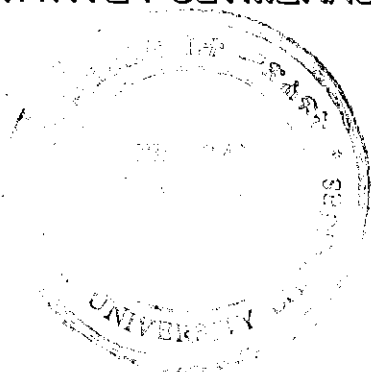


**ANALYSIS OF TESTICULAR GROWTH FACTORS BY
QUANTITATIVE POLYMERASE CHAIN REACTION**

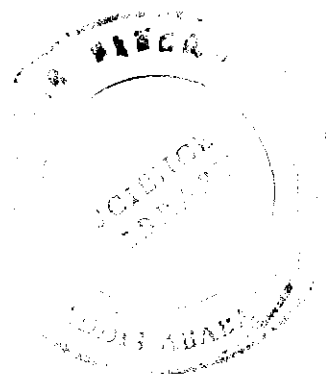


**A THESIS PRESENTED TO THE SCHOOL OF GRADUATE STUDIES
ADDIS ABABA UNIVERSITY
IN PARTIAL FULFILMENT OF THE REQUIREMENT OF
THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY**

by

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Addis Ababa, February ,1996



ACKNOWLEDGMENT

I am deeply indebted to my advisors Dr. Yisehak Worku, AAU-FM, Dept. of Biochemistry, Dr Mikael Holst and Dr Olle Soder, Karolinska Hospital, and other for their invaluable effort in educating me in their project and training me with the techniques, and for all their efforts to finalise the experiments, correcting the thesis. I thank the staff members of Department of Biochemistry for their endeavour and support during the study period. I am deeply indebted to all members of the Department of Paediatric Endocrinology, Karolinska Hospital for their enthusiastic participation in my training and their familial and friendly hospitality during the training period at Karoliniska. I am also grateful for Dr Hakan Meurner of AHRI for allowing me to use the lab. facility during sample preparation and processing. At last I would like to express my gratitude to my families and friends for their continuous moral support in the process and brought the work to completion.

- This study was conducted in collaboration between Department of Biochemistry AAU-FM and Department of Paediatric Endocrinology, Karolinska Hospital, and sponsored by SAREC.

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Abbreviations

A	Adenine
ACTH	Adrenocorticotrophic hormone
cAMP	cyclic Adenosine Mono Phosphate
AP-1	Activating Protein - 1
AU	Adenine-Uracil
C	Cytosine
CRF	Corticotrophin Releasing Factor
DEPC	Di ethyl pyrocarbonate
DNA	Deoxyribonucleic acid
c-DNA	complementary- DNA
ds-DNA	double stranded- DNA
ss-DNA	single stranded- DNA
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immuno Sorbent Assay
FGF	Fibroblast Growth Factor
FSH	Follicle Stimulating Hormone
G	Guanosine
GRE	Glucocorticoid Response Element
IL-1	Interleukin-1
ICE	Interleukin-1 Converting enzyme
IL-1R	IL-1 Receptor
rIL-1	recombinant IL-1
tIL-1	Testicular IL-1
IL-6	Interleukin-6
KD	Kilo Dalton
LH	Luteinizing Hormone
LHRH	Luteinizing Hormone Releasing Hormone
NF	Nuclear Factor
NGF	Nerve Growth Factor
NTP	Nucleotide triphosphate
dNTP	deoxyribonucleotide triphosphate
LPS	Lipopolysaccharide
PCR	Polymerase Chain Reaction
PGE-2	Prostaglandin E-2
PK-A	Protein Kinase- A
PLA-2	Phospholipase A-2
RIA	Radioimmuno assay
RNA	Ribonucleic Acid
mRNA	messenger RNA
RT-PCR	Revers transcription-PCR
TAE	Tris Acetate EDTA
Taq	Termus aquaticus
TE	Tris EDTA
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
UV	Ultra Violet

ABSTRACT

Expression of testicular Interleukin-1 α was analysed both at the level of biologically active peptide and its encoding mRNA, using a sensitive bioassay, Thymocyte proliferation assay, and Polymerase Chain Reaction. In order to analyse the relative change in the level of IL-1 α mRNA expression a Quantitative PCR technique was developed involving an internal standard IL-1 α RNA construct derived from rat macrophage IL-1 α cDNA preparation.

The IL-1 α mRNA expression during postnatal rat development could be detected by PCR as early as 20 days, postnatal, and The intensity of amplified cDNA band was found to increase with increasing days of postnatal age.

By using Quantitative PCR, a relative increase in the level of amplified IL-1 α cDNA could be demonstrated starting from day 20, and this increase in the level of amplified cDNA is found to reach a sustained high level from 30 to 60 postnatal days of rat testicular development. This study shows that the level of IL-1 α mRNA rises in parallel to the level of IL-1 α peptide during maturity, previously demonstrated, and suggests that it is controlled at the level of mRNA expression, probably at transcriptional level.

The role of testosterone in the regulation of tIL-1 α was analysed using rats treated with, EDS with and without testosterone replacement, and androgen receptor blocker, cyproterone acetate. The result showed that EDS pretreatment resulted in an increase in the level of both IL-1 α bioactivity and the level of its mRNA. Exogenous testosterone replacement along with EDS treatment significantly reduced the effect of EDS treatment on the expression of tIL-1 α peptide and mRNA. Cyproterone acetate treatment in contrast resulted in a decrease in the IL-1 α mRNA levels.

This study shows that tIL-1 α expression is developmentally regulated at the level of its mRNA expression. It also suggests that testosterone regulates testicular IL-1 α expression at the level of transcription. This finding further strengthens the importance of testicular IL-1 α as a paracrine growth factor in the regulation of testicular development and function.

CHAPTER ONE

1 INTRODUCTION

Growth factors are molecules that stimulate cell proliferation and differentiation, often identified by measuring their stimulatory effect on DNA replication. Most of these molecules are proteins and an increasing number of tissues are found to produce such factors. One of the earliest discovered was epidermal growth factor (EGF), followed by nerve growth factor (NGF) and fibroblast growth factor (FGF). To date approximately over 100 growth factors with growth promoting ability have been detected. Growth factors are known to act mainly through paracrine and/ or autocrine mechanisms via specific receptors.

1.1 Testicular Growth Factors

The testis is the site production of numerous growth factors. One example of such a molecule is inhibin, a hormone whose importance was defined long before a reliable bioassay was developed. It is a feed back inhibitor of follicle stimulating hormone (FSH) (Skinner *et al.*, 1989).

Several growth factors that were previously known to exist in many other tissues have also been discovered in the gonads. These factors include EGF, transforming growth factor (TGF) α and β , NGF and interleukin-1 (IL-1) (Mullaney *et al.*, 1992 and 1993; Aeyr-lievre *et al.*, 1988; Soder *et al.*, 1991). To date over forty growth factors are known to be expressed by the testis and suggested to play a paracrine role (Bellve and Zheng, 1989; Ackland *et al.*, 1992).

In an effort to define the paracrine or autocrine role of growth factors, three criteria had been proposed (Ackland *et al.*, 1992).

- I To demonstrate that a molecule is expressed by a given tissue and not coming from elsewhere. This requires detection of a growth factor or identification of its' mRNA in isolated cell culture or tissue.
- II Local production of the growth factor is regulated by physiological status of the tissue.
- III Identification of the growth factor receptor or the receptor's mRNA in the tissue that Produces the growth factor.

The mitogenic role of some of these growth factors: EFG, TGF and IL-6 on adult rat testis was demonstrated by *in vitro* and *in vivo* induction of cell proliferation and differentiation (Mullaney *et al.*, 1992; Parvinen *et al.*, 1992). Recently, IL-1 like activity was also discovered in the rat testis (Khan *et al.*, 1987; Soder *et al.*, 1988). Since then the molecule is well characterised and its mRNA is detected in various rat organs except the kidney. This study led to the proposition of IL-1 α as a paracrine intratesticular growth factor (Pollanen *et al.*, 1986; Parvinen *et al.*, 1991; Soder *et al.*, 1991).

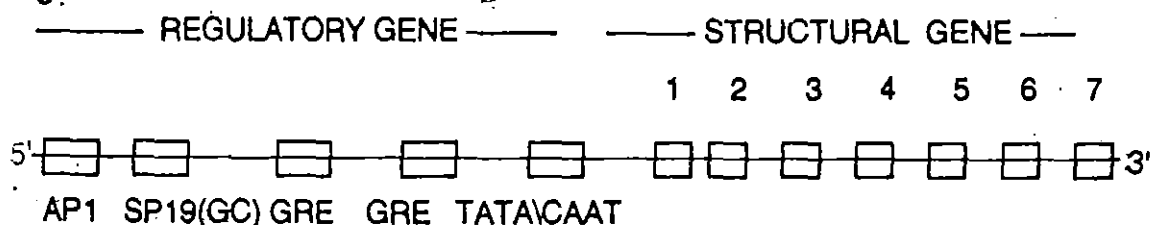
1.2 Molecular and Gene Structure of IL-1

Interleukin-1 (IL-1) is a polypeptide growth factor that exist in two isoforms: IL-1 α and IL-1 β (Clarck *et al.*, 1986). The newly synthesized intracellular IL-1 proteins have a molecular weight of 31KD. Proteolytic cleavage of both precursors produce mature bioactive IL-1 protein. Human short IL-1 α contains 159 amino acids and IL-1 β is 152

amino acids (March *et al.*, 1985) with a molecular weight of 17 KD. Similar data has also been obtained for the mouse (Lomedico *et al.*, 1984). IL-1 α and IL-1 β display 26% amino acid sequence homology and their identity is mostly restricted to a stretch of 36 amino acids known as a receptor site (Dinarelo, 1988). X-ray structural analysis has demonstrated that IL-1 β has a core of antiparallel β -pleated sheets and 11 surface loops. Comparative analysis of amino acid sequence of IL-1 β 's and IL-1 α 's suggests that these molecules are likely to possess similar if not identical β -pleated sheets (Terharné *et al.*, 1990). In man isoelectric focusing studies had revealed isoelectric point of IL-1 α and IL-1 β of 5 and 7, respectively (Mizel, 1989).

Genomic studies in man had shown that IL-1 α and β are located on chromosome 2 at positions q 13 and q 21 respectively (Arai *et al.*, 1990). The gene encoding both molecules possess seven exons and six introns (Fig.1.1). The two molecules have two identical 5' flanking regulatory sequences: a multiple tandem GC sequence (SP-1 binding site) and glucocorticoid response element. On the other hand IL-1 β has consensus TATA and CAAT sequences whereas IL-1 α has only got TATA like and CAAT like sequences. Finally, IL-1 α but not β has AP.-1 binding sites (Clark *et al.*, 1986 and Auron, 1994).

Fig. 1.1 The Structure of IL-1 gene



GRE glucocorticoids response element, AP.-1 and SP-1 are binding sites for AP.-1 and SP-1 regulatory proteins, respectively. The promoters are shown in TATA /CAAT, or TATA/CAAT like sequence. The exons are numbered (Clark *et al.*, 1989 and Arai *et al.*, 1990).

1.3. Biosynthesis of IL-1

Using mRNA detection techniques or measurement of bioactivity, several laboratories had shown the presence of IL-1 in macrophages, fibroblasts, keratinocytes, Langerhan's cells of the skin, astrocytes, micro glial cells of the brain, endothelial cells, gastrointestinal tract and testis (Dinarello, 1988; Mizel, 1989; Granholm *et al.*, 1992b; and Soder *et al.*, 1991).

As mentioned earlier IL-1 α pre mRNA is spliced to produce mature mRNA. Translation of the latter produces 31 KD protein (Saklativala *et al.*, 1985 and Akira *et al.*, 1990). The product may be directly inserted into plasma membrane as myristoylated IL-1 α , or its' truncated version is secreted into extracellular space (Akira *et al.*, 1990; Bakouche *et al.*, 1987; Dinarello, 1994a; Kurt-jones *et al.*, 1985). In the testis, it is secreted bidirectional i.e. both into the lumen of the seminiferous tubules and the interstitial space (Soder *et al.*, 1991). However, unlike most secretory proteins this molecule is devoid of signal peptide (Mizel, 1989; Dinarello, 1988). Experiments had shown the 31 KD protein suffers a single site cleavage. IL-1 β is processed by similar mechanism utilising Interleukin-1 converting Enzyme (ICE) (Wilson *et al.*, 1994). IL-1 α remains on the plasma membrane or in the cell (Kurt-jones *et al.*, 1985; Bakouche *et al.*, 1987). Since IL-1 does not possess signal peptide it does not visit the Golgi apparatus and hence it is not glycosylated.

1.4. Regulation of IL-1 Expression

The production and steady state concentration of an eukaryotic protein depends on several factors. These factors are onset and rate of transcription, posttranslational processing of pre-mRNA, delivery of mRNA to the cytoplasm, mRNA stability, rate of translation (Darnel, 1982) and protein degradation. While these factors do not equally apply to all proteins, transcriptional regulation appears to be the most important (Drapkin *et al.*, 1993). However detection and analysis of IL-1 mRNA and protein has revealed that this molecule is synthesized constitutively (Syed *et al.*, 1988). There is a great variation in the amount of IL-1 synthesized by various tissues. For example, activated macrophages express a large amount whereas testis produces a small amount (Bahl and Foreman, 1994; Letournel-Bouland *et al.*, 1994; Wong *et al.*, 1993). Other studies have shown that IL-1 production may increase or decrease with age (Syed *et al.*, 1988). In fact, determination of the effect of age on IL-1 synthesis is one of the objectives of this work.

