TRANSCRIPTIONAL REGULATION OF THE RAT ATRIAL NATRIURETIC FACTOR GENE

by

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SUMMARY

Atrial natriuretic factor (ANF), a 28 amino acid peptide hormone, is the major secretory product of the heart. Because of its diuretic, natriuretic and vasodilating activities, this peptide may be involved in the maintenance of proper fluid and electrolyte balance and blood pressure control. In order to study the transcriptional regulation of ANF, we have isolated the rat ANF gene and we have established a system of cardiocytes in primary cell culture for studies on the hormonal, tissue-specific and developmental regulation of the ANF gene. Using this *in vitro* system, we have demonstrated that thyroid hormone increases ANF mRNA levels about 2- to 4-fold in atrial and ventricular cells in primary cardiocyte cell cultures, respectively. Similarly, glucocorticoids augment by about 3-fold both atrial and ventricular ANF mRNA levels in cardiac cells in culture. Glucocorticoids exert this effect at the transcriptional level probably via the binding of glucocorticoid receptor to a DNA element in the distal 5'-flanking sequences of the gene as suggested by DNA-mediated transfection studies in cardiocyte cultures. In order to better understand the mechanisms governing the cardiac-specific as well as developmental expression of the ANF gene, we have analyzed ANF promoter sequences by transient transfection studies in primary cardiocyte cultures. Our data show that the ANF promoter is active only in cells of cardiac origin. Moreover, up to -1.6 kb of 5' upstream sequences are necessary for full expression of the ANF gene in cardiac cells. Within these sequences, two particular elements, a proximal and a distal, are necessary for full ANF transcriptional activity. The proximal element can confer cardiac specificity to an otherwise non tissue-specific heterologous promoter. Further upstream sequences, between -2.5 and -1.6 kb appear to be implicated in the developmental control of ANF gene expression, as assessed by differential activity in 1 and 4 day old cultures. Thus, we have shown that the ANF gene is subject to at least three distinct levels of transcriptional control each of which is mediated by specific DNA elements located in the 5'-flanking sequences of the gene.

RÉSUMÉ

Le facteur natriuretique des oreillettes (ANF) est une hormone peptidique de 28 acides amines qui est secretee par le coeur et qui possede une activite diuretique, natriuretique et vasodilatatrice. Nous avons isole et sequence le gene encodant l'ANF dans le but d'etudier sa regulation transcriptionnelle. Nous avons etabli un systeme de cardiocytes en cultures primaires pour etudier les mecanismes de regulation qui s'exercent sur le gene de l'ANF au cours du developpement, ainsi que ceux qui determinent sa specificite cardiaque et sa reponse aux hormones. Nous avons ainsi demontre que les hormones thyroïdiennes augmentent d'environ 2 a 4 fois les niveaux d'ARNm dans les cellules auriculaires et ventriculaires en culture primaire. En outre, les glucocorticoïdes produisent une augmentation d'environ 3 fois des niveaux d'ARNm de l'ANF dans les cellules auriculaires et ventriculaires *in vitro*. Cet effet transcriptionnel semble être medie par la liaison du recepteur des glucocorticoïdes a un site de liaison du recepteur (GRE) situe dans les sequences 5' du gene. Afin d'identifier les sequences d'ADN qui determinent la specificite cardiaque et le niveau d'expression au cours du developpement, nous avons etudie le promoteur du gene de rat de l'ANF par transfection dans des cardiocytes en culture primaire. Nos resultats ont demontre que le promoteur de l'ANF n'est actif que dans les cellules cardiaques. Jusqu'a -1.6 kb des sequences du promoteur sont necessaires pour sa reconnaissance dans les cellules cardiaques et deux sous-elements presents dans cette region semblent conferer cette activite. Un de ces elements confere une activite tissu-specifique a un promoteur heterologue. Enfin, en utilisant des cultures provenant de rats de differents âges, nous avons constate que les sequences distales, entre -2.5 et -1.6 kb, semblent impliquées dans le contrôle de l'expression du gene au cours du développement. Le gène de l'ANF est donc sujet a au moins trois niveaux de contrôle distincts qui semblent tous être conferes par des sequences regulatrices specifiques situées dans la region 5' de ce gène.

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ABBREVIATIONS

aa:	amino acid
Ab:	antibody
ANF.	atrial natriuretic factor
ATP:	adenosine triphosphate
AVP:	arginine vasopressin
bp.	base pairs
cAMP:	3', 5'-cyclic adenosine monophosphate
CAT:	catecholamine acetyl transferase
cDNA:	complementary DNA
cGMP:	3',5'-cyclic guanosine monophosphate
CRF:	corticotropin releasing factor
Da:	daltons
DE:	distal element
DEX:	dexamethasone
DNA:	deoxyribonucleic acid
DOCA.	deoxycorticosterone
GR.	glucocorticoid receptor
GRE.	glucocorticoid response element
hGH	human growth hormone
HRE:	hormone response element
HSV:	Herpes simplex virus
Ir:	immunoreactive
kDa.	kilodaltons
Luc	luciferase
MMTV:	mouse mammary tumor virus
mRNA:	messenger ribonucleic acid
PE:	proximal element
PND.	pronatriodilatin*
RSV:	Raus sarcoma virus
SHR:	spontaneously hypertensive rats
SHRSP:	spontaneously hypertensive rats stroke prone
SV40:	Simian virus 40

T_3:	thyroid hormone
TK:	thymidine kinase
TPA:	12-O-tetra-decanoylphorbol 13-acetate
TR:	thyroid hormone receptor
TRE:	thyroid hormone element

*PND is used in certain chapters (2 and 3) and refers to the previous nomenclature for pro-ANF.

To my parents,

Bruno and Luigina

The true way goes over a rope which is not stretched at any great height but just above the ground.

It seems more designed to make people stumble than to be walked upon.

Kafka

Learning is nothing without cultivated manners, but when the two are combined in a woman you have one of the most exquisite products of civilization.

Maurois

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I would also like to express my appreciation to all the members of the laboratory, past and present, for their collaboration, comradeship and good humour. In particular, I wish to extend my gratitude to Marc-André Valiquette for his eager help in the preparation of cell cultures, Michel Chamberland for his many helpful hints and Danielle Gagnon for her assistance in the completion of my project.

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Finally, I wish to thank Lise Laroche for her expertise, professionalism and inexhaustible patience in the preparation of this thesis.

PREFACE

The present thesis, consisting of 6 chapters, describes the genomic structure and transcriptional regulation of the rat ANF gene. Chapter 1 is a literature review which can be subdivided into three major subsections. The first section deals with the purification of ANF, its primary structure, its biosynthesis and processing. The biological actions and regulation of this peptide are equally discussed. The second section deals with the molecular biology of ANF. What is known about its gene structure and the various levels of control of the ANF gene such as its developmental and hormonal regulation is included. The extraatrial sites of ANF mRNA synthesis are also discussed. The third section consists of a general review on eukaryotic gene regulation. The cis- and trans-acting elements that control transcription are discussed. A look at two important levels of transcriptional regulation, hormonal and cell-type restricted, concludes Chapter 1.

Chapters 2 to 5, inclusively, are comprised of scientific articles, in their original form, with some very minor modifications, in accordance with the McGill University Guidelines Concerning Thesis Preparation (Section 7) which reads as follows: "The candidate has the option, subject to the approval of the Department, of including as part of the thesis the text, or duplicated published text, of an original paper, or papers. In this case the thesis must still conform to all other requirements explained in Guidelines Concerning Thesis Preparation. Additional material (procedural and design data as well as descriptions of equipment) must be provided in sufficient detail (e.g. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature

review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interests of cohesion."

In Chapters 2 and 3, the term pronatriodilatin (PND) appears frequently to describe the pro-ANF moiety. However, following specific recommendations by a special advisory Committee, the term PND was dropped and ANF thereinafter used to refer to the gene and its pro-hormone. Thus, in all other sections (Introduction, Chapters 4, 5 and Discussion), only ANF is used to describe the gene encoding this peptide. It is also important to note that in all manuscripts (Chapters 2 to 5), the laboratory director and co-director, Dr. J. Drouin and Dr. M. Nemer, respectively, appear as co-authors.

Since, an examination of genetic sequence elements can offer certain clues as to the presence of common regulatory elements or particular sequence motifs, the characterization of ANF genomic sequences could serve as a useful tool towards the improved understanding of cardiovascular endocrine function at the molecular level. Thus, Chapter 2, **The Gene for Rat Atrial Natriuretic Factor**, is published work (*J. Biol. Chem.* 260, 1985:4568-4571) that describes the isolation and characterization of the rat ANF gene. Authors in addition to myself and my directors include members of the laboratory of Dr. P. Davies who isolated the rat ANF genomic clone. I was responsible for the subcloning, sequence determination and analysis of this clone.

Because of the documented notable effects of T_3 on the cardiovascular system and on ANF peptide levels, a study was undertaken to assess the effect of thyroid hormones on cardiac ANF mRNA synthesis. Chapter 3, **Thyroid Hormone Stimulates Rat Pronatriodilatin mRNA Levels in Primary Cardiocyte Cultures**

(*Biochem Biophys Res Commun.* 146, 1987.1336-1341), describes the regulation of cardiac ANF gene expression, by thyroid hormones, in both atria and ventricles in an *in vitro* system of primary cardiocyte cultures which I have established.

The mounting evidence for glucocorticoid regulation of ANF peptide synthesis and these hormones' generalized effects on the cardiac tissue incited us to pursue a detailed study on the effects of these corticosteroids on ANF gene expression. Hence, in Chapter 4, **A Distal Cis-Acting Promoter Element Mediates Glucocorticoid Stimulation of Cardiac ANF Gene Transcription**, the same *in vitro* system was used to evaluate the biochemical nature and the kinetics of glucocorticoid regulation of the ANF gene. The pharmacological properties of the response were characterized. The transcriptional nature of this regulation is confirmed through transient DNA-mediated gene transfer assays in the primary cardiocyte cultures. DNase I footprinting and gel retardation studies were executed by Dr. Y. L. Sun. Dr. T. Schmidt provided us with the glucocorticoid receptor protein. This work has recently been submitted.

Given the surprising conservation of DNA sequence elements among several species and the obvious involvement of ANF gene 5'-flanking sequences in the hormonal regulation of ANF gene expression, it was imperative to establish whether the cardiac-specific and developmental expression of the gene could equally be attributed to any particular sequence elements. Moreover, the very limited knowledge regarding the cis- and trans-acting factors that modulate cardiac-muscle gene expression further stimulated such studies since the ANF gene serves as an excellent and unique model towards this end. Chapter 5, **Identification of Cis-Acting Elements Involved in Cardiac-Specific and Developmental Expression of the Rat ANF Gene**, is a detailed study of the cis-

acting DNA elements of the ANF gene that mediate its tissue-specific and developmental pattern of expression. For this work, 5' deletion mutants were prepared by Dr. I. Lihrmann. These DNA chimaerae were tested in transient cell transfection assays. Dr I. Lihrmann performed the transfection studies in PC12 cells. This manuscript has recently been submitted

Finally, Chapter 6 includes a detailed discussion on the ANF gene regarding its hormonal and developmental regulation A description of future prospects for studies on the ANF gene is also included. A general bibliography follows chapter 6 and includes the references cited in the general introduction and discussion. References for each individual article (Chapters 2 to 5) are included within the respective chapter.

LIST OF PAPERS

CHAPTER 2: THE GENE FOR RAT ATRIAL NATRIURETIC FACTOR - Stefania Argentin, Mona Nemer, Jacques Drouin, Gary K Scott, Brian P Kennedy, and Peter L. Davies
The Journal of Biological Chemistry 260(8):4568-4571, 1985

CHAPTER 3: THYROID HORMONE STIMULATES RAT PRONATRIODILATIN mRNA LEVELS IN PRIMARY CARDIOCYTE CULTURES - Stefania Argentin, Jacques Drouin, and Mona Nemer
Biochemical and Biophysical Research Communications 146(3):1336-1341, 1987.

CHAPTER 4: A DISTAL CIS-ACTING PROMOTER ELEMENT MEDIATES GLUCOCORTICOID STIMULATION OF CARDIAC ANF GENE TRANSCRIPTION - Stefania Argentin, Yu Lin Sun, Tom Schmidt, Jacques Drouin, and Mona Nemer
Submitted 1990.

CHAPTER 5: IDENTIFICATION OF CIS-ACTING ELEMENTS INVOLVED IN CARDIAC-SPECIFIC AND DEVELOPMENTAL EXPRESSION OF THE RAT ANF GENE - Stefania Argentin, Isabelle Lihrmann, Jacques Drouin, and Mona Nemer
Submitted 1990.

CHAPTER I

INTRODUCTION

1.1 BIOCHEMISTRY OF ANF

1.1.1 Historical Background

In 1956, Bruno Kisch first described the presence of small electron dense bodies in the atrium of the guinea pig heart (Kisch, 1956) Later, similar findings were reported in bovine, rat, and human cardiac atria (Kisch, 1959, Bompiani et al., 1959, Battig et al., 1961) Although only the occasional presence of atrial specific granules was described in mammalian ventricles (Kisch, 1965, Palade, 1961), the ventricles of lower vertebrates were found to contain morphologically similar granules (Bencosme and Beyer, 1971) A more detailed characterization of the atrial specific granules, carried out by Jamieson and Pallade (1964), suggested that the granules were distinct from other known cellular bodies of liposomal nature The close association of the granules with an extensive Golgi complex, and the presence of a similar material in both the Golgi cisternae and the granules themselves strongly suggested that they were of secretory nature (Jamieson and Pallade, 1964) It was not until 1976, two decades later, that a possible role for the atrial specific granules in fluid and sodium balance was established when Marie and collaborators observed that atrial granularity was correlated with variations in salt and water balance (Marie et al 1976) This hypothesis was confirmed by the memorable experiment performed by de Bold (1981) and his colleagues, demonstrating that the supernatants of atrial but not ventricular homogenates could elicit a rapid natriuresis and diuresis These activities were later ascribed specifically to a substance stored within the atrial granules (de Bold et al., 1982, Garcia et al., 1982), and termed atrial natriuretic factor, or ANF

1.1.2 Purification and Identification of the ANF Peptides

The purification of atrial natriuretic factor (ANF) from the atria of both rat and human cardiac tissue suggested that the biological activities associated with ANF resided in a peptide of approximately 3000 daltons, although the existence of longer 5 and 13 kDa forms were reported (Kangawa et al., 1984, 1984a, Kangawa and Matsuo, 1984b, Thibault et al., 1983, 1984, Grammer et al., 1983, Trippodo et al., 1983). Furthermore, it was soon discovered that a potent vasorelaxant activity could also be elicited from atrial but not ventricular tissue extracts. Relaxation of both vascular and non-vascular smooth muscle preparations was detected in the presence of a protease sensitive atrial substance (Currie et al., 1983). Progressive purification of this activity showed it co-chromatographed with the 3 kDa natriuretic activity in all chromatographic systems tested (Grammer et al., 1983).

As more groups reported the complete amino acid sequence of the 3 to 5 kDa rat and human peptides, it became apparent that various amino-terminal extensions of the same peptide were being isolated (Seidah et al., 1984, Kangawa et al., 1984, 1984a, Kangawa and Matsuo 1984b, Currie et al., 1983a; Thibault et al., 1984), suggesting that the low molecular weight ANF peptides were products of a larger precursor molecule. Nonetheless, the 3 kDa, 28 aa carboxy-terminal peptide, appeared to possess all of the biological activity and was identical in both the rat and human, with the exception of a single amino acid substitution at position 110 where methionine (Met) replaces isoleucine (Ile) in human ANF (Fig. 1.1) (Thibault et al., 1984, Kangawa et al., 1984). In both species, the biologically active peptide consists of a molecule having a disulfide linkage between 2 cysteine residues (Kangawa et al., 1984, 1984a), creating a ring structure which appears to be essential for its activity (Misono et al., 1984). It was not until 1987 that Thibault

and collaborators succeeded in purifying the intact 126 aa ANF precursor molecule from rat atria. The precursor was detected by immunocytochemistry in all granules, and was the predominant molecular weight form of ANF in the atria (Thibault et al., 1987).

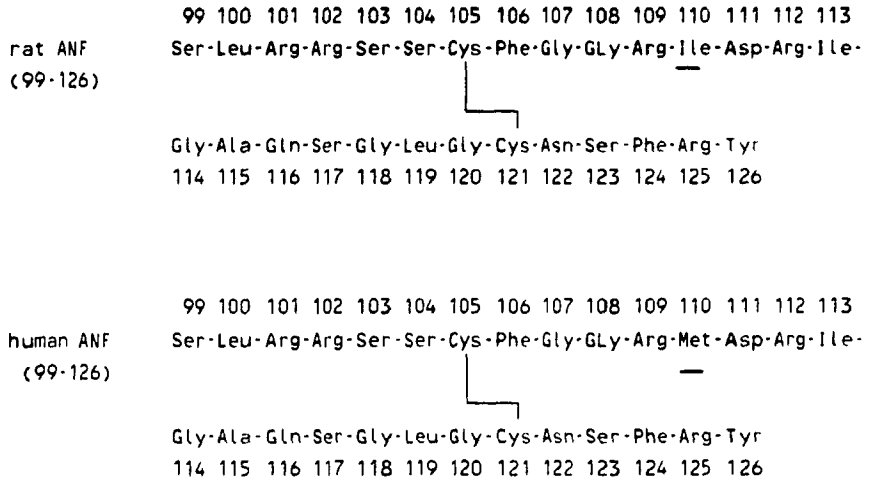

Figure 1.1. Amino acid sequence comparison of rat and human ANF peptides The carboxy terminal amino acids 99 to 126 of the pro-ANF molecule are illustrated. The cysteine residues forming a disulfide linkage are indicated and amino acid 110 is underlined.

Following the development of a specific radioimmunoassay, the endocrine nature of ANF was confirmed by the identification of ANF in the plasma (Gutkowska et al., 1984, Tanaka et al., 1984). The plasma derived immunoreactivity had a similar mobility in reverse phase-high performance liquid chromatography (RP-HPLC) as the low molecular weight atrial peptide and corresponded to the carboxy-terminal of the high molecular weight species (ANF_{1-126}) (Schwartz et al., 1985, Thibault et al., 1985).

1.1.3 Processing and Release of ANF

In several species, including human, it is now well established that the circulating form of ANF is the biologically active ANF_{99-126} (Gutkowska et al., 1984; Tanaka et al., 1984; Currie et al., 1984). Circulating ANF has an extremely short half life of about 30 sec in the rat and 25 min. in man (Luft et al., 1986; Yandle et al., 1985) It appears to be metabolized primarily in the kidney with a very high metabolic clearance rate (Hayashi et al., 1987). The heart stores the prohormone form of ANF_{1-126} but secretes the processed peptide (ANF_{99-126}) (Fig. 1.2, Schwartz et al., 1985; Thibault et al., 1986).

In contrast, cultured neonatal atrial and ventricular cardiocytes both store and secrete the uncleaved precursor (Bloch et al., 1985; Glembotski et al.; 1985, Bloch et al., 1986), suggesting that maturation of the peptide is a post-secretory event However, while serum has been shown to effect proteolysis, neither whole blood nor plasma can efficiently cleave the substrate, making the circulatory system an unlikely physiologically significant site of peptide maturation (Michener et al., 1986; Gibson et al., 1987).

Several observations tend to suggest that ANF peptide processing occurs within the cardiac tissue. In support of this view is the finding that ANF_{1-126} is effectively processed when reperfused through an isolated rat heart (Michener et al., 1986). In addition, in the normal state, the intact ANF_{1-126} precursor has never been detected in the circulation while both N- and C-terminal metabolites are increased in blood in response to certain secretagogues such as d-arginine vasopressin (d-AVP) (Michener et al., 1986). Recently, it has been shown that in the presence of glucocorticoids, cultured atrial myocytes possess the ability to

properly process ANF_{1-126} to a form chromatographically indistinguishable from ANF_{99-126} (Shields and Glembotski, 1988). This study suggests that the cardiocytes themselves or adjacent support cells have the intrinsic ability to effect proteolysis perhaps in conditions favoring the expression of an enzymatic component. To further support this view, immunocryoultramicrotomy studies have shown that the peptide travels uncleaved from the Golgi complex to the mature secretory granules. This suggests that maturation of the peptide is either directly associated with exocytosis of the granular contents or mediated by the environment directly adjacent to the cardiocytes, within the extracellular space (Thibault et al., 1989a).

1.1.4 Biological Actions of ANF

In addition to its natriuretic, diuretic and vasodilatory properties, ANF acts on many tissues and affects various hormones. Overall, the actions of ANF all contribute to the lowering of blood pressure and the maintenance of cardiovascular homeostasis.

At the level of the kidney, ANF stimulates glomerular filtration rate (GFR) (Cogan, 1986), promoting the excretion of water, sodium, chloride, magnesium, calcium and phosphate ions (Winquist, 1987). Although ANF concentrations in the physiological range in man (20.9 pmol/l) can produce a significant natriuresis and diuresis (Anderson et al., 1987), the natriuretic activity of ANF tends to decrease somewhat at higher doses or after prolonged infusion due to the ensuing hypotension that results in decreased renal perfusion pressure (Seymour et al., 1985; Granger et al., 1986). Renal vasodilation (Wakitani et al., 1985), redistribution of renal blood flow (Borenstein et al., 1983), and modulation of effects on sodium handling in the distal nephron segment (Zeidel and Brenner, 1987), all presumably

contribute to the actions of ANF on the kidney.

At the level of the vasculature, ANF can lower blood pressure in both normal and hypertensive subjects (Cody et al., 1986; Tikkanen et al., 1985a). *In vitro*, ANF relaxes isolated vascular smooth muscle segments (Garcia et al., 1985). The relaxant effect is more prominent in the presence of vasoconstrictors like angiotensin II or noradrenaline (Winquist, 1987). *In vivo*, both atrial extracts or synthetic ANF peptides can elicit a decrease in cardiac output and peripheral resistance (Ackermann et al., 1984; Hirata et al., 1985). ANF-mediated vascular relaxation is associated with increments in cGMP (Winquist et al., 1984). Isolated veins are generally unresponsive to ANF (Winquist, 1987).

ANF has also been shown to have various effects on other hormonal systems. In particular, ANF decreases both basal and stimulated aldosterone release at the level of the adrenal by inhibiting early steps of the steroidogenic pathway (Kudo and Baird, 1984; De Lean et al., 1984; Chartier et al., 1984) and, *in vivo*, elevated plasma aldosterone concentrations are reduced following ANF infusion (Atarashi et al., 1985). There have been reports of an inhibitory effect on glucocorticoid synthesis by the zona fasciculata cells (De Léan et al., 1984; Racz et al., 1985), although most investigators report little or no effect on cortisone secretion (Atarashi et al., 1984; Campbell et al., 1985). Although cGMP increases seem to be associated with the inhibitory effect on steroidogenesis (Higuchi et al., 1986), the addition of a cGMP derivative does not seem to mimic the action of ANF (Elliott and Goodfriend, 1986).

At levels eliciting natriuresis and hypotension, ANF inhibits renin secretion (Winquist, 1987). Elevations in tissue cGMP levels have been observed in parallel with this action (Kurtz et al., 1986). Indeed, administration of ANF antibodies

with this action (Kurtz et al., 1986). Indeed, administration of ANF antibodies results in increased plasma renin activity in rats (Naruse et al., 1985).

Vasopressin release from the posterior pituitary is also inhibited by ANF (Samson, 1985). At high ANF concentrations, testosterone production by mouse interstitial cells is stimulated (Bex and Corbin, 1985). On the other hand, decreased progesterone secretion has been demonstrated in murine Leydig tumor cells in response to ANF (Pandey et al., 1985).

1.1.5 Regulation of ANF Secretion

Extensive studies have shown that the strongest stimulus for ANF secretion is atrial distension. This effect has been observed in both intact animals and in isolated hearts (Dietz, 1984; Ledsome et al., 1986). Several physiological states also produce increments in plasma ANF levels, including head-out water immersion (Epstein et al., 1987) and changes in body posture (Larose et al., 1985). It has been shown that episodes of atrial or ventricular tachycardia are associated with elevated plasma ANF levels (Yamaji et al., 1985; Tikkanen et al., 1985). This effect has also been observed *in vitro* where the frequency of contraction of isolated atria directly influences ANF secretion (Schiebinger and Linden, 1986). In addition, acute as well as chronic volume expansion is a strong stimulus for ANF release (Petterson et al., 1986; Metzler et al., 1987) as is acute isotonic saline infusion (Lang et al., 1985). Moreover, several pathological conditions lead to increased circulating ANF levels: chronic renal failure (Rascher et al., 1985), myocardial infarction (Tomoda, 1988), congestive heart failure (Burnett et al., 1986; Ding et al., 1987; Cody et al., 1986) and hypertensive states (Imada et al., 1985; Snajdar and Rapps, 1986; Mercadier et al., 1989).

Several humoral factors are associated with the stimulation of ANF secretion. In certain studies both α and β adrenergic agonists increase the release of ANF from perfused hearts and isolated atria (Ruskoaho and Leppaluoto, 1988, Schiebinger 1987), and *in vivo* infusion of noradrenaline to human subjects elicits a significant rise in plasma ANF levels (Sanfield et al., 1987). However, the relative roles of α and β adrenergic stimulation have not been entirely clarified since β adrenergic stimulation is ineffective in eliciting a stimulatory response in several cases (Shields and Glembotski, 1989, Sonnenberg and Veress, 1984, Currie and Newman, 1986). In an early publication, Sonnenberg and Veress have reported that activators of the phosphoinositide system including α-1 adrenergic and muscarinic cholinergic agonists are efficient secretagogues for ANF. These results have been corroborated by more recent work showing that activators of protein kinase C, and calcium ionophores do indeed mediate ANF release while protein kinase A activators are inhibitory (Shields and Glembotski, 1989, Matsubara et al., 1988, Iida and Page, 1988, Ruskoaho et al., 1985). In addition to these neurotransmitters, opioids and several anaesthetics have been shown to augment plasma ANF levels in intact rats (Horky et al., 1985, Gutkowska et al., 1988, Chen et al., 1989).

Certain hormonal substances that modulate ANF synthesis and/or release have also been identified. *In vitro*, atrial cell cultures respond to dexamethasone, testosterone and thyroid hormone (T_3) stimulation by increased ANF synthesis and secretion (Matsubara et al., 1987). This observation has been confirmed *in vivo* where hyperthyroid states are associated with elevated plasma ANF levels (Kohno et al., 1986, Gardner et al., 1987a). Similarly, *in vivo* studies have shown that glucocorticoid administration or elevated plasma cortisol levels correlate with elevated plasma ANF both in rats and humans (Yamaji et al., 1988; Gardner et al.,

1986) Furthermore, it appears that ANF could be involved in the mineralocorticoid "escape" mechanism since the rise in plasma ANF levels following mineralocorticoid administration closely parallels this phenomenon (Grekin et al., 1986; Ballermann et al., 1986). The direct effects of certain physiological stimuli and of steroid and thyroid hormones on ANF gene transcription, are discussed below.

1.1.6 Localization of Extraatrial ANF peptides

Although the atria are the major sites of ANF synthesis ANF immunoreactivity has been found in numerous extraatrial tissues (reviewed by Gutkowska and Nemer, 1989a) Early reports described the detection of ANF by immunocytochemical studies in the rat salivary glands (Cantin et al., 1984) Similarly, immunoreactive ANF has been identified in certain vascular tissues such as the pulmonary vein and vena cava (Toshimori et al., 1988, Larsen et al., 1987 1988, Asai et al., 1987), where in rodents these blood vessels contain extensions of cardiac muscle cells

High molecular weight immunoreactive ANF peptide was also found in ventricular tissue (Nemer et al., 1986) In the ventricles, unlike the atria the peptide is not accumulated in secretory granules but is rapidly secreted through a constitutive pathway (Bloch et al., 1986) In this tissue, it is particularly subject to various physiological and hormonal stimuli, resulting in significant variations in peptide synthesis

In the lung, both high and low molecular weight peptides have been detected, both in the rat and human (Gutkowska et al., 1987; Sirois and Gutkowska 1988) Furthermore, secretion of the 28 amino acid ANF from lung tissue has been

demonstrated from pneumocytes in primary cell culture and in perfusates from isolated rat lungs (Gutkowska et al., 1987b, Matsubara et al. 1988a) A role for pulmonary ANF in certain lung disorders may be possible as it has been shown to confer a protective effect against pulmonary edema (Inomata et al., 1987; Imamura et al. 1988).

The ANF peptide has also been identified in numerous sites of the peripheral and central nervous systems. Using sensitive radioimmunoassays and immunocytochemical techniques, ANF-like immunoreactivity has been reported in several structures of the sympathetic and parasympathetic autonomic nervous systems (Debinski et al., 1986, 1987a; Morii et al., 1987) and in several locations of the brain (Inagami et al., 1989) The highest peptide concentration was found in the rat hypothalamus and was estimated to be 10^4 to 10^5 times less abundant than in atria (Tanaka et al., 1984; Morii et al. 1985, Zamir et al., 1986, Imada et al., 1985) A survey on the distribution of brain ANF revealed it to be present extensively throughout several regions including the midbrain, cortex, olfactory bulb, thalamus and pontine medullary region (Morii et al., 1985). In contrast to pro-ANF in the atria, brain ANF appears to be stored in a low molecular weight form corresponding to 24 and 25 amino acid species ($ANF_{102/103-126}$) (Shiono et al. 1986), however, this question still remains controversial Although it has been shown that the secretion of low molecular weight ANF is predominant from the hypothalamus *in vitro* (Tanaka and Inagami, 1986), a recent report demonstrates the presence of considerable pro-ANF in perfusates from rat hypothalamic explants (Nissen et al., 1989) Some activities associated with the central administration of ANF include inhibition of non-stimulated and angiotensin II-stimulated dipsogenesis in rats (Antunes-Rodrigues et al. 1985; Masotto et al., 1985), inhibition

of dehydration and hemorrhage-induced vasopressin release (Samson, 1985), inhibition of firing of vasopressin neurons (Standaert et al., 1987), inhibition of acetylcholine-induced CRF release from the hypothalamus (Takao et al., 1988) and stimulation of natriuresis and diuresis in conscious hydrated rats (Israel et al., 1988).

