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Regulating the Activity of Steroid Hormones From Start to Finish: Transcriptional Regulation of Human P450scc and End-Organ Modulation of Cortisol by 11B-Hydroxysteroid Dehydrogenase

by

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DISSERTATION

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This thesis is dedicated to my wife, Jackie, who indulged my pursuit of a PhD and at the same time put it all in perspective.

Preface

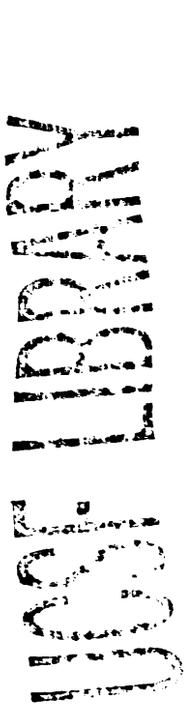
The body of this thesis is based on four manuscripts that are published:

Chapter 1: Moore, C.C.D, S.T. Brenatano and W.L. Miller. 1990. Human P450scc gene transcription is induced by cyclic AMP and repressed by 12-*O*-tetradeconylphorbol-13-acetate and A23187 through independent *cis* elements. Mol. Cell. Biol. 10:6013-6023

Chapter 2: Moore, C.C.D. and W.L. Miller. 1992. Regulating the regulators: The role of transcriptional regulation in steroid hormone biosynthesis. J. Steroid Biochem. Mol. Biol. 40:517-525

Chapter 3: Moore, C.C.D., D.W. Hum and W.L. Miller. 1992. Characterization of the human P450scc gene promoter in placental JEG-3 cells: Evidence for a cAMP and negative regulatory element and tissue-specific regulation. Mol. Endocrinol. 6: 2045-2058

Chapter 5: Moore, C.C.D., S.H. Mellon, J. Murai, P.K. Siiteri and W.L. Miller. 1993. The hepatic form of squirrel monkey 11β-hydroxysteroid dehydrogenase does not protect the renal mineralocorticoid receptor. Endocrinology 133: 368-375



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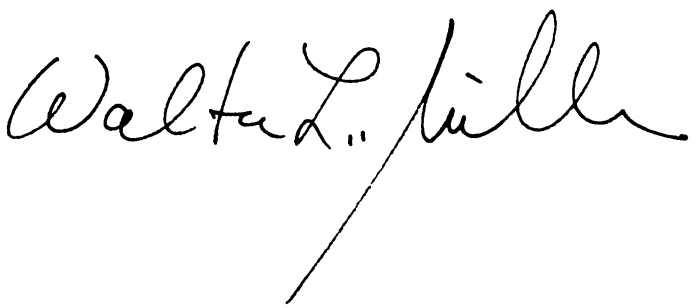
Abstract

Regulating the Activity of Steroid Hormones From Start to Finish: Transcriptional Regulation of Human P450scc and End-Organ Modulation of Cortisol by 11β-Hydroxysteroid Dehydrogenase

Chris C.D. Moore

Steroid hormones are critical signals governing homeostasis, sexual differentiation and reproduction. Understanding how circulating concentrations of these hormones are regulated is important for understanding their physiology and disorders of their production causing human diseases. Long-term regulation of steroidogenesis occurs principally by transcriptional regulation of the gene for the first, and rate-limiting step catalyzed by the cholesterol side-chain cleavage enzyme P450scc. The principal hormones that control P450scc expression are specific to each steroidogenic tissue, but work via one of two second messenger pathways; either by cAMP or by Ca^{++} and protein kinase C (PKC). In murine adrenal Y1 cells transfected with the human P450scc promoter fused to the bacterial gene for chloramphenicol acetyl transferase (CAT), the cAMP agonist forskolin increased CAT activity by 900% while mobilizing Ca^{++} combined with activation of PKC reduced CAT activity to 15% of control. These two pathways work independently and use different *cis*-acting DNA elements: cAMP induction by sequences between -605 and -2327, Ca^{++}/PKC repression by multiple elements between -89 and -343. In human placental JEG-3 choriocarcinoma cells basal and hormonally regulated expression of both CAT and firefly luciferase reporters was mediated by different P450scc *cis*-elements from those we found in adrenal cells. Basal expression in JEG-3 cells involves the complex interplay of activating and repressing sequences. Induction by cAMP localized to sequences between -89 to -108, and was direct as cycloheximide did not block induction. Gel-mobility shift assays and DNAse I footprinting experiments identified five protein:DNA complexes that correlate with the functional reporter assays.

Steroid hormone concentrations are not only regulated at synthesis but also at the end-organ. One example is the conversion (and inactivation) of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase (11β-HSD). This prevents cortisol, a potent mineralocorticoid, from overwhelming signalling by the physiological mineralocorticoid, aldosterone. Squirrel monkeys have markedly elevated serum cortisol without ill-effects. We tested the hypothesis that increased abundance and/or activity of 11β-HSD protected this primate from their relative hypercortisolism. Using an oligonucleotide probe we cloned the gene and cDNA for the squirrel monkey hepatic 11β-HSD. Tissue abundance of the mRNA was similar or less than that seen in the rat or in humans. Enzyme kinetic analysis of the squirrel monkey enzyme expressed in Chinese hamster ovary cells showed only a slight increase in efficiency compared to the human enzyme. Taken together these data do not support a role for this enzyme in protecting the squirrel monkey from elevated cortisol levels or in providing specificity for aldosterone action in the kidney.



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Introduction

Steroid hormones are necessary for maintaining physiological homeostasis, sex differentiation and for reproduction. They are broadly classified by their actions; glucocorticoids such as cortisol that modulate glucose metabolism and the immune system; mineralocorticoids such as aldosterone that regulate salt and water balance; and the sex steroids testosterone, estradiol and progesterone that are crucial for proper sexual differentiation and reproductive function. Their importance is apparent from diseases in steroid hormone metabolism such as congenital adrenal hyperplasia (CAH) (reviewed in Miller and Levine, 1987). CAH displays three types of disruptions seen in diseases of steriod hormone metabolism: the lack of a particular steroid, the overabundance of a steroid, and the synthesis of a steroid at an innapropriate time. Patients with this disease usually have defects in 21-hydroxylation leading to deficiencies in cortisol and aldosterone resulting in poor stress tolerance and electrolyte abnormalities respectively. Precursors to the 21-hydroxylation step build-up and spill-over into alternate, normally relatively quiescent pathways and produce an overabundance of adrenal androgens. If this occurs during gestation in female embryos the resulting innappropriate levels of androgens causes masculinization of the fetus. Another disruption in steroid hormone physiology is seen in diseases caused by receptor defects as seen in testicular feminization syndrome (Tfm) where lesions in the androgen receptor causes androgen resistance and genetic males (XY) have a female phenotype. To understand the physiology of these hormones, and their role in such diseases, we must know how they are synthesized, how they elicit their effects in the end-organ, and how their effective circulating concentrations are regulated.

With the application of molecular biology our understanding of steroid hormone biosynthesis progressed tremendously over the last decade (Reviewed in Miller, 1988). Most of the steroidogenic enzymes have been cloned and belong to one of two families, the predominant one being the cytochrome P450's. This group includes the side chain cleavage enzyme (P450scc), 17-hydroxylase,17-20 lyase (P450c17), 21-hydroxylase (P450c21),

aromatase (P450aro), and the two forms of 11b-hydroxylase (P450c11). The other family of enzymes, the dehydrogenases, includes 11b-hydroxysteroid dehydrogenase (11b-HSD), 3b-hydroxysteroid dehydrogenase (3b-HSD), and 17b-hydroxysteroid dehydrogenase (17b-HSD). These enzymes are related to each other and to a very diverse set of evolutionarily conserved dehydrogenase enzymes (Agarwal, et al., 1989).

This past decade also saw enormous advances in our understanding of how steroids regulate the functions of their target tissues. All the steroid hormone receptors isolated to date belong to a family of proteins that contain a highly conserved zinc finger DNA binding domain, and function as *trans*-acting transcription factors (Reviewed in Beato, 1989). This family also includes the receptors for the non-steroid hormones retinoic acid, thyroid hormone, and vitamin D. These intracellular receptors have a tripartite functional structure, including a steroid binding domain, a zinc finger DNA binding domain, and a transcriptional *trans*-activating domain. The binding of the appropriate steroid hormone activates the receptor, allowing for high affinity, specific binding to specific DNA sequences via the zinc-finger domain. The binding of the activated receptor permits the *trans*-activating domain to increase the rate of transcription of the associated promoter by mechanisms that are poorly understood.

The third aspect of steroid hormone biology, that of regulating the effective circulating concentrations of steroids, is the topic of my thesis. Such regulation occurs at several levels, from controlling the rate at which steroids are synthesized, modulating the amount of hormone that is free by altering the concentration of steroid binding proteins, modifying the steroid before it reaches the target cell or, finally, changing the rate which a steroid is cleared. While all these mechanisms have a role in controlling steroid hormone levels the most significant step is that of their synthesis. This is because steroids are not stored within cells for secretion upon stimulation by the appropriate secretagogue but rather are secreted as they are made, and the various hormones that control steroidogenesis do so mostly by altering the rate of steroid hormone synthesis. This is the topic of Part I of my

thesis which describes experiments dissecting the molecular mechanisms by which basal and hormonally regulated transcription of the P450scc gene, a key component of the steroid hormone synthesis pathway, is controlled in two steroidogenic cell lines.

As mentioned above the biological activity of steroids can be modulated by modifying them before they reach their target cells. A steroid the target tissue does not respond to can be converted to one that it does. For example in the genital tubercle (the embryological precursor for the penis and clitoris) the enzyme 5a-reductase converts testosterone, a weak androgen in this tissue, into a more active androgen, dihydrotestosterone. In male embryos this causes the tubercle to form a penis. Alternatively, deactivating a steroid hormone can block or modulate the effect of that steroid on the target tissue. A controversial example of this is the conversion, by the enzyme 11b-HSD, of cortisol which binds to both the glucocorticoid and mineralocorticoid receptor to its keto derivative, cortisone, which binds to neither receptor. This is important in tissues that must respond to aldosterone, as the pharmacological and pathological data suggests such tissues are overwhelmed by normal circulating cortisol concentrations and cannot be regulated by aldosterone when such inactivation does not occur (Stewart, et. al., 1988). To explore the physiological role of 11b-HSD in deactivating cortisol we cloned and expressed the hepatic form of 11b-HSD from the squirrel monkey (*Saimari scireus*), an experiment of nature that partially resists its very high circulating cortisol concentrations by increased 11b-HSD activity. The results from these experiments are presented in Part II of this thesis.

PART I

Transcriptional Regulation of the Human P450scc Gene in Adrenal and Placental Cells

Steroids, unlike protein and peptide hormones, are not stored and then released when the cell is stimulated by the appropriate signal, but instead are secreted as they are synthesized. Thus, signals that modulate steroid hormone levels do so by regulating the rate of steroid hormone synthesis. Understanding this process is crucial for understanding how circulating levels of steroids are established and their subsequent regulation of end-organ function.

In this respect P450scc is especially important as it is the first, and rate-limiting step in the synthesis of all steroid hormones (Stone and Hechter, 1955). All steroids originate from cholesterol which is first converted to pregnenolone by P450scc in three separate, sequential reactions. These are hydroxylation of carbon 20 then carbon 22, and finally the physiologically irreversible scission of the C20 to C22 bond yielding both pregnenolone and isocaproic acid from cholesterol. Each step requires a pair of electrons donated from NADPH, via the mitochondrial flavoprotein protein adrenodoxin reductase (AR) and the iron-sulfer protein adrenodoxin (Adx). These proteins also donate electrons to the other mitochondrial steroidogenic enzyme P450c11. As AR and Adx are not tightly associated with P450scc their transport of electrons is slowed by diffusion (Harikrishna, et al., 1993) and may, along with the need for three seperate reactions, result in the very slow V_{max} of about 1 mole of pregnenelone from cholesterol per second per mole of P450scc (Duque, et al., 1980). As P450scc is the first and committed step in steroidogensis, as well as rate limiting, understanding its regulation is key to understanding regulation of circulating steroid hormone levels.

The regulation of steroid hormone synthesis occurs in two phases, acute and chronic. The acute response occurs within seconds to minutes after stimulation of a

steroidogenic tissue by the appropriate hormone and cannot be blocked by inhibiting protein synthesis. This response results from the activation of pre-existing components of the synthetic pathway causing mobilization of cholesterol to the inner mitochondrial membrane, generation of pregnenolone by P450scc, and then subsequent modifications by the other steroidogenic enzymes leading to the final product(s). By contrast the chronic activation of steroidogenesis requires transcription and translation, and increases the abundance of the components necessary for steroid hormone biosynthesis. This process can occur within minutes to hours, or proceed over days or weeks in the face of prolonged stimulation by a trophic hormone such as ACTH on the adrenal cortex (Simpson, et al., 1988).

There are several mechanisms by which catalysis of cholesterol to pregnenolone by P450scc could be regulated for either the acute phase, the chronic phase, or in some cases both. These would include: 1) altering the concentration of the substrate, cholesterol, that is available to P450scc; 2) allosteric interactions or covalent modification of the P450scc enzyme itself; 3) modifying the activity of P450scc by changing the delivery of electrons via AR and Adx; or 4) increasing the amount of P450scc enzyme that is available for the reaction. Of these mechanisms, the first two could alter steroid hormone synthesis acutely as they require little time to act, however the last two mechanisms would require more time, on the order of hours, to be effective.

The delivery of cholesterol to the inner mitochondrial membrane for catalysis by P450scc is a complex process involving a growing number of proteins, and is also an important site for regulation. The proteins involved include cholesterol esterase, which converts the cholesterol esters in cytoplasmic lipid droplets to cholesterol, sterol carrier peptide 2 (SCP_2), which facillitates cholesterol transport to the mitochondria (Billheimer, et al., 1990; Yamamoto, et al., 1991), steroidogenic activator peptide (SAP), which stimulates the side chain cleavage reaction (Pederson and Brownie, 1987), and endozepine and its receptor the peripheral benzodiazepine receptor, that may be involved in movement

of cholesterol across the mitochondrial membrane (Besman, et al., 1989; Yanagibashi, et al., 1988; and reviewed in Papadopoulos, 1993). The mechanisms used by these proteins and much of their regulation is poorly understood at present.

Allosteric interactions and covalent modification are common mechanisms for regulating enzymatic activity (especially in metabolic pathways) however, there is no evidence to date for such regulation of P450scc.

If delivery of electrons limits P450scc activity then increasing the rate of electron transfer by AR and Adx could regulate the rate of steroidogenesis. Fusing the protein sequences of P450scc AR and Adx together resulted in about a five-fold increase in P450scc activity compared with coexpression of equimolar amounts of each protein expressed individually, suggesting electron transport is important (Harikrishna, et al., 1993). Teatment with activators of the cAMP pathway increased the abundance of Adx coordinately with P450scc in both granulosa and adrenal cells (Voutlainen, et al., 1988) as well as in placental cells (Picado-Leonard, et al., 1988). However, production of Adx protein was unchanged after 24 hours and Adx mRNA declined to less than 50% of control in placental JEG-3 cells treated with 8-Br-cAMP (Brentano, et al., 1992). Thus, it is unclear how significantly electron delivery to P450scc limits its enzymatic activity - especially during chronic stimulation by hormones linked to cAMP production.

The final mechanism listed above for increasing P450scc activity is an increase in it abundance. All the hormones that regulate steroidogenesis via cAMP increase the amount of P450scc protein and mRNA in their respective target tissues (Chung, et al., 1986; DiBlasio, et al., 1987; Golos, et al., 1987; Picado-Leonard, et al., 1988; Voutilainen and Miller, 1987; Voutilainen, et al., 1986). Prolonged treatment with AII, however reduces P450scc abundance despite its potent acute effect of stimulating aldosterone synthesis and secretion (Cozza, et al., 1990; Enyedi, et al., 1985; Mason, et al., 1986; McAllister and Hornsby, 1988).

There are several mechanisms by which P450scc abundance could be altered and thus long-term steroid hormone biosynthesis regulated. These mechanisms include changes in gene transcription, altered stability of the mRNA, changes in translation of the mRNA, and changes in the stability of the protein. Although examples for regulation by the last three mechanisms exist for other proteins (reviewed in Jackson, 1993) the most common mechanism by which protein abundance is altered is by changes in gene transcription.

Eukaryotic gene transcription is carried out by one of three RNA polymerase molecules, RNA Pol I, II or III. The promoters for genes that encode proteins, such as P450scc are transcribed by RNA Pol II, as are the promoters for certain small nuclear RNA's. The promoters for RNA Pol II, as well as those for I and III, contain two fundamental types of elements; basal elements and modulating elements. The basal elements dictate RNA polymerase specificity, selecting from Pol I, II and III, and directing low levels of basal transcription. In the case of RNA Pol II these include the TATA box, and sometimes an initiation sequence (Inr) that spans the start site of gene transcription. The modulating elements are usually short inverted repeat sequences located upstream of the transcription initiation site; they increase or decrease the transcription determined by the basal elements. It is the combination of these two types of elements, and the factors that bind them, that determines the level of transcription by a particular gene, and the tissue in which that transcription occurs.

None of the RNA polymerase molecules bind to their cognate promoters directly, but rather the basal elements are recognized by specific transcription factors that in turn recruit the appropriate RNA polymerase (reviewed in Hernandez, N., 1993). In the case of RNA Pol II promoters these factors can be purified chromatographically and were initially called transcription factor fraction II D (TFIID). A key component of this fraction is the protein that initially binds the TATA sequence, TATA binding protein (TBP), which is apparently a universal component found in the initiation complexes of RNA Pol I and III as well. While TBP is sufficient to direct basal RNA Pol II transcription, other proteins in the

TFIID fraction, called co-activators or trancription activating factors (TAF's), are required to dictate RNA Pol II specificity, and to modulate transcription by any upstream modifying elements that control tissue-specificity as well as developmental and hormonal regulation.

The modifying elements are a rapidly expanding set of *cis*-acting DNA elements that are recognized by their respective *trans*-acting DNA binding proteins. These proteins perform a variety of functions essential in defining cellular phenotype and function including tissue-specificity of gene expression, developmental regulation and hormonal regulation. An example of the latter is the steroid hormone family of receptors mentioned earlier that , in response to ligand, bind specifically to their DNA element and modulate transcription. Very little is known about the molecular mechanism(s) by which these modulating elements and their respective transcription factors modulate transcription by RNA Pol II via the TAF's (reviewed in Hernandez, 1993), but potential mechanisms include: 1) facilitating or stabilizing the binding of TBF to the TATA element; 2) facilitate assembly of the transcription initiation complex; and 3) direct or indirect interaction with TBP. Regardless of the mechanism used, defining the element(s) and their respective *trans*-acting DNA binding protein(s) provides a great deal of information about how individual genes are regulated, particularly in linking hormonal signals from outside the cell to alterations in the cell's function.

