize the yield of the 56 kDa protein for large scale isolation, the effect of salt concentration on the extraction was studied. Ammonium bicarbonate was chosen as the salt because of its volatility upon lyophilization. The lyophilized homogenates were free of excess salts which would interfere with electrophoresis. Several combinations of salt, protease inhibitor and reducing agent were used to extract the 56 kDa protein from GSF. The abundance of the actin spot was used as a standard for comparison purposes. The 56 kDa protein was found to be extracted and focused best with 0.18 M ammonium bicarbonate and 1 mM PMSF (Fig. 12 B), in comparison to extraction with 0.5 M ammonium bicarbonate and 1 mM PMSF (Fig. 12 A). PMSF was used even though no changes were seen in its presence or absence. However, PMSF was used to avoid the possibilities of: i) degraded proteins migrating to the position of the 56 kDa protein or ii) partial degradation of the 56 kDa protein. The inclusion of DTT resulted in an increase of the size the 56 kDa protein doublet (data not shown), which reflects the amount of protein. However, DTT also resulted in a decrease in the total number of spots, creating the possibility that other proteins might be focused to the same coordinates as the 56 kDa protein and thus DTT was not used for future experiments.

B. Relationship between the 56 kDa protein doublet proteins

The fact that the 56 kDa spots were usually found together and had a similar molecular size suggested that they were structurally related. To test this possibility, several spots of the 56 kDa pI 6.5 (left) and pI 6.7 (right) were excised from Coomassie Blue stained 2D-gels. Their peptide patterns were compared after partial hydrolysis with *Staphylococcus aureus* V8 protease (data not shown) and with chymotrypsin (Fig. 13). The peptide bands stained by silver were virtually identical when compared by eye inspection and after densitometric scanning. This suggested that they represented the same protein with internal molecular modifications which resulted in different isoelectric points as seen by 2D-PAGE analysis. Whether the modification(s) were inherent in the protein or were an artifact of the method of analysis was further analyzed. Furthermore, any modification identified might also point towards a function for the protein.

1. Is the 56 kDa protein a phosphoprotein?

The difference in the isoelectric points between the two 56 kDa proteins may be due to charge differences coming from the addition of phosphate groups. Each 100 mm dish of GSF were incubated with 80 μ Ci of [32 P]orthophosphate for 2h at 37°C. The cells were pelleted,

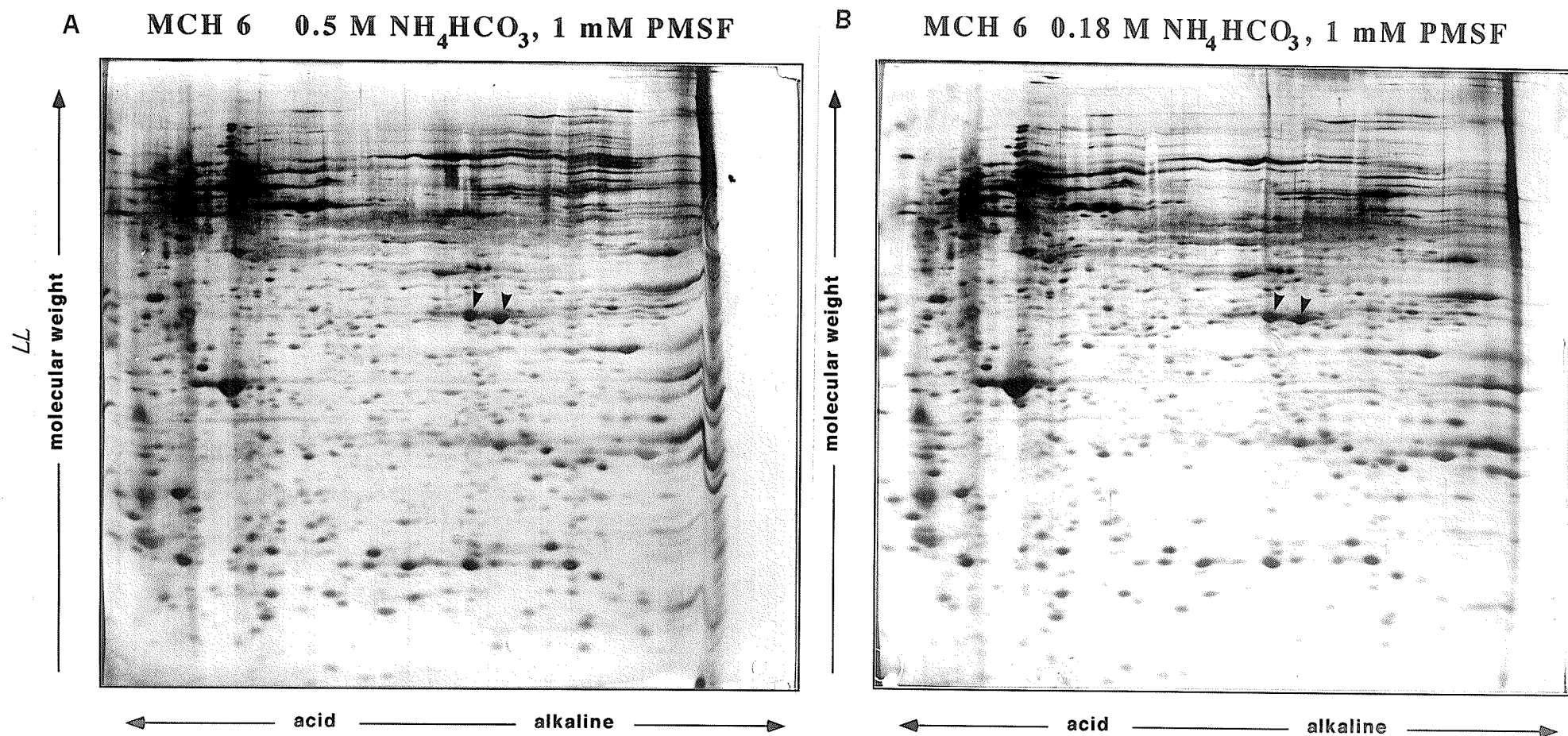


Figure 12. Proteins from strain MCH 6 separated by 2D-PAGE and the gel stained by silver. A. proteins extracted with 0.5 M ammonium bicarbonate and 1 mM PMSF; B. proteins extracted with 0.18 M ammonium bicarbonate and 1 mM PMSF.

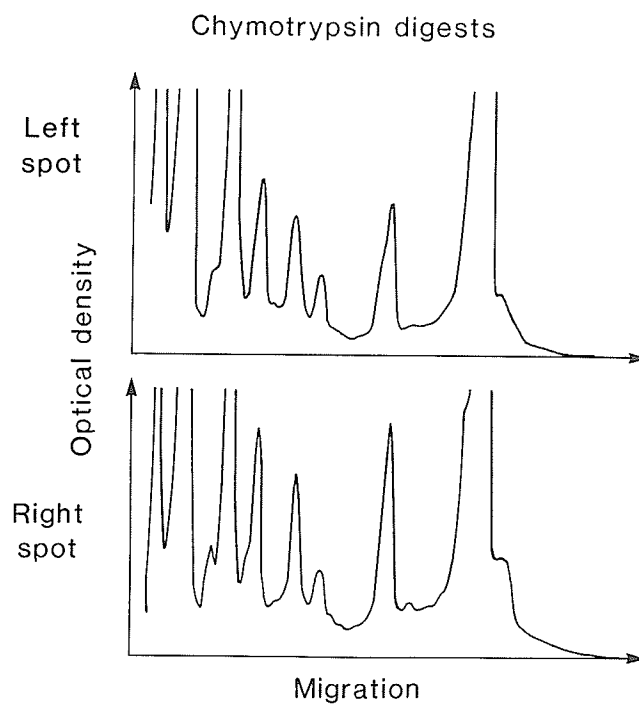


Figure 13. Peptide patterns after limited proteolysis of the 56 kDa/pI6.7 (right) and 56 kDa/pI6.5 (left) proteins with chymotrypsin resolution by SDS-PAGE. Shown are densitometric scans of silver stained peptide bands from the chymotrypsin digests.

homogenized and lipoproteins were extracted. The water soluble proteins were lyophilized and analyzed by 2D-PAGE. The gel was dried and exposed for autoradiography for 3 days (Fig. 14). Many proteins were phosphorylated but the 56 kDa protein was not found to be a phosphoprotein by this analysis.

2. Is the 56 kDa protein glycosylated?

Lectins with varying sugar specificities were used to stain glycoproteins on Western blots of GSF proteins. Fourteen different lectins were used on separate strips of blotted proteins separated by SDS-PAGE (Fig. 15). Only one lectin PHA-E from *Phaseolus vulgaris* detected glycoproteins in the molecular mass range of 50 - 60 kDa. This lectin was used to bind to a Western blot of GSF proteins fractionated by 2D-PAGE. The only glycoproteins stained were those with acidic isoelectric points (Fig. 16). Thus, the 56 kDa protein doublet is found not to be bound by 14 different lectins with a variety of specificities for sugar moieties.

3. Is the 56 kDa protein a heat shock protein?

Due to the abundance of the 56 kDa protein and the identification of heat shock proteins of similar molecular mass that associate with steroid receptor complexes (Tai *et al.*, 1986), its expression was analyzed for heat shock response. Cells were incubated at 37°C or 41°C for 4 h in the presence of 50 μ Ci each of [14 C]glycine and [14 C]leucine. Two cell strains were used, one which expressed the 56 kDa protein abundantly and one which did not express the protein as determined by silver staining. The proteins from these two cell strains were analyzed by 2D-PAGE and the gels treated for fluorography. Both fluorograms (Fig. 17 A and B) revealed several heat shock proteins with molecular sizes in the range of 90, 80, 70 and 30 kDa. These may represent some of the classical heat shock response proteins. However, the 56 kDa protein was not labeled, implying that it was not a heat shock protein.

4. Is the doublet due to an artifact of the method of analysis?

The 56 kDa protein pI 6.7 (right spot) was excised from Coomassie Blue stained two-dimensional gels and the protein eluted from the gel according to Hunkapillar *et al.* (1988). The resultant protein was re-run by 2D-PAGE and the gel stained with silver revealing the characteristic 56 kDa protein doublet (data not shown). This may indicate that the generation of the pI 6.5 (left spot) may be an artifact of the method of analysis. This is not without reason as the denaturing agent used in 2D-PAGE is urea at 8 - 9 M. This creates the possibility that urea degrades to cyanate

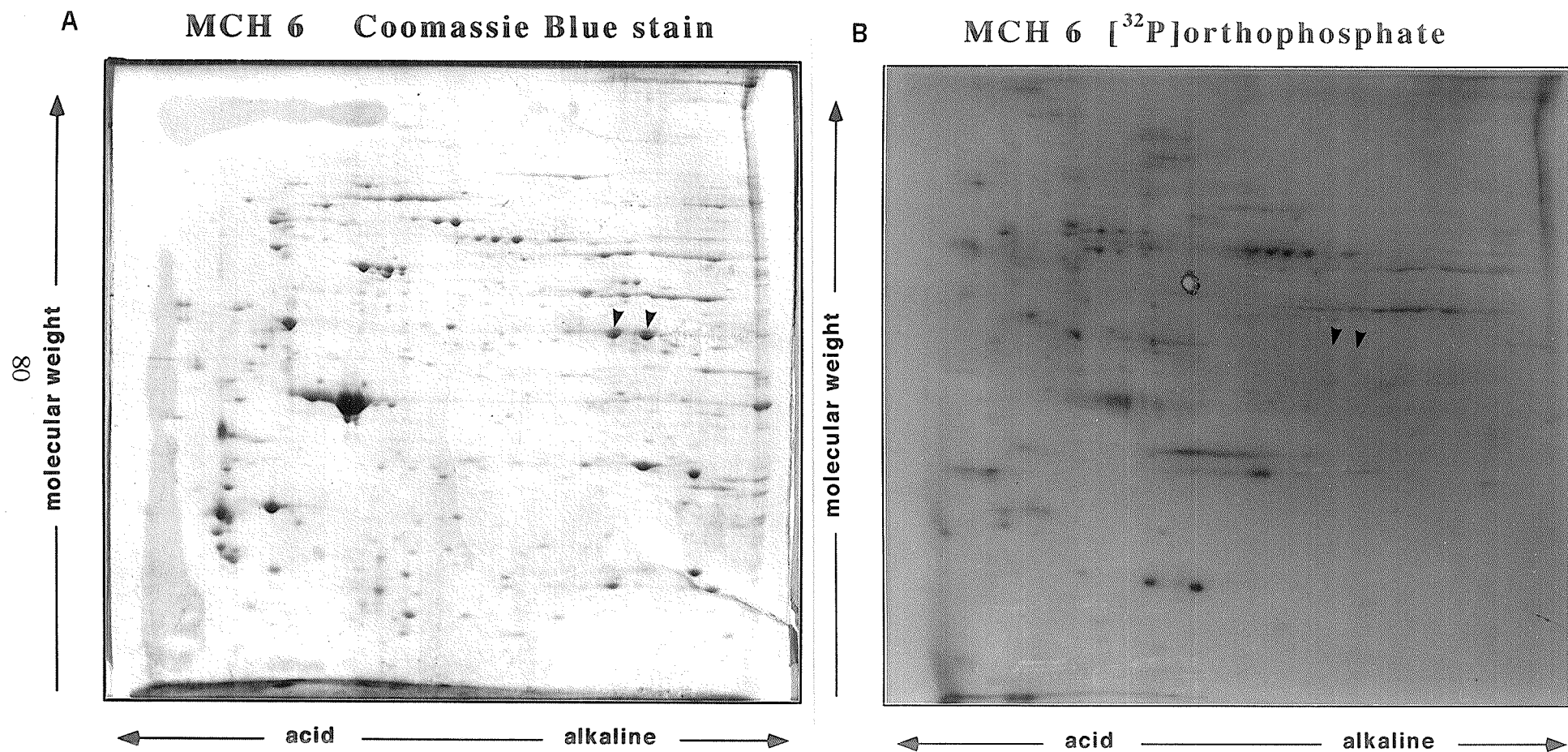


Figure 14. Phosphorylation analysis of genital skin fibroblast proteins. A. Coomassie Blue stained gel after 2D-PAGE of MCH 6 proteins labeled with [^{32}P]orthophosphate. B. autoradiogram of ^{32}P -labeled proteins from MCH 6.

MCH 6 lectin binding proteins

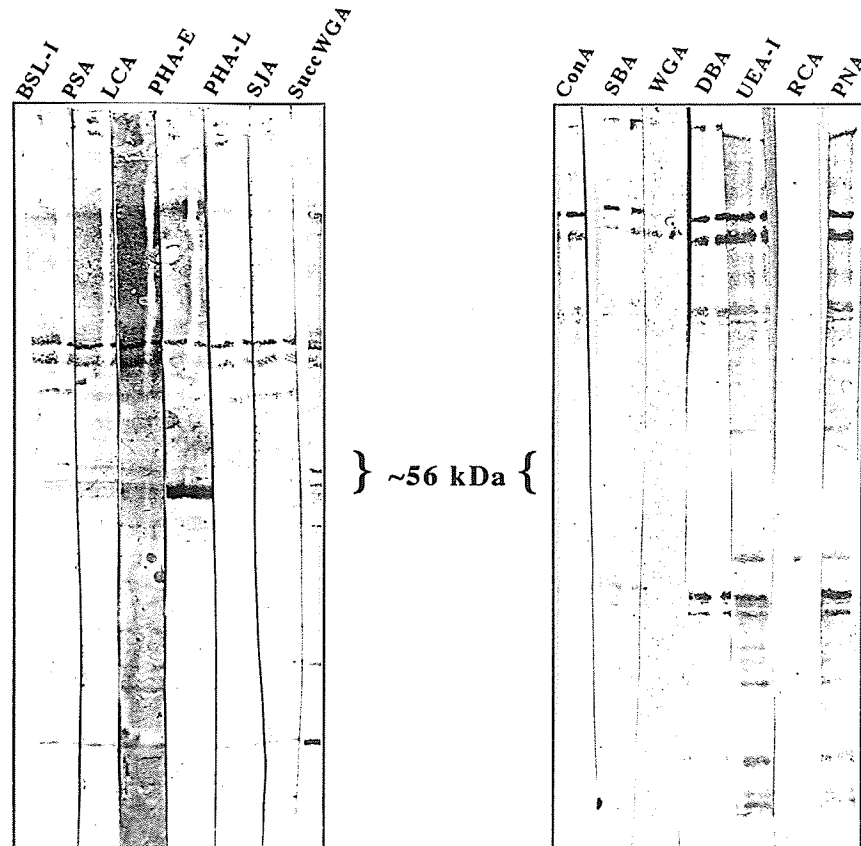


Figure 15. Analysis of lectin binding proteins in genital skin fibroblasts. SDS-PAGE immunoblots of genital skin fibroblast strain MCH 6 were probed with various lectins. Molecular weight markers were stained with india ink (not shown).

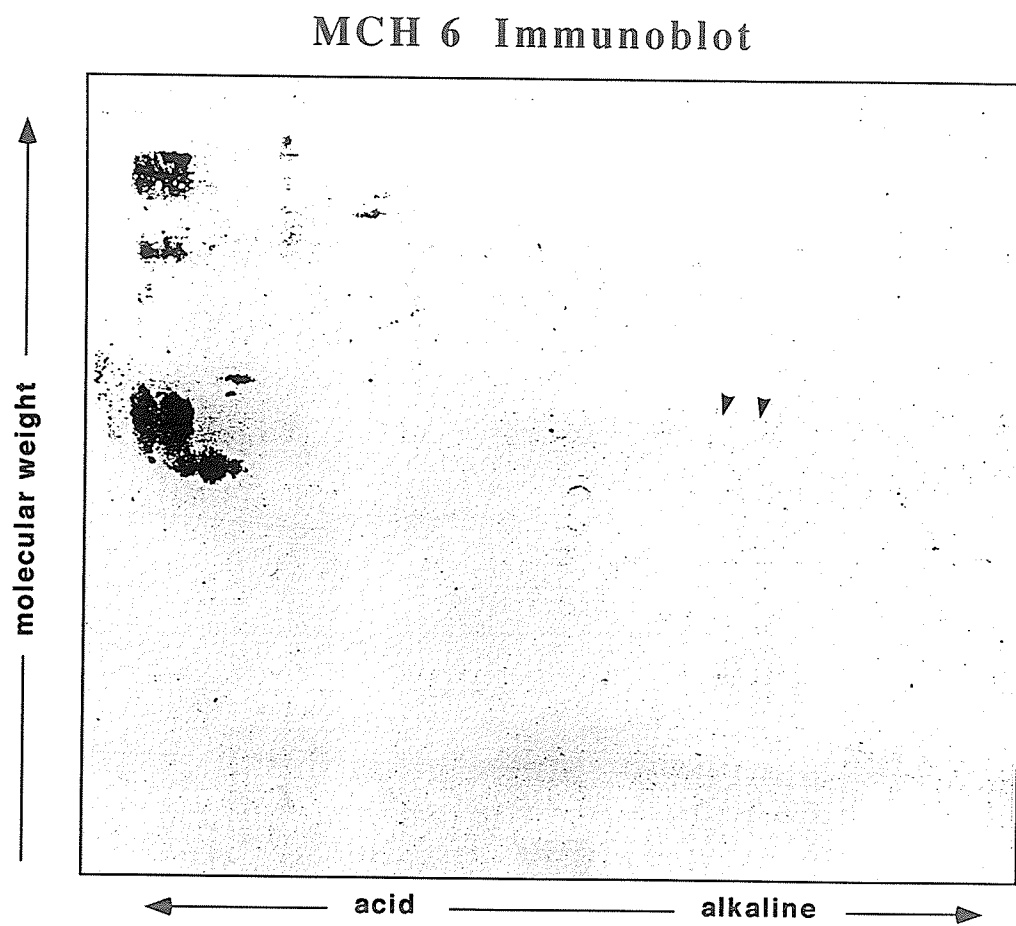


Figure 16. 2D-PAGE immunoblot of MCH 6 proteins detected with lectin PHA-E from *Phaseolus vulgaris*. Arrows mark the coordinates for the 56 kDa protein.

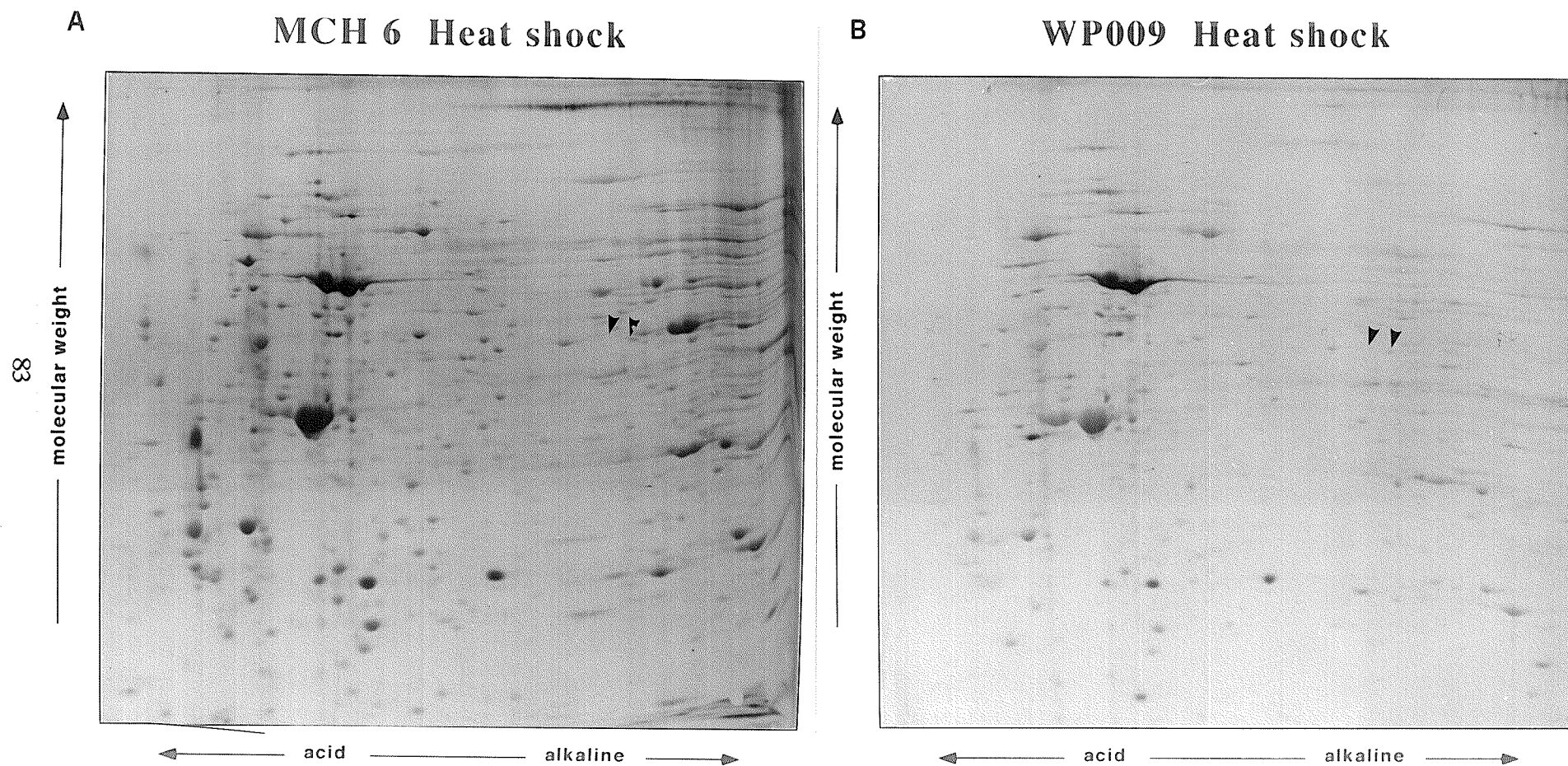


Figure 17. Analysis of heat shock proteins in skin fibroblasts. Proteins were labeled with ^{14}C -amino acids for 4 h at 41°C and fractionated by 2D-PAGE prior to fluorography. A. Genital skin fibroblasts strain MCH 6; B. Non-genital skin fibroblast strain WP009.

resulting in carbamylation of proteins. This seen as the characteristic “beads on a string” appearance of protein spots with identical molecular mass on two-dimensional protein maps (O’Farrell, 1975). This possibility was not pursued with further experimentation. The 56 kDa protein is usually seen as a doublet, and, at most, as a triplet (more left than the pI 6.5 left spot upon photolytic labeling and discussed later, but with apparently larger protein spots as molecular mass increases in the left spots).

However, it is known that ALDH 1 is N-terminally blocked by a post-translational modification resulting in removal of the first methionine and acetylation of the next serine (Hempel *et al.*, 1984). The 56 kDa protein doublet may represent the two species before and after modification and thus would not be an artifact. The result described here could be due to better focusing of the proteins from the isolated right pI 6.7 spot to the pI 6.5 spot position. Furthermore, the N-terminal block would account for the fact that several attempts at N-terminal peptide sequencing, for generating degenerate oligonucleotides for cloning purposes, had failed.

5. Is the 56 kDa protein abundance due to its stability?

GSF cells were incubated with [^{14}C]leucine for 36 h then the cells were washed and incubated in medium without radioactivity to “chase” the label for varying amounts of time. Over the period of a 4 h chase, no significant decrease in the amount of label in the 56 kDa protein spots was detected (Fig. 18). Other proteins had a significant decrease in label over the same time period. This would indicate that the 56 kDa protein was very stable with a half-life much longer than 4 h. The stability of the 56 kDa protein may allow sufficient accumulation to account for the abundance of the protein doublet.

C. Immunological analyses of the 56 kDa protein

Production of an antiserum was necessary to allow immunological analyses of the 56 kDa protein, but most importantly for use in expression screening of cDNA libraries to clone the cDNA for the 56 kDa protein. This became necessary when N-terminal peptide sequencing had failed and internal peptide sequencing of fragments generated by cyanogen-bromide cleavage at internal methionines required much more protein than we could purify by excising spots from 2D-gels. The glass fiber filter sequencing technology available at the time required at least 100 - 500 pmol of a 56 kDa protein, although today it can be done with 1 to 10 pmol of protein (Wadsworth *et al.* 1992).