In addition to these neuronal tissues, ANF immunoreactivity was discovered in both anterior (McKenzie et al., 1985) and posterior hypophyses (Gutkowska et al., 1987a). While both the high and low molecular weight peptides were detected in the anterior pituitary (Gutkowska and Cantin, 1988a), only the 28 amino acid form was found in the posterior gland (Gutkowska et al., 1987a). This is consistent with the production of ANF by the hypothalamus, like other posterior pituitary hormones such as oxytocin and vasopressin. In the anterior pituitary, ANF immunoreactivity appears to be associated with the gonadotrophs (McKenzie et al., 1985). Although the actions of ANF in this tissue remain to be determined, an inhibitory effect of ANF on vasopressin release by the posterior pituitary has been reported (Obana et al., 1985, Januszewicz et al., 1986).

In the peripheral nervous system, ANF was demonstrated in several ganglia of the autonomic system (Debinski et al., 1986, 1987a, Morii et al., 1987) and in the spinal cord (Morii et al., 1987). The low molecular weight peptide was found to be the predominant form in all the tissues tested. ANF was found to inhibit norepinephrine release during sympathetic nerve stimulation in rat mesenteric arteries (Nakamaru and Inagami, 1986) as well as to partially inhibit catecholamine synthesis by rat superior cervical ganglia (Debinski et al., 1987).

The identification of ANF immunoreactivity in cells of the adrenal medulla (McKenzie et al., 1985, Mukoyama et al., 1988, Morel et al., 1988) led to the

the mature peptide were detected in the chromaffin cell granules. Likewise, both forms are co-secreted by these cells (Nguyen et al., 1988). Interestingly, ANF peptide synthesis in these cells is stimulated by phorbol esters and forskolin, activators of protein kinases C and A, respectively (Pruss and Zamir, 1987; Nguyen et al., 1988). Although the role of ANF in the adrenal is not clear, possible paracrine functions of ANF include the modulation of noradrenaline release (Drewett et al., 1988) and inhibition of aldosterone synthesis in the adrenal cortex (De Léan et al., 1984).

A small quantity of ANF has been detected in the digestive system. High molecular weight pro-ANF was detected in the guinea pig intestine (Vollmar et al., 1988) and in rat stomach and small intestine (Vuolteenaho et al., 1988). The presence of the high molecular weight species suggests local synthesis of ANF. The exact role of ANF in this tissue remains unclear.

Other peripheral tissues were found to contain ANF. For example, in the thymus the presence of high molecular weight precursor was reported (Vollmar and Schulz, 1988b). Rabbit ovary and bovine corpus luteum were found to contain both high and low molecular weight forms (Kim et al., 1989; Vollmar et al., 1988a). Since ANF has been shown to diminish progesterone secretion from testicular Leydig cells (Pandey et al., 1985), a similar function in the ovary might be postulated.

Finally, in addition to blood, ANF has been detected in urine where it has been shown to increase in congestive heart failure and to return to normal levels following treatment (Ando et al., 1988).

1.1.7 The ANF-Receptors and Signal Transduction Systems

Early observations that atrial homogenates or synthetic ANF peptides stimulated cyclic 3', 5' guanosine monophosphate (cGMP) levels in whole animals, crude adrenal membrane preparations or isolated vascular segments (Hamet et al., 1984; Waldman et al., 1985, Winquist et al., 1984) led to the conclusion that cGMP may act as a second messenger of ANF action. The correlation between ANF-induced stimulation of cGMP levels and the distribution of particulate guanylate cyclase suggested that this enzyme and not the soluble guanylate cyclase, was implicated (Tremblay et al., 1985, Winquist et al., 1984) In fact, it was shown that detergent dispersion of particulate guanylate cyclase resulted in a preparation that retained sensitivity to ANF activation (Tremblay et al., 1985a), suggesting that the receptor molecule and guanylate cyclase activity were either in close association or part of the same entity

Cross-linking studies with iodinated-ANF (^{125}I-ANF) allowed the identification of two types of ANF cell surface binding sites. A low molecular weight receptor with an apparent molecular weight of 60-70 kDa was radiolabelled from cultured bovine smooth muscle cells as well as rabbit aorta membranes (Schenk et al., 1985; Vandlen et al., 1985) Similar studies also revealed the existence of a high molecular weight receptor of 120-180 kDa in various tissues such as rabbit and bovine aortas and rat and bovine adrenal cortex (Vandlen et al 1985; Schenk et al., 1985; Misono et al., 1985) Competition studies with native ANF peptides and modified or truncated analogs showed the two receptors were pharmacologically distinct. The high molecular weight receptor was shown to have more stringent constraints for binding of ANF while the more abundant low molecular weight subtype could bind ANF analogs of varying biological potencies

(Olins et al. 1988. Meloche et al. 1987. Leitman et al. 1986). Furthermore. activation of guanylate cyclase and cGMP formation appeared to be associated solely with the high molecular form of the ANF-receptor (Leitman et al.. 1985).

Purification of the ANF receptor proteins by various groups showed that guanylate cyclase activity indeed co-purifies only with the high molecular weight 120-180 kDa ANF-receptor. and that ANF binding and guanylate cyclase activities reside within the same polypeptide (Meloche et al. 1988; Kuno. 1986; Takayanagi et al.. 1987. Arenjanyil et al.. 1987)

A more detailed characterization of the two ANF receptor subtypes has recently become available with the cloning of the cyclase uncoupled. low molecular weight receptor (ANF-C receptor) as well as both bovine and rat cyclase-linked high molecular weight ANF receptors (ANF A and B receptors) (Fuller et al. 1988; Chinkers et al.. 1989. Lowe et al. 1989. Chang et al.. 1989. Schulz et al. 1989) The isolation of a cDNA clone corresponding to the bovine ANF-C receptor showed it encoded a polypeptide containing a single potential transmembrane domain. Binding of ANF and truncated or modified analogs to this receptor is consistent with the ligand binding specificity previously described for the low molecular weight ANF receptor subtype The functional role attributed to these receptors is one of a clearance site. possibly providing a buffering system against large fluctuations in plasma ANF levels (Maack et al. 1987). hence the name clearance or C-receptor.

Both rat and human biologically functional. cyclase-linked receptors were also recently cloned The isolation and characterization of both rat and human cDNA clones revealed the presence of two distinct receptor subtypes. named ANF-A and ANF-B receptors with different ligand binding specificities (Chang et al..

1989, Schulz et al., 1989) These cDNA's all encode mature proteins of 1048-1057 aa, having molecular weights of about 115 kDa and containing single membrane spanning regions. In both the rat and human ANF A and B receptors, there lies within the intracellular portion of the protein two domains a protein kinase-like domain and the guanylate cyclase catalytic domain (Lowe et al., 1989, Schulz et al., 1989) Since deletion mutagenesis studies revealed that the removal of the protein kinase domain from the wild type receptor resulted in ligand independent constitutive guanylate cyclase activity, it has been postulated that the protein kinase domain is involved in the repression of guanylate cyclase activity. Ligand binding seems to mediate a conformational change to overcome this inhibitory interaction ATP binding seems to enhance the conformational change required for catalytic function (Chinkers and Garbers, 1989a)

The direct activation of guanylate cyclase activity by ANF receptor binding is representative of a new paradigm in signal transduction systems cGMP is believed to be the direct mediator of smooth muscle relaxation (Winquist et al 1984 Fiscus et al., 1985) However, it is still unclear whether it is directly involved in mediating diuresis and natriuresis in the kidney, although particulate guanylate cyclase activity is tightly correlated to ANF binding sites in the renal segments (Tremblay et al., 1985, De Lean et al. 1985) In the adrenal, the inhibition of aldosterone synthesis does not appear to be directly mediated via cGMP elevation (Elliot and Goodfriend, 1986, Matsuoka et al., 1987), although ANF was shown to stimulate particulate guanylate cyclase in this tissue More work is needed to further elucidate the mechanism by which cGMP modifies the appropriate effectors that produce a physiological end response

1.2 MOLECULAR BIOLOGY OF ANF

1.2.1 Structure of the ANF Gene

Complementary DNA's (cDNA's) for pro-ANF have been cloned and sequenced in various species including the rat, human, dog and rabbit (Maki et al., 1984, Seidman et al., 1984, Yamanaka et al., 1984, Zivin et al., 1984, Nakayama et al. 1984, Oikawa et al., 1984, 1985) The structure of the pre-pro ANF as deduced from the nucleotide sequence of these cloned cDNA's includes a hydrophobic stretch of 23-25 amino acids, depending on the species, providing the signal for cotranslational transport through the endoplasmic reticulum

The remaining prohormone in all species is then constituted of an additional 126 amino acids, the carboxy-terminal of which generates the biologically active ANF peptide Finally, an additional pair of arginine residues are present at the carboxy-terminal extremity of the rabbit, rat and mouse prohormones which must be cleaved at very early processing stages as they have never been detected in the stored pro-ANF molecule (Fig 1.2)

A comparison of the amino acid sequences between species has shown that 73.8 % of pro-ANF amino acids are identical in the five species, and this homology increases to 93.3% when comparing the last 45 carboxy-terminal amino acids (Oikawa et al., 1985).

The human, rat, mouse, and bovine genomic clones of the pre-pro-ANF have also been isolated and sequenced (Nemer et al., 1984; Greenberg et al., 1984; Maki et al., 1984a, Seidman et al., 1984a; Argentin et al., 1985, Vlasuk et al., 1986) These ANF genes are present as single copies in the haploid genome, the human ANF gene has been localized to the distal short arm of chromosome 1 in band 1p36 while the mouse gene is found on chromosome 4 (Yang-Feng et al., 1985)

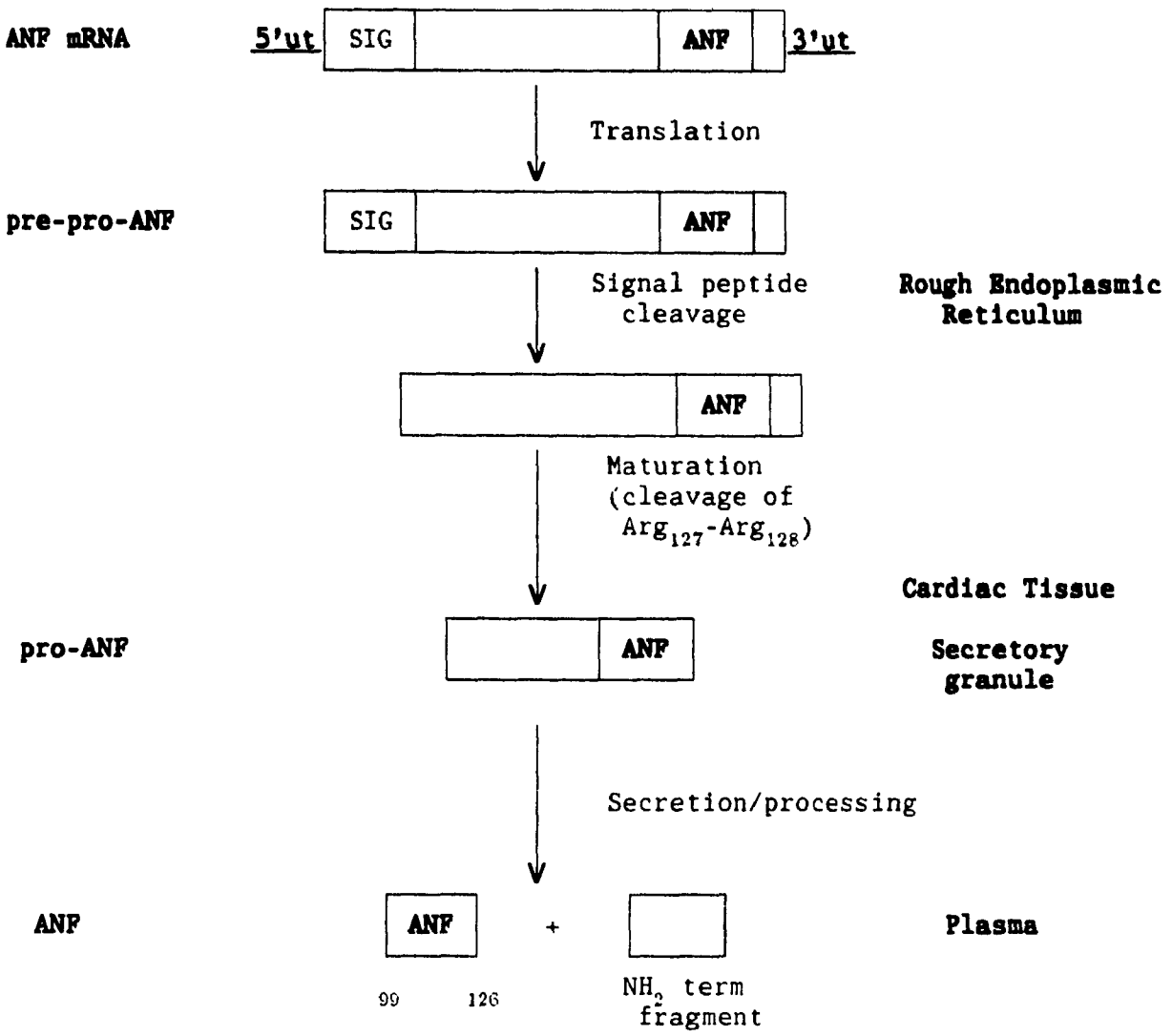

Figure 1.2 Translation and processing of the ANF pre-prohormone Translation of ANF mRNA and peptide maturation steps of the pro-ANF in cardiac tissue are shown. Abbreviations: sig, signal peptide; ut, untranslated sequences.

In all species the gene consists of a similar structural organization of 3 exons separated by two introns. The first exon encodes the 5' untranslated sequences, the signal peptide and the first few amino acids of the prohormone

The second exon encodes the remainder of the prohormone, including the biologically active portion of the peptide, while the third exon encodes the last one or three amino acids, depending on the species. For example, in the rat, mouse and rabbit the terminal Tyr-Arg-Arg residues are translated while in the human and dog, only the final Tyr is present due a point mutation converting the Arg into a stop codon. A more detailed description of the rat ANF gene is to be found in Chapter 2 of the present manuscript.

The evolutionary conservation and hence the putative functional importance of the ANF gene sequences was assessed by DNA sequence comparisons between the rat, human, and bovine genes (Argentin et al., 1985; Vlasuk et al., 1986). These analyses show a significant conservation of both structural coding sequences as well as non-coding 5'-flanking sequences; while there appears to be no evolutionary pressure on intron sequences. The conservation of the 5'-flanking sequences is suggestive of conserved regulatory mechanisms for ANF synthesis accross species.

1.2.2 Tissues Expressing the ANF Gene

The availability of ANF cDNA clones has permitted the identification of ANF gene transcripts in various tissues. In adult mammals, the major site of ANF synthesis is the cardiac atria, and it has now been well established that in the rat, the transcript accounts for about 1 to 3% of total atrial mRNA (Nakayama et al., 1984; Nemer et al., 1988). Later, it was unequivocally demonstrated using *in situ* hybridization and Northern blot analyses, that the ventricles also express the ANF gene at a level of about 1% of that in the atria in the adult rat and human (Nemer et al., 1986; Gardner et al., 1986a; Mercadier et al., 1989a). The level of ventricular ANF mRNA synthesis is subject to both hormonal and non-hormonal modulation as

discussed in the following sections That the ventricular ANF peptide contributes to the circulating plasma levels is demonstrated by a recent study showing that ventricular ANF secretion is important in cardiomyopathic hamsters with severe heart failure (Thibault et al., 1989). Since the ventricles release ANF constitutively (Bloch et al., 1986) while the atria store the peptide in granules, the modulation of ventricular ANF synthesis can result in a significant contribution to plasma ANF levels given this tissue's large mass

The detection of immunoreactive ANF in various extra-cardiac tissues has prompted the examination of these tissues as *bona fide* sites of ANF peptide synthesis. Among these, the central nervous system has been extensively studied for the presence of ANF gene transcripts, particularly since ANF does not cross the blood-brain barrier Indeed, the hypothalamus and pontine brainstem are two tissues in which ANF gene transcripts have been detected and where levels of ANF mRNA have been estimated to lie in the range of 0.5 to 1% of atrial ANF mRNA levels (Gardner et al., 1987; Nemer et al., 1988) In addition, the cerebral cortex, cerebellum and thalamus also appear to contain ANF mRNA but only at about 0.1% of atrial levels (Gardner et al., 1987). At all these sites, the transcripts appear to be of the same length and possess the same 5' terminus as the atrial mRNA

Another tissue where ANF transcripts have been detected both by *in situ* hybridization and S_1 nuclease, mapping analyses is the anterior pituitary (Gardner et al., 1986, Nemer et al., 1988). Here the ANF mRNA are less than 1% of atrial levels and appear to arise from the gonadotrophs In addition, *in situ* hybridization studies have revealed the presence of ANF mRNA in about 15% of adrenal medullary cells (Morel et al., 1988; Nemer et al., 1988).

TABLE 1.1 TISSUES EXPRESSING THE ANF GENE

	ANF mRNA	IrANF
Atrium	+++++	++++
Ventricle	+++	+
Aortic arch	+	+
Adrenal Medulla	+	++
Anterior pituitary	+	+
Hypothalamus	++	+
Pontine brainstem	+	+
Cerebral cortex	+	+
Cerebellum	+	+
Thalamus	+	+
Lung	+	+
Ovary	+	+
Olfactory bulb	+	+

ANF mRNA has also been detected in the aortic arch and thoracic aorta (Gardner et al., 1987b; Gutkowska and Nemer, 1989a). Similarly, within several pulmonary vesicles containing an extension of atrial myocardium in the rat, *in situ* hybridization studies have demonstrated the presence of ANF in atrial-like granules (Springall et al., 1988). Furthermore, using various techniques ANF

transcripts have been detected in pulmonary tissue (Gardner et al., 1986, Nemer et al. 1988; Gutkowska et al., 1989). Lung tissue contains less than 1% of atrial ANF mRNA and is subject to regulation as can be seen in the model of congestive heart failure where pulmonary ANF mRNA is induced (Gutkowska et al. 1989).

Finally both ANF peptides and transcripts have been detected in the rat olfactory bulb, and ovaries (Gutkowska and Nemer, 1989a) Transcripts identical to those found in the cardiac atria have been observed in the olfactory bulb In conclusion, the extra-cardiac localization of ANF peptides and transcripts show there is both gene transcription and mRNA translation at these sites In some cases, secretion of the peptide has been demonstrated

1.2.3 Developmental Expression of the ANF Gene

Although in the adult heart ANF gene expression is significantly higher in the atria than in the ventricular tissue, it undergoes, throughout the course of development, a temporal pattern of expression Studies on the early maturation stages of the rat heart have shown that the earliest time point for ANF gene transcription is on gestational day 8 (E8) (Zeller et al., 1987) Expression at this time is localized to a subpopulation of myocardial cells in the endocardial layer of the heart. By day E9, prominent labelling of both atria and the primitive ventricle is evident, while complete septation of the heart and establishment of the fetal circulatory system at day E14 are accompanied by the presence of ANF mRNA in both atria and ventricles (Zeller et al., 1987) Following birth, ventricular ANF mRNA levels are at their highest on day 1, and atrial and ventricular ANF mRNA levels are approximately equivalent (Wu et al. 1988, Wei et al., 1987, Bloch et al. 1986).

While ventricular ANF rapidly drops to near adult levels within the first week post-partum, atrial ANF mRNA's gradually rise to reach maximal levels within the first two months after birth (Chapter 5; Wei et al, 1987; Wu et al., 1988). The immunoreactive cardiac ANF content closely parallels the temporal pattern of ANF gene expression. Immunocytochemical studies have shown that while positive immunostaining of the atrial tissue for ANF was homogeneous, a more restrictive pattern of immunocytochemical staining prevailed in the ventricular tissue where ANF containing myocytes were primarily limited to the subendocardium (Bloch et al 1986; Wu et al., 1988) The plasma ANF levels were also assessed at different time points on or around the day of birth, and it was observed that plasma ANF levels in the fetus and within the first two days following birth are significantly elevated but rapidly drop thereafter (Wei et al., 1987, Wu et al., 1988) Since there is a drop in ventricular ANF gene expression, it is tempting to speculate that the high levels of fetal and neonatal plasma ANF are due to the elevated ventricular tissue content of the peptide Certain factors, including the drastic change in the pattern of blood flow and hormonal alterations following birth could contribute to enhanced secretion by this tissue.

When similar studies were conducted using human fetal tissues, atrial ANF mRNA levels were found to be some 5 to 10 times more elevated than in ventricular tissue between 13 and 29 weeks of gestation (Gardner et al., 1989, Mercadier et al., 1989a). In the human adult as in the rat, atrial ANF mRNA levels are about 100 times more abundant than ventricular levels (Mercadier et al., 1989a) Similar patterns of distribution of ANF immunoreactivity are evident in both the rat and human hearts, where ventricular ANF staining is concentrated to the subendocardium in both species (Gardner et al., 1989).

1.2.4 Regulation of ANF Gene Expression

ANF gene expression is modulated by a series of hormonal and non-hormonal factors. In conjunction with the isolation of a rat ANF cDNA, Nakayama and his collaborators (1984) reported that atrial ANF mRNA levels underwent a significant decrease as a consequence of water deprivation in rats. This observation was later confirmed by others who showed 2- to 3-fold drops in atrial ANF mRNA following several days of dehydration (Takayanagi et al., 1985; Zisfein et al., 1986). Plasma ANF levels also dropped in the dehydrated state, indicating that changes in ANF mRNA levels closely parallel alterations in plasma ANF levels. Conversely, salt loading in the dehydrated rats resulted in a concomittant rise in ANF mRNA levels (Takayanagi et al., 1985).

A strong stimulus for ANF release from the atria is known to be cardiac muscle distension (Dietz, 1984). Hence, several studies were undertaken to examine the effects of volume overload on atrial and ventricular ANF gene expression in the rat. The results of such studies indicate that both chronic and acute volume overload result in small, albeit significant increases in atrial ANF mRNA levels with more notable increases in left ventricular ANF mRNA levels (Lattion et al, 1986). When overload is induced by suprarenal aortic coarctation to increase intraventricular pressure, ANF immunoreactivity in the ventricular cells increases (Gu et al., 1989). Following this manoeuvre, membrane bound electron dense granules begin to appear increasing proportionately with the number of days after coarctation. *In situ* hybridization, in this model, reveals increased ventricular staining with increased pressure and duration of overload. In a different study where experimental hypertension was induced by coarctation of the aorta, a

biphasic increase in left ventricular ANF gene expression was observed. The first increase of about 20-fold was independent of stable hypertension and hypertrophy while the second peak, which was 10-fold above control values, was achieved following the establishment of stable hypertension and hypertrophy (Mercadier et al. 1989) The authors conclude that the initial induction of the ANF gene may be due to the marked increase in left-end diastolic pressure shortly following coarctation It must be noted, however, that myocardial infarct may be a complication of the model It remains clear, however, that in this model ANF gene activation is a complex phenomenon and a multifactorial response

In the model of the spontaneously hypertensive rats (SHR) and the stroke prone SHR (SHRSP) a progressive increase in ventricular ANF mRNA was observed with the establishment of hypertension and hypertrophy (Arai et al. 1988) while no significant differences were observed in atrial ANF mRNA content At 6 weeks of age, before the establishment of hypertension, 4-fold increases in ventricular ANF gene expression were observed in the SHRSP rats as compared to controls. The increase rose to 7- and 40-fold for the SHR and SHRSP, respectively, at 27 weeks of age Tissue ANF protein contents in the ventricles were also increased In this study, it is the onset of hypertrophy that appears to accompany ventricular ANF gene induction. Interestingly, in our laboratory, it has been found that in 4 week old SHR, ventricular ANF mRNA were significantly decreased compared to the normal animals (Lavigne, 1988a) This observation suggests that initially, ventricular ANF gene expression may be deficient in SHR Similarly, in cardiomyopathic hamsters with heart failure (Thibault et al., 1989) and in humans with ventricular hypertrophy (Nemer et al., 1988), ventricular ANF gene expression is induced about 100-fold In the former, atrial ANF mRNA levels

are somewhat reduced Ten-fold increases of ANF mRNA levels in the ventricles of young cardiomyopathic hamsters have been observed at the onset of hypertrophy but prior to ventricular pressure increases (Thibault et al., 1989). Thus cardiac cell growth appears to be a significant stimulus for ANF gene reinduction in the adult. In humans, myocardial infarction is also associated with ventricular ANF gene activation with no effect on atrial ANF mRNA (Galipeau et al., 1988)

In the arginine vasopressin (AVP) deficient Brattleboro rat, ANF mRNA and peptides in both atria and ventricles are increased (Lavigne et al., 1988) The 5% increase in plasma osmolality and sodium content in these rats probably accounts for the induction of ANF gene expression (2-fold) in this model

Thus, these studies all tend to suggest that the ventricular tissue is recruited for ANF gene transcription and peptide synthesis following a strong stimulus In fact ventricular tissue is presumably more sensitive to induction than atrial tissue and the left ventricle appears to be somewhat more responsive to strong stimuli In several models of hypertrophy, such as deoxycorticosterone (DOCA)-salt treatment aortocaval fistula, abdominal aortic constriction or spontaneous biventricular hypertrophy (Lattion et al., 1986, Day et al., 1987, Lee et al., 1988) left ventricular ANF mRNA levels are significantly induced with little or no effect on right ventricular ANF mRNA levels However, in the case of a slowly progressing spontaneous biventricular hypertrophy, right ventricular ANF mRNA levels are also induced (Lee et al., 1988) This observation suggests that both ventricular chambers are equally subject to activation of ANF gene expression

ANF gene transcription has also been shown to be under hormonal control Glucocorticoids stimulate ANF gene transcription both *in vivo* and *in vitro* (Chapter

4 Nemer et al in preparation, Day et al., 1987, Gardner et al., 1986, 1988) In primary cardiocyte cell cultures, glucocorticoid induction of ANF gene transcription is mediated directly via cardiac glucocorticoid receptor (Chapter 4) While the gene is stimulated about 3-fold *in vivo*, in whole animal studies, glucocorticoids induce ANF gene expression, approximately 2- to 3-fold in the ventricular tissue and about 2-fold in the atria (Day et al., 1987, Gardner et al., 1986) Plasma ANF levels concurrently rise following dexamethasone (DEX) treatment in the intact rat (Gardner et al., 1986), and ANF secretion by primary cardiocyte cultures is increased by DEX treatment (Matsubara et al., 1987). While some studies suggest that mineralocorticoids might also modulate atrial ANF mRNA levels, it is likely that such an effect is secondary to volume expansion in the intact animal (Ballermann et al., 1986) The absence of a stimulation of ANF gene expression in primary cardiocyte cultures when treated with aldosterone (our unpublished results) supports this hypothesis Hence the implication of ANF in the mineralocorticoid escape phenomenon is probably secondary to the volume expansion and sodium retention that ensues mineralocorticoid administration.

The ANF gene is also induced by thyroid hormone (T_3) (Chapter 3) Our *in vivo* studies have shown that both atrial and ventricular cells respond to T_3 treatment by 2- and 4-fold increases of ANF mRNA synthesis. Cardiocytes in primary cell culture also secrete more ANF when stimulated with T_3 (Matsubara et al., 1987) *In vivo*, hyperthyroidism in rats results in about 2-fold increases in total atrial and ventricular ANF mRNA content Conversely, hypothyroidism elicits 2- and 3-fold drops in right atrial and total ventricular ANF mRNA contents, respectively (Ladenson et al., 1988) Although T_3 is known to have various effects on myocardial protein synthesis (Morkin et al., 1983; Dillman et al., 1983), the

observed effects of T_3 on both atrial and ventricular cells in culture indicate a direct action of this hormone on ANF mRNA synthesis (Chapter 3, Gardner et al., 1987a). In fact, T_3 also elicits a 2-fold increase in media ANF immunoreactivity from cultured cardiocytes (Gardner et al., 1987a, Matsubara et al., 1987).

We have recently found that calcium ionophore stimulates cardiac ANF mRNA levels (Argentin et al., in preparation). This observation has recently been reported by others, and it was demonstrated that calcium ionophore (A23187) and phorbol ester (TPA), either alone or in combination increased atrial and ventricular ANF mRNA levels 2- and 3.5-fold, respectively when cells were treated *in vitro* (Lapointe et al 1990). The secretion of ANF is already known to be promoted by these treatments (Matsubara et al., 1988; Shields and Glembotski 1989). Thus, ANF gene regulation involves a complex interplay of multihormonal and multifactorial controls

1.3. GENE TRANSCRIPTION IN MAMMALIAN CELLS

1.3.1 Cis- and Trans-Acting Factors in Eukaryotic Transcription

It is a prime interest of eukaryotic molecular biology to understand the mechanisms by which specific genes are expressed in a tissue-specific and or temporal manner The introduction of mutations into cloned genes has allowed the identification of cis-acting DNA sequences necessary for transcriptional regulation. More recently, several techniques have been developed for the characterization, purification and, in some cases, cloning of cDNAs corresponding to proteins that specifically bind to these regulatory DNA sequences Thus, at present, the mechanism by which specific protein-DNA interactions regulate gene expression is

under intensive investigation

Studies on the structural and functional organization of several eukaryotic genes have revealed the presence of some well conserved features which are important for transcriptional control In the immediate vicinity of a gene's transcriptional start site lies a control region generally referred to as the promoter. Usually about 100 bp in length, promoters ensure accurate and efficient initiation of transcription, they include the AT rich TATA motif and a cap site (Dynan and Tjian, 1985, McKnight et al., 1986). It is noteworthy that certain promoters, such as those for "housekeeping" genes, are devoid of TATA boxes but contain GC rich elements instead (Bird, 1986) An additional series of sequence elements, upstream of the TATA box have also been identified. Some of these are quite common, such as the CCAAT box which is found in a multitude of genes, and the GGGCGG element (Dynan and Tjian, 1985) Mutations at these sites are often associated with decreased promoter strength (Myers et al., 1986, Dynan et al, 1985) In addition to these upstream promoter elements (UPE's), other less ubiquitous motifs that help increase the rate of transcription have also been identified within the first 100 bp of several genes (Maniatis et al, 1987). For example, saturation mutagenesis studies of the β-globin promoter allowed the precise localization of a CACCC box at -95 bp in addition to a CCAAT box at -80 bp Nucleotide substitutions within each of these motifs resulted in dramatic drops of transcriptional activity (Myers et al., 1986) Similarly, in the chicken ovalbumin gene, an upstream promoter element which is the binding site of the COUP and S300II proteins, is necessary for the efficient transcription (Sagami et al, 1986)

Certain ubiquitous transcriptional factors necessary for transcription initiation have been identified Although RNA polymerase II cannot alone

accurately recognize the site of initiation, several other factors participate in the recognition of minimal promoter sequences. In Hela cell extracts at least five chromatographic fractions are necessary for the formation of a transcriptional pre-initiation complex on the human adenovirus major late promoter (Saltzman and Weinmann, 1989). Although the functions of TFII A, B, D, E and F are still at present not entirely clear, it is known that TFII D is the only factor that binds DNA, recognizing the TATA box (Saltzman and Weinmann, 1989). Currently, a model involving the sequential assembly of these factors to form a pre-initiation complex is described. Many other transcription factors are promoter specific and interact with cis-acting control elements For example, the Simian virus 40 (SV40) early promoter contains a TATA box and six randomly arranged GC boxes (GGGCGG) shown to bind the promoter specific Sp1 factor. Originally identified from Hela cell extract (Dynan and Tjian, 1983), Sp1 binding sites exist in many viral and cellular promoters. Two Sp1 binding sites are found within the Herpes simplex virus (HSV) thymidine kinase (TK) promoter, contributing to full promoter activity (Jones et al., 1985). Three such sites are also found in the mouse dihydrofolate reductase gene (Dynan et al., 1986) This factor has been cloned from a Hela cell cDNA library and its sequence shows it to be a member of the zinc-finger DNA-binding protein family (Kadonaga et al., 1987).