That changes in the rate of RNA Pol II transcription is the predominant mechanism used to alter the abundance of most steroidogenic enzymes, including P450scc, was first demonstrated by nuclear run-off assays of primary bovine adrenal cells stimulated by ACTH (John, et al., 1986). This assay directly measures the rate of gene transcription by determining the relative number of RNA polymerase II molecules bound to the promoter when the nucleii are harvested. These results were confirmed later by the use of transfected reporter plasmids using a variety of steroidogenic enzyme gene promoters in several different steroidogenic cells (reviewed in Moore and Miller, 1992). Thus, transcriptional

regulation, especially that for the rate limiting enzyme P450scc, is important for understanding the long-term regulation of circulating steroid hormone concentrations.

The hormones that regulate steroidogenesis are different in each steroidogenic tissue. In the adrenal the predominant hormones are ACTH, which regulates glucocorticoid synthesis, and angiotensin II (AII), which controls the production of aldosterone. These two hormones work by distinctly different mechanisms. The ACTH receptor belongs to a family of hormone receptors that are linked to G-proteins and activate adenylate cyclase to increase intracellular cAMP and activate protein kinase A (PKA) (reviewed in Bockaert, 1991). By contrast the AII receptor is linked to the production of inositol triphosphate (IP_3) and diacyl glycerol resulting in increased flux of Ca^{++} and activation of protein kinase C (PKC) (reviewed in Barrett, et al., 1989). Regulation of steroidogenesis in the other steroidogenic tissues is predominantly by the gonadotropins, follicle stimulating hormone (FSH) and leutenizing hormone (LH) in the gonads, and human chorionic gonadotropin in the placenta. As with ACTH, these hormones all work through receptors that are linked to cAMP production via a G-protein and adenylate cyclase (reviewed in Bockaert, 1991).

While much was known about the early steps in the cAMP second messenger cascade, the connection with alterations in gene transcription was virtually unknown until the recent cloning of the cAMP response element binding protein (CREB) (Hoeffler et al., 1988). CREB is the prototype member of a family (bZIP) of *trans*-acting DNA binding proteins that contain a basic domain, a region with a high concentration of positively amino acids, and a leucine zipper, a domain with leucines at every seventh position (the heptad repeat) that mediates dimerization (reviewed in Meyer and Habener, 1993). These proteins all bind as either homo or heterodimers to variations of the consensus CRE modifying element, an 8 base inverted repeat, 5'-TGACGTCA-3' found in the promoters of many genes whose transcription increases in response to increases in intracellular cAMP, including that for the murine steroidogenic enzyme P45011b-hydroxylase (Rice, et al., 1989). The mechanism(s) by which CREB modulates gene transcription in response to

changes in intracellular cAMP is unclear but apparently involves phosphorylation of CREB itself by PKA. This phosphorylation is necessary, but insufficient, for transactivation by CREB in some circumstances, but may trigger a cascade of phosphorylation events by other kinases with resulting transactivation. Turning the system off may involve dephosphorylation by phosphatases, possibly by protein phosphatase-1.

Given that P450scc mRNA and protein abundance are altered by hormones that increase intracellular cAMP (ACTH, FSH, LH and hCG), CREB (or another member of the bZIP family) is a likely candidate for regulating P450scc transcription as it does for murine P450c11. Alternatively cAMP regulation of P450scc transcription may be mediated by a non-CREB protein through sequences with no homology to the consensus CRE as is the case for both bovine P450c11 and P450c17, (Lund, et al., 1990; Honda, et al., 1990) as well as for the human P450c21 promoter (Kagawa and Waterman, 1990).

The cAMP pathway is also implicated in controlling basal, unstimulated levels of steroidogenic enzymes in adrenal cells. Unstimulated levels of murine P450c21, P450c11 and P450scc are all compromised in adrenal Y1 cells that have mutations in their PKA; the degree of compromise correlating with the severity of the PKA mutation (Handler et al., 1988; Mouw, et al., 1989; Wong, et al., 1989). Of the three genes studied, P450scc was the least affected (Wong, et al., 1989). In addition, point mutations of the DNA sequence implicated in cAMP responsiveness in the murine P450c11 promoter reduced both stimulated and unstimulated levels of transcription, (Mouw, et al., 1989). These results imply that basal activity is regulated by the basal level of cAMP in adrenal cells and this basal activity works via the same *cis*-acting DNA element that confers hormonal regulation

Production of aldosterone by the adrenal zona glomerulosa is hormonally regulated principally by angiotensin II, the end-product of an enzymatic cascade that begins with the release of renin by cells in the kidney juxtaglomerular apparatus. Renin then converts plasma angiotensinogen, produced by the liver, into angiotensin I which is then converted by angiotensin converting enzyme into the final active hormone AII. AII acts on adrenal

glomerulosa cells through the angiotensin type-1 receptor which is coupled to inositol phosphate metabolism, presumably via a G-protein (Sasaki et al., 1991). The acute response by adrenal glomerulosa cells to AII is a rapid, marked increase in aldosterone production, however many of these studies were limited to less than 120 minutes (Kojima, et al., 1984). Recent work studying the chronic effects of AII treatment found a profound decrease in aldosterone production after 24 to 48 hours, as well as a decrease in P450scc mRNA and protein (Mason, et al., 1986; McAllister and Hornsby, 1988). Such a biphasic response was proposed to function in modulating both the amounts and types of steroids produced by the adrenal gland when chronically stimulated by AII (McAllister and Hornsby, 1988).

The decrease in P450scc mRNA and protein after chronic AII treatment could reflect a direct change in transcription, or one of the other, rarer mechanisms discussed earlier, or due to desensitization of the protein kinase C pathway. This latter phenomenon is seen with prolonged activation of the PKC pathway, especially with the phorbol ester agonist TPA (Hoeffler, et al., 1989). If basal transcription of the P450scc gene is maintained by the basal level of PKC activity (as seen in the cAMP pathway discussed above) then such desensitization could decrease basal P450scc transcription.

While much is known about the control of steroidogenesis by AII it is unknown if AII alters gene transcription as nuclear run-off assays analogous to those done for cAMP were not done. Assuming that AII does alter transcription of P450scc, how might this regulation be mediated? While not as well defined as the pathway for cAMP regulation, the PKC pathway probably works in an analogous fashion. Binding of AII to the AII type-1 receptor increases intracellular Ca^{++} and diacylglycerol and activates PKC, which results in the phosphorylation, either directly or indirectly, of DNA binding proteins that in turn alter gene transcription. A number of the DNA binding proteins that mediate action of the PKC pathway, and their respective binding sites, are known. These include the c-fos/c-jun heterodimer (Activating Protein-1, AP1), AP2, AP3 and NFkB. As some of the *cis*-acting

DNA elements these factors bind to were initially defined by their conferring transcriptional activation in response to TPA they were called TPA response elements or TRE's (reviewed in Karin, 1989). TheseTRE's had DNA consensus sequences of either TGA$^{C}/_{G}$TCA, which binds AP1, AP2 or AP3, or CCCCAGGC which binds AP2. Other *cis*-acting DNA elements include the serum response element (SRE), CLE2 and GC, the latter two sites mediating regulation of the granulocyte/macrophage colony stimulating factor promoter by both TPA and the calcium ionophore A23187 (Miyatake, et al., 1988) - the combination most effective in regulating adrenal aldosterone production.

While many components of the two intracellular signalling pathways, cAMP and Ca^{++}/IP3, are unique, the pathways do not function independently. There is accumulating evidence for "cross-talk" all they way down to the level of the transcription factors. Adenylate cyclase is inhibited by treatment with AII in rat adrenal cells (Woodcock and Johnston, 1984) apparently via the G-protein, G_i (Woodcock and Johnston, 1986) as *Bordetella pertussis* toxin blocks inhibition. The transcription factor CREB contains a consensus PKC phosphorylation site, however its role in regulating CREB activity is controversial (Meyer and Habener, 1993). At the level of the cis-acting DNA elements, activation of either pathway can converge on either CRE's, TRE's or both (Hoeffler, et al., 1989; Imagawa et al., 1987; and reviewed by Karin, 1989). The interactions at all these levels makes it important to distinguish direct, independent regulation of P450scc by AII or ACTH versus indirect, modulation of the other signalling pathway.

Regulating transcription is also important for determining the tissue-specific expression of P450scc and other steroidogenic enzymes. In contrast to many other promoters whose tissue-specificity limits them to a single tissue, P450scc is expressed in more than one tissue; the adrenal cortex, gonads, placenta and possibly regions of the brain, as well as several other tissues not usually considered steroidogenic. These tissues arise from different embryological origins, thus understanding the tissue-specific

expression of P450scc should provide insights into mechanisms for establishing appropriate tissue specific gene expression.

For studying the transcriptional regulation of steroidogenic enzyme genes we are fortunate in having cell lines that make steroids and are hormonally responsive in a manner consistent with their tissues of origin. These are the very well characterized mouse adrenal Y-1 cell line, the human placental JEG-3 cell line, and the mouse Leydig cell line MA-10. All three cell lines express their endogenous P450scc gene, and its abundance is altered by agonists of the cAMP pathway and agonists of the PKC pathway.

To study the transcriptional regulation of the human P450scc gene we used the standard approach of fusing various portions of its 5' flanking DNA to an easily assayed reporter construction, either chloramphenical acetyl transferase (CAT) or firefly luciferase. By transfecting these plasmids into the above cell lines we hoped to address the following general questions. What are the *cis*-acting DNA elements and their cognate *trans*-acting DNA binding proteins that mediate basal transcription, tissue-specific transcription, and hormonal regulation of the human P450scc promoter? Are the *cis*-elements and *trans*-acting proteins the same, or different, in the various steroidogenic tissues? And finally, do the different steroidogenic enzyme gene promoters use factors in common, or is each promoter regulated independently by different factors.

Chapter 1

Human P450scc Gene Transcription is Induced by Cyclic AMP and Repressed by 12-*O*-Tetradecanoylphorbol-13-Acetate and A23187 Through Independent *cis*-Elements

Chris C.D. Moore, Steven T. Brentano, and Walter L. Miller

Abstract

The long-term regulation of mammalian steroid hormone synthesis occurs principally by transcriptional regulation of the gene for the rate limiting cholesterol side-chain cleavage enzyme, P450scc. Adrenal steroidogenesis is regulated by two tropic hormones: corticotropin (ACTH), which works via cAMP and protein kinase A, and angiotensin II (AII) which works via Ca^{++} and protein kinase C. Forskolin and 8-Br-cAMP stimulate while prolonged treatment with a phorbol ester (TPA) and calcium ionophore (A23187) additively suppress accumulation of endogenous P450scc mRNA in transformed murine adrenal Y1 cells. In Y1 cells transfected with 2500 bp of the human P450scc promoter fused to the bacterial CAT gene, forskolin increases CAT activity 900% while combined TPA plus A23187 reduces CAT activity to 15% of control. Forskolin induced the P450scc promoter as rapidly as a promoter containing two cAMP response elements fused to an SV40 promoter, a system known to respond directly. Basal expression was increased by sequences between -89 and -152, and increased further by sequences between -685 and -2500. This upstream region also conferred inducibility by cAMP and contains one consensus TPA response element (TRE) as well as six sequences with high homology to the TRE consensus. TPA plus A23187 transiently increased CAT activity before repressing it, reflecting the complex actions of AII *in vivo*. Repression by prolonged treatment with TPA plus A23187 was mediated by multiple elements between -89 and -343. The induction of CAT activity by forskolin was not diminished by treatment with TPA plus A23187 nor did the regions of the promoter responsible for regulation by the two pathways co-isolate. Thus, the human P450scc gene is repressed by TPA plus A23187 by mechanisms and sequences independent of those mediating induction by cAMP.

Introduction

Steroid hormones,which work by controlling transcription of specific genes, are critical regulators of physiological processes (Beato, 1989). The first, rate limiting, and hormonally regulated step in the generation of steroid hormones is the conversion of cholesterol to pregnenolone by the cholesterol side chain cleavage enzyme, P450scc. This mitochondrial cytochrome P450 enzyme recieves electrons from NADPH via two protein intermediates, a flavoprotein, adrenodoxin reductase, and an iron-sulfer protein, adrenodoxin. Using these electrons, P450scc catalyzes three sequential reactions - 22 hydroxylation, 20 hydroxylation, and $C_{20,22}$ bond cleavage - apparently at a single active site (reviewed in Miller, 1988). As side chain cleavage is the rate-limiting step in steroidogenesis (Stone and Hechter, 1955) it is important to understand both the hormonal regulation and tissue-specific expression of this gene.

The developmental patterns of expression and hormonal regulation of P450scc are specific to each steroidogenic tissue. Increased steroidogenesis and accumulation of P450scc mRNA are stimulated by adrenocorticotropic hormone (ACTH) in the human adrenal zonae fasciculata and reticularis, by luteinizing hormone (LH) and follicle stimulating hormone (FSH) in human ovarian granulosa cells, and by LH and human chorionic gonadotropin (hCG) in human testicular leydig cells and placental cytotrophoblasts (Chung, et al., 1986; DiBlasio, et al., 1987; Golos, et al., 1987; Picado-Leonard, et al., 1988; Voutilainen and Miller, 1987; Voutilainen, et al., 1986). In all these cases the stimulatory hormone binds a cell surface receptor that activates a G-protein (G_s) to increase intracellular cAMP. This in turn increases transcription of the P450scc gene (Inoue, et al., 1988; Mellon and Vaisse, 1989). By contrast, angiotensin II (A II) acutely stimulates mineralocorticoid production in the adrenal zona glomerulosa by alterations of intracellular Ca^{++} and activation of protein kinase C (PKC) (reviewed in Barrett, et al., 1989). However, prolonged stimulation with AII or agents that activate the PKC pathway

repress the quantities and activities of steroidogenic enzymes in cultured human fetal adrenal cells (Mason, et al., 1986; McAllister and Hornsby, 1988) or repress steroidogenesis in both rat and bovine cultured adrenal cells (Cozza, et al., 1990; Enyedi, et al., 1985). Thus adrenal steroidogenesis is complex and is regulated by at least two different intracellular second messenger systems.

The regions of the P450scc promoter responsible for cAMP-induction and basal expression remain poorly characterized. In transient transfections of mouse adrenal Y1 tumour cells, 5.4kb of 5' flanking DNA from the human P450scc gene mediated cAMP induction of bacterial chloramphenicol acetyl transferase (CAT) expression to 750% of control, but the responsible sequences were not localized (Inoue, et al., 1988). The region between -2500 and -600 of the human promoter conferred strong basal expression in the adrenocortical Y1 cells but not in human placental cytotrophoblast JEG3 cells (Chung, et al., 1989), suggesting it might contain an adrenal-specific element. Shorter fragments of the bovine P450scc promoter fused to different reporter genes gave internally inconsistent data and responded weakly in Y1 cells treated with cAMP or forskolin (about 2 fold) (Ahlgren, et al., 1990).

The mechanism by which cAMP regulates P450scc mRNA is controversial. In primary cultures of bovine adrenal cells, induction of bovine P450scc gene transcription by cAMP appeared to be slow and to require protein synthesis (John, et al., 1986). These authors suggested that cAMP induced expression of a protein that then activated transcription of P450scc. We and others have shown that cAMP induction of P450scc can be rapid, and independent of protein synthesis (Golos, et al., 1987; Mellon and Vaisse, 1989). While it was possible these differences were species-specific, tissue-specific, or both, the group reporting a requirement for protein synthesis was unable to confirm this in either Y1 cells or primary cultures of bovine adrenal cells transiently transfected with bovine P450scc/CAT constructs (Ahlgren, et al., 1990). Thus the mechanism for cAMP-mediated induction is unclear , and the *cis*-acting sequences responsible for either

basal or cAMP-induction have not been localized. Furthermore, the transcriptional regulation of the P450scc gene by the Ca^{++}/PKC second messenger pathway has not been studied.

In this study we characterize the regions of the human P450scc promoter necessary for basal expression and for transcriptional regulation mediated by both cAMP and Ca^{++}/PKC. Basal expression is significantly increased by two separate regions of 5' flanking DNA. The upstream region (-2500 to -685) is also responsible for cAMP induction and contains the proposed adrenal-specific element (Chung, et al., 1989). The kinetics of the cAMP induction are rapid, suggesting a direct effect on transcription. The promoter region between -343 and -89 contains multiple elements that function as novel, hormonally dependent, transcriptional repressors that respond strongly to a combination of A23187, a Ca^{++} ionophore, and TPA, a phorbol ester. As the regions responsible for induction by cAMP and for repression by the Ca^{++}/PKC pathway do not colocalize, the two second messenger pathways must alter P450scc transcription by independent mechanisms.

Results

Expression and regulation of the endogenous murine P450scc mRNA in Y1 cells

Y1 cells are a stably transformed cell line derived from a mouse adrenocortical tumour (Yasumura, et al., 1966). These cells possess many features of adrenal cortex cells including the presence of high affinity receptors for both ACTH and A II, and they respond to these hormones with increases in steroidogenesis (Begeot, et al., 1987; Pierson, 1967). Although Y1 cells lack most adrenal steroidogenic enzymes, they express P450scc activity (Schimmer, 1979). To determine the suitability of Y1 cells for studying transcriptional regulation of the human P450scc gene we treated them with several known regulators of adrenal steroidogenesis and measured the response of the endogenous murine P450scc mRNA. Northern blots of Y1 cell mRNA probed with a rat probe for P450scc show that both forskolin, a cAMP agonist, (Figure 1.1) and 8-Br-cAMP (data not shown) strongly increased the abundance of P450scc mRNA. By contrast prolonged treatment with both the Ca^{++} ionophore A23187 and the phorbol ester TPA diminished mouse P450scc mRNA abundance in Y1 cells; furthermore the effects of these drugs were additive (Figure 1.1A). This decrease was not due to a general inhibition of transcription or to selective cell death: reprobing the northern blots showed no effect on nuclear lamin (Figure 1.1B), and staining Y1 cells treated with TPA plus A23187 with the vital dye trypan blue showed no effect on cell viability (data not shown). In Y1 cells treated with combinations of forskolin, TPA, and A23187, the PKC agonists reduced the abundance of P450scc mRNA, but did not abolish forskolin induction (Figure 1.1A).

Expression and regulation of a transfected human P450scc CAT fusion construct

To study the human P450scc promoter we constructed a series of plasmids containing progressively shorter segments of the 5' flanking DNA from the human P450scc gene fused to the bacterial chloramphenicol acetyl transferase (CAT) reporter gene (Figure 1.2).

The parental vector, pAnΔCAT, contains an SV40 polyadenylation signal upstream from the human P450scc sequences to reduce spurious transcription generated within the vector (Brentano, et al., 1990). CAT activity from this promoterless vector was seldom detected above background, therefore the CAT data for basal expression are normalized to the shortest plasmid, pAnscc-89CAT.

As extracellular Ca^{++} is an important regulator of steroidogenesis (Barrett, et al., 1989) we determined if our transient transfection protocol, which utilizes $Ca(PO_4)_2$:DNA coprecipitation, could artifactually alter transcription of P450scc. Y1 cells were transfected or mock-transfected and then treated with A23187, TPA and forskolin. Northern blots of the endogenous murine P450scc mRNA show that the transfection protocol has no significant quantitative effect on the regulation of murine P450scc by the various drug treatments (Figure 3).