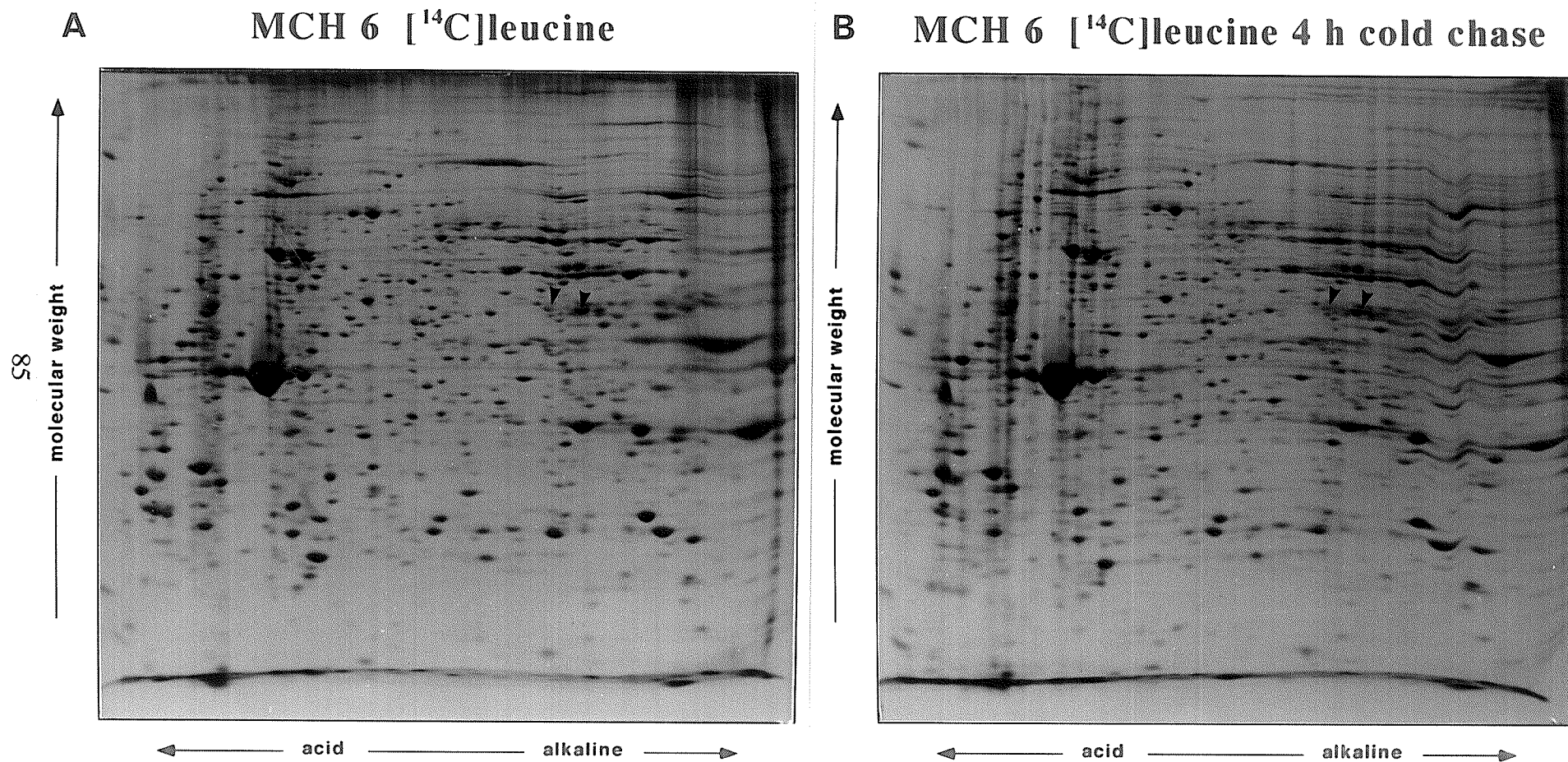


Figure 18. Labeling and chase of [^{14}C]leucine labeled 56 kDa protein in genital skin fibroblast stain MCH 6. Proteins were labeled for 36 h with [^{14}C]leucine and radiolabel chased with radioinert leucine medium for various lengths of time. A. autoradiogram after 36 h label; B. autoradiogram after 4 h chase of the radiolabel.

1. Generation of a polyclonal antiserum against the 56 kDa protein

Normal GSF strain MCH 6 was chosen for purification of the 56 kDa protein by 2D-PAGE because the spots were expressed at the 4+ abundant level and this strain grew relatively well (for the vast number of cells required). Due to the analytical nature of the technique, two hundred fractionations were performed, spots excised and pooled in order to collect enough spots. The section of 3 IEF gels corresponding to the approximate pI for the 56 kDa protein doublet was run into one SDS-PAGE gel (Fig. 19 B). The gel was briefly stained, using the method of Hunkapillar *et al.* (1988), and the 56 kDa protein spots excised and stored. The larger pI 6.7 'right spot' (Nickel *et al.* 1988) was used for immunizing a rabbit as it contained approximately 10 fold more protein than the pI 6.5 'left spot'. The immune response was tested on dot blots (data not shown) and on Western immunoblots of 2D-gels (Fig. 20). The blot shows that a dilution of 1:500 of the total rabbit serum was specific for the 56 kDa protein. This result also confirmed that the two spots were structurally related as this antiserum, against the pI 6.7 spot, detected the pI 6.5 (left spot) equally well.

2. Preliminary analysis of subcellular localization of the 56 kDa protein

The antiserum allowed the analysis of the subcellular localization of the 56 kDa protein to determine whether its location would be consistent with its function as a nuclear receptor. The antiserum was used on immunoblots of nuclei from MCH 6 separated by 2D-PAGE (Fig. 21). The results clearly showed that the 56 kDa protein did not appear in the nuclear fraction. However, some cross-reactive material with acidic isoelectric points were detected with the antiserum. The molecular mass of these proteins, 60 - 70 kDa and 100 - 110 kDa, fell into the sizes of proteins identified as the androgen receptor with molecular mass of 100 kDa (Johnson *et al.* 1982) and 60 kDa (Chang *et al.* 1982), and for proteins associated with steroid receptor complexes (Tai *et al.* 1986). This suggested a structural relationship of the 56 kDa protein with the androgen receptor, and more possibly with nuclear proteins.

3. Immunoprecipitation of *in vitro* translated proteins

Nickel *et al.* (1988) proposed that the abundance of the 56 kDa protein doublet was due to accumulation of a degradative product of a larger protein species, namely the androgen receptor. This idea had been used to explain the source of high affinity androgen-receptor proteins with similar molecular size and isoelectric points in steer seminal vesicle (Chang *et al.* 1982), rat (Brinkmann *et al.*, 1986) and human (Murthy *et al.* 1984) prostate, and human liver (Bannister *et*

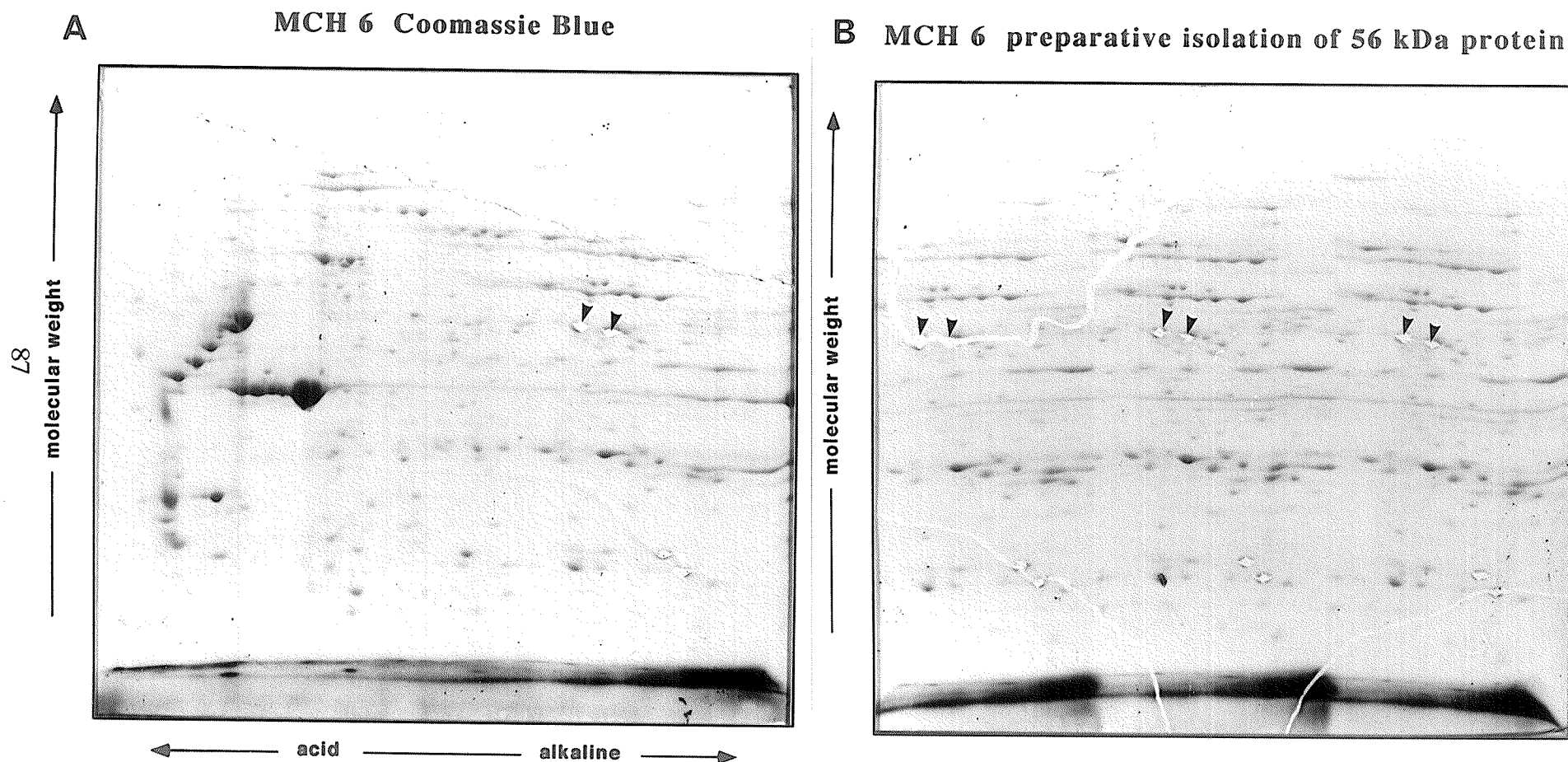


Figure 19. Preparative isolation of the 56 kDa protein. A. 2D-PAGE analysis of proteins from genital skin fibroblast strain MCH 6 B. The section of three isoelectric focusing tube gels corresponding to the approximate isoelectric point for the 56 kDa protein was run into one SDS gel. The gels were briefly stained with Coomassie Blue and the spots excised leaving the vacant spots at the coordinates where the 56 kDa protein doublet was present.

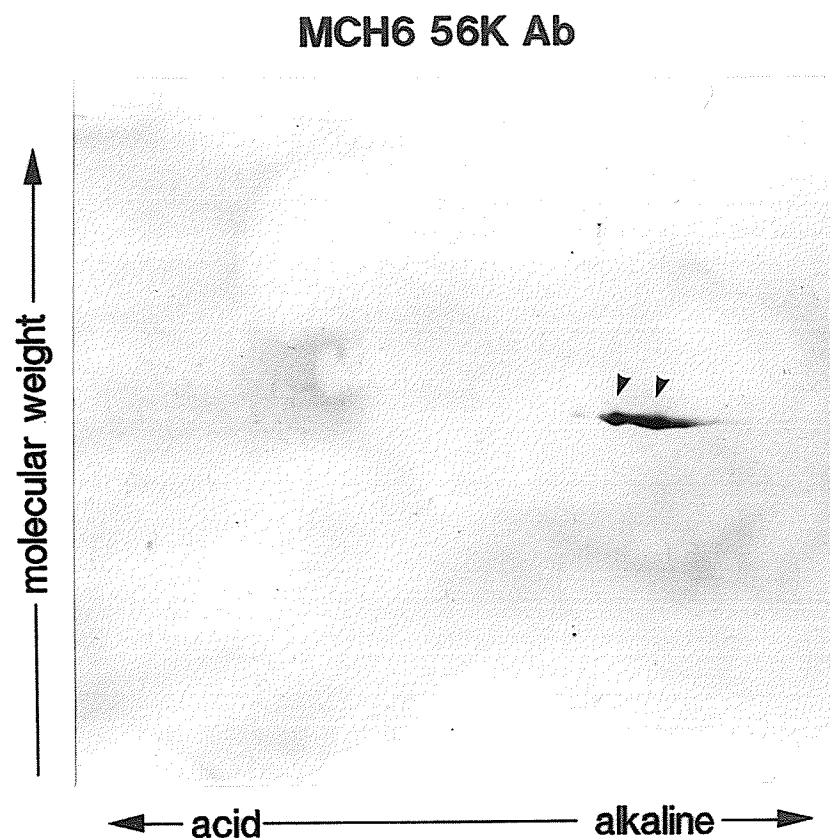


Figure 20. Immunoblot of MCH 6 cell lysate. Total cellular proteins were transferred from a two dimensional gel to nitrocellulose and reacted with the anti-56 kDa rabbit antiserum. The binding of specific antibodies were visualized by developing for alkaline phosphatase activity of the second antibody, goat anti-rabbit IgG alkaline phosphatase conjugate. The 56 kDa protein doublet is marked by arrows.

MCH6 Nuclei : 56K Ab + Leup + Pepst

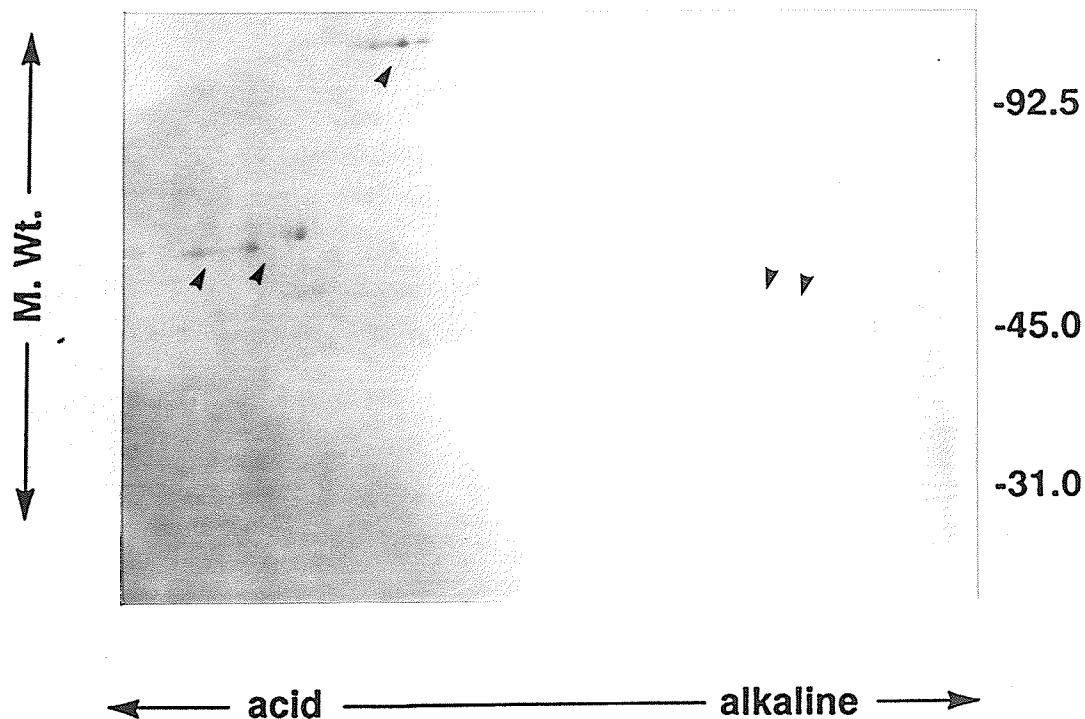


Figure 21. Immunoblot of nuclear proteins from genital skin fibroblast strain MCH 6. Nuclei were isolated from MCH 6, the proteins were separated by 2D-PAGE and electroblotted onto nitrocellulose. The blot was reacted with the anti-56 kDa antiserum.

al. 1985). To determine whether the 56 kDa protein was synthesized directly from its own mRNA or as a larger precursor protein which was subsequently degraded to a molecular mass of 56,000 daltons, its synthesis was tested by *in vitro* translation and immunoprecipitation of the translated products. A 2-D PAGE map of total translation products clearly revealed the 56 kDa protein doublet (Fig. 22 A). This was confirmed by immunoprecipitation using the antiserum and again analyzing the precipitated products by 2D-PAGE (Fig. 22 B). Thus, the 56 kDa protein was synthesized from its own mature mRNA species and not as a larger precursor protein.

4. Cross-reactivity and partial purification of antiserum

The antiserum showed some more cross-reactive material when a dilution of only 1:200 (Fig. 23) was used against proteins from MCH 6 on SDS-PAGE immunoblots. Bands from many molecular size ranges were detected with different intensities. Furthermore, serum from different bleedings had different specificities and affinities to these different bands. Glutamate dehydrogenase from beef liver (MW 53 K) was used as a molecular weight standard. This high degree of cross-reactivity would be detrimental to expression screening of cDNA libraries. An attempt was made to partially purify the antiserum to the 56 kDa protein away from the other antibodies to the cross-reactive material. The antiserum was diluted 1:200 and adsorbed to immunoblots of proteins from non-GSF strain WP009 which did not express the 56 kDa protein but was of fibroblast origin. Two successive cycles of adsorptions were performed on one serum stock (labeled 17.X) as it had been judged to have the least cross-reactive material (Fig. 23). The resultant antiserum was diluted 1:500 and used against a composite Western immunoblot consisting of a strip of SDS-PAGE gel and a 2D-PAGE gel of proteins from MCH 6 (Fig. 24). The blot shows that the antiserum specifically detects the 56 kDa doublet on the 2D-gel and a band of identical molecular size on the SDS-gel but also a little cross-reactive material (MW ~70 K). This antiserum was used for screening of cDNA expression libraries.

D. Relationship of the 56 kDa protein to the AR

Nickel *et al.* (1988) reported that the presence or absence of the 6.7 pI/ 56 kDa protein in GSF could serve as a new marker of the genetic heterogeneity within the class of complete androgen insensitivity patients. A close structural and/or functional relationship with the classical AR was also postulated because the protein was not found in GSF from ten of 12 unrelated subjects with receptor-negative complete androgen insensitivity. However, the abundant presence of the protein in the GSF of two subjects with complete insensitivity and receptor-binding negative suggested that it was not androgen-induced. Furthermore, they were able to show that the 56 kDa protein

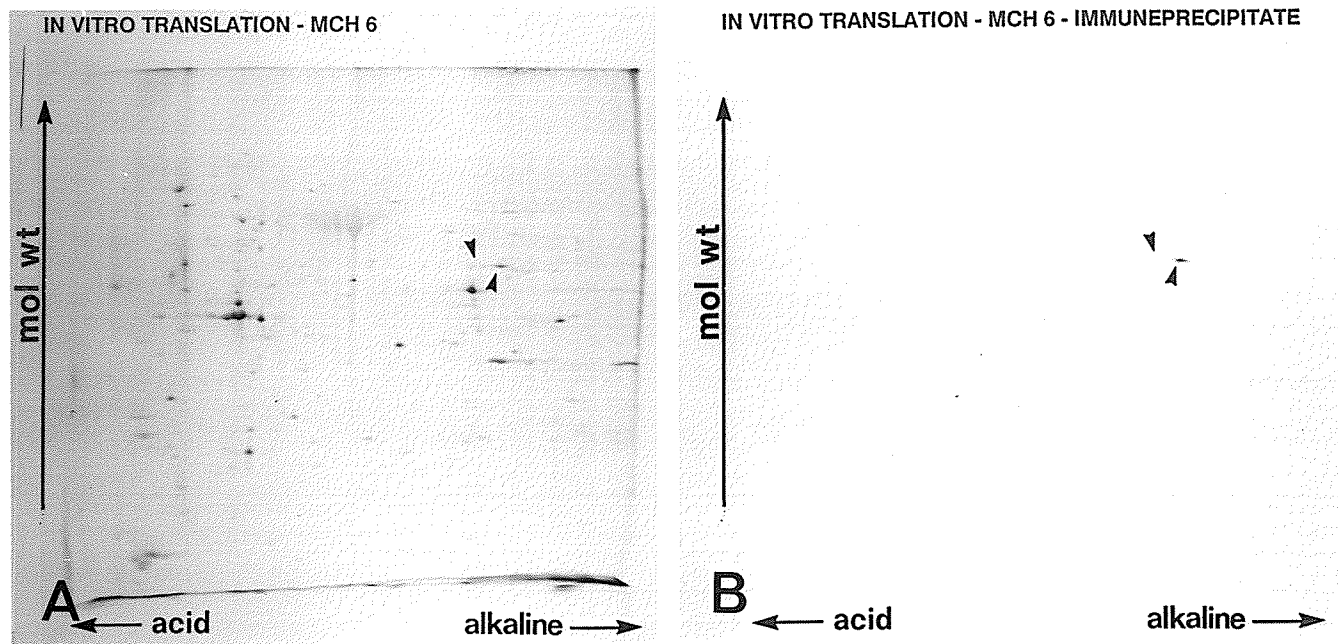


Figure 22. *In vitro* translation of mRNA isolated from genital akin fibroblasts strain MCH 6. A: Autoradiogram of total translation products labeled with [^{35}S]methionine and separated on 2D-PAGE. B: Autoradiogram of immunoprecipitate of the translation products separated by 2D-PAGE. Arrowheads point to the 56 kDa protein doublets.

MCH 6 immunoblot: antisera showing cross-reactivity

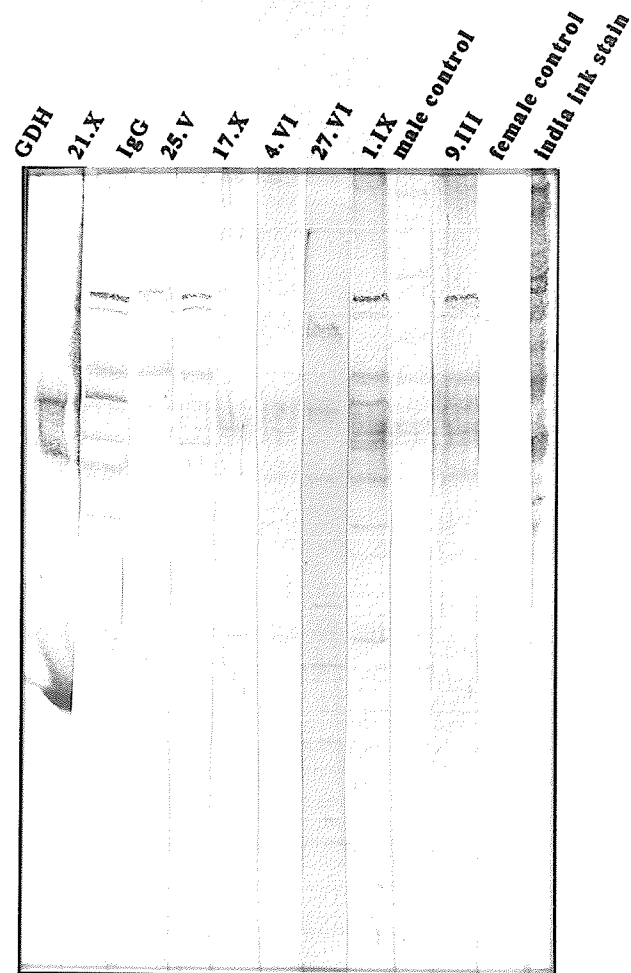


Figure 23. Cross-reactivity of the anti-56 kDa antiserum to proteins from genital skin fibroblasts. Strips of SDS-PAGE immunoblots of proteins from strain MCH 6 detected with a 1:200 dilution of the anti-56 kDa antiserum from different bleedings of the rabbit. Glutamate dehydrogenase (GDH from beef liver, 53 kDa) is used as standard.

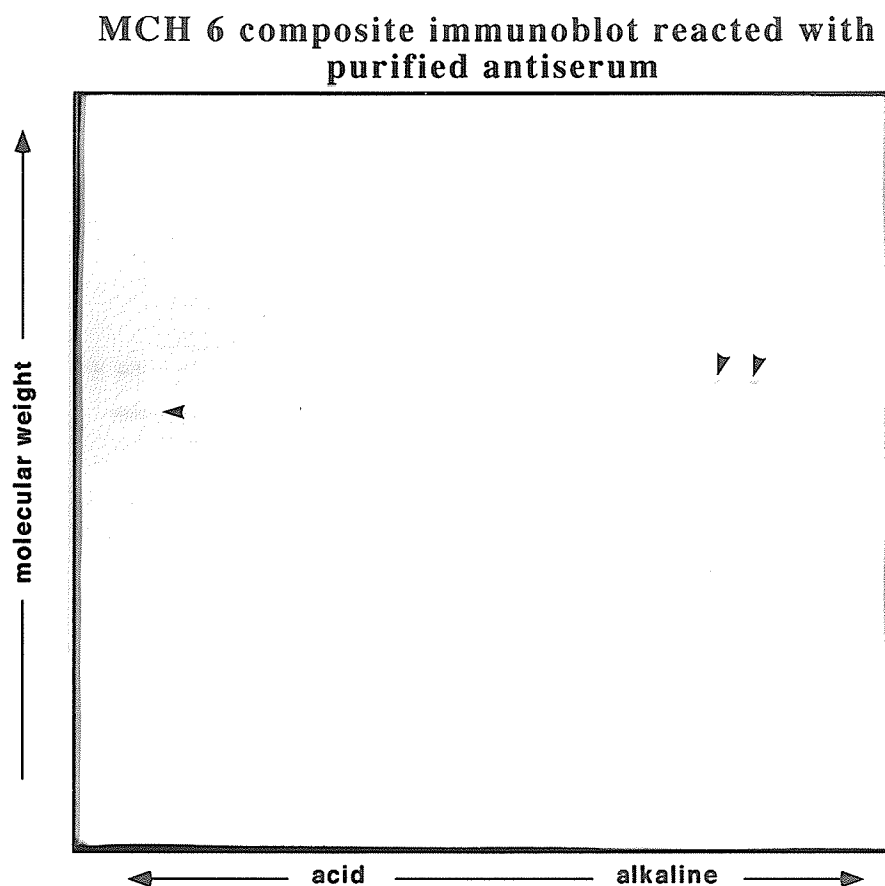


Figure 24. Partial purification of the anti-56 kDa antiserum. The antiserum was successively adsorbed onto immunoblots of proteins from a non-genital skin fibroblast strain WPOO9, which does not express the 56 kDa protein. The partially purified antiserum was then used to detect proteins from a composite immunoblot of a strip of SDS-gel and a 2D-gel of proteins from genital skin fibroblast strain MCH 6. Arrows mark the 56 kDa doublet and the 56 kDa band.

could be photolytically labeled with 50 nM [^3H]MT. This finding was compatible with the notion that the 56 kDa protein had a common structural or genetic ancestry with the AR (Kovacs, Turney, 1988; Nickel *et al.* 1988). This relationship was the subject of further analyses.

1. Covalent photoaffinity labeling of the 56 kDa protein with an androgen analog

The photoligand methyltrienolone was used to label GSF proteins in order to determine whether the 56 kDa protein could bind androgens with high-affinity. GSF were incubated with 8 nM [^3H]MT for 48 h followed by photolysis, 2D-PAGE and treatment of the gel for fluorography. After exposure for 30 days, only two faint spots appeared on the fluorogram and these were superimposed over the 56 kDa doublet (data not shown). In addition, re-exposure of the fluorogram for 253 days still resulted in the same two spots which again superimposed on the 56 kDa proteins of pI 6.5 and 6.7 which had been stained with Coomassie Blue (Fig. 25). Thus, the 56 kDa protein can selectively bind MT, but apparently with a lower binding affinity than that of the classical AR ($K_d \sim 1$ nM) as determined by Scatchard analysis.

2. Specificity of androgen binding to the 56 kDa protein

The faint signal and long exposure time of the fluorogram in the original MT labeling experiment (Fig. 25) was difficult to reproduce. However, the same result was obtained after exposure of the fluorogram for only 33 days when the MT concentration was raised to 50 nM (Fig. 26 A). Labeling of the spots was suppressed in the presence of a 200-fold molar excess of radioinert MT (Fig. 26 B). Furthermore, overexposure of these same two gels for 201 days revealed that the two 56 kDa spots are labeled very strongly and there was weak non-specific labeling of other abundant proteins (Fig. 26 C). The excess radioinert MT clearly suppresses the specific labeling of the 56 kDa proteins but incompletely (Fig. 26 D). The non-specific labeling of other proteins is not suppressed. However, the overexposed fluorograms reveal two additional specifically labeled spots of less abundance and of approximately 110 kDa (small arrows, Fig. 26 C, D) which could represent the classical AR itself (unfortunately, this result was not reproducible). Inclusion of a 500-fold molar excess of the glucocorticoid triamcinolone acetonide (an anti-androgen which would interfere with binding to the classical androgen receptor), did not alter the labeling of the 56 kDa protein with MT (data not shown). Thus, photolytic labeling with the androgen analog MT is specific and selective for the 56 kDa protein in preference over all other proteins in genital skin fibroblasts' cytosol.