1.4.1. Transcriptional Regulation

Stimulation of IL-1 producing cells such as monocytes by endotoxin or phagocytosis increases the production of IL-1 (Letournel-Bouland *et al.*, 1994; Shadiack *et al.*, 1994). Rapid rise in IL-1 primary transcript could be observed as early as 30 minutes after stimulation by tumour necrosis factor α (TNF α) or LPS. The rise in mRNA levels, IL- 1 α and IL- 1 β lasted up to eight hours (Turner *et al.*, 1989). On the other hand inhibition of transcription by actinomycin blocks the stimulatory effect of LPS on mRNA production, but increases its' stability (Green, 1991). It follows from the above that transcriptional regulation plays an important role in IL-1 production.

As described earlier (sec. 1.2), the regulatory sequence of IL-1 α gene has TATA like and CAAT like regulatory elements; therefore most if not all the known transcription factors (Drapkin *et al.*, 1993) will be used to initiate synthesis of pre-mRNA from this gene. These genes also possess regulatory elements very distant from the initiation site therefore enhanced by them i.e. AP.-1, SP-1 and GRE (Dinarello, 1988; Akira, 1990).

1.4.2 Post Transcriptional Regulation

Eukaryotic mRNA concentration and stability can be regulated by hormonal factors. At the molecular level this can occur by several mechanisms including protection of the mRNA by polyadenylation, synthesis of polyA binding protein or destabilisation by AU rich 3'-untranslated region (Nielsen *et al.*, 1990;). Sequencing studies have shown that cytokine genes including IL-1 have stretch of 3'-AU region that enhance their instability (Mizel, 1989; Arai *et al.*, 1990). On the other hand, proteins that specifically bind AU rich regions were recently discovered (Shaw *et al.*, 1986; Caput *et al.*, 1986; Malter *et al.*, 1989). However, the role of these proteins in stabilizing IL1 mRNA and the process that controls the concentration of these proteins are unclear.

1.4.3 Developmental and Hormonal Regulation

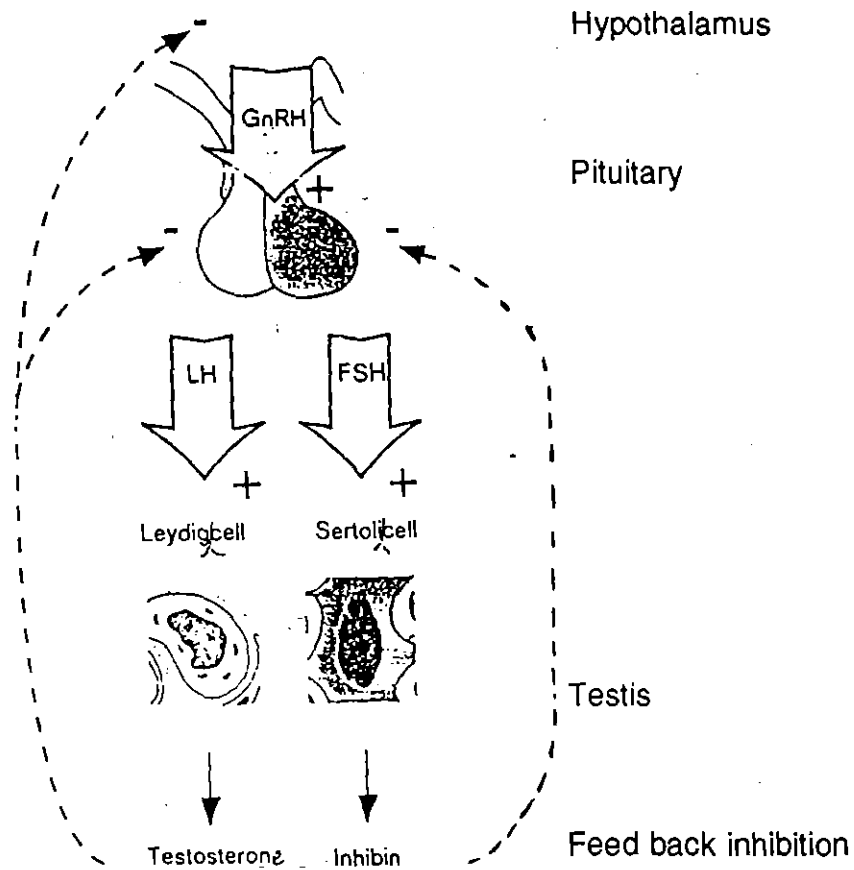
Expression of IL-1 α and other cytokines decrease during thymocyte maturation and restarts at a latter stage. RT-PCR based studies have shown that this fluctuation is due to transcriptional changes. Synthesis of IL-1 mRNA decreases during early

differentiation but increases during late maturation (Moore *et al.*, 1993). It is clear from the example given above that IL-1 production could be developmentally regulated. In view of the above, it is one of the objectives of this study to investigate the role of developmental stages on production of testicular IL-1 α .

IL-1 production is inhibited by prostaglandins (Maccio *et al.*, 1994) and IL-4 (Wong *et al.*, 1993). This effect appears to occur via reduction of mRNA synthesis. In addition to the above, glucocorticoids also decrease IL-1 expression (Lee *et al.*, 1988; Bateman *et al.*, 1989). Furthermore, *in vivo* and *in vitro* work had shown that steroid hormones such as testosterone and progesterone are also known to inhibit IL-1 α production (Jarrous and Kaempfer, 1994; Da Silva *et al.*, 1994). However, the mechanism by which this inhibition occurs i.e. whether it is at the level of mRNA expression or its peptide production is not known. In fact the third question this work addresses is to define the effect of testosterone on production of IL-1 α .

Several endocrine tissues (hypothalamus, pituitary and gonads) are involved in the induction and regulation of spermatogenesis. The hypothalamus stimulates the pituitary to release luteinizing hormone (LH). LH stimulates Leydig cells to produce testosterone. In turn testosterone is a feedback inhibitor of LH and LHRH (Fig. 2). Similarly FSH of the pituitary stimulates Sertoli cells. The latter feedback inhibits FSH production by releasing inhibin. (Fig.1. 2).

Fig.1.2 Organs and hormones involved in Regulation of Spermatogenesis



The different cell types of the testis: Leydig cells, Sertoli cells, peritubular myoid cells and germ cells are regulated by hormones (table 1.1). It is clear from table-1.1 that germ cells have no receptors for gonadotropin. There is no information supporting the presence of testosterone or IL-1 receptor either. On the other hand, germ cells proliferate and differentiate within pockets generated by Sertoli cells (Fig.1.2). Taking the anatomic feature described above and paucity of data on germ cells' receptors, it is tempting to speculate spermatogenesis is directly regulated by secretions of Sertoli cells but only indirectly by testosterone.

Table -1.1 Testicular Cells types and hormonal responsiveness

Cell type	Hormone responsiveness (a)
1. Tubular compartment	
Sertoli cells	• FSH, Testosterone
Peritubular cells	Testosterone
Premiotic germ cells	(b)
Postmiotic germ cells	
2. Interstitial compartment	
Leydig cells	LH,
Testicular macrophages	(b)
Peritubular myoid cells	Testosterone

N.B. (a) Based on presence of hormonal receptor and/or biological response to hormonal stimulation
 (b) Convincing evidence for hormonal responsiveness is lacking.

In addition to pituitary's FSH, Sertoli cells are regulated by testosterone from Leydig cells and peritubular factors that modulate Sertoli cell function i.e. P-Mod-S. While the effect of testosterone on Sertoli cell is less significant, this androgen can stimulate peritubular myoid cells to produce P-Mod-S; a factor with stimulating effects on Sertoli cells (Skinner, 1989).

Sertoli cells are thought to produce IL-1. In the testis, this cytokine is known to induce two effects: Suppress the production of testosterone from Leydig cells and stimulation of spermatogonial division (Sec 1.6). On the other hand, the effect of IL-1, if any, on P-Mod-S production is unknown. It is clear from the above that several questions require future work.

1. Why dose IL-1 show apparently conflicting effect on Leydig cells and germ cells ?
2. What is the role of IL-1 on peritubular myoid cells?
3. What is the mechanism by which spermatogenesis is regulated?

Any investigation that addresses the fore-mentioned questions require improvement in methods of mRNA quantitation. In this context, to date it has not been possible to measure testicular IL-1 mRNA with acceptable accuracy hence the need for this work.

1.5 Mechanism of Action of IL-1

There are two types IL-1 receptors (IL-1R). The type I receptor (IL-1R), which thought to be a signalling receptor is a glycoprotein of 80KD (Bazan, 1993; Kolesnick and Golde, 1994; Kishimoto *et al.*, 1994; Sims and Dower, 1994). It is anchored on the plasma membrane with extracellular, membrane spanning and intracellular domains containing 319, 21 and 217 amino acids respectively (Arai *et al.*, 1990). IL-1 α or β signal through this type I receptor displaying an affinity of 10^9 - 10^{10} M⁻¹ (Wong *et al.*, 1993). The number of type I IL-1 receptors per responsive cell show a great variation. For instance human hepatoma cells (Hep-2) has 540 where as monocytes (U 937) have <10 receptor per cell.

The type I IL-1 receptor lacks tyrosine kinase activity (Dinarelo, 1988). It does not trigger tyrosine phosphorylation or display protein kinase C activation. On the other hand stimulation of cells by IL-1 increases pKa activity (Bazan, 1993; Kolesnick and Golde., 1994; Kishimoto *et al.*, 1994) and utilises a novel signalling pathway employing sphingomyelin. In the case of T-lymphocytes, the rise in c-AMP leads to activation of protein kinase A and Nuclear Factor NF-K β (Dinarelo, 1994a). The last molecule binds the following target sequence (GGGATTTTCC) there by transmitting IL-1 induced stimulation of transcription.

1.6 Activities of IL-1

As is the case with other cytokines, IL-1 is a multifunctional hormone-like factor (Akira *et al.*, 1990, Dinarello, 1994b). It is important in cellular immune regulation, proliferation of smooth muscle cells, and inflammatory reaction (Dinarello, 1988). IL-1 promotes activation and differentiation of B and T-Lymphocytes (Mizel *et al.*, 1989). Likewise it is important in proliferation and activation of fibroblasts (Akira, 1990).

IL-1 activates synovial cells and chondrocytes to produce prostaglandin E₂ (PGE₂), collagenase and Phospholipase A₂ (PLA₂). In addition to the above it brings about degranulation of granulocytes and attracts lymphocytes to inflamed tissue (Mizel *et al.*, 1989). It has conflicting role on systemic circulation. Initially it decreases arterial blood pressure but later the blood pressure increases. It promotes vascular congestion and clot formation. In the brain, it induces the synthesis and secretion of many hypothalamic and pituitary peptide including endorphins, Corticotropin releasing factor (CRF), adrenocorticotrophic hormone (ACTH) and somatostatin (Lee *et al.*, 1988; Bateman *et al.*, 1989). Likewise hepatocytes incubated with IL-1 increase the biosynthesis of acute phase proteins such as complement factor C3, metalothionins, C-reactive protein and Amyloid protein A, but decrease the biosynthesis of albumin (Dinarello, 1988 and Akira, 1990).

The physiological role of testicular IL-1 (tIL-1) was investigated using *in vivo* and *in vitro* experiments. Administration of recombinant IL-1 α to hypophysectomized animals stimulated spermatogonial division (Pollanen *et al.*, 1989). Moreover, in rats, the production of IL-1 correlates with onset of puberty (Syed *et al.*, 1988). *In vitro* studies using recombinant IL-1 (rIL-1) also demonstrated proliferation of germ cells (Parvinen *et al.*, 1991). Taken together the description given above suggests that tIL-1 is functionally important factor in spermatogenesis.

1.7 Methods for Analysing Gene Expression

Gene expression can be studied by measuring the quantity of transcribed RNA or by measuring the quantity of proteins synthesized. The latter can be measured directly as the case is with enzyme activity or indirectly by monitoring biological activity of hormones, RIA and ELISA.

1.7.1 The Need for Reverse Transcriptase-Polymerase Chain Reaction

As mentioned earlier (sec 1.4) gene expression is regulated at several levels: onset of transcription, processing pre-mRNA, transport of mRNA from the nucleus to the cytoplasm and rate of mRNA degradation.

Several hybridization techniques can be used to measure RNA. These methods included dot blot, insitu or soluble hybridization and Northern blot (Berger *et al.*, 1987). As far as sensitivity of these methods is concerned insitu hybridization can detect 10-100 target RNA molecules/cell whereas the rest fail to detect $<10^5$ molecules/cell. However, only Northern blot can distinguish size difference between full length and partially degraded transcript. Taken together the problems cited above stimulated a desire to develop a new technique to measure target mRNA. This new technique depends on amplification of mRNA by Reverse Transcription - Polymerase Chain Reaction (RT-PCR).

1.7.2 Polymerase Chain Reaction

PCR is a cell free method by which a particular DNA sequence is amplified. Given the mechanism of DNA synthesis, conducting PCR requires three essential steps : Denaturation of ds - DNA, primer annealing and primer extension.