Y1 cells were then transiently transfected with pAnscc-2500CAT or the control plasmid pAnTK-109CAT and treated with forskolin, TPA, and A23187. The changes in CAT activity (Figure 1.4) were identical to the changes seen in murine P450scc mRNA abundance (Figure 1.1). Thus many, if not all, the regulatory elements responsible for transcriptional regulation lay within the 2500 bp fragment used.

Testing for interactions between the two pathways required knowing the maximal responses for each, therefore, we performed dose response and time-course experiments (Figure 1.5). Forskolin induced P450scc promoter-dependent CAT activity maximally at $3x10^{-6}$ M, and half-maximally at about 10^{-6} M (Figure 1.5A). High concentrations of TPA can desensitize the PKC pathway (Hoeffler, et al., 1989), probably accounting for the rise in CAT activity with TPA concentrations greater than 10^{-7} M (Figure 1.5B). To test this, Y1 cells were pretreated with 300 nM TPA for 12 hours to desensitize the PKC pathway and then treated with TPA plus forskolin. This resulted in complete abolition of the inhibition normally seen with effective doses of TPA (30 nM) (Table 1.1). The kinetics of the response to TPA plus A23187 are biphasic (Figure 1.5C): CAT activity is induced

mildly at 60 min, falls back to basal values by 90 min, and decreases below basal thereafter.

Mechanism for cAMP induction of the P450scc promoter

The kinetics of forskolin induction of pAnscc-2500CAT (Figure 1.6) suggests that the induction of the human P450scc promoter in Y1 cells by cAMP is rapid and direct. We compared expression of our pAnP450scc-2500CAT with a promoter that responds rapidly and directly to cAMP. This construct contains two tandem copies of the CRE from the gene for the α–subunit of human chorionic gonadotropin (hCGα) fused to an SV40 promoter driving CAT expression (Fenstermaker, et al., 1989). This CRE contains the consensus sequence, TGACGTCA, which responds rapidly and directly to changes in intracellular cAMP (Bokar, et al., 1988; Jameson and Lindell, 1988; Jameson, et al., 1989; Milsted, et al., 1987). The temporal regulation of the human P450scc promoter by cAMP is even more rapid than that of the CGα CRE (Figure 1.6). Whether this induction is direct or requires protein synthesis is unknown as Y1 cells treated with the protein synthesis inhibitor cycloheximide at 40 μM increased the abundance of the murine P450scc mRNA to the same level as that seen with forskolin alone (data not shown).

Regulation by forskolin in the presence of TPA and A23187

Northern analysis (Figure 1.1A) shows that TPA or A23187 treatment of cultures also treated with forskolin reduces the abundance of endogenous Y1 P450scc mRNA compared to forskolin treatment alone. The effect is strongest when TPA and A23187 are combined. To determine if some of this repression is mediated by compromising the cAMP pathway, we measured forskolin induction of pAnscc-2500CAT in the presence or absence of TPA and A23187. The induction of CAT activity by forskolin is not changed by TPA and A23187 (Table 1.2), suggesting that little if any repression is due to an interaction between the two second messenger pathways.

Promoter regions important for basal expression of the human P450scc gene

The series of deletion constructions (Figure 1.2) was transiently transfected into Y1 cells and assayed for CAT activity (Figure 1.7A). The basal expression of all the deletion plasmids was detectable above background. Adding sequences to -152 bp increased transcription 600% above the level for the shortest construct, pAnscc-89CAT. Adding more 5' sequence to -685 bp reduced basal activity slightly, but the addition of sequences between -685 and -2500 bp increased basal activity further to about 1000% of pAnscc-89CAT. Thus, two regions of the promoter appear to contribute significant basal activity: The first is between bases -89 and -152 and the second is between bases -685 and -2500.

Promoter regions necessary for induction by cAMP and repression by TPA plus A23187

To identify regions involved in hormonally regulated transcription, Y1 cells transfected with the series of deletion plasmids were treated with forskolin (Figure 1.7B) or TPA plus A23187 (Figure 1.7C). Forskolin induced CAT activity of all the constructions, but only the activity of pAnscc-2500CAT was greater than the negative control, pAnTK-109CAT. These results isolate the principal *cis*-element(s) for cAMP induction within the -685 to -2500 bp fragment. In transfected Y1 cells treated with TPA plus A23187, significant repression below that seen for the control plasmid pAnTK-109CAT begins with pAnscc-152CAT and decreases further with constructs pAnscc-267CAT and pAnscc-343CAT (Figure 1.7C). This indicates that repression by TPA plus A23187 is mediated by multiple elements between -89 and -343.

RNase protection experiments (Figure 1.8) demonstrate that the deletion constructs initiated transcription from the correct site for both basal and drug-regulated expression. The clustered family of bands surrounding the correct initiation site in the RNase protection experiments was also seen with human P450scc mRNA extracted from JEG-3 cells (data

not shown). The RNase protection data in Figure 8B confirm that forskolin did not increase transcription from deletion plasmids smaller than pAnscc-2500CAT as shown in Figure 1.7B, as they are not induced more than the internal control, pAnRSVCAT. The apparent forskolin induction of the shorter deletion plasmids as well as the two control plasmids, pAnTK-109CAT and pAnRSVCAT, may be due to a general increase in RNA Pol II transcription and/or an effect of forskolin on the transfection protocol in Y1 cells. Similiarly, RNase protection experiments (Figure 1.8C) confirm that transcription is initiated from the correct site and is repressed by treatment with TPA plus A23187 as shown in Figure 7C. The specificity of this repression of the human P450scc promoter by TPA plus A23187 is demonstrated by the increased repression seen with constructs longer than pAnscc-89CAT, by the relatively smaller repression seen with the pAnTK-109CAT control, and by the induction of the pAnRSVCAT internal control plasmid used in the RNase protection experiments. As the transfection control plasmid RSVβGAL contains the same RSV promoter fragment which is induced by treatment with TPA plus A23187 (Figure 1.8C) the slight repression of pAnTK-109CAT and pAnscc-89CAT is probably an artifact of normalizing the data.

Discussion

Y1 cells as a model system for analyzing transcriptional regulation of the hP450scc gene

Primary cultures of human adrenocortical cells are not readily used for studying transcription of the human P450scc gene, and a stably transformed human adrenocortical cell line does not exist. Y1 murine adrenocortical cells are well-characterized and respond to agonists of steroidogenesis similiarly to murine, bovine and human adrenocortical cells and have been used to analyze transcriptional regulation of several steroidogenic enzymes (Ahlgren, et al., 1990; Chung, et al., 1989; Handler, et al., 1988; Morohashi, et al., 1987; Rice, et al., 1989). Since the endogenous murine P450scc gene responded to treatment with forskolin, TPA and A23187 (Figure 1.1) Y1 cells should be useful for analyzing transcriptional regulation of the transfected human P450scc gene.

Promoter regions necessary for basal transcription

All of our human P450scc deletion constructions gave detectable CAT activity in transiently transfected Y1 cells. The shortest construct, pAnscc-89CAT contains a TATA box and a possible CAAT motif (CATT at -63) as well as a sequence between -86 and -71 that closely resembles the "basal transcription element" recently described for the P450c gene expressed in human liver (Yanagida, et al., 1990). Constructions containing bases between -152 and -685 of the human P450scc 5' flanking DNA were all transcribed at between 400% and 600% of the pAnscc-89CAT construct. This indicates an element for basal expression lies between -89 and -152. A likely candidate is the sequence between bases -117 and -108, GGGGAGGAGC, which matches at 9 out of 10 bases with the SP-1 consensus, $^{G}/_{T}GGGCGG^{G}/_{A}{}^{G}/_{A}{}^{C}/_{T}$ (Dynan, et al., 1986).

Basal expression increased further with the region between -685 and -2500, which is also necessary for cAMP induction. The colocalization of these two functions is also seen in the genes for the murine P450c21, murine P450c11, and murine P450scc (Handler,

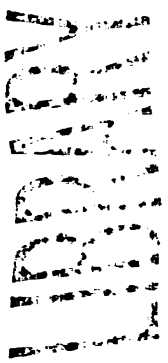
et al., 1988; Mouw, et al., 1989; Wong, et al., 1989). In those experiments basal expression correlated with the activity of the type I cAMP-dependent protein kinase A (PKA) in Y1 cells with a variety of PKA mutations. In addition, point mutations in the putative cAMP-response element in the murine P450c11 promoter eliminated basal activity (Mouw, et al., 1989). These results suggested that basal activity of the PKA pathway helps dictate the basal level of expression of these genes (Handler, et al., 1988). The murine P450scc gene was only partially sensitive to the diminished activity of PKA in the various Y1 PKA mutants (Wong, et al., 1989). Similiarly basal expression of the human P450scc promoter is not severly compromised in the shorter P450scc/CAT constructs that lack the cAMP response region -2327 to -685.

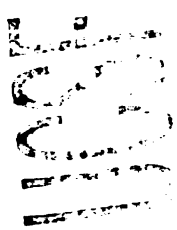
Promoter regions involved in cAMP regulation

Several hormones that regulate steroidogenesis utilize cAMP as a second messenger. cAMP can regulate gene transcription through *cis*-acting cAMP-response elements (CRE, consensus binding site TGACGTCA) which bind a family of related cAMP response element binding proteins (CREB) (Gonzalez, et al., 1989; Hoeffler, et al., 1988) as well as a number of other proteins (discussed in (Dean, et al., 1990)). Alternatively cAMP can induce transcription through *cis*-acting TREs, (consensus binding sites CCCCAGGC or TGAC/GTCA) (Hoeffler, et al., 1989; Imagawa, et al., 1987), for review see (Karin, 1989). The cAMP-responsive region of the human P450scc promoter (bases -685 to -2500) lacks sequences similiar to the consensus CRE but does contain one consensus TRE sequence, TGAGTCA, between bases -686 to -693 as well as several other homologous sequences. Whether these TRE sequences mediate cAMP induction of the human P450scc promoter is unknown. Other genes for steroidogenic enzymes, including murine P450c21 and bovine P450c17 , are also regulated by cAMP but lack both consensus CRE or TRE sequences (Handler, et al., 1988; Lund, et al., 1990). In contrast cAMP induction of both

the murine P450c11 and human P450c17 genes appear to involve CRE or CRE-like sequences (Brentano, et al., 1990; Rice, et al., 1989)].

An alternate, indirect pathway has been proposed to explain the apparently slow cAMP induction kinetics and cycloheximide sensitivity of the bovine P450scc gene (John, et al., 1986). However, experiments with the human P450scc gene in human granulosa cells (Golos, et al., 1987) and with the murine P450scc gene in mouse leydig MA10 cells (Mellon and Vaisse, 1989) did not demonstrate any sensitivity of P450scc to inhibitors of protein synthesis. Our comparison of the kinetics of forskolin induction of the human P450scc promoter with those of the CRE in the hCGα promoter indicate that the human P450scc promoter is stimulated very rapidly with a $t_{1/2}$ of about 6 hours. Similiarly, the bovine P450scc promoter transiently transfected into Y1 cells was induced rapidly and was not impaired by inhibitors of protein synthesis (Ahlgren, et al., 1990). The rapid induction we observe is consistent with a direct activation of human P450scc transcription by cAMP in Y1 cells.





Promoter elements involved in Ca^{++}/PKC regulation of P450scc transcription

A variety of experiments indicate that the response of adrenal cells to AII is biphasic. Short term stimulation (0.5 h to 2.0 h) of bovine adrenal cells with AII or the combination of TPA plus A23187 rapidly increases aldosterone synthesis and secretion (Kojima, et al., 1984), however the effects on P450scc synthesis are unknown. In contrast, long-term stimulation (24 to 48 h) of primary cultured human fetal adrenal cells with TPA lowered the abundance of P450scc mRNA and protein and blocked the ability of cAMP to increase the abundance of P450scc (Mason, et al., 1986; McAllister and Hornsby, 1988). The kinetics for the response of the human P450scc promoter to treatment with TPA plus A23187 is consistent with a such a biphasic response. One hour treatment of the transiently transfected pAnscc-2500CAT construction with TPA plus A23187 resulted in a mild induction, but longer incubations (2.0 h to 12.0 h) strongly repressed transcription. Such

repression of adrenal steroidogenesis by the PKC pathway may modify both the amounts and types of steroids produced by the adrenal (McAllister and Hornsby, 1988).

Although cAMP and PKA may play a role in basal transcription of the human P450scc gene it is unlikely that repression by TPA plus A23187 indicates the loss of analogous basal induction by the Ca++/PKC pathway. The pretreatment experiment (Table 1.1) shows that desensitization of PKC abolishes TPA repression, but does not reduce CAT activity below basal. Thus TPA repression requires an intact PKC pathway, and a desensitized PKC pathway does not compromise basal expression.

Repression of P450scc promoter activity by TPA plus A23187 does not involve inhibition of the cAMP/PKA pathway. Such an interaction was an attractive hypothesis, given the apparent role of cAMP/PKA in determining basal expression of P450scc, the known affect of AII to inhibit adenylate cyclase via a G-protein (Woodcock and Johnston, 1984), and the convergence of the PKA and PKC pathways on both CRE and TRE *cis*-acting elements (Hoeffler, et al., 1989; Imagawa, et al., 1987). However, induction by cAMP and repression by TPA plus A23187 map to seperate *cis*-acting regions of the human P450scc promoter, thus an interaction between these pathways is not responsible for repression by TPA plus A23187.

Repression by TPA plus A23187 is mediated by multiple DNA:protein interactions as repression increases progressively with longer promoter fragments between -89 and -343 bp. Whether this is because TPA and A23187 work through seperate *cis*-elements or converge through a common *cis*-element in multiple regions of the promoter is unknown. No repressive *cis*-acting elements responsive to TPA plus A23187 have been described, however several *cis*-acting elements including binding sites for Jun/AP-1, AP-2, AP-3, NFkB, and the SRE mediate transcriptional activation by TPA (Angel, et al., 1987; Chiu, et al., 1987; Imagawa, et al., 1987; Lee, et al., 1987; Sen and Baltimore, 1986). TPA combined with A23187 also activates the murine granulocyte-macrophage colony stimulating factor promoter through the CLE2 and GC elements (Miyatake, et al., 1988).

The human P450scc promoter region between -89 and -343 contains several regions of limited homology to some of these activation sequences but their role, if any, in repression of the human P450scc promoter by TPA plus A23187 is unknown.

Most models for transcriptional repression involve either interference with an activating protein(s) or direct interaction with the RNA polymerase II transcription complex (Miyatake, et al., 1988). We have eliminated the possibility that repression of the human P450scc promoter by TPA plus A23187 is through interference with the cAMP response element(s): the two effects map to distinctly different regions of the promoter and removal of the cAMP response element does not eliminate repression by TPA plus A23187. Another possibility is that TPA plus A23187 represses the activity of an unidentified basal transcription factor(s). Part of the region that contains the repression elements responsive to TPA plus A23187 also contains a basal activation element(s). Furthermore, the repression by TPA plus A23187 to 15 to 20% of control in pAnscc-343CAT is just enough to account for the 500% increase in basal activity conferred by sequences between -89 and -152. Thus the repression may be a reversal of the activation associated with this region of the promoter analagous to glucocorticoid repression of the bovine prolactin and the human glycoprotein α-subunit promoters (Akerblom, et al., 1988; Sakai, et al., 1988). If this is true a simple binding site competition model, as proposed for glucocorticoid repression (Akerblom, et al., 1988; Sakai, et al., 1988), cannot explain all the repression of P450scc because the basal activation and the effects of TPA plus A23187 map to the same as well as different regions of the promoter. This could be explained by some form of protein:protein interaction between the two types of elements.

Materials and Methods

Tissue Culture

Y1 cells, a generous gift from Dr B. Schimmer, were maintained in 50% DME H16/ 50% Ham's F12 with 15% heat-inactivated horse serum, 2.5% fetal calf serum and 50 µg/ml gentamycin at 37°C in 5% CO_2. For hormonal treatment the cells were switched to medium supplemented with only 0.5% fetal calf serum and 50 µg/ml gentamycin. Unless indicated otherwise, treatments lasted for 12 hours using 20 µM for forskolin, 500 µM for A23187,30 nM for TPA or 1 mM for 8-Br-cAMP. Forskolin, A23187, TPA and 8-Br-cAMP were all purchased from Sigma.

RNA Isolation

Cells were harvested from the tissue culture plates with phosphate buffered saline free of Ca^{++} and Mg^{++} (PBS/CMF), and pelleted by a brief spin in a clinical centrifuge, and lysed with 1.0 ml of 5M guanidinium isothiocyanate, 50 mM Tris pH 7.8, 0.5% sarkosyl, 0.1 g/ml $CsCl_2$ and 10% β-mercaptoethanol. Total cellular RNA was isolated by pelleting through a 500 µl cushion of 5.7 M $CsCl_2$, 100 mM EDTA. The RNA pellet was resuspended in 300 µl of 10mM Tris pH 7.5, 0.1 mM EDTA and 1% $NaDodSO_4$, extracted once with a 1:1 solution of buffered phenol:chloroform (pH 7.0) and then extracted with 200 µl of ether saturated with water before precipitation with 1/10 volume of 3 M sodium acetate and 2.2 volumes of ice cold ethanol.

RNA Transfer Blots

The appropriate amount of RNA was pelleted and then resuspended in 16 µl of 1 M glyoxal, 50% dimethylsulfoxide, and 10 mM NaH_2PO_4. RNA was denatured in this solution at 50°C for 60 minutes, cooled to room temperature and loaded with 4 µl of 50% glycerol, 0.01 M NaH_2PO_4, and 0.4% bromophenol blue into a 1.0% agarose, 10 mM

NaH_2PO_4, pH 7.0 gel. The samples were electropheresed at 40-50 V until the bromophenol blue migrated 8.0 cm and were then transferred for 24 hours to Hybond-N (Amersham) membranes. The transferred RNA was crosslinked to the membrane using UV irradiation (1200 μjoules using a Stratagene UV 'StrataLinker'). Membranes were prehybridized overnight at 42°C in 50% formamide, 5X SSC (0.15 M NaCl, 0.015 M NaCitrate), 5X Denhardt's solution (2% ficoll, 2% polyvinylpyrollidone, 2% bovine serum albumin), 50 mM NaH_2PO_4, pH 8.0, 0.5% $NaDodSO_4$ and 250 μg/ml of Torula yeast RNA and 250 μg/ml herring sperm DNA. Hybridization was for 24 hours in the same buffer except at pH 7.0 and containing only 100 μg/ml of Torula RNA and of herring sperm DNA with $1x10^6$ cpm/ml of probe generated by random priming from a cDNA template. Templates were gel purified fragments of the full-length human P450scc cDNA (Chung, et al., 1986) a 1.2 kb fragment of the rat P450scc cDNA (Goldring, et al., 1987) and a 700 bp Hind III fragment of the human lamin A cDNA (Fisher, et al., 1986). Nonspecific hybridization was removed by washing either in 0.1X SSC/1% $NaDodSO_4$ or 0.5X SSC as indicated for 15 minutes at room temperature followed by two 30 minute washes at 55°C. Autoradiography was done using one intensifying screen at -70°C.