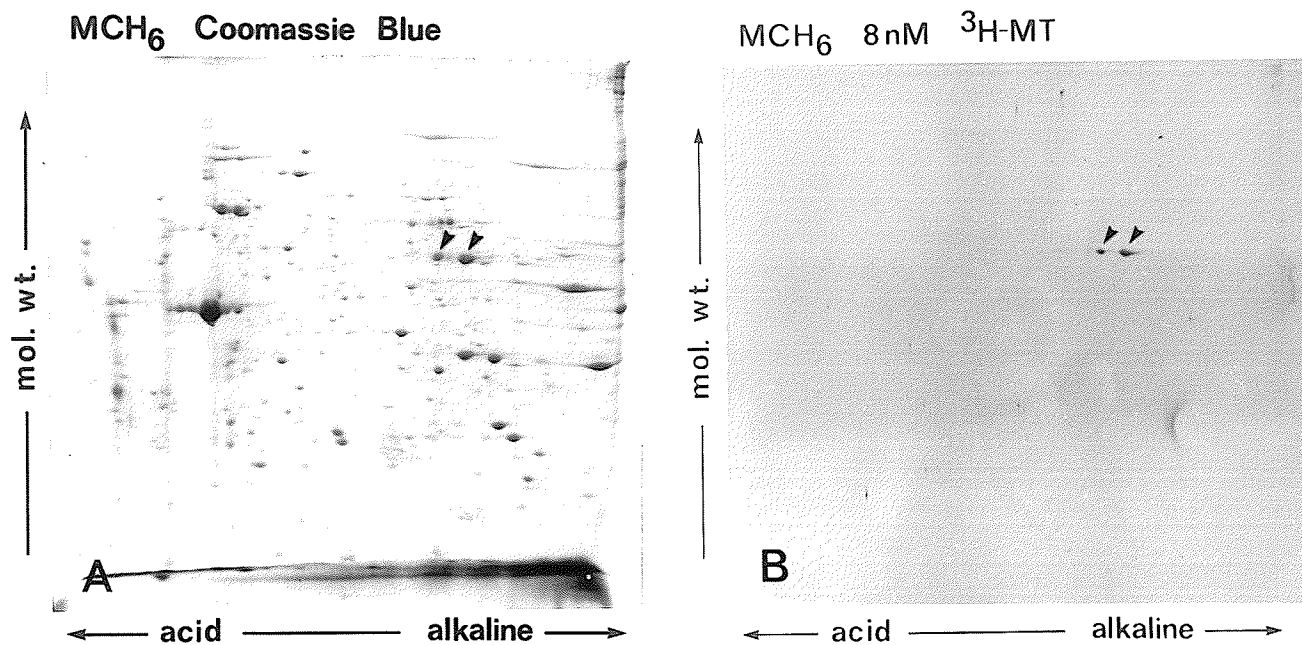


Figure 25. Protein maps of genital skin fibroblasts, strain MCH 6 and identification of proteins which have been covalently labeled with [³H]MT after photolysis. A: Coomassie Blue stained gel, B: Fluorogram exposed for 253 days at -75°C after cells were incubated for 48h in 8nM [³H]MT and photolysed. Arrows mark the 56 kDa Doublet (from Wrogemann et.al. 1988).

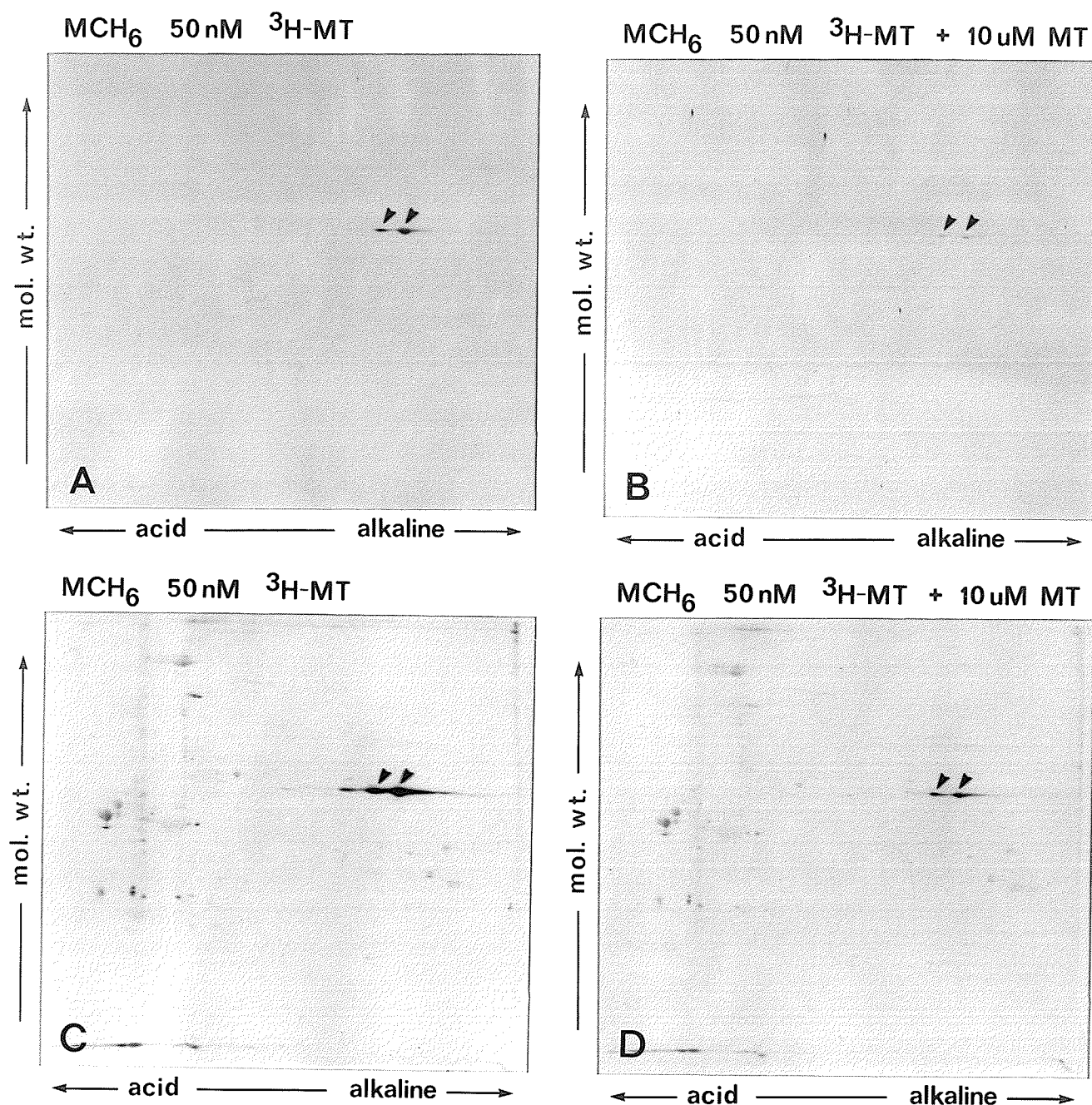


Figure 26. Specific and non-specific labeling of proteins by photolysis of strain MCH 6 genital skin fibroblasts. Proteins from strain MCH 6 were photolysed with 50 nM ^3H -MT in A and C; or with 50 nM ^3H -MT $\pm$ 200-fold excess radioinert MT in B and D. Exposure: 33 Days in A and B; 201 days in C and D. Separation by 2D-PAGE and overexposure of fluorograms allow clear distinction between specifically labeled proteins (completed by excess cold ligand, arrows) and non-specifically labeled proteins (unsaturable with ligand, all other spots in C and D) (from Wrogemann et.al. 1988).

3. Competition of MT labeling with other steroids

Competition of the MT binding to the 56 kDa protein with other steroids may reveal the affinity of androgen-binding to the 56 kDa protein. A hierarchy of affinity for steroids, similar to that reported for the AR (Keenan *et al.* 1984), was found for competition of the MT photolytic labeling of the 56 kDa protein. Dihydrotestosterone was identified as the most potent competitor and hydrocortisone the least (Table 7). The labeling efficiency was determined by scintillation counting of gel spots excised from 2D-gels. This covalent labeling efficiency was calculated to be approximately 0.125 % assuming that one molecule of ligand would bind per molecule of 56 kDa protein. This would account for the long exposures required for the fluorograms and possibly reflects the instability of the photoligand under ultraviolet light and may not reflect the true affinity of the ligand for the 56 kDa protein.

4. Photolytic labeling of GSF from subjects with CAIS due to androgen receptor defects

Nickel *et al.* (1988) had characterized the 56 kDa protein in GSF from several subjects with CAIS due to negligible androgen receptor binding activity. The GSF from some of these patients were subjected to photolytic labeling with MT to determine whether the MT labeling could represent the labeling of the classical high-affinity low capacity mature androgen receptor. Nine cell strains were found not to express the 56 kDa protein and exhibited no specific MT labeling by photolysis (Table 8). However, strain TRL, which lacked AR binding activity (Kaufman *et al.* 1976) but expressed the 56 kDa protein (Nickel *et al.* 1988), could be labeled with [3 H]MT (Fig. 27 A). Additionally, this specific labeling was found only in the 56 kDa protein and could be suppressed with a 200-fold molar excess of radioinert ligand (Fig. 27 B). Conversely, cell strain KIL, which has close-to-normal AR binding activity but no 56 kDa protein (Nickel *et al.* 1988), did not have any specifically labeled proteins even when using 50 nM photolytic ligand (data not shown). Thus, while the 56 kDa protein expression and MT labeling generally correlate with the high affinity AR binding activity (Table 8), analysis of the latter two cell strains indicate that the photolytic labeling of the 56 kDa protein was not a reflection of the classical high-affinity low-capacity binding activity. This was in contrast to that reported by Kovacs and Turney (1988). They found under native conditions, that a 58 kDa protein, labeled with [3 H]dihydrotestosterone bromoacetate, was indistinguishable from the native [3 H]DHT-labeled androgen receptor. This 58 kDa protein, was subsequently shown to be identical to the 56 kDa protein. These two observations were contradictory and required more analysis of the characteristics of the 56 kDa protein in the context of an androgen receptor.

Table 7. Competition by other steroids of the photolytic labeling of the 56 kDa protein with [^3H]MT.

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

Cells of strain MCH 6 were incubated for 48 h with 50 nM [^3H]MT in the absence (control) or presence of a 500-fold molar excess of radioinert competing steroids. The cells were photolysed, fractionated by 2D-PAGE, exposed for fluorography, and the peak label intensity over the 56 kDa protein quantified by densitometric scanning of the X-ray film.

Table 8. Androgen receptor (AR), the 56 kDa protein status, and photolytic labeling of genital skin fibroblasts from patients with complete androgen insensitivity syndrome due to receptor defects and normal controls.

Cell Strain	AR-Binding (fmol/mg protein)	56 kDa Protein Status*	MT photolytic labeling	Number of analyses for protein status
598	5	-	-	4
2379	5	-	-	8
6779	1	-	-	8
8481	1	-	-	7
31082	2	-	-	5
A5879	2	-	-	18
CVL	0	-	-	8
JRL	0	-	-	13
LEL	1	-	-	4
TML	1	-	-	6
NHL	1	4+	+	11
TRL	1	3+	+	10
30284	17	-	-	4
KIL	11	-	-	4
DB	1	-	+	15
8812	0	1+	+	10
Normal Controls				
MCH6	24	4+	+	>100
MCH49	24	3+	+	>25

Spot status as determined on silver stained 2D-gels.

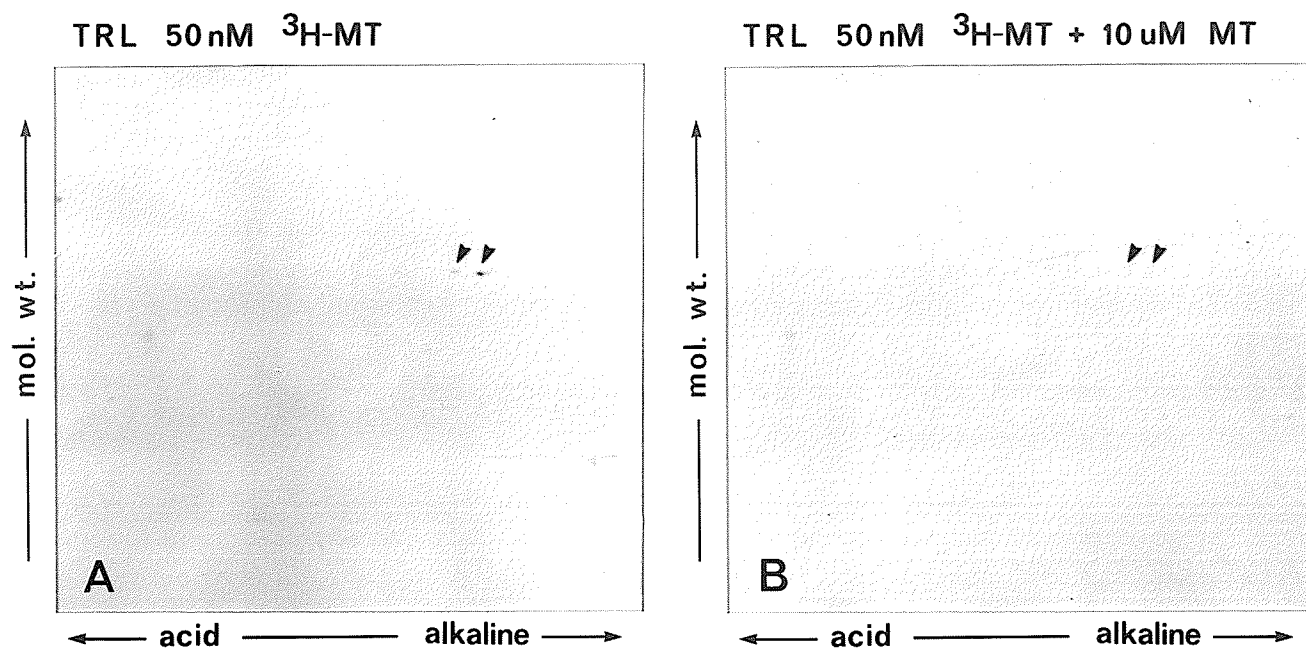


Figure 27. Photoaffinity labeling of genital skin fibroblast strain TRL. Strain TRL from a patient with complete androgen insensitivity is receptor binding negative and 56 kDa protein positive. Photoaffinity labeling conditions were as in Figure 20 A and 20 B. The exposure time was 112 days (from Wrogemann *et.al.* 1988).

5. Labeling during "upregulation" conditions

Preincubation of GSF with MT for 24 h or more caused more than a doubling of the MT-receptor activity over basal (1 h) levels. This was termed "upregulation" of AR-binding activity (Pinsky *et al.* 1983; Kaufman *et al.* 1981). To test whether this in turn resulted in an increase in covalent binding of ligand to the 56 kDa protein, GSF were incubated with [3 H]MT from 2 h (no "upregulation") to 48 h (maximum "upregulation") (Fig. 28). The results strongly indicate that subjecting the cells to photolysis after 2 h of incubation with MT results in maximal labeling of the 56 kDa protein. There is no increase in MT labeling of the 56 kDa protein at "maximal upregulation" of the AR and in fact, the amount of labeling actually decreased. The result clearly showed that the 56 kDa protein was not "upregulated" like the AR. Furthermore, the expression of the 56 kDa protein has not been found to be influenced by androgens after incubation of GSF and non-GSF with testosterone or DHT in the presence and absence of the anti-androgen cyproterone acetate (Nickel *et al.* 1988; Wrogemann *et al.* 1988). Thus, the 56 kDa protein does not appear to be induced by androgens in GSF or non-GSF cells. However, the 56 kDa protein has never been tested at the transcriptional level by more specific experimentation.

6. Affinity of androgen for photolytic labeling of the 56 kDa protein

Conventional charcoal binding studies and Scatchard plot analyses of GSF do not reveal a low-affinity large-capacity component which could represent the 56 kDa protein. Presumably, this was because the ligand has a higher affinity for charcoal than for the 56 kDa protein. Mibolerone, another synthetic androgen analog (Bannister *et al.* 1988), was found to label specifically the 56 kDa protein with almost 10 times better efficiency than with MT (Fig. 29). Fluorograms need only be exposed for as little as 2 days to visualize the labeled 56 kDa proteins. Furthermore, labeling with MB is sufficiently specific and selective for the 56 kDa protein that the analysis can be done by one-dimensional SDS-PAGE. This allowed for the determination of the concentrations of ligand required to saturate the 56 kDa protein with androgen under conditions of photolytic labeling. At 2 nM MB, which would saturate the classical AR (Gottlieb *et al.* 1987), labeling of the 56 kDa protein is observed but competition with a 200-fold molar excess of radioinert MB does not suppress the labeling (Fig. 30). This may reflect a larger capacity of sites for binding than one observes for the high-affinity low-capacity AR. Densitometric analysis of this fluorogram at different exposure times, to avoid film saturation, revealed that half-maximal saturation was only achieved at approximately 4 μ M MB (Fig. 31, plotted as a saturation plot (and not a Sactchard) because no analysis of the free vs bound counts were determined). This was in accord with our previous observation using methyltrienolone. Because ligand molecules and receptor proteins may

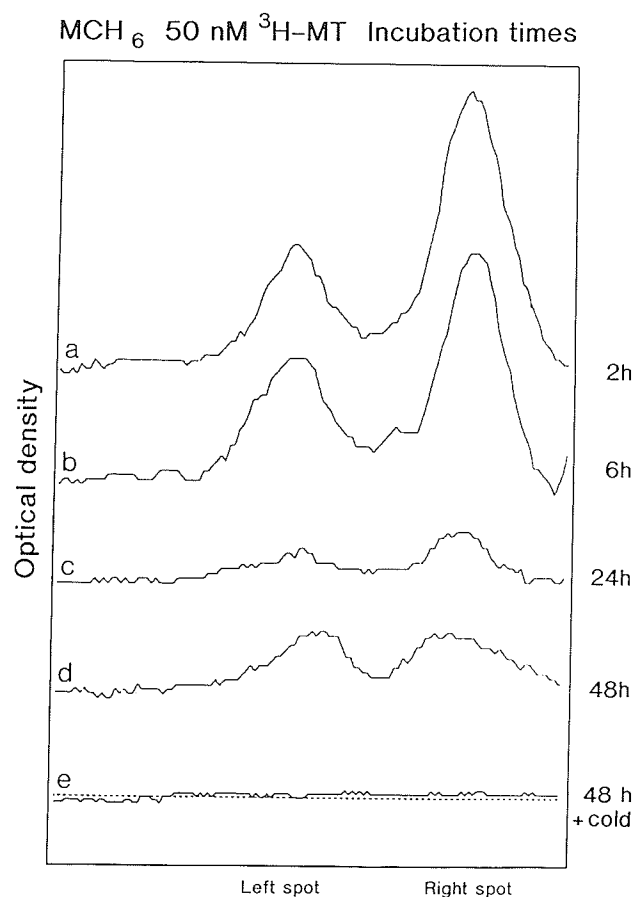


Figure 28. Effect of incubation with ligand on photoaffinity labeling of proteins from genital skin fibroblast strain MCH 6. Densitometric scans of fluorograms. Other conditions as in Figure 20A, except in a: 2 h of incubation with 50 nM [³H]MT; b: 6 h incubation; c: 24 h incubation; d: 48 h incubation; E: 48 h incubation with inclusion of 200-fold excess radioinert MT. The exposure time was for 9 days (from Wrogemann et.al. 1988).

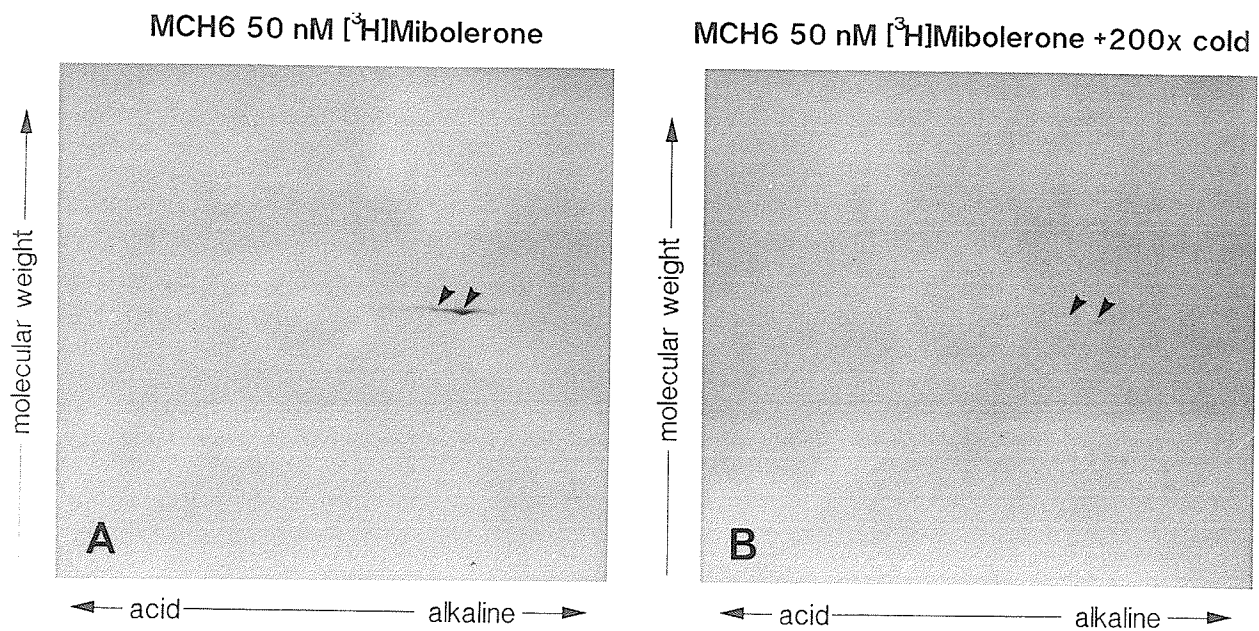


Figure 29. Photoaffinity labeling with [3 H]mibolerone of strain MCH 6 genital skin fibroblasts. A: Cells labeled *in situ* with 50 nM [3 H]mibolerone, separated by 2D-PAGE and exposed by fluorography. B: As in A, but with the inclusion of 200-fold excess of radioinert mibolerone. The exposure time was for 44 h. Arrowheads point to the positions of the 56 kDa protein doublets (from Pereira et.al. 1990).

56K [³H]Mibolerone Labelled

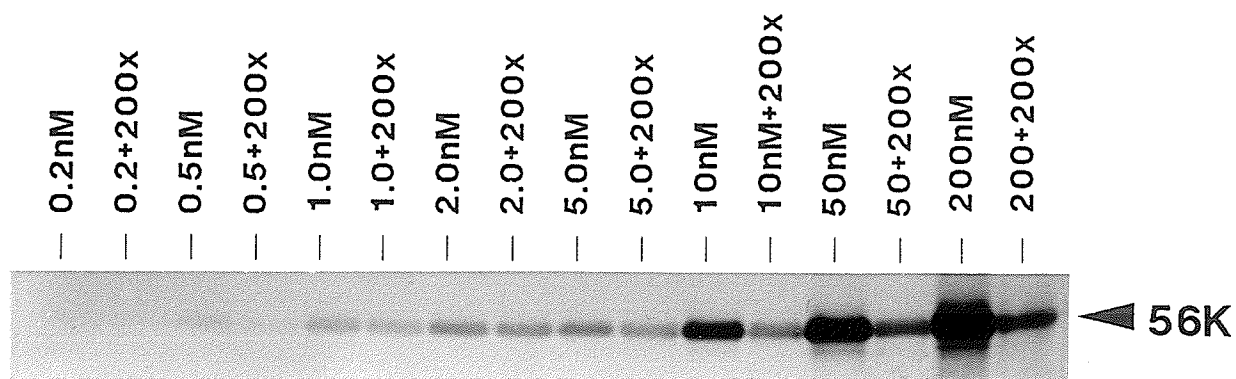


Figure 30. Effect of mibolerone concentration on covalent labeling of the 56 kDa protein by photolysis. MCH 6 genital skin fibroblasts were labeled by photolysis *in situ* with increasing concentrations of [³H]mibolerone ± a 200-fold molar excess of radioinert ligand, followed by SDS-gel electrophoresis and fluorography (from Pereira et.al. 1990).

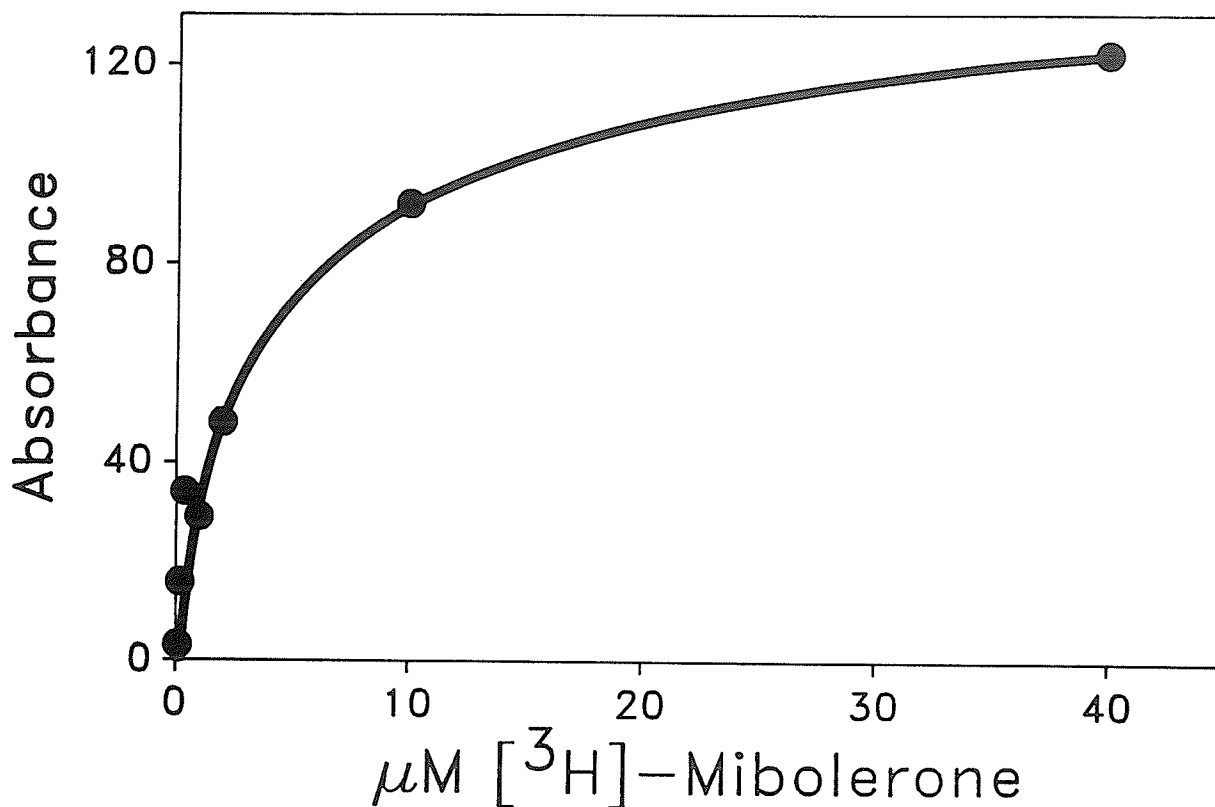


Figure 31. Saturation curve for the covalent labeling of the 56 kDa protein with [^3H]mibolerone. Densitometric scans of absorbancy peaks of the fluorogram of Figure 24 expressed in arbitrary absorption units. Only lanes with 200-fold molar excess of radioinert mibolerone were scanned for this graph. Half-maximal saturation is reached at approximately 4 μM mibolerone (from Pereira et.al. 1990).

degenerate during the 10 min exposure to ultra-violet light, it is possible that the photolytic covalent labeling underestimates the true affinity for the ligand. However, if the affinity of this protein was identical to that of the androgen receptor, binding studies should have revealed a larger binding capacity than the 38 fmol/mg protein observed by non-covalent labeling in cell strain MCH 6. Similarly, if binding affinity was only somewhat lower, Scatchard analysis should have revealed a second component of lower affinity in genital skin fibroblasts; this is not observed at concentrations up to 40 nM (Keenan *et al.* 1984). Hence, most of the excess androgen-binding activity measured in genital skin fibroblasts by photolytic labeling must represent binding to a very low affinity androgen-binding protein that is not recognized by non-covalent dihydrotestosterone labeling. This may reflect a larger capacity of sites for steroid binding to the 56 kDa protein and clearly distances itself from the high-affinity low-capacity androgen-binding ability of the classical androgen receptor.