Successful amplification of a target DNA would require a very careful control of the three steps (Siaki *et al.*, 1988; Keohavong and Thilly, 1989; Innis and Gelfand, 1990). Denaturation of ds-DNA is conducted by thermal dissociation (90- 95 oC). Primer annealing is conducted by lowering the temperature to 55°C. Care is taken to avoid reassociation of the template strands by dilution and using higher concentration of primers. However, primer concentration should be kept sufficiently low to avoid primer-primer dimer formation. Finally, extension of a template primer duplex is conducted by Taq DNA polymerase, an enzyme from *Thermus Aquaticus*. At 95 °C, this enzyme has $t_{0.5}$ of 40 minutes (Innis and Gelfand, 1990).

Amplification of both sense and antisense strands of ds-DNA require two primers. One of these primers known as upstream primer is similar to 5' end of the sense strand and complementary to antisense strand whereas the second primer is complementary to 3' end of the sense strand. It is necessary to note that a lot of work was conducted to optimize assay conditions i.e. temperature, time needed for each of the three steps mentioned above, buffer pH, concentrations of enzyme, template, primer, $MgCl_2$, KCl, dNTP, non-ionic detergent (Tween 20) and albumin or gelatin. Innis and Gelfand (1990) recommended it is necessary to conduct optimization tests for all PCR steps mentioned above before searching for target DNA or RNA.

1.7.3 Reverse Transcription - Polymerase Chain Reaction

In theory, reverse transcription of mRNA and amplification of the resulting SS-cDNA can be used to detect the presence of gene transcript. In practice, this method was used for numerous applications including detection of low copy number mRNA <10 copies / cell and number of other uses (sec.1.7.4).

A lot of effort went into developing the theoretical basis (Raeymaekers, 1993) and practical methods of titrating mRNA (Chelly, *et al.*, 1988; and Becken-Andre and Hahlbroch, 1989). This effort led to derivation of a formula that links yield (y) of RT-PCR with initial concentration of mRNA (Eqn1), Chelly *et al.*, 1988). It is clear from equation 2, that the efficiency of amplification can be calculated from a graph of log y versus n.

$$y = A(1 + E)^n \quad \text{Eqn 1}$$

$$\text{Log } y = \text{Log}A + n\text{Log}(1+E) \quad \text{Eqn 2}$$

$$\frac{A_{\text{target}}}{A_{\text{ref}}} = \frac{Y_{\text{target}}}{Y_{\text{ref}}} \quad \text{Eqn 3}$$

Abbreviations: A, Amount of RNA before Amplification,

E Efficiency of Reaction,

n The number of cycles,

Y yield.

It is not possible to obtain the initial amount of target mRNA (A_{target}) from Eqn 2. This problem is resolved by coamplification of target RNA and a known amount of 'reporter' RNA (Becken-Andre and Hahlbroch, 1989). Careful studies (Chelly *et al.*, 1988) had shown the target and 'reporter' RNA may possess identical efficiencies. It follows from the above that the initial amount of target RNA can be calculated from Eqn.3. If amplification of target and reporter mRNA is conducted in different tubes, any difference in temperature, dNTP, Mg^{2+} , inhibitory pyrophosphates, concentration of primer and sequence length of mRNA could lead to a large difference in efficiency.

This problem can be ameliorated by Competitive RT - PCR (Wang and Mark 1990): a process in which both reporter and target sequence are in the same tube hence subjected to identical factors. Even under this condition, only sequences that differ by a restriction site but otherwise identical would display equal efficiency (Innis and Gelfand, 1990). On the other hand length difference could entail difference in efficiency. However, it is possible to optimize the conditions such that efficiency is identical in order to obtain a good estimate of the amount of target mRNA using competitive PCR. In this study the target molecule is rat testicular IL-1 α mRNA whereas the reporter mRNA is obtained by *in vitro* transcription from a shortened IL-1 α cDNA. The latter was constructed by removal of 120 bp (158ⁿ to 277ⁿ) from IL-1 α cDNA.

1.7.4 Importance of PCR and RT-PCR

PCR has been used for numerous applications. Some of these applications are detection of parasites such as plasmodium falciparum, viruses such as hepatitis B in biological samples (Norder *et al.*, 1992) and forensic DNA typing (Saiki *et al.*, 1988). Recombinant-PCR was also used to link promoter sequences to DNA of interest (Higuchi, 1990). The latter method is useful for large scale production of proteins.

RT-PCR is used for expression analyses in eukaryotic cells. These methods include: detection and quantitation of mRNA from single cells or a given tissue (Kawasaki, 1990). mRNA and analysis of mRNA stability (Belyavsky *et al.*, 1989; Pfeifer *et al.*, 1989; Gilland *et al.*, 1990). In this thesis, RT-PCR is used to study regulation of tIL-1 α mRNA synthesis.

CHAPTER TWO

2 OBJECTIVES AND SIGNIFICANCE OF THE STUDY

2.1. OBJECTIVES OF THE STUDY

2.1.1 General Objectives

1. Development of a quantitative PCR that can allow precise determinations of a specific mRNA: IL-1 α
2. Analyse the hormonal and developmental control of testicular IL-1 expression.

2.1.2 Specific Objectives

1. Establishment of a quantitative PCR method by using an internal standard.
2. Identification and estimation of the level of testicular IL-1 α peptide and mRNA
3. Evaluation of the effects of obliteration of intratesticular testosterone production on the levels of IL-1 α peptide and mRNA.
4. Evaluate the effects of an androgen receptor antagonist on the level of IL- 1 α expression.

2.2 SIGNIFICANCE OF THE STUDY

This project helps to establish a sensitive and accurate method of measuring mRNA. Such a method is important both in diagnosis as well as in monitoring therapy by measuring the effects of drugs on concentration of gene product(s). In this study, the method was used to measure tIL-1 α expression to evaluate its role on testicular development and function .

Chapter Three Materials and Methods

3.1 Materials

3.1.1 Rat Testis

Testis from two groups of rats were used. The first group of rats, aged 10, 20, 25, 30, 45 and 60 days, were used to study effect of developmental stages on IL-1 α synthesis. The second group of (60 to 90 days old) adult rats were used either as control subjects or to study effect of drugs on IL-1 α production.

The drugs used were cyproterone acetate (Androgen receptor antagonist), EDS (Ethane-Dimethane-Sulfonate), a reagent that is selectively cytotoxic to the Leydig cells, and obliterates testosterone production. All these chemicals are hydrophobic hence they were prepared as sesame oil solution and were injected intramuscularly, with the exception of EDS which was dissolved in Dimethylsulphoxide : water, 7:3 (W:V), and injected intraperitoneally. What is displayed below (table 3.1) is the protocol used to administer drugs and each group of animals consisted of five adult rats. After 22 days the rats were sacrificed by carbon dioxide suffocation and testis was excised, weighed and stored at -20°C or used immediately. These samples were used to prepare total RNA for analysis of IL-1 α mRNA expression.

Table 3.1 Drug Administration Regimen

Treatment Group	Injection protocol
Control	0.6 ml of Sesame oil/kg was injected on day 1, 6 and 16
EDS treated	75 mg/kg as a single dose from a solution containing 52.5 mg of EDS/ml on day 1.
EDS + testosterone	EDS as above, plus 140mg/kg of testosterone from a solution containing 250 mg per ml of testosterone on day 1, 6, 16.
Cyproterone acetate	90 mg/kg from a solution containing 250 mg/ml of cyproterone acetate every day.

3.1.2 Preparation of Rat Thymocytes

4 to 8 weeks old, substrain of NMRI/KI rats (Granholm *et al.*, 1992a) were killed by carbon dioxide suffocation. The thymus was excised and thymocyte suspension was prepared in Alfa modified Eagle minimum Essential Medium (α MEM) supplemented with Phytohemagglutinin (5 μ g/ml). The cells were counted using automated cell counter, and suspension of 5×10^6 cells/ml was used in thymocyte proliferation assay as previously described by Granholm *et al* 1992.

3.1.3. Reagents

Guanidine Isothiocyanate, Cesium Chloride, Sodium Lauryl Sarcosate were bought from Sigma Co. St. Louis, Mo. Reverse transcription kit was procured from Pharmacia, Uppsala, Sweden. Taq DNA-polymerase, dNTPs, PCR buffer and Magnesium Chloride were from Boehringer Mannheim, Mannheim, Germany. In vitro transcription kit and RNA \sim sin were from Promega, Madison, WI. Oligonucleotide primers were purchased from Inovagene, Lund, Sweden. Rat Macrophage RNA was prepared in the laboratory as described in section 3.2.2.

3.1.3.1 Stock and Working Solution

The Procured enzymes and inhibitor had concentrations stated below. Molony Murine Leukemia Virus (M_oM_uLV) Reverse Transcriptase had activity of 200 units/ μ l. Taq DNA polymerase was obtained from various supplies hence at different activities. Promega's stock T4-RNA polymerase was supplied at 19 units/ μ l where as RNA \sim sin from the same source was at concentration of 200 units/ μ l.

3.1.3.2 RNA Extraction

- a. 1% Diethylpyrocarbonate (DEPC) in double distilled water. This solvent was used to prepare all solutions.
- b. Stock solution of 20 % Sodium lauryl Sarcosinate was prepared. Homogenization buffer was made by diluting this solution to 2%.
- c. 5.7 M Cesium Chloride
- d. 99 % β -mercaptoethanol that was diluted to 1% was used during homogenization.
- e. 4 M Guanidine Isothiocyanate (GT)

3.1.3.3 Reverse Transcription-Polymerase Chain Reaction

- a. 5x Reverse transcriptase buffer containing 250mM Tris HCl pH8.3, 375mM Potassium chloride (KCl), 15 mM Magnesium chloride ($MgCl_2$) and 0.1M Dithiothritol (DTT).
- b. 10 x PCR buffer composed of 100mM Tris-HCl (pH 8.4), 500mM KCl, 0.1% gelatin
- c. Stock solution of 25mM $MgCl_2$ was prepared and was used at final concentrations of 1.5 - 2mM
- d. A solution containing 10mM each of dNTP was prepared in double distilled water.

3.1.3.4 In vitro transcription Buffer

- a. 5x In vitro transcription buffer contained 40mM Tris-HCl, 6mM $MgCl_2$, 10mM dithiothritol (DTT) and 2mM Spermidine.
- b. 10mM Ribonucleotide triphosphates were prepared in double distilled water.

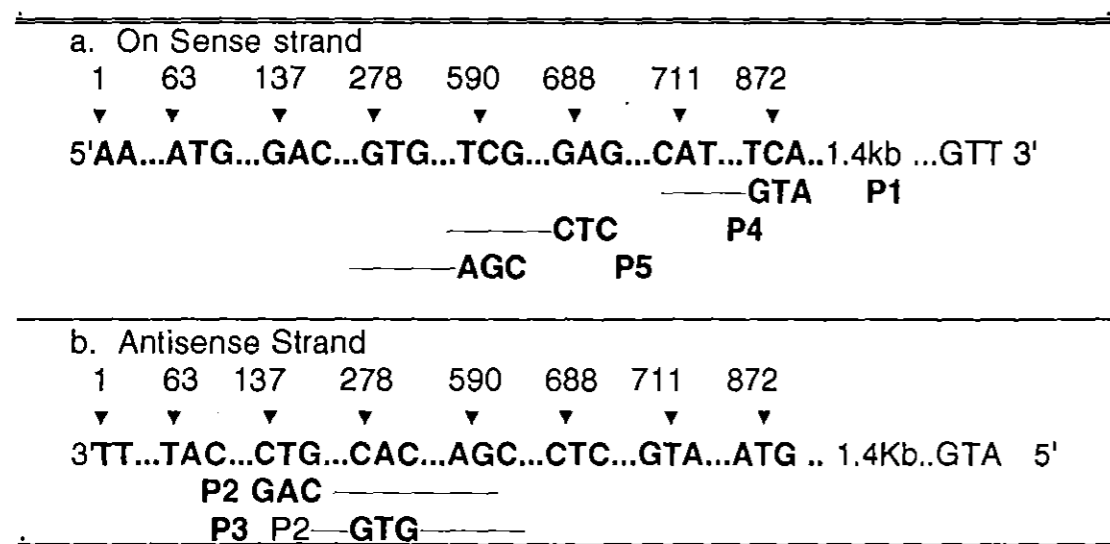
3.1.3.5 Electrophoresis

- Buffer - 50 x Tris-Acetate-EDTA (TAE) buffer contained 2M Tris-HCl (pH 7.5), 50mM EDTA, 250 mM Sodium acetate.
- Gel - High quality agarose supplied by FMC Bioproducts, Rockland, ME, was used to prepare 2% gel.

3.1.3.6 Oligonucleotide primers

IL-1 α cDNA is 2.1Kb long molecule. The entire sequence was scanned for the presence of 18 to 43 nucleotides that possess minimal secondary structure and high replication efficiency. This effort led to selection of five primers (Fig. 3.1) and a T7-bacteriophage promoter carrying primer (P6). All were synthesized and supplied by Inovagene, Lund, Sweden. The six primers possess 50-60% G/C bases, 3' non-palindromic sequence as described by Sheffield *et al.*, (1989). Preliminary tests had also shown these primers possess excellent efficiency. Table 3.2 and Fig. 3.1 convey sequence as well as positions where the primers anneal to on IL-1 α mRNA or strands of cDNA.

Fig. 3.1 Annealing Position of Oligonucleotide primers on IL-1 α cDNA



Note: P3 is made by linking nucleotide sequence (137 to 157) of IL-1 α cDNA to nucleotides (277 to 298) of the same sequence thus deleting 120 bps of IL-1 cDNA that lie between bases 157 and 278. Ref: Nishida *et al.*, 1989.