Plasmids

The construction of our CAT expression vector pACAT is described elsewhere (Brentano, et al., 1990) and was modified further by the deletion of sequences from the *Nde* I site to the polyadenylation signal and the removal of the polylinker sequence between the *Xba* I and *Sph* I sites to yield pAnΔCAT S/X (Figure 1.2A).

A plasmid containing about 2,500 bp of the 5' flanking and untranslated DNA for the human P450scc gene was generously provided by Dr Bon-chu Chung. This plasmid was used to generate the series of 5' deletion constructs (Figure 1.2B) using unique or rare restriction sites for internal deletions. Each deletion was created such that a *Kpn*I site was always located on the 5' end. The 3' end of all the constructs was defined by ligating the

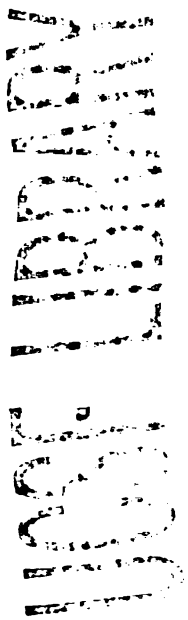
Pvu II site at +49 to a filled-in *Bam* HI site, thus regenerating the *Bam* HI site. Each deletion clone was digested with *Kpn*I and *Bam* HI, the fragment purified by gel electrophoresis, and ligated into the *Kpn*I and *Bam* HI sites of the pAnΔCAT X/S vector. The resulting P450scc/CAT plasmids are designated as pAn-"X"-CAT where "X" designates the 5' end of the construct, in base pairs, relative to the transcriptional initiation site of human P450scc (Figure 1.2B). pAnRSVCAT was generated by cloning the 587 bp *Hin*d III fragment of the RSV promoter/enhancer from RSVβ-gal (Edlund, et al., 1985) into the *Hin*d III site of pAnΔCAT. Similiarly the *Bam*HI to *Bgl*II fragment of TKCAT that contains the herpes simplex virus thymidine kinase promoter out to -109 bp relative to the transcriptional start site was cloned into the pAnΔCAT *Bam*HI site to generate pAnTK-109CAT. All constructs were confirmed by restriction mapping and by sequencing across all cloning junctions.

Transfections

Plasmids were isolated using the Triton X-100 cleared lysate protocol, purified by one cycle of equilibrium density centrifugation through $CsCl_2$, treated with RNase A and proteinase K and extracted several times with phenol:chloroform (50:50, pH 7.0) Cells were transfected with a slightly modified calcium phosphate coprecipitation procedure of Chen and Okayama (Chen and Okayama, 1988) using HEPES instead of BES buffer. Precipitates contained 15 μg of DNA which ,unless otherwise indicated, consisted of 10 μg of experimental CAT plasmid with 5 μg of the transfection control plasmid, RSVβGal (Edlund, et al., 1985). DNA precipitates were left on the cells for 12 hours at 37°C in 2.5% CO_2 then the transfection medium was replaced with medium containing the appropriate hormone(s).

CAT Assays

The cells were harvested with 10 ml of PBS/CMF, pelleted, rinsed with 1.0 ml PBS, pelleted again and resuspended in 100 µl of 250 mM Tris pH 7.5, 0.1% Triton X-100. Cells were lysed by incubating on ice for 5 minutes and vortexing vigorously several times; cellular debris was then removed by pelleting in a microfuge at 4°C for 10 minutes. From this cleared extract 50 µl was used for the β-galactosidase assay (Edlund, et al., 1985) and 50 µl used for the two-phase CAT assay (Eastman, 1987; Neumann, et al., 1987). A standard curve was always performed with purified CAT enzyme (Sigma) to assure that experimental values fell within the linear range of the assay. Background was determined by a mock-transfected extract and removed from the experimental CAT values, which were than normalized for transfection efficiency with the β-galactosidase data. Each experimental treatment was performed in triplicate with independent DNA precipitates and repeated at least three times with at least three different plasmid preparations. Unless indicated otherwise, the values are presented as the mean of three or more experiments +/- SEM.



RNase Protections

RNA was harvested as described above. Templates for transcribing the RNA probes were generated by cloning the appropriate promoter fragment into Bluescript vectors in the antisense orientation to the T7 promoter and then linearized at an appropriate restriction enzyme site (Figure 7). Probes were synthesized in a 25 µl volume containing 1 µg linearized template, 400 µM CTP,ATP and GTP, 50 µCi UTP (800 Ci/mmole, Amersham), 25 U placental ribonuclease inhibitor (RNasin, Promega) 10 U T7 polymerase, and 5 µl of 5X T7 buffer supplied with the enzyme. The transcription reaction was done at 37°C for 30 minutes followed by one phenol/chloroform extraction, ethanol precipitation, and purification of the full-length transcripts by gel electrophoresis on

denaturing 5% polyacrylamide/7 M Urea gels. RNA samples were precipitated with ethanol and resuspended in 30 µl of 80% formamide, 400 mM NaCl, 40 mM PIPES pH 6.7, and 1 mM EDTA containing $5x10^5$ cpm of control probe (pAnRSV-CAT) and $5x10^5$ cpm of experimental probe. Hybridization was done overnight at either 55°C or 58°C after which 300 µl of ice cold 10 mM Tris pH 7.5, 1 mM EDTA, 300 mM NaCl, 0.35-0.7 U DNase free RNase (Boehringer Mannheim), and 0.5 µg RNase T1 was added and then incubated at either 30°C or 37°C for 60 minutes. The samples were then treated with 20 µg of proteinase K, 1 µg tRNA, and $NaDodSO_4$ to 1% for 30 minutes at 65°C, extracted with phenol/chloroform, and precipitated with ethanol twice. The pellets were resuspended in 3 µl of 90% formamide 0.5 mM EDTA, 0.04% xylene cyanol, and 0.04% bromophenol blue, electrophoresed on a denaturing 7% polyacrylamide/8 M urea gel, and autoradiographed as above.

Acknowledgments

We thank Dr Bon-chu Chung (Academia Sinica, Taipei, Republic of China) for the 2500 bp fragment of the human P450scc 5' flanking DNA, Dr Bernard Schimmer (U. of Toronto, Canada) for the Y1 cells, Dr John Nilson (Case Western Reserve U., Cleveland OH) for the plasmid phα18x23'SV1CAT and Dr Tim Reudelhuber (U. of California, San Francisco CA) for pTKCAT and RSVβGal. We also thank the various members of the Metabolic Research Unit for valuable discussions and helpful suggestions. Supported by March of Dimes predoctoral fellowship #18-88-25 to CCDM, NIH postdoctoral fellowship F 32 DK08221 to STB and March of Dimes Grant 6-396 and NIH Grants DK 37922 and DK 42154 to WLM.

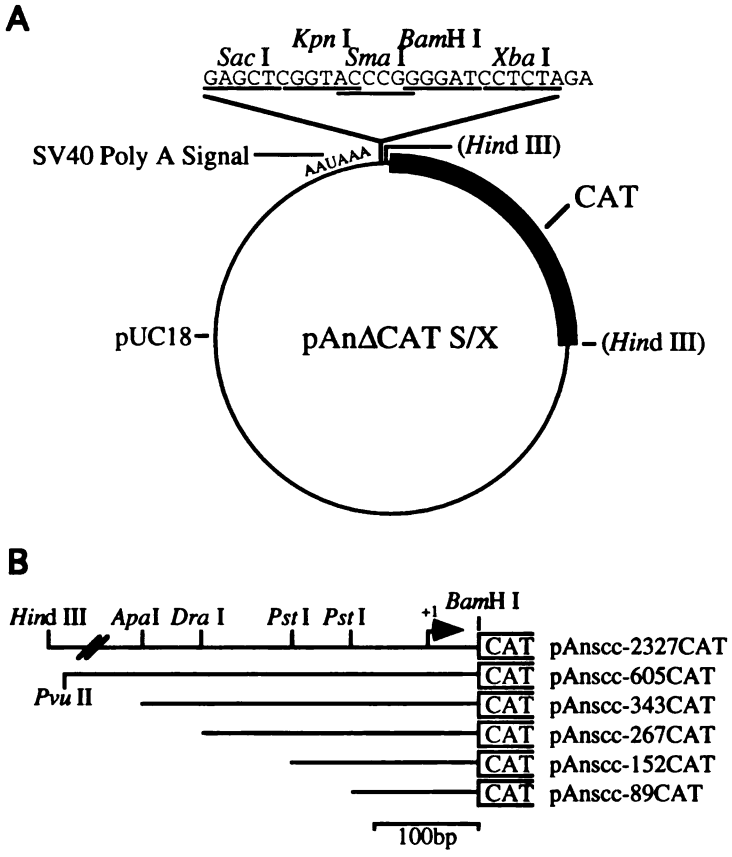


Figure 1.1 Schematic representation of pAnscc"X"CAT 5' deletion plasmids. (A) The parent vector pAnΔCAT S/X contains the CAT sequence, the SV40 early polyadenylation signal cloned in the sense orientation relative to the CAT coding sequence, and the *Sac*I to *Bam*HI sites of the pUC18 polylinker. (B) The series of human P450scc deletion plasmids used in this study with the largest P450scc fragment ending at the *Hin*dIII site at about -2500 base pairs upstream from the transcriptional initiation site (+1) indicated by the arrow. The plasmids were generated in Bluescript™ vectors using the sites indicated such that *Bam*HI and *Kpn*I sites defined the 3' and 5' ends respectively and were used to clone into pAnΔCAT S/X.

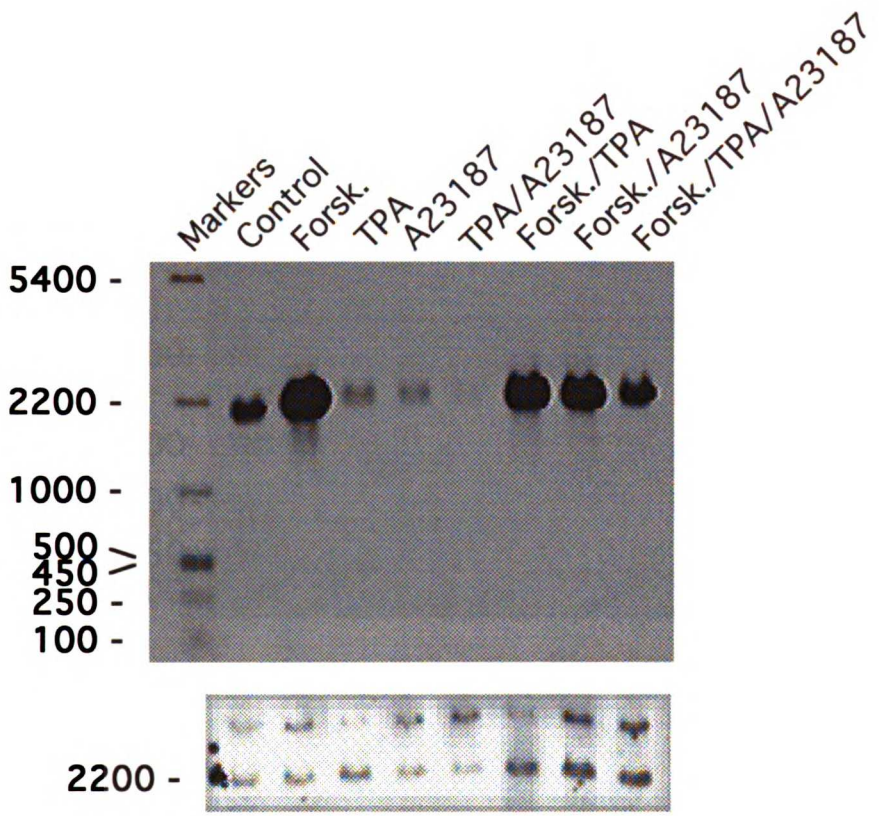


Figure 1.2 Regulation of murine P450scc mRNA in Y1 cells. Y1 cells were grown to a density of about 10^7 cells/10 cm dish and then exposed to the indicated drugs for 12 hours (forskolin, 2.0×10^{-5} M; TPA, 3.0×10^{-8} M; A23187, 5.0×10^{-7} M). Total cellular mRNA was prepared and 20 µg samples subjected to northern analysis. (A) The blot was probed with a 1.2 kb fragment of the rat P450scc cDNA and washed in 0.1X SSC at 55°C. (B) The blot was washed and then reprobed with a 750bp *Hin*dIII fragment of the human nuclear lamin cDNA and washed in 0.5X SSC at 55°C. Molecular size markers are end-labeled *Hin*dIII cut bacteriophage PM2 .

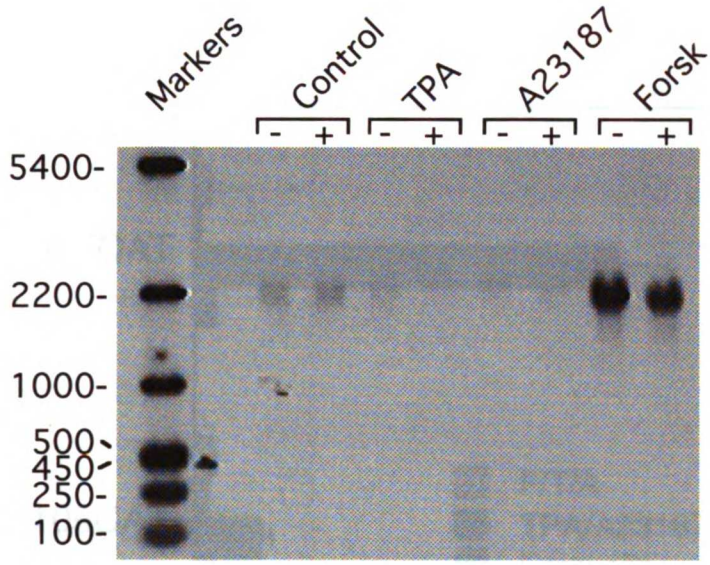


Figure 1.3 Effect of the $Ca(PO_4)_2$ transfection protocol on P450scc regulation. Northern analysis of 20 µg endogenous murine P450scc mRNA from Y1 cells which were transfected or mock transfected and then treated with forskolin, TPA or A23187 as in figure 1. The blot was probed with a 1.2 kb fragment of the rat P450scc cDNA and washed in 0.1X SSC at 55°C. Molecular size markers are end-labeled *Hin*dIII cut bacteriophage PM2.

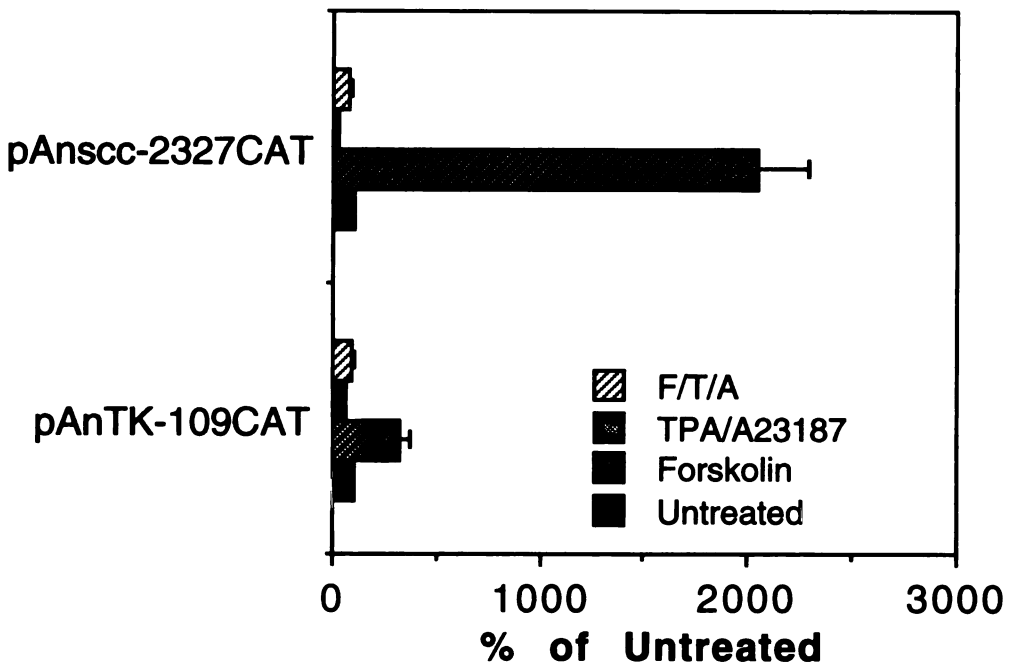


Figure 1.4. Regulated expression of transiently transfected human P450scc promoter. Y1 cells were transiently transfected with either pAnscc-2327CAT or the control plasmid pAnTKCAT and treated with forskolin , TPA plus A23187 or the combination of all three as in figure 1. The data are from one experiment done in triplicate and presented as the mean % change from untreated +/- SEM.

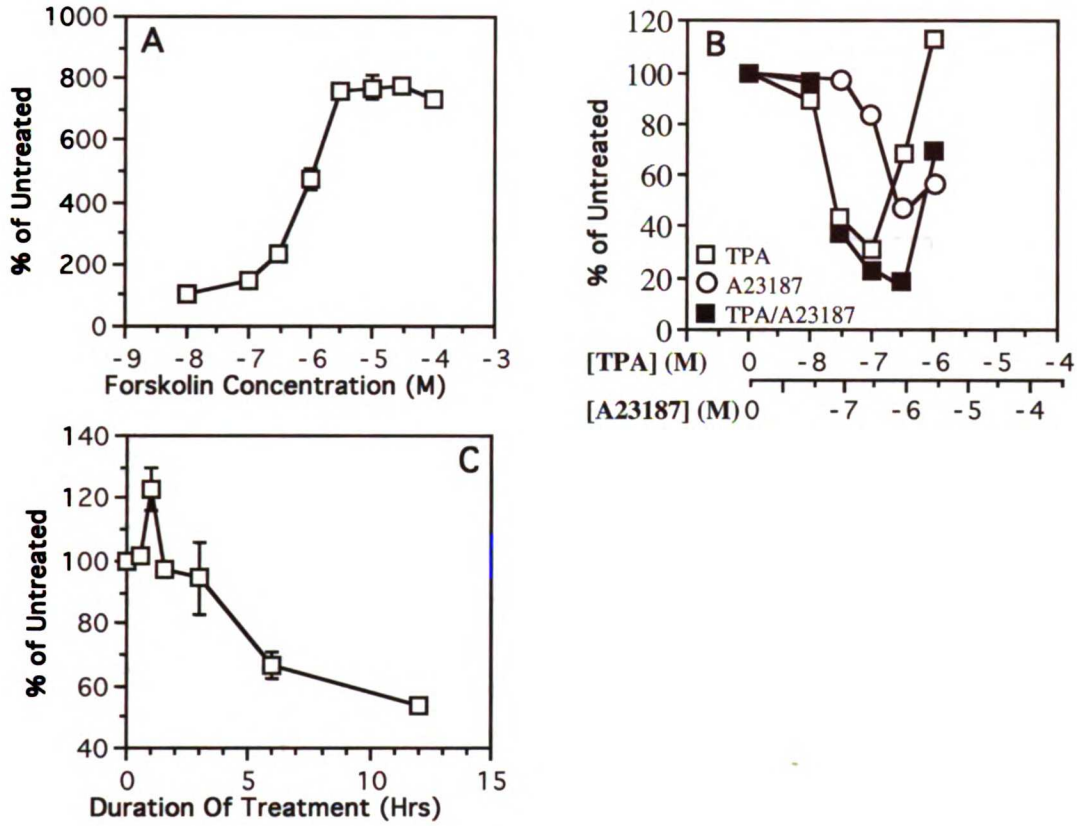


Figure 1.5. Dose response and time course experiments. Y1 cells were transiently transfected in triplicate with pAnscc-2500CAT, treated as described below and then CAT extracts analyzed. (A) Dose response for forskolin induction of pAnscc-2500CAT. (B) Dose responses for treatment with TPA, A23187 or the combination of TPA plus A23187. This experiment was done in the presence of 2.0×10^{-5} M forskolin to prevent repression below background. (C) Induction kinetics for treatment with both TPA plus A23187 (TPA, 3.0×10^{-8} M; A23187, 5.0×10^{-7} M). Values are the mean % change +/- SEM from the appropriate untreated extract for one experiment.