7. Identity of the 56 kDa protein with the 58 kDa protein labeled with DHTBrAc

In separate studies, the compound DHTBrAc had been reported to covalently bind to androgen receptors from several species (Chang *et al.* 1982, 1984). This ligand has also been reported to bind with relatively high affinity to a putative androgen receptor from human GSF (Kovacs and Turney, 1988). The radioactive ligand covalently labeled a single 58 kDa protein that was absent from normal non-GSF or GSF from a subject with 'receptor-negative' androgen insensitivity. Furthermore, under non-denaturing conditions, the 58 kDa protein which was covalently radiolabeled by DHTBrAc had been found to be indistinguishable from the authentic high affinity [³H]DHT labeled androgen receptor on the basis of Stokes' radius, sedimentation coefficient, isoelectric point (as determined by chromatofocusing) or hydrophobicity (Kovacs *et al.* 1989). Kovacs *et al.* (1989) concluded that the 58 kDa protein could represent the accumulation of a smaller (precursor or degradative) form of the AR. Thus, a comparison of the 56 kDa protein and the 58 kDa protein was necessary. A preparation of the 58 kDa protein, covalently labeled by [³H]DHTBrAc, was found to migrate identically to the 56 kDa protein, which was photolytically radiolabeled with [³H]MB, when fractionated by SDS-PAGE (Fig. 32). Furthermore, the antiserum towards the 56 kDa protein was used on Western immunoblots of 2D-PAGE fractionations of the 58 kDa protein radiolabeled by [³H]DHTBrAc. The 58 kDa protein was found to react to the antiserum and to migrate to the same coordinates as the 56 kDa protein doublet (Fig. 33 A & B). The Western immunoblots were treated for fluorography and exposed to X-ray film. The resultant fluorogram revealed two spots which perfectly superimposed over the spots

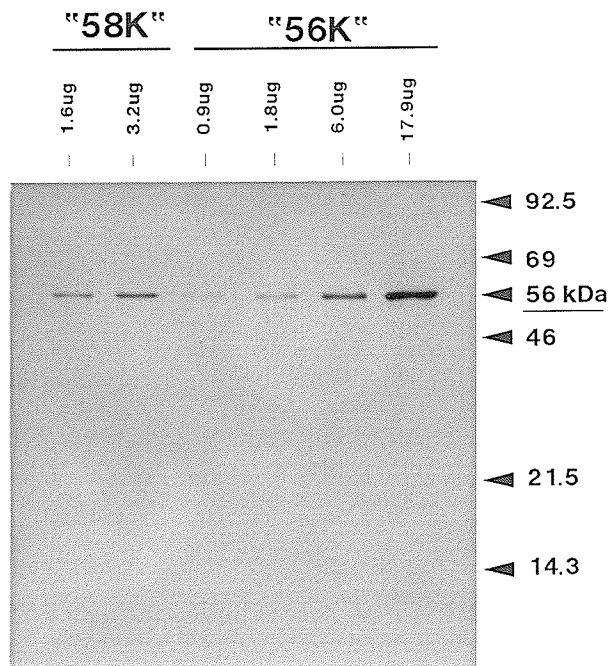


Figure 32. Fluorogram of SDS-gel electrophoresis of a normal genital skin fibroblast preparation labeled with 5 nM [^3H]DHTBrAc (lanes 1-2, marked "58 K") and of a normal genital skin fibroblast cell lysate labeled with 50 nM [^3H]mibolerone (lanes 3-6, marked "56 K"). The 56 kDa band is marked (from Belsham et.al., 1989).

Comparison of 58K and 56K proteins

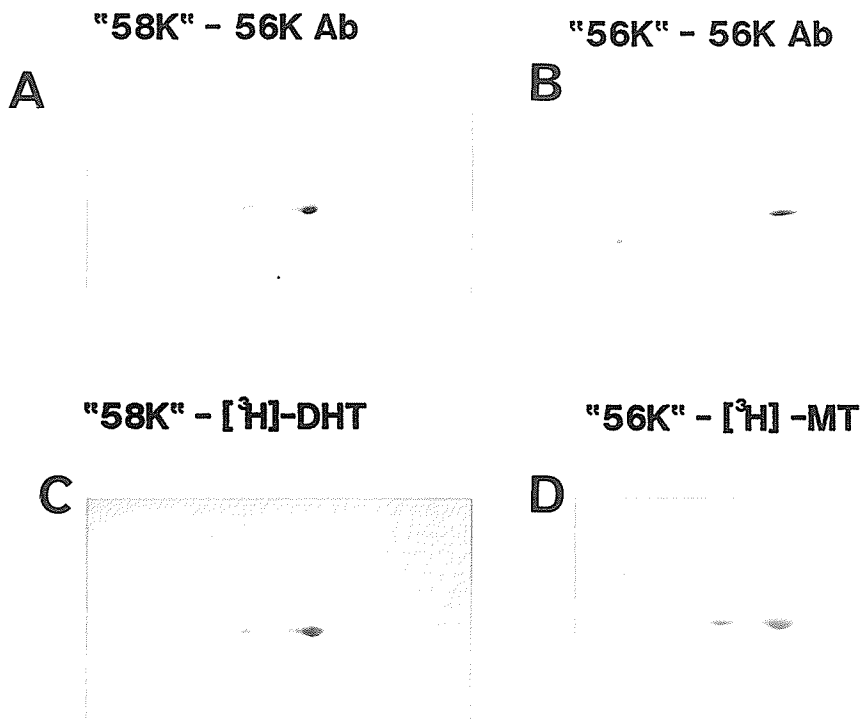


Figure 33. Two-dimensional gel electrophoresis, immunoblots and fluorography of [³H]DHTBrAc labeled or [³H]methyltrienolone labeled human genital skin fibroblast proteins. A: Immunoblot of "58 kDa" [³H]DHTBrAc labeled cell extract reacted with anti 56 kDa antiserum. B: Immunoblot of strain MCH6 genital skin fibroblasts reacted with anti 56 kDa antiserum. C: Fluorogram of the immunoblot shown in A. D: Fluorogram of strain MCH 6 genital skin fibroblasts labeled with [³H]methyltrienolone (from Belsham et.al. 1989).

detected by the antiserum in both the 58 kDa and in the 56 kDa Western immunoblots (Fig. 33 C & D). It was concluded that the 56/58 kDa proteins were indistinguishable.

Thus, the findings of the two different laboratories regarding the characteristics of the same protein were diametrically in opposition. The resolution of these differences would require additional evidence on the origin of the protein.

Another protein with estimated molecular weight 59 K had also been found to be associated with several steroid hormone receptor complexes but has no apparent ligand binding activity (Tai *et al.* 1986, Koutsilieris *et al.* 1988). This protein was found to have a pI of 5.4 and to be clearly different from the 56/58 kDa protein by 2D-PAGE. Furthermore, antibodies against the 59 K protein did not recognize the 56 kDa species nor did the antiserum against the 56 kDa protein recognize the 59 K species (data not shown).

8. Presence of 56 kDa protein in GSF of a patient with complete deletion of AR gene

In the course of screening subjects from 27 families with CAIS by a variety of molecular and genetic methods, Pinsky *et al.* (1989) discovered a patient with a complete deletion of the AR coding region. This allowed us to study the 56 kDa protein in the absence of any AR. GSF from this patient were analyzed for the expression of the 56 kDa protein. GSF from this patient were analyzed by 2D-PAGE and stained with silver revealing the 56 kDa protein by its coordinates (data not shown). As expected, the same protein was labeled specifically by 3 - 5 nM [3 H]DHTBrAc, whether analyzed by SDS-PAGE (not shown) or by 2D-PAGE (Fig 34 A) as compared to a control MCH 6 labeled in the same manner (Fig. 34 B). The labeling of the protein was eliminated in the presence of 200-fold molar excess of radioinert DHTBrAc. Thus, Trifiro *et al.* (1991) concluded that the 56 kDa protein could not represent the accumulation of a slowly turned-over degradation product of the AR gene itself. Nevertheless, there remained a correlation between the two proteins and only the cloning of the cDNA for 56 kDa protein could provide an explanation.

E. Identity of the 56 kDa protein

During this same time, the identity of the 56 kDa protein was being determined from another point of view. We had previously reported that the 56 kDa protein was not androgen induced, was not the AR itself nor a degradation product of the AR, but bound androgen. Furthermore, with few exceptions, expression of the 56 kDa protein maybe associated with an intact AR gene for its

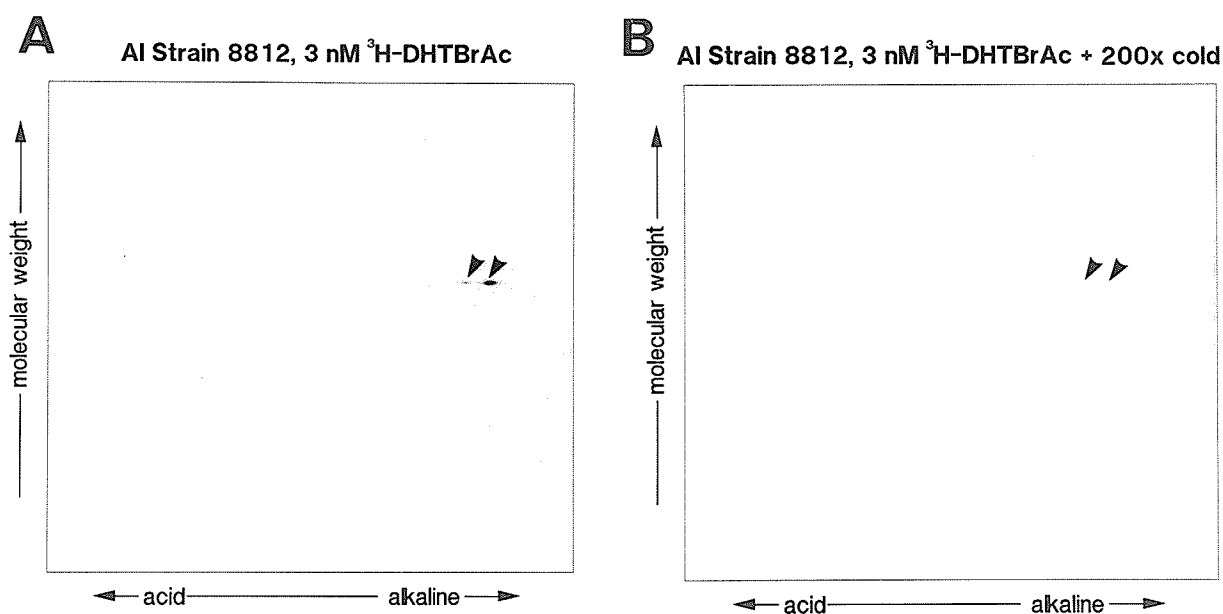


Figure 34. Affinity labeling of GSF from a subject with a complete deletion of the coding region for the AR (8812) and a control (MCH 6). (A) the 56/58 kDa androgen-binding protein after SDS-PAGE of cultured genital skin fibroblast cytosol covalently labeled by 5 nM [^3H]DHT-BrAc. 8812 is the subject: (B) The 56/58 kDa androgen-binding protein in its doublet form (pI 6.7 6.5) after 2D-PAGE of control genital skin fibroblasts covalently labeled by 3 nM [^3H]DHTBrAc (from Trifiro et.al. 1991).

expression. To account for these differences, the following possibilities were considered: (i) the 56 kDa protein was a stable degradation product of the full-length AR; (ii) it was encoded by the AR gene, but was synthesized as a separate protein by use of different promoters, different splicing and/or different polyadenylation sites, or different translation start sites; or (iii) it was a distinct 'orphan' member of the steroid/thyroid/retinoic acid receptor superfamily of ligand-activated, DNA-binding, transcription-regulating protein whose authentic ligand(s) remained to be identified (Wrogemann *et al.* 1988; O'Malley, 1990). Cloning and sequencing of the cDNA of the 56 kDa protein would allow comparison to the AR gene which had been cloned by this time (Chang *et al.* 1988; Lubahn *et al.* 1988).

1. Quantitation and quality of libraries

As mentioned earlier, expression screening with antibodies was chosen because of a lack of N-terminal amino acid sequence required for synthesizing a degenerate set of oligonucleotides for hybridization screening. Thus, cDNA libraries were constructed in bacteriophage expression vectors and screened with the partially-purified antiserum. The three libraries that were constructed were of different qualities and had different quantities of plaque forming units (Table 9). When analyzing the first and second strand fragment sizes it can clearly be seen that the library constructed in λ gt11 has an overall small average size as compared to fragments from the Lambda ZAP II library construction (Fig. 35). The average size of the largest fragments in the first library is ~2.1 kb whereas the largest fragments for the ZAP II library are of ~5.5 kb and the average size fragments ~1.9 kb. This marked contrast of sizes reflected the average size of inserts that were isolated from each of the libraries. The ZAP II library has been used successfully by another researcher to isolate the 5'-end of an ATPase inhibitor by PCR amplification (Triggs-Raine, unpublished data) attesting to the many full-length sequences in the library. This clone was found to have more 5'-end sequence than that previously published.

2. Isolation of positive clones with the purified antiserum

The first libraries in the expression vectors λ gt11 and Lambda ZAP were immunoscreened with the partially-purified antiserum. The Lambda ZAP library yielded no positives but the λ gt11 library yielded several positives. The positives were plaque purified by successive screenings and the inserts subcloned into plasmid vectors for sequencing and generation of probes for Northern analyses.

Table 9. Quantitation and quality of cDNA libraries.

DNA	# plaques	# non-recom.	Dil.	Total pfu	pfu/ml	pfu/ μ g
λ gt11	172.5	1.5(0.9%)	10^4	8.65×10^5	1.73×10^6	1.73×10^7
ZAP	99.5	2.5(2.5%)	10^4	4.98×10^5	9.95×10^5	9.95×10^6
ZAP II	147	2.0(1.3%)	10^4	7.25×10^5	3.63×10^5	3.63×10^6

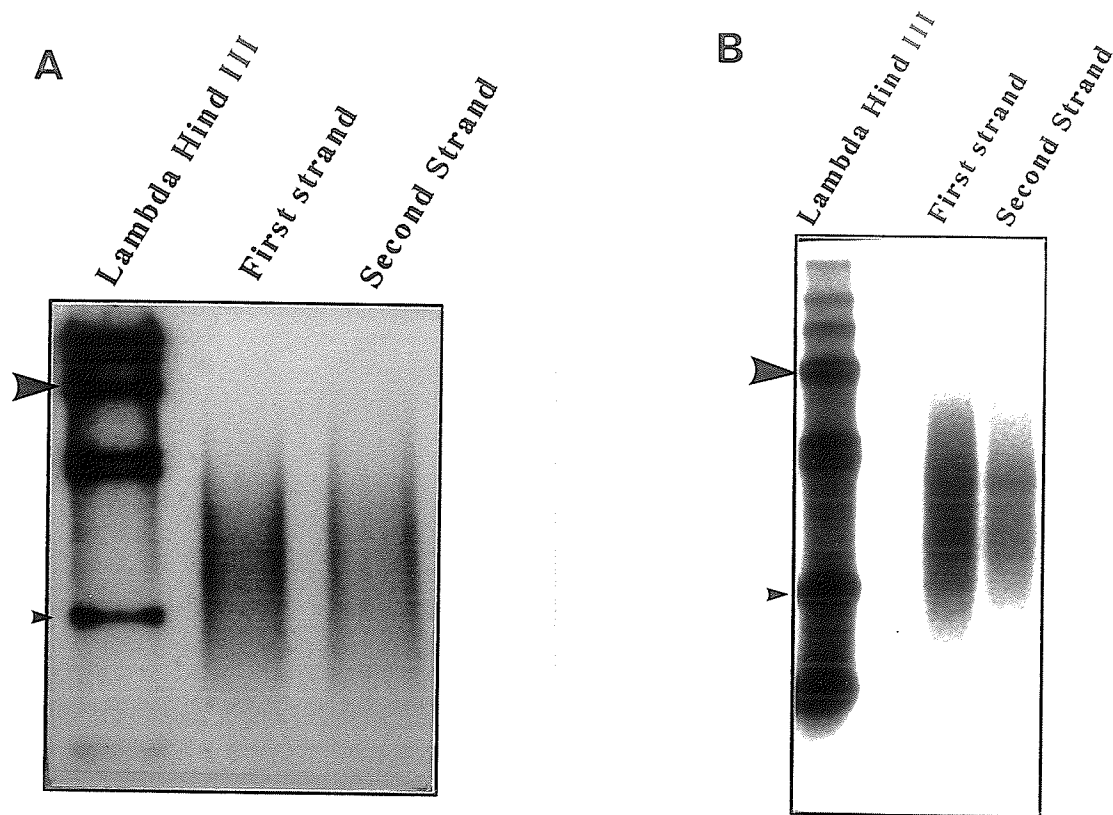


Figure 35. Alkaline gel electrophoresis of cDNA synthesized from mRNA isolated from strain MCH 6. A. Complementary DNA synthesized for λ gt11 library: lane 1. Hind III digests of λ DNA are used as molecular weight markers; lane 2. First strand synthesis; lane 3. Second strand synthesis. B. Complementary DNA synthesis for Lambda ZAP II library: lane 1. Hind III digests of λ DNA are used as molecular weight markers (Large arrowhead 4361 bp, small arrowhead 564 bp); lane 2. First strand synthesis; lane 3. Second strand synthesis.

3. Confirmation of the cDNA clones

Positive clones for the 56 kDa protein were determined by three criteria: i) mRNA detected by Northern blot analysis by clones would reflect quantitatively the level of detectable 56 kDa protein by silver stain on 2D-gels; ii) the clones needed to select for a mRNA species which faithfully translated into the 56 kDa protein as seen on a 1D and 2D-gel; iii) the translated protein must be recognizable by the antiserum.

a. Northern blot analysis

One of the clones (pI9.13.35) detected contained a 1.3 kb insert. This clone was used as a probe on Northern blots of poly A⁺ RNA from a variety of normal and mutant cell strains (Fig. 36). A 2.2 kb message was detected only in cell strains which expressed the 56 kDa protein. A constant band (1.8 kb) was also detected in all cell strains and did not quantitatively nor qualitatively reflect the expression of the 56 kDa protein. However, upon further subcloning of the insert and reprobing another blot with 1 kb of the clone (Fig. 37), the 1.8 kb band was eliminated. The expression of the 2.2 kb message was found to qualitatively parallel the expression of the 56 kDa protein in the different cell strains. Furthermore, the quantity of this message in the specific cell strains also paralleled the amount of the 56 kDa protein as detected by silver stain on 2D-gels.

b. In vitro translation of mRNA hybrid-selected with clone PUMB1

The 1 kb clone was coined PUMB1 (**P**rotein from the **U**niversity of **M**anitoba (**MB**) **1**) and registered with the Human Gene Mapping committee. PUMB1 was used for hybrid selection of mRNA from strain MCH 6 for *in vitro* translation to determine whether it coded for a 56 kDa protein. The cDNA from PUMB1 was subcloned into pUC 19. This clone (pI9.7.35) was used to select mRNA isolated from MCH 6. A chicken β -actin and pUC 19 plasmid were used as controls of the selection and *in vitro* translation reactions. The selected mRNA was translated *in vitro* and the products fractionated by SDS-PAGE (Fig. 38). Plasmid pI9.7.35, containing the cDNA from clone PUMB1, selected a mRNA species which translated into a 56 kDa protein. This result can be seen in the lanes for both the 3 h and overnight selection of mRNA. Whereas, the lanes with no plasmid added only reveal a constant 46 kDa band (which is a product of the reticulocyte lysate translation mix and is present in all lanes), and the control plasmid pUC 19 lanes selected a 45 kDa band similar in size to the abundant actin band. The chicken β -actin plasmid selected many different messages but with a very prominent and abundant translation product at 45 kDa, which is the approximate size of actin. Two-dimensional PAGE analysis of aliquots of the

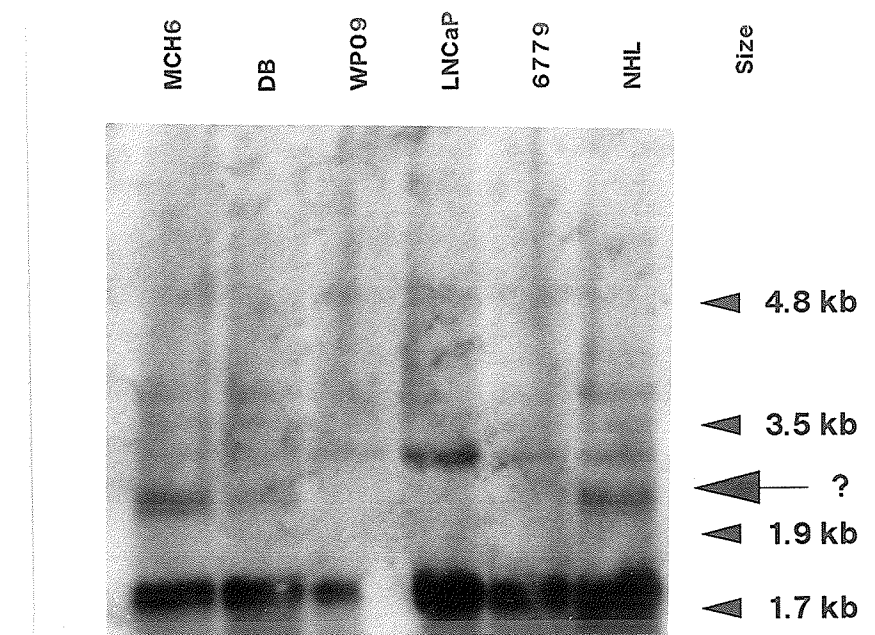


Figure 36. Northern blot analysis of poly (A)⁺ RNA from a variety of mutant and normal cell strains. lane 1, GSF of a normal individual (MCH 6); lane 2, GSF from an androgen insensitive patient (DB); lane 3, non-GSF from a normal individual (WP09); lane 4, Lymph Node Carcinoma of the Prostate cell line (LNCaP) which over expresses the androgen receptor; lanes 5 and 6 are GSF from androgen insensitive patients 6779 and NHL, respectively.

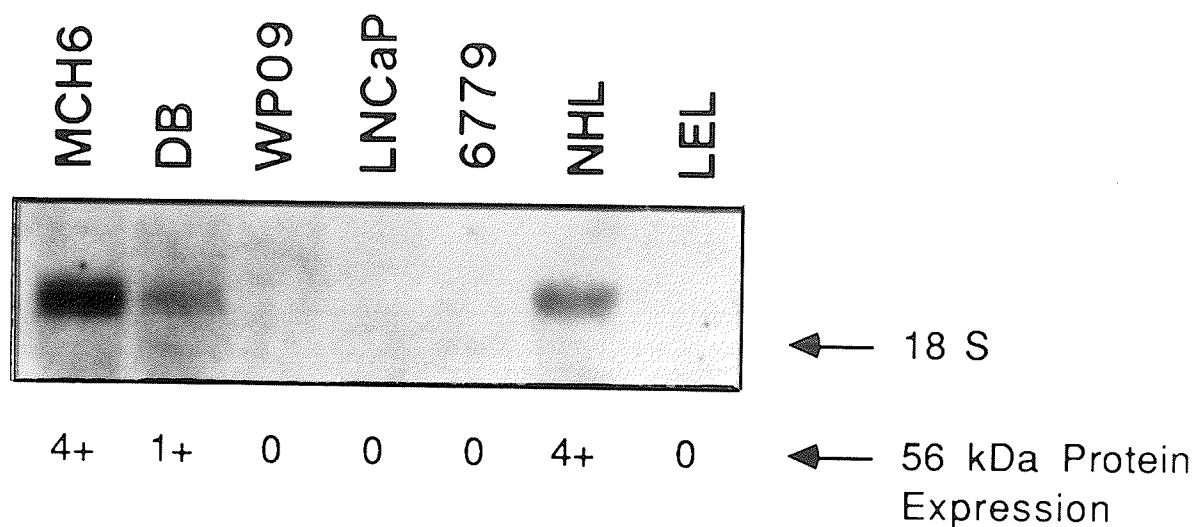


Figure 37. Northern Blot Analysis of PUMB 1 mRNA extracted from: 1, GSF of a normal individual (MCH 6); 2, GSF from an androgen insensitive patient (DB); 3, non-GSF from a normal individual (WP09); 4, Lymph Node Carcinoma of the Prostate cell line (LNCaP) which over expresses the androgen receptor; 5, 6, and 7 are GSF from androgen insensitive patients 6779, NHL, and LEL respectively. The expression of the 2.2 kb message parallels the expression of the 56 kDa protein as illustrated by the scores from 0 (not detected) to 4+ (most strongly expressed).

Hybrid-Selection and in Vitro Translation of MCH6 mRNA With Plasmid 19.7.35

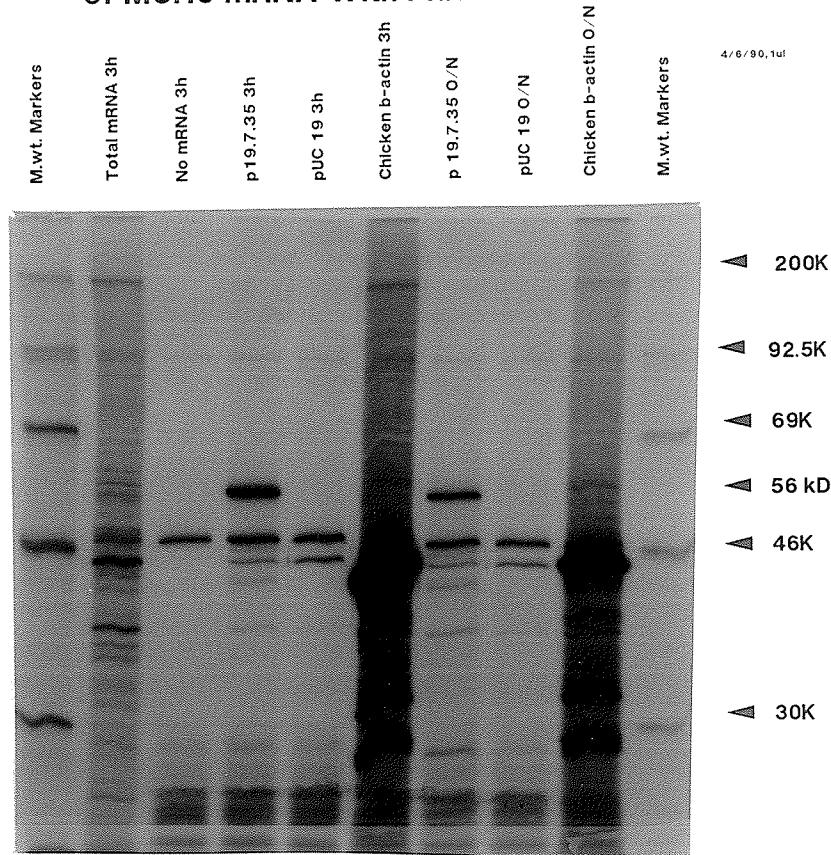


Figure 38. *In vitro* translation of mRNA hybrid selected with clone p19.7.35 from normal genital skin fibroblast strain MCH 6. Translation products are shown from: lane 2. total mRNA; lane 3. blank control with no mRNA added to the *in vitro* translation reaction; lane 4. mRNA selected for 3 h with clone p19.7.35; lane 5. mRNA selected for 3 h with pUC 19 vector; lane 6 mRNA selected for 3 h with chicken β -actin clone; lane 7. mRNA selected overnight with clone p19.7.35; lane 8. mRNA selected overnight with pUC 19 vector; lane 9. mRNA selected overnight with chicken β -actin clone; lanes 1 and 10. ^{14}C -molecular weight markers in kilodaltons.

translation products selected with clone pI9.7.35 reveal the characteristic 56 kDa protein doublet at the proper coordinates (Fig. 39 B). The translation products from the pUC 19 vector selection only show a 45 kDa protein spot and the actin spot, found in all translation reactions (Fig. 39 C). Finally, the translation products from total mRNA are shown for comparison (Fig. 39 A). Thus, the PUMB1 cDNA clone that identified a 2.2 kb message, which paralleled the expression of the 56 kDa protein as determined by silver stained 2D-PAGE of GSF, encoded for a 56,000 dalton protein doublet similar to the 56 kDa protein.

c. Immunoprecipitation of *in vitro* translated proteins

The proteins translated *in vitro* from mRNA selected by clone PUMB1 were immunoprecipitated with a partially purified antiserum. The same experiment was performed as described for the *in vitro* translation of hybrid selected mRNA but the translated products were immunoprecipitated using the antiserum to the 56 kDa protein. The translated products from the hybrid selection are shown in comparison to the immunoprecipitated translation products (Fig. 40). It can be clearly seen that the only protein immunoprecipitated by the antiserum has a molecular mass of 56,000 daltons. Thus, this reconfirms that the PUMB1 clone selected a mRNA species which translated into a 56 kDa protein that is specifically immunoprecipitated by the antiserum to the 56 kDa protein from GSF.