The frequently occurring CCAAT box is recognized by a multiplicity of transcription factors. NFI/CTF consists of a family of CCAAT-box binding polypeptides able to activate transcription *in vitro* (Jones et al., 1985, Jones et al., 1987); a different CCAAT binding protein, CBP or C/EBP, isolated from rat liver, has distinct biochemical properties (Graves et al., 1986) Additional CCAAT element binding proteins, such as CP1 and CP2, that preferentially bind the α-globin and γ-fibrinogen promoters have been identified from Hela cell extracts (Chodah et al., 1988). CP1 and CTF/NFI can be distinguished on the basis of their binding affinities for different CCAAT box permutations Furthermore, the two

factors are antigenically distinct and thus probably do not arise from closely related genes (Mitchell and Tjian, 1989). Similarly, CTF NFI and C EBP are encoded by two separate genes (Landschulz et al., 1988; Santoro et al., 1988). While C/EBP belongs to the leucine zipper family of transcriptional factors, CTF NFI cannot be classified in any of the current subgroups (Landschulz et al., 1988a; Johnson and McKnight, 1989). Biochemical evidence supports the notion that there exists a variety of CCAAT box binding factors and that more than one type might exist within the same cell (Johnson and McKnight, 1989).

While proximal promoter elements do contribute to proper and efficient transcriptional initiation, the contribution of additional modules is required for an enhanced transcription rate (Dynan, 1989). Such elements, known as enhancers, are classically recognized as DNA motifs that increase the rate of transcription of cis-linked promoters, act in an orientation and position independent manner and can activate heterologous promoters (Atchison, 1988). The SV40 enhancer serves as a classical example, possessing all of the above properties (McKnight and Tjian, 1986). In SV40, two 72 bp tandem repeats mediate a distance and orientation-independent enhancement of transcription. The binding of nuclear proteins to enhancer motifs has best been demonstrated within SV40. The transcription factor AP-1 binds to the conserved TGANTC/AA site in the SV40 enhancer as well as in several cellular genes. Indeed, AP-1 sites modulate phorbol ester induction of collagenase, stromelysin and metallothioneinII_A (metII_A) genes (Angel et al., 1987). It was rapidly realized that the AP-1 factor was a heterodimeric product of c-*jun* and c-*fos* proto-oncogenes. Both proteins contain a leucine repeat region, and it is postulated that the proteins dimerize via the leucine-zipper domain (Landschulz et al., 1988a). The distinct transcription factor AP-2 binds the consensus CCCCAGGC, occurring in several genes such as hmetII_A and collagenase genes. This factor is also sensitive to phorbol ester induction as well as cAMP elevating agents (Imagawa et al., 1987; Chiu et al., 1987).

While most viral enhancers are functional in all cell types, it is not uncommon for certain eukaryotic enhancers to function in a cell-specific manner, such as those found in the immunoglobulin heavy and light chain genes (Gillies et al., 1983, Gerster et al., 1987). For example, the frequently seen octamer motif, ATGCAAAT, has been shown to possess enhancer activity as well as to confer cell specificity (Ondek et al., 1987, Falkner and Zachan, 1984). This motif is found within the enhancers of SV40 and in the immunoglobulin heavy chain (IgH) enhancer and promoter. When binding of proteins to the immunoglobulin gene octamer was first demonstrated, two binding activities were observed, one specific to lymphoid cells and one ubiquitous (Singh et al., 1986). This is an example of two indistinguishable proteins binding to the same motif and behaving as positive activators of transcription in either a lymphoid-specific or ubiquitous manner (Scheidereit et al., 1987, Fletcher et al., 1987).

Enhancers can also be of the inducible type, functioning only in response to specific stimuli such as growth factors, second messengers, heavy metals or steroids. Such enhancers have been identified in a number of genes including *c-fos* (Treisman, 1985), pro-enkephalin (Comb et al., 1988) and metallothionein genes (Karin et al., 1984). Usually it can be shown that very short 10 to 15 bp DNA elements can mediate an induction upon both homologous and heterologous promoters (Maniatis et al., 1987; Elder et al., 1988; Comb et al., 1988). Furthermore, synergy between multiple regulatory elements is not uncommon and has been demonstrated for glucocorticoid and cAMP regulatory elements (Danesch et al. 1987; Comb et al., 1988).

While the presence of negative cis-acting DNA elements has been well studied in lower eukaryotes such as yeast (Brand et al., 1985), mounting evidence

points to the existence of such negative elements or silencers in higher eukaryotic genes as well (Kuhl et al., 1987). For example, a negative regulatory element is responsible for β-interferon gene repression in the uninduced state, whereas induction by viral agents or cytokines is mediated by the same or an adjacent DNA element (Fujita et al., 1988). The identified negative element has been shown to repress transcription from a heterologous promoter (Goodbourn et all. 1986). Similarly it has been postulated that the embryonic skeletal myosin heavy-chain gene is cell-specifically regulated by both positive and negative regulatory elements (Bouvagnet et al., 1987). In the rat growth hormone gene (GH), cis-acting silencer elements are thought to restrict GH expression to pituitary cells (Larsen et al., 1986a). The ability of the repressive elements to act on a heterologous promoter suggests these sequences contain a mammalian silencer. Indeed, extinction of gene expression in pituitary-fibroblast cell hybrids, accompanied by activation of the silencer and loss of specific nuclear factor binding, supports the notion that basic mechanisms prevent expression of inappropriate genes in inappropriate cells (Tripputi et al., 1988).

Various models have been put forth for the mechanisms of both positive and negative gene regulation. Some of the events implicated might include the activation or induction of positive and negative transcription factors or, alternatively, the inactivation or displacement of activators and repressors. Studies on prokaryotic repressor-operator interactions have helped generate a model for negative transcriptional effects. Binding of a repressor in close proximity to the transcription initiation unit, preventing access by RNA polymerase, is an example of negative regulation (Ptashne, 1988). Alternatively, protein-protein interactions, rather than direct contact with DNA, may result in negative transcriptional effects

as is the case for GAL80 and GAL4 (Ma and Ptashne, 1987). A third mechanism may be the direct competition of positive and negative transcription factors for overlapping DNA sequences as has already been observed in yeast (Nasmyth and Shore, 1987). In higher eukaryotes, the following examples might be considered. The loss of a positive transcriptional factor following fusion of fibroblasts and pituitary cells supports the notion that a positive trans-activator protein is repressed following cell fusion (McCormick et al., 1988). On the other hand, it is clear that in the case of most hormone dependent enhancers, an activation of transcription results from binding of an "activated" receptor to specific cis-acting elements (Beato et al., 1989). In contrast, hormone-mediated repression has been attributed to the displacement of a positive trans-activator, such as a cAMP responsive factor (CREB), by the steroid receptor (Akerblom et al., 1988). Similarly, the competition between thyroid and estrogen receptors for binding to the estrogen response element can result in a transcriptional inhibition (Glass et al., 1988). Finally, post-translational modifications may equally be involved in mediating proper activating or repressing functions. For example, several transcription factors require phosphorylation for activity. Cyclic AMP responsive element binding proteins (CREB) exists as a mixture of inactive monomers and active dimers, phophorylation favors dimerization and hence activity (Mitchell and Tjian, 1989). Similarly, Sp1, a heavily glycosylated protein, is inactive following treatment with wheat germ agglutinin, without altering its DNA binding activity (Mitchell et al., 1989). The future characterization of the nuclear proteins involved in cell-type, temporal and inducible gene expression will undoubtedly offer novel and invaluable information necessary for the elucidation of the various mechanistic features of gene transcription in eukaryotic cells.

1.3.2 Regulation of Tissue-Specific Gene Expression

Tissue-specific expression of several genes has been associated with the presence of tissue specific transcription factors These include several liver (Frain et al., 1990), pituitary (Bodner et al., 1988), lymphoid cell (Scheidereit et al., 1987) and muscle specific factors Growth hormone factor 1 (GHF-1) or Pit-1 is a pituitary-specific transcription factor that plays an important role in the expression of the growth hormone (GH) and prolactin (Prl) genes (Castrillo et al., 1989; Ingraham et al., 1988, Bodner and Karin, 1987, Mangalam et al., 1988). Pit-1 is found in lactotrophs, somatotrophs and thyrotrophs (Bodner et al., 1988, Ingraham et al., 1988, Crenshaw et al., 1989) although this protein binds only to specific sites in both the GH and Prl promoters. In the growth hormone gene, two Pit-1 binding sites are located within the proximal promoter region These sites are crucial for full tissue-specific expression of the gene both *in vitro* (Nelson et al. 1986) and *in vivo* (Lira et al 1988) The situation is more complex in the prolactin gene where there are two clusters each containing four Pit-1 binding sites It has been suggested that the distal cluster is mainly responsible for tissue-specific Prl expression (Nelson et al., 1986 1988) although the requirement of only the proximal region has equally been documented (Cao et al.,1987). Recently using a transgenic animal model, it has been concluded that either the distal region fused to a herpes thymidine kinase (TK) minimal promoter or the proximal region alone is capable of directing low level expression in the pituitary (Crenshaw et al., 1989) However, a synergistic interaction between the distal and proximal elements was necessary for high level expression in the transgenes Interestingly, removal of the distal enhancer from its normal context led to expression in thyrotrophs The presence of endogenous Pit-1 in thyrotrophs and somatotrophs and the absence of

Prl expression in these cells suggest that restrictive mechanisms mediate the cell-specific expression of Pit (Crenshaw et al., 1989)

Pit-1 has been cloned and characterized (Bodner et al., 1988, Ingraham et al. 1988) and its functional domains identified (Theill et al., 1989) Pit-1 is a homeodomain protein with sequences distantly related to the Drosophila homeotic genes. A helix-turn-helix region is characteristic of the homeodomain (Struhl 1989) Moreover, a POU domain, conserved among several transcriptional and developmental factors including OTF-1, OTF-2 and unc-86 (Herr et al., 1988), is also found in Pit-1 The observation that GH gene extinction in fibroblast-pituitary cell hybrids is accompanied by a loss of GHF-1 protein and mRNA supports the role of this protein as a positive cell-specific trans-activator of the GH gene (McCormick et al., 1988)

Lymphoid-cell specific, octamer-binding factor (OTF-2), necessary for immunoglobulin gene expression, has been purified and characterized (Scheidereit et al., 1987) The same octamer motif can be found in a variety of promoters and bind to an ubiquitous factor (OTF-1) (Fletcher et al., 1987) When present in the light and heavy chain promoters, however, this motif has been shown to confer lymphoid-specific expression *in vivo* (Wirth et al. 1987) The purified OTF-2 protein can activate transcription in non-lymphoid cell extracts and bind to the octamer element, producing footprints indistinguishable from those produced by OTF-1 (Scheidereit et al. 1987) Clearly, distinct promoter features governing cell-specific versus ubiquitous factor binding, in this model, appear crucial in determining the transcription of the proper gene in the proper cell type

Several liver-specific gene have been studied and consequently a few liver-specific transcriptional factors have been identified In the human albumin gene

three distinct cis-acting domains have been defined a negative regulatory region, an enhancer that interacts with liver-specific factor LF-B1 and a promoter (Frain et al., 1990) Removal of the LF-B1 binding domain results in a significant drop of activity. Similarly, in the human α1-antitrypsin gene (α1AT), two functional domains have been defined by mutational analyses (De Simone et al., 1987), both of which are required for tissue-specific expression in hepatoma cells Two factors identified from liver nuclear extracts, were shown to bind the α1AT cis-acting domains LF-A1 and LF-B1 In the case of LF-B1, a good correlation between binding and transcriptional activation were observed (Hardon et al., 1988)

1.3.3 Muscle-Specific Gene Expression

Relatively, little is know about the tissue-specific expression of muscle genes and particularly cardiac specific genes where the absence of cardiac-specific cell lines has been a major drawback Nonetheless, several cardiac-specific genes are expressed in certain embryonic skeletal muscle cells or myoblastic cell lines and can thus be studied These include myosin heavy and light chain, cardiac-actin troponin and muscle creatine kinase genes

A well studied system is that of the human cardiac α-actin gene This gene encodes the major actin in adult mammalian heart but is also expressed at early stages of muscle development (Miwa and Kedes, 1987) Fine deletion mutagenesis and linker scanning studies in C2 myogenic cells have revealed the importance of two CArG box ($CC(A+T\ rich)_6GG$) motifs in the proximal regulatory region of the α-actin gene The CArG motif is highly conserved in the 5'-flanking sequences of cardiac actin in several species (Miwa and Kedes, 1987) Maximal promoter activity is dependent on the integrity of these motifs, contributing to tissue-specific

expression of the gene. Binding to the proximal CArG box by positive trans-acting factors from both muscle and non-muscle nuclear extracts has already been shown (Gustafson et al., 1988). Hence, the CArG box binding protein (CBP) appears to be an ubiquitous factor that might mediate muscle specificity through some higher order interaction with additional ancillary factors or via specific post-translational modifications, rendering the protein active solely in muscle cells (Gustafson et al., 1988). It is interesting to note that the CArG box is equivalent to the serum response element and binding of serum response factor (SRF) to CArG has been demonstrated (Gustafson et al., 1988). In fact, the binding of a total of five different factors on the cardiac α-actin gene promoter illustrates the complex nature of the interactions that might be involved in the gene's regulation (Gustafson and Kedes, 1989).

Similar studies on the rat embryonic myosin heavy-chain gene in C2 myotubes and myoblasts have revealed the binding of a positive transcription factor (NFe) to a cis-acting element in the 5'-flanking sequences of the gene (Yu and Nadal-Ginard, 1989). Although this factor is not tissue-specific due to its presence in myotubes, myoblasts and Hela cells, the authors claim it contributes to full promoter activity in myotubes and myoblasts. Meanwhile, in the case of the muscle creatine kinase (MCK) gene, a myocyte-specific nuclear factor was identified (MEF-1) that was required for enhancer activity in skeletal myoblasts (Buskin and Hauschka, 1989). Although, the role of this element in cardiac muscle has not been assessed in culture, a transgenic mouse model with various 5' promoter elements showed that the MEF-1 binding site also activates expression in cardiac muscle (Johnson et al., 1989a). MEF-1 is identical to or closely related to MyoD, a myogenic determination factor. However, this factor is present only in

skeletal muscle (Tapscott et al., 1988), thus, the authors suggest that in cardiac muscle, the MEF-1 enhancer element may be recognized by a related cardiac protein (Johnson et al., 1989a). Interestingly, in this system, of the two enhancer regions analyzed, one was functional only in skeletal muscle while the other was functional in both cardiac and skeletal muscles (Johnson et al., 1989a). Similarly, differential regulation of the cardiac troponin T (cTNT) gene in skeletal and cardiac muscle has been demonstrated (Mar and Ordahl, 1988). While only 129 nucleotides upstream from the cTNT initiation site are sufficient for skeletal muscle expression in embryonic skeletal muscle cultures, additional upstream elements are necessary for cardiac cell expression in embryonic heart cell cultures (Mar et al., 1988a). The cardiac-specific element resides within a fragment containing sequences between -268 and -201 bp. Other sequences, still further upstream, contribute to muscle-specific enhancer activity, but are not required for basal expression (Mar et al., 1988a).

Recently, several skeletal muscle-specific DNA binding factors that are capable of altering the fate of mesodermal cells by activating the myogenic phenotype have been reported (Tapscott et al., 1988; Weintraub et al., 1989; Wright et al., 1989). MyoD, myogenin and myf-5 gene products are restricted exclusively to skeletal muscle (Edmonson and Olson, 1989; Weintraub et al., 1989; Braun et al., 1989). Certain structural similarities are retained among these proteins including a basic region and a region of homology to the c-*myc* oncogene. Deletion mutagenesis has revealed that a 68 aa segment containing these regions of MyoD are sufficient for conversion of fibroblasts to muscle cells (Tapscott et al., 1988). It is interesting to note that these same regions are important for site-specific DNA binding. Indeed, the characteristic structures of these proteins consisting of a

putative helix-loop-helix motif followed directly by a basic region has led to the suggestion that these proteins belong to a novel class of DNA transcriptional factors (Murre et al., 1989). The helix-loop-helix domain has the potential to form two amphipathic helices separated by an intervening loop. Other members of this novel family include members of the Drosophila *achaete-scute* and *twist* gene family and immunoglobulin κE2 binding proteins (Murre et al., 1989). This region also appears to be essential for dimerization.

Transfection of MyoD or myogenin cDNAs into fibroblasts leads to the expression of several muscle-specific genes such as myosin-heavy chain and, in fusion-promoting conditions, muscle creatine kinase and troponin-T (Edmonson and Olson, 1989). Recently, it has been demonstrated that the myocyte-specific enhancer binding protein (MEF-1), necessary for full transcriptional activity of the MCK gene, is antigenically related to MyoD (Buskin and Hauschka, 1989; Lassar et al., 1989). In fact, MyoD has also been shown to bind to the MCK enhancer regions (Lassar et al., 1989). However, MyoD is not present in all the cell lines that express the MCK gene, and it has been reported that MyoD is not involved in the expression of the δ-subunit gene of the murine acetylcholine receptor (Lassar et al., 1989; Baldwin and Burden, 1989). Thus several unanswered questions remain regarding the factors involved in the determination of muscle differentiation and gene expression. For example, it has been proposed that the pattern of myogenin expression in cultured cells is consistent with a role in regulating the decision to differentiate. Simple models based on the transient expression of myogenin versus the constitutive expression of MyoD in myoblasts predict that myogenin has a role in the regulation of determination (Wright et al., 1989). However, other MyoD-related proteins, such as myd (Pinney et al., 1988) and myf-5 (Braun et al., 1989)

TABLE 1.2 CLASSIFICATION OF TRANSCRIPTIONAL REGULATORY PROTEINS IN VERTEBRATES

	Structural Feature	Reference
Glucocorticoid receptor	ZF	Beato, 1989
Thyroid hormone receptor	ZF	Beato, 1989
Estrogen receptor	ZF	Beato, 1989
Progesterone	ZF	Beato, 1989
Sp1	ZF	Kadonaga et al., 1987
c-*jun*	LZ	Maki et al., 1987
v-*fos*	LZ	Van Beveren et al., 1983
C EBP	LZ	Landschulz et al., 1988
OTF-1	HTH/POU	Sturm et al., 1988
OTF-2	HTH/POU	Scheidereit et al., 1987
Pit-1	HTH/POU	Ingraham et al., 1988
c-*myc*	HLH	Murre et al., 1989
MyoD	HLH	Murre et al., 1989
CTF/NF-1	n.d.	Santoro et al., 1988

Abbreviations used: ZF, zinc finger; LZ, leucine zipper; HTH, helix-turn-helix; POU, POU domain protein; HLH, helix-loop-helix

might be equally implicated in this function. Unfortunately, no such cardiac muscle-specific regulatory factors have thus far been identified, and the molecular mechanisms governing cardiac myogenic determination are as of yet unknown.

1.3.4 Steroid and Thyroid Hormone Regulation of Gene Transcription

The recent isolation and characterization of several nuclear receptor cDNAs has revealed the existence of a superfamily of proteins that include functionally diverse receptors. Members of this family include glucocorticoid, progesterone, androgen, estrogen, mineralocorticoid, thyroid hormone, retinoic acid and vitamin D_3 receptors (Beato, 1989). Remarkably, all these receptors retain a similar general organization and certain common structural features. For example, each receptor protein can be subdivided into three main domains: a variable N-terminal domain that appears to have a modulatory effect on trans-activation, a C-terminal domain with ligand binding, transactivation, dimerization and nuclear translocation functions and a central cystein-rich DNA-binding domain (Beato, 1989). The DNA-binding domain of these receptors is characterized by a pair of so-called "zinc-fingers", consisting of 4 cysteines coordinated with a zinc atom in a tetrahedral formation (Freedman et al., 1988, Severne et al., 1988).

The most extensively studied steroid receptor is the glucocorticoid receptor although recent evidence supports similar mechanisms of action for other members of this receptor family (reviewed by Beato, 1989). Indeed, it has now been well established that nuclear receptors, such as the glucocorticoid receptor, bind to high affinity binding sites, referred to as hormone responsive elements (HREs) in the chromatin. It is generally held that the receptor-DNA interaction mediates the hormonal response. For example, in the presence of glucocorticoids, transcription of the mouse mammary tumor virus (MMTV) DNA at the correct site of initiation is rapidly and selectively increased (Yamamoto, 1985). In gene transfer studies, glucocorticoids mediate a 50-fold induction of MMTV transcription when the HRE is present (Chandler et al., 1983). Consistent with the notion that hormonal

regulation of gene transcription might involve sequence-specific receptor-DNA interactions. binding of partially purified glucocorticoid receptor (GR) to the HRE has been demonstrated both *in vivo* and *in vitro* (Willman and Beato. 1986; Becker et al.. 1986). It is interesting to note that while no clear consensus sequences for progesterone. androgen or mineralocorticoid responsiveness have yet been defined. the 15 bp glucocorticoid responsive element (GRE) can mediate induction by progesterone. androgens and mineralocorticoids (Cato et al. 1986; Darbre et al.. 1986; Cato et al. 1987. Strahle et al. 1987. Cato and Weinmann. 1988a). The differential modulation by the appropriate hormone may be accounted for by the abundance of the receptor. the unique contact each receptor might have with the HRE and or by the contribution of other trans-acting factors to a specific hormone-receptor interaction (Cato et al. 1988. Chalepakis et al.. 1988)

In addition to the viral promoter HRE. several cellular steroid-regulated genes have been described and include the tyrosine amino transferase (TAT) (Jantzen et al. 1987). human growth hormone (hGH) (Slater et al.. 1985). chicken lysozyme (chLys) (Renkawitz et al. 1984) and the human metallothionein IIA genes (Karin et al.. 1984). Comparison of the sequences within the glucocorticoid receptor binding sites has revealed the presence of a well-conserved 15 bp motif (Fig 1.3) Methylation protection and interference. and DNase I footprinting experiments have pointed to specific residues within this sequence that are important for glucocorticoid receptor binding (Scheidereit and Beato. 1984). The GRE appears to act as a typical hormone responsive enhancer and thus to function at varying distances from the site of transcription initiation In some cases. it is located within just several hundred bp from the site of initiation (Renkawitz et al.. 1984). in others it may be as far as several kilobases away (Cato et al. 1984;

Danesch et al., 1987), while in the hGH gene it is located within the transcription unit (Slater et al., 1985). There does, however, appear to be a positional constraint for functional cooperative interactions between receptor molecules and or other essential transcription factors (Schüle et al., 1988). In the case of the TAT gene, functional cooperativity has been demonstrated between two GREs (Jantzen et al., 1987). It has also been demonstrated that when a 15 bp GRE is placed at a distal position relative to the Herpes simplex virus thymidine kinase (HSV-TK) minimal promoter, hormone-mediated induction requires the binding site of an additional transcription factor in the vicinity of the GRE (Strähle et al., 1988)

MMTV	-181	GTTACAAACTGTTCT	-67	Ham et al., 1988
MMTV	-128	GGTATCAAATGTTCT	-114	Ham et al., 1988
TAT	-2508	TGTACAGGATGTTCT	-2495	Jantzen et al., 1987
hGH	+93	GGCACAATGTGTCCT	+107	Slater et al., 1985
$hMetII_A$	-262	GGTACACTGTGTCCT	-248	Karin et al., 1984

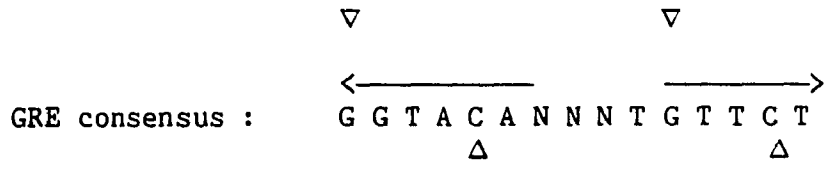

Figure 1.3 - Alignment of GREs identified in several cellular genes. A dyad axis of symmetry is indicated by the arrows. Interfering methylated guanine residues are indicated by arrowheads. (Beato, 1989).

Indeed, it has been shown that both the progesterone and glucocorticoid receptors

act synergistically with several other transcription factors including the CCAAT box, Sp1, octamer (OTF), nuclear (NF I) and CACCC box binding factors (Strähle et al., 1988; Schüle et al., 1988). A second glucocorticoid or progesterone binding site can also act synergistically (Schüle et al., 1988). However, hormone inducibility in different cell lines varies with the transcription binding site and hence with transcription factor presence and/or abundance (Strähle et al., 1988). This probably explains the differential hormone inducibility of various genes in different cell lines.

It is interesting to note that the glucocorticoid receptor also acts as a hormone-dependent negative transcriptional regulator. Sequences located upstream of the bovine prolactin (bPrl) (Camper et al., 1985; Sakai et al., 1988), the α-subunit of the glycoprotein hormone (Akerblom et al., 1988) and the rat pro-opiomelanocorticotropin (POMC) genes (Drouin et al., 1989) are able to confer glucocorticoid-dependent repression to linked reporter genes. Mutagenesis studies have shown that specific receptor-binding sites mediate negative regulation of Prl, α-subunit and POMC gene expression (Sakai et al., 1988; Drouin et al., 1989). Such negative glucocorticoid responsive elements (nGREs) bear minimal resemblance to the positive GRE consensus sequence. It has been speculated that GR binding to the nGREs might alter the conformation of the receptor, causing it to act as a repressor. Alternatively, the GR-nGRE interaction may occlude the binding of a transcriptional activator protein, thereby bringing about a repression (Akerblom et al., 1988).

Finally, recent investigations have uncovered some of the binding characteries of the glucocorticoid receptor. In the inactive state, the 94 kDa monomeric receptor protein is usually associated with heat stock protein 90 (hsp90)

(Denis et al., 1988). In the presence of hormone, the ligand-activated receptor can form a homodimer (Wrange et al., 1989), it is the dimeric form that usually binds a GRE (Tsai et al., 1988; Eriksson and Wrange, 1990)

The thyroid hormone receptors constitute a distinct subfamily with greater similarity with the retinoic acid receptor Two forms of thyroid hormone receptor (TR) have been isolated and cloned, the α and β TR (Weinberger et al., 1986, Sap et al., 1986; Thompson et al., 1987, Benbrook and Pfahe, 1987) TR are encoded by the cellular homologue of the viral erbA oncogene (c-erbA) The α- and β-subtypes have been mapped to human chromosomes 17 and 3, respectively (Bradley et al., 1989) Alternative splicing of the αTR gene in the rat gives rise to a functional rTRα_1 and a non-functional rTRα_2 (Bradley et al., 1989) The latter form is unable to mediate T_3 dependent gene regulation (Izumo and Mahdavi, 1988) Similarly, it has been suggested that rTRβ_1 and rTRβ_2 receptors arise from the same gene where rTRβ_2 transcripts are restricted to the anterior pituitary (Hodin et al., 1989) In the heart, expression of both α- and β-subtypes has been detected (Thompson et al., 1987; Koenig et al., 1988) It is interesting to note that the human hTRα_1 hTRα_2 and the hTRβ receptors all possess similar affinities for hormone ligands (Nakai et al., 1988) The molecular mechanism of thyroid-hormone mediated gene regulation is similar to that outlined for steroids (Thompson and Evans, 1989) The receptor has a nuclear localization and mediates hormone actions through direct DNA binding (Sap et al., 1986). Few thyroid hormone responsive genes have been studied; they include hypophyseal prolactin and growth hormone genes and muscular myosin heavy chain genes In the rat growth hormone gene (rGH) an element within the first 200 bp of upstream sequences is sufficient for thyroid hormone induction of gene transcription (Flug et al., 1987, Larsen et al 1986)

Thyroid hormone receptor binding to the rGH thyroid hormone responsive element, TRE, has been demonstrated *in vitro* (Koenig et al., 1987; Glass et al., 1987). The TR has also been shown to bind the TRE of the rat thyrotropin β (rTSHβ) gene, exerting a negative transcriptional regulation (Darling et al., 1989). As with the glucocorticoid receptor, T_3-mediated transcriptional regulation appears to be cell-type dependent. Indeed, in non rGH expressing cells, such as mouse fibroblasts, T_3 responsiveness is absent upon transfection of a rGH TRE-containing plasmid, despite the existence of TR (Larsen et al., 1986). Alternatively, the response may be variable upon the cell-type tested as in the case of different pituitary cell lines where either inhibition or stimulation of prolactin gene transcription in response to T_3 is observed (Stanley, 1989).