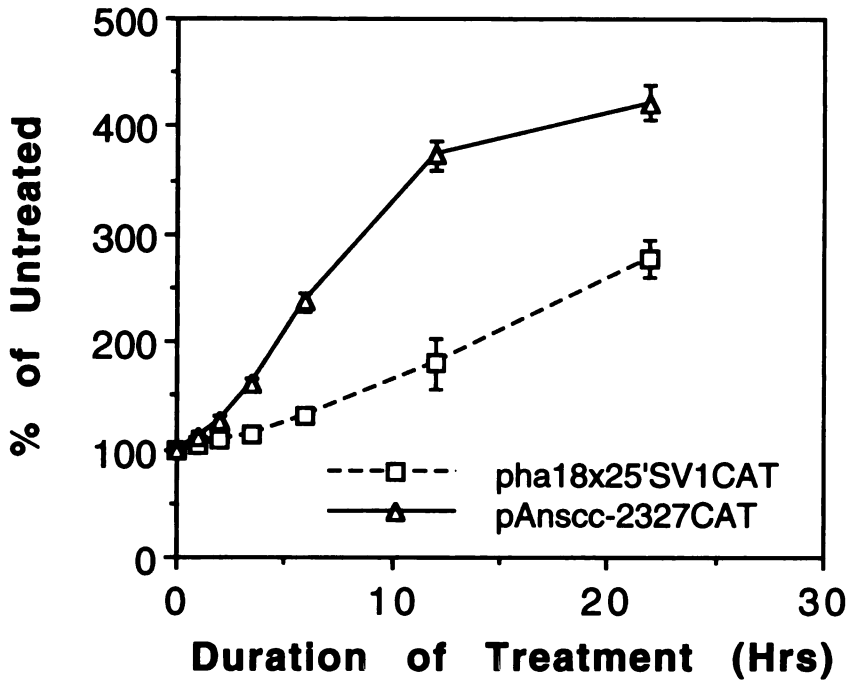


Figure 1.6. Comparison of the cAMP induction kinetics of pAnscc-2500CAT with the human glycoprotein hormone α-subunit CRE. Y1 cells were transiently transfected with either pAnscc-2500CAT or phα18x23'SV1CAT, which contains two tandem copies of the α-subunit CRE. Forskolin (2.0×10^{-5} M) treatment for the longest duration (22 hours) began 12 hours after transfection. Values are the mean % change +/- SEM from the appropriate untreated extract for one experiment.

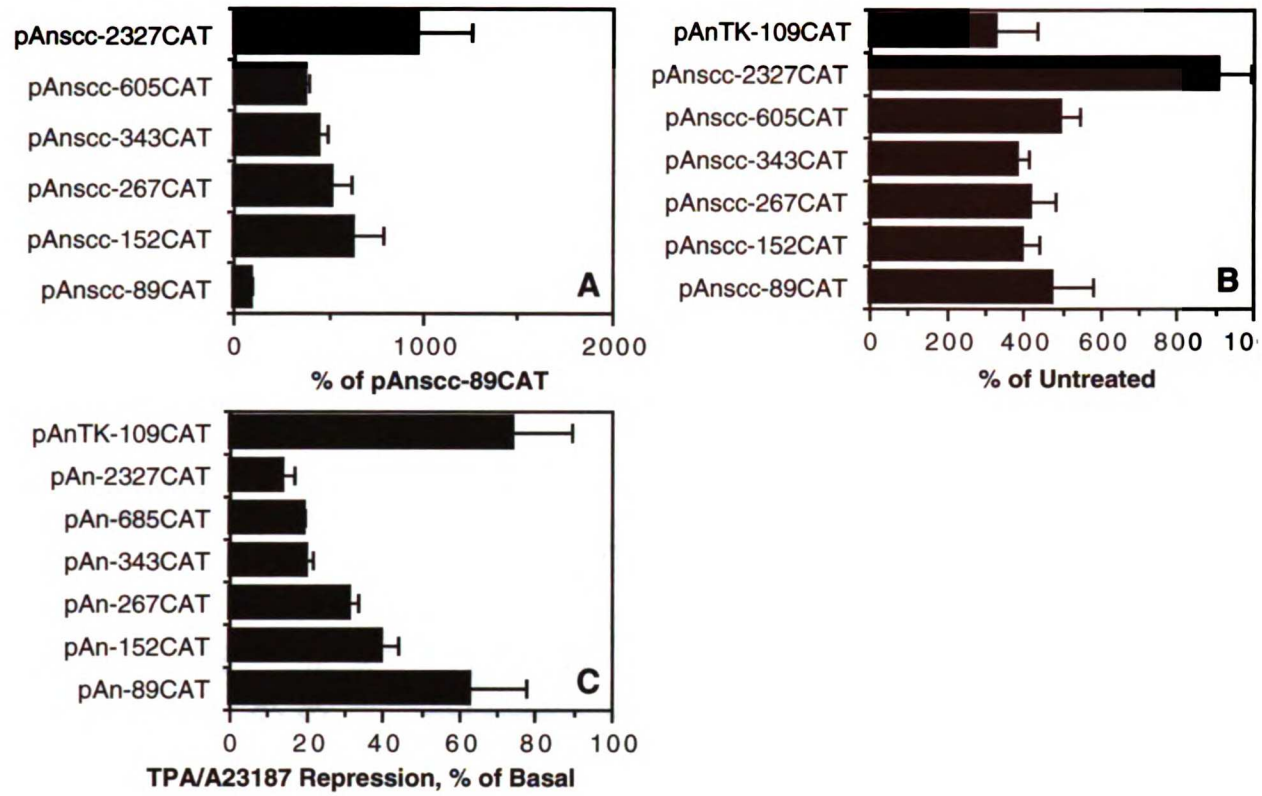
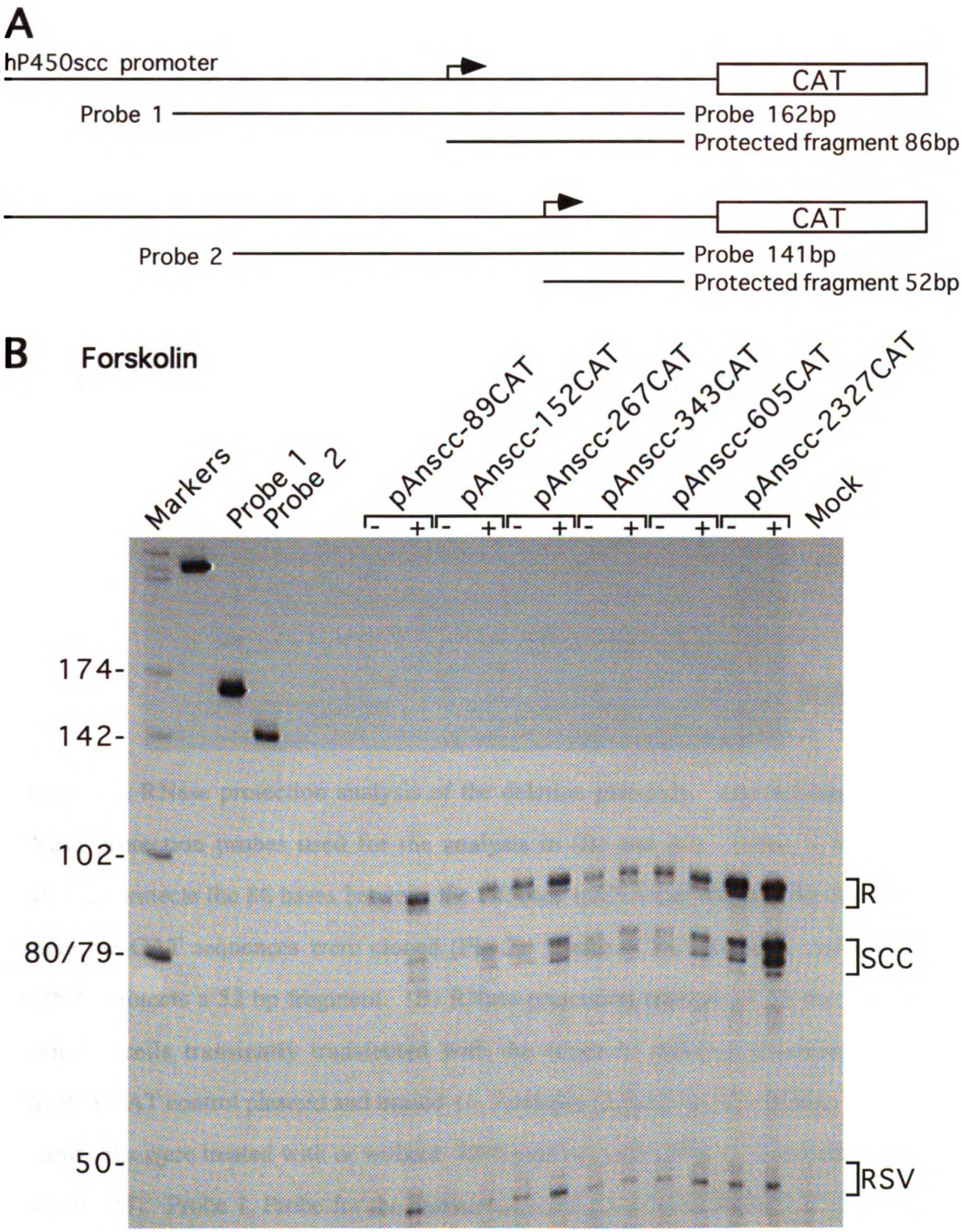


Figure 1.7 Basal and regulated expression levels for the human P450scc"X"CAT deletion plasmids. Y1 cells were transfected with the indicated plasmids in triplicate and CAT assays performed on cell extracts after a 12 hour incubation in medium with or without drugs. (A) Basal level of expression of transiently transfected deletion plasmids in Y1 cells. Values are the % difference from the shortest construct, pAnscc-89CAT and represent the mean +/- SEM of at least three seperate transfections. (B) Forskolin induction of the various deletion plasmids expressed as the mean % change +/- the SEM from the same construct not treated with forskolin from at least three seperate transfections. (C) Repression by TPA plus A23187 of the deletion plasmids expressed as the mean % change +/- the SEM from the same construct not treated with TPA plus A23187 from at least three seperate transfections.


A
hP450scc promoter
CAT
Probe 1
Probe 162bp
Protected fragment 86bp
CAT
Probe 2
Probe 141bp
Protected fragment 52bp
B
Forskolin
Markers
Probe 1
Probe 2
pAnscc-89CAT
pAnscc-152CAT
pAnscc-267CAT
pAnscc-343CAT
pAnscc-605CAT
pAnscc-2327CAT
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174-
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80/79-
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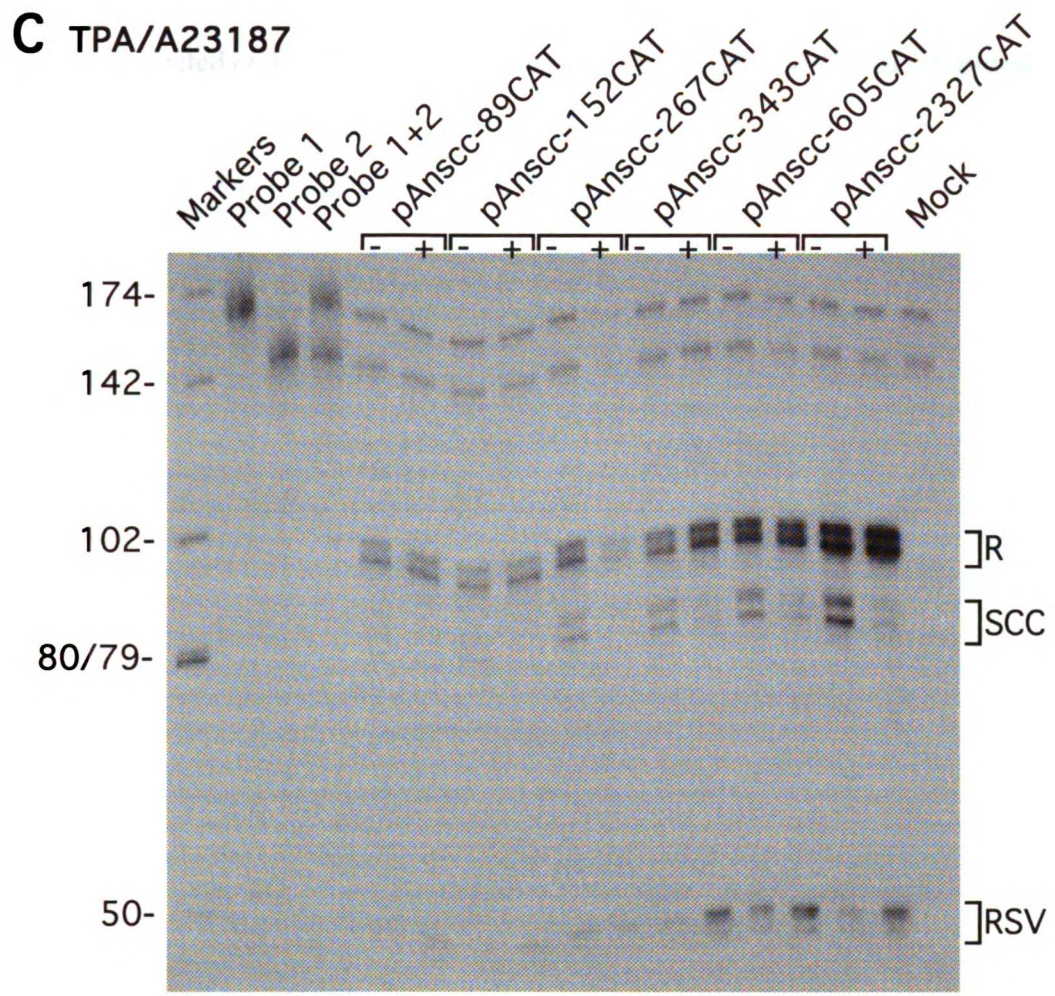


Figure 1.8. RNase protection analysis of the deletion plasmids. (A) Schematic of the RNase protection probes used for the analysis in (B) and (C). Probe 1, for human P450scc, protects the 86 bases between the P450scc mRNA cap site and the *Bam*HI site where the CAT sequences were cloned (Fig 2). Probe 2, for the Rous sarcoma virus mRNA, protects a 52 bp fragment. (B) RNase protection analysis of 20 mg of mRNA from Y1 cells transiently transfected with the series of deletion plasmids plus the pAnRSVCAT control plasmid and treated +/- forskolin (2.0×10^{-5}). (C) Similiar to panel B except cells were treated with or without TPA plus A23187 (TPA, 3.0×10^{-8} M; A23187, 5.0×10^{-7} M). Probe 1, Probe for the human scc/CAT transcripts; Probe 2, Probe for the RSVCAT internal control transcripts; R, Readthrough transcripts and/or probe annealed to the transfected plasmid; SCC, Correctly initiated transcripts from the scc promoter; RSV,

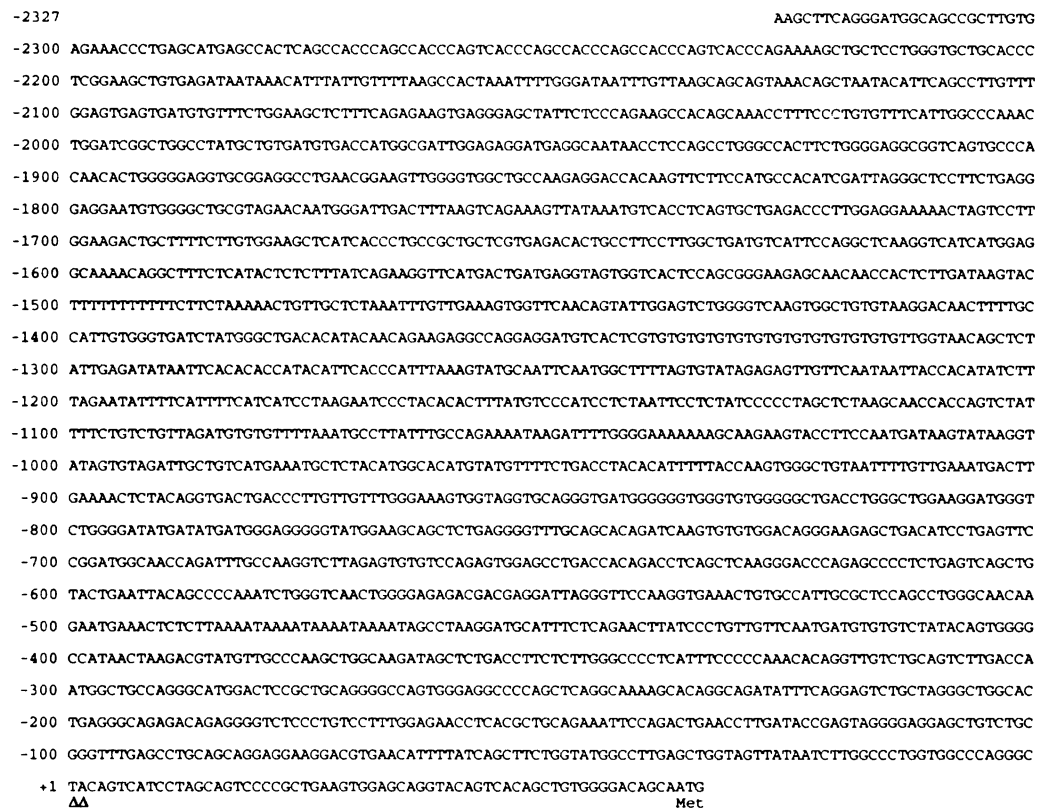


Figure 1.9. Sequence for the human gene for P450scc from -2327 bp relative to the transcriptional start site to the translation initiation codon, ATG. Both strands were sequenced by dideoxy-chain termination of double stranded templates. The sequence out to -605 coresponds exactly to that of Morohashi et al. (Morohashi, et al., 1987), except that our sequence contains two fewer TAAAA repeats between bases -467 and -486. Arrowheads mark the sites of transcriptional initiation as determined by Inoue et al. (Inoue, et al., 1988) and Chung et al. (Chung, et al., 1989). The CAAT and TATA motifs are underlined.