Table 3.2 Sequences of Oligonucleotide primers

Primer	Sequence of primer	Annealing position		Importance
		position on cDNA	Nucleotide number on cDNA	
P ₁	5'ATGAGTTTTGGTGTTC CG 3'	Down stream	710	RT of reporter RNA from Macrophage IL-1 α mRNA
P ₂	5'GACCATCTGTCTCTGAATCAG3'	upstream	137-157	PCR of Reporter cDNA and testicular RNA
P ₃	5GACCATCTGTCTCTGAATCAGGTGGTCAGCAATCAAAC 3'	Upstream	P2+27 7-298	PCR of Truncated molecule
P ₄	5'CTC CTT CAG CAA CAC AGG 3'	Downstream	671-688	RT of Reporter RNA and Testicular RNA
P ₅	5'CGA TGA GTA GGC CAT ACA TGT C 3'	Downstream	569-590	PCR of reporter and testicular cDNA
P ₆	<u>5TAATACGACTCACTATAGGGAGAGACCATCTGTCTCTGAATCAG3</u>	T7 promoter +(21 base) of P ₃	T7 promoter+P2	Linking T7 promoter to Truncated cDNA

Note (1) Ref:Nishida et al (1989)

(2) The 23 base bacteriophage promoter sequence in P₆ is underlined

3.2 Methods

3.2.1 Thymocyte Proliferation Assay

5 x 10⁶ cells/ml suspension of thymocytes were dispensed in 100µl aliquots into multiwell plates. To these wells, 10µls of serially diluted testicular homogenate (1:1, 1:4, 1:16, 1:64, and 1:256) were added and the plate was incubated at 37°C for 46 hours. This was followed by addition of [³H] thymidine (0.5µci in 10 µl) and the incubation was continued for two hours. The cells were collected, on glass fibre filter, by using an automated cell harvester and were mixed with 2ml scintillation cocktail for β-counting. The amount of radioactivity from [³H] thymidine that is incorporated by the thymocytes was taken as a measure of the thymocyte proliferation in response to the level of IL-1 α in the sample homogenate, as described by Granholm *et al.*, 1992.

3.2.2 RNA Preparation

Macrophages express a large quantity of IL-1α mRNA. This source was therefore used to extract RNA needed to construct a reporter IL-1α cDNA. Total RNA from testicular samples was prepared as follows: 1- 0.5 gram of the samples were homogenized in 5 volumes of homogenization buffer containing: 50% Guanidine isothiocyanate, 2% Na-Sarconate, and 1%β Mercapto ethanol. 2.Two to three ml of the homogenate is layered on 5.7M cesium chloride in 5ml Beckman centrifuge tubes and centrifuged in SW 55 rotor at 35000g (aproximatly 100,000 xg) at 20°C for 18 hours. The RNA pellet was then extracted once in phenol: chloroform: isoamyl alcohol (25:24:1) and once in chloroform: isoamyl alcohol (24:1), and precipitated over night by 95% ethanol at -20°C, as described by Berger 1987. The precipitate was then dissolved in Tris - EDTA Buffer to a concentration of approximately 0.33 µg/ul.

3.2.3 Reverse Transcription

Reverse transcription was conducted in 20 μ l of assay volume containing, 5x RT buffer, 4 μ l; 10mM of each dNTPs, 1 μ l; RNA-sin, 1.5 μ l; 0.1M dithiothritol, 2 μ l; RNA sample 1 μ l; Primer (17nM), 3 μ l; reverse transcriptase 2 μ l; DEPC water to make 20 μ l, as described by Kawasaki (1990).

The assay mixture was subjected to 70 $^{\circ}$ C for 10 minutes to anneal RNA and its' primer. The process was continued at 37 $^{\circ}$ C for 2 minutes, followed by addition of 400 units of reverse transcriptase and incubated at 45 $^{\circ}$ C for 2 hours. The reaction was terminated by lowering the temperature to 4 $^{\circ}$ C until this cDNA preparation was directly used for PCR.

3.2.4 Polymerase Chain Reaction

The following ingredients were added to a total volume of 50 μ ls: 20 μ l of c-DNA preparation (sec.3.2.3), 5 μ l of 10X PCR buffer, 5 μ l of primer(stock 5 μ M), 5 μ ls of dNTPs and 4 μ l of MgCl₂ (25mM) and 0.3 μ ls of Taq DNA polymerase (2 units) and distilled water was added to adjust the volume. The reaction was then conducted for 35 to 40 cycles to amplification using PHC thermal cycler (Techne, Cambridge UK) The amplification cycles were: denaturation at 95 $^{\circ}$ C for one minute and primer annealing at 55 $^{\circ}$ C to 61 $^{\circ}$ C for 30 seconds, primer extension at 72 $^{\circ}$ C -3 minutes as described by Innis and Gelfland (1990). The reaction was then terminated by cooling the mixture to 4 $^{\circ}$ C.

3.2.5 In vitro Transcription

In vitro transcription of reporter c-DNA was carried out in 50 μ l of reaction volume containing 20 μ l of 5x in vitro transcription buffer, 20 μ l of ribonucleotide triphosphate (stock concentration of each NTP was 10 mM), 0.7 μ g cDNA template and 1 μ l of RNA polymerase (19 units) and in the presence 5 μ l of 5 μ ci [³H-UTP]. The total volume was worked up by 1% DEPC containing distilled water. The reaction was carried out at 37 $^{\circ}$ C for 60 minutes and terminated by cooling to 4C, as described by Holland and Innis (1990).

3.2.6 Column Chromatography of RNA

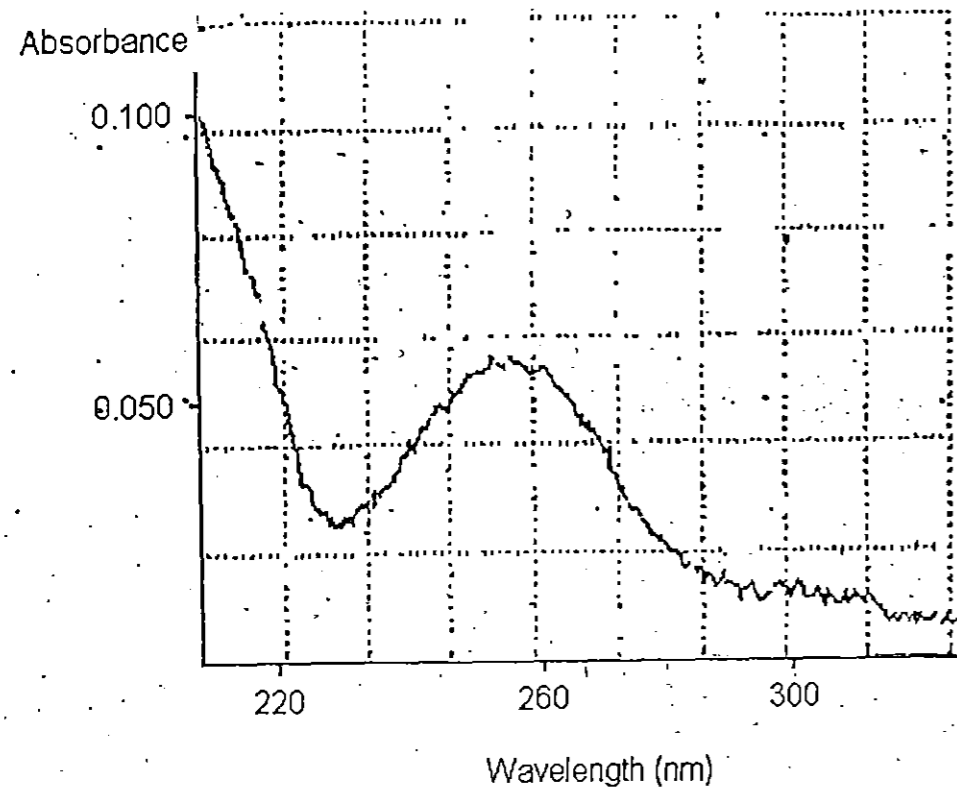
Sphadex g 50 (Pharmacia, Uppsala, Sweden)column was equilibrated with buffer containing 0.2 % SDS, 25mM Tris-HCl, pH 7.5, 50mM EDTA. The samples were applied, 300 μ l fractions were collected and radioactivity measured as described in section 3.2.10.

3.2.7 Analysis and purification of PCR products

Low gelling temperature agarose gel (2% W:V) was prepared in 1%TAE buffer. The agarose gel was submerged in TAE buffer and the PCR samples were applied. This was followed by electrophoresis at 75 V for 1.5 hours . PCR products were then isolated from the low gelling temperature agarose Using "Magic Prep" (Promega Corp., Madison, WI) resin as recommended by the supplier. The PCR products visible in UV after ethidium bromide staining were cut out from the agarose gel and melted in the presence of "Magic prep" resin to degrade the agarose gel. The DNA was then eluted from the resin in a spin column and the products were precipitated in 95 % ethanol.

3.2.8 Spectrophotometric Determination of RNA

The purity of RNA preparation (Sec.3.2.2) can be checked by UV absorbance at 260nm (Fig.3.2). In the absence of impurity the molecule has a peak at about 260nm with little absorbance elsewhere for the wavelength interval shown. In addition to the above OD readings are also used to quantify RNA as described by Burger 1987.

Fig.3.2 Spectrophotometric Profile of RNA

A typical spectrophotometric profile of RNA preparation showing a peak absorbance at 260 nm.

3.2.9 Fluorescent Photography

Electrophoresis was conducted on agarose gel that was cast with 0.1% Ethidium Bromide. In the presence of this dye, polynucleic acids (DNA/RNA) fluoresce when excited by UV light, hence the use of this property to obtain fluorescent photography.

3.2.10 Scintillation Counting

Scintillation counting was used to measure the following synthetic processes. Thymocyte proliferation (Sec. 3.2.1), in vitro transcription (Sec 3.2.5). In the former experiments dried glass fibre filters from the thymocyte proliferation were mixed with 4ml of scintillation cocktail and the total radioactivity was measured. In vitro transcription products were measured as follows: After agarose gel electrophoresis, the gel was cut in 1.5 mm slices, 5 ml of scintillation cocktail was added and radioactivity measured with Beckman scintillation Counter.

CHAPTER FOUR RESULT

4.1. Construction and Invitro production of IL-1 α mRNA

4.1.1 Synthesis of IL-1 α cDNA

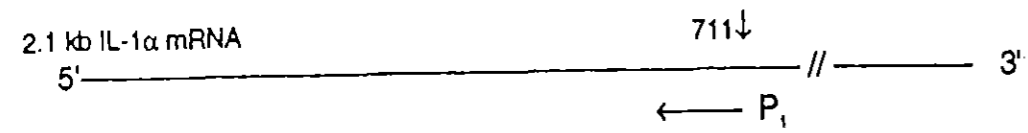
One microgram purified IL-1 α mRNA or total macrophage RNA was annealed with primer P1 (Fig 3.1) and the duplex was subjected to reverse transcription (sec. 3.2.3). A 711 base long antisense strand of IL-1 α cDNA was then subjected to 35 to 40 cycles of PCR using primers P2 and P4 (Sec. 3.2.4, Fig. 4.1). The product is a 575 bp long cDNA which is here after referred to as 'full length' IL-1 α cDNA.

Inspection of Fig. 4.1 reveals minor 3'- over hangs both on sense and antisense strands. In reality these unpaired nucleotides are removed by Taq polymerase during the amplification of cDNA. The product of the aforementioned RT/PCR is a full length 575 bp long IL-1 α cDNA.

4.1.2 Preparation of Reporter cDNA

Reporter cDNA was constructed by deletion of 120 bp i.e. bases 158th to 227th from the full length rat IL-1 α cDNA. The aforementioned was achieved by p3; a primer which was designed by ligating 21 nucleotides that correspond to position 137-157 at the 5' end of the full length cDNA with 19 nucleotides corresponding to positions 277 to 295 of 2.1 kb IL-1 α cDNA sequence. As a result of the deletion described above a 432 bp long cDNA known as reporter IL-1 α cDNA was constructed, (Fig. 4.2) and purified from low melting agarose gel as described in sec. 3.2.7.