In cardiac and skeletal muscles, members of the myosin heavy chain (MHC) multigene family are under differential control in response to thyroid hormone (Izumo et al., 1986, Chizzonite and Zak, 1984). Indeed, a heterogeneity in the responses to thyroid hormone has been observed in different muscle types. The opposite effects of thyroid hormone on α- and β-MHC gene expression in ventricular myocardium (Lompre et al., 1984) and the distinct responses to thyroid hormones of the β-MHC gene observed in atrial versus ventricular tissues (Izumo et al., 1986), clearly illustrate the complexity of this system. Similarly, the α-MHC gene is independent of thyroid hormone in the atrium but highly dependent on T_3 in the ventricle (Izumo and Mahdavi, 1988). More recently, the identification of a cis-acting element responsible for T_3 responsiveness in the α-MHC gene has been reported (Izumo and Mahdavi, 1988). Using a myogenic cell line, 5'-flanking sequences between -161 and -71 bp of the α-MHC gene were found to confer T_3 inducibility. Moreover, the TRα_1 isoform was identified as the transcription factor

involved

Although an analysis of the TREs from various T_3 responsive genes has not permitted the identification of a definitive TRE consensus sequence. Brent et al (1989) do propose a consensus TR binding site based on *in vitro* binding studies of receptor to rGH TRE mutant oligonucleotides: AGGT(C A)A in at least two copies. The authors show by DNA mutagenesis studies that the rGH TRE consists of 3 subdomains (A, B and C) where domains A and B are imperfect direct repeats and C is a divergent inverted copy (Brent et al., 1989)

Interestingly, in the α-MHC gene, a conserved 13 nucleotide core element is found in the non-coding strand within the T_3 responsive sequences (Izumo and Mahdavi 1988). As with the glucocorticoid, progesterone, androgen and mineralocorticoid subfamily, cross-binding within the thyroid, retinoic acid (RAR) and estrogen (ER) receptors on similar HREs has been reported. Indeed, the RAR has been shown to bind TREs (Umesono et al., 1988, Graupner et al., 1989) and TR can equally bind an estrogen responsive element (Glass et al., 1988). This promiscuity in nuclear receptor-DNA interactions suggests that specificity in gene regulation may be achieved by some higher order interactions among receptor and regulatory proteins or may be related to the subtle variations in the organization of the individual DNA binding sites.

CHAPTER 2

THE GENE FOR RAT ATRIAL NATRIURETIC FACTOR

SUMMARY

Atrial natriuretic factor (ANF), a peptide hormone recently isolated from heart atria appears to play an important role in the regulation of extracellular fluid volume and blood pressure. Indeed, natural and synthetic ANF rapidly and markedly stimulate natriuresis and diuresis and produce smooth muscle relaxation. Consistent with the hypothesis that ANF is a novel hormone, it was recently shown that ANF is present in circulation and high affinity membrane receptors specific for ANF have been described in renal, vascular, and adrenal tissues. These important biological activities suggest that conditions like hypertension could be associated with defective ANF gene expression. We and others have shown by cDNA cloning that ANF is part of a larger precursor, pronatriodilatin (PND). We now describe the isolation and structural analysis of the rat PND gene. Southern blot analysis of rat genomic DNA suggests the presence of a single PND gene per haploid genome. The PND coding sequences are interrupted by two short introns. A long alternating purine-pyrimidine tract $(GT)_9GATG(GT)_{5-}$ is found 111 base pairs downstream of the polyadenylation site, such sequences could adopt Z-DNA configuration, and they have been associated with sequences that appear very active in intergenic recombination. Comparison of the rat and human PND genomic sequences shows highest homology in 5'-flanking as well as in coding sequences. The rat PND gene will be a useful model to study the physiology and pathology of this important regulator of the cardiovascular system.

INTRODUCTION

The finding of potent natriuretic, diuretic, and vasorelaxant peptides in heart atria opens a new and potentially very important field of investigation for the understanding of cardiovascular homeostasis in health and disease (1, 2). The

structure of these recently isolated peptides was determined (3-8), and synthetic atrial natriuretic factor $(ANF^1)_{8-33}$ is equipotent with the natural peptide (3). Antibodies raised against synthetic ANF were used 1) to prove by immunocytochemistry that ANF is contained in atrial secretory granules (9) and 2) to establish by radioimmunoassay the presence of ANF in circulation (10, 11). Further support for the hormonal nature of ANF is provided by the finding of specific high-affinity receptor sites for ANF in renal, vascular, and adrenal tissues (12, 13). In the latter ANF receptor binding parallels inhibition of steroid production.

Implication of ANF in diseased states was first suggested by reports that atrial ANF activity is reduced in spontaneously hypertensive rats (14) and in Bio 14.6 hamsters which are subject to hereditary cardiomyopathy (15). Administration of synthetic ANF to spontaneously (16) and to experimentally (17) hypertensive rats lowers blood pressure back to control levels. These results point to a possible link between ANF expression and the development of hypertension and some cardiomyopathies. Molecular analysis of ANF gene structure and transcription will help define mechanisms involved in the control of ANF expression.

Molecular cloning and sequencing of rat (18-23) and human (18, 24, 25) ANF encoding cDNAs revealed that ANF is present at the carboxyl end of a larger precursor molecule, pronatriodilatin (PND), elsewhere referred to as proANF (20-23) or procardionatrin (19). We now report the isolation and complete structure of the rat pronatriodilatin (rPND) gene.

EXPERIMENTAL PROCEDURES

Genomic DNA Cloning - Genomic DNA was prepared from the testes of a mature Sprague-Dawley rat (26). Standard molecular cloning techniques were used

(27) Rat DNA partially digested with *Sau*3a was fractionated by size on a 10-40% sucrose gradient. *Sau*3a restriction fragments in the range 13-20 kb were ligated with the annealed arms of *Bam*HI-digested λ vector Charon 30 DNA. DNA was packaged (Amersham) into phage λ particles that were used to infect *Escherichia coli* K802. The resulting library was screened without amplification for hybridization to nick-translated rat PND cDNA. Positive plaques were purified and a 4.2 kb *Eco*RI fragment containing the entire rat PND gene was subcloned in the *Eco*RI site of pUC9.

DNA Sequence Determination - The DNA sequence was determined by the dideoxy method (28). Single-stranded DNA templates were obtained by subcloning in M13mp8 and mp9 (29), fragments of the 4.2 kb *Eco*RI fragment produced with *Bgl*II + *Pst*I, *Bgl*II+*Eco*RI, *Bgl*II+*Pst*I, *Hind*III+*Eco*RI and *Hind*III+*Pst*I. A specific oligonucleotide primer (15-mer) was synthesized by the phosphotriester method and used for sequencing in the 5'-flanking region. DNA sequences were compiled and analyzed with computer programs adapted from Staden (30) or developed at the Institut de recherches cliniques de Montreal by M. Mikhail for a Vax 750 Computer.

RESULTS AND DISCUSSION

Rat genomic DNA was analyzed by Southern blotting to characterize the PND gene sequences (Fig. 21). Nick-translated rPND cDNA hybridized to a single band per digest (size in kb given in parentheses) *Bam*HI (12.5), *Eco*RI (4.2), *Hind*III (4.1), *Kpn*I (5.2), *Sst*I (10.5), and *Xba*I (8.4). This simple pattern taken together with the intensity of hybridization suggests the rPND gene is present as a singe copy per haploid genome. When 4×10^5 phages from a Sprague-Dawley rat

genomic DNA library were screened by hybridization with the same probe three different recombinants, λJ1-3, were isolated. Altogether, 27 kb of rat genomic DNA were mapped from the overlap of phages λJ1 and λJ3 (Fig. 2.2a). The insert of phage λJ2 is entirely contained within λJ3. The 4.2 kb *Eco*RI fragment which contains all sequences hybridizing wit the rPND cDNA probe was subcloned in pUC9 for further analysis. Subfragments of the rPND cDNA clone generated by *Bgl*II digestion were used as probes to map the 5' and 3' ends of the gene (Fig. 2.2b). The 2.4 kb region between the *Eco*RI and central *Bgl*II sites was sequenced by the dideoxy chain termination method (28). The sequencing strategy is illustrated in Fig. 2.2d.

The rPND gene spans 1.3 kb including two short introns of 104 and 391 bp that are flanked by typical exon-intron junction sequences (Fig. 2.3). The first exon contains 5'-untranslated sequences and sequences coding for the signal peptide and the first 16 amino acids of the pro-hormone. The central exon contains sequences for most of the pro-hormone including ANF. The third exon encodes the last three amino acids which are not important for biological activity (31) and the 3'-untranslated region. The site of transcription initiation which was determined earlier by primer-extension on rat (18) and human (32) PND mRNA lies 30 bp downstream of a perfect TATAAA sequence. Two AATAAA sequences are present in the 3'-untranslated region about 20 and 30 bp upstream of polyadenylation sites. three different cloned cDNAs (19-23) provide evidence for heterogeneous polyadenylation in the region *bracketed* by *arrows* in Fig. 2.3. An alternating purine-pyrimidine tract $(GT)_9GATG(GT)_{27}$ is present 111 bp downstream of the polyadenylation site. Another $(GT)_n$ tract is present 3' of this one in the 1 kb *Kpn*I-*Eco*RI fragment (Fig. 2b) as revealed by hybridization with synthetic $(GT)_{12}$

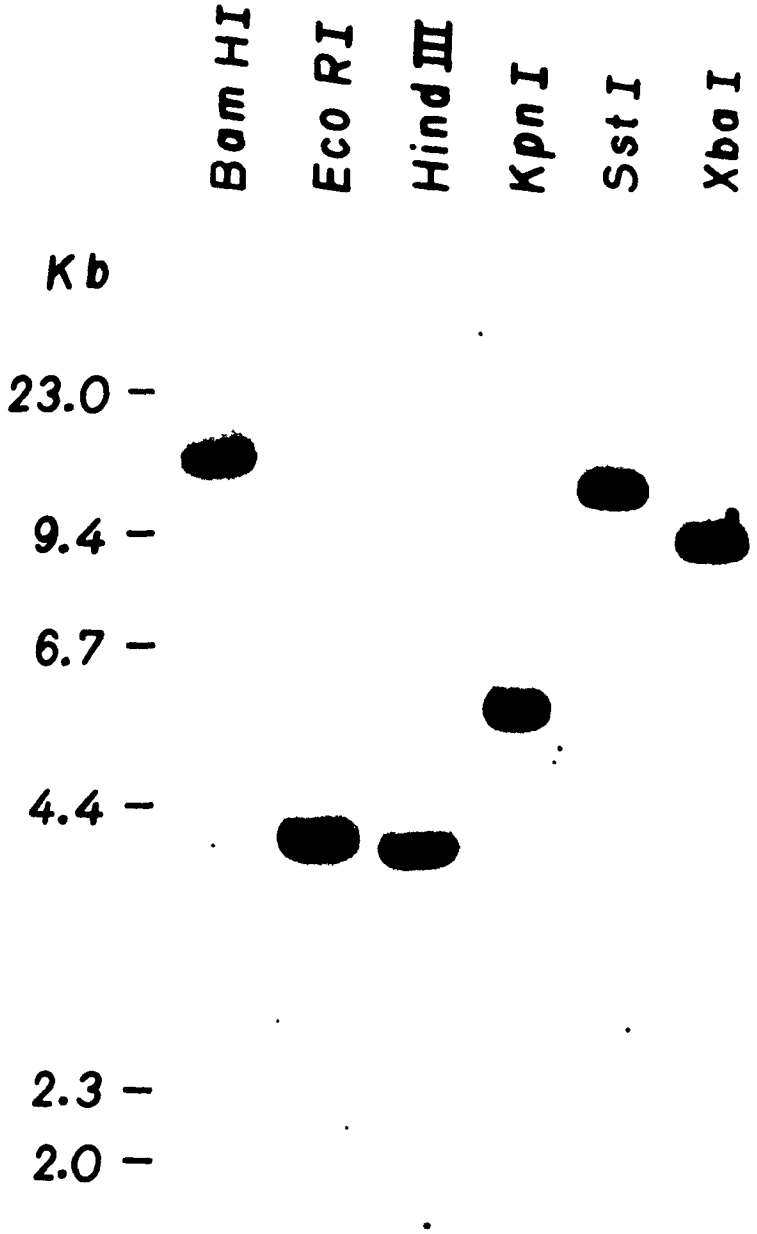

Figure 2.1 Analysis of pronatriodilatin sequences in rat genomic DNA. Sprague-Dawley rat testes DNA digested to completion with indicated restriction endonucleases was separated by electrophoresis on a 1% agarose gel. PND sequences were detected by hybridization with the nick-translated rPND cDNA. DNA marker sizes are shown on left.

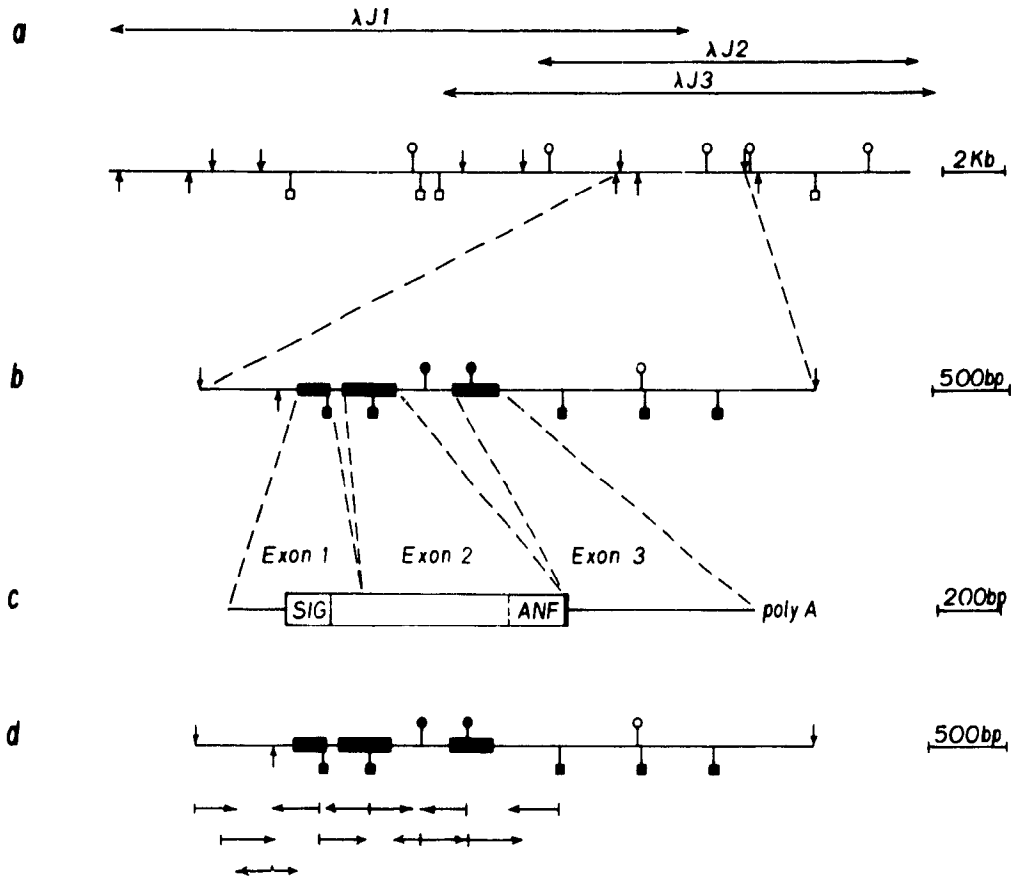

Figure 2.2 Structure of the rat pronatriodilatin gene. *a*. Rat genomic DNA restriction map deduced from restriction mapping by double digestion of three recombinant λ phages, λJ1-3. The following symbols are used for restriction enzyme cleavage sites *Eco*RI (), *Hind*III (), *Kpn*I (), and *Bam*HI () *b*. Detailed map of 4.2 kb *Eco*RI restriction fragment containing PND exon sequences (bars). These sequences were localized by hybridization with cDNA probes and by DNA sequencing. Symbols used *Pst*I (●) and *Bgl*II (■) *c*. Schematic diagram of rPND mRNA showing regions present in each of the three exons. The translated portion of the mRNA is shown as an *open box* which includes the signal peptide sequence (SIG) and the ANF sequence. The ANF sequence is preceded by a *broken vertical line* to indicate the putative nature of this processing site (3-8). *d*. Strategy used for DNA sequence determination. *Arrows below* the diagram of the 4.2 kb *Eco*RI fragment (as in b) indicate the length and direction of sequence determination.

(not shown). Such sequences were found in regions of the fetal human globin genes that are thought to undergo gene conversion (33, 34) and their possible Z-DNA conformation could promote recombinational events (35, 36). $A(GT)_8$

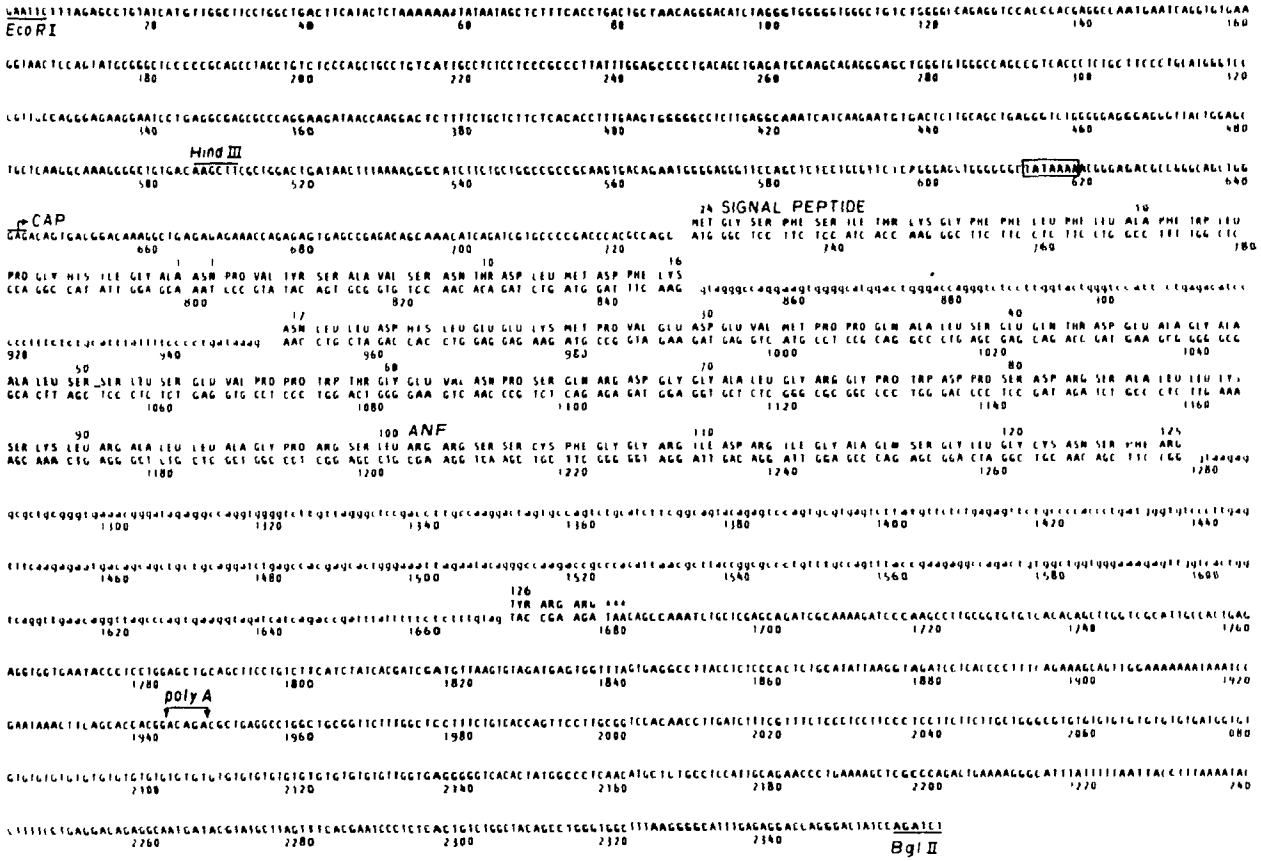

Figure 2.3 DNA sequence of the rat pronatriodilatin gene. The sequence was determined from the *Eco*RI site lying 640-bp upstream from the site of initiation of transcription to the *Bgl*II site 415 bp after the polyadenylation site. The exon-intron junctions and reading frames for translation were assigned by comparison with the cDNA sequence (18, 19). Intron sequences are shown in *lower case*. Amino acids are numbered positively for the PND precursor and negatively for the putative signal peptide sequence. The TATA box is *boxed*. The site of initiation of transcription (CAP) and polyadenylation (poly A) are indicated by *vertical arrows*.

sequence is present in the mouse PND gene at an equivalent position (37), however, hybridization of our human PND genomic clone (32) with the $(GT)_{12}$ probe does not suggest the presence of a similar GT tract in the human gene

Comparison of the rPND gene sequence with the human PND (32) sequence reveals a higher homology in the 5'-flanking region than in the introns, the 3'-untranslated region, or even the signal peptide-coding region (Fig 2.4) This sequence conservation might reflect a selective pressure to maintain important regulatory sequences As it is thought that the only structural constraints on signal peptide function are appropriate length and hydrophobicity (38), it is not surprising to find that sequences encoding the PND signal peptides are not highly conserved Similarly, the high conservation of sequences encoding the N-terminal region of PND suggests that these sequences play an important role, it was suggested that a peptide containing these sequences, cardiodilatin, has only vasorelaxant activity without the natriuretic and diuretic activities associated with ANF (39) The large intron of the rPND gene is shorter than its human counterpart by roughly the length of the two 300-bp Alu repeated sequences found in the latter, indicating that the insertion of these sequences in the human gene is a relatively recent event Furthermore, significant sequence conservation is only found at the ends of this intron No sequences homologous to B1 sequences the rat equivalent of Alu repeated sequences, are found in the large rPND intron

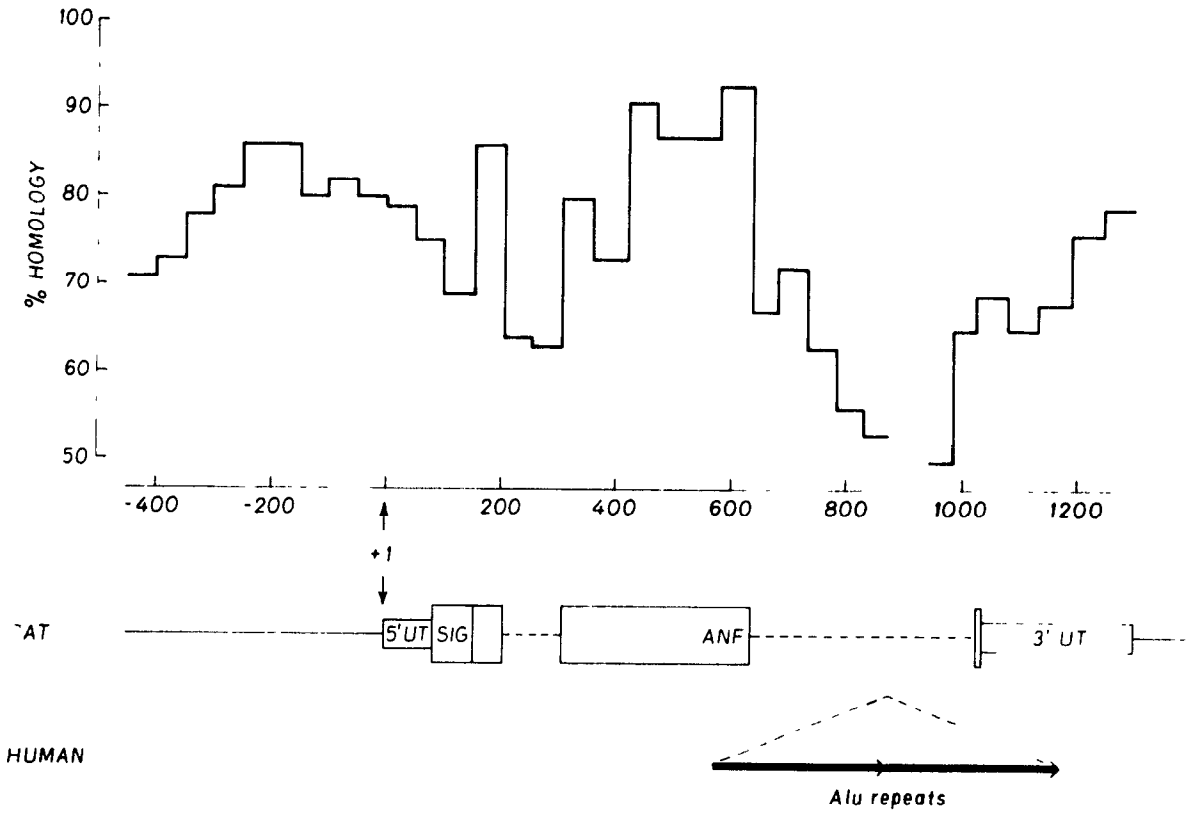

Figure 2.4 Homology of rat and human (32) PND gene sequences. Percentage homology was computed from alignment of the two sequences with an algorithm maximizing homology with the introduction of a minimal number of gaps (counted as a mismatch). Using this algorithm, two unrelated mammalian gene sequences have background homology of about 50%. Percentage homology is plotted against the length (in bp) of the rPND DNA sequence (50-bp window) numbered from the site of initiation for transcription (+1). The rat PND gene is illustrated schematically *below* to show relations between homology and regions of the sequences. The human PND gene has a similar organization except for the presence of two Alu repeated sequences in the large intron. Abbreviations are as in Fig. 2.2. 5'- and 3'-UT are 5' and 3'-untranslated mRNA sequences.

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CHAPTER 3

THYROID HORMONE STIMULATES RAT PRONATRIODILATIN mRNA LEVELS IN PRIMARY CARDIOCYTE CULTURES

SUMMARY

Pronatriodilatin (PND) is the precursor for atrial natriurectic factor (ANF), a hormone which plays an important role in cardiovascular homeostasis. Since the effects of thyroid hormone (T_3) on the cardiovascular and renal systems appear to mimic those elicited by ANF, we studied the effect of T_3 on PND gene expression using rat neonatal cardiocytes in primary cultures. Treatment of cardiocytes for 48 h with T_3 (5×10^{-9} M) results in a maximal increase in PND mRNA levels, this increase is 2-fold in atrial and 4-fold in ventricular cell cultures. These results taken together with a previous report showing decreased plasma ANF in hypothyroid and increased plasma ANF in hyperthyroid rats suggest that at least some of the cardiovascular and renal effects of T_3 may be mediated by a T_3-dependent increase in PND gene expression.

INTRODUCTION

Atrial natriurectic factor (ANF) is a 28 amino acid peptide hormone derived from a 126 amino acid precursor, pronatriodilatin (PND), which is synthesized and secreted by cardiac tissues (1, 2). The primary actions of ANF include the relaxation of peripheral vascular tone and the regulation of fluid and electrolyte balance through its diuretic and natriuretic effects on the kidney (1). Thus ANF appears to be an important modulator of cardiovascular homeostasis.

In order to better understand the physiological role of ANF, we have been studying the interaction of ANF with other regulators of the cardiovascular system, particularly thyroid hormone. The effects of thyroid hormone on the cardiovascular and renal systems are well documented (3). For example, hyperthyroid states are characterized by decreased peripheral vascular resistance and increased renal blood flow (3). On the other hand, hypothyroid states are

associated with increased peripheral vascular resistance, decreased glomerular filtration rate and increased total body sodium (4)

Since the effects elicited by thyroid hormone on the cardiovascular and renal systems seem to mimic those induced by ANF, we hypothesized that ANF could mediate the action of thyroid hormone This hypothesis is supported by recent data showing that plasma ANF levels are elevated in hyperthyroid rats (5) and decreased in both hypothyroid rats (5) and humans (6) We therefore tested the direct effect of thyroid hormone on PND gene expression using primary cardiocyte cultures In this report we show that thyroid hormone specifically stimulates PND mRNA levels in atrial and ventricular cardiocyte cultures

MATERIALS AND METHODS

Primary Cultures. Primary cardiocyte cultures were prepared using four day old Sprague-Dawley rats (Charles River) The atria and lower half of the ventricles were removed aseptically and placed separately in Joklik's modified Eagle's medium (Gibco) The tissues were then minced and subjected to three sequential digestions of 20, 15 and 10 minutes each in 1% collagenase (Cooper Biomedical) Bovine fetal calf serum (28 5%) was added and the cells were filtered through a 100 μm nylon mesh The filtrate was centrifuged (2 minutes), and the resulting cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) with 15% fetal calf serum (FCS, Gibco) Atrial cells were plated at a density of 0 8 - 1 0 X 10^6 cells 9.6 cm_2 well (Falcon) while ventricular cells were plated at a density of 2 5 - 3 0 X 10^6, 25 cm^2 flask (Falcon) After 24-48 h the time required for the cells to adhere, the medium was replaced by a serum free, hormone supplemented synthetic medium, as described by Mohamed et al (7) In order to test the effect of thyroid hormone on PND synthesis, cells were kept in

the synthetic medium without thyroxine (T_4) Triiodothyronine (T_3) at a concentration of 10^{-7} M or 5 X 10^{-9} was then added for the indicated time

Northern Blot Analysis At the end of the experiment, cytoplasmic RNA was extracted from control and T_3 treated cells using isotonic buffer and 1% Nonidet P-40 as previously described (8) Northern blots were performed in the standard manner (9) except that 0 2 μM Nytran membranes (Schleicher and Schuell) were used The membranes were prehybridized and hybridized in an aqueous solution containing heparin (10). Hybridizations were carried out overnight at 65°C. at a probe concentration of about 1 X 10^6 cpm ml The radioactive probes used were prepared by random priming (11) and labelled to a specific activity of about 0 5 X 10^9 dmp μg DNA A 600 bp rat PND cDNA clone (12) was used to detect PND mRNA and a rat brain β-actin cDNA probe (13) was used as an internal control In order to quantitate PND mRNA levels, autoradiograms were scanned with a densitometer (BioRad, model 620) coupled to an integrator (Varian CDS 401)

RESULTS

The effect of T_3 on PND gene expression was evaluated by measuring PND mRNA levels using Northern blot hybridization to a rat PND cDNA clone (12) Treatment of neonatal cardiocytes with T_3 at 10^{-7} or 5 X 10^{-9} M led to a maximal 2-fold increase in PND mRNA levels in atrial and 4-fold increase in ventricular cells (Fig 3 1 and Table 3 1) A similar effect was also observed by treating the cells with thyroxine (T_4) at a concentration of 10^{-7}M The specificity of the T_3 mediated increase in PND mRNA levels was assessed by hybridization of the same blots to a rat β-actin probe, no effect of T_3 on cytoplasmic β γ-actin mRNA was observed (Fig 3 1) The effect of T_3 on PND mRNA level was apparent at 12 h

(earliest point examined) and maximal at 48 h (Table 3.1) in agreement with other reports on the time course of T_3 action in cultured cells (14). The stimulatory effect of T_3 was near maximal at a concentration of 5×10^{-9} M which correlates very well with the affinity of T_3 for its nuclear receptor (Kd of 0.3 nM, ref. 14). This in turn suggests that the effect of T_3 on PND gene expression may be at the nuclear or transcriptional level.