Table 1.1: Effect of Protein Kinase C desensitization on TPA repression of the P450scc promoter.

TPA Pretreatment	Treatment	Relative CAT activity (% of Control +/- SEM)
none	Control	100
none	Forskolin	344 +/- 22
none	Forskolin & TPA	148 +/- 10
300nM/12hr	Forskolin & TPA	298 +/- 16

Table 1.1: Y1 cells were transiently transfected in triplicate with pAnscc-2500CAT. 12 hours after adding the DNA the cells were rinsed and then treated with or without 300nM TPA for 12 hours and then the media were switched to that indicated for an additional 12 hours before harvesting cell extracts for CAT assays. Values are the average of triplicate treatments +/- the SEM.

Table 1.2: Degree of forskolin induction of pAnscc-2500CAT in the presence vs absence of TPA + A23187.

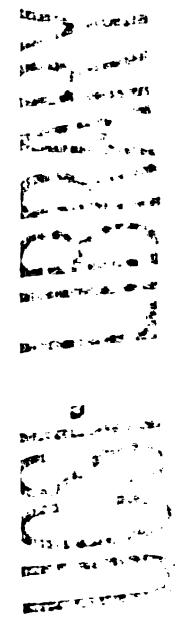
Treatment	Control	Relative CAT activity (% of Control +/- SEM)
Forskolin	Untreated	983 +/- 95
Forskolin +TPA+A23187	TPA + A23187	841 +/- 56

Table 1.2: Y1 cells were transiently transfected with pAnscc-2500CAT and treated with or without forskolin and with or without the combination of TPA and A23187. The values expressed represent induction by forskolin over untreated and induction by forskolin in the prescence of TPA/A23187 over the repressed level seen by TPA/A23187 alone.

Chapter 2

The Role of Transcriptional Regulation in Steroid Hormone Biosynthesis

Chris C.D. Moore and Walter L. Miller

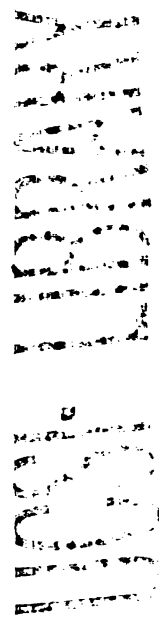


Summary

The regulated expression of the genes encoding the various steroidogenic enzymes is a crucial component in the control of steroid hormone biosynthesis. Tissue-specific transcription of each of the steroidogenic enzyme genes determines the array of enzymes present within a steroidogenic tissue, and therefore the types of steroid hormones the tissue produces. Transcriptional regulation also determines developmental changes in the steroid hormones synthesized by steroidogenic tissues and for the quantitative regulation of steroid hormones necessary for reproduction and for maintaining physiological homeostasis. The molecular mechanisms governing transcriptional regulation of steroidogenic enzyme genes is now being studied. The results so far indicate that, like most other genes, transcription of steroidogenic enzyme genes is regulated by *cis*-elements in the 5' flanking DNA of the genes that bind *trans*-acting proteins found in the nucleus. Several types of *cis*-elements have been identified: elements responsible for basal transcription, for induction by cAMP, and for both basal and cAMP induction. Some of the basal *cis*-elements identified may have a role in tissue-specific transcription of certain steroidogenic enzyme genes in steroidogenic tissues. We have also identified regions in both the human P450scc and human P450c17 promoters that repress transcription when activated by the Ca^{++}/ protein kinase C intracellular second messenger system used by angiotensin II. This review summarizes our current understanding of transcriptional regulation of the steroidogenic enzyme genes.

Introduction

Steroid hormones are important regulators of many physiological processes. While the mechanisms by which steroids signal their target cells are relatively well understood (reviewed in (Beato, 1989)) the equally important regulation of their synthesis is less clear (Miller, 1988; Waterman and Simpson, 1989). A key component in the control of steroid hormone synthesis is the regulated expression (ie transcription) of the genes encoding enzymes needed to produce a particular steroid hormone. This transcriptional control is responsible for the tissue-specific production of steroids, for the developmental regulation of steroid hormone production and for the quantitative regulation needed for reproduction and maintaining physiological homeostasis.

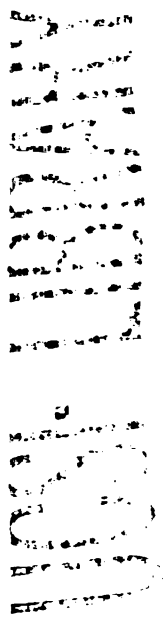


The isolation of cDNA and genomic clones for most of the enzymes involved in steroidogenesis has provided the reagents necessary for detailed analysis of the molecular mechanisms, particularly gene transcription, responsible for tissue-specific gene expression, developmental programming and quantitative regulation. From studies of the 5' flanking DNA of a variety of the steroidogenic enzyme genes, a picture is emerging in which a complex array of known *cis*-acting DNA sequence elements (*cis*-elements) and *trans*-acting nuclear proteins, as well as previously undescribed *cis*-elements and proteins, are responsible for transcriptional regulation of these genes.

The following review summarizes this information with a focus on two key enzymes in the pathway of steroidogenesis: P450scc, the cholesterol side chain cleavage enzyme, and P450c17 which mediates 17-hydroxylase and 17,20-lyase activities. P450scc is a mitochondrial cytochrome P450 enzyme that catalyzes the conversion of cholesterol to pregnenelone by three sequential reactions, all mediated on one active site. P450scc is the first and rate limiting step in steroid hormone synthesis; therefore, it is a critical quantitative regulator of steroidogenesis. P450c17 is a microsomal cytochrome P450 enzyme that catalyzes two reactions, 17 hydroxylation and 17,20 carbon bond scission, again on one active site. P450c17 is an important qualitative regulator as it occupies a crucial fork in the

pathways of steroidogenesis such that expression and regulation of its two enzymatic activities directs pregnenelone to mineralocorticoids (neither activity); to glucocorticoids (17α-hydroxylation but not C-17,20 cleavage); or to sex steroids (both 17α-hydroxylase and C-17,20 cleavage). The identity, function, hormonal control, and genetics of each of the enzymatic components in steroid hormone synthesis have been reviewed recently (Miller, 1988).

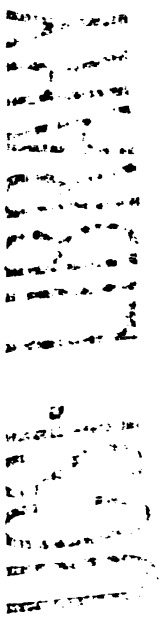
Most of the work described in the following review was done by analyzing the expression and regulation of transfected reporter constructions. Control of transcription is usually mediated by the actions of *trans*-acting proteins, such as steroid hormone receptors, which bind to specific DNA sequences (*cis*-elements) in the 5' flanking DNA of a gene. This can be studied by fusing portions of the 5' flanking DNA and promoter from the gene of interest in front of the gene for an easily assayed reporter gene such as bacterial chloramphenicol acetyl transferase (CAT). These constructions are then introduced (transfected) into cultured cells using one of a variety of chemical or physical techniques, usually by coprecipitation with calcium phosphate. When this DNA construction reaches the nucleus of the transfected cells, the DNA of interest, which was fused in front of the CAT sequences, drives transcription of the CAT gene. The resulting CAT mRNA is translated into CAT protein, which can be measured using an easy and very sensitive assay. Since the half-life of the CAT mRNA is very short, the level of CAT activity accurately reflects the transcriptional power of the sequences fused in front of the CAT reporter.



Using this approach several groups have identified regions of the 5' flanking DNA of various steroidogenic enzyme genes that are important for basal, unstimulated transcription, for regulated expression by either cAMP or Ca^{++}/protein kinase C (PKC), or for both basal and cAMP regulated transcription.

Basal Transcription

Reporter constructions driven by varying amounts of 5' flanking DNA from a number of steroidogenic enzyme genes are expressed when transfected into steroidogenic mouse adrenal Y1 cells. Such expression demonstrates that a *cis*-element(s) that confers basal transcription must exist within the 5' flanking DNA used; if the DNA used lacks such a *cis*-element no reporter activity would be seen. By deleting portions of the flanking DNA inserted in front of the reporter gene (usually progressively from the 5' end) the regions necessary for basal and regulated expression of a gene can be identified. The results of such experiments with a variety of steroidogenic enzyme genes are summarized in Figure 2.I. In some cases more precise experiments have isolated the *cis*-elements responsible for basal and regulated expression and are summarized in both Figure 2.I and in Table 2.I. Table 2.I also lists *cis*-elements that have a proposed role in transcriptional regulation based on their sequence homology to known *cis*-elements, to regions in the 5' flanking DNA of the corresponding gene in different species, or to regions in other steroidogenic enzyme genes.



The basal *cis*-elements identified so far fall into two categories. The first category of *cis*-elements only confers basal transcriptional activity. The second category of *cis*-elements is multifunctional, as they confer both basal activity and regulation by cAMP. The role of cAMP regulated *cis*-elements in conferring both basal activity and cAMP regulation was first observed with the classic cAMP response element (CRE) in the promoter for the rat gene encoding PEPCK (Delegeane, et al., 1987). This work demonstrated that basal promoter activity was partially determined by the presence of the CRE and by the basal concentration of intracellular cAMP.

To study the role of cAMP in basal expression of steroidogenic enzyme genes, several groups have used a set of mutant cell lines created from the Y1 adrenocortical tumor cell line. These mutant cell lines differ in the ability of cAMP to activate protein kinase A (PKA). In one of these PKA-deficient cell lines, the basal expression of transfected

reporter constructions driven by the murine P450c21 promoter was significantly reduced (Handler, et al., 1988). Basal expression of the endogenous genes for murine P450scc and P450c11 in the set of mutant cell lines was directly correlated with the severity of the PKA mutation (Wong, et al., 1989). The gene for P450c11 was the most sensitive to reduced PKA activity, as it was not detected in the severest mutants. By contrast, both the endogenous P450scc gene and the transfected P450c21 gene do not have an absolute requirement for PKA activity as their basal expression was reduced, but not eliminated, in the same cell lines that did not express P450c11 (Handler, et al., 1988; Wong, et al., 1989). These results strongly suggest that the basal state of the cAMP intracellular signalling pathway is important for basal expression of these steroidogenic enzyme genes.

While basal activity and cAMP-inducibility have been co-localized to the same regions of 5' flanking DNA (see Figure 2.1) of the human (Moore, et al., 1990) and bovine (Ahlgren, et al., 1990) P450scc genes and the murine P450c21 gene, the *cis*-elements responsible have not been isolated so this dual function has not yet been studied. This is not the case for the murine P450c11 gene, in which mutation of the classic CRE element (described below) markedly decreased basal reporter activity in addition to eliminating cAMP-inducibility (Rice, et al., 1989).

When a reporter construction driven by the murine P450scc promoter was transfected into the Y1 cell lines with PKA mutations, the basal activity was not reduced relative to that seen in the wild type Y1 cells (Rice, et al., 1990). As expression of the endogenous murine P450scc gene was reduced in these mutants another cAMP regulated *cis*-element with basal activity must lie outside the 5' flanking DNA used in the reporter construction. One possibility is that the *cis*-element is further upstream, as is seen in the human P450scc gene (Moore, et al., 1990).

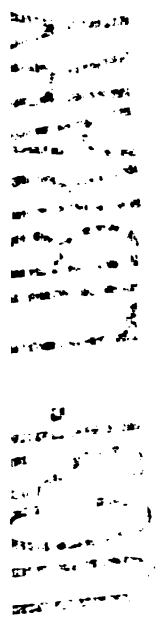
Basal *cis*-elements belonging to the first category (cAMP-independent) have been identified at bases -50, -73 and -126 of the murine P450scc promoter (Table 2.I)(Rice, et al., 1990) (Note that bases upstream from the site of transcription initiation site (+1) are

assigned negative numbers, while those downstream receive positive numbers). Mutations of these *cis*-elements severely reduced basal expression, but had no apparent effect on regulation by cAMP (Rice, et al., 1990). Similarly, the *cis*-elements in the the murine P450c11 promoter at -380 and -320 (Table 2.I) also belong to this category, as mutations in these *cis*-elements reduced basal expression but had no effect on cAMP regulation (Bogerd, et al., 1990). In the murine P450c21 promoter a large number of basal *cis*-elements have been identified. However, none of these *cis*-elements were directly tested for a role in cAMP regulation so they may or may not be cAMP-independent basal *cis*-elements (Rice, et al., 1990).

Other candidates for cAMP-independent basal *cis*-elements include the potential SP1 binding sites in the human P450scc (Moore, et al., 1990) and bovine P450scc (Ahlgren, et al., 1990) promoters. SP1 is a ubiquitous *trans*-acting protein with a role in the transcription of a number of viral and cellular genes, usually (but not exclusively) associated with "housekeeping" functions. SP1 has not been reported to mediate regulation by cAMP. In the human P450scc promoter the possible SP1 sequence is at -117 (Moore, et al., 1990); in the bovine P450scc promoter a homologous sequence is found at -109 (Table I) and a second consensus sequence is also found at -68 (Ahlgren, et al., 1990). It is not yet known if these putative SP1 sites are functional.

Another sequence with homology to a known basal transcription *cis*-element is found in the promoters for both human P450scc at -86 and human P450c21 at -67 (Yanagida, et al., 1990). These *cis*-elements resemble the basal transcription element (BTE) identified in the gene for xenobiotic-metabolizing rat P450c (Yanagida, et al., 1990). This BTE confers basal transcriptional activity and is also required for full induction by the *cis*-acting xenobiotic response elements (Yanagida, et al., 1990). The functional role of these *cis*-elements in the basal transcription of the genes for steroidogenic P450's is not yet known.

The basal transcription *cis*-elements have not yet been defined in either the human or bovine P450c17 genes, either by functional assays or by sequence homology to known *cis*-elements. However, basal expression is clearly seen with the reporter constructions used in transfection studies for both these genes (Brentano, et al., 1990; Lund, et al., 1990); therefore, the 5' flanking DNA used must contain basal transcription *cis*-elements.



Tissue-Specific Transcription

The presence or absence of a given steroidogenic enzyme in a specific steroidogenic tissue is determined at the level of transcription. Northern blots and more sensitive RNase protection assays for a variety of the key steroidogenic enzymes demonstrate that they are found only in the specific tissues that require their presence for the synthesis of the appropriate steroids (Mellon and Miller, 1989; Miller, 1989). It is this regulated expression that results in the correct array of enzymes to produce cortisol in the human adrenal while the rat adrenal produces corticosterone, or that is responsible for the different steroidogenic potentials of ovarian granulosa and theca cells.

Very little is known about the mechanisms that determine the tissue-specific expression of the individual steroidogenic enzymes. In other systems, such as somatotrope-specific expression of growth hormone, the presence or absence of one *trans*-acting protein, termed GHF-1 or Pit-1, confers tissue-specific expression (Bodner, et al., 1988; Ingraham, et al., 1988). To explain the various arrays of steroidogenic enzymes in different steroidogenic tissues by this mechanism would require a complex array of such *trans*-acting proteins whose presence, absence, and possibly abundance would determine which array of steroidogenic enzymes is expressed.

The best system for studying tissue-specific expression of steroidogenic enzyme genes is P450scc, because various transformed steroidogenic cell lines continue to express it, while non-steroidogenic cell lines do not. Several groups have shown that reporter constructions driven by human (Chung, et al., 1989; Inoue, et al., 1988; Moore, et al., 1990), murine (Rice, et al., 1990), and bovine (Ahlgren, et al., 1990) P450scc 5' flanking DNA are expressed when transfected into adrenal Y1 cells. Such constructions also work in other steroidogenic cell lines such as human JEG-3 choriocarcinoma cells (Chung, et al., 1989); C.C.D. Moore and W.L. Miller, unpub obs) and in testicular MA-10 cells (Rice, et al., 1990; C.C.D. Moore and W.L. Miller, unpub obs). By contrast, transient transfection of a variety of non-steroidogenic cell lines such as L929, HTC, Hepa I, HeLa

I, CHO or Cos-I cells (Chung, et al., 1989; Inoue, et al., 1988; C.C.D. Moore and W.L. Miller, unpub obs) results in low or undetectable expression of the P450scc promoter. These results suggest that tissue-specific regulation of P450scc expression is determined, at least in part, by a *cis*-element(s) lying within the 5' flanking DNA of the P450scc gene used in the reporter constructions, as the reporters are expressed only in the appropriate cell lines.

While the transient transfection strategy used in the studies described above can provide informative results, variations in transfection efficiency and in the expression of control constructions in different cell lines can diminish the sensitivity and reliability of the data. To avoid these problems, we are using pools of stably transfected cell lines to study the tissue specificity of the human P450scc promoter. As the selection process results in a final population only of transfected cells, the efficiency of the initial transfection no longer influences the reporter assay. Our preliminary experiments show that constructions containing up to -2327bp of 5' flanking DNA from the human P450scc gene are expressed very well in Y1 cells, but that expression is undetectable in Cos-I cells (C.C.D. Moore and W.L. Miller, unpub obs). This confirms our previous results with transient transfection analysis (Moore, et al., 1990). Rice *et al.* (Rice, et al., 1990) found that Y1 cells stably transfected with reporter constructions driven by the murine P450scc promoter expressed this construction efficiently and that it was responsive to cAMP. By contrast, this same construction was expressed relatively poorly and was not responsive to cAMP when stably transfected into testicular MA-10 cells. Thus tissue specificity is probably conferred by multiple *cis*-elements interacting with different *trans*-acting proteins in the various steroidogenic tissues, although a role for *cis*-elements and proteins common to multiple steroidogenic tissues remains possible, and is discussed below.

Direct testing for *cis*-elements that confer tissue-specific expression of the steroidogenic enzyme genes has not been done; however, a variety of *cis*-elements necessary for basal expression has been described. As *cis*-elements that confer tissue

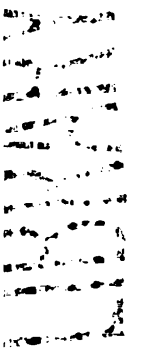
specificity will also confer basal activity, the basal *cis*-elements described above are good candidates for tissue specificity elements. Strong candidates for steroidogenic tissue specificity are the basal *cis*-elements found in the murine genes for both P450scc (at -73) (Rice, et al., 1990) and P450c21 (at -74 and -214) (Rice, et al., 1990) and in the murine P450c11 gene (at -32) (Bogerd, et al., 1990). These *cis*-elements also have sequence homology to regions in the genes for human P450scc at -772 and P450c11 at -241 and in bovine P450scc at -834 (see Table 2.I).