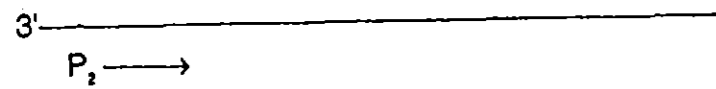
Fig. 4.1 Synthesis of full length IL-1 α cDNA
 a) Reverse transcription (RT)



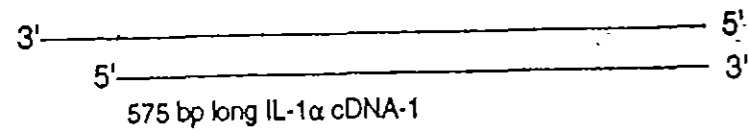
↓ RT

b) PCR

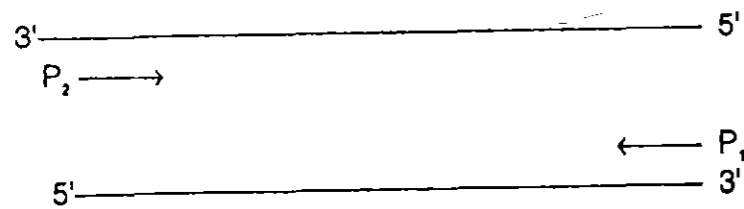
711 bp, ss IL-1 α cDNA
 ↓137



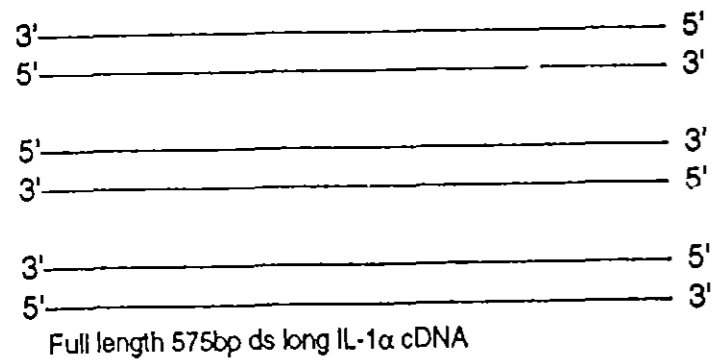
↓ cycle 1



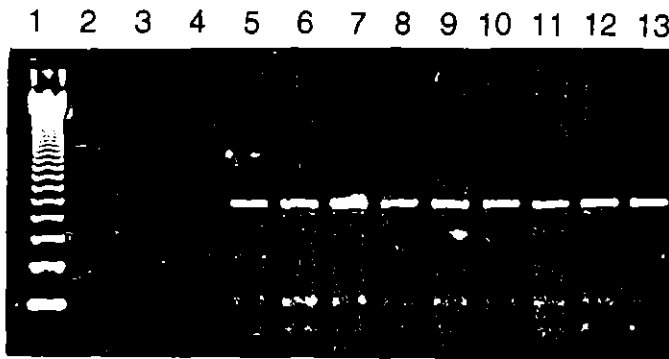
↓ denature



↓ 35 to 40 cycles



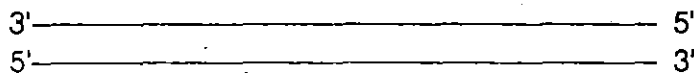
c) Agarose gel profile displaying the quality of amplified IL-1 α cDNA



Lane 1: Molecular size standard (123 bp DNA ladder, GIBCO BRL), fragments are 123, 246, 369, etc. Lanes 2-4: Negative controls. Lanes 5-13. Amplified product, 575 bp.

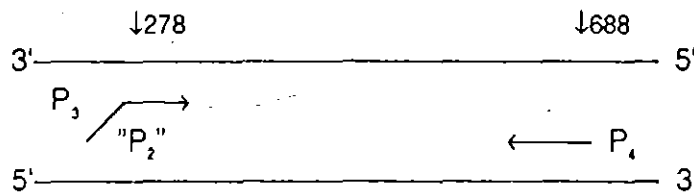
Fig. 4.2 Schematic view of construction of 432 bp long reporter cDNA

a. Construction



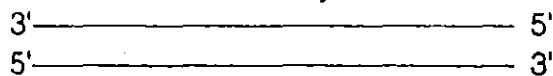
Full length 575bp ds long IL-1 α cDNA

↓ denature



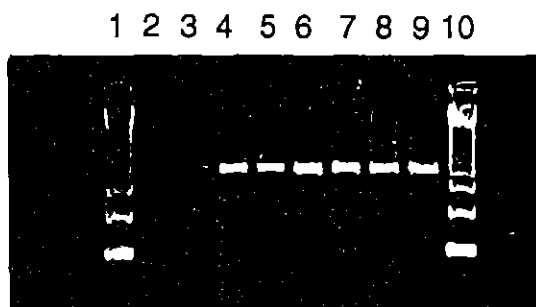
Full length 575bp ds long IL-1 α cDNA

↓ 35 to 40 cycles



432 bp reporter IL-1 α cDNA containing P₂ sequence

b. Agarose gel electrophoresis showing purity of reporter cDNA

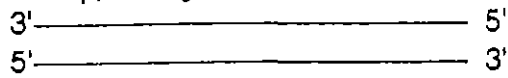


Lane 1 and 10: 123 bp DNA ladder Lane 2: Negative control. Lanes 3-9: Amplified product, 432 bp.

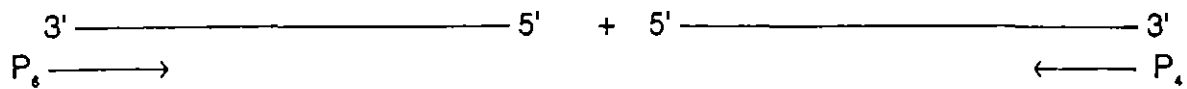
Fig. 4.3 Cloning of T7 promoter linked reporter cDNA

a. Construction

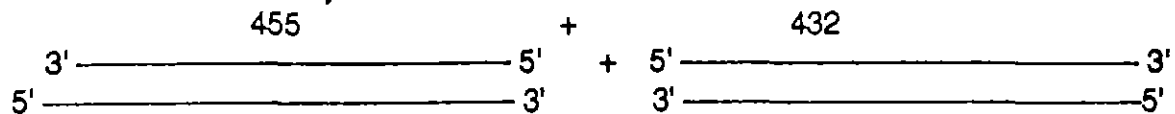
432 bp, full length cDNA



↓ denature



↓ Cycle 1

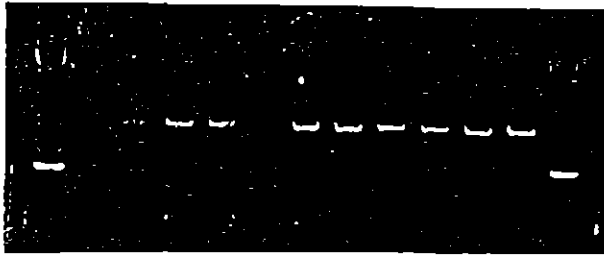


↓ 35 to 40 cycles

455 bp T7 promoter linked cDNA

b. Agarose gel electrophoresis showing purity of T7 promoter linked cDNA

1 2 3 4 5 6 7 8 9 10 11 12 13



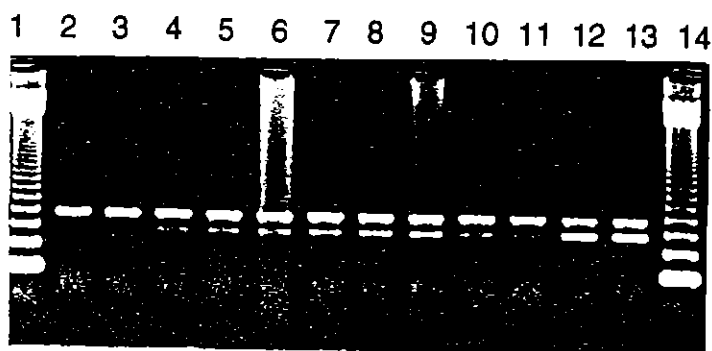
Lane 1 and 13: 123 bp DNA ladder Lane 6: Negative control.
 Lanes 2-5 and 7-12: Amplified product, 455 bp (lane 2: nonspecific amplification).

4.1.5 Identification of IL-1 α mRNA Expression

To assess the purity of the product both full length and truncated mRNAs were subjected to RT-PCR using combination of primers P2 & P4. This was followed by electrophoresis of the products on ethidium bromide stained agarose gel. Fluorescent Photographs of this gel (Fig. 4.4) revealed 453bp and 333bp long products corresponding to full length and truncated IL-1 α cDNA, respectively. The two cDNAs mentioned above reveal a difference of 120bp i.e. the expected difference in length between full length and truncated mRNAs that were subjected to RT-PCR, with increasing concentration of truncated (reporter) IL-1 α RNA. The full length product was gradually reduced. Adilution of the truncated IL-1 α mRNA stock

solution to 1/10,000 gave comparable amount of the two products indicating the concentration of 1.3 pg/ μ l of IL-1 α mRNA in the RNA preparation of that experiment. Activated rat macrophages were used as IL-1 producing positive controls in all experiments. The RT-PCR product from this tissue has previously been shown to be identical with rat IL-1 α utilising digestion with restriction enzymes Msp I and Pst I (Granholm 1992).

Fig. 4.4. Gel profile of Size difference between full length and truncated mRNAs



Lane 1 and 14: 123 bp DNA ladder. Lane 2-3: Amplified product from testis total RNA (1 μ g) without reporter mRNA. Lanes 4-13: Same as lane 2-3 with increasing amounts of reporter mRNA: lane 4-5: 0.13 pg/ μ l, lane 6-7: 0.20 pg/ μ l, lane 8-9: 0.26 pg/ μ l, lane 10-11: 0.50 pg/ μ l, lane 12-13: 1.3 pg/ μ l.

4.2 Ontogeny of IL-1 α

The presence or absence of IL-1 α mRNA in seminiferous tubules of 10 to 60 days old rats was tested using total RNA preparation obtained as described in sec. 3.2.2, and 1.0 μ g of total RNA was subjected to RT-PCR (sec. 4.1.1). At the end of the assay, 10 μ l of the sample was applied on ethidium bromide laced agarose gel to conduct electrophoresis (sec. 3.2.7). Fluorescent films of these gels (Fig. 4.5), demonstrate that young rats (0 to 15 days) do not express IL-1 α mRNA, while older rats 20 to 60 days expressed IL-1 α mRNA.

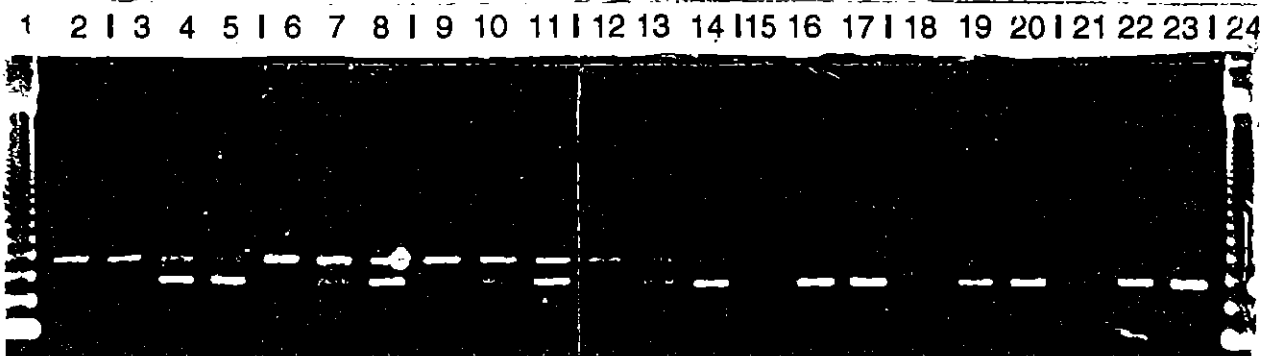
For coamplification of the reporter mRNA and IL-1 α mRNA from testicular RNA extract, cDNA synthesis was performed with RT primer P4. RT-PCR reaction was then conducted for 35-40 cycles. The result was then analysed by agarose gel electrophoresis and visualized by fluorescent photography (Fig 4.5 and Table 4.1). The data displayed can lead to the following conclusions:

1. 15 days old or younger rats do not have measurable quantity of testicular IL-1 α mRNA
2. Testicular IL-1 mRNA was detected in 20 days old rats. It was 0.26 pg / μ g of total RNA extract of testicular tissue.
3. In parallel with pubertal development the amount of testicular IL-1 α mRNA increased (Table 4.1).

Table 4.1 Ontogeny of tIL-1 α mRNA expression in rat testis.

Age of rats (days)	Amounts of tIL1 α mRNA (arbitrary units)
10	-
15	-
20	+
30	+++
40	++++
50	++++
60	+++

Fig. 4.5 Electrophoretic quantitative profile of reporter and testicular IL-1 α mRNA



Lanes 1 and 24: 123 bp DNA ladder. Lane 2: Amplified product from adult rat testis total RNA (1 μ g) without reporter mRNA. Lanes 3-5: Amplified testicular cDNA of 60 days old rats, lanes 3-5; 50 days, lanes 6-8; 40 days, lanes 9-11; 30 days, lanes 12-14; 20 days, lanes 15-17; 15 days, lanes 18-20; 10 days, lanes 21-23, with increasing amounts of reporter mRNA: lanes 3, 6, 9, 12, 15, 18, 21: 0.26 pg/ μ l; lanes 4, 7, 10, 13, 16, 19, 22: 1.3 pg/ μ l; lanes 5, 8, 11, 14, 17, 20, 23: 2.6 pg/ μ l.

4.3. Effect of Testosterone on IL-1 α Production

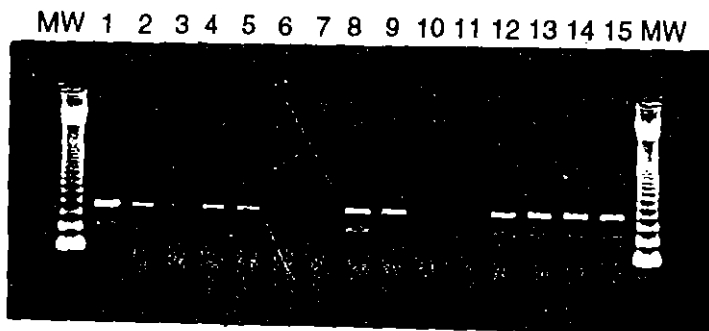
The role of testosterone on expression of IL-1 α can be monitored by titrating the quantity of IL-1 α mRNA, or by measuring the biological activity of this cytokine. The former is quantified as described in sec. 4.2, whereas biological activity of IL-1 α is measured as its' proliferative effect on Thymocytes (sec. 3.2.1).

4.3.1 Effect of Testosterone and EDS on IL-1 α mRNA expression

Testes were excised from five control and five EDS treated rats (sec. 3.1.1). This was followed by isolation of total RNA (sec. 3.2.2) from each testis.