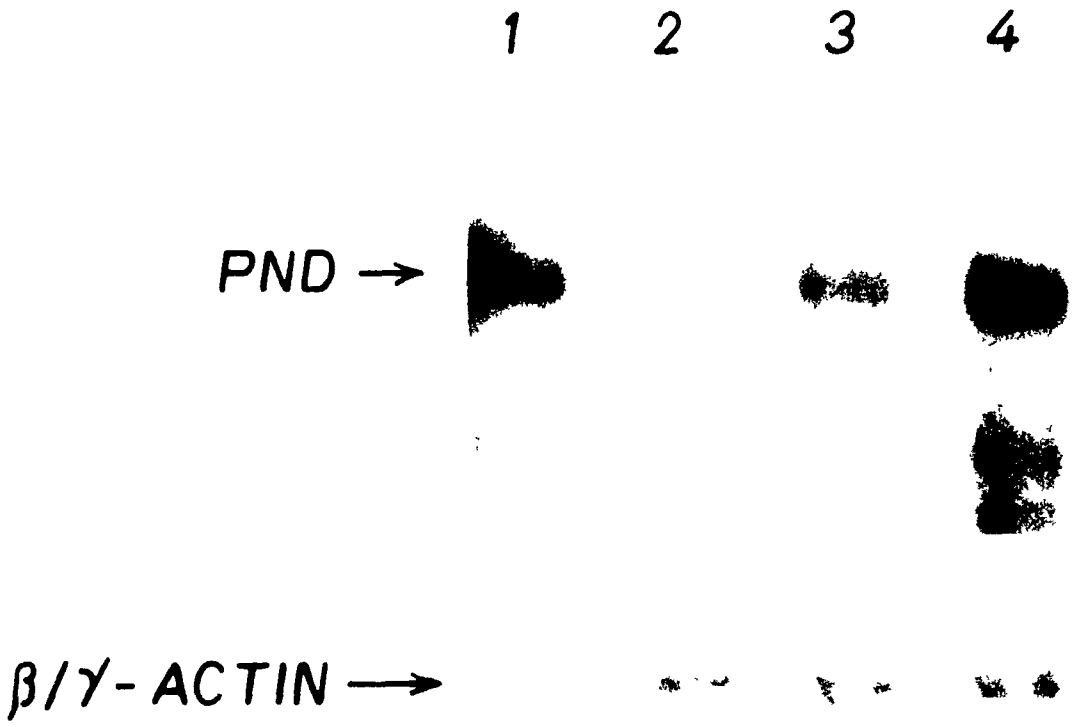

Figure 3.1. Northern blot showing the effect of T_3 on ventricular PND mRNA levels (top panel) in primary cardiocyte cultures. Hybridization to a β-actin probe was used as an internal control and is shown in the lower panel. Lane 1 contains 3 μg of total RNA from rat ventricles. Lanes 2-4 contain 6 μg of total RNA from untreated control cells (lane 2) and from cells treated with 5×10^{-9} M T_3 for 24 h (lane 3), and 48 h (lane 4). RNA from control and experimental samples was extracted at the same time.

TABLE 3 1 EFFECT OF T_3 ON PND mRNA LEVELS

		TIME (h)	PND ACTIN mRNA†
Atria	Control	0	1.00 ± 0.3
	T_3 10^{-7}M	24	2.09 ± 0 61 *
	T_3 10^{-7}M	48	2.51 ± 1.02 *
	T_3 $5x10^{-9}$M	48	1 93 ± 0.61 *
	T_4 10^{-9}M	48	1 67 ± 0 15 *
Ventricles	Control	0	1 00 ± 0 2
	T_3 $5x10^{-9}$M	24	2 90 (n=1)
	T_3 $5x10^{-9}$M	48	4.02 ± 0 42 *
	T_3 10^{-7}M	12	1 72 ± 0 4
	T_3 10^{-7}M	24	1 59 ± 0 27
	T_3 10^{-7}M	48	3 89 ± 0.78 *

† values represent the ratios of PND actin mRNA and are expressed as means ± SEM of four different determinations
* $p < 0.05$

DISCUSSION

We have used rat neonatal primary cardiocyte cultures to study the regulation of PND gene expression by thyroid hormone Because thyroid hormone has numerous effects on the cardiovascular and renal systems, direct effects of the hormone on PND gene expression may be difficult to assess in the intact animal The *in vitro* system offers the possibility to study the effect of various hormones on PND synthesis at the heart level, this system has already been used to document the effects of glucocorticoids on cardiac PND mRNA levels (15). Furthermore,

measurements of mRNA levels are a better indication of ANF synthesis than measurements of peptide tissue content, which reflect the difference between synthesis and release

In this report, we show that thyroid hormone treatment of cultured cardiocytes leads to 2- to 4-fold increase in PND mRNA levels This result, taken together with the two reports showing increased plasma ANF levels in hyperthyroid rats (5) and decreased plasma ANF levels in hypothyroid rats (5) and man (6) indicates that thyroid hormone directly affects cardiac PND gene expression in the rat and possibly in man Since thyroid hormone action is believed to be mediated by specific nuclear receptors (14) whose structure has recently been elucidated (16, 17), our results suggest that thyroid hormone affects PND gene expression at the transcriptional level

Although thyroid hormone treatment results in stimulation of PND mRNA levels both in atrial and ventricular cardiocytes, the magnitude of stimulation is different for the two tissues A similar situation has been reported for the differential effect of thyroid hormone on atrial and ventricular isomyosins in the rat (18) Taken together, these results indicate that ventricles are more responsive to thyroid hormone than atria perhaps as a result of varying levels of thyroid hormone receptors in the two tissues

In conclusion, the data presented in this report indicate that thyroid hormone acts on the heart not only to modulate the composition of myosin isoenzymes (18, 19) but also to stimulate the synthesis of ANF, its major secretory product The stimulation of ANF synthesis can explain at least some of the known cardiovascular and renal effects of thyroid hormone

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CHAPTER 4

A DISTAL CIS-ACTING PROMOTER ELEMENT MEDIATES GLUCOCORTICOID STIMULATION OF CARDIAC ANF GENE TRANSCRIPTION

SUMMARY

While the heart appears to be a target for glucocorticoids, specific cardiac genes which are glucocorticoid sensitive have not yet been extensively studied. In this report, we show that transcription of the atrial natriuretic factor (ANF) gene, which encodes the heart major secretory product, is induced by glucocorticoids in rat primary cardiocyte cultures. In both atrial and ventricular cells, ANF mRNA levels are increased 2- to 4-fold in a time- and dose-dependent manner. This response to glucocorticoids is completely abolished by the specific antagonist RU486. DNA-mediated gene transfer studies indicate that glucocorticoids affect ANF gene transcription via a glucocorticoid response element located (GRE) in the distal 5'-flanking sequences of the rat ANF gene at -960 bp. DNase I footprinting experiments revealed a binding site for purified glucocorticoid receptor within the ANF GRE. Mobility shift assays and competition experiments showed that binding of the glucocorticoid receptor to the ANF sequence results in a DNA-protein complex similar to that of the well studied mammary tumor virus (MTV) GRE. The stimulation of ANF transcription by glucocorticoids could mediate some of the effects of glucocorticoids on the cardiovascular system and identifies the first cardiac gene which is under glucocorticoid control.

INTRODUCTION

Several studies have suggested that glucocorticoids, which affect the cardiovascular system in several ways (1), may act directly on cardiac tissues and alter the expression of cardiac genes. Indeed, glucocorticoids affect cardiac contractility (2, 3) and cardiac weight (4). In the heart, glucocorticoids have also been shown to induce several cardiac proteins whose identity is still unknown (5, 6,

7) Glucocorticoid effects are mediated by specific intracellular receptors, and, such high affinity glucocorticoid binding sites have been documented in cardiac tissues (8, 9) Thus, it appears that the heart is a target for glucocorticoid action although little is known about specific cardiac genes which are glucocorticoid sensitive.

We and others (9, 10, 11, 12) have reported that glucocorticoid administration to intact or adrenalectomized rats induces atrial and ventricular expression of atrial natriuretic factor (ANF), the heart major secretory product (13) Other studies have shown that glucocorticoids stimulate secretion of ANF peptides in primary cardiocyte cultures (14 15), suggesting that the ANF gene might be one of the glucocorticoid sensitive cardiac genes

Glucocorticoids can affect gene expression at various levels including transcriptional and post-transcriptional stages. Transcriptional stimulation by glucocorticoids involves binding of the glucocorticoid receptor to specific DNA elements usually present in the upstream region of target genes (16) In the present report, we examined the mechanisms involved in glucocorticoid stimulation of ANF gene expression using DNA-mediated gene transfer into primary cardiocyte cultures and *in vitro* binding of purified glucocorticoid receptor to upstream regions of the rat ANF gene. Our results indicate that glucocorticoids stimulate cardiac-specific transcription of the rat ANF gene via a distal promoter element which contains a glucocorticoid receptor binding site

MATERIALS AND METHODS

Cell Cultures Primary cardiocyte cultures were prepared using neonatal Sprague-Dawley rats (Charles River) as previously described (17) Atrial and

ventricular cells were plated at a density of 1 x 10^5 cells cm^2 in DMEM and 15% FCS, following a pre-plating step to reduce fibroblast contamination. For pre-plating, cells were incubated in culture dishes twice for 30 min and the unattached, myocyte enriched supernatant was recuperated and plated in Primaria culture dishes (Falcon). Myocyte cultures were about 80% homogeneous as judged by staining with an antiANF antibody (Peninsula Labs). After 24 h, the time required for the cells to adhere, the medium was replaced by a serum free, hormone supplemented synthetic medium, as previously described (17). In order to test the effect of steroid hormones on ANF synthesis, cells were kept in the synthetic medium without thyroxine (T_4) and hydrocortisone (HC) for 48 h. Dexamethasone and RU486 at varying a concentrations were then added for the indicated times.

At this time point, both steroid free and steroid stimulated cells displayed spontaneous contractility and were arranged in clusters of beating myocytes, forming extensive networks as expected for such cells.

RNA Extraction and Analysis Total RNA was extracted from cardiocyte cultures using the Nonidet P-40 procedure as previously described (17). Northern blots were performed as before (17) using 0.2 μm Nytran membranes (Schleicher and Schuell). The membranes were prehybridized and hybridized in an aqueous solution containing heparin. Hybridizations were carried out overnight at 65°C, at a probe concentration of about 1 x 10^6 cpm/ml. The radioactive probes used were prepared by random priming (18) and labelled to a specific activity of about 1.5 x 10^9 dpm/μg DNA. A 600 bp rat ANF cDNA clone (19) was used to detect ANF mRNA and a rat brain β-actin cDNA probe (20) was used as internal control. In order to quantitate ANF mRNA levels, autoradiograms were scanned with a densitometer (BioRad, model 620) coupled to an integrator (Varian CDS 401).

Immunocytochemistry. In order to assess the homogeneity of the myocyte cultures, selected culture dishes were fixed in Bouin and stored with a polyclonal antibody raised against rat ANF_{99-126} (Peninsula Labs), or with normal rabbit serum as previously described (21, 22)

Plasmid Constructions. A previously subcloned (23) 703 bp rat ANF promoter fragment (*Eco*RI$_{-640}$ *Nhe*I$_{+40}$) containing *Nhe* polylinkers was inserted into the *Nhe*I site of the promoterless pOGH vector (24) which contains sequences encoding the human growth hormone (hGH) gene An *Eco*RI fragment containing sequences from -3700 to -640 bp was excised from a 12 kb *Bam*HI genomic fragment (23) and inserted in the *Eco*RI site of the ANF_{-640}-GH plasmid The resulting plasmid (ANF_{-3700}-GH), containing 3.7 kb of 5' upstream sequences, was then digested by appropriate restriction enzymes to generate various 5' deletions

Transient Cell Transfection Assays. DNA was introduced into the primary cardiocyte cultures and other cell lines using a modification of the calcium phosphate precipitation technique (25) DNA concentrations in the linear range of transfection efficiency were chosen Two μg of DNA ml of medium was used to transfect cells The formation of a fine DNA Calcium phosphate precipitate was allowed to form by incubating the cells overnight at 37°C and 3% CO_2 The next day, cells were fed with the appropriate medium The cell media was collected 48-72 h after transfection for hGH assays In selected experiments, RSV-luciferase was used as internal control and luciferase activity was assayed in cell extracts using a LKB luminometer as previously described (26)

hGH Immunoassays. The hGH secreted into the cell culture medium was measured directly from 100 μl aliquots of medium after spinning down any cellular debris hGH immunoassays were performed using the Allegro hGH kit (Joldon),

following manufacturers instructions.

Receptor Footprinting. A 332 bp *SnaB*I-*Hind*III fragment of the ANF promoter, corresponding to sequences -1029 to -697 bp, was 5' end-labeled at the *SnaB*I site using [γ^{-32}-P]ATP and T_4 polynucleotide kinase. Purified rat liver GR was obtained as a previously described (27). The binding reaction, DNase digestion and gel electrophoresis were performed essentially as described by LeFebre et al. (28) except that binding reactions were carried out in 0.02% Triton, 0.16% BSA, 4 mM DTT, 50 mM NaCl, 20 M TrisHCl (pH 7.4), 1 mM EDTA and 12% glycerol. Typically, binding reactions were carried out with 1 fmole of labeled DNA and varying amounts of purified receptor. The amount of receptor was quantitated using a recently developed radioimmunoassay (29).

Gel Retardation Assays. A 32 bp double strand oligonucleotide corresponding to -948 to -979 bp of the rat ANF promoter (5'-AGACTGCCTGTTTGTGTTCTGAGGATGCCAGA-3') and a mouse mammary tumor virus (mMTV) GRE probe containing sequences -191 to -159 bp (5'-GTTTATGGTTACAAACTGTTCTTAAAACAAGG-3') (30) were both 5' end-labeled as described above. Gel retardation assays with both ANF-GRE and MMTV-GRE probes were performed according to the protocol of Eriksson and Wrange (31).

Methylation Interference. Double-stranded oligonucleotides, 5' end-labeled on one strand, were subjected to DMS treatment for 5 min (32). Methylated probes were used for binding and gel retardation assays as described above. Protein-bound and free DNA were excised from the gel, eluted and cleaved according to standard protocols.

RESULTS

Immunocytochemical Staining of Cardiocyte Cultures. In order to assess to homogeneity of both atrial and ventricular cultures, staining with a specific anti ANF antibody was performed on cells growing in hormone supplemented medium As can be seen in Fig 4.1, the vast majority of the cells in culture were arranged in clusters, had a morphology typical of cardiac myocytes and stained positively for ANF These cultures were previously shown to contain endogenous ANF mRNA that can be modulated by thyroid hormone (17)

Glucocorticoid Regulation of ANF mRNA Levels in Cardiac Cells. We and others (9-12) have shown that glucocorticoids increase the level of ANF mRNA in atria and ventricles of intact or adrenalectomized rats In order to determine whether the effects of glucocorticoids on cardiac ANF mRNA levels are due to a direct action on ANF-expressing cells, we tested the effect of various glucocorticoids on atrial and ventricular neonate cardiocytes maintained in primary culture.

Since tissue culture medium contains 14 μM hydrocortisone, we first assessed the effect of removal of glucocorticoids from the medium on ANF mRNA levels. As illustrated in Fig. 4.2a for ventricular cells, removal of steroids from the culture media lead to a progressive decrease in ANF mRNA levels in both atrial and ventricular cell cultures, this decrease was maximal after 48 h of culture in steroid-deprived medium when ANF mRNA levels were 4 times lower than in cells cultured with steroid-supplemented medium Glucocorticoid withdrawal had no visible effect on cardiocyte morphology, size or number and there was no effect on the amount of total RNA extracted from dishes treated or not with hydrocortisone Furthermore, the glucocorticoid sensitivity was specific to the ANF gene as there

Figure 4.1. Immunocytochemical staining of ventricular (a) and atrial (c) cells with an antiANF antibody Non-immune normal rabbit serum is used in ventricular (b) and atrial (d) cells as a negative control.

was no detectable effect on cytoplasmic β/γ-actin or on α-actin mRNA (Fig 4.4a).

Addition of 10^{-7} M dexamethasone (DEX) or 10^{-6} M hydrocortisone for 24 h to steroid deprived cultures reversed the effect of glucocorticoid withdrawal and produced a 3-fold increase in the steady state level of ANF mRNA When cells were exposed to 10^{-7} M dexamethasone for varying amounts of time (Fig 4.2b), ANF mRNA levels were maximally induced after 6 hours of treatment (the earliest time point examined) in ventricular cells (Fig. 4.2b) and after 12 h of treatment (the earliest time point examined) in atrial cells (data not shown).

The dose dependence of ANF mRNA stimulation by DEX was determined by incubation of cardiac myocyte primary cultures with increasing concentrations of DEX for 48 h In ventricular cells, a 6 nM concentration of DEX was required to produce a half-maximal stimulation of ANF mRNA levels (Fig. 4.3a). In atrial cells, the apparent ED_{50} was higher at 15 nM (Fig 4.3b) This suggests that ventricular cells are more sensitive to glucocorticoids than their atrial counterpart

Glucocorticoid Receptors and Dexamethasone Stimulation of ANF Gene Expression To determine whether DEX stimulation of ANF gene expression was mediated by cardiac glucocorticoid receptors, we used the glucocorticoid antagonist RU486 which is known to compete glucocorticoid action at the receptor level (33).

Treatment of cardiomyocyte cultures with RU486 alone for 24 h did not modify ANF mRNA levels (Fig 4.4). On the other hand treatment of cells with either DEX or hydrocortisone resulted in a similar 3-fold increase in ANF mRNA levels Simultaneous treatment with RU486 (10^{-5} M) and DEX (10^{-7} M) blocked the DEX mediated increase of ANF mRNA in both atrial and ventricular cells (Fig 4.4b). These data are consistent with a glucocorticoid receptor mediated effect and, taken together with the observed rapid stimulation of mRNA levels, suggest a

a

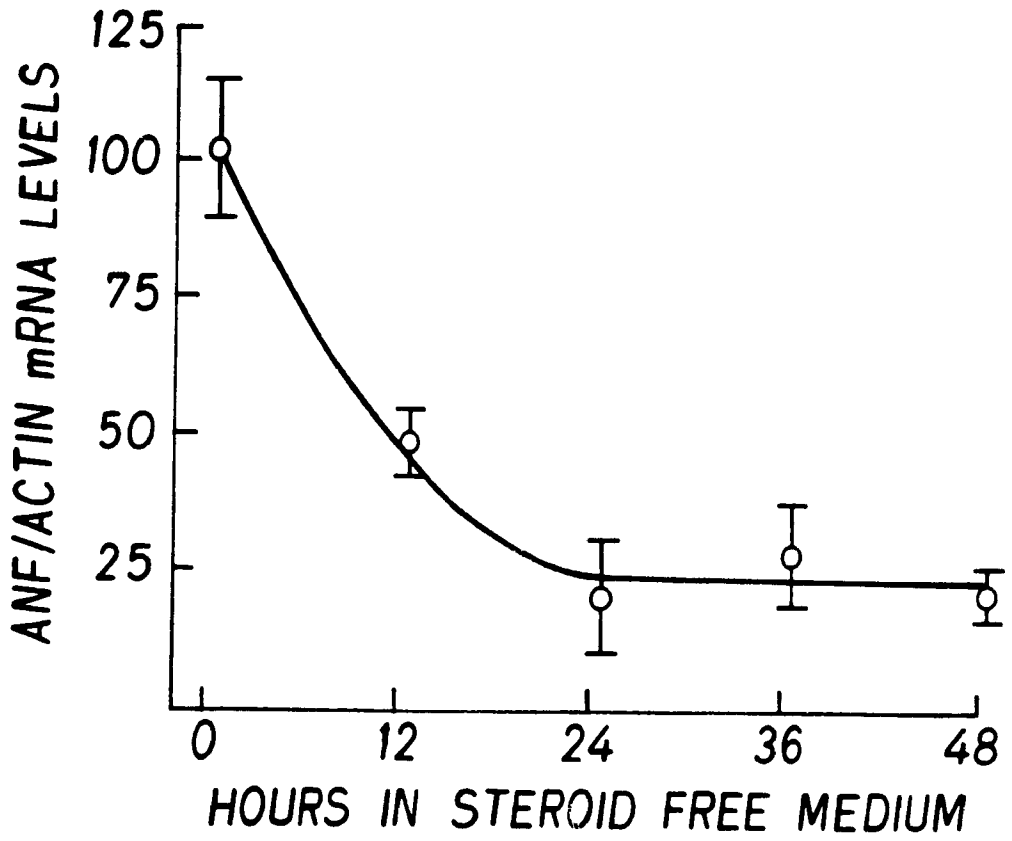


Figure 4.2. Effect of glucocorticoids on steady state ANF and β/γ actin mRNA levels were quantitated by densitometry following Northern blot hybridization **a)** Effect of hydrocortisone removal from culture media. Steroid-supplemented medium contained 5 μg/ml (14 μM) hydrocortisone (17).

b

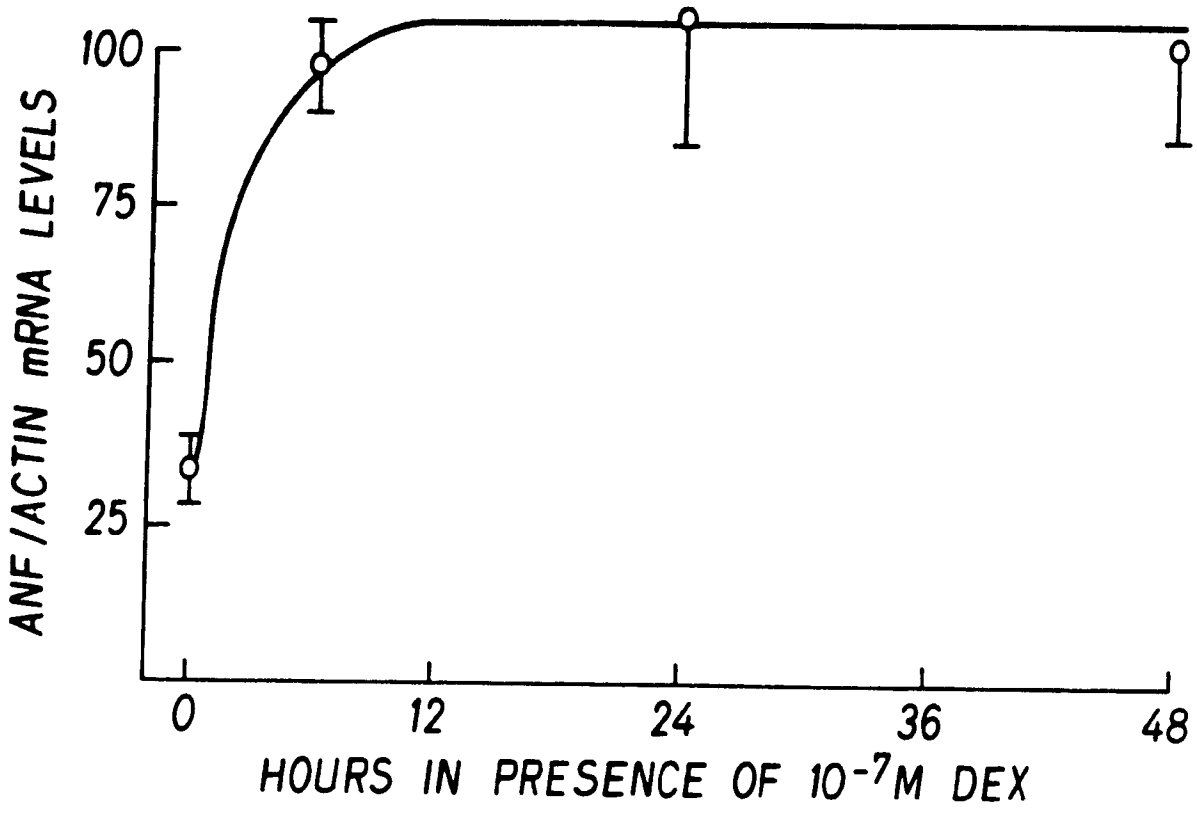
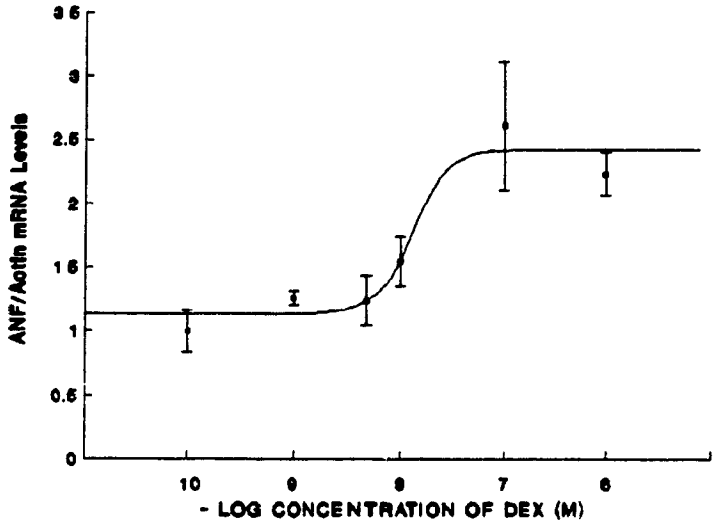


Figure 4.2b) Effect of DEX on ANF mRNA levels. Following 48 h of deinduction, cells were stimulated with 10^{-7} M DEX for various time periods.

a



b

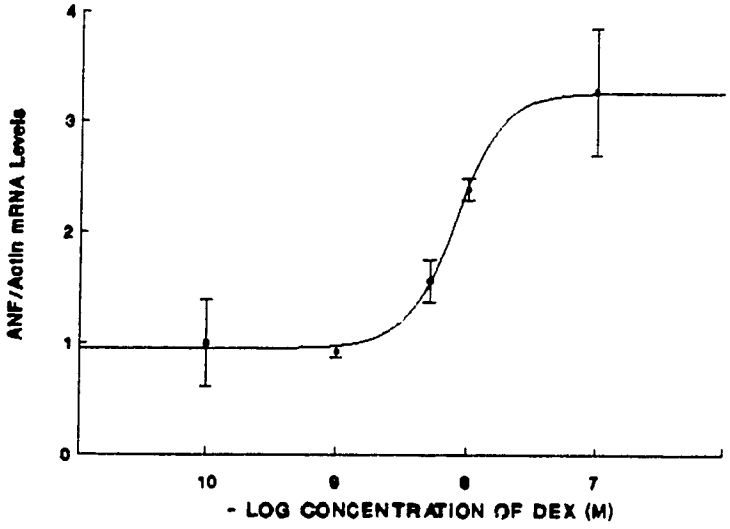


Figure 4.3. Dose-dependent induction of ANF mRNA levels in both atrial (a) and ventricular (b) cells. Cells were stimulated with increasing concentrations of DEX for 24 hours. An ED_{50} of about 15 and 6 nM is seen in atrial (a) and ventricular (b) cells, respectively.

transcriptional regulation of ANF gene expression by glucocorticoids.

Effect of Glucocorticoids on ANF Promoter Activity. In order to determine whether glucocorticoid mediated induction of ANF mRNA levels was due to transcriptional activation of the ANF gene, we tested the effect of glucocorticoids on rat ANF promoter fragments transfected in primary cardiocyte cultures. The activity of a 3.7 kb promoter fragment was induced 2-fold by a 24 h DEX treatment of transiently transfected ventricular cultures (Table 1). The same treatment produced no effect on the activity of viral promoters such as RSV (Table 1) or TK (not shown) and there was no effect of DEX on the activity of the promoterless pOGH plasmid (not shown). These results indicated that a least part of the glucocorticoid effect was at the transcriptional level.