Thus, the results of this series of experiments provided strong evidence that we had the cDNA for the 56 kDa protein found in genital skin fibroblasts.

d. Sequence of the PUMB1 clone

The cDNA sequence of clone PUMB1 was determined by the Sanger dideoxy chain termination method from single stranded M13 subclones. The sequence (Fig. 41 A) has an open reading frame of 812 bp (Fig 41 B). The sequencing strategy allowed complete sequencing of the 998 bp of cDNA (Fig. 42) by using two internal oligonucleotide primers deduced from the sequence when it was available (not shown in the figure). When compared to the sequences in GenBank, it was found to be identical with human aldehyde dehydrogenase 1 (EC 1.2.1.3) from 748 to 1746 bp (Fig. 42). Aldehyde dehydrogenase 1 is the cytoplasmic isoenzyme (ALDH1) which has been cloned and sequenced from human liver (Hsu *et al.* 1985). Thus, this clone was a partial clone which coded for the carboxy terminal 247 amino acids of ALDH 1 and for 230 nucleotides of the 3'-untranslated message. This also implied that the oligo-(dT) priming for the reverse transcription reaction did not prime at the native poly (A) tail but in fact primed from a short 'A tract'

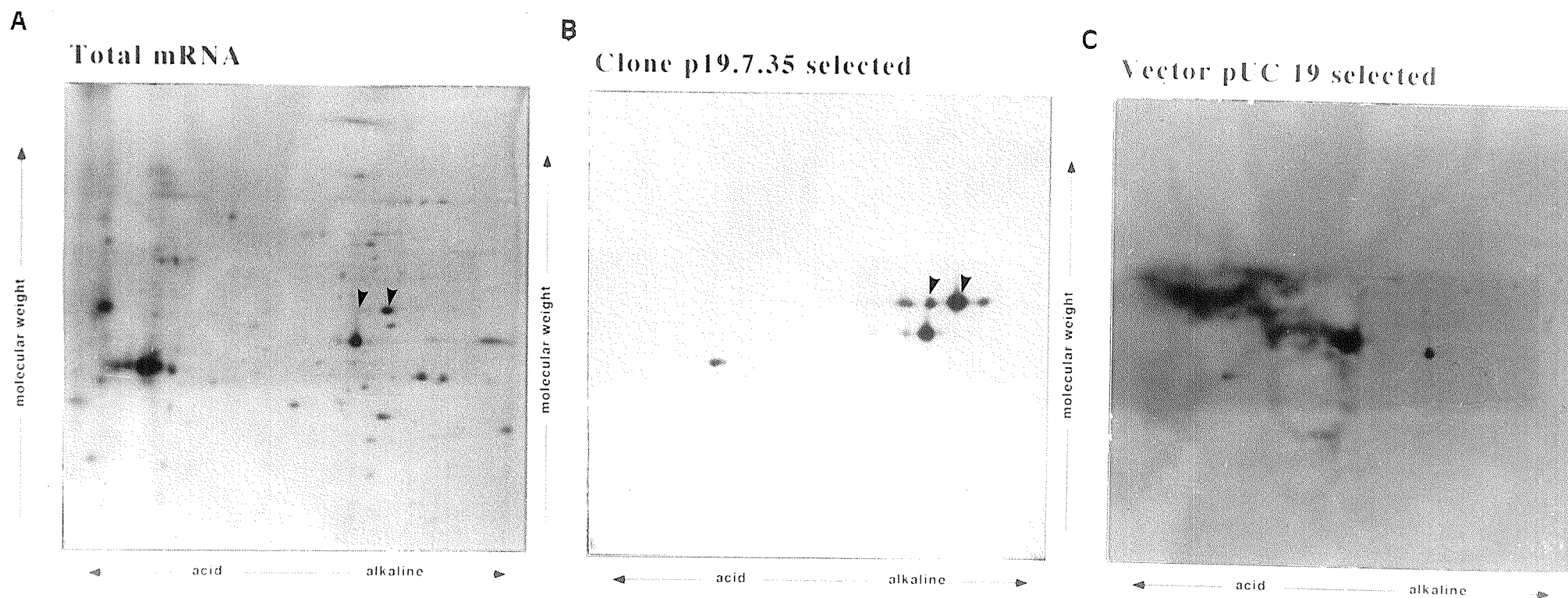


Figure 39. Two-dimensional gel electrophoresis of translation products from *in vitro* translation of hybrid selected messages. A. total mRNA translation products; B. mRNA selected for 3 h with clone p19.7.35; C. mRNA selected for 3 h with pUC 19 vector.

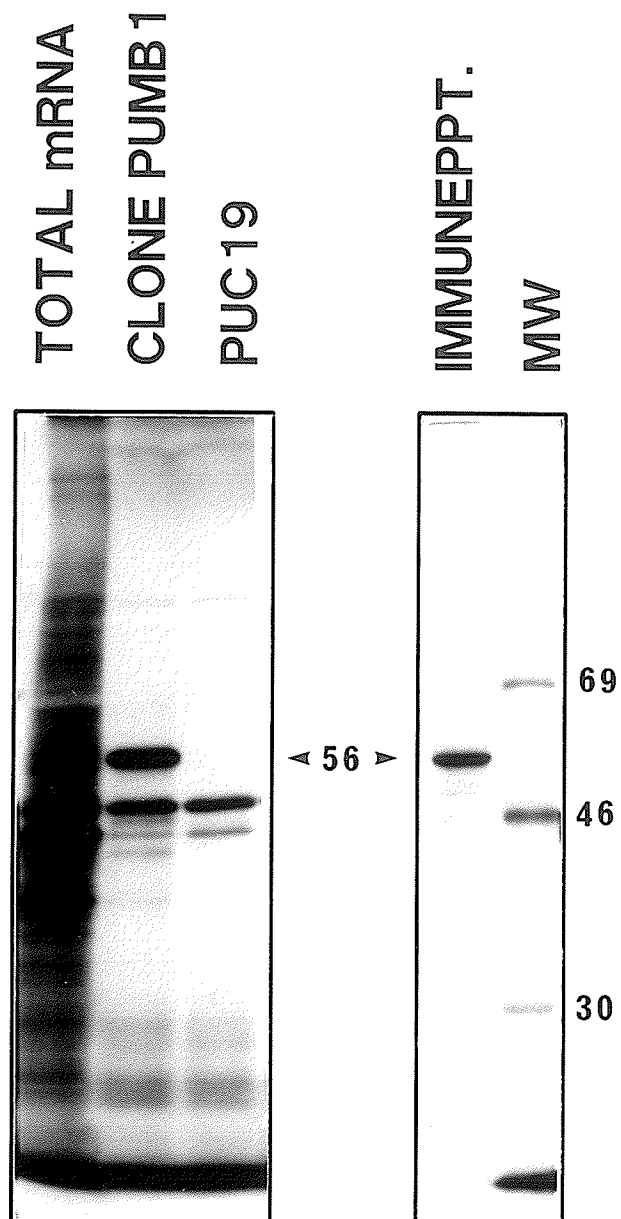


Figure 40. Immunoprecipitation of *in vitro* translation products from mRNA hybrid selected from normal GSF. Lane 1, translation of total mRNA; lane 2, hybrid selected with cDNA clone PUMB 1; lane 3, hybrid selected with the vector pUC19; lane 4, immunoprecipitate of the translation products of lane 2; lane 5, molecular weight markers in kilodaltons.

A. Complete nucleotide sequence of clone PUMB 1.

		10		20		30		40			50		60		70		80	
1	CCATTTCTTC	TCACATGGAT	ATAGACAAAG	TAGCCTTCAC	AGGATCAACA	GAGGTGGCA	AGTTGATCAA	AGAAGCTGCC	80									
81	GGGAAAAGCA	ATCTGAAGAG	GGTGACCCTG	GAGCTTGGAG	GAAAGAGCCC	TTGCATTGTG	TTAGCTGATG	CCGACTTGGA	160									
161	CAATGCTGTT	GAATTTGCAC	ACCATGGGGT	ATTCTACCAC	CAGGGCCAGT	GTTGTATAGC	CGCATCCAGG	ATTTTGTGG	240									
241	AAGAATCAAT	TTATGATGAG	TTTGTTCGAA	GGAGTGTGA	GCGGGCTAAG	AAGTATATCC	TTGGAAATCC	TCTGACCCCA	320									
321	GGAGTCACTC	AAGGCCCTCA	GATTGACAAG	GAACAATATG	ATAAAATACT	TGACCTCAT	GAGAGTGGGA	AGAAAGAAGG	400									
401	GGCCAAACTG	GAATGTGGAG	GAGGCCCGTG	GGGGAATAAA	GGCTACTTTG	TCCAGCCCAC	AGTGTCTCT	AATGTTACAG	480									
481	ATGAGATGCG	CATTGCCAAA	GAGGAGATT	TTGGACCAGT	GCAGCAAATC	ATGAAGTTTA	AATCTTTAGA	TGACGTGATC	560									
561	AAAAGAGCAA	ACAATACTTT	CTATGGCTTA	TCAGCAGGAG	TGTTTACCAA	AGACATTGAT	AAAGCCATAA	CAATCTCCTC	640									
641	TGCTCTGCAG	GCAGGAACAG	TGTGGGTGAA	TTGCTATGGC	GTGGTAAGTG	CCCAGTGCCC	CTTTGGTGGA	TTCAAGATGT	720									
721	CTGGAATGG	AAGAGAACTG	GGAGAGTACG	GTTTCCATGG	ATATACAGAG	GTCAAAACAG	TCACAGTGAA	AATCTCTCAG	800									
801	AAGAACTCAT	AAAGAAAATA	CAAGAGTGGA	GAGAAGCTCT	TCAATAGCTA	AGCATCTCCT	TACAGTCACT	AATATAGTAG	880									
881	ATTTTAAAGA	CAAAATTTT	CTTTCCTTGA	TTTTTTTAA	ACATAAGCTA	AATCATATTA	GTATTAAATAC	TACCCATAGA	960									
961	AAACTTGACA	TGTAGCTTCT	TCTGAAAGAA	TTATTTGCC					999									
		10		20		30		40			50		60		70		80	

B. Schematic representation of the 812 nucleotide open reading frame 3.

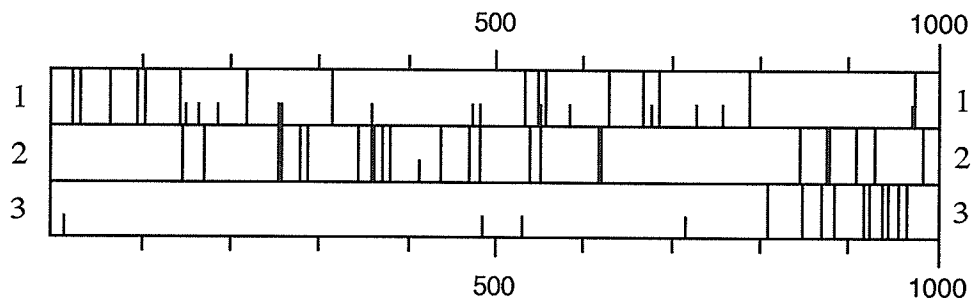


Figure 41. Complete sequence of clone PUMB 1. A. Nucleotide sequence of clone PUMB 1; B. Schematic representation of the 812 nucleotide in the third open reading frame.

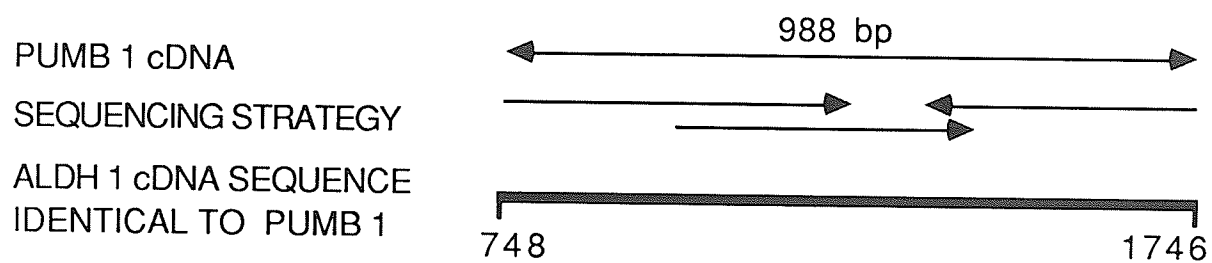


Figure 42. Schematic representation of cDNA sequencing strategy of clone PUMB1 also showing the 988 nucleotide sequence in comparison to the human cytosolic ALDH 1 sequence.

(AAATAAAAAAGA) at position 1746 - 1757 (not shown). This was not considered to be unusual as the poly (dT) primer may prime at any poly (A) tract. Furthermore, the 300 bp of the original clone was also sequenced and found to be identical to the 16S mitochondrial gene from nucleotides 2955 - 3207 (data not shown). This would further imply that the original clone had two inserts. This indicated that an under-estimation of the number of ligatable ends for the ligation reaction caused some blunt-ended cDNA to be ligated together without vector.

4. Aldehyde dehydrogenase activity associated with the 56 kDa protein

Normal GSF were tested for aldehyde dehydrogenase activity using a zymogram. Genital skin fibroblasts MCH 6 cytosol was separated on non-denaturing isoelectric focusing gels and stained for aldehyde dehydrogenase activity with acetaldehyde and benzaldehyde as substrates. A single prominent activity band was detected with an isoelectric point of 5.1 (Fig. 43). In the same experiment, the cells had been labeled *in situ* with 5 nM [³H]DHTBrAc ± 200-fold molar excess of radioinert ligand. After staining and photography, the gel from each lane was sliced and the pieces counted in a scintillation counter. The specific counts were found to comigrate with the activity band developed for aldehyde dehydrogenase activity. Specific labeling of the only protein in GSF cytosol comigrates with the aldehyde dehydrogenase activity. Our previous results have shown that the only specifically labeled protein readily detectable in GSF is the 56 kDa protein. Thus, the 56 kDa protein was identified as an aldehyde dehydrogenase.

5. X-chromosome dosage analysis of cDNA

The ALDH 1 gene was known to be localized to chromosome 9q21 (Hsu *et al.* 1986), however, the clone isolated from cDNA libraries was only a partial clone representing the about half the complete coding region of ALDH 1. The family of isozymes of aldehyde dehydrogenases are quite homologous in this region and this created the possibility that the partial clone from GSF represented another isozyme species. It was postulated that if the locus of this new isozyme were closely linked to the AR gene, some mutations affecting the AR might also affect the isozyme locus in some individuals. Since the AR locus was assigned to the X-chromosome, the PUMB 1 clone was used to probe Southern blots to determine whether it was linked to the X-chromosome. The 998 bp of clone pI9.7.35 was used to probe a Taq I digested genomic DNA from hybrid cell lines with 1 - 5 X-chromosomes or from peripheral white blood cells from several unrelated males and females (Fig. 44 A). The three bands identified have a constant intensity in all lanes whether there was 1, 2 or 5 X-chromosomes. This blot was also probed with the X-linked probe from the

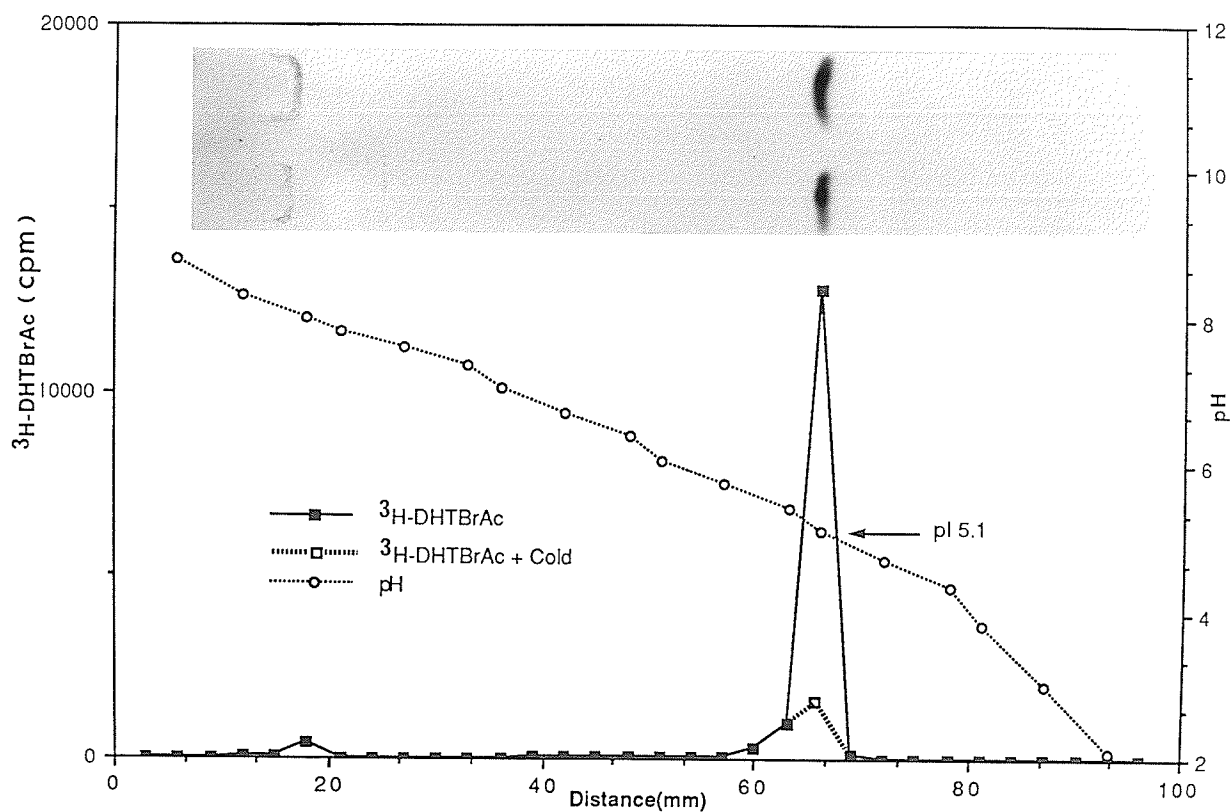
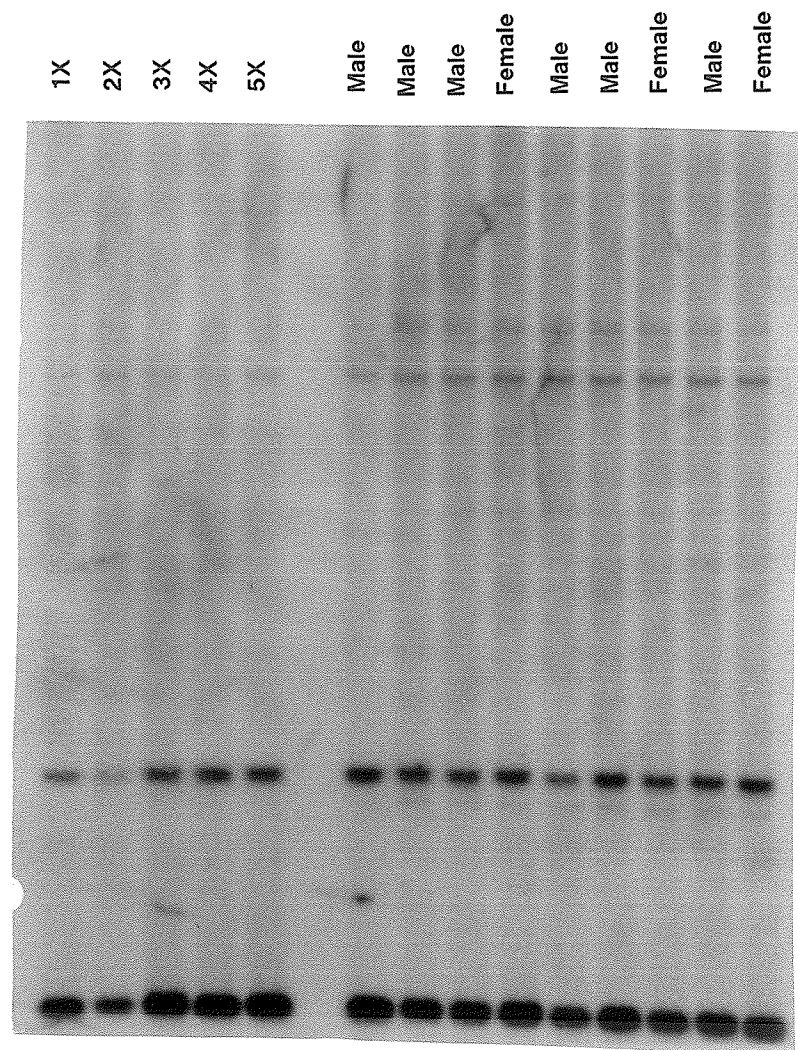


Figure 43. Aldehyde dehydrogenase activity associated with [^3H]DHTBrAc labeled GSF cytosol from the normal individual MCH6. Activity gel is shown as an insert. Lane A, cells labeled with 5 nM [^3H]DHTBrAc; lane B, as in A but with the inclusion of 200-fold molar excess of radioinert DHTBrAc. The pH 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).

X-Chromosome Dosage With cDNA p19.7.35 (Taq I)

Blot 14B

A



B

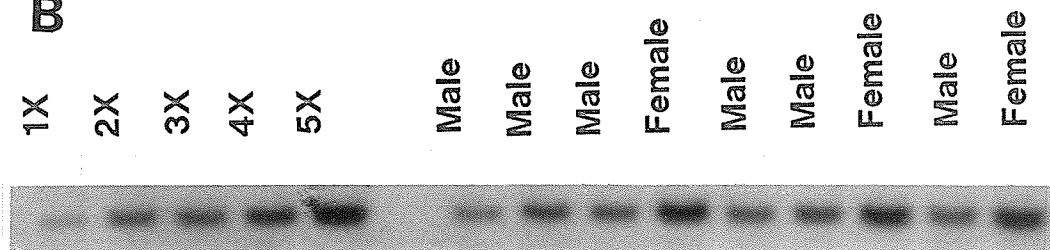


Figure 44. X-chromosome dosage analysis of ALDH 1. A. The 0.7 kb of clone p19.7.35 was used to probe a Taq I digested genomic DNA from hybrid cell lines with 1 to 5 X-chromosomes or from peripheral white blood cells from several unrelated males and females; B. A probe from the X-linked Duchenne muscular dystrophy gene (cDMD8) is used as a control of X-chromosome dosage on the same blot.

dystrophin gene (DMD) (Fig. 44 B). This probe clearly shows X-chromosome dosage on the same blot. Thus, the ALDH 1 gene is not linked to the X-chromosome.

6. RFLP analysis

Several ALDH 1 variants had been described in Orientals and Caucasians which displayed normal activity and lower rate of utilization of substrates and much decreased activity (Yoshida *et al.* 1983; Kanayama *et al.* 1984). Subsequently, Yoshida *et al.* (1989) reported that restriction fragment length polymorphisms (RFLP) existed in some racial groups. In particular, Oriental and Caucasian subjects, who were sensitive to alcohol and displayed a flushing response (turning red in the face) were polymorphic at the ALDH 1 locus with Taq I and Msp I alleles. Southern blots were made with Hind III, Eco RI, Taq I, and Msp I digests of genomic DNA from 3 controls and 25 subjects with CAIS (Fig. 45, Msp I not shown). These blots were probed with a 0.7 kb fragment of the ALDH 1 cDNA, using a similar 3' region as Yoshida *et al.* (1989). All blots reveal constant banding patterns across all lanes whether samples were from control or patients. Thus, these RFLP haplotypes did not exist in CAIS patients as determined with the 0.7 kb ALDH 1 3'-end probe.

7. Tissue distribution of the ALDH 1 mRNA

Northern blots were made from mRNA isolated from different rat tissues. Using the same 3'-end probe as for the Southern blots, all tissues except spleen in the rat had a species of mRNA that cross-hybridized to this probe under low stringency washes (washing at 50 °C with 1 x SSC and 0.5 % SDS), after longer exposure of the blot (Fig. 46 A). The bands are of slightly different sizes in the different tissues which probably reflects the many isoenzyme forms of aldehyde dehydrogenases in mammals.