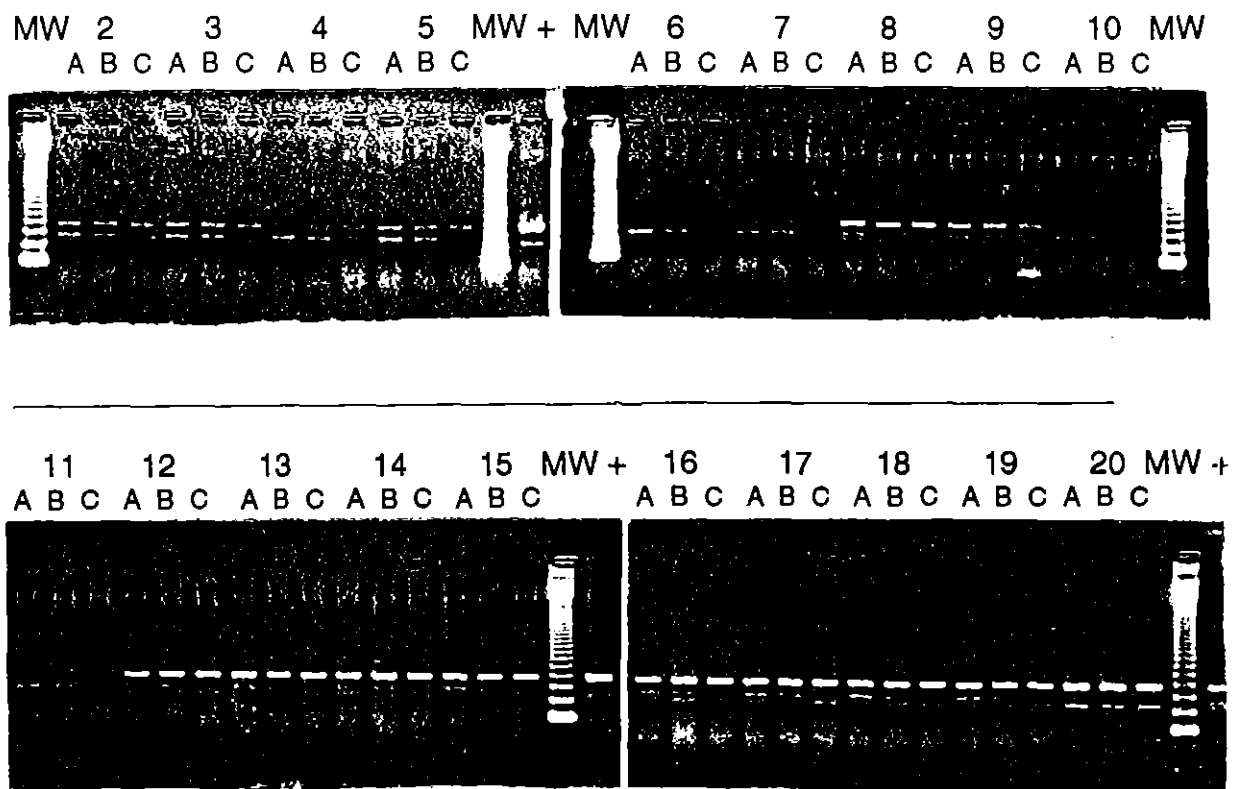
RT-PCR was then conducted on 1 μ g of total RNA from each of the control and experimental sample as described in sec. 4.2.2. Fig. 4.6 displays an experiment in which the presence of IL-1 α mRNA was investigated. The result showed high level of IL-1 α mRNA in all treated animals except lane 11. In addition to this qualitative test, quantitative RT-PCR was also conducted in the presence of reporter IL-1 α RNA serial dilution (Fig 4.7).

Fig. 4.6 Electrophoretic profile of IL-1 α cDNA obtained from control or EDS; EDS+testosterone; and cyproterone acetate treated rats.



Lanes labelled MW: 123 bp DNA ladder. Lane 1: Amplified product from activated rat macrophages (1 μ g). Lanes 2-15: Amplified testicular cDNA of adult rats treated with cyproterone acetate, lanes 2-5; EDS + testosterone, lanes 6-10; EDS alone, lanes 11-15.

Fig. 4.7 Electrophoretic quantitative profile of IL1 RT-PCR products from the Control; EDS; EDS + testosterone; and cyproterone acetate treated rats.



Lanes labelled MW: 123 bp DNA ladder. Lanes labelled +: Amplified product from activated rat macrophages (1 μ g) without reporter mRNA. Lanes 2-20: Amplified testicular cDNA of adult rats treated with cyproterone acetate, lanes 2-5; EDS + testosterone, lanes 6-10; EDS alone, lanes 11-15 and controls, lanes 16-20. Lanes 2-20 was from samples with increasing amounts of reporter mRNA: lanes 3 2A, 3A, 4A...20A: 2.6 μ g/ μ l; lanes 2B, 3B, 4B...20B: 1.3 μ g/ μ l; lanes 2C, 3C, 4C...20C: 0.26 μ g/ μ l.

4.3.4 Effect of testosterone on IL1 α bioactivity

Testes were collected from controls and EDS treated animals. Each testis was homogenised in saline (1:4 W/V) in a Turrax homogeniser.

10 μ l of control or experimental testicular homogenate was mixed with 100 μ l of 5×10^6 cells/ml. The effect of testicular extract was then assayed as described in sec. 3.2.1. The result of this experiment is displayed in Fig. 4.8. It is clear from

4.1.3 Linking reporter cDNA with T-7 bacteriophage promoter

Reporter cDNA was ligated with T7 bacteriophage promoter sequence from 1 µg of template using the steps described below. During the first cycle of PCR the primer-P6, that was constructed by linking 23 nucleotide long minimal bacteriophage promoter sequence and the first 21 nucleotides of the sense strand of reporter cDNA, was annealed with the antisense strand. The 3'-end of the antisense strand extends towards the 5' end of the T 7 promoter, while the primer extends towards the 5'-end of the reporter sequence thus producing 455bp long cDNA. Simultaneously, primer P4 leads to production of 432bp long cDNA that is identical with the original template.

PCR of 455bp long cDNA with P4 and P6 leads to amplification of this template. On the other hand the 432bp template leads to regeneration of its self with P4 but enrichment of 455bp product on strands that anneals with P6 (Fig. 4.3). Finally, the T7 promoter-linked reporter cDNA was purified as described in sec. 3.2.7.

4.1.4 Production of Reporter mRNA

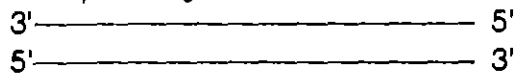
Approximately 1.0 µg of T7 promoter linked cDNA (455bp long molecule) was the template used to synthesize 432 base long reporter mRNA as described in Sec.3.2.5.

The quantity of reporter mRNA synthesized from T7 promoter containing truncated IL-1 α cDNA can be measured by two approaches: with or without [3 H]CTP. In the absence of [3 H]CTP, mRNA is recovered from *in vitro* transcription mix using method described in sec 3.2.6 and quantified by UV spectrophotometry (sec.3.2.8). In the presence of [3 H]CTP sample of *in vitro* transcription was subjected to agarose gel electrophoresis and quantitated as described in sec.3.2.10. Both spectrophotometry and scintillation counting revealed similar quantity, 0.5-1.5 µg of reporter IL-1 α mRNA. The reporter mRNA was dissolved in TE buffer (10mM Tris-PH 7.5, 1mM EDTA) such that a concentration of 13 ng was attained (stock solution).

Fig. 4.3 Cloning of T7 promoter linked reporter cDNA

a. Construction

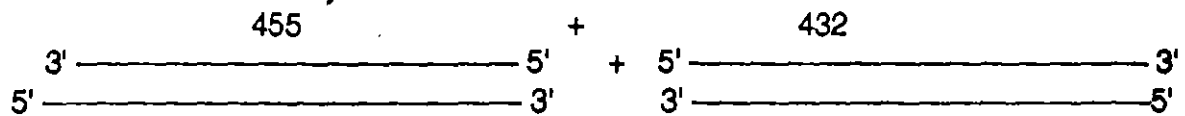
432 bp, full length cDNA



↓ denature



↓ Cycle 1

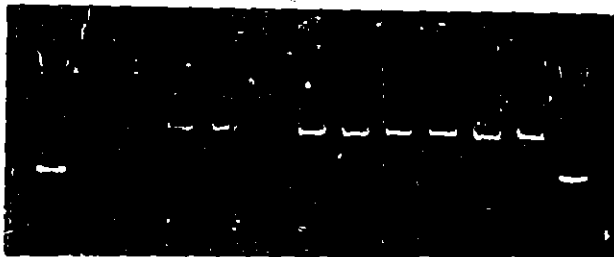


↓ 35 to 40 cycles

455 bp T7 promoter linked cDNA

b. Agarose gel electrophoresis showing purity of T7 promoter linked cDNA

1 2 3 4 5 6 7 8 9 10 11 12 13



Lane 1 and 13: 123 bp DNA ladder Lane 6: Negative control.

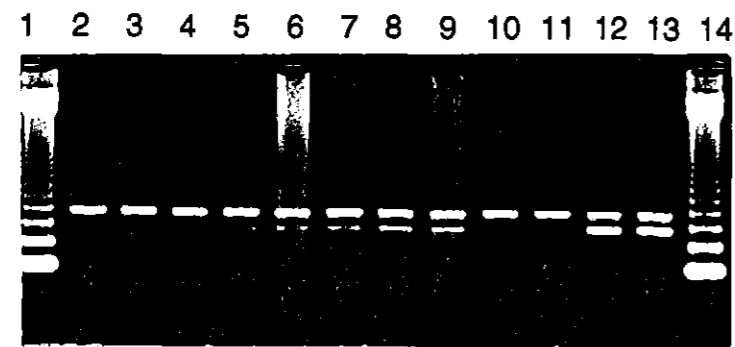
Lanes 2-5 and 7-12: Amplified product, 455 bp (lane 2: nonspecific amplification).

4.1.5 Identification of IL1 α mRNA Expression

To assess the purity of the product both full length and truncated mRNAs were subjected to RT-PCR using combination of primers P2 & P4. This was followed by electrophoresis of the products on ethidium bromide stained agarose gel. Fluorescent Photographs of this gel (Fig. 4.4) revealed 453bp and 333bp long products corresponding to full length and truncated IL-1 α cDNA, respectively. The two cDNAs mentioned above reveal a difference of 120bp i.e. the expected difference in length between full length and truncated mRNAs that were subjected to RT-PCR. With increasing concentration of truncated (reporter) IL1 α RNA. The full length product was gradually reduced. A dilution of the truncated IL-1 α mRNA stock

solution to 1/10,000 gave comparable amount of the two products indicating the concentration of 1.3 pg/ μ l of IL-1 α mRNA in the RNA preparation of that experiment. Activated rat macrophages were used as IL-1 producing positive controls in all experiments. The RT-PCR product from this tissue has previously been shown to be identical with rat IL-1 α utilising digestion with restriction enzymes Msp I and Pst I (Granholm 1992).

Fig. 4.4. Gel profile of Size difference between full length and truncated mRNAs



Lane 1 and 14: 123 bp DNA ladder. Lane 2-3: Amplified product from testis total RNA (1 μ g) without reporter mRNA. Lanes 4-13: Same as lane 2-3 with increasing amounts of reporter mRNA: lane 4-5: 0.13 pg/ μ l, lane 6-7: 0.20 pg/ μ l, lane 8-9: 0.26 pg/ μ l, lane 10-11: 0.50 pg/ μ l, lane 12-13: 1.3 pg/ μ l.

4.2 Ontogeny of IL1 α

The presence or absence of IL-1 α mRNA in seminiferous tubules of 10 to 60 days old rats was tested using total RNA preparation obtained as described in sec. 3.2.2, and 1.0 μ g of total RNA was subjected to RT-PCR (sec. 4.1.1). At the end of the assay, 10 μ l of the sample was applied on ethidium bromide laced agarose gel to conduct electrophoresis (sec. 3.2.7). Fluorescent films of these gels (Fig. 4.5), demonstrate that young rats (0 to 15 days) do not express IL1 α mRNA, while older rats 20 to 60 days expressed IL-1 α mRNA.

For coamplification of the reporter mRNA and IL-1 α mRNA from testicular RNA extract, cDNA synthesis was performed with RT primer P4. RT-PCR reaction was then conducted for 35-40 cycles. The result was then analysed by agarose gel electrophoresis and visualized by fluorescent photography (Fig. 4.5 and Table 4.1).

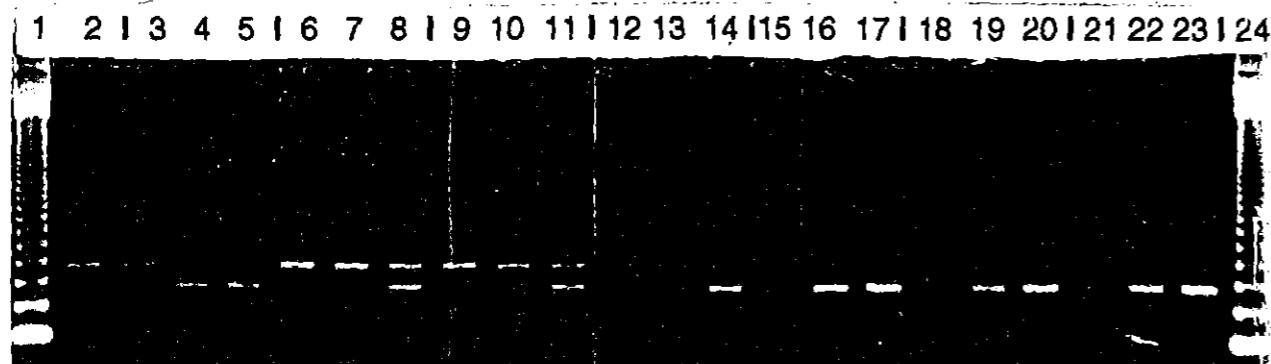
The data displayed can lead to the following conclusions:

1. 15 days old or younger rats do not have measurable quantity of testicular IL-1 α mRNA
2. Testicular IL-1 mRNA was detected in 20 days old rats. It was 0.26 pg / μ g of total RNA extract of testicular tissue.
3. In parallel with pubertal development the amount of testicular IL-1 α mRNA increased (Table 4.1).