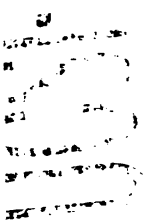
To determine if these *cis*-elements confer tissue specificity by interacting with *trans*-acting proteins expressed only in steroidogenic cell lines, several groups have used very sensitive techniques for detecting protein:DNA interactions. Although only a limited number of cell lines was assayed, the *cis*-element at -320 that confers basal activity in the murine P450c11 gene binds a protein(s) found only in extracts from adrenal Y1 and murine testicular MA-10 cells (Bogerd, et al., 1990). In addition, a *cis*-element at -140 in the murine P450c21 promoter that has a sequence similar to the P450c11 -320 *cis*-element, also binds protein only from Y1 and MA-10 cells (Rice, et al., 1990). However, similar *cis*-elements from murine P450scc at -73 and the *cis*-elements at -74 and -214 in murine P450c21 (see Table 2.I) also bind a protein(s) from HeLa and PC12 cells (Rice, et al., 1990) (Rice, et al., 1990). The amount of this protein appears to be reduced in the non-steroidogenic HeLa and PC12 cells compared to the steroidogenic Y1 and MA-10 cells. Whether all these *cis*-elements with homologous sequence bind the same protein(s) but with varying affinities (and thus confusing the issue of tissue specificity) or to different proteins is unknown.

Other basal *cis*-elements found in the human P450scc and murine P450c11 genes may also have a role in tissue specificity. For example, the protein(s) that binds the murine P450scc *cis*-element at -50 is found only in Y1 and MA-10 cell extracts (Rice, et al., 1990), and the *cis*-element at -380 in the murine P450c11 gene apparently binds a protein

found only in Y1 cell extracts. However, as the authors note, the weakness of this interaction makes this conclusion suspect (Bogerd, et al., 1990).

Some of the remaining *cis*-elements with basal activity are unlikely to have a role in tissue specificity. For example, sequences in the human and bovine P450scc promoters resemble the consensus DNA binding site for SP1. However, since SP1 is expressed in all cell types it may be necessary but not sufficient for tissue specificity. Similarly, the CRE element in the P450c11 gene that confers both basal activity and responsiveness to cAMP is also unlikely to confer tissue specificity since CREB, the *trans*-acting protein that binds to the CRE, is expressed in a wide variety of tissues. The regions responsible for the combined basal and cAMP responsive activity in the genes for murine P450c21, murine and bovine P450scc, and bovine P450c17 do not contain *cis*-elements with significant homology to the CRE and may interact with another tissue-specific protein(s) that confers both cAMP regulation and basal activity.





Hormonal Regulation

The production of steroids is regulated by several hormones in a pattern specific to each steroidogenic tissue. Examples exist for hormonal regulation by steroids themselves and by several intracellular signalling pathways; cGMP by atrial natriueretic peptide, cAMP by tropic hormones such as ACTH and gonadotropins, and intracellular Ca^{++} by angiotensin II. The regulation of steroidogenic enzyme gene transcription by cAMP is the best studied of these systems, although we have recently described regions of the human P450scc and P450c17 promoters that are repressed by agents that mimic the action of angiotensin II (Brentano, et al., 1990; Moore, et al., 1990).

Regulation by both cAMP and Ca^{++} occurs at many levels and can be divided into two phases. The first is an acute phase where steroid hormone production is altered rapidly, within minutes, and depends upon activation of pre-existing proteins to alter the synthesis of steroids from their precursors. The second, chronic phase , involves altered abundance of the steroidogenic enzymes and other components needed to increase or decrease the capacity of the tissue to produce the appropriate steroids.

Although mechanisms other than transcription can account for alterations in the abundance of a protein, direct evidence for increased transcription for a number of steroidogenic enzyme genes (P450scc, c21, c11, and c17) was initially described in bovine adrenal cells treated with ACTH (John, et al., 1986). More recently transcriptional runoff assays demonstrate that transcription of P450scc is also regulated by 8-Br-cAMP in testicular MA-10 cells (Mellon and Vaisse, 1989). In addition, transfection analysis of a reporter gene driven by either human P450scc (Moore, et al., 1990) or P450c17 (Brentano, et al., 1990) 5' flanking DNA revealed that their transcription is regulated by agents that mimic the actions of ACTH and angiotensin II in adrenal Y1 cells.

In most systems studied, the hormonal regulation of gene transcription is mediated by *trans*-acting proteins that bind to *cis*-elements in the 5' flanking DNA of the gene to alter transcription by RNA polymerase II. Such appears to be the case for hormonal

regulation of the steroidogenic enzyme genes studied so far. Figure 2.1 and Table 2.1 summarize the regions and *cis*-elements identified so far that confer regulation by cAMP and Ca^{++}/PKC on the various steroidogenic enzyme promoters.

The cAMP-regulated transcription of many genes involves one or more *cis*-elements that resemble the consensus, TGACGTCA. These cAMP response elements (CRE) are high-affinity binding sites for the *trans*-acting protein CREB (cAMP response element binding protein) (reviewed in (Karin, 1989)). CREB is apparently phosphorylated by protein kinase A in response to increased intracellular cAMP (Gonzalez, et al., 1989), in turn CREB increases gene transcription by RNA polymerase II by an as yet undetermined mechanism. Among the steroidogenic enzyme genes studied to date, a functional, classic CRE element has been identified only in the promoter of the murine P450c11 gene (Rice, et al., 1989). The region of the human P450scc promoter conferring inducibility by cAMP contains the sequence (TGATGTCA) that matches the CRE consensus at 7 out of 8 bases (Moore, et al., 1990), where the underlined base indicates the mismatch. In one study this sequence bound CREB poorly (Bokar, et al., 1988), however the sequences flanking this altered CRE in that study differ from those in the human P450scc gene, and are also important for determining CRE function (Hoeffler, et al., 1988). Thus the role of this CRE-like element in the human P450scc gene is unknown. cAMP regulation of the human P450c17 gene may also utilize a CRE-like sequence. The similar sequence TGAGCTCA lies within the 80 bases of the promoter in a region that confers some induction by cAMP, as shown by transient transfection analysis (Brentano, et al., 1990).

In other steroidogenic enzyme genes that respond to cAMP, no *cis*-elements with significant homology to the CRE, or to other *cis*-elements that confer cAMP regulation, have been found in the promoter regions responsible for cAMP induction. Within these regions only the similar *cis*-elements at -166 and -243 in the bovine genes for P450scc and P450c17, respectively were proposed to have a role in cAMP regulation but function of these *cis*-elements has not yet been demonstrated (Ahlgren, et al., 1990).

In contrast to the direct mechanism by which cAMP regulates transcription of genes with the classic CRE element that binds CREB, an indirect mechanism was proposed for the action of cAMP on bovine steroidogenic enzyme gene transcription (reviewed in (Waterman and Simpson, 1989)). In this model, cAMP would induce the expression of a labile protein(s), which in turn induced transcription of these genes. This was proposed to explain the apparently delayed cAMP induction kinetics of steroidogenic enzyme genes and the sensitivity of this induction in bovine adrenal cells to inhibition of protein synthesis by cycloheximide (John, et al., 1986). However, recent experiments rule out this model. cAMP induction of P450scc is not sensitive to inhibitors of protein synthesis in either human granulosa cells (Golos, et al., 1987) or in mouse testicular Leydig MA-10 cells (Mellon and Vaisse, 1989) and cAMP induction of adrenodoxin is similarly insensitive in placental JEG-3 cells (Picado-Leonard, et al., 1988). Also, cAMP induces transcription from the promoters of human (Moore, et al., 1990) or bovine (Ahlgren, et al., 1990) P450scc and bovine P450c17 (Lund, et al., 1990) with very rapid kinetics that are more consistent with a direct effect of cAMP. Furthermore, inhibiting protein synthesis with cycloheximide did not block cAMP induction of the transiently transfected reporter constructions driven by either the P450scc (Ahlgren, et al., 1990) or P450c17 (Lund, et al., 1990) promoters in either Y1 cells or in primary cultures of bovine adrenal cells. Thus, direct induction of P450scc and P450c17 transcription by a pre-existing protein(s) is probably responsible for cAMP regulation.

The role of protein kinase A (PKA) in induction by cAMP was studied using the adrenal Y1 PKA mutant cell lines described above. The degree of induction by cAMP for the endogenous genes for P450scc and P450c11 was significantly impaired in the mutant cell lines relative to the wild type Y1 cells (Wong, et al., 1989). Most of the induction by cAMP could be recovered by reverting the mutant phenotype by transfecting the cells with functional copies of the PKA subunits (Wong, et al., 1989). Thus, PKA is a critical component in the regulation of steroidogenic enzyme transcription in adrenal cells.

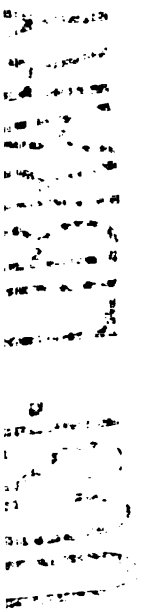
Another *cis*-acting element that can confer cAMP induction is the TPA-response element (TRE), the DNA sequence that responds to both phorbol esters such as TPA and cAMP (reviewed in Karin, 1989). TRE's bind transcription factor AP1, a heterodimer of the c-*jun* and c-*fos* proteins. The cAMP-responsive region of the human P450scc gene contains a sequence identical to the TRE consensus, TGAG/CTCA at -611 (Moore, et al., 1990). A role for this *cis*-element, or for the potential CRE at -1633, in cAMP regulation of the human P450scc gene will remain unknown until we complete further functional analysis of this region of the promoter. The absence of sequences homologous to the *cis*-elements known to confer cAMP regulation in the cAMP regulated promoters for bovine P450c17, bovine P450scc, and murine P450c21 genes suggests that cAMP regulation of these genes involves other *cis*-acting elements that may or may not bind presently known *trans*-acting proteins.

Angiotensin II regulates adrenal steroidogenesis by altering intracellular Ca^{++} concentrations and activating protein kinase C. In studies of both the human P450scc and human P450c17 promoters we used agents that mimic the actions of angiotensin II in the adrenal (reviewed in Barrett, et al., 1989). Treatment of Y1 adrenal cells with a combination of the Ca^{++} ionophore A23187 and the phorbol ester TPA markedly reduced P450scc mRNA abundance after 12 hours (Moore, et al., 1990). Treatment with TPA and A23187 of Y1 adrenal cells transiently transfected with a CAT reporter driven by the human P450scc promoter also repressed CAT activity. Similarly, the human P450c17 promoter was repressed by treatment with TPA (Brentano, et al., 1990). Thus both the human P450scc and P450c17 promoters contain *cis*-elements that repress transcription after prolonged exposure to agonists of the PKC pathway.

While it might appear surprising that agents mimicking the action of angiotensin II would repress transcription of steroidogenic enzyme genes, our results are consistent with recent experiments studying the chronic effects of angiotensin II on adrenal steroidogenesis (Mason, et al., 1986; McAllister and Hornsby, 1988) (Naseeruddin and Hornsby, 1990).

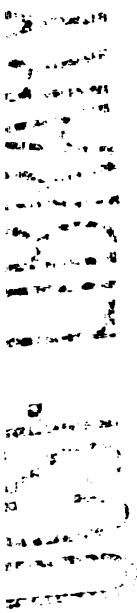
Our time course experiment revealed that short term, acute treatment with these agents increased transcription of the P450scc reporter construction at 60 minutes (Moore, et al., 1990), consistent with numerous short-term studies that studied the effects of AII on adrenal steroidogenesis. The molecular mechanism for this regulation is complex. The temporal regulation described above first involves an acute increase in transcription followed by repression of transcription after chronic treatment with A23187 and TPA. The sequences of the human P450scc gene responsible for this complex behavior are not confined to one region but are spread over a large region between -343 and -89 bp. This suggests that multiple *cis*-elements are involved. No common *cis*-elements are found within this region of the human P450scc promoter, nor are any homologies apparent between this region and the region that confers repression by TPA on the human P450c17 promoter.

Transcriptional repression is a poorly understood phenomenon in higher eukaryotes; few examples exist, especially for hormonally regulated repression. The repressive *cis*-elements in the human P450scc gene mapped to regions different from those responsible for basal or cAMP regulated expression. Thus, a simple model in which the *trans*-acting proteins are competing for the same *cis*-elements cannot explain this repression. The results are consistent with protein:protein interactions between the *trans*-acting proteins mediating repression and those conferring basal promoter activity (Moore, et al., 1990).



Conclusions

The regulation of steroid hormone biosynthesis is an important component for the endocrine function of steroids. Hormonal control of steroidogenesis includes an acute, immediate response and a chronic, prolonged response. The acute response to a tropic hormone occurs within minutes and relies on pre-existing proteins. The chronic response of a steroidogenic tissue to prolonged tropic stimulation involves altering the expression of the genes encoding the steroidogenic enzymes, primarily by regulating transcription. Transcriptional regulation is also responsible for the tissue-specific expression and developmental regulation that results in the ability of a steroidogenic tissue to produce the correct steroids in the appropriate tissue at the right time. We are just beginning to understand the molecular mechanisms that regulate steroidogenic enzyme gene transcription and the role of *trans*-acting protein factors with their cognate *cis*-acting DNA elements within their promoters. Further understanding of the mechanisms that control transcription of these genes will require more detailed analysis of these *trans*-acting proteins and *cis*-acting DNA elements using many of the techniques mentioned in this review. Better understanding of the control for each of the individual promoters will provide the groundwork for understanding the complex, integrated regulation of the enzyme arrays required for tissue-specific, developmental, and quantitative regulation of steroid hormone production in individual steroidogenic tissues.



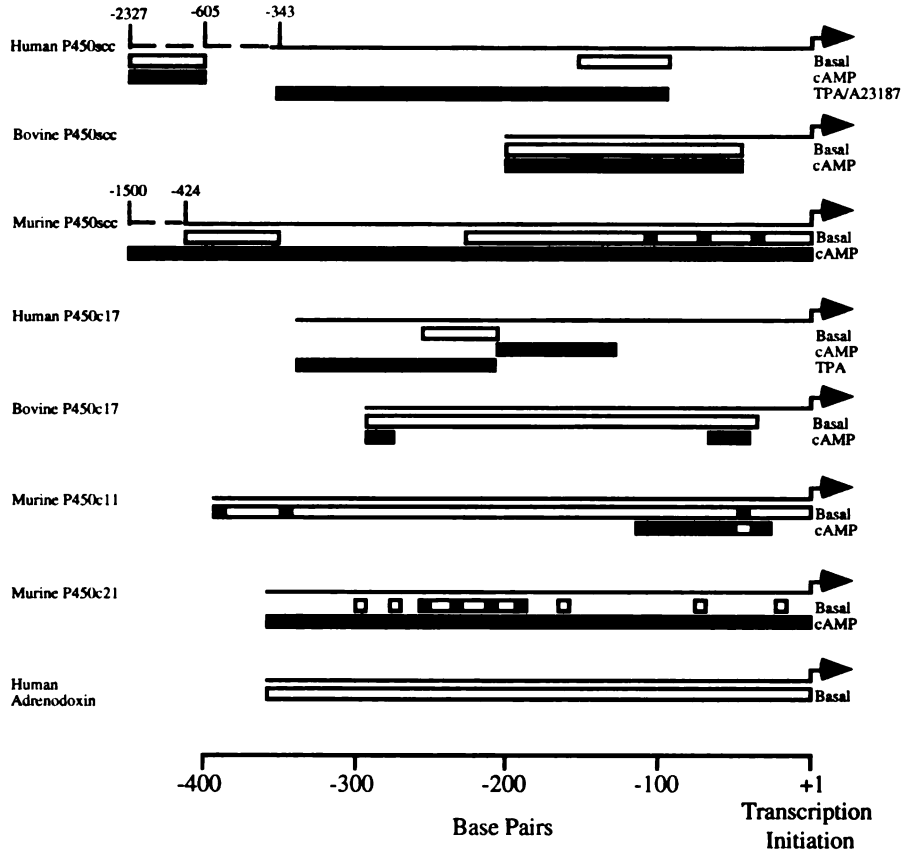


Figure 2.1. Regions of the steroidogenic enzyme promoter important for transcriptional regulation. The boxed regions confer basal or regulated transcriptional activity to transfected DNA constructions containing a reporter gene. Most of the regions were identified by progressive deletions through the 5' flanking DNA. Smaller boxes were shown to mediate the designated activity by a functional assay and are also listed in Table I.

GENE	SEQUENCE	FUNCTION[1]	REFERENCES
bovine P450scc	-68 CCGCCC	Homology to SP1 consensus	(Ahlgren, et al., 1990)
human P450scc	-117 GGGGAGGAGC	Homology to SP1 consensus	(Moore, et al., 1990)
bovine P450scc	-109 TGGGAGGAGC	Homology to SP1 consensus and to sequence in human P450scc	(Moore, et al., 1990)
human P450scc	-1633 TGATGTCA	Homology to CRE	(Moore, et al., 1990)
	-611 TGAGTCA	Homology to TRE	(Moore, et al., 1990)
murine P450scc	-50 CTCTTAGCCTTGAGCTG	Basal, binds SF-1	(Rice, et al., 1990)
murine P450scc	-126 TCTGAGTTTGGGAG	Basal, binds SF-3	(Rice, et al., 1990)
murine P450scc	-325 TAGCCATGAGTCAGGAG	Homology to TRE consensus and binds protein in Y1 extracts	(Rice, et al., 1990)
human P450scc	-86 AGCAGGAGGAAGGAC	These sequences resemble each other and	(Yanagida, et al., 1990)
human P450c21	-67 AGCAGGAGGGATGGC	sequences in xenobiotic P450 genes.	
human P450c17	-287 GGAGTCA	Homology to TRE and to negative	(Brentano, et al., 1990)
	-256 GGAGTCA	regulatory element in HIV LTR	(Brentano, et al., 1990)
	-164 TGAGCCAG	Homology to TRE	(Brentano, et al., 1990)
	-110 TGAGCTCA	Homology to CRE	(Brentano, et al., 1990)
bovine P450scc	-166 TTGATG	Homology to sequence in bovine P450c17	(Ahlgren, et al., 1990)
bovine P450c17	-243 TTGATGGACAGTGAGCAAG	Lies within cAMP responsive region and interacts with nuclear proteins	(Lund, et al., 1990)
murine P450c11	-380 CCTTAGATTTATTTTCACT Basal		(Bogerd, et al., 1990)
murine P450c11	-56 TGACGTGA	cAMP regulation	(Rice, et al., 1989)
murine P450c21	-74 AGGTCA	Basal	(Rice, et al., 1990)
murine P450c21	-214 AGGTCA	Basal	
murine P450scc	-73 GGGGAGGTCACCGCTCCA	Basal, binds SF-2	(Rice, et al., 1990)
murine P450c21	-150 GATTCTCCAAGGCT	Footprint	(Rice, et al., 1990)
murine P450c11	-320 ACCAAGGTCTTCTATGATATC	Basal. These sequences are all homologous	
bovine P450c21	-251 AGGTCA	to each other and to elements in murine	
human P450c21	-241 AGGTCA	c11, scc and c21 with basal activity	
bovine P450scc	-834 AAGGTCA	(Bogerd, et al., 1990; Rice, et al., 1990;	
human P450scc	-772 CCAAGGTCTT	Rice et al, 1990). In the case of the c21 genes the homology extends further than shown across species (Parker, et al., 1986).	
murine P450c21	-128 CTGTGCCAATGTGAAA	Basal	(Rice, et al., 1990)
murine P450c21	-177 GATGGTCCCATC	Basal	(Rice, et al., 1990)
murine P450c21	-185 TTCTTCTTGA	Basal	(Rice, et al., 1990)
murine P450c21	-237 AGTTAGGAAACT	Basal	(Rice, et al., 1990)
murine P450c21	-259 TCCGCCTACT	Basal	(Rice, et al., 1990)
murine P450c21	-288 TGGTGGGAGGGG	Basal	(Rice, et al., 1990)

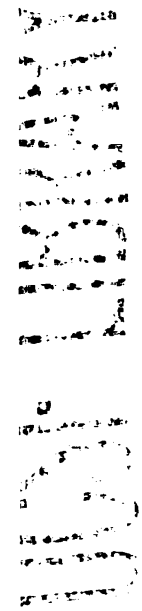
[1] If the function has been demonstrated it is listed, otherwise the homology to known elements, or to regions of other steroidogenic genes, is given and functionality awaits demonstration.