Furthermore, the expression of ALDH 1 was determined for some cell lines commonly used for hormone regulation or transfections (Fig. 46 B). Using the same 3' end probe, the message for ALDH 1 is found to be absent from the mouse fibroblast NIH3T3, rat hepatoma H4IIE and African green monkey COS1 but expressed strongly in human GSF MCH 6, human hepatoma HepG2, African green monkey CV1 and marginally expressed in African green monkey COS7 cells. It is interesting that CV1 expresses the message strongly whereas the two COS cell lines derived from CV1 have drastically different expressions of the message. These cell lines may be used in the analysis of the hormonal regulation of the ALDH 1 gene. All the experiments performed with GSF never resulted in any response of the ALDH 1 gene to steroids, however, it

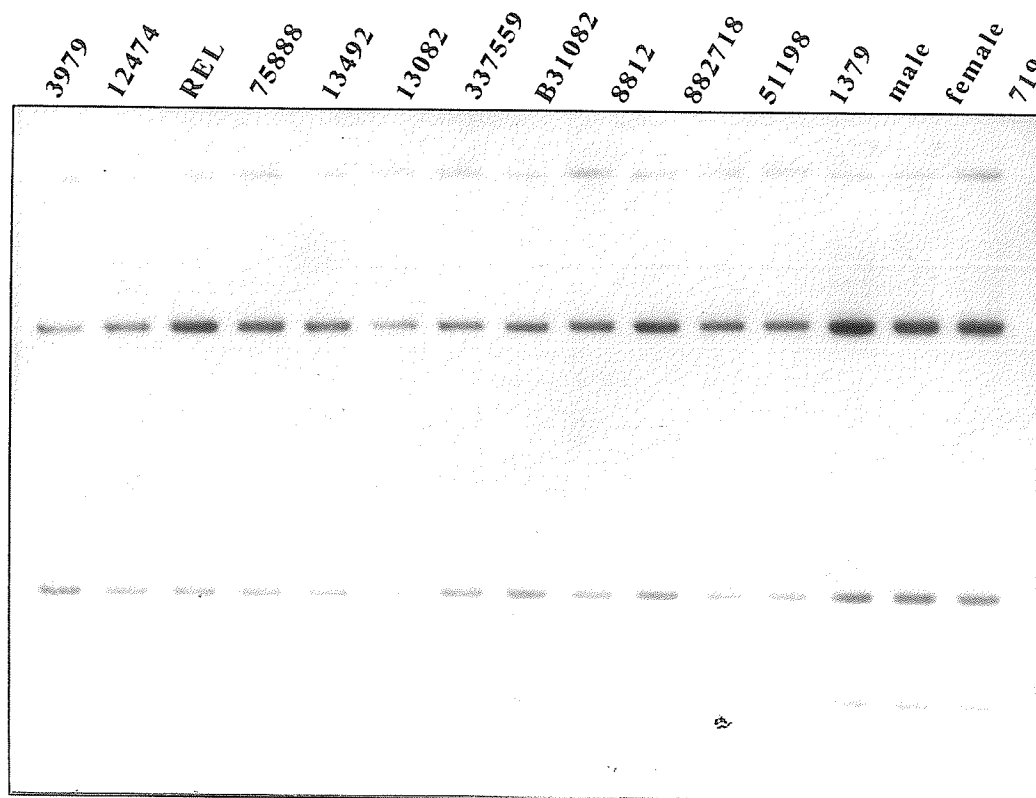
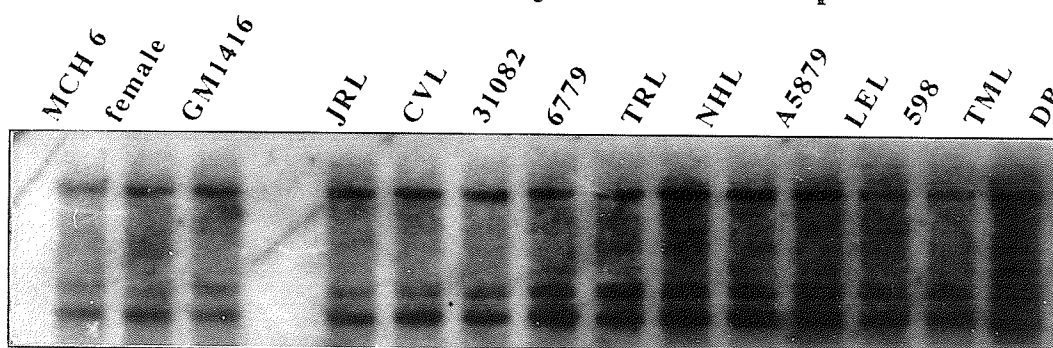
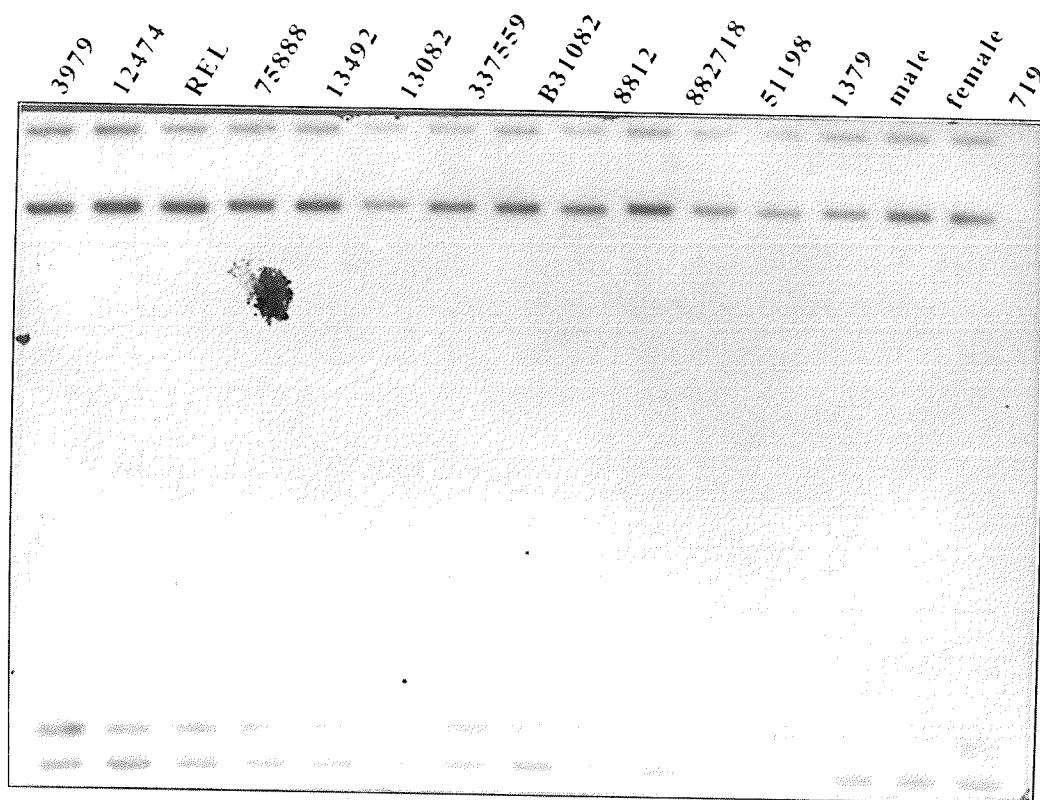
A**Hind III RFLP analysis of CAIS patients****B****Eco RI RFLP analysis of CAIS patients**

Figure 45. Restriction fragment length polymorphism analysis of controls or of subjects with complete androgen insensitivity syndrome using a ALDH 1 probe. A. Southern blot of Hind III digested genomic DNA; B. Southern blot of Eco RI digested genomic DNA; C. Southern blot of Taq I digested genomic DNA (on following page).

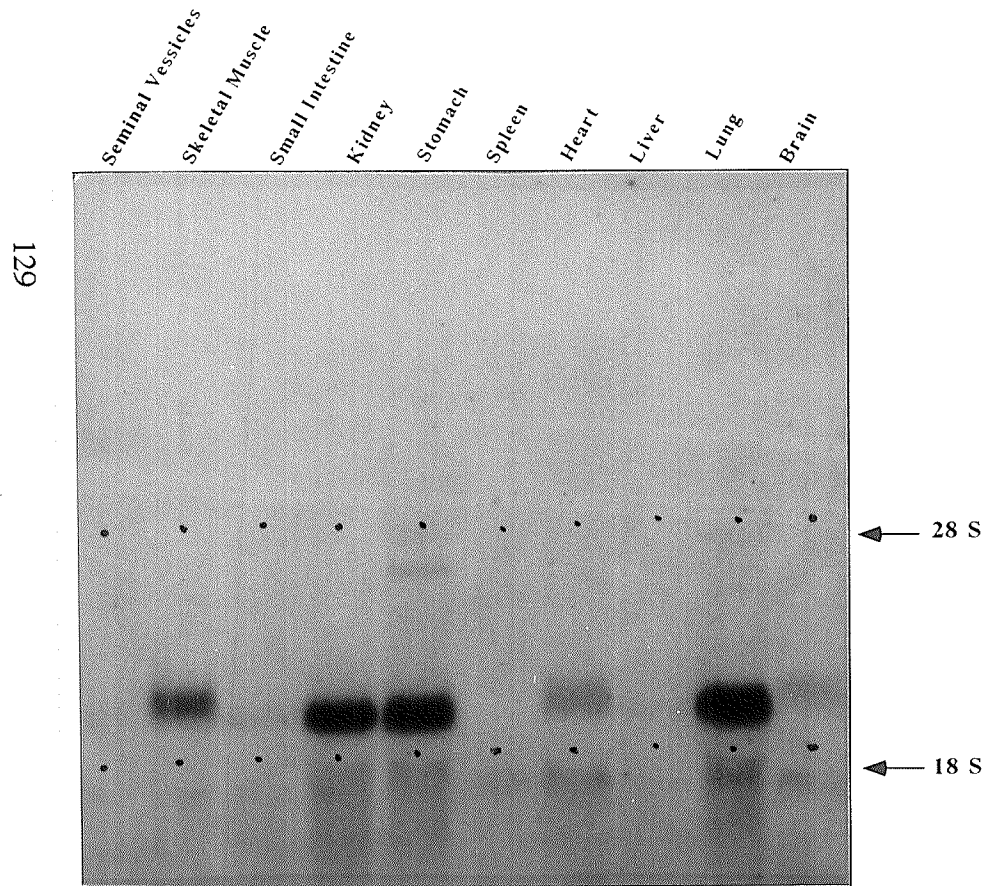
C

Taq I RFLP analysis of CAIS patients



A

ALDH 1 expression in rat tissues



B

ALDH 1 expression in established cell lines

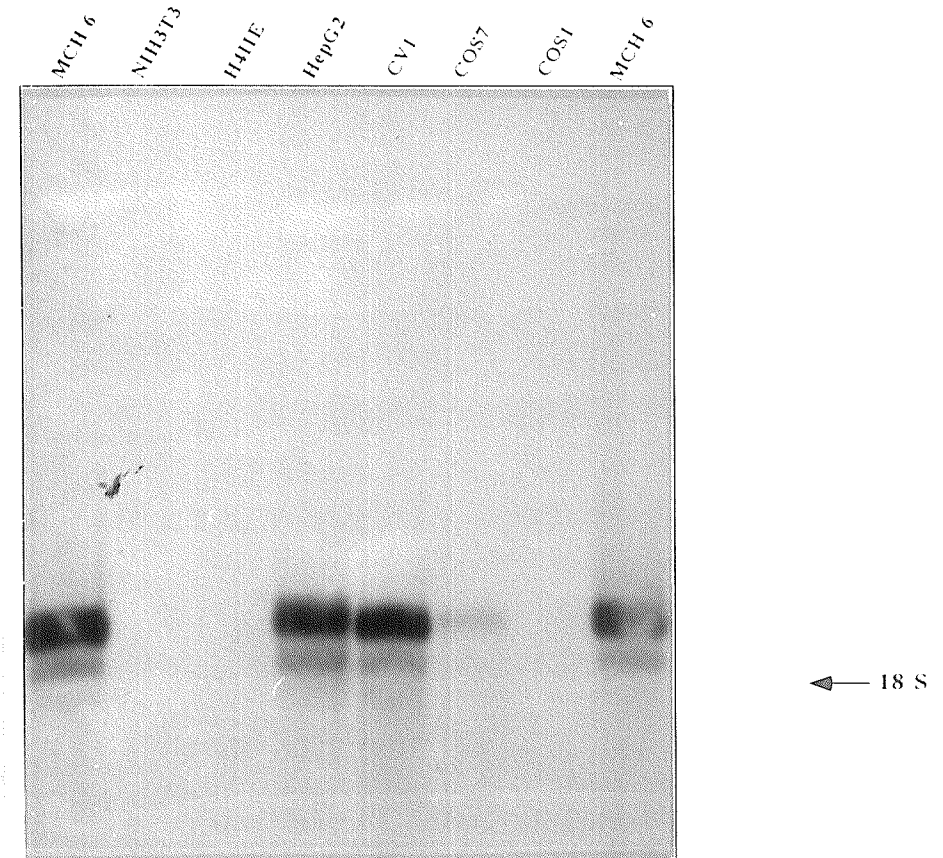


Figure 46. Northern blot analysis of rat tissues and established cell lines with ALDH 1. The blots are probed with a 0.7 kb 3'-end fragment from ALDH 1 coding region. A. Rat tissues; B. Cell lines.

has been discovered that androgen regulated genes are not inducible in GSF (Wilson *et al.* 1991). Thus, knowing the expression of ALDH 1 in these cell lines will become invaluable to test its response to different exogenously added compounds (androgens and other steroids, xenobiotics, etc.).

8. Cloning of the full-length cDNA for the 56 kDa protein

The rationale for isolating the full-length cDNA for the 56 kDa protein in GSF was two fold: (i) as mentioned before, it was not known whether the gene coding for the GSF enzyme was identical in sequence to that of the liver isoenzyme cloned by Hsu *et al.* (1985); and (ii) consequently, how the functional enzyme produced by this gene was affected by the binding of androgens. To accomplish this, a cDNA library was constructed in Lambda ZAP II using mRNA from strain MCH 6. The library was screened first with oligonucleotides from the 5' region of the 998 bp clone (PUMB1). Positive clones were rescreened with the 0.7 kb fragment of the clone to confirm their identity. Clones that were still positive were plaque purified to homogeneity. The clones were subcloned by the *in vivo* excision of the pBluescript plasmid containing the cloned insert. Thirty clones were identified in this manner. The plasmid clones were digested with EcoRI to release the inserts, a Southern blot made of the fragments and hybridized with the 0.7 kb ALDH 1 fragment to determine which clones were still positive. Eighteen clones showed strong hybridization to the probe with several clones having identical sized inserts (data not shown). The clones were sequenced using the M13 universal primers after subcloning the 5' end into a Bluescript vector and using internal oligonucleotide primers as before. Two clones were found to have a complete cDNA for ALDH 1, as compared to the published sequence. The two clones have a 5' end sequence which is identical to the reported sequence to -37 and -30 nucleotides from the start of translation of the ALDH 1 mRNA (Fig. 47), where -53 was the full-length sequence as determined by primer extension analysis (Hsu *et al.* 1989). The two sequences are completely identical except for a two nucleotide discrepancy in the 3' untranslated end which could be a sequencing artifact. The full-length sequence of one of the clones (ZAP 50) with its 1533 nucleotide open reading frame and 501 amino acid sequence is shown in Fig. 48. The sequences of both clones were found to be identical to the ALDH 1 coding sequence. Thus, the 56 kDa protein from GSF is found to be completely identical to the human cytosolic aldehyde dehydrogenase from human liver.

ALDH 1: 5'-53 ATCAGAACCAAATTGCTGAGCCAGTCACC.....ATG**AT**(A)₁₈ 3'
 ZAP 43: 5' - 37 TGAGCCAGTCACC.....ATG**ATT**(A)₁₆3'
 ZAP 50: 5' - 30 GTCACC.....ATG**AA**(A)₁₈ 3'

Figure 47. Comparison of sequences from the 5'- and 3'-ends of the published sequence (Hsu et.al. 1989) and those from two full-length cDNA clones.

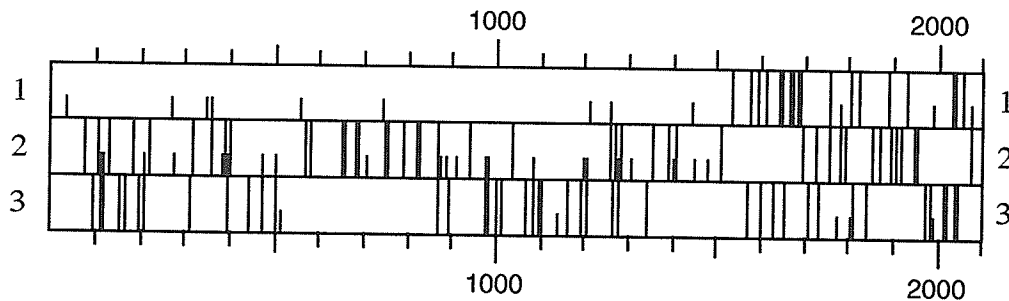
A. Complete sequence of clone ZAP 50.

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      | 10      | 20      | 30      | 40      | 50      | 60      | 70      | 80
1  GTCACCTGTG TTCCAGGAGC CGAATCAGAA ATGTCATCCT CAGGCACGCC AGACTTA OCT GTCCCTACTCA CCGATTTGAA 80
81 GATTCAATAT ACTAAGATCT TCATAAA CAA TGAATGGCAT GATTCAATGA GTGGCAAGAA ATTTCTGTCT TTTAATCTG 160
161 CAACTGAGGA GGAGCTCTGC CAGGTAGAAG AAGAGATAA GGAGGATGTT GACAAAGCAG TGAAGGCCGC AAGACAGGCT 240
241 TTT CAGAT TG GATCTCCGTG GCGTACTATG GATGCTTC CG AGAGGGGGCG ACTATATAC AAGTTGGCTG ATTTAATCGA 320
321 AAGAGATCGT CTGCTGCTGG CGACAATGGA GTCAATGAAT GGTGGA AAAC TCTATTC CAA TGCATATCTG AATGATT TAG 400
401 CAGGCTGCAT CAAAACATTG CCGTACTGTG CAGGTTGGGC TGACAAGATC CAGGCGCTA CAATACCAAT TGATGGAAAT 480
481 TTT TTTACAT ATACAAGACA TGAACCTATT GGTGTATGTG GCCAAATCAT TCCTTGG AAT TTC CGTTGG TTATGCTCAT 560
561 TTGGAAGATA GGGCTGCAC TGAAGCTGTG AAACACAGTG GTTGTCAAAC CAGCAGAGCA AACTCTCTC ACTGCTCTCC 640
641 ACGTGGCATC TTTAATAAA GAGCGCAGGT TTCTCTCTGG AGTAGTGAAT ATTTCTCTG GTTATGGGC TCAGCA GSG 720
721 GCAGCCATTT CTCTCACA T GGATATAGAC AAAGTAGCCT TCACAGATC AACAGAGGT GCGAAGTTGA TCAAGAGAGC 800
801 TGC OGGGAA AGCAATCTGA AGAGGTTGAC CCGTGAGCTT GGAGGAAAGA GCGCTGCAT TGTGTTAGCT GATGCGACT 880
881 TGGACAATGC TGTGAATT T GCACACCATG GGGTATCTA CCACCA GGC CAGTGTGTA TAGCGCATC CAGGATTTT 960
961 GTGGAAGAA T CAATTTATGA TGA GTTTGTT CGAAGGAGTG TTGAGCGGC TAAGAAGTAT ATCTTTGSA ATCTCTGAC 1040
1041 CCCAGGAGTC ACTCAAGGCC CTCAGATTGA CAAGGAACAA TATGATAAA TACTTGACCT CATTGAGAGT GGAAGAAAG 1120
1121 AAGGGGCCAA ACTGGAATGT GGAAGAGGCC CCGTGGSGAA TAAAGGCTAC TTTGTCCAGC CCACAGTGT CTCTAATGTT 1200
1201 ACAGATGAGA TGGCATTTGC CAAAGAGGAG ATTTTGGAC CAGTGCAGCA AATCATGAAG TTTAAATCTT TAGATGACGT 1280
1281 GATCAAAAGA GCAACAAATA CTTTCTATGG CTTATCAGCA GGAGTGT TTA CCAAAGCAT TGATAAAGCC ATAACAATCT 1360
1361 CCTCTGCTCT GAGGCAAGGA ACAGTGTGGG TGAATTGCTA TGGCGTGGTA AGTGC CAGT GCCCTTTG TGATTTCAG 1440
1441 ATGCTCTGGA ATGGAAAGGA ACTGGGAGAG TACGGTTTCC ATGGATATAC AGAGGTCAAA ACAGTCACAG TGAATATCTC 1520
1521 TCAGAAAGAC TCATAAGAA AATACAAAG TGGAGAGAAAG CTCTTCAATA GCTAAGCATC TCTTACAGT CACTAATATA 1600
1601 GTAGATTTTA AAGACAAAAT TTTCTTTTTC TTGATTTTTT TTAACATAA GCTAAATCAT ATTAGTATTA ATACTACCA 1680
1681 TAGAAAAC TT GACATGTAGC TTTCTTCTGAA AGAATTTATTT GCCTTCTGAA ATGTGACCC CAAGTCTAT CTTAATAAA 1760
1761 AAAAGACAAA TTGGATGTA TGA TCTCTCT AGCTTTGPCA TAGTTATGTG ATTTCTCTT GTAGCTACTT TCGAGGATA 1840
1841 ATAATTTTAT AGAAAAGGAA CAGTTGCATT TAGCTTCTTT CCCTTAGTGA CTCTTGAAGT ACTTAACATA CAGGTTAACT 1920
1921 GCAGAGTAAA TTGCTCTGTT CCGAGTAGTT ATAAAGTCCT TGGACTGT TT TGAAAAGTTT CCTAGGATGT CATGTCGCT 2000
2001 TGTCAAAAGA AATAATCCT GTAAATTTA GCTGTAAACT GAATATAAG CTTAATAAAA ACAACCTTGC ATGATAAAA 2080
2081 AAAAAAAA AAAA
      | 10      | 20      | 30      | 40      | 50      | 60      | 70      | 80

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B. 1533 nucleotide open reading frame.