Table 4.1 Ontogeny of tIL1 α mRNA expression in rat testis.

Age of rats (days)	Amounts of tIL1 α mRNA (arbitrary units)
10	-
15	-
20	+
30	+++
40	++++
50	++++
60	+++

Fig. 4.5 Electrophoretic quantitative profile of reporter and testicular IL-1 α mRNA



Lanes 1 and 24: 123 bp DNA ladder. Lane 2: Amplified product from adult rat testis total RNA (1 μ g) without reporter mRNA. Lanes 3-5: Amplified testicular cDNA of 60 days old rats, lanes 3-5; 50 days, lanes 6-8; 40 days, lanes 9-11; 30 days, lanes 12-14; 20 days, lanes 15-17; 15 days, lanes 18-20; 10 days, lanes 21-23, with increasing amounts of reporter mRNA: lanes 3, 6, 9, 12, 15, 18, 21: 0.26 pg/ μ l; lanes 4, 7, 10, 13, 16, 19, 22: 1.3 pg/ μ l; lanes 5, 8, 11, 14, 17, 20, 23: 2.6 pg/ μ l.

4.3. Effect of Testosterone on IL-1 α Production

The role of testosterone on expression of IL-1 α can be monitored by titrating the quantity of IL-1 α mRNA, or by measuring the biological activity of this cytokine. The former is quantified as described in sec. 4.2, whereas biological activity of IL-1 α is measured as its' proliferative effect on Thymocytes (sec. 3.2.1).

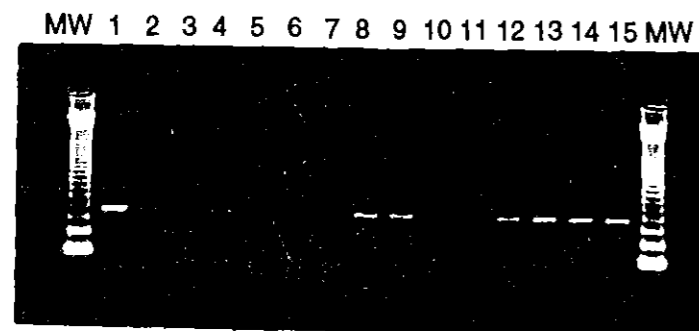
4.3.1 Effect of Testosterone and EDS on IL-1 α mRNA expression

Testes were excised from five control and five EDS treated rats (sec. 3.1.1).

This was followed by isolation of total RNA (sec. 3.2.2) from each testis.

RT-PCR was then conducted on 1ug of total RNA from each of the control and experimental sample as described in sec. 4.2.2. Fig. 4.6 displays an experiment in which the presence of IL1 α mRNA was investigated. The result showed high level of IL-1 α mRNA in all treated animals except lane 11. In addition to this qualitative test, quantitative RT-PCR was also conducted in the presence of reporter IL-1 α RNA serial dilution (Fig 4.7).

Fig. 4.6 Electrophoretic profile of IL-1 α cDNA obtained from control or EDS; EDS+testosterone; and cyproterone acetate treated rats.



Lanes labelled MW: 123 bp DNA ladder. Lane 1: Amplified product from activated rat macrophages (1 μ g). Lanes 2-15: Amplified testicular cDNA of adult rats treated with cyproterone acetate, lanes 2-5; EDS + testosterone, lanes 6-10; EDS alone, lanes 11-15.

The data displayed above was used to estimate the amount of target mRNA in controls and EDS treated rats. Inspection of fluorescent intensity (Fig. 4.7) revealed control rats had IL1 α mRNA content that corresponds to 4X of the highest concentration of reporter mRNA indicating a concentration of 10 ug/ul. On the other hand fluorescent band intensity of EDS treated rats corresponds 5X reporter mRNA corresponding to 13 μ g / ul (No 11 remains negative). From the quantitative data presented above that suppression of testosterone production enhances synthesis of IL-1 α mRNA

4.3.2 Effect of Testosterone Replacement

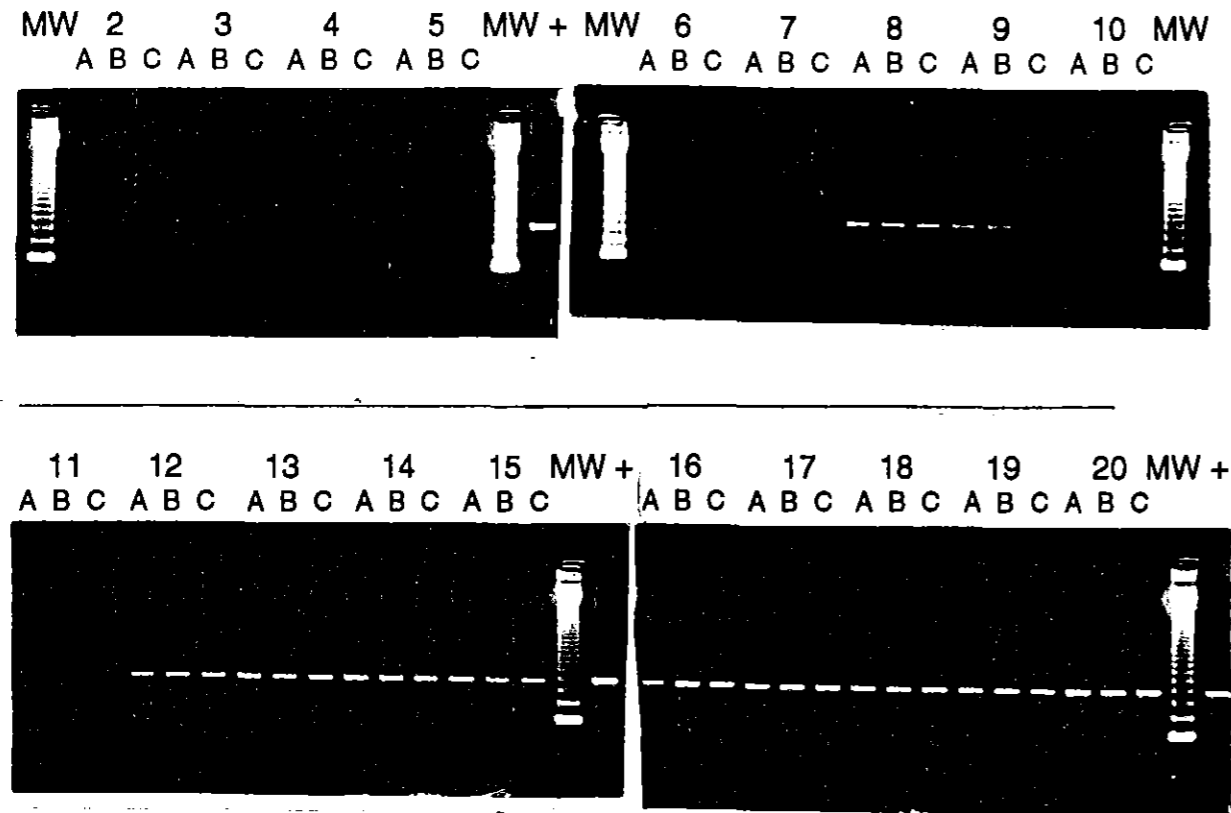
RNA samples were harvested from rats treated with EDS alone, or EDS+testosterone (Sec.3.1.1). This was followed by quantitative RT-PCR. Comparison of fluorescent band intensity from these samples (Fig. 4.6) indicated a tendency to reduce IL-1 α mRNA in groups that had received testosterone. Quantitative RT-PCR (fig4.7) revealed that mRNA from rats treated with EDS and testosterone, were 5-10 fold lower than from rats treated with EDS alone, (one sample i.e No 8 showed stronger signal). This data reinforced the conclusion that suppression of testosterone formation enhances production of IL1 α mRNA.

4.3.3 Effect of cyproterone acetate on IL-1 α mRNA Expression

The effect of Androcure was based on five control and drug treated rats (Sec.3.1.1). Testes were collected, total RNA harvested and quantitative RT-PCR conducted as described in sec. 4.3.1 and 4.3.2. The result is displayed in Fig. 4.6 and Fig 4.7 .

Comparison of fluorescent band intensities reveals that there is more IL-1 α mRNA in control samples than in samples from cyproterone acetate treated animals. The hypothetical reasons for such a paradox will be discussed in Chapter 5.

Fig. 4.7 Electrophoretic quantitative profile of IL1 RT-PCR products from the Control; EDS; EDS + testosterone; and cyproterone acetate treated rats.



Lanes labelled MW: 123 bp DNA ladder. Lanes labelled +: Amplified product from activated rat macrophages (1 µg) without reporter mRNA. Lanes 2-20: Amplified testicular cDNA of adult rats treated with cyproterone acetate, lanes 2-5; EDS + testosterone, lanes 6-10; EDS alone, lanes 11-15 and controls, lanes 16-20. Lanes 2-20 was from samples with increasing amounts of reporter mRNA: lanes 3, 2A, 3A, 4A...20A: 2.6 pg/µl; lanes 2B, 3B, 4B...20B: 1.3 pg/µl; lanes 2C, 3C, 4C...20C: 0.26 pg/µl.

4.3.4 Effect of testosterone on IL1 α bioactivity

Testes were collected from controls and EDS treated animals. Each testis was homogenised in saline (1:4 W/V) in a Turrax homogeniser.

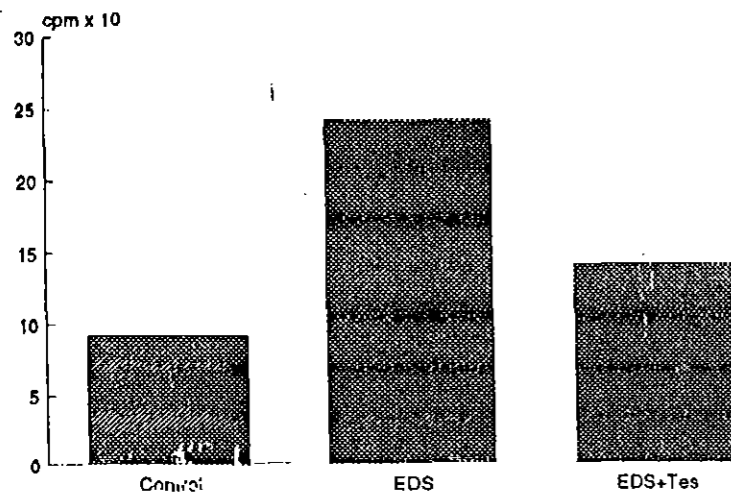
10 µl of control or experimental testicular homogenate was mixed with 100µl of 5×10^6 cells/ml. The effect of testicular extract was then assayed as described in sec. 3.2.1. The result of this experiment is displayed in Fig. 4.8. It is clear from

this figure that suppression of testosterone is accompanied by 2-3 fold increase in thymocyte proliferative activity of IL-1 α , i.e. increased production of this cytokine.

On the other hand if EDS treated rats were subjected to testosterone replacement therapy the following results were obtained:

1. IL-1 α production in the presence of both EDS/Testosterone is 1.5 fold higher than control.
2. IL-1 α production in the presence of EDS/Testosterone is only 60% of the amount when EDS alone was used.

Fig. 4.8 Effect of EDS and Testosterone on IL1 α production

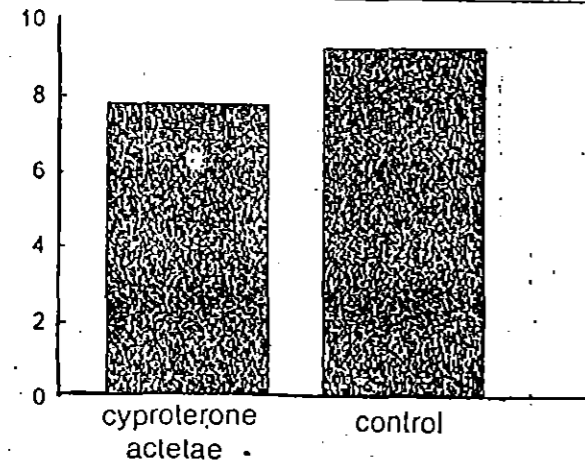


IL1 bioassay done on EDS, EDS/testosterone and control groups of rats. Data points are in group mean cpmx10 (\pm SEM) of 3 H thymidine incorporated in thymocyte cultures incubated in the presence of testicular homogenate. Compared to the level of IL1 bioactivity in the control (9.92 \pm 0.72), the value in the EDS treated groups (24.21 \pm 3.75) was significantly higher. The level of IL-1 activity in the group treated with EDS + testosterone (14.16 \pm 1.93) was significantly lower than the value in the EDS treated group.