We next mapped the cis-acting element required for glucocorticoid response using a series of 5' promoter deletions The same stimulatory effect of DEX was observed on promoter fragments containing at least 1 kb of upstream sequences; however, a deletion at -700 bp completely abolished the DEX effect (Table 1). These results suggested that the glucocorticoid response element (GRE) was located between -700 at -1000 bp

Binding of Purified Glucocorticoid Receptor to the ANF GRE. Next, we examined whether sequences between -700 and -1000 bp contained glucocorticoid receptor binding (GRE) sites. In footprinting experiments, purified rat liver glucocorticoid receptor (27) bound an element located between -952 and -976 bp (Fig. 4.5). This element showed sequence homology with consensus GREs (34); in particular, the TGTTCT hexamer motif which is invariably present in all GREs is completely conserved in the ANF GRE. Binding of the GR to the ANF element was further investigated using mobility shift assays. A 32 bp double stranded

a

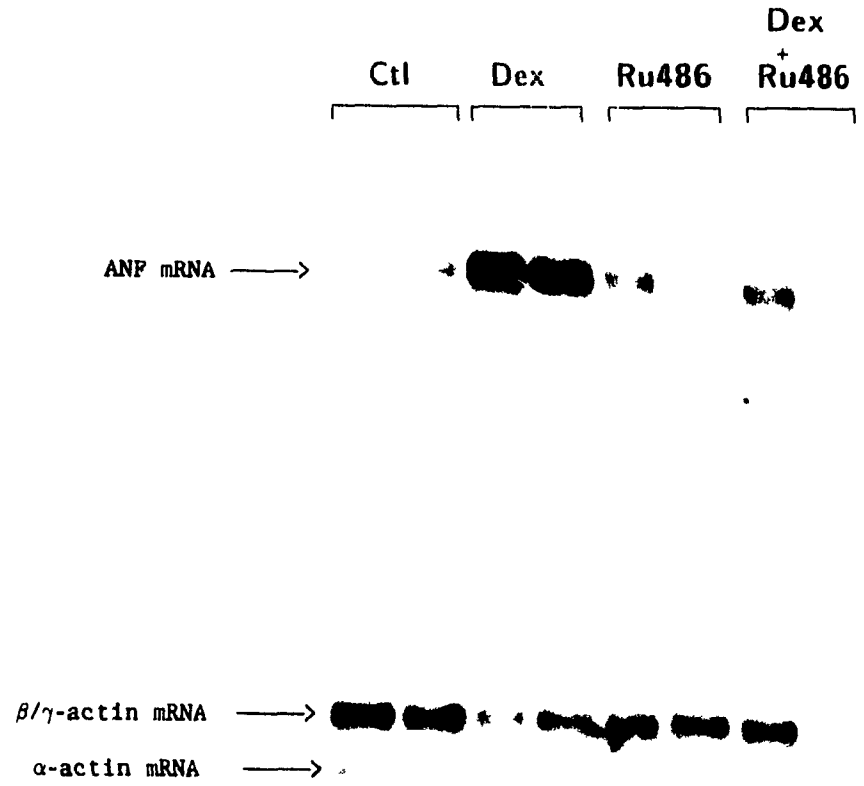


Figure 4.4 Effect of RU486 on DEX-mediated induction of ANF mRNA levels in atrial cells. Following deinduction, cells were treated for 24 h with 10^{-7} M DEX or 10^{-5} M RU486 or both. 10^{-5} M RU486 alone has no effect on ANF mRNA but completely abolishes the DEX-mediated induction. **a)** Autoradiogram of a Northern blot showing the hybridization of 1 μg of total atrial RNA to an ANF cDNA probe and subsequently to a β γ actin cDNA as internal control

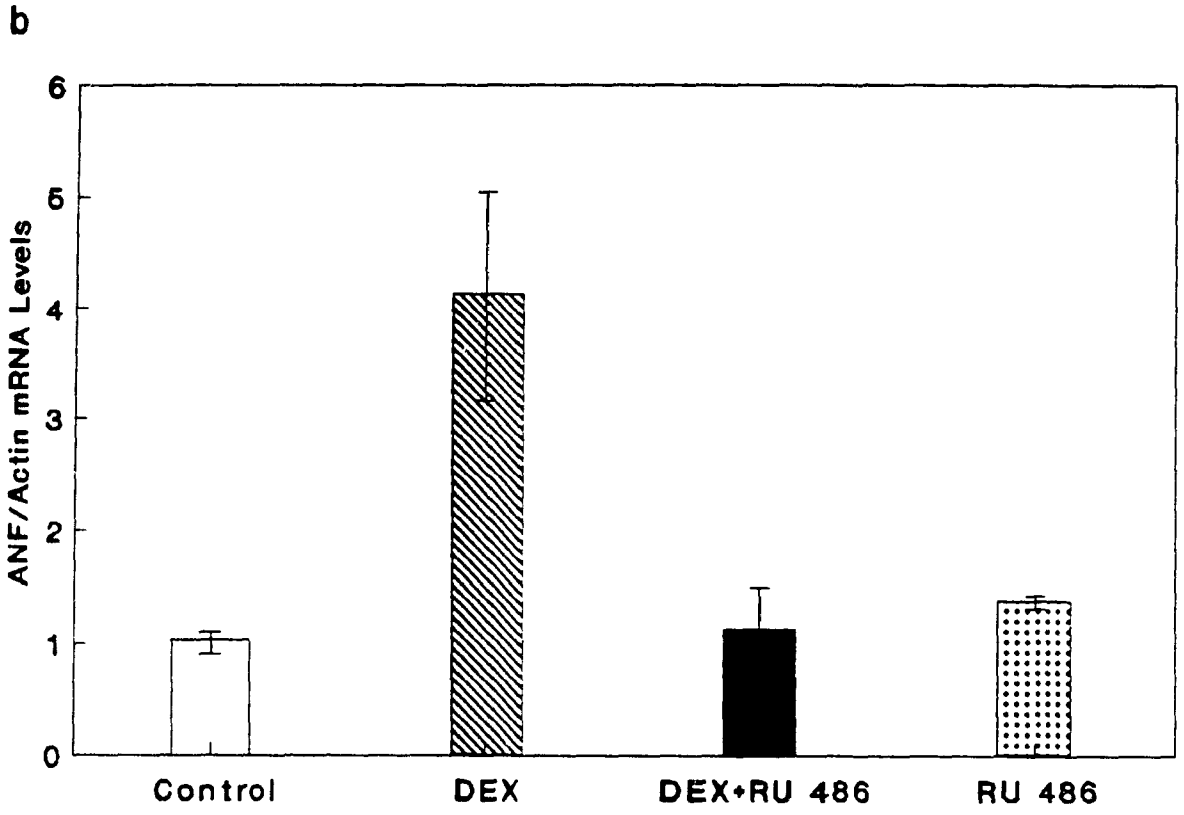
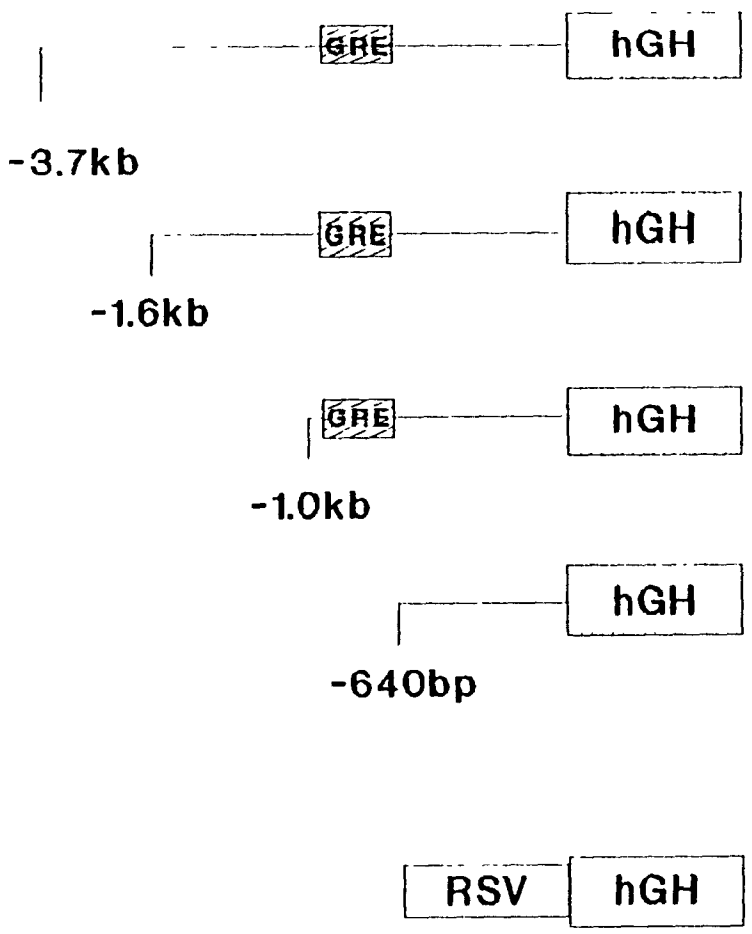


Figure 4.4b) Densitometric analysis of the autoradiogram. The data presented are from one experiment that illustrates results obtained on at least three separate occasions both in atrial and ventricular cells

TABLE 4.1 Glucocorticoid responsiveness of various 5' deletion mutants of ANF promoter



	- DEX	+ DEX	+DEX/-DEX
-3.7kb	0.74 ± 0.02 (n=2)	1.45 ± 0.07 (n=2)	2.0
-1.6kb	1.23 ± 0.23 (n=2)	1.98 ± 0.12 (n=3)	1.7
-1.0kb	0.91 ± 0.06 (n=6)	1.63 ± 0.14 (n=6)	1.8
-640bp	0.98 ± 0.09 (n=6)	1.00 ± 0.13 (n=6)	1.0
RSV	2.10 ± 0.05 (n=2)	1.83 ± 0.02 (n=2)	1.1

Secretion of hGH was taken as a measurement of transfected gene activity. Values are represented relative to -640ANF-GH ± S.E.M. of the number of indicated determinations

oligonucleotide containing the GR footprint on the ANF promoter was used along with an oligonucleotide of similar length corresponding to the -191 to -159 bp GRE of the mouse mammary tumor virus (MTV, ref. 30) Binding of GR to both oligonucleotides (Fig. 4 6a) produced a DNA-protein complex of similar mobility which would correspond to binding of a GR dimer to the DNA (31) An additional, lower migrating complex was observed with the ANF oligonucleotide; this complex was seen in some experiments with the MTV GRE albeit to a much lower extent (not shown). Addition of a polyclonal antibody specific to the rat GR (35) to the binding reaction resulted in an upward shift of both bands (Fig 4 6b) indicating that both complexes contained GR Furthermore, both bands were readily competed by a 3-fold molar excess of MTV GRE oligonucleotide (Fig 4 6d).

Finally, methylation interference studies showed that only methylation of the two guanosine residues present within the hexamer interferes with binding of GR to the ANF sequences, interestingly, both the upper (B_2) and lower (B_1) complexes showed an identical pattern of interference which was very similar to that obtained with the MTV GRE (Fig 4.7). Together, all these data suggest that GR binds to the ANF sequence in dimeric (B_2) and monomeric forms

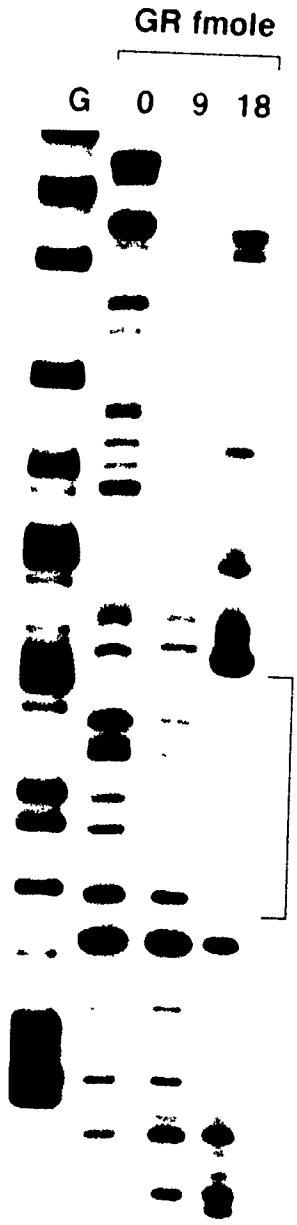


Figure 4.5 Polyacrylamide gel showing DNase I footprinting of purified GR on the distal ANF promoter fragment The various lanes represent a G-ladder of free DNA (G) and DNase I digestion of DNA bound by 0, 9 and 16 fmole of GR, respectively. The ANF fragment was 5' end labeled at the *Sna*BI site (-1029 bp). The gel corresponds to the non-coding strand

a

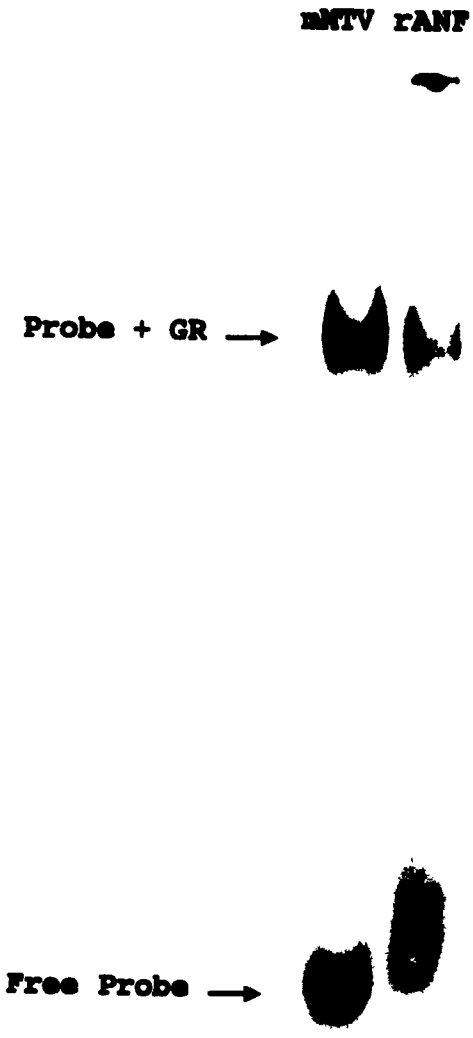


Figure 4.6 Analysis of GR binding to the ANF GRE using gel shift assays a) Comparison of GR complexes obtained with synthetic oligonucleotides corresponding to the mMTV-GRE (-191 to -159 bp) and ANF-GRE (-948 to -979 bp)

b

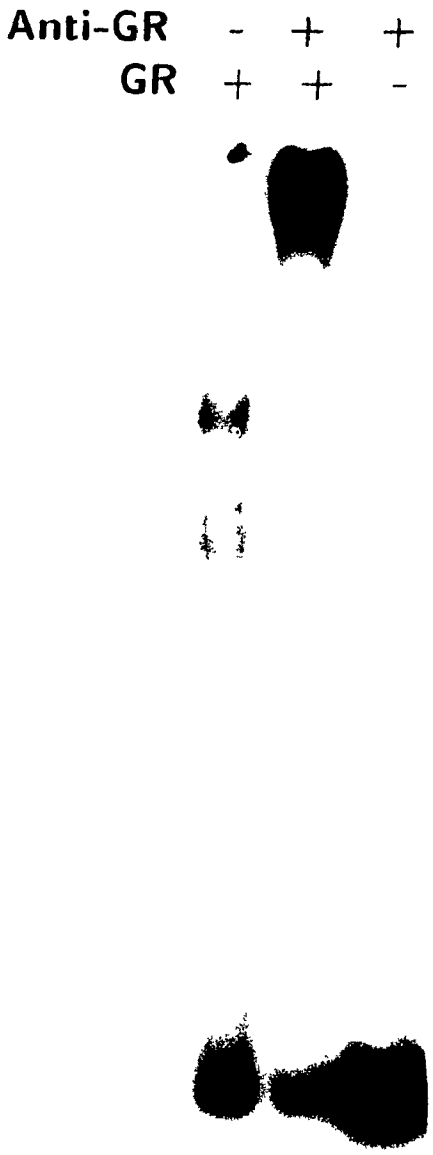


Figure 4.6b) Addition of anti-GR antibody to ANF-GRE·GR complexes cause an upward mobility shift of the complexes, indicating the presence of GR molecules

c

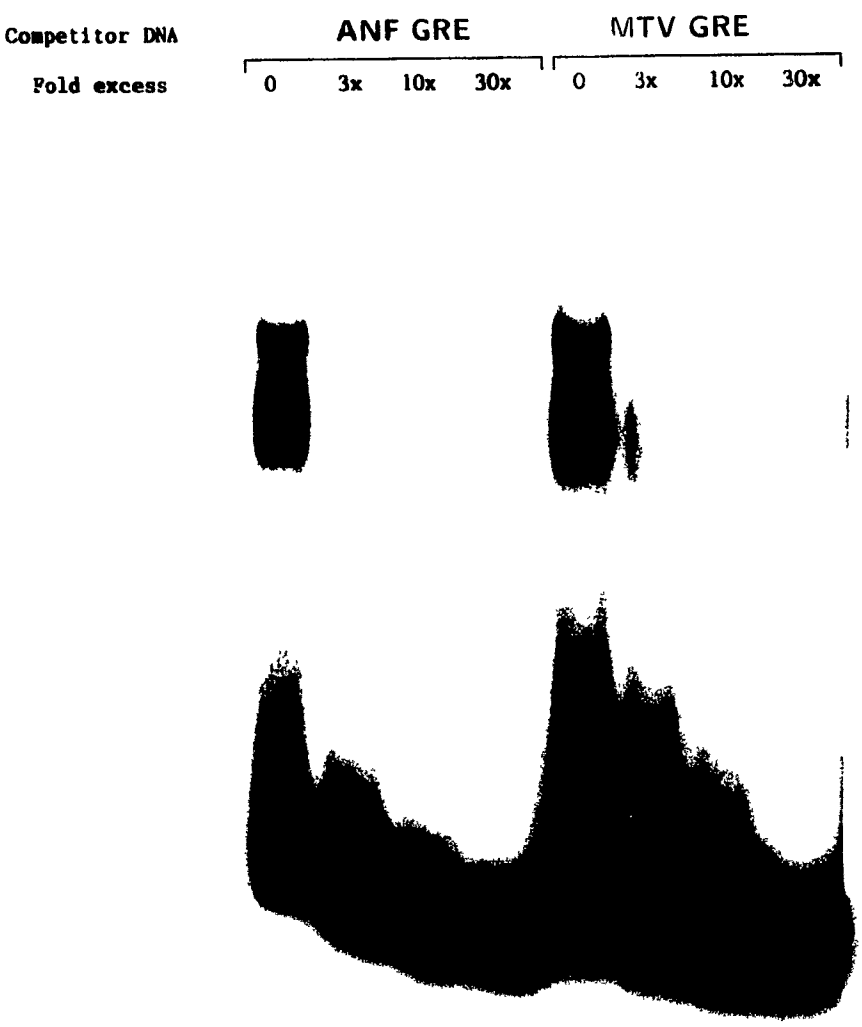


Figure 4.6c) Binding of GR to the ANF GRE is competed by the MTV-GRE. Binding reactions were carried out using 5 fmole of labelled ANF-GR in presence of the indicated amount of competitor DNA. Both ANF and MTV GREs compete equally well for GR binding

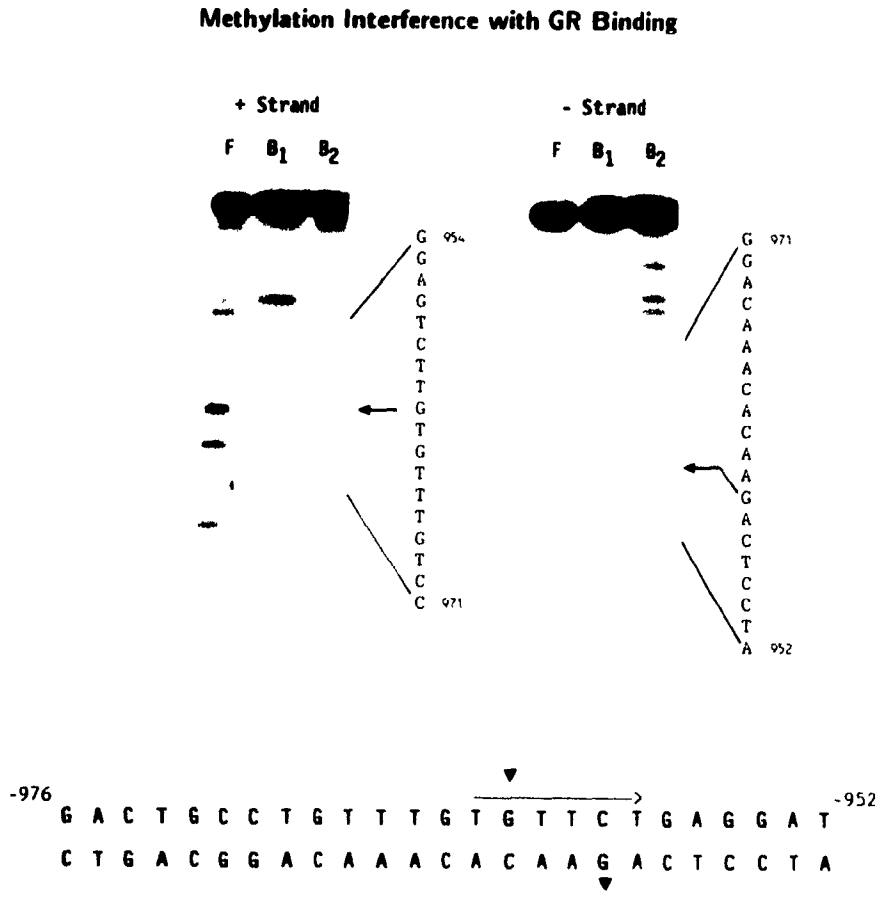


Figure 4.7. The techniques used for methylation interference are detailed in Material and Methods. F is free DNA, B_1 and B_2 correspond to the lower and upper bands, respectively, obtained following binding of GR to the ANF-GRE. G residues interfering with GR binding are indicated by an arrowhead in the bottom panel

DISCUSSION

Atrial natriuretic factor (ANF), a recently identified cardiac hormone, appears to play an important role in blood pressure regulation. ANF lowers blood pressure by its action on multiple target tissues including the adrenal gland where ANF inhibits both mineralo- and glucocorticoid production (36). The data presented in this paper indicate that glucocorticoids in turn directly alter ANF gene expression and that at least part of this effect occurs at a transcriptional level.

In order to study the direct effect of glucocorticoids on the ANF gene, we used a primary cardiocyte culture system where ANF mRNA and peptides are readily detected. Because of possible differences in the regulation of ventricular versus atrial ANF gene expression, atrial and ventricular myocytes were cultured separately. The quantitative difference observed between atrial and ventricular ANF mRNA levels at the neonatal stage are maintained when these cells are cultured. Indeed, there is about a 5- to 8-fold difference in the amount of ANF gene transcripts both in cultured cells and in age-matched neonatal rats *in vivo* (data not shown). Treatment of these cultures with DEX lead to a significant 3- to 4-fold increase of ANF mRNA levels both in atrial and ventricular cells. The effect of DEX was both time and dose dependent and completely abolished by the specific glucocortiocid antagonist RU486. Both DEX deinduction and induction were rapid: a 75% drop in expression was achieved in less than 48 h, and a subsequent maximal induction was observed after only 6-12 h of stimulation.

Although both atrial and ventricular cells responded to DEX stimulation, ventricular cells displayed increased sensitivity to hormonal stimulation as revealed by the difference in the observed ED_{50}'s in the two tissues. This differential

sensitivity of the ANF gene to hormonal stimulation in the two heart compartments is consistent with the in vivo data showing a larger glucocorticoid induction of ANF mRNA in ventricles (11 and Nemer et al., in preparation). A similar increased sensitivity to glucocorticoid induction of ANF release has been reported by others in ventricular cell cultures (14). This difference is not due to changes in glucocorticoid receptor levels or affinity in ventricular tissues as similar levels of receptor protein with identical ligand affinity are present in atria or ventricles (Nemer and Antakly, in preparation). A similar differential sensitivity to glucocorticoids has been reported for the induction of the liver tyrosine amino transferase (TAT) gene in two different hepatoma cell lines (37) that had similar glucocorticoid receptor levels (38). Interestingly, regulation of the ANF gene in atrial and ventricular cells displays differential sensitivity to other hormones like thyroid hormone (17) suggesting the presence of a factor in ventricular cells which is permissive for hormonal regulation of the ANF gene.

In order to test whether stimulation of ANF mRNA levels occurs at a transcriptional level, we tested glucocorticoid responsiveness of various ANF promoter fragments. Glucocorticoids increased by 2 fold the activity of several ANF promoter fragments containing at least 1.0 kb of upstream sequences in cardiac cells, suggesting that at least part of the glucocorticoid stimulation of ANF gene expression is at the transcription level This result is in agreement with an independent study which also suggested a transcriptional effect of glucocorticoids on the ANF gene based on *in vitro* transcription assays (39) and with preliminary data suggesting that a 2.4 kb fragment of the rat ANF promoter is responsive to glucocorticoids (40). The discrepancy between the 2 fold effect observed on ANF promoter activity and the 3 fold stimulation of endogenous ANF mRNA levels,

raises the possibility that glucocorticoid response elements within the gene may contribute to full glucocorticoid effect. In this regard, it is worth mentioning that a sequence with high homology to classical GREs is present in the second intron of the ANF gene (23, 41). Whether this element contributes to regulation of the ANF gene by glucocorticoids remains to be tested. Alternatively, glucocorticoids may act at a transcriptional as well as a post transcriptional level to regulate ANF gene expression, an effect of glucocorticoids on ANF mRNA stability has indeed been suggested by others (39). It is worth mentioning that glucocorticoid stimulation of ANF promoter activity is restricted to cardiac cells. Indeed, no effect of the hormone could be detected on ANF promoter activity in several non cardiac cell lines such as adrenal chromaffin cells (PC12), pituitary somatotrophs (GH_3) and fibroblasts (L). This suggests that glucocorticoid responsiveness of the ANF promoter requires the presence of cardiac specific trans-acting factor(s). These factors may be stimulated by the hormone or they may cooperatively interact with the glucocorticoid receptor. Such tissue specific hormonal regulation has been observed for some hepatic and pituitary genes like tyrosine amino transferase (42) and prolactin (43). In the case of prolactin, it was shown that the estrogen receptor binds to a distal promoter element and interacts cooperatively with the pituitary-specific transcription factor, Pit-1, to induce prolactin expression. Whether the glucocorticoid receptor interacts with a putative cardiac-specific factor that binds both the proximal and distal elements of the ANF promoter, (our unpublished data) will be further investigated.

Finally, *in vitro*, binding studies using purified rat liver glucocorticoid receptor revealed high affinity binding of the receptor to an element within the promoter region which is required for glucocorticoid stimulation. Gel shift and

methylation assays confirmed the specificity of this binding Interestingly, glucocorticoid receptors bind to the ANF sequence in both dimeric and monomeric forms. Indeed, most of the well studied GREs contain three base pairs, the trinucleotide ACA, upstream of the conserved TGTTCT hexamer (34). This sequence is part of an imperfect second hexamer that is thought to bind a second GR monomer on the same side of the DNA helix. It is interesting to note that, in the ANF GRE, the ACA trinucleotide is present on the opposite DNA strand relative to the TGTTCT hexamer This results in the presence within the GRE of an inverted repeat instead of a dyad symmetry.

```
                          ∇
MMTV GRE  GGTACAnnnTGTTCT
          CAATGTnnnACAAGA
               ∇          ∇

                          ∇
ANF GRE   GCCTGTnnnTGTTCT
          CGGACAnnnACAAGA
                          ∇
```

While there is no report on positive GREs with such a feature, glucocorticoid receptor binding sites that considerably diverge from the consensus GRE have been documented for negatively regulated genes (44, 45) Thus, GR is capable of binding to degenerate DNA consensus sequences. Whether the ANF-GRE can mediate transcriptional activation by glucocorticoids is presently being tested.

In conclusion, the present study provides evidence for transcriptional regulation of the ANF gene by glucocorticoids via a distal promoter element which contains a binding site for the glucocorticoid receptor The molecular events leading to the increased expression of the ANF gene in situations of hormonal

imbalance, such as in Cushing's disease (46), may explain some of the poorly understood effects of glucocorticoids on the cardiovascular system.

ACKNOWLEDGEMENT

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CHAPTER 5

IDENTIFICATION OF CIS-ACTING ELEMENTS INVOLVED IN CARDIAC-SPECIFIC AND DEVELOPMENTAL EXPRESSION OF THE RAT ANF GENE

ABSTRACT

Expression of atrial natriuretic factor (ANF), a 28 aa peptide hormone synthesized predominantly in cardiac tissues, is under differential developmental regulation in atria and ventricles. In order to study molecular mechanisms involved in cardiac-specific and developmental expression of ANF, we have analyzed cis-acting elements of the rat ANF gene. Using transient transfections into primary neonatal rat cardiocyte cultures and a fusion gene containing ANF 5'-flanking sequences linked to the human growth hormone (hGH) gene as reporter, we have established that cis-acting elements within the gene's 5'-flanking sequences confer cardiac-specific expression to the ANF gene Progressive 5' deletions revealed that 1.6 kb of upstream sequences were sufficient for maximal expression in cardiac cells. Furthermore, two elements located between -1 6 and -1.0 kb (distal element) and -640 and -136 bp (proximal element), were identified as being necessary for full promoter activity in these cells The importance of PE was confirmed by using an internal deletion mutant (deleted from -700 to -136 bp) All the 5' deletion mutants of the ANF gene are nactive in non-cardiac cells with the exception of the shortest deletion (to -136 bp) The promoter activity observed in L cells with the internal deletion mutant (-700 and -136 bp) suggests the presence of a repressor element upstream of -136 bp which would be active solely in non-cardiac cells. Both PE and DE increased the activity of the thymidine kinase (TK) promoter in cardiac cells Thus, the cell-type and developmental control of ANF gene expression appears to be mediated by a complex interaction of various 5'-flanking elements.

INTRODUCTION

Eukaryotic gene expression is controlled by cis-acting DNA elements usually

found within the 5'-flanking regions of a gene (Maniatis et al., 1987; McKnight et al., 1982). Recent studies suggest that tissue-specific gene expression is determined by an interaction of such cis-acting elements with specific trans-acting factors (for review see Mitchell and Tjian). Although the mechanisms governing the tissue specific expression of several genes, such as growth hormone and immunoglobulin genes (Castrillo et al., 1989; Scheidereit et al., 1987), are becoming clearer, the events underlying the expression of cardiac muscle-specific genes is not well understood and is currently under intensive investigation. Of particular interest are the cardiac actin, troponin and muscle creatine kinase genes (Miwa et al., 1987; Muscat et al., 1988; Mar et al., 1988). Although certain cis-acting sequences have been attributed to the cardiac specific expression of the latter two (Johnson et al., 1989; Mar et al., 1988a), cardiac-specific trans-acting factors have yet to be identified.

The atrial natriuretic factor gene (ANF) encodes a potent endogenous diuretic, natriuretic and vasodilating peptide that is secreted from the heart in response to various physiological stimuli (for review see Baxter and Gardner, 1988). In the adult heart, ANF is predominantly synthesized and stored in secretory granules of the atria although it is also present at much lower levels in the ventricles, where peptide storage is not very prominent. Indeed, Northern blot analysis has revealed that ANF mRNA is present at levels about 100-fold lower in the ventricles than in the atria of adult rats (Nemer et al., 1986). In the newborn rat, however, atrial and ventricular ANF mRNA levels are almost equivalent (Bloch et al., 1986).