C. 501 amino acid sequence.

```

      | 10      | 20      | 30      | 40      | 50      | 60      | 70      | 80
1  MSSSGTDLPL VLITDLRIQY TKIFINNEWH DSVSGKKFPV FNPATEEELC QVEEGDKEDV DKA VKAARQA FQIGSEWR TM 80
81 DASERGRLLY KLADLI ERDR LLLATME SMN GGLKLYSNAYL NDLACGIKTL RYCAGWADKI QGRTIPIDGN FFTYTRHEPI 160
161 GVCQGIIPWN EPLVMLIWKI GPALSGNTV VVKPAEQ TPL TALHVASLIK EAGFPFGWVN IVPGYGPTAG AAISSHMDID 240
241 KVAFTGSTEV GKLIKEAAGK SNLKRVTLEL GKSFPICVLA DADLDNAVEF AHG VFYHQG QOCIAASRIF VEESIYDEFV 320
321 RRSVERAKKY ILLGNLTPGV TQSPQIDKEQ YDKILDLIES GKKEGAKLEC GGGFWGNRGY FVQPTVFSNV TDEMRLAKEE 400
401 IFGPVQQIMK FKSLDDVVKR ANNTFYGLSA GVFTKIDKA ITISSALQAG TVWVNCYGVV SAQCPFGGFK MSGNGRELGE 480
481 YGFHGYTEVK TVTVKISQKN SZ
      | 10      | 20      | 30      | 40      | 50      | 60      | 70      | 80

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Figure 48. Complementary DNA sequence of clone ZAP 50. A. The complete cDNA of 2099 nucleotides including 5'-end untranslated 30 nucleotides, the full-length mRNA is identical to that reported by Hsu et.al. (1989); B. The 1533 open reading frame found in the first frame; C. The 501 amino acids coded by the open reading frame.

F. Regulation of the ALDH 1 gene

The observation of the “absence” of the 56 kDa protein in 12/14 GSF of AIS patients, its “presence” in all 23 GSF of control strains, and its “absence” in 30/32 non-GSF cell strains was based on the identification of the protein on two-dimensional denaturing gels stained for proteins (Nickel *et al.* 1988). Although this finding has been confirmed in two other labs (Kovacs, Turney, 1988; Trifiro *et al.* 1991b) this “absence” only means “below the level of detection” of the protein stains. Thus, we have been reevaluating this “absence” with more sensitive techniques: the covalent photolytic labeling of the 56 kDa protein by non-metabolizable androgen analogs MT (R1881) (Wrogemann *et al.* 1988), Mibolerone (Pereira *et al.* 1990), and specific covalent labeling with [³H]dihydrotestosterone bromoacetate (DHTBrAc) (Trifiro *et al.* 1991b). Now that the 56 kDa protein is identified as an aldehyde dehydrogenase, Northern blot analyses with cDNA probes and the activity staining for aldehyde dehydrogenase in cell extracts separated by non-denaturing isoelectric focusing (Pereira *et al.* 1991) will allow another level of analysis of patient GSF. Further analyses would help to decide to what extent the expression of the gene was suppressed in various patients and point to whether the expression was regulated transcriptionally, translationally or at both levels in genital skin fibroblasts. The literature (Lindahl, 1992) indicated that ALDH1 was regulated at the level of transcription.

1. Northern blot analysis with ALDH 1

The cDNA encompassing exons 1 to 6 of ALDH 1 was used as a probe for Northern blots of mRNA from controls or from subjects with CAIS. It can clearly be seen that the expression of the ALDH 1 message was found in many GSF strains (Fig. 49 B), whether from control or subjects with CAIS. Some patients, like non-GSF controls, had no message. These experiments revealed a spectrum of expression levels of the mRNA for the 56 kDa protein. As can be seen in Figure 37, the expression of the ALDH 1 message paralleled the expression of the 56 kDa protein as detected by silver stain on 2D-gels. These results indicate that the expression of the 56 kDa protein was also expressed in a spectrum of levels. The expression of ALDH 1 is shown in contrast to the expression of the androgen receptor messages in these same patients (Fig. 49 A). However, the molecular defects in the androgen receptor in many of these patients have not been determined and cannot be used for correlation analysis. Nevertheless, the original conclusion (Nickel *et al.* 1988) that the presence or absence of the 56 kDa protein on two dimensional gels stained for proteins was a marker for heterogeneous spectrum of defects in patients with CAIS, must be updated. Thus, it must be stated that the 56 kDa protein was under-expressed in most patients with CAIS. These

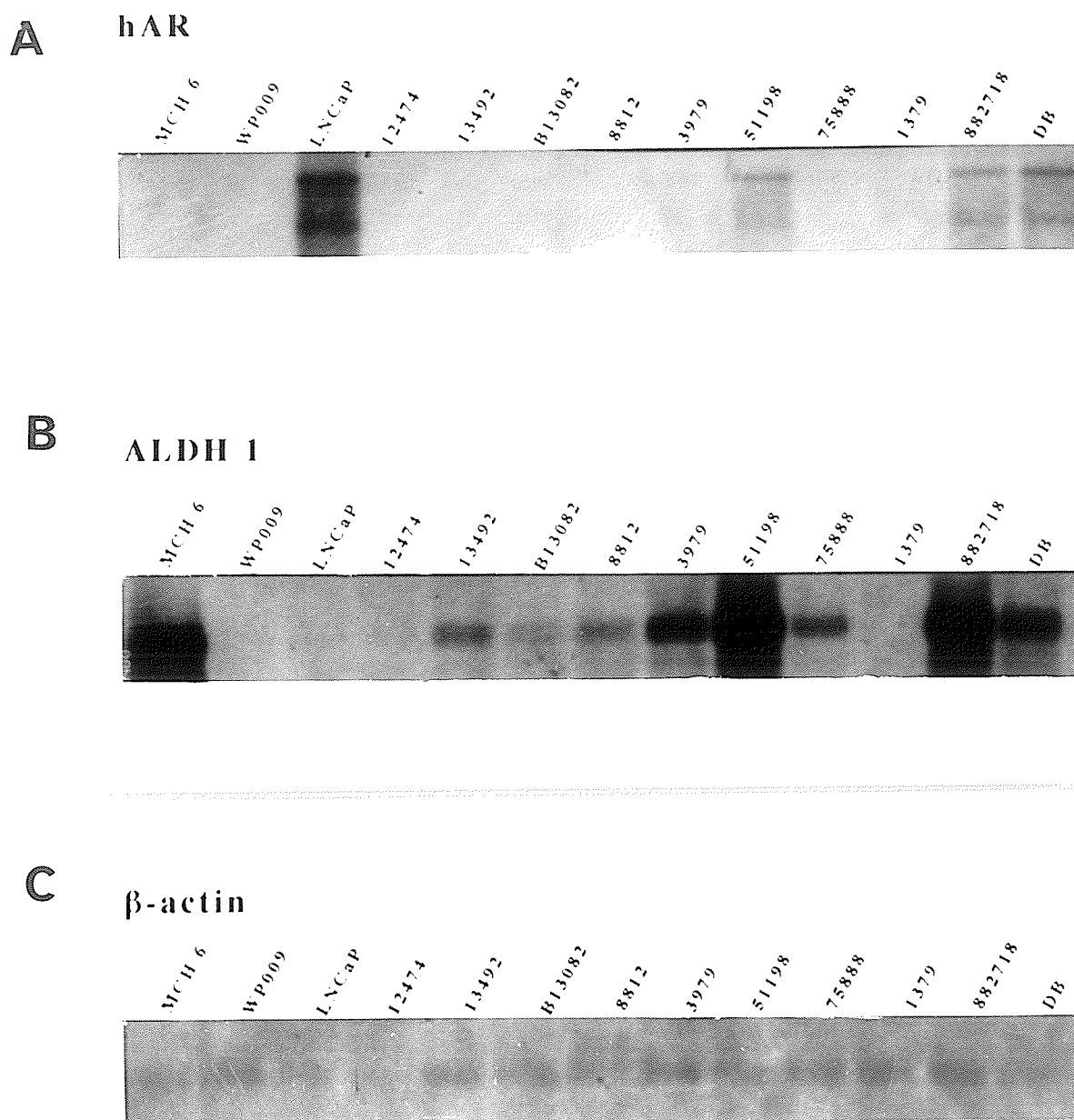


Figure 49. Northern blot analysis of mRNA from genital skin fibroblasts from normal (MCH 6) and patients with CAIS, from non-genital skin fibroblast (WPO09), and from LNCaP prostate carcinoma cells. A. Blot probed with human androgen receptor; B. Blot probed with ALDH 1; C. Blot probed with chicken β -actin for normalization of samples loaded in each lane.

analyses are enlarging the catalog of the baseline expression of the 56 kDa aldehyde dehydrogenase in our myriad of patient and normal samples, supporting the observation that its expression was altered in CAIS.

However, Yoshida *et al.* (1993) have pointed out that the alcohol dehydrogenase class 1 gene (AHD1) is also expressed in a genital skin fibroblast strain from an AIS patient, which also expresses ALDH 1. Whereas, AHD1 was not expressed in most CAIS patients as found for ALDH 1. Other marker genes used, ALDH 2, ALDH 3, glucose-6-phosphate dehydrogenase and actin, were found not to be different in normal and patient fibroblasts. The expression of the class 1 AHD gene(s) are under tissue-specific and developmental control (Ikuta, Yoshida, 1986) including regulation by androgens (Berger, Watson, 1989). Yoshida *et al.* (1993) proposed that these results indicate that the mode of tissue-and/or development-dependent regulatory mechanism has been altered in this particular cell line, and the cell line does not represent the original patient's genital cells. This also points to the possible transcriptional mode of regulation of the ALDH 1 gene by tissue specific and/or developmentally regulated factors.

2. Aldehyde dehydrogenase activity stain

Activity staining for aldehyde dehydrogenase, in cell extracts from GSF from controls and subjects with CAIS separated by non-denaturing isoelectric focusing, revealed that expression of ALDH 1 mRNA did not always result in aldehyde dehydrogenase activity (Fig. 50 A). The amount of 56 kDa protein in cell strain DB was not detectable by silver staining, could only be detected by photolytic labeling and the gene produced a low amount of ALDH 1 mRNA (Fig. 37), but has no detectable aldehyde dehydrogenase activity. Whereas NHL+, which expresses the 56 kDa abundantly at 4+ by protein stain, photolytic labeling or by mRNA, does have aldehyde dehydrogenase activity (Fig. 50 B). Could the ALDH 1 in DB be a variant aldehyde dehydrogenase with negligible activity? This experiment was not specific or sensitive enough to determine this. Furthermore, it was only performed once, and will have to be repeated. It might still be worth analyzing for the Msp I polymorphism found for the ALDH 1 locus in this patients' DNA.

Nevertheless, there does not seem to be a strict correlation of the specific androgen receptor mutations with respect to the resultant receptor phenotype and the expression of the ALDH1 message nor aldehyde dehydrogenase activities. Thus, the activity of the resultant aldehyde dehydrogenase in patient GSF must be a consideration in the determination of the role of ALDH 1 in CAIS.

A Aldehyde Dehydrogenase Activity in genital skin fibroblasts

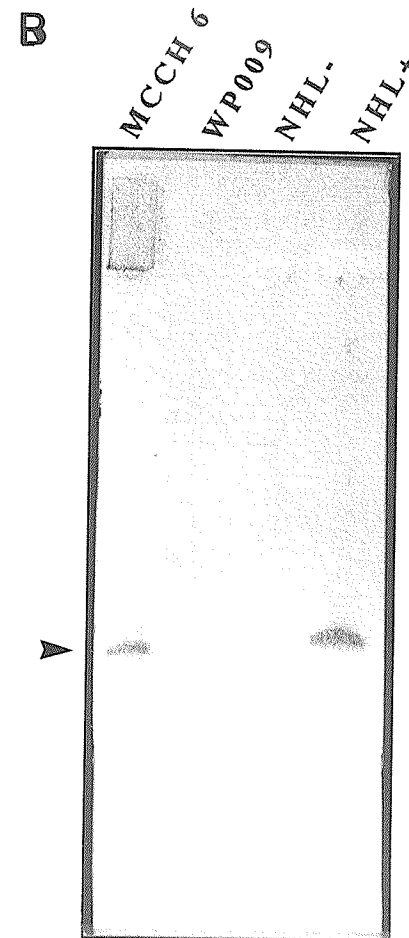
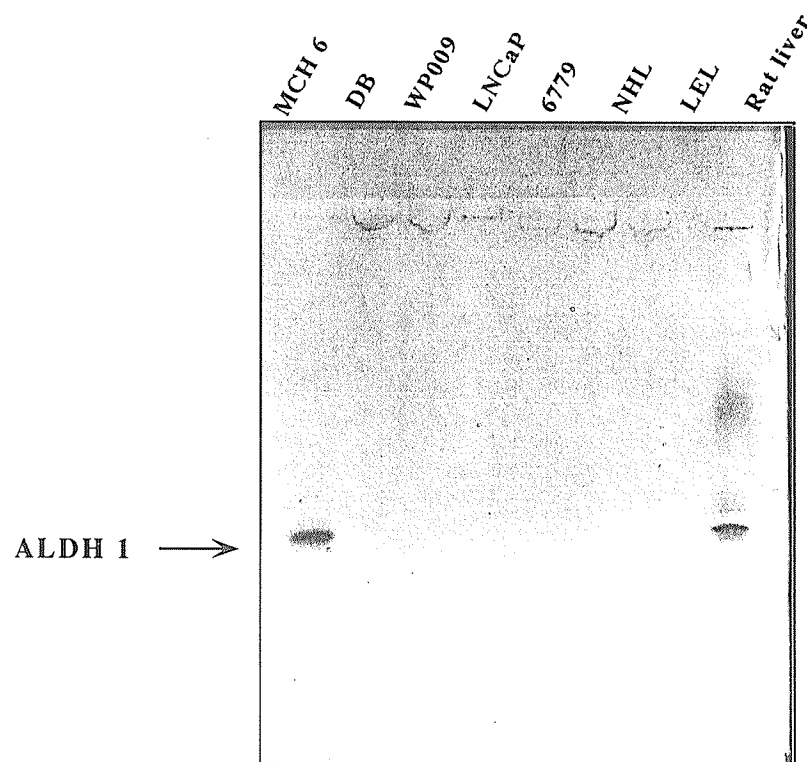


Figure 50. Activity staining for aldehyde dehydrogenase. (A) Cell extracts from GSF from controls and subjects with CAIS or from non-genital skin fibroblast (WPOO9), from LNCaP prostate carcinoma cells, and from rat liver homogenate. (B) Aldehyde dehydrogenase activity in cell strain NHL+ as compared to Control genital and non-genital skin fibroblasts and NHL- strain. The arrow points to the aldehyde dehydrogenase activity stain.

3. Cloning of the genomic 5'-flanking sequence

The 5' flanking region of genes are known to have sequences which are important for the regulation of its expression. The genomic 5'-promoter region of the ALDH 1 gene was isolated by PCR amplification and cloned into a pCRII vector by way of their complementary (T:A) overhanging nucleotides. The sequence for the 5' and 3' primers used was deduced from the published genomic sequence (Hsu *et al.* 1989). Primers used were ALDH 1 G I (5' GAATTCCCTAAAAGTCCTGCTGGCT 3') and ALDH 1 G II (5' GATTCGGCTCCTGGAAC 3') (Fig. 52). These primers had melting temperatures of 74°C and 64°C, respectively. The PCR reaction was optimized by varying the annealing temperature from 55 - 60 °C and the molar concentrations of the primers. The 723 bp fragment was amplified best using 20 - 25 pmol of each primer, 1.5 mM MgCl₂ and annealing at 57°C (Fig. 51). The fragment was sequenced from both directions using Sequenase® and end-labeled primers. The sequence was found to be identical to the reported sequence (Hsu *et al.* 1989) (Fig. 52).

4. Consensus *trans*-acting factor binding sites

An analysis of the 5' promoter region of ALDH 1 gene for the presence of cis-acting DNA elements which are targets for transcriptional activation and induction by transcription factors was performed using a computer program (DNA strider by Marck, 1989) and comparing known sequences. Several consensus cis-acting elements have been identified (Fig. 52). These include: three glucocorticoid response element half-sites; three GATA transcription factor binding sites (one of which coincides with the TATA box); a steroid response element from 11 β -hydroxylase; three AP-1 binding sites; an ETS oncogene binding site; a CAAT enhancer binding protein element; an octamer binding site; and a TATA-box binding site. No CACACA element was identified (up to -690 bp from the start of transcription), as proposed by Lindahl (1992), however, analysis of the 5' regions for other class 1 genes, like the mouse Ahd2 gene (Rongnoparut, Weaver, 1991) and the rat phenobarbital-inducible genes (Dunn *et al.* 1989), both have this sequence in their 5' promoter region. It has been shown that the noncanonical TATA sequence of the chicken β -globin gene (GATAAA) serves as a site for GATA-1 action. In this manner, GATA-1 functions to confer basal erythroid specificity to the promoter (Orkin, 1990). The zinc-finger transcription factor GATA-1 (previously known as GF-1, NF-E1 or Eryf 1 (Pevny *et al.* 1991) binds to GATA consensus elements in regulatory regions of the α - and β -globin gene clusters and other erythroid cell-specific genes. GATA-1 is found in abundance in erythroid cells, megakaryocytes, mast cells and in the testes, but not in other blood lineages or in other non-haemopoietic cells. GATA-2 has recently been found to have a more ubiquitous expression which includes GSF (Lee *et al.* 1991).

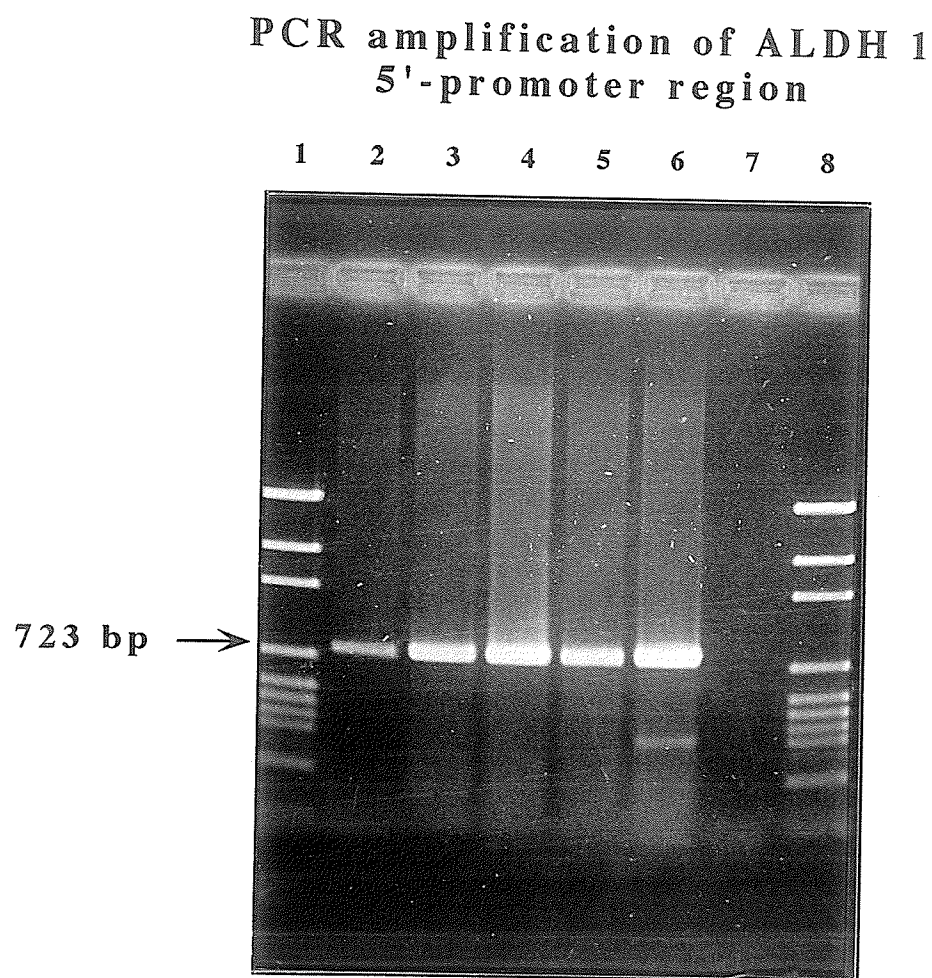


Figure 51. Polymerase chain reaction amplification of the 5'-promoter region of ALDH 1. Agarose gel electrophoresis of amplifications products using: lane 2. 50 ng of each primer; lane 3. 100 ng of each primer; lane 4. 200 ng of each primer; lane 5. 300 ng of each primer; lane 6. 400 ng of each primer; lane 7. 50 pmol of each primer but without any genomic DNA added; lanes 1 and 7. Hinf I, Rsa I and Sin I digests of pGEM-3 DNA as molecular weight markers.

ALDH 1 5' FLANKING REGION

-723

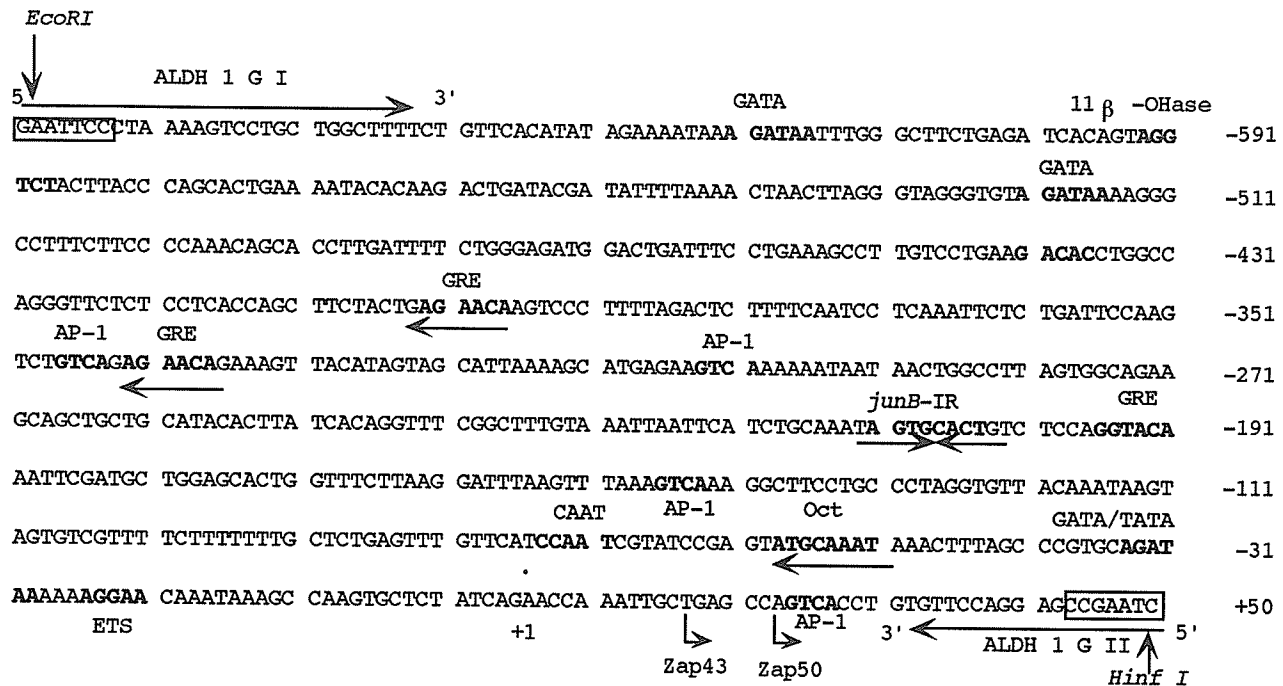


Figure 52. Sequence of the 5'-flanking region of ALDH 1. Complete sequence of the polymerase chain reaction amplified products. Consensus DNA binding motifs shown in bold type, long arrows showing 5' and 3'-end primers, arrows showing direction of consensus cis-acting elements, (↗) showing start of the two cDNA full-length clones, and (•) solid dot showing the start of transcription. GRE: Glucocorticoid response element (Nucleic Acids Res. 16:5263-5276, 1988); OCT: Octamer binding protein binding element (Science 245:371-378, 1989); ETS: ETS oncogene binding site (Nature 346:191-193, 1990); CAAT: CAAT enhancer binding protein element (Cell 32:695-709, 1983); GATA (JBC 266:16188-16192, 1991); *junB*-IR: *junB* inverted repeat binding protein (Nucleic Acids Res.19:775-781, 1991); TATA-box: TATA-binding protein element (Cell 18:1285-1297, 1979); AP-1: AP-1 binding element (Science 245:371-378, 1989); 11β-OHase: 11β-hydroxylase steroid response element (Molec. Endocrinol. 5:1552-1561, 1991).

Efficient activation in erythroid cells requires multiple GATA binding elements whereas a single element is sufficient for non-erythroid expression (Evans, Felsenfeld, 1991). In agreement with this, we have found three possible GATA binding sites (AGATAA) suggesting GATA binding may be an element which conveys expression of ALDH 1 in red blood cells and in genital skin fibroblasts. In addition, androgen response elements have been shown to be very similar to glucocorticoid response elements (Cato *et al.* 1988; Ham *et al.* 1988). Lastly, it has been found that the glucocorticoid receptor binds GATA-1 and interferes with its function before any interaction with DNA in mouse erythroleukimia (MEL) cells (Chang *et al.* 1993).

Delineation of the possible role of any of these consensus cis-acting elements *in vitro* and *in vivo* would help to define more specifically the normal and aberrant control of the ALDH1 gene. However, all these elements together show that the regulation of the ALDH 1 promoter may be quite complex. Thus, in a start towards analysis of whether any of these elements actively participate in the regulation of ALDH 1, DNA-protein interaction assays have been pursued.

5. DNA-protein electrophoretic mobility shift analysis

a. Nuclear proteins bind to the 5' flanking region of ALDH 1

A 157 bp 5'-most fragment (-723 to -566) has been analyzed for DNA-protein interactions using the electrophoretic mobility shift assay (Fig. 53). The fragments were retarded by proteins from nuclear extracts from cells that express the 56 kDa protein (GSF, MCH 6), and those that do not (non-GSF, WP009) or from chicken immature erythrocytes. There were two major gel retardation (GR) bands (GR I and GR II) which were specifically retarded. The distribution of the two different species of proteins were different between the extracts, i.e. WP009, 8812 and the chicken immature erythrocyte extracts mostly had GR II whereas, MCH 6 and DB extracts mostly had GR I.

b. Specificity of band-shift GR II

A band-shift similar to GR II (found most strongly in chicken immature erythrocyte, 8812 and WP009) can be shown to be a specific shift with WP009 nuclear extracts, as an example (Fig 54, right panel), and a smaller fragment (-723 to -645). The gels contained a similar percentage of crosslinker allowing the comparison of bands with different sized fragments when run to the same distance from the bottom of the gel. The band was seen to be shifted with 0.4 μ g of extract (lane

ALDH 1 promoter region DNA-protein mobility shift analysis using -723 to -566

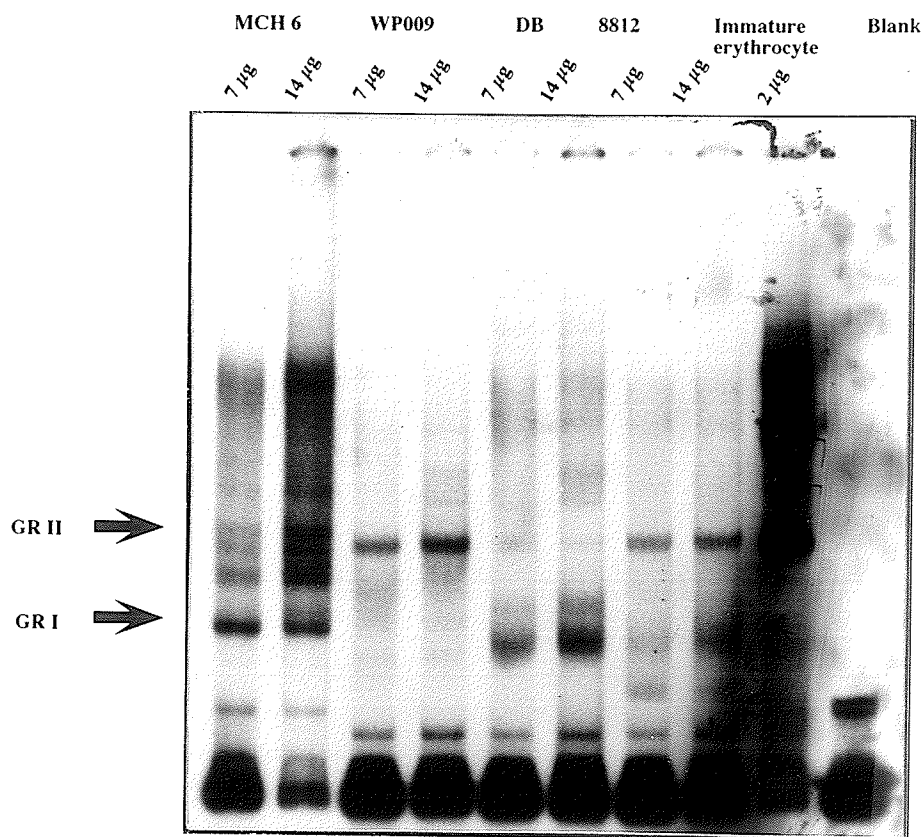


Figure 53. DNA-protein electrophoretic mobility shift analysis with 5' flanking region of ALDH 1. A 157 bp 5'-most (-723 to -566) of the PCR amplified fragment was used with nuclear extracts from GSF from normal (MCH 6) and from patients with CAIS (DB, 8812), non-GSF (WPO09) or from chicken immature erythrocytes.

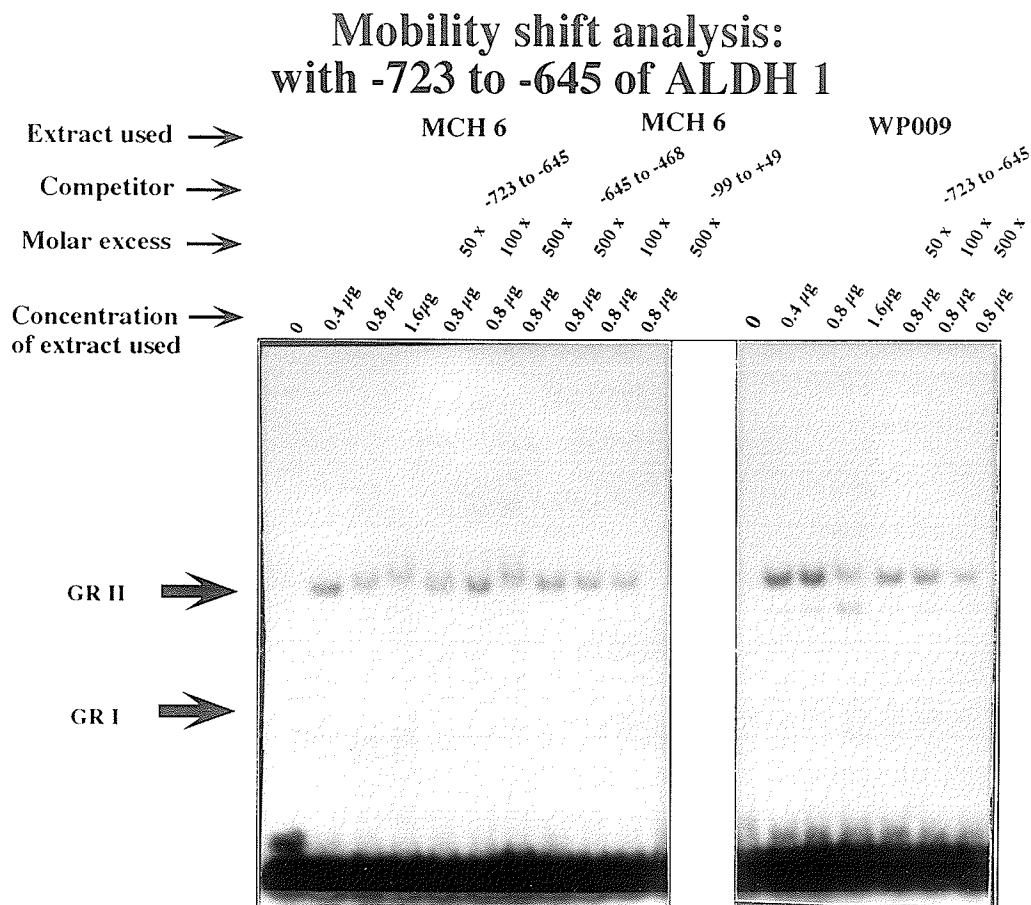


Figure 54. Mobility shift analysis with 78 bp (-723 to -645) fragment from the 5' flanking region of ALDH 1, using nuclear extracts from genital skin fibroblasts (left panel) and non-genital skin fibroblasts (right panel). Lanes 1 to 4, in the left panel, increasing amounts of MCH 6 extract; lanes 5 to 10 using 0.8 µg MCH 6 extract and lanes 5 to 7 competition with increasing molar excesses of the same but non-radioactive fragment (-723 to 645); lane 8 competition with 500-fold molar excess of non-identical fragment (-645 to -468); lanes 9 to 10 competition with increasing amount of another non-identical fragment (-99 to +49). The right panel shows lanes 1 to 5 with increasing amounts of WPOO9 nuclear extract; lanes 5 to 7 using 0.8 µg of extract and competition with molar excesses of the same but non-radioactive fragment (-723 to 645).

2, counting from left to right) and could be partially competed away (Lanes 5 - 7) with a 500-fold molar excess of the identical but radioinert fragment.

On the same gel (left panel) it can be seen that nuclear extracts from MCH 6 also have this specific band-shift (lanes 2 - 8). However, this band again cannot be specifically competed away even with the use of a 500-fold molar excess of the identical but radioinert fragment, nor with other non-identical fragments from the promoter region.

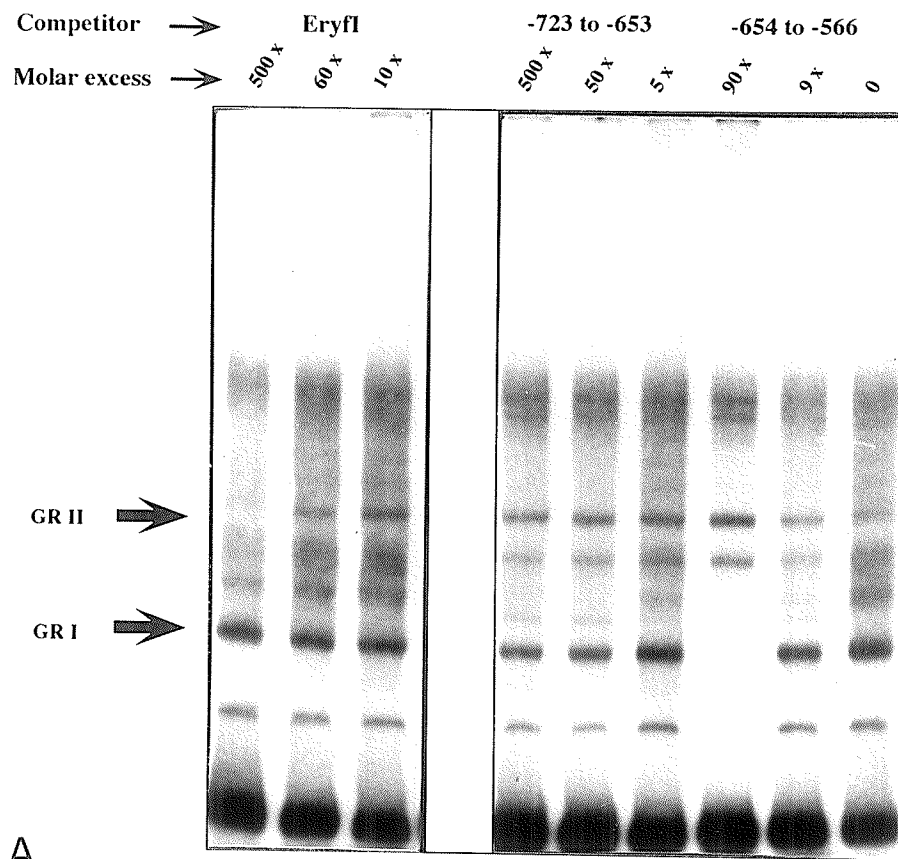
This lack of complete competition with even a 500-fold molar excess may be due to the following: i) the competition experiment was performed by adding the labeled and the non-radioactive fragment to the side of the microcentrifuge tube and mixing them to begin the reaction. This may have allowed enough radioactive fragment to bind to the protein factor(s) with enough avidity hampering complete competition to be seen; ii) there may be more protein factor available to bind than the small amount of fragment used to bind; iii) the fragments were insufficiently purified leaving residues of agarose/acrylamide which interfere with the binding and competition (Dr. Susan Kaspar, personal communication).

c. Specificity of band-shift GR I

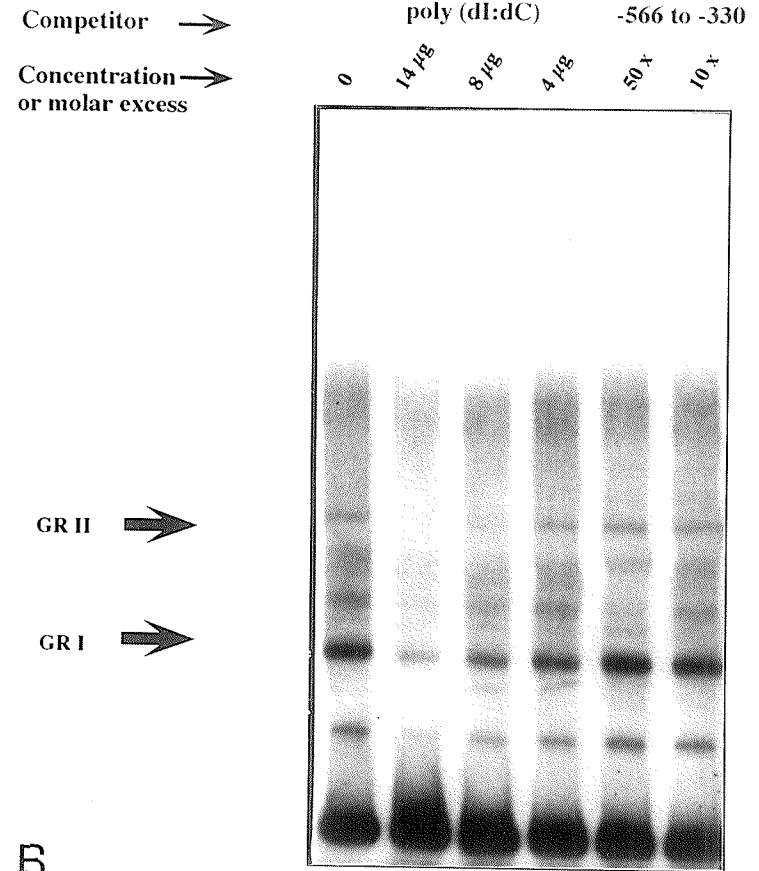
A band-shift similar to GR I could be seen to be specific (lane 1 counting from right to left, Fig. 55 A) when extracts of MCH 6 were used with a 3'-end fragment (-654 to 566) of the 157 bp and no competitor. An almost complete competition of the radioactive band-shift was seen with 90-fold molar excesses of the identical but radioinert fragment (lane 3). Lanes 4 - 6 show that the 5'-end fragment that bound a band similar in size to GR II cannot compete away this specific band nor can an oligonucleotide specific for a GATA 1 element lanes (left panel, lanes 1 - 3). The GATA 1 (Eryf1) element seemed to compete away a second band similar to GR II in lane 9 but non-specific competitor (dI:dC) also suppressed binding to this fragment (Fig. 55 B, lane 5 from right). The GR I band-shift was also seen to be a specific-shift (in this figure) and was not competed with an overwhelming excess (14 μ g compared to the normal 1 to 2 μ g) of the non-specific competitor poly (dI:dC). Lanes 3 - 5 show that the band-shift was specific whereas bands above were competed away. Lanes 1 and 2 show that a fragment 3' of the 157 bp cannot compete away this specific shift.

Thus, these mobility shift experiments revealed two species of protein-DNA interactions bound to a 157 bp fragment (-723 to -566) of the ALDH 1 promoter. When the 157 bp fragment was cut into smaller fragments it was found that GR II was bound by the 5'-end (-723 to -645) and GR I

Mobility shift analysis: MCH 6 nuclear extracts and -654 to -566



A



B

Figure 55. Mobility shift analysis with nuclear extracts from MCH 6 and fragment -654 to -566 from the 5' flanking region of ALDH 1. A. The right panel shows competition of the specific band-shift with increasing molar excesses of the same but radioinert fragment (lanes 1 to 3, counting from right to left); lanes 4 to 6 show no competition of any bands with a fragment (-723 to -653) 3' to the fragment used in the shift experiment; The left panel shows a lack of competition of the specifically shifted GR I band with an oligonucleotide specific for GATA 1 (EryfI). B. The specificity of the GR I band is seen even in the presence of 14 µg of non-specific competitor DNA (lane 5, counting from right to left); and again lack of competition with a fragment (-566 to -330) 3' to the fragment used in the shift experiment.

was bound by the 3'-end (-654 to -566) of the 157 bp fragment. These protein-DNA interactions were fragment-specific as they could only be competed away by molar excesses of the same fragment. The interactions were extract-specific as each was found to be more abundant in a specific set of extracts with neither extracts tested having equal amounts of the two species. *In vitro* footprinting of the 5'-end of the 723 bp of the ALDH 1 promoter was used to reveal the specific nucleotides that were protected by any specific protein-DNA interactions. Using a 515 bp end-labeled fragment (-723 to -208) and nuclear extracts from GSF DB, *in vitro* footprint analysis revealed several protein-DNA interactions (data not shown). Several DNase I hypersensitive site were created, several regions were protected from DNase I digestions, and several minor DNase I sensitive sites created. Two of the sites protected resided near two of the consensus GATA binding sites. This indicated that it may be a result of protection of the DNA sequence by the presence of GATA transcription factor binding near the two regions region.

These results suggested that the 5' promoter region of ALDH 1 gene was bound by protein factors from nuclear extracts and is a start to the dissection of its complicated expression in GSF from patients with complete androgen insensitivity due to receptor defects and from normal.

IV. Summary

Having analyzed the processes of normal sexual development with respect to the hormones required, the androgen receptor and its mutations, and the resultant biochemical and sexual phenotypes observed, there still remains a void of information on the complete process(s) required for sexual differentiation and development. The absence of the 56 kDa protein in genital skin fibroblasts from patients with complete androgen insensitivity syndrome provided an opportunity to examine a protein that may have a role in the pathogenesis of this syndrome.

This relatively abundant 56 kDa protein in genital skin fibroblasts can be identified by its coordinates on a two-dimensional gel stained by Coomassie Blue or silver, and from an autoradiogram of [^{14}C] or [^3H] amino acids or [^{35}S]methionine labeled proteins from genital skin fibroblasts. The protein is normally found as a 56 kDa protein doublet with isoelectric points of 6.5 (left spot) and 6.7 (right spot). We have shown that the normal expression of the 56 kDa protein can vary from being expressed abundantly (MCH 6, 4+) to being expressed with less abundance (FXF, 1+). The protein, however, has generally not been found in non-genital skin fibroblasts by all methods of analysis.