In addition to the above, the effect of blocking testosterone receptor was also tested using cyproterone acetate (testosterone antagonist). This was done using rats treated with Androcure as described in Sec. 3.1.1. Other processes: excision of testes, homogenisation of testes and thymocyte proliferation test was conducted as described above. The result is displayed in Fig.4.9. It was shown that IL-1 α production in the presence of cyproterone acetate is approximately 90% of the amount produced by controls.

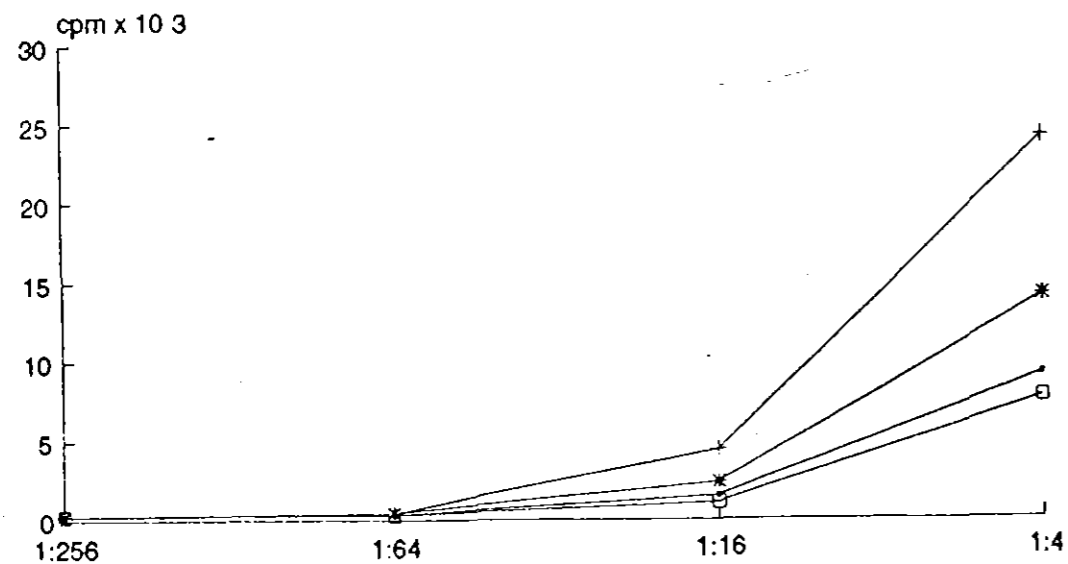
All IL-1 bioactivity data was analysed by monitoring the dose response curve. A typical dose response curve produced by testicular extract is shown in Fig. 4.10

Fig. 4.9 Effect of cyproterone acetate on IL-1 α production



The data points shown are in group mean $\text{cpm} \times 10^3$ (\pm SEM) of ^3H thymidine incorporated in thymocyte cultures incubated in the presence of testicular homogenate from the group treated with cyproterone acetate (7.74 ± 0.49), and the control (9.92 ± 0.72) groups of rats. No significant difference was observed between the two groups

Fig. 4.10 Dose Response Curve showing [^3H] Thymidine Incorporation into murine thymocytes as a function of dilution of testicular extracts.



Dose response curve showing the thymocyte proliferative response to serial dilutions of testicular extracts from control, (—■—), EDS treated, (—×—); EDS/testosterone (*—*) and cyproterone acetate treated groups (—□—) of rats. The plotted data indicate the mean level of ^3H thymidine incorporated in duplicate thymocyte cultures used in the assay.

CHAPTER FIVE DISCUSSION

Expression of Interleukin-1 α (IL-1 α) in the rat testis during development and its hormonal control by testosterone was analysed using quantitative polymerase chain reaction to make a relative estimation of IL-1 α mRNA; and thymocyte proliferation assay to determine the level of biologically active peptide.

In order to determine IL-1 α mRNA levels, a quantitative PCR method was developed using an internal standard RNA. This Internal standard was developed by in vitro transcription from a cDNA construct which is a 120 bp shorter version of the native rat macrophage IL-1 α cDNA preparation. As shown in the result section 4.1 coamplification of the serial dilutions of the internal standard transcript with the same amount of IL-1 α mRNA allowed to make a relative determination of the target IL-1 α mRNA. The internal standard transcript was designed in such a way that it minimizes all the variables that causes difference in the efficiency, hence the rate of amplifications of the target IL-1 α mRNA and the standard transcripts (see sec.4.1). These include such variables as differences in their sequences, the nature of primers used in the RT and PCR steps, and difference in the lengths of the target IL-1 α mRNA and the standard transcript (Wang *et al.*, 1990; Burmeister *et al.*, 1991; Raeymaekers, 1991).

In order to minimize the sequence difference between the target IL-1 α mRNA and the standard transcript, the cDNA used to construct the standard transcript was IL-1 α cDNA, prepared using primers specific for IL-1 α from rat macrophage RNA by RT-PCR, and checked to have a defined restriction enzyme pattern of IL-1 α cDNA. It has been previously shown that significant sequence difference can affect the efficiency of amplification (Chelly *et al.*, 1988; Becker-Andre and Hahlbroch, 1989). This is mainly due to the fact that the amount of temperature

required for complete denaturation (dissociation of double strands) of the different sequences differ. Hence resulting in different rate of denaturation of the target and the standard cDNAs during coamplification. In this study, effect of sequence difference is minimized since the 432 nucleotides of standard transcript used is designed to have sequence that is identical to the 432 base pairs of the target (IL-1 α) mRNA .

Primer annealing step is also the other important step adding to the variability in PCR amplification rate. Even in a PCR mixture with the same ionic strength and subjected to the same annealing temperature, primers that differ in their sequences have been shown to display difference in their rate and specificity with which they hybridize to their complementary site (Innis and Gefland, 1990). In this context, the standard IL-1 α transcript was constructed to have the common RT primer site, and both of the two PCR primer sites, hence allowing coamplification of both the target IL-1 α mRNA and the standard transcript using the same pair of primers. In this regard, both the target and the standard IL-1 α transcripts that are coamplified in the same PCR mixture are expected to be amplified with the same rate of primer annealing during revers transcription and subsequent PCR cycles.

In the present work the size difference 120 bp is used to distinguish the standard cDNA coamplified from the target IL-1 α cDNA. This size difference which is revealed by agarose gel electrophoresis, is another important variable that may differentiate the rate of amplification of internal standards used in quantitative PCR. This may lead to a question as to whether the standard transcript and the target IL-1 α sequences differing by 120 bp have the same efficiency of amplification. Several works have previously addressed this question by keeping

all other known variables constant and analysing rate and efficiency PCR amplification of DNA sequences differing by lengths. Chelly *et al.*, (1988) and Gilland *et al.*, (1990), have shown that size difference by up to 150 bp or by a small intron has no effect on the efficiency hence the rate of PCR amplification.

Quantitative PCR performed by making two and five fold serial dilutions of the 120 bases short internal standard transcript and coamplifying it with IL-1 α mRNA, is shown to have a sensitivity distinguishing as low as two fold differences in the concentrations of the target IL-1 α mRNA (see sec. 4.2). Previous works by Innis and Hahlbroch (1989) and Izutani *et al.* (1994) have shown sensitivities (of two fold and three to four fold difference) of quantitative RT-PCR experiments applied on IL-2, IL-8 and other gene transcripts. The quantitative PCR technique in this project has a comparable sensitivity (distinguishing 2 fold differences). The method is applied to make a relative determination of testicular IL-1 α mRNA expression during postnatal rat development, and to analyse the hormonal effect by testosterone.

This study shows the expression of IL-1 α mRNA to become detectable at the age of 20 days and the mRNA level progressively increases from day 30 to day 40 to reach a higher plateau level at day 60. The production of testicular Interleukin-1 α during postnatal rat development has been previously identified in the testis by using thymocyte proliferation bioassay; and its production correlates with puberty during maturation. The present finding demonstrated that the testicular IL-1 α mRNA expression rises in parallel with the level of biologically active IL-1 α peptide. Moore *et al.* (1993) have shown a change in the level of IL-1 α and β expression during thymocyte development. This change in the amount of IL-1 α production is due to an early increase in its mRNA level (up regulation),

which, during late thymocyte maturation was down regulated at the level of transcription. The pattern of IL-1 α mRNA expression and its correlation with the production of IL-1 protein suggested that the onset and developmental expression of IL-1 α is regulated through a rise in the level of its RNA expression at about the age of 15 postnatal days of rats by transcriptional initiation.

The regulation of tIL-1 α production by testosterone was also examined both at the level of IL-1 α mRNA and its encoded peptide. This experiment involves *In vitro* obliteration of intra testicular testosterone production by treating rats with EDS, an agent that selectively kills testosterone producing cells, leydig cells. EDS treatment and subsequent analysis of tIL-1 α bioactivity showed that obliteration of intratesticular testosterone resulted in a significant rise in the level of bioactive IL-1 α peptide. The level of tIL-1 α mRNA was also increased, however to lesser extent. This suggests testosterone produced from the Leydig cells inhibites tIL-1 α expression.

Leydig cells are known to produce several growth factors that regulate functions of sertoli cell, peritubular myoid cells, and germ cell differentiation (Bellve and Zheng, 1989; and Lamb, 1993). Removal of the leydig cells by EDS treatment could, hence, obliterate the production of these non androgenic products of the leydig cells. This leads to a question: could the increase in the expression of testicular IL-1 α that results during EDS treatment exclusively be due to testosterone removal, or due to additional effect of obliteration of non androgenic products of the leydig cells. This question was approached by analysing the IL-1 α expression in a group of rats that are treated with EDS and supplimented with exogenous testosterone. Testosterone replacement in this group of rats has significantly reduced tIL-1 α bio-activity and tIL-1 α mRNA production. This finding

shows that the rise in the level of IL-1 α expression during EDS treatment is largely due to removal of intratesticular testosterone production. However compared to the level in the untreated control, the IL-1 α peptide production during testosterone replacement is still higher than that in the control. Following this observation, in the EDS treated groups, that a high dose of testosterone administration could not reduce the level of IL-1 α expression to the control level, the following alternate explanations can be considered: First, it could be due to the fact that a high level of intratesticular testosterone concentration can not be reached with exogenous administration of high dose testosterone. Secondly, other non-androgenic products of the leydig cells, the production of which are removed by EDS treatment and not replaced, may also be involved in the negative regulation of IL-1 α expression. The result from RT-PCR experiment which shows markedly reduced level of IL-1 α mRNA makes it tempting to speculate that large part of the EDS stimulatory effect is achieved at posttranscriptional level.

Testosterone action is mediated by a nuclear Androgen receptor, a member of steroid receptor family. Hormone activated steroid receptors bind to specific DNA sequence of the gene which acts as transcriptional enhancer. In order to investigate whether this effect of testosterone on IL-1 α expression involves activation of androgen receptor, IL-1 α peptide and mRNA levels were analysed in a group of rats that are treated with androgen receptor antagonist, Cyproterone acetate. The result has shown that cyproterone acetate treatment and the resulting inhibition of androgen receptor action markedly reduced IL-1 α mRNA and also inhibited the peptide level. This result may therefore be a paradox to the result of the previous experiment which showed that testosterone inhibits IL-1 α expression. On these basis the following explanations can be given.

Firstly, it has been previously shown that testosterone concentration higher than what is required to saturate intratesticular androgen receptor is needed for normal testicular function. This suggests the possibility that testosterone may act by mechanisms other than through androgen receptor to inhibit IL-1 α expression. Taken together the negative regulatory effect of testosterone may not only involve androgen receptor activation.

Secondly, It has also been known that activated steroid receptors regulate gene transcription in concert with other regulatory proteins in the primary cellular response, and/or by altering the expression of these regulatory proteins (in the secondary cellular response). Therefore, inhibition of androgene receptor in this study might have left or enhanced the combined inhibitory effect of the other regulatory proteins on IL-1 α gene transcription. Similarly it might have increased the expression of inhibitory factors that normally are suppressed by testosterone, or decreased the expression of regulatory factors that normally stimulate IL-1 α mRNA expression.

The third possible explanation may, on the other hand, be that systemic cyproterone acetate treatment may not completely compete with the high intratesticular level of testosterone, but still inhibits the androgen receptor in the pituitary and elsewhere by competing with testosterone from the circulation. Androgen receptor inhibition in the pituitary will therefore block the feed back inhibitory effect of testosterone and result in an increase in the production of gonadotropin. This rise in gonadotropin level due to cyproterone acetate treatment may further increases the synthesis and production of testosterone in the testis more than the physiological level and ultimately suppress the expression of IL-1 α mRNA and peptide in the testis.

Although a complete androgen response element (ARE) could not be found at present, other steroids and retinoic acid are previously shown to regulate the expression of both IL-1 α and IL-1 β through their receptors, both at the level of transcription and postranscriptionally, mainly at the mRNA stability and processing of the primary transcript. It has been known that a primary cellular response of the target cell to hormone activation of steroid receptors mainly result in change at the level of transcription.

The present study suggests that the production of testicular IL-1 α is regulated by testosterone at the level of its mRNA expression possibly at the level of transcription initiation.

In addition stimulation of cells with testosterone and other steroids is also known to regulate expression of some genes post transcriptionally as a secondary cellular response. Jarous and Kaempfer, (1994) have described that human IL-1 α genes are also regulated at the level of processing of their primary transcripts by activated retinoic acid receptor, another member of the steroid receptors family. However relative determination of tIL-1 RNA, after in vivo pretreatment in the present work could not allow to study the change in the pre mRNA processing or the half life of IL-1 α mRNA as a function of the testosterone withdrawal and replacement. Therefore, the present data could not exclude the possibility that testosterone may act at these post transcriptional steps of IL-1 α gene expression.

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