We have isolated and sequenced both the rat and human ANF genes and have shown that a high degree of homology exists between the two species in their proximal 5'-flanking sequences, suggesting the presence of putative well conserved

regulatory elements within this region (Argentin et al., 1985). In order to address the questions of tissue specificity and developmental control of ANF expression, we undertook an analysis of the rat ANF gene promoter. Using 5' and internal deletion mutants as well as chimeric constructs containing certain regions of the rat ANF gene linked to a heterologous promoter, we were able to identify two elements that are responsible for promoter activity in cardiac cells, whereas they appear to be inactive in other cells. Thus, we have defined two cardiac-specific regulatory domains within the ANF gene

MATERIALS AND METHODS

Cell Culture. Atrial and ventricular cardiocyte cultures were prepared from 1 day old and 4 day old Sprague Dawley rats (Charles River). Atrial and ventricular tissues were aseptically removed and washed with Joklik's modified Eagle's medium (Gibco) The tissues were then minced and subjected to three sequential digestions of 30, 20 and 10 min. each in 0.1% collagenase (Cooper Biomedicals) To stop the enzymatic digestion, cold fetal calf serum was added to a final concentration of 28.5%. Undigested tissue remnants were removed by filtering through a 100 μM nylon mesh The cell-containing filtrate was then centrifuged and the resulting cell pellet was resuspended in Dulbeco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum To eliminate fibroblasts, the cells were pre-plated for 2-30 minute periods, whereby the unattached cardiocyte-enriched cells were collected The cardiac cells were plated in Primaria (Falcon) plates at a density of 1 x 10^5 cells/cm^2. To maintain cardiac-enriched differentiated cell cultures, the serum supplemented medium was replaced 16 h later by a serum-free hormonally-defined medium (Mohamed et al. 1983)

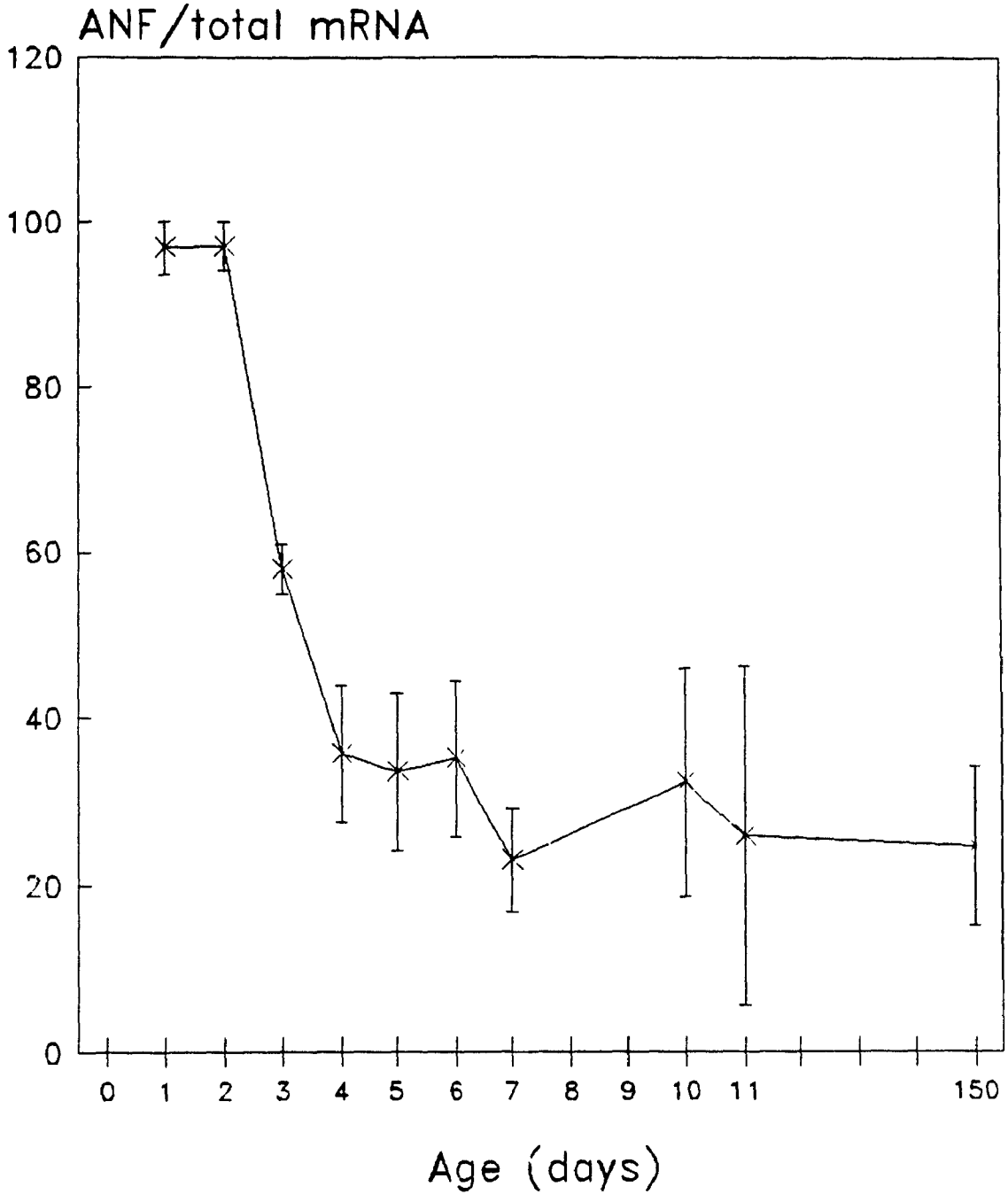


Figure 5.1 Postnatal pattern of ANF gene expression in ventricular tissue ANF mRNA levels, corrected for total mRNA using an oligo dT probe, are plotted on the y-axis as a function of age. Each value represents the average of at least 4 separate determinations.

Figure 5.2 Immunocytochemical staining of atrial and ventricular cells with an anti-ANF antibody. A 1:1000 dilution was used for atrial cells (a) and 1:500 dilution was used for ventricular cells (b). Non-immune serum served as a negative control in a plate containing ventricular cells (c). Positive immunostaining is apparent in both atrial and ventricular cells.

Non-myocyte enriched cell cultures were obtained by maintaining the cells in a mitogen-rich medium containing 15% FCS. (Kohtz et al., 1989).

Cells were transfected about 24 h after the initial plating. L and PC12 cells were plated at a density of about 0.2 x 10^5 cells/cm^2 and maintained in DMEM medium supplemented with 10% FCS or DME supplemented with 5% FCS and SerX-tend (Hana). Fresh medium was added to the cells 4-5 h prior to transfection.

Plasmid Constructions. A previously subcloned 703 bp ANF promoter fragment from position -640 to +40, containing XbaI polylinkers, was inserted into the XbaI site of the promoterless pOGH vector, containing sequences encoding the human growth hormone (hGH) gene (Selden et al., 1986). This plasmid (-0.64ANF-GH) was tested and found to have significant, but low promoter activity, thus, further 5' sequences were obtained and subcloned as follows. An *Eco*RI fragment containing sequences between -3700 and -640 bp was excised from a 12 kb *Bam*HI genomic fragment (Argentin et al., 1985), purified on agarose gel and inserted into the *Eco*RI site of -0.64ANF-GH. The resulting plasmid (-3.7ANF-GH), containing 3.7 kb of 5' upstream sequences was then digested by various restriction enzymes to obtain the 5' deletion and internal deletion mutants (Fig. 5.4). For heterologous promoter constructs, two different fragments of the ANF promoter were subcloned into the polylinker site of two plasmids containing short Herpes simplex virus thymidine kinase gene (TK) promoters (-81 and -109 bp) fused to the luciferase cDNA (De Wet et al., 1987). Briefly, -2.4ANF-GH was digested with *Hin*dII and *Sna*bI (positions -1600 to -1000). The blunt ended *Hin*dII-*Sna*bI fragment was then inserted and ligated to the *Sma*I site of pTK81-luc and pTK109-luc. Similarly, *Hin*dIII digested -2.4ANF-GH was used to produce a fragment with positions -700 to -136. This element was inserted into the *Hin*dIII sites of pTK81-luc and

pTK109-luc. Standard procedures were used to identify recombinants containing both orientations of inserted ANF fragments (Maniatis et al., 1982)

Transient Expression Assays. To introduce foreign DNA into cells, a modification of the calcium phosphate precipitation technique was employed (Chen et al., 1987) DNA concentrations in the linear range of transfection efficiency were chosen 2-4 μg of DNA/ml of medium were used to transfect cardiac cells. The formation of a fine DNA-calcium phosphate precipitate was allowed to form by incubating the cells overnight at 37 ^{o}C and 3% CO_2. The next day, cells were fed with the appropriate medium Cell media and cell extracts were collected 48-72 h after transfection for hGH assays and luciferase assays, respectively.

hGH Immunoassays. The hGH secreted into the cell culture medium was measured directly from 100 μl aliquots of medium after spinning down any cellular debris hGH immunoassays were performed by the Allegro hGH kit (Joldon), following the manufacturers intructions.

Luciferase Cell extracts were obtained by scraping cells in a luciferase lysis buffer (0.1 M KPO_4 (pH7.8) and 1 mM DTT), spinning down the cell pellet and then lysing cells in the same buffer containing 0.5% NP40. Cells were resuspended and left on ice 5 minutes; cellular debris were spun down at 14K for 15 min at 4 ^{o}C. The resulting supernatant was stored at -20^{o}C until luciferase assay.

Luciferase activity was determined as follows in cell extracts . A 50 μl reaction containing 0.1 M KPO4 (pH 7.8), 0.005M ATP, 0.01M MgCl2 and the cell extract was added to a luminometer cuvette To this, 100 μl of 0.001M luciferin was injected, and light emission was automatically recorded. Total protein in the extracts was determined by the method of Bradford (1976)

RNA Analysis. RNA was prepared by the guanidium thiocyanate phenol extraction method described by Chomczynski et al., 1987. Total mRNA was quantitated by hybridization with [^{32}P] labelled oligodT$_{12-18}$ (Suggs et al., 1981). Northern blots were performed as previously described (Nemer et al., 1986) and autoradiographs were quantitated by densitometric scanning.

Immunocytochemistry. Atrial and ventricular cells were fixed with 4% p-formaldehyde. After dehydration in graded alcohols, the cells were preincubated for 10 min at 37 °C in 10% normal goat serum. A polyclonal rabbit antiANF-Ab was then applied overnight at 4 °C at 1:500 and 1:1000 dilutions in ventricular and atrial cells, respectively. Non-immune serum at the appropriate dilution was used as a negative control. The Vectastain ABC kit (Vector Lab., Burlingame, CA) was used to reveal antiANF staining as follows. The cells were treated with biotinylated goat anti rabbit IgG at a 1:100 dilution for 30 min at room temperature followed by the Vectastain A and B reagents (Avidin DH and Biotinylated Hoseradish Peroxidase H, respectively) each at a 1:100 dilution, also for 30 min. at room temperature. Finally the peroxidase substrate diaminobenzidine tetrahydrochloride (DAB) was added (.015%) along with .003% hydrogen peroxide, and cells were incubated in the dark at room temperature for 6-8 min.

RESULTS

Developmental Expression of the Rat ANF Gene. In order to establish the pattern of atrial and ventricular ANF gene expression during cardiac development we analyzed ANF mRNA levels as a function of age in rats. On the day of birth, ANF mRNA levels are about 5 to 8 times higher in atria compared to ventricles

Thereafter, ventricular ANF mRNA levels drop rapidly within the first week while atrial ANF mRNA levels rise, resulting in the 100-fold difference in ANF mRNA levels in these two tissues in the adult rat (Nemer et al., 1986; Fig. 5.1); at day 4, ventricular ANF mRNA levels resemble adult levels. In atrial cells, ANF mRNA levels appear to rise about 2-fold overall throughout the course of development (data not shown). In keeping with the *in vivo* pattern of ANF expression, in cell culture, there is about a 4- to 5-fold difference in the level of ANF mRNA between atrial and ventricular cells of 1 d old rats, this value rises to about 8-fold in 4 day old rats (data not shown). Since the *in vivo* model of cardiocytes in primary cell culture closely resembles the tissue *in vivo*, we used the system of cardiocytes from 1 and 4 day old rats to study the ANF promoter in the developmental expression of ANF

In order to assess the homogeneity of primary cardiocyte cultures, cells were stained with an anti-ANF antibody Immunocytochemical analysis of the cell cultures showed that a positive immunoreaction is produced in almost all cells (about 70-80%) in both the atrial (Fig 5.2a) and ventricular (Fig. 5.2b) cultures. In control wells, treatment of cells with non-immune serum showed no staining (Fig. 5.2c) As expected, ventricular cells which secrete ANF constitutively (Bloch et al., 1986), gave a weaker immunocytochemical staining than atrial cells. In addition, the general apperance of the cell cultures confirms the finding that cardiocytes make up a large fraction of the plated cells since about 80% of the cells display spontaneous contractility and the typical network formation of beating cell clusters

Cardiac-Specific Expression of the ANF Gene is Determined by 5'-Flanking Sequences. Transient transfection assays were used to localize rat ANF promoter

sequences involved in the tissue-specific expression of the gene A plasmid containing 3.7 kb of 5' upstream sequences linked to the human growth hormone (hGH) reporter gene was tested along with a positive control plasmid, containing the viral Herpes simplex virus thimidine kinase (TK) promoter (Fig 5 3 and 5 4)

These plasmids were introduced into various cells: atrial and ventricular primary cell cultures of neonatal rats, non-myocyte enriched primary cell cultures (mostly fibroblasts), fibroblast L cells, and adrenal medullary pheochromocytoma PC12 cells The results from these assays indicate that the plasmid containing 3.7 kb of 5' upstream sequences (-3 7ANF-GH) contains sequences required for cardiac-specific expression of the ANF gene (Fig. 5 3) As expected, the promoter is about 5-fold less active in ventricular versus atrial cells On the other hand, this promoter was almost inactive in the non-ANF expressing cells

Localization of Cis-Acting Elements Governing Tissue-Restricted and Developmental Expression of ANF. In order to localize the DNA elements responsible for cardiac-specific expression of ANF, we constructed a series of 5' deletion mutants of the ANF promoter (Fig. 5 4) These plasmids were introduced along with pRSV-Luc, as an internal control, into eight cell types Atrial and ventricular cardiocytes, from both 1 and 4 day old neonatal rats, were tested to determine whether cis-acting DNA sequences were responsible for the developmental expression of ANF. In addition, cardiac non-myocytes, L cells and PC12 were tested to assess the tissue specificity of the ANF promoter

The 5' deletion mutants appear to be inactive or exhibit very low transcriptional activity in the non-ANF expressing cells, such as cardiac non-myocytes, L an PC12 cells (Fig. 5.5) The -1.6ANF-GH plasmid is at least 7 5 times

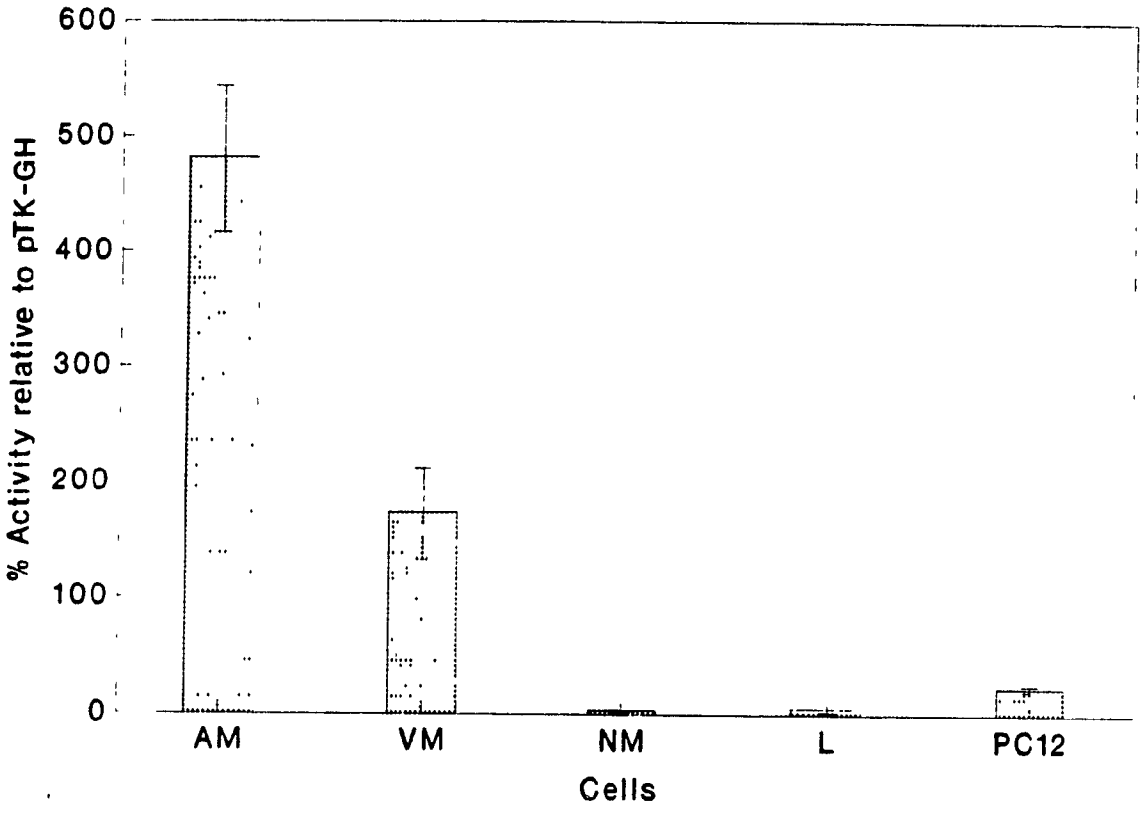


Figure 5.3 Cardiac-specific activity of the ANF promoter. The histogram represents activity of the full ANF promoter (-3.7ANF-GH) relative to viral promoter activity (pTK-GH) which is taken as 100%. Each point represents the mean ± SEM of several determinations: 1 day old atrial myocytes (AM) n = 5, 1 d old ventricular myocytes (VM) n = 6, cardiac non-myocytes (NM) n = 4, fibroblast L cells (L) n = 3, adrenal medullary cells (PC12) n = 10.

more active in the ventricular myocytes than in the non-cardiac cells. This difference rises to at least 16-fold when non-cardiac cells are compared to atrial myocytes (data not shown). The smallest deletion mutant, however, -0.14ANF-GH, exhibits higher promoter activity in the L cells and in the fibroblast enriched cardiac cells (Fig 5.5). This activity may suggest the presence of a repressor

element upstream of -136 bp, active in certain non-expressing cells or to the presence of sequences that contribute to the maintenance of cardiac-restricted expression since activity in cardiac cells is also reduced with this deletion mutant Indeed, this rise in transcriptional activity with the -136 bp deletion mutant can be used as an indication of fibroblast or non-myocyte proliferation in cardiocyte cultures.In answering the question of promoter-dependent developmental regulation of ANF, our data clearly shows a greater overall distal promoter activity in the 1 d old rats versus the 4 d old rats in ventricular cells (Fig 5.6a and b) A significant difference of about 3-fold ($p < 0.001$) in promoter activity was observed for most 5' deletion mutants between the 1 and 4 day old ventricular cells No such significant difference in ANF promoter activity was observed in the 1 and 4 day old atrial cells. This is consistent with the developmental pattern of expression observed in the endogenous ANF mRNA levels (Fig 5 1)

In the cardiac cells, the same general expression curve is obtained for both atrial and ventricular cells and shows that -1.6 kb of upstream sequences produce maximal transcriptional activity in both cell types (Fig. 5.5) As is best exemplified by the ventricular cells and confirmed in the atrial cells, promoter activity drops 2- to 3-fold ($p < 0.001$) when sequences up to -1.0 kb are deleted in both 1 and 4 d old rats. A second 2- to 3-fold drop in activity ($p < 0.001$) is observed when sequences between -700 bp and -136 bp are deleted at both ages Similar results are observed in the atrial cells. Thus, two regions, a distal one between -1 6 and -1 0 kb (DE) and a proximal one between -700 and -136 bp (PE) contribute to promoter activity in cardiac cells. Furthermore, a slight but consistent and significant ($p < 0.001$) repression appears to be contributed by the sequences between -2.5 and -1.6 kb

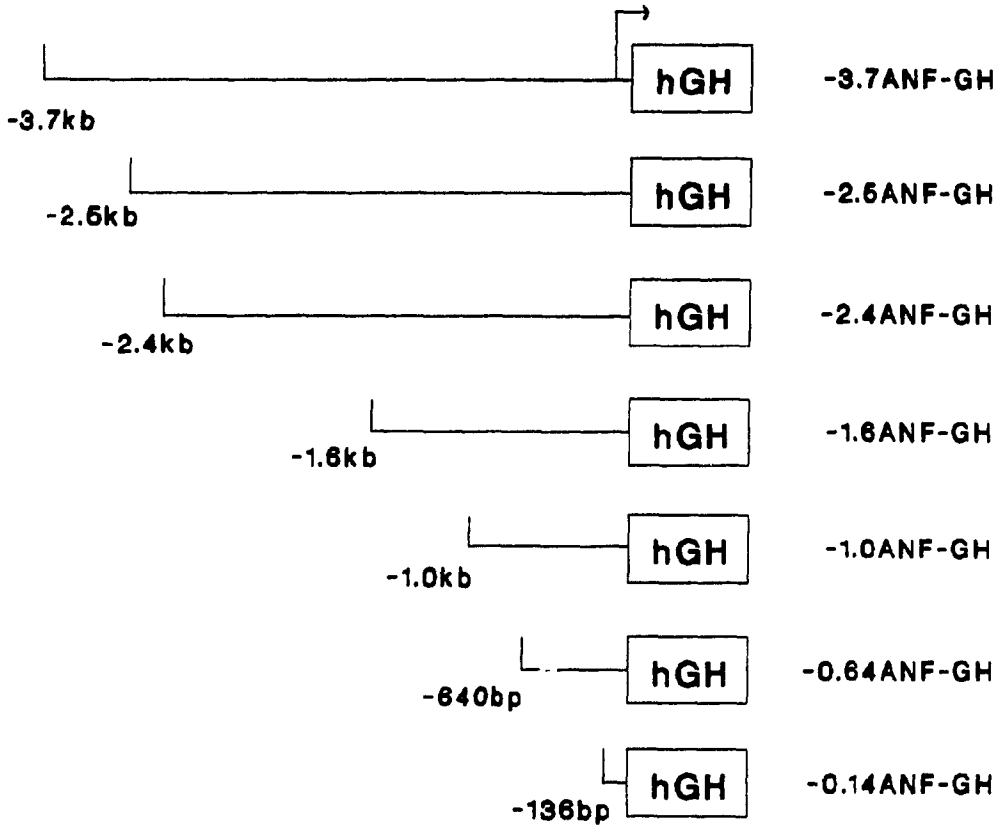


Figure 5.4 5' deletion fragments of the rat ANF promoter. Various restriction enzymes were used to generate a series of sequential 5' deletion mutants of the rat ANF promoter. These constructs, directing human growth hormone (hGH) reporter gene expression, were tested in various cell types.

To confirm the importance of the proximal promoter element in cardiac expression, an internal deletion was obtained between -700 and -136 bp from a plasmid containing 2.4 kb of 5' upstream sequences (-2.4ANF-GH) (Table 5.1). As can be seen from Table 5.1 this deletion resulted in a 4- to 5-fold drop in ANF promoter activity in cardiac cells, indicating the presence of an important positive regulatory element within this DNA fragment. In L cells, this same deletion led to an increase of about 2-fold in promoter activity, supporting the hypothesis that a negative regulatory element upstream of -136 bp is active in certain non-cardiac cells.

Heterologous Promoter Constructs. In order to determine whether the proximal and distal promoter elements can confer cardiac-specific expression DNA fragments corresponding to PE and DE were inserted upstream of two different TK-luciferase reporter genes which contained either 81 or 109 bp TK 5'-flanking sequences. Similar results were obtained with both reporters, data for pTK81-luc are presented in Table 5.2. These constructs were tested by transfection in both cardiac and L cells. Luciferase activity was corrected for protein content and transfection efficiency was assessed with an internal control plasmid pRSV-GH. Only the proximal ANF gene fragment (-700 to -134 bp) increased significantly the activity of the reporter (Table 5.2). This increase was only observed in cardiac cells and in only one orientation of the ANF fragment. In addition, both fragment produced a slight increase in cardiac and L cells, in one or both orientations. These results indicate that at least the proximal and possibly the distal element (PE and DE) can mediate a tissue-specific activating function upon a heterologus promoter.

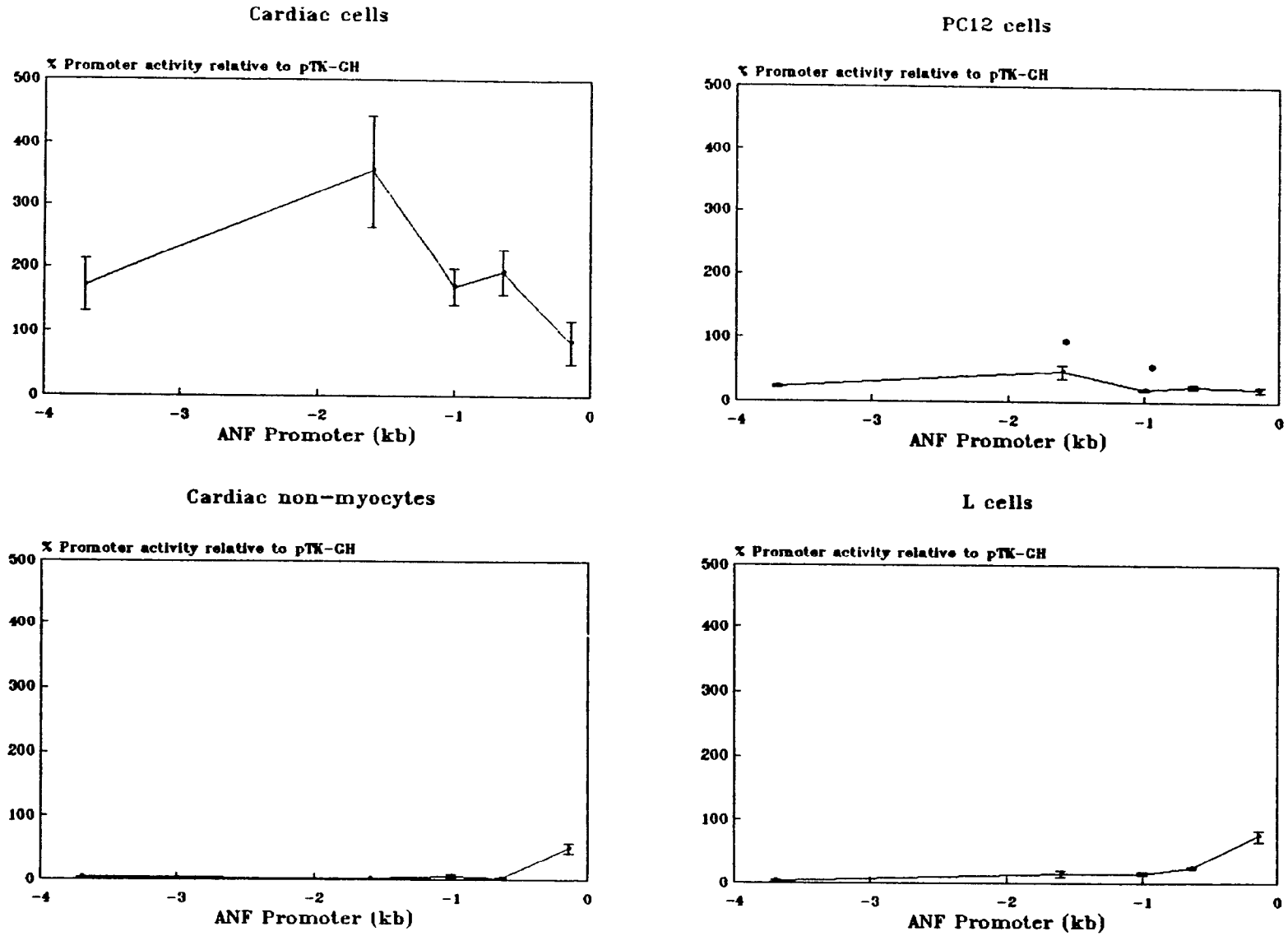


Figure 5.5 Activities of ANF 5' deletion mutants in various cell types. The 5' deletion mutants were tested in 1 d old ventricular myocytes (cardiac cells), cardiac non-myocytes, PC12 cells and L cells. % promoter activity is given relative to viral promoter activity (pTK-CH) which is taken as 100%. Each point represents the mean ± SEM of several determinations: cardiac cells n = 6, cardiac non-myocytes n = 4, PC12 cells n = 4 to 12, except * where n = 2, L cells n = 3 to 4.

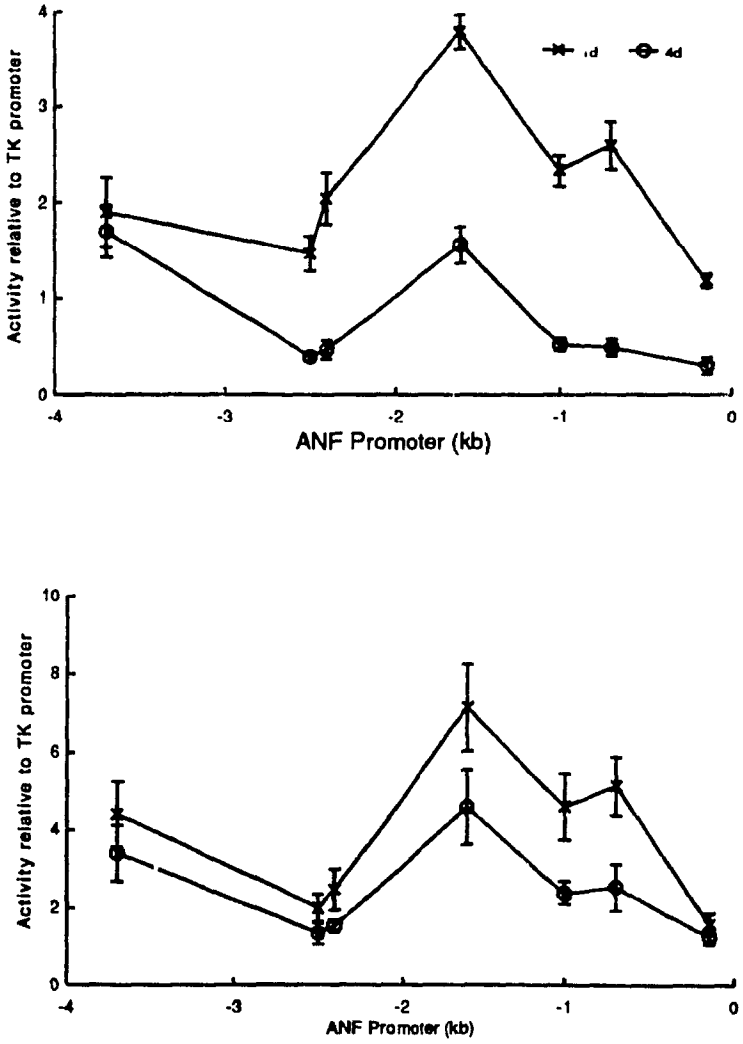
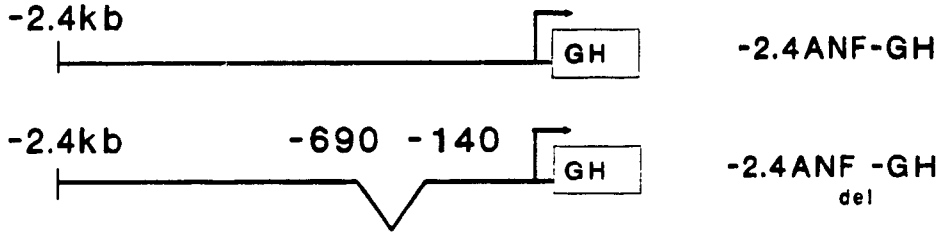


Figure 5.6 Transcriptional activities of ANF 5' deletion mutants in 1 and 4 d old atrial and ventricular cells. A series of 5' deletion mutants were tested in 1 day old and 4 day old ventricular (a) and atrial (b) cells. Transcriptional activity, represented on the y-axis, is given relative to the TK promoter. Each point represents the mean ± SEM of n = 3 to 6 values for ventricular cells, and n = 4 to 14 values for atrial cells. The data were compiled from at least three separate experiments.