We have shown that the two species of the 56 kDa protein doublet are identical. The two spots have been found to have virtually identical peptide fragments upon partial proteolytic digestion with chymotrypsin suggesting that only minor structural modifications result in the difference in isoelectric points between the two spots. The cause of this subtle difference still remains unknown as the proteins have been found not to be phosphorylated nor glycosylated. Whether the molecular difference(s) were due to an artifact of the method of analysis (carbamylation by urea) could not be dismissed. In addition, we have successfully produced a polyclonal antiserum in rabbit from a partially purified fraction of the 56 kDa protein (Pereira *et al.* 1990). This antiserum, made to the pI 6.7 (right spot) 56 kDa protein, recognizes both protein species on Western immunoblots. This has helped to confirm that the two proteins have structural identity. The 56 kDa protein is not a heat shock protein and its long half-life probably contributes to its abundance. In addition, *in vitro* translation of mRNA from genital skin fibroblasts and immunoprecipitation of the translation products yielded a protein of molecular mass 56,000 daltons. This would suggest that the 56 kDa protein was synthesized directly and was unlikely a stable degradative product of a larger precursor molecule.

The 56 kDa protein as stained by silver on two-dimensional gels is missing in genital skin fibroblasts of most patients with complete androgen insensitivity syndrome (Nickel *et al.* 1988). These patients have a defective androgen receptor and it was suggested that the 56 kDa protein might be related to the androgen receptor protein itself. The 56 kDa protein from GSF from these patients was specifically labeled with methyltrienolone. The concentration for the half-maximal saturation of ligand required to bind the 56 kDa protein under conditions of photolytic labeling with mibolerone was found to be approximately 4 μ M. Thus, while the detection of the 56 kDa protein and methyltrienolone labeling generally correlated with the high affinity androgen receptor binding activity, the photolytic labeling in patients with a defective androgen receptor was clearly not to the classical androgen receptor.

Subsequently, in a collaborative study, we were able to show that the 58 kDa androgen receptor protein (Kovacs *et al.* 1989) was indistinguishable from the 56 kDa protein by one or two dimensional gel electrophoresis and on immunoblots detected with the partially purified antiserum (Belsham *et al.*, 1989). These finding strengthened the postulate of a structural and/or genetic relationship between the classical androgen receptor and the 56/58 kDa androgen-binding protein.

Although this protein has molecular characteristics very similar to some previously ascribed to the androgen receptor (Chang *et al.* 1982; Tai *et al.* 1986), this protein could not be the classical receptor because: i) its concentration is too high; ii) its affinity for androgen is too low; iii) its size is too small for the intact androgen receptor monomer (Johnson *et al.* 1987); iv) it does not show upregulation as does the androgen-binding activity; four patients with androgen receptor defects and minimal androgen-binding activity express the 56 kDa protein in similar quantities to normal cells and with photolytic binding characteristics indistinguishable from controls. Furthermore, these same two cell strains with defective androgen receptors exclude the possibility that the 56 kDa protein is an androgen induced protein.

To accommodate these findings, we postulated that the 56 kDa protein shared some sequences with the androgen receptor protein. Thus, the gene for the 56 kDa protein and that for the androgen receptor could have evolved from a common ancestor or these proteins could share the same gene but were produced by use of separate promoters, differential splicing of mRNA and/or through different polyadenylation sites (Leff *et al.* 1986).

Subsequently, we discovered that the 56 kDa protein was expressed in genital skin fibroblasts from a patient with complete androgen insensitivity syndrome due to a complete deletion of the androgen receptor gene (Trifiro *et al.* 1991). This conclusively determined that the 56 kDa protein

was not synthesized from the androgen receptor gene, the resolution of which required the cloning and sequencing of the cDNA for the 56 kDa protein.

We constructed cDNA libraries and successfully cloned a partial cDNA for the 56 kDa protein (Pereira *et al.* 1991). The proof for this was several fold: i) a probe of the clone detected a 2.2 kb message which paralleled the expression of the 56 kDa protein in the cell strains as determined by two-dimensional gels; ii) *in vitro* translation of mRNA hybrid-selected with the clone produced a protein of molecular mass 56,000 daltons; iii) this same protein was specifically immunoprecipitated with the partially purified antiserum to the 56 kDa protein; iv) separation of the translation products by two-dimensional gel electrophoresis revealed the characteristic 56 kDa doublet at the proper coordinates; v) sequencing of this cDNA and comparing it to the GenBank database identified it to be homologous to a previously cloned human cytosolic aldehyde dehydrogenase (ALDH 1, EC 1.2.1.3), and the specific labeling of proteins in genital skin fibroblasts comigrated with the activity band developed for aldehyde dehydrogenase activity on non-denaturing isoelectric focusing gels; vi) the subunit size on denaturing gels, the isoelectric point on non-denaturing gels and the message size for the 56 kDa protein all concur with those for the enzyme; vii) data on the enzyme also correspond with the observation that ALDH 1 was neither expressed in skin fibroblasts which must now be specified as non-genital skin fibroblasts. Additionally, this clone was found not to be linked to the X-Chromosome, unlike the androgen receptor. These results further established that the 56 kDa protein was not synthesized from the androgen receptor gene nor a degradative product of the receptor protein. The full-length cDNA for the 56 kDa protein was ultimately cloned and identified to be completely identical to the human cytosolic aldehyde dehydrogenase gene from human liver.

Analysis of Northern blots of mRNA from genital skin fibroblasts from patients with complete androgen insensitivity syndrome with an ALDH 1 cDNA fragment reveal a spectrum of expression levels of the 56 kDa protein. This implies that the previous analysis, based on silver staining of two-dimensional gels, was not sufficiently sensitive to determine that the 56 kDa protein was under-expressed and not missing in some genital skin fibroblast strains from patients with complete androgen insensitivity syndrome as previously reported.

However, the fact that one genital skin fibroblast strain, which expresses a low amount (1+) of the 56 kDa protein, did not have aldehyde dehydrogenase activity (as determined once by zymogram activity staining) will have to be reexamined. Only a larger analysis of many more patient cell strains for ALDH 1 activity will be necessary to make a conclusion of the functionality of the ALDH 1 enzyme in patient cells. Restriction fragment length polymorphisms at the ALDH 1

locus, found for some alcohol-flushers, was not identified in subjects with complete androgen insensitivity syndrome, and could not explain the lack of ALDH 1 activity in these patients' GSF.

Based on our results (Nickel *et al.* 1988; Pereira *et al.* 1991), one other group has analyzed ALDH 1 message and protein (by immunostaining) expression in genital skin fibroblasts from normal and from patients with complete androgen insensitivity and receptor-negative binding activity (Yoshida *et al.* 1993). They found the same correlation of the absence of ALDH 1 in most patient cells but also identified a message and protein in one patient with androgen insensitivity syndrome. Furthermore, they found a one-to-one correlation of ALDH 1 message to protein as we have proposed. They also concurred with the initial findings (Nickel *et al.* 1988), that ALDH 1 is a marker of the heterogeneous spectrum of defects found in androgen insensitivity syndrome but they did not analyze the molecular defects in androgen receptor gene from their patient genital skin fibroblasts.

The molecular defects in the androgen receptor for some of our patients have been identified (Trifiro *et al.* 1991; Prior *et al.* 1992). Upon analysis, no strict correlation could be identified in these GSF strains between the mutations in the androgen receptors and the receptor phenotypes with the expressions of the ALDH1 message or of its activities.

These facts underscore the multifactorial nature of phenotypic sexual development, and the determination of the role of ALDH 1 in normal cells and in cells with a defective androgen receptor required an analysis of the mechanism of regulation of its gene.

Analysis of the 723 bp 5'-flanking the ALDH 1 gene has revealed several cis-acting DNA elements. These include: three glucocorticoid response element half-sites; three GATA transcription factor binding sites (one of which coincides with the TATA box); a steroid response element from 11 β -hydroxylase; three AP-1 binding sites; an ETS oncogene binding site; a CAAT enhancer binding protein element; an octamer binding site; and a TATA-box binding site.

Initial experiments have concentrated on defining the minimal regions of the promoter which have consensus cis-acting binding sites. Electrophoretic mobility shift experiments with fragments of a 157 bp region have revealed proteins that bind specifically to this region. The fragments were retarded by proteins from nuclear extracts from cells that express the 56 kDa protein (GSF, MCH 6), and those that do not (non-GSF, WP009) or from chicken immature erythrocytes. Two major bands (GR I and GR II) were specifically retarded, with the distribution of the two species of proteins varying between the different nuclear extracts. This may suggest that different protein

factors could be bound to this promoter region in the different cell strains. Chicken immature erythrocytes have high expression of GATA-1 factor (Penner, Davie, 1992) and this fragment has two elements identical to the consensus GATA binding element. When the 5'-most 157 bp was cut into smaller fragments it was found that a band similar to GR II was retarded by the 5'-end fragment and another band similar to GR I was retarded by the 3'-end fragment. A GATA-1 oligonucleotide was unable to compete for the binding of either band-shift.

Further analyses will reveal whether the presence of the different trans-acting factors are responsible for the regulation of the 56 kDa protein expression in genital skin fibroblasts from normals and patients with complete androgen insensitivity syndrome.

V. Future Implications

The identification of the 56 kDa protein as an aldehyde dehydrogenase led us to consider what role it might play in genital skin fibroblast cells. It is known that the ALDH 1 enzyme can oxidize retinal to retinoic acid (Ambroziak, Pietruszko, 1991; Lee *et al.* 1991; Connor, Smit, 1987; Leo *et al.* 1989; McCaffery *et al.* 1991; McCaffery *et al.* 1992; McCaffery, Dräger, 1993). Already in 1962 a "steroid-sensitive" aldehyde dehydrogenase was described which could oxidize retinene to retinoic acid (Futterman, 1962; Elder & Topper, 1962). In a study of human ALDH isozymes, Lee *et al.* (1991) reported that ALDH1, but not other ALDH isozymes, oxidized retinal. The K_m of purified ALDH1 for retinal has been estimated to be about $0.06 \mu M$ by analysis of single reaction curves at pH 7.5 (Yoshida *et al.* 1993) which is compatible with the retinal concentration in tissues which is estimated to be about $0.1 \mu M$ or less (McCormick, Napali, 1982).

The alcohol form of vitamin A (retinol) can be regarded as a physiologically significant substrate for vertebrate alcohol dehydrogenase. Retinol has a long-chain aliphatic alcohol group and has long been known to be a very good substrate for mammalian alcohol dehydrogenase (Bliss, 1951; Zachman, Olson, 1961; Mezey, Holt, 1971; Van Thiel *et al.* 1974). A mechanism for the regulation of retinoic acid synthesis involving the feedback induction of AHD3 gene (by retinoic acid), which converts retinol to retinal, has been proposed (Zgombic, Duester, 1993; Duester *et al.* 1991). Retinal produced from retinol oxidation is generally required by vertebrates for vision (Futterman, 1963; Wald, 1951), but more importantly, vertebrates require retinal for the aldehyde dehydrogenase catalyzed synthesis of retinoic acid, a known regulator of gene expression (Lee *et al.* 1991b; Glass *et al.* 1991).

The effects of retinoic acid are transduced through nuclear retinoic acid receptors (RAR) which, in the presence of ligand(s), are transformed into transcription factors able to bind DNA near the promoters of some genes (Glass *et al.* 1991). The retinoic acid receptor family include several classes (RAR- α , RAR- β , RAR- γ and RXR) and within each class are subclasses (RAR- α 1, RAR- α 2 etc.), and the complexes thus produced act as extremely potent transcriptional regulators for the genes which are involved in embryonal development and cell differentiation (Ragsdale, Brockes, 1991). Differential expression of these receptors is undoubtedly important for correct transduction of the retinoic acid signal in various target tissues (Dollé *et al.* 1989). Retinoic acid is known to induce several genes encoding homeobox-containing proteins which regulate embryonic pattern formation by activating transcription of another set of genes in a morphogenetic cascade (Simeone *et al.* 1990). Genes encoding extracellular matrix proteins needed for differentiation of many cells,

such as laminin B1 and collagen, are also induced by retinoic acid (Vasios *et al.* 1989). Finally, the RAR β gene is induced by retinoic acid in teratocarcinoma and hepatoma cell lines (Hu, Gudas, 1990; De The *et al.* 1990) suggesting that the autoregulation of this retinoic acid receptor species is crucial to the morphogenic differentiation process.

A Hypothesis on the role of ALDH1 in the pathogenesis of complete androgen insensitivity syndrome

It is obvious from the complete androgen insensitive phenotype that numerous genes must have an altered expression to result in the female phenotype in contrast to the wild-type male. Overall, we think that altered transcription of the many unknown genes is brought about by a cascade of transcription factors altered in their level of expression and/or activities. Some of these factors may also regulate each others' expression levels as exemplified by the first transcription factor which regulates this hierarchical cascade, the androgen receptor. In complete androgen insensitivity, the mutant receptor results in an altered expression of specific genes involved in sexual differentiation or other transcription factors cumulatively resulting in a different sexual phenotype, namely female. We know virtually nothing about which genes might be involved. However, one possible gene is ALDH 1 which we have found to be under-expressed in GSF of CAIS patients. This is presented in the central column of the scheme in Figure 56. Another group, Yoshida *et al.* (1993) have proposed a very similar model based on observations and those of others (Nickel *et al.* 1988; Pereira *et al.* 1991). As this enzyme could produce retinoic acid, a known morphogen, the altered production of retinoic acid could be responsible for the altered transcription of many other genes through mediation of the retinoic acid receptors.

The androgen-binding activity of the enzyme is of possible significance as well. It has been suggested that the steroid-binding capacity of the class 1 enzyme may play a role in the dorso-ventral segregation of the mouse *AHD2* (dorsal retina) and a retinal dehydrogenase (ventral retina) in the embryonic retina: it might represent a mechanism to suppress expression of the ventral enzymes in cells (dorsal retina) that contain *AHD2* (McCaffery, Dräger, 1993) by increasing its activity. Previous data for the enzyme (Elder & Topper, 1962) suggests inhibition of retinoic acid formation by androgens. However, using purified preparations of the enzymes ALDH 1 and ALDH2, Yoshida *et al.* (1993) have shown that there is an approximately 30 % activation of retinal oxidation by ALDH1 in the presence of 5 μ M dihydrotestosterone. No oxidation was observed with ALDH 2. If ALDH 1 is under-expressed in complete androgen insensitivity as we have found, then this would have an effect of diminishing the production of the morphogen retinoic acid.

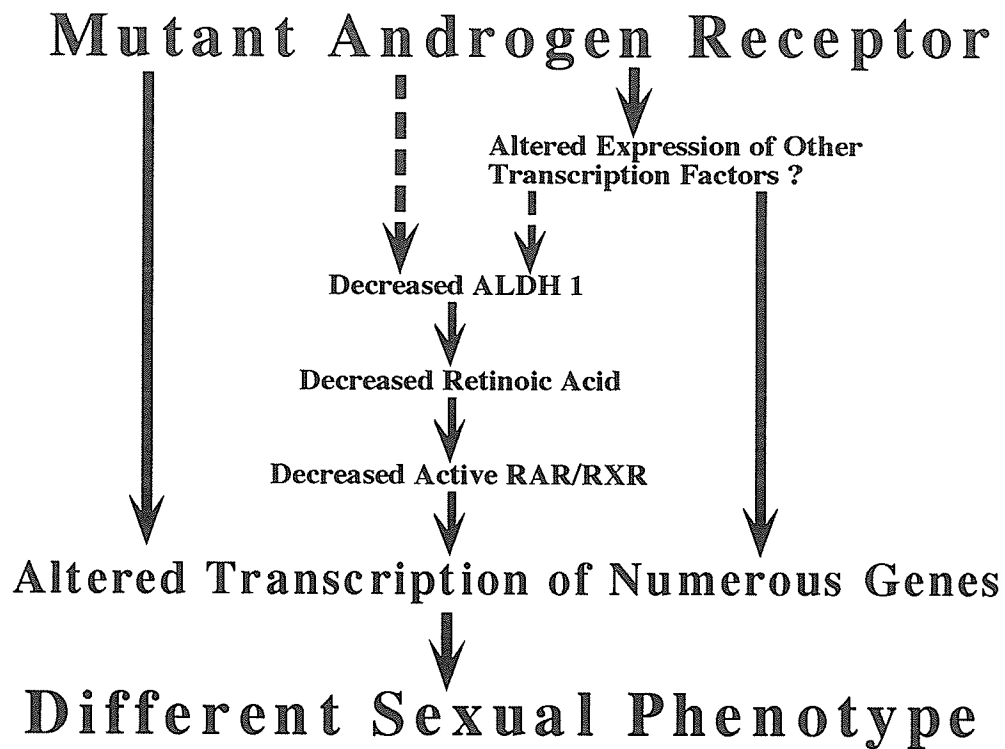


Figure 56. Speculations on the molecular pathogenesis of complete androgen insensitivity syndrome.

Thus, the scheme in Figure 56 leads to the following hypothesis:

Aldehyde dehydrogenase is required for the development of the male (female) phenotype by producing the morphogen retinoic acid in precursor tissues at the appropriate time during development.

From Table 8 it is apparent that two AIS patients do express the protein normally, yet they do exhibit the full clinical picture of complete androgen insensitivity. If the expression of ALDH 1 in GSF faithfully reflects the situation in the anlagen for the external genitalia during the critical stages of development, then the hypothesis presented above is untenable. Thus, it is important to analyze for the expression of ALDH 1 at the time prior to differentiation of the indifferent sexual anlagen.

Furthermore, at this time we do not understand the mechanism by which mutations in the androgen receptor gene lead to the under-expression of ALDH1. The most obvious mechanism, one of conventional androgen induction, does not appear to operate in GSF, as we could never influence the expression of ALDH1 by culture conditions with androgens, antiandrogens or serum-free media. Furthermore, the normal expression of ALDH1 in the two exceptional patients (Table 8) provides strong genetic evidence against a simple mechanism of androgen dependence for the expression of ALDH1. The 5'-promoter region is currently being analyzed for sequence-specific DNA-binding proteins by gel mobility shift assays. Differences in band shifts between cell strains that express ALDH1 and those that do not have been observed in preliminary experiments. Thus, we speculate that the control of expression is influenced by other transcription factors, some of which may be directly controlled by the androgen receptor, and possibly other epigenetic mechanisms not yet understood.

The attractiveness of the hypothesis that ALDH 1 plays a pivotal role in the development of the altered phenotype in complete androgen insensitivity is that it can be tested. It would be difficult to test in man, because it would be relatively meaningless to try this in human genital skin fibroblasts, where we fortuitously found the under-expression of ALDH 1 (Nickel *et al.* 1988; Pereira *et al.* 1991). Rather, it is important to test this in the precursor tissue or anlagen for the internal and external genitalia at just the right time during embryogenesis. Therefore, the hypothesis should be tested using *situ* hybridization techniques in an animal model of complete androgen insensitivity syndrome, the *Tfm* mouse. Of special interest will be the anlagen for external genitalia identified as the genital tubercle (Dollé *et al.* 1990), the sexually undifferentiated precursor of the male and female external genitalia. In support of our scheme above is the recent finding that the various retinoic acid receptor transcripts, especially for the α -isoform can be found in these precursor

structures at 12.5 to 14.5 days post-fertilization (Dollé *et al.* 1990). It should be possible to test whether ALDH1 transcripts can be found in these structures at the same time and whether clear differences will be noticeable in *Tfm* vs. wild-type and male vs. female animals.

Lastly, if the results are positive, they would also lend support to the *in vitro* observations of a function for ALDH 1, that is, it may be one aldehyde dehydrogenase isoform that can produce retinoic acid *in vivo* at a time prior to differentiation of the anlagen to the external genitalia.

VI. Conclusions

Characterization of the 56 kDa androgen-binding protein has revealed that:

1. The two species of the 56 kDa protein doublet are identical and did not arise due to phosphorylation nor glycosylation.
2. The 56 kDa protein was not found to be a heat shock protein and its abundance is probably due to a long half-life.
3. The 56 kDa protein has low-affinity but high-capacity androgen-binding ability.
4. Isolation and analysis of the cDNA encoding the 56 kDa protein has revealed that it is identical to the human liver cytosolic aldehyde dehydrogenase 1 isozyme and that it is under-expressed in genital skin fibroblasts from patients with complete androgen insensitivity.
5. This proved that the 56 kDa protein is neither synthesized from the androgen receptor nor was it a stable degradation product of the receptor protein.
6. Nuclear proteins, which are differentially expressed in cells which have the 56 kDa protein present and in those that do not, are found to bind to a portion of the 5' flanking region of ALDH 1.
7. A mechanism is proposed for the role of ALDH 1 in the development of the different sexual phenotypes and in the pathogenesis of complete androgen insensitivity syndrome.
8. We postulate that one of its biological roles is to produce the morphogen retinoic acid in the precursor tissues of the sex organs at the appropriate time during sexual development.

VII. References

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