Table 2.I. Functional and potentially functional *cis*-acting DNA elements in the promoters of steroidogenic enzyme genes. The sequences are presented 5' to 3' reading towards the transcriptional initiation site (+1). The number preceding the sequence is the 5' most base of the sequence. Bases underlined indicate that sequence homology exists with other sequences listed in the table. Such homologous sequences are grouped together.

Chapter 3

Identification of Positive and Negative Placenta-Specific Basal Elements and a Cyclic Adenosine 3',5'-Monophosphate Response Element in the Human Gene for P450scc

Chris C.D. Moore, Dean W. Hum, and Walter L. Miller



Abstract

The chronic regulation of steroiodgenesis is mediated principally by transcriptional regulation of the genes encoding the various steroidogenic enzymes. The cholesterol side-chain cleavage enzyme, P450scc, is rate-limiting and hormonally regulated in a tissue-specific fashion. Human placental steroidogenesis is regulated by LH and hCG through increased intracellular cAMP, and forskolin or 8-Br-cAMP increase the abundance of human P450scc mRNA in human JEG-3 choriocarcinoma cells. We transfected JEG-3 cells with 24 promoter/reporter constructions to examine the tissue-specific and hormonally induced transcription of the human P450scc gene in these cells. A reporter construction containing only bases -79 to +49 of the human P450scc gene was expressed in JEG-3 cells. This basal expression was increased by four elements, especially by a powerful element between -152 to -142. Adding DNA sequences to -177 suppressed the basal expression seen with the -152 construction, indicating that a repressor element lies between -177 and -152. Thus, basal expression of the human P450scc gene in JEG-3 cells is mediated by the interplay of several separate *cis*-acting DNA elements. Forskolin induction was conferred by sequences between -108 and -89. The mechanism for cAMP induction appears to be direct as this induction is rapid and is not blocked by inhibiting protein synthesis with cycloheximide. Gel-mobility shift experiments identified six specific DNA:protein complexes. Five of these complexes correlate closely with the basal transcription activities identified by the reporter assays. The powerful basal element, the repressor element, and the cAMP element differ from those identified by similar experiments in mouse adrenal Y1 cells suggesting that the human P450scc gene is regulated by the tissue-specific use of different regulatory elements.

Introduction

Steroid hormones, which work primarily by controlling transcription of specific genes, are critical regulators of many physiological processes. The first and rate limiting step in the production of all steroid hormones is the conversion of cholesterol to pregnenolone by the cholesterol side chain cleavage enzyme, P450scc. This mitochondrial cytochrome P450 enzyme receives electrons from NADPH via two intermediates, the flavoprotein adrenodoxin reductase, and the iron-sulfur protein adrenodoxin (Miller, 1988). P450scc catalyzes three sequential reactions - 22 hydroxylation, 20 hydroxylation, and $C_{20,22}$ bond cleavage at a single active site (reviewed in ref. (Miller, 1988)). Human P450scc is encoded by a single, unique gene (Morohashi, et al., 1987) formally termed CYP11A (Nebert, et al., 1991) on chromosome 15q23-q24 (Sparkes, et al., 1991). As side chain cleavage is the rate-limiting step in steroidogenesis it is important to understand both the hormonal regulation and tissue-specific expression of the gene encoding P450scc.

Human steroidogenic tissues express P450scc in different developmental patterns (Voutilainen and Miller, 1986; Voutilainen and Miller, 1988; Waterman and Simpson, 1989) and in response to different hormonal stimuli (Miller, 1988; Miller, 1989; Waterman and Simpson, 1989); thus transcriptional regulation of the P450scc gene is complex. Steroidogenesis and accumulation of human P450scc mRNA are stimulated by LH and CG in human placental cytotrophoblasts and testicular Leydig cells, by ACTH in the human adrenal zonae fasciculata and reticularis, and by LH and FSH in human ovarian granulosa cells (Chung, et al., 1986; DiBlasio, et al., 1987; Golos, et al., 1987; Picado-Leonard, et al., 1988; Ringler, et al., 1989; Voutilainen and Miller, 1987; Voutilainen, et al., 1986). In all of these cases the stimulatory hormone binds a cell-surface receptor that activates a G-protein (G_s) to increase intracellular cAMP, which increases transcription of the P450scc gene (Ahlgren, et al., 1990; Brentano and Miller, 1992; Chung, et al., 1989; Inoue, et al., 1988; John, et al., 1986; Mellon and Vaisse, 1989; Moore, et al., 1990; Rice, et al., 1990). By contrast, angiotensin II acutely stimulates mineralocorticoid production in the adrenal

zona glomerulosa by alterations of intracellular Ca^{++} and activation of protein kinase C (PKC) (reviewed in ref. (Brentano and Miller, 1992)). Prolonged activation of these pathways, however, represses transcription of the P450scc gene (Moore, et al., 1990). Thus, regulation of steroidogenesis is a tissue-specific process and is regulated by a variety of hormones working through at least two different intracellular second messenger systems.

The placenta produces P450scc mRNA (Chung, et al., 1986; Ringler, et al., 1989) which it uses in producing progesterone, but in contrast to adrenal steroidogenesis, little is known about the expression and regulation of steroidogenic enzymes in the placenta. JEG-3 cells are a stable transformed human placental cytotrophoblast cell line (Kohler and Bridson, 1971) that retains many characteristics of human cytotrophoblast biology (Ringler and Strauss, 1990). They contain P450scc and its mRNA, and synthesize pregnenolone in a hormonally responsive fashion similarly to isolated cytotrophoblast cells (Golos, et al., 1987; Picado-Leonard, et al., 1988; Ringler, et al., 1989). Thus JEG-3 cells should contain the nuclear proteins needed for placental-specific and hormonally-responsive expression of the human P450scc gene.

The role of protein synthesis in cAMP-mediated induction of steroidogenesis has been controversial. In bovine adrenal cells ACTH or cAMP induce transcription of the genes for several steroidogenic enzymes, but that induction is blocked by inhibiting protein synthesis with cycloheximide (John, et al., 1986). These results suggested that cAMP first increases the transcription and translation of a factor required for transcription of the steroidogenic enzymes (Waterman and Simpson, 1989). However, this model is not supported by more recent results. Although cAMP-induced accumulation of P450scc mRNA is also slow and inhibited by cycloheximide in human JEG-3 cells (Picado-Leonard, et al., 1988) and cytotrophoblasts (Ringler, et al., 1989) as it is in bovine adrenal cells, P450scc mRNA is unaffected by cycloheximide in human ovarian granulosa cells (Golos, et al., 1987) or mouse Leydig MA-10 cells (Mellon and Vaisse, 1989) and neither

adrenodoxin (Picado-Leonard, et al., 1988; Ringler, et al., 1989) nor adrenodoxin reductase (Brentano, et al., 1992) follows the pattern of P450scc in human placental cells. Transcription of the P450scc gene in mouse MA-10 cells is insensitive to cycloheximide (Mellon and Vaisse, 1989), and cAMP induction of reporter plasmids controlled by the human (Moore, et al., 1990) and bovine (Ahlgren, et al., 1990) P450scc promoters was rapid, inconsistent with the need for protein synthesis before induction. Thus cAMP may induce steroidogenesis by different mechanisms in different cell types.

We recently identified regions in the human P450scc promoter that confer basal expression, cAMP induction, and repression by the Ca^{++}/PKC pathway when transfected into mouse adrenal Y1 cells (Moore, et al., 1990). To determine if these regions serve similar functions in human placental cells, we transiently transfected JEG-3 cells with reporter constructions and found three regions important for basal transcription. Sufficient *cis*-acting elements lie between -79 and +49 to drive correctly initiated basal transcription. At least three, and possibly four basal elements lie between -79 to -152, the most powerful lying between -152 to -142. A potent repressor element lies upstream between -177 and -152. Regulation by cAMP was mediated by sequences between -108 and -89. cAMP induction was not blocked by inhibiting protein synthesis, thus induction of the P450scc promoter by cAMP appears to be direct. Gel mobility shift experiments identified six specific DNA:protein complexes between JEG-3 nuclear proteins and fragments of the P450scc promoter. Five of these interactions co-localize with regions identified by the reporter assays as important for basal, or cAMP regulated transcription, or both.

These results differ profoundly from results in the murine adrenal Y1 cells. In Y1 cells sequences between -152 to -89 function as a basal element that is much less powerful than in JEG-3 cells, and no repressor activity was found beyond -152 (Moore, et al., 1990). In Y1 cells sequences between -1697 and -1558 confer cAMP responsiveness (Inoue, et al., 1991), whereas in JEG-3 cells cAMP regulation lies between -108 to -89. Thus, the human P450scc gene is regulated by a very complex *cis*-acting transcription

apparatus that is utilized differently by two steroidogenic tissues, suggesting that different *trans*-acting factors may serve similar functions in these two cell types.

Results

Regulation of endogenous P450scc mRNA in JEG-3 cells.

To determine how the human P450scc gene is regulated in JEG-3 cells, we treated them with known or suspected regulators of placental steroidogenesis and measured endogenous P450scc mRNA by Northern blots. Treatment with $1.2x10^{-5}$ M forskolin, (Fig. 3.1A), or $1.0x10^{-3}$ M 8-Br-cAMP (not shown) for 12 h increased the abundance of P450scc mRNA compared to vehicle (EtOH) alone. The calcium ionophore A23187 ($5.0x10^{-7}$ M) reduced P450scc mRNA or had no effect, while the phorbol ester, TPA ($3.0x10^{-8}$ M), had no effect compared to vehicle (DMSO) alone. TPA plus forskolin increased P450scc mRNA abundance above either forskolin alone or TPA alone, but TPA plus A23187 was not consistently different from the DMSO control. Reprobing the blots for human nuclear lamin A (Fisher, et al., 1986) showed that forskolin increased lamin A mRNA consistent with the known slight inductive effect of cAMP on lamin, but the induction was less than that for P450scc, indicating that the effect of forskolin on the P450scc mRNA abundance is specific (Fig. 3.1B).

Regulation of P450scc promoter/CAT reporter plasmids.

To determine if the 5' flanking DNA of the human P450scc gene regulates transcription in placental cells we transiently transfected JEG-3 cells with a promoter/reporter construction containing bases -605 to +49 relative to the transcriptional start site. Basal CAT activity from pAnscc-605CAT was significantly above background and about 20% of the control plasmid, pAnTK-109CAT (Fig. 3.2). Treatment with forskolin increased the P450scc-driven CAT expression by about 750%, but had no effect

on the expression of pAnTK-109CAT. Thus, *cis*-acting DNA regulatory elements required for basal expression and for forskolin regulation of human P450scc gene transcription lie between -605 to +49.

To identify the *cis*-acting DNA elements controlling placental transcription of P450scc we transiently transfected JEG-3 cells with six P450scc/CAT constructions containing progressive deletions of the 5' flanking DNA and measured the resulting CAT activity (Fig. 3.3A). All of these deletion constructions, including the shortest construction, pAnscc-89CAT, produced CAT activity at least 1.5 to 2 times greater than background. Adding sequences to -152 increased the basal promoter activity to about 12,600 % over that seen with pAnscc-89CAT. This tremendous increase in basal promoter activity was repressed to 550 % of pAnscc-89CAT by adding 5' sequences to -257. Longer constructions had basal expression similar to that of pAnscc-257CAT, except a slight reduction was seen with pAnscc-2327CAT. These results indicate that a functional promoter lies within -89 to +49, a powerful basal transcription element lies between -89 and -152 and a repressor element lies between -152 and -257.

To characterize forskolin regulation of the P450scc promoter, and to maximize the sensitivity of the assay, we examined the time course and dose-response of forskolin induction of pAnscc-605CAT. Induction reached maximal at 3.0×10^{-6} M then plateaued (Fig. 3.3B), thus 6.0×10^{-6} M forskolin was used for subsequent experiments. Forskolin rapidly increased CAT activity, and although it was still approaching equilibrium at 24 hours; half of this induction occurred by about 7 hours (Fig. 3.3C). This is similar to the 6 hour time for half-maximal induction seen with this promoter in Y1 cells (Moore, et al., 1990), and faster than the $t_{1/2}$ of about 15 hours for the CREB-activated human chorionic gonadotropin α-chain promoter in JEG-3 cells (Jameson and Lindell, 1988; Milsted, et al., 1987). Such rapid induction by cAMP is consistent with the direct activation of P450scc transcription by pre-existing proteins.

To locate cAMP-responsive regions of the P450scc promoter we transiently transfected JEG-3 cells with the same plasmids used in Fig. 3.3A. The negative control plasmid, pAnTK-109CAT, did not respond to treatment with forskolin (Fig. 3.3D). Although a small response was seen with the shortest promoter fragment, pAnscc-89CAT, adding sequences to -152 had little effect. However, all plasmids with sequences beyond -152 responded to forskolin by 440% to 600% over the corresponding untreated samples, suggesting that a cAMP responsive element lies between -257 and -152.

To confirm the location of the elements conferring basal induction, basal repression, and cAMP-responsiveness identified by deletional mutagenesis, we built two internal deletion plasmids that retain sequences between -86 and + 49 and between -343 to -253, but lack the regions apparently responsible for basal expression (-148 to -86), or for basal repression and cAMP induction (-252 to -153) (Fig 3.4A). Basal expression from pAnsccΔ148/86CAT, which lacks bases -148 to -86, was markedly reduced, confirming the location of a basal element in this region (Fig. 3.4B). Basal expression from pAnsccΔ252/153CAT, which lacks bases -252 to -153, was greatly increased (Fig. 3.4B), confirming the presence of a basal repression element in this region. However, pAnsccΔ148/86CAT, which should retain the putative cAMP-responsive element identified by the deletional mutagenesis, responded poorly to forskolin, while pAnsccΔ252/153 was induced about 400% (Fig. 3.4C), implying that a cAMP response element lies between -148 and -86, rather than between -252 and -153.

Regulation of P450scc promoter/luciferase reporter plasmids.

To resolve this discrepancy in the location of cAMP-responsive element and to locate basal induction and repression elements more accurately, we built higher resolution deletion plasmids using *Bal* 31 digestion or PCR and fused these to a more sensitive luciferase reporter system. Basal activity of these deletion/luciferase plasmids (Fig. 3.5A) is qualitatively very similar to the results with the deletion/CAT plasmids in Fig. 3.3A.

Greatly increased basal activity is found with the -152 plasmid that is then repressed by adding sequences beyond -152. The increased resolution of the deletion/luciferase plasmids more clearly defines the locations of these basal elements, and suggests that several elements are involved. A slight increase in basal activity is seen by adding sequences from -79 to -110 and a further increase is seen between -110 to -127. The greatest increase was again with the -152 construction, to 2700% of the p-79LUC control. Repression of this tremendous basal activity seen with p-152LUC is mediated mostly by the sequences between -152 to -177, though further repression is seen with p-206LUC and p-222LUC. As was seen with the CAT plasmids the longest construction, p-2327LUC, also has reduced basal expression. Thus, basal transcription of the human P450scc gene in placental JEG-3 cells is conferred by the actions and interplay of many *cis*-elements, some conferring increased transcription and others repressing transcription.

JEG-3 cells transfected with the fifteen deletion/luciferase plasmids were then treated with $6x10^{-6}$M forskolin (Fig. 3.5B), resolving the apparent discrepancy between the results of the deletion mutants and internal deletions fused to CAT (Figs. 3.3D and 3.4). Forskolin induced both p-79LUC and p-89LUC between 350% and 400% over untreated, which is not much greater than the 310% non-specific induction seen with the promoterless luciferase vector p-ΔLUC. This non-specific induction was not seen with the CAT constructions and may reflect the use of pAnRSVCAT to normalize the data, as pAnRSVCAT activity decreased between 50% and 75% in response to forskolin. Addition of sequences between -89 and -108 tripled induction over untreated, indicating that a cAMP-responsive element lies in this region. Addition of sequences to -127 reduced forskolin responsiveness slightly to about double the control value, but, with the notable exception of p-152LUC, all plasmids containing 127 or more bases of the P450scc promoter had essentially the same responsiveness to forskolin. This is the same result seen with the CAT constructions, where pAnscc-152CAT responded poorly but all longer constructions gave a 3-fold increase in CAT activity over the pAnTK-109 control (Fig.

3.3D). The forskolin insensitivity of the -152 constructions could indicate a negative element between -142 and -152. However, we believe it is more likely that the -152 construction, which exhibits such great basal activity (Fig. 3.3A and 3.5A) is being transcribed maximally so that forskolin cannot amplify this transcription further. Thus the initial deletion mutants with CAT (Fig. 3.3) detected cAMP responsive sequences with constructions longer than -152, but examining only -89 and -152 constructions failed to detect the forskolin-responsive element between -89 and -108. The data in Fig. 3.5 also show an inverse relationship between basal activity and responsiveness to forskolin. As both the CAT and luciferase assays were well within their linear ranges, an interaction between basal promoter activity and induction by cAMP is likely.

To verify that the LUC activity originated from correctly initiated transcripts we did RNase protection assays for the chimeric P450scc/LUC mRNA (Fig. 3.6). Transcripts initiated from the principal cap site of P450scc will protect 684 bases of probe, including 636 bases of LUC vector mRNA and 48 bases of the 5' untranslated region of P450scc mRNA. Transcripts spuriously initiated in the vector or correctly initiated mRNA transcribed throughout the full 5 kb circumference of the plasmid would protect 766 bases of the probe. The promoterless plasmid ΔLUC yields a band of protected luciferase RNA, despite the two polyadenylation sites inserted upstream from the polylinker cloning site