Table 5.1 Effect of proximal element deletion in cardiac and L cells.



Plasmid	hGH activity*(ng/ml)	
	cardiac cells	Fibroblasts L cells
-2.4ANF-GH	1.97	0.90
-2.4ANF$_{del}$-GH	0.42	2.73

Table 5.1 The deletion of nucleotides from -640 to -140 bp results in the removal of the proximal element. The activity of this deletion mutant in ventricular cardiocytes and fibroblast L cells is given as ng/ml of hGH. The values represent an average of at least 4 separate determinations.

Table 5.2 Activity of heterologous promoter constructs in cardiac cells and fibroblasts.

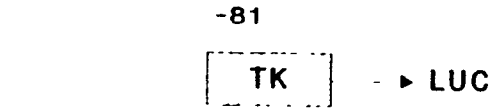
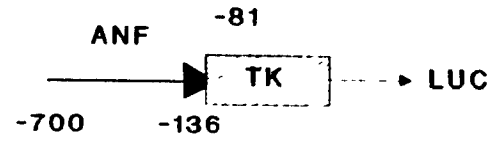
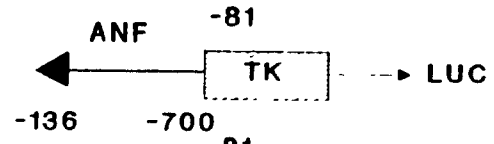
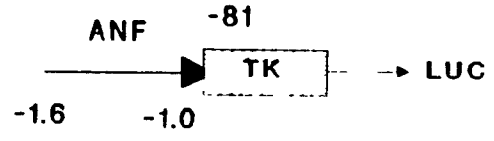
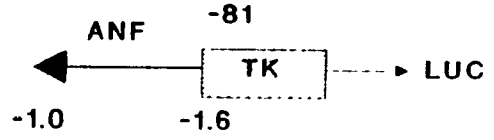
Plasmids	Cardiac cells	Fibroblasts
	1.0 ± 0.23	1.0 ± 0.30
	3.01 ± 0.28	2.89 ± 0.89
	13.85 ± 1.76	3.34 ± 0.71
	2.30 ± 0.21	1.90 ± 0.92
	0.93 ± 0.36	1.87 ± 0.54

Table 5.2 Heterologous promoter constructs were tested in both 1 and 4 day old ventricular cells and fibroblast L cells. Values shown for cardiac cells represent results obtained in 1 day old ventricular cells. Values represent the mean ± SEM of n = 3 to 4 determinations for cardiac cells and n = 3 to 6 for L cells from at least two separate experiments.

DISCUSSION

Using DNA-mediated gene transfer studies, we have shown that activity of the ANF gene promoter is restricted to cells of cardiac origin, specifically to the atrial and ventricular myocytes of the heart (Fig. 5.3). Expression in non-myocytes such as L, PC12 and non-myocyte enriched (mostly fibroblasts) primary cardiac cell cultures is significantly lower than in cardiac cells Only 1.6 kb of ANF 5'-upstream sequences are sufficient for promoter activity in cardiac cells, and successive deletions downstream of -1 6 kb result in further reductions of ANF promoter activity (Fig 5 6).

Since primary cell cultures contain a heterogeneous cell population, several precautions were taken in order to maintain a high proportion of cardiocytes in these cultures. Reduction of non-myocyte cell proliferation was achieved through a combination of techniques including differential plating, the use a a serum-free hormonally defined medium and high plating density (Blondel et al., 1976; Claycomb, 1980; Mohamed et al., 1983; Libby, 1984; Millart et al, 1986; Speicher et al. 1981) In our cultures, both atrial and ventricular myocytes displayed spontaneous contractile activity and were arranged in the typical cluster-like fashion expected of such cells in culture. The estimate by simple observation that 70-80% of the cells were myocytes was confirmed by immunocytochemical staining of these cells for ANF (Fig 5 2). In contrast, cells maintained in a mitogen-rich medium containing 15% fetal calf serum were morphogically very different and exhibited significant proliferation of non-myocyte cells In these cells, ANF promoter activity was undetectable (Fig. 5.3 and 5.5), but like in fibroblast cells, expression of the shortest deletion mutant was significant (Fig. 5.5). With respect to this observation, a recent report (Seidman et al 1988) suggested that ANF

promoter activity is non-existent in neonatal ventricular cells while 640 bp of 5' sequences result in virtually background chloramphenicol acetyl transferase (CAT) reporter gene expression in atrial cells. The use of mitogen-rich culture conditions favoring the proliferation of non-myocytes could explain both the absence of ANF promoter activity in neonatal ventricles, that are known to express ANF, and the very low level of CAT activity in atrial cells Furthermore, the use of the CAT reporter gene assay, which is about 10-fold less sensitive than the hGH reporter gene assay (Selden et al., 1986), could also have contributed to the very low promoter activities obtained in the cardiac cells. A report by Wu et al (1989), where the human ANF gene promoter (hANF) was tested in a similar way, also described undetectable CAT activity in ventricular cells However, these investigators also made use of culture conditions that favor fibroblast or non-myocyte proliferation

The ANF gene follows a developmental pattern of expression as we and others (Wu et al. 1998; Wei et al., 1987; Bloch et al 1986) have shown, and it is clear that in the neonatal state, both atrial and ventricular cells express the gene at similar levels In light of this, the absence of ANF promoter activity in ventricular cells, as described by Seidman et al and Wu et al., is unexpected We have tested both 1 and 4 day old atrial as well as ventricular cell cultures, and variations in the levels of promoter dependence were observed in agreement with ANF's temporal pattern of expression. Indeed, the region between -2.5 and -1.6 kb seems to be implicated in the developmental regulation of the gene by mediating a certain degree of repressor function In 4 d old rats, these sequences seem to contribute to a pronounced suppression of distal ANF promoter activity As expected from *in vivo* observations, in atrial cells, similar levels of promoter

activity were observed in the 1 and 4 d old cells

Our data also show that 1.6 kb 5'-flanking sequences are required for expression in cardiac cells. Two elements appear to contribute significantly to this activity, each producing a 2- to 3-fold enhancement, these are situated between -1.6 and -1.0 kb (DE) and between -640 and -136 bp (PE) While it has been shown that the human gene requires a single proximal tissue-specific positive regulatory element, located within the first 400 bp of 5' upstream sequences (Wu et al., 1989; Field et al., 1988), our work suggests that the rat gene requires additional 5'-flanking sequences. In agreement with our results, a previous study concluded that sequences between -2.4 and -0.64 kb contain important regulatory sequences, but the low CAT activities measured in those experiments prevented further conclusions to be made regarding the role of the proximal regions of the promoter (Seidman et al., 1988)

The two positive regulatory regions that we have identified were compared for sequence similarity to the human ANF gene, and we have observed that the single positive proximal element (hPE) identified in the human gene (Lapointe et al., 1988) has a high degree of homology with short sequence elements found within both PE and DE. It can be noted that a relatively well conserved octanucleotide sequence is present in both the PE and DE of the rat gene.

```
   -364
PE      T G G G T G T G G
   -1137 * * * * *  * * *
DE      T G G G T A T G G
```

Extensive sequence analyses have also revealed the presence of other sequences having significant similarity with some of the muscle-specific promoter motifs that have been characterized. A perfect CArg box, CCAAATAAGG, is

located on the non-coding strand at about -410 bp and a second less well conserved element, CCAAAGAAGA, is located at -1.6 kb (Miwa et al., 1987). These coincide with the PE and DE, respectively. Two sequences resembling the cardiac troponin T heptamer, CATTCCT, were found at -2757 and -3058 bp (Mar et al., 1988). Whether these elements contribute to the cardiac-specific expression of ANF will be determined by more direct mutational analyses.

The differential activity observed for the PE fragment in cardiac cells and in non-myocyte cultures and L cells was intriguing. The consistent and significant increase in activity observed in the latter two cell types after deletion of PE led us to propose the presence of a repressor just upstream of -136 bp. This hypothesis was further supported by the internal deletion mutant of PE (Table 5.1). On the other hand, in both atrial and ventricular cells, this same PE fragment conferred positive regulatory activity. The presence of a negative regulatory sequence active only in certain non-ANF expressing cells is interesting, however, a similar phenomenon was found to exist in the embryonic skeletal myosin heavy-chain gene promoter (Bouvagnet et al., 1987) and in the rat growth hormone (rGH) gene (Larsen et al., 1986). This mechanism may be implicated in the extinction of ANF gene expression in non-expressing cells as has been suggested in the case of rGH (Tripputi et al., 1988). It is tempting to speculate that such a repressor might be active exclusively in cells of mesodermal origin such as fibroblasts. The notion that such cells have the potential of conversion to cells of myogenic lineage by the introduction of the skeletal muscle specific MyoD protein (Tapscott et al. 1988), allows one to suppose that the repressor might be involved in the maintenance of these cells' phenotype.

In an attempt to better assess the functional role of the PE and DE

heterologous promoter constructs containing the PE and DE in both orientations in front of pTK81 and pTK109 minimal promoters were prepared The PE was found to confer a positive regulatory effect on both minimal TK promoters, when tested in cardiac cells. The greater magnitude of enhancement observed with this element in the 3' to 5' orientation might be a consequence of favorable positional interactions between the ANF and TK promoter elements. The low but significant activation of transcription obtained in cardiac cells with DE is consistent with an enhancer-like activity residing within this domain as well However, the similar activity detected in L cells suggested that this activity might not be cell-specific. It is possible to envisage a cooperative interaction between the DE and PE elements. Indeed, the requirement for cooperative interactions has already been demonstrated in the case of OTF-2, where both octamer and heptamer elements of the immunoglobulin promoters are required for efficient binding (Poellinger et al., 1989). In hindsight, it might not have been very surprising to find a modest enhancer-like activity in L cells since the PE fragment contains the ubiquitous enhancer octamer motif that can activate transcription via the binding of the OTF-1 transcriptional factor (Fletcher et al., 1987) We have evidence, by gel retardation studies, that the octa-motif is protected in both cardiac and non-cardiac cells, but the protein-DNA complexes observed with different tissue extracts are different (A. Ardati, unpublished observations). No transcriptional activation upon the heterologous promoter was observed with the DE in L cells Thus, these observations are consistent with a cardiac-specific transcriptional activator binding to the PE and possibly DE elements

In conclusion, we have found that the rat ANF gene is transcriptionally regulated by 5' cis-acting elements. The temporal pattern of ANF gene expression

seems to be under the control of upstream distal 5'-flanking sequences while two distinct elements, a proximal and distal one, appear to be necessary for cardiac-restricted and high level expression of the gene in both atrial and ventricular cells. These two elements probably function in a co-operative manner to elicit full transcriptional activity from the ANF promoter in the proper cell type.

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CHAPTER 6

DISCUSSION

6.1 GENERAL DISCUSSION AND CONCLUSION

The present work describes the structure and transcriptional regulation of the rat atrial natriuretic factor gene. The results obtained in this study provide a deeper insight into the multifactorial modes of ANF gene expression. We have observed at least three distinct levels of ANF gene regulation: hormonal, developmental and tissue-restricted. The complex nature of ANF gene control and its interesting pattern of developmental regulation in cardiac tissue provide an excellent model for the study of cardiac muscle-specific gene expression.

6.1.1 The ANF Gene

We have shown that the rat ANF gene consists of three exons separated by two short introns (Chapter 2). Most of the well conserved sequence elements generally found in eukaryotic genes are present in the ANF gene. Typical exon-intron junction sequences are found as well as a perfect TATAAA box at -30 bp, although a consensus CCAAT box element is absent. The presence of several alternating purine-pyrimidine tracts both in the 3' downstream and in the 5'-flanking sequences at about -1.2 kb present an interesting characteristic of this gene. The exact role of such sequences, capable of assuming a Z-DNA configuration, remains unclear at this time although a possible involvement in recombinatorial or deletion events has been postulated (Nordheim an Rich, 1983; Wells et al., 1982, Freund et al., 1989). It is interesting to note that an alternating purine-pyrimidine tract has also been observed within an intron of the brain natriuretic peptide (BNP) (our unpublished results). This cardiac and brain peptide is a new member of the family of natriuretic peptides. The presence of such sequence similarities suggest it is a member of the natriuretic hormone family. Indeed, the presence of an alternating purine-pyrimidine tract has also been

reported in the mouse gene (Seidman et al., 1984) although none seems to be present in the human gene (Argentin et al., 1985). Since flanking 5' or 3' bovine ANF gene sequences are not available, it is difficult to assess the presence of purine-pyrimidine tracts in this species; although the presence of such sequences cannot be ruled out (Vlasuk et al., 1986; Seidman et al., 1984, Nemer et al., 1984).

The maintenance of high sequence homology between similar genes in different mammalian species is not surprising. Protein-coding, but not intron or 3' untranslated sequences, are well conserved as would be expected for the evolutionary pressures exerted on a biologically active peptide (Chapter 2; Vlasuck et al., 1986). For example, over 81% sequence homology is maintained within the second exon, peptide coding sequences between bovine versus human, rat and mouse species (Vlasuk et al., 1986). On the other hand, presence of homologies in the promoter suggests a pressure to conserve regulatory sequences. In the 5'-flanking region, over 70% sequence homology is retained between bovine versus the rat, mouse and human species (Vlasuk et al., 1986). Between the human and rat genes, the homology rises to close to 90% for the 5'-flanking regions (Chapter 2). This may be indicative of the presence of important regulatory elements within this region. Indeed, a stretch of very well conserved nucleotides can be found at the level of the putative CCAAT box (GAAT) (Chapter 2, Nemer et al., 1984, Seidman et al., 1984; Vlasuck et al, 1986). It is noteworthy that DNase I footprinting studies carried out in our laboratory have demonstrated binding of nuclear proteins to this element in the rat ANF gene. Similarly, a well-conserved sequence motif that may have a functional role in ANF gene expression is found in both rat and human genes (PE2) (Chapter 5). Thus, DNA sequence analyses have been useful for predicting putative genetic regulatory elements in the ANF

gene

6.1.2 Thyroid Hormone Regulation of ANF Gene Expression

In order to study the direct effect of various hormones on ANF mRNA levels, an *in vitro* system of primary cardiocyte cultures was established. Such an easily controlled system allows to test the direct action of various agents at the level of the heart In this system, clear induction of ANF mRNA levels by thyroid hormone (T_3) was observed. The physiological significance of this result is supported by the observation that *in vivo*, the hyperthyroid state is characterized by effects that mimic those induced by ANF, such as decreased peripheral vascular resistance and increased renal blood flow (Morkin et al., 1983). Moreover, in whole animal studies, both cardiac ANF mRNA and plasma ANF levels are elevated in the hyperthyroid state (Ladenson et al., 1988; Kohno et al., 1986; Gardner et al., 1987). Thus, the direct effect of T_3 at the cardiac level appears to be translated into higher circulating ANF levels and consequent peripheral actions.

The finding that T_3 directly affects the ANF gene is not surprising since other cardiac genes are regulated, either positively or negatively, by thyroid hormone. The multigene family of α- and β-myosin heavy-chain (MHC) genes serves as an example of cardiac genes that are differentially regulated by thyroid hormone (Izumo et al., 1986). Moreover, a heterogeneity in the responses to thyroid hormone is displayed by the various myosin isoforms when tested in different muscle types (Lompre et al., 1984; Izumo et al., 1986). Thus, the MHC genes are affected by T_3 in a complex and highly tissue-specific manner (Gustafson et al., 1986) The absence of a response to thyroid hormone by the cardiac α-actin demonstrates that the effect of T_3 is not a generalized one (Winegrad et al., 1990). Given the current model for the mechanism of thyroid hormone action in the

nucleus (Thompson and Evans, 1989), it is highly probable that cardiac thyroid hormone receptors (Thompson and Evans, 1989; Koenig et al., 1988, Osty et al., 1988) mediate T_3 induction via specific thyroid hormone response elements (TREs) in the DNA. The presence of both α and β thyroid hormone receptor expression in cardiac tissue has been documented and would support this hypothesis (Thompson and Evans, 1989; Koenig et al.,1988). Since only steady state mRNA levels were measured in the case of ANF (Chapter 3), an effect of T_3 on either transcriptional activation of the gene or mRNA stabilization may be postulated. However, given the early onset of ANF mRNA induction observed (12 h), the direct interaction of the thyroid hormone receptor with an ANF TRE might be considered. Since the TRE consensus sequences that have been characterized remain considerably degenerate (Brent et al., 1989), it is difficult to assess the presence of such an element in the rat ANF gene. DNA-mediated gene transfer and receptor binding studies with ANF promoter sequences might reveal the presence of a functional thyroid hormone responsive motif within this gene. The importance of 5'-flanking sequences in thyroid hormone mediated regulation of the α-myosin heavy-chain gene in fetal heart cells has already been demonstrated (Gustafson et al., 1987), and the TRE has been mapped to specific 5' sequences (Izumo and Mahdavi, 1988).

6.1.3 Glucocorticoid Regulation of ANF Gene Expression

Glucocorticoids mediate various effects on the cardiovascular system. More specifically, ANF peptide secretion in primary cardiocyte cultures and ANF plasma levels in rats are elevated following dexamethasone treatment (Matsubara et al., 1987, Day et al., 1987, Gardner et al., 1986). We have shown that glucocorticoids do indeed modulate ANF gene transcription in myocardial cells (Nemer et al., 1987 abs; Argentin et al., 1987 abs; Chapter 4). This effect has also been observed *in*

vivo (Gardner et al., 1986; Nemer et al., in preparation), where adrenalectomized and intact rats respond to DEX treatment with increased cardiac ANF mRNA levels. The ventricular tissue seems to be more responsive to glucocorticoid stimulation *in vivo* (Day et al., 1987). Although similar levels of induction are observed *in vitro*, a differential sensitivity to glucocorticoids between atrial and ventricular tissue was observed (Chapter 4). This is not uncommon and has equally been observed in response to T_3 (Chapter 3). Similarly, β-MHC gene regulation in response to T_3 is notably more important in ventricular than in atrial tissue (Izumo et al., 1986).

The DEX effect on ANF mRNA accumulation is maximal at a very early time point (6 hours) (Chapter 4), suggesting that glucocorticoids affect ANF gene transcription. DNA-mediated gene transfer studies with various 5' deletion mutants of the ANF promoter confirmed this hypothesis. Binding of the glucocorticoid receptor to a glucocorticoid responsive element (GRE), suggests that transcriptional activation results from the binding of GR to ANF sequences. Although an average 3-fold induction of endogenous ANF mRNA is observed in the *in vitro* system of cardiocyte cultures, only a 2-fold induction in reporter gene expression in response to DEX is observed in the cell transfection system. This observation suggests that glucocorticoids may have an mRNA stabilizing effect in addition to a direct transcriptional activation. Such a stabilizing effect has been independently suggested by others (Gardner et al., 1988). Alternatively, a second GRE consensus sequence, situated in the second intron of both rat and human genes, might contribute to ANF transcriptional activation in the presence of DEX, although this hypothesis remains to be tested.

From our data, a model for hormone-dependent ANF gene activation may be envisaged Binding of the GR to the upstream GR binding site, probably through interactions with other nuclear proteins (Ptashne, 1988), enhances transcription from the ANF promoter The observation that a nuclear protein binds to a site in very close proximity to the GRE lends some support to this notion (our unpublished observations). Indeed, the absence of hormone-dependent gene activation in non-cardiac cell lines supports the idea that other cell-specific nuclear proteins are required for this response. The possibility of a cooperativity between the GR and other nuclear factors has already been demonstrated (Strähle et al., 1988). In fact, the requirement for cooperative interactions might also help explain the differential sensitivity of atrial versus ventricular tissues to glucocorticoids. The greater abundance or availability of an auxiliary factor might help increase transcriptional induction by glucocorticoids in ventricular cells. Interestingly, a similar situation was observed in response to thyroid hormone where ventricular tissue is again subject to an increased hormonal sensitivity.

6.1.4 Cardiac-Specific and Developmental Expression of the Rat ANF Gene

Recently, the cis-acting elements regulating several cardiac-specific genes have been studied (Mar and Ordahl, 1988, Johnson et al., 1989a) However, since most of these genes encoding contractile proteins, such as cardiac α-actin and myosin genes, are also expressed in certain myoblastic cell lines and skeletal muscle they have been essentially studied in non-cardiac muscle cells (Miwa et al., 1987, Gustafson and Kedes, 1989). Consequently, cardiac-specific nuclear factors have not been well studied The ANF gene which is highly specific to cardiac muscle cells, serves as an excellent model for the study of cardiac-specific nuclear factors and cis-acting elements. Indeed, the ANF promoter is inactive in other muscle cells

such as H9C2 embryonic myoblasts or skeletal muscle cells (our unpublished observations).

In the present work, transfection in primary cardiocyte cultures with ANF-hGH hybrid genes has permitted the assessment of putative regulatory regions that might be involved in the cell-type and developmentally-restricted mode of ANF gene expression. The marked nucleotide sequence homology in the 5'-flanking regions of the human and rodent genes strongly supports the notion that certain well-conserved DNA elements might have such a regulatory function (Chapter 2). Our studies confirm this hypothesis as maximal ANF gene transcription requires at least 1.6 kb of 5'-flanking sequences (Chapter 5). The cis-acting elements that contribute to cardiac-cell restricted expression appear to be localized within these 1.6 kb and can further be dissected into proximal and distal regulatory elements (Chapter 5).

It is interesting to note that for the human ANF gene, only the first 400 bp are sufficient for the proper atrial-specific expression of the gene in transfection and transgenic animal studies (Wu et al., 1989; Field, 1988) while ventricular expression was undetectable in these reports. It remains unclear whether the absence of ventricular reporter gene expression was due to a lack of sensitivity of the assay or insufficient promoter activity for ventricular expression (Wu et al., 1989; Field, 1988). In the transgenic animal model, however, ventricular ANF expression was observed only early on in development (Field, 1988). Perhaps the presence of a distal promoter element, as observed in the rat gene, may be required for full and hence detectable transcriptional activity in the ventricles. The repetition of a well conserved sequence motif that binds cardiac specific nuclear proteins in both the proximal and distal elements of the rat gene support this

notion. Such a requirement for cooperativity would not be surprising given the synergism required between several cis-acting elements for full transcriptional activity in the case of several other promoters including the prolactin gene (Crenshaw et al., 1989).

The presence of silencer elements, active in both atrial and ventricular cells, and at both ages tested, at varying degrees, was surprising At present, we have not studied nuclear protein binding to these sequences nor have we tested the activity of these sequences in a heterologous promoter system Nonetheless, repressor activity associated with these sequences (-2.5 to -1.6 kb) is significant. No such effect has been reported for the human ANF gene although this question was not well investigated (Lapointe et et al., 1988). On the other hand, a small but possibly significant repressor activity was observable with the 2.5 kb ANF 5' deletion mutant of the rat ANF promoter when tested in atrial cells (Seidman et al., 1988). While an *in vivo* function for these elements has not yet been demonstrated, such negative cis-acting elements have been described in other muscle-specific genes such as the myosin heavy chain and troponin genes (Bouvagnet et al., 1987; Mar et al., 1988a). The suppression of ventricular ANF gene expression with age might be attributed to these ANF 5'-flanking elements Indeed, in 4 d old rats, sequences between -2.5 and -1.6 kb appear to mediate a suppression on the distal and proximal activating elements, rendering them about 3-fold less active in the older rats than in the 1 d old animals The involvement of these sequences in developmental regulation *in vivo* can probably best be tested in a transgenic animal model.

Finally, we have observed an unusual action of the proximal fragment in certain non-cardiac cells such as cardiac non-myocytes and L cells The 5' deletion

of the proximal fragment results in a marked activation of ANF promoter activity in non-myocytes and L cells (Chapter 5). Similarly, the internal deletion of proximal fragment gives rise to the same effect. Unfortunately, it could not be reproduced in the heterologous promoter system, suggesting it is a promoter-specific phenomenon Possibly, the proper position and context of specific trans-acting factors is necessary to observe this function. Alternatively, the homologous promoter or a different heterelogous promoter possessing different basal activity might be necessary to observe this effect. Indeed, the protection of the proximal element motif (PE2) in non-cardiac cells, such as L cells, by a factor different from that seen in the heart has been observed. Although the exact molecular mechanisms of this effect are unknown, it is possible that a distinct or modified transcriptional factor may be responsible for this differential effect in certain non-cardiac cells. In contrast, no such activity was observed in other differentiated cells such as GH3 or PC12 cells

In light of the data herein presented along with more recent *in vitro* DNA binding studies (A. Ardati, unpublished observations), it is possible to propose a tentative model for the mechanism of ANF gene regulation that is summarized in Figure 6.1. We can see, from this cartoon, that the ANF gene appears to be regulated by a complex interplay of positive and negative regulatory elements: a tissue-specific proximal fragment, an activating distal fragment, a 5'-flanking repressor fragment (possibly involved in development) and a glucocorticoid responsive element.

Clearly, there remain many unanswered questions regarding the regulation of this unique heart-specific gene, that deserve further investigation. While we now have some clues as to the DNA and protein elements involved in ANF gene

control (Fig. 6.1), much more work needs to be done to fully understand the precise mechanisms and interactions that are involved.

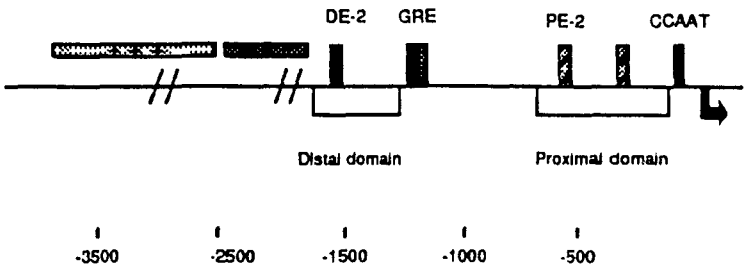

Figure 6.1. Model for transcriptional regulation of the ANF gene. PE-2 and DE-2 refer to binding sites for cardiac-specific nuclear proteins and GRE refers to putative glucocorticoid response element + and - refer to activating and repressing domains, respectively.

6.1.5 Future Prospects for Studies on the ANF Gene

The discovery of the ANF gene, encoding one of very few non-contractile cardiac proteins, has led to a rapid unravelling of its biochemical, pharmacological and molecular genetic properties. In dealing with the latter, we have been able to demonstrate that ANF is a complex gene subject to multiple levels of regulation. The temporal pattern of ANF gene expression, its sensitivity to both hormonal and physiological factors and its restriction to cardiac cells make for an excellent model of study. For example, the availability of cardiac cell cultures that represent distinct stages of differentiation allow for the use of the ANF gene as a useful tool in studies of cardiac cell growth. Likewise, events leading to cardiac hypertrophy, as mediated by growth factors or other agents, may equally be examined using the ANF gene marker. The molecular events triggering these

particular cellular states can thus be examined more closely.

On a narrower scope, as a direct consequence of the present work, future work may be directed towards finer studies on the hormone and cardiac-specific regulatory regions that have been identified. We already have evidence for the binding of tissue-specific nuclear proteins to sequence elements within the DE and PE. The use of fine deletion or linker scanning mutants of the nuclear protein binding sites (PE2 and DE2) might help reinforce a functional role for these elements. Loss of nuclear factor binding on mutated PE2 and DE2 motifs, accompanied by loss of function would help confirm the current hypothesis that cardiac specific nuclear proteins bind to these elements to direct cardiac-specific gene expression. Since no cardiac-specific nuclear transcription factor has yet been identified, a great contribution to the understanding of muscle-specific gene expression would be the isolation and characterization of such a factor.

Furthermore, a great deal of work could be directed towards the understanding of the repressor sequences between -2.5 and -1.6 kb, that seem to modulate the temporal pattern of ANF gene expression. More refined deletion mutants along with DNase I footprinting and gel retardation studies on these sequences are required to precisely pinpoint the elements involved. It would ultimately be interesting to follow ANF gene expression in transgenic mice where these sequences have been altered. Defective developmental control of ANF gene expression as a result of such manipulations would confirm their role in development.

Finally, to date, it remains unclear as to whether thyroid hormone action is mediated via thyroid hormone receptor binding to a thyroid hormone response element (TRE) in the ANF gene. Although the early onset of T_3 action on ANF

mRNA induction (Chapter 3) suggests to a direct transcriptional activation. DNA-mediated transfection studies in cardiocytes, similar to those carried out in Chapter 4, would help identify a TRE.

In conclusion, the ANF gene serves as an excellent model for the study of various molecular aspects of cardiac tissue. Mechanisms governing cardiac cell growth, differentiation, hormone responsiveness and cell-specific gene activation are all questions that deserve further investigation and that may consequently be answered using the ANF gene as a tool

CLAIMS TO ORIGINALITY

The present work describes for the first time:

A) The genomic sequence of the rat atrial natriuretic factor gene

B) The establishment of an *in vitro* model consisting of primary atrial and ventricular cardiocyte cultures that express the ANF gene

C) The thyroid hormone-mediated induction of ANF mRNA levels in the *in vitro* cardiocyte culture system

D) The glucocorticoid-mediated induction of ANF mRNA through a direct effect at the cardiocyte level

E) The requirement of a distal glucocorticoid response element in the ANF gene 5'-flanking sequences for a transcriptional response to glucocorticoids

F) The requirement of ANF cis-acting sequences for cardiac-specific expression of the gene

G) The localization of two specific cis-acting domains in the 5'-flanking region contributing to high level cardiac-specific expression of the ANF gene

H) The presence of a regulatory domain in the 5'-flanking region of the ANF gene contributing to the differential pattern of ANF promoter activity at different stages of development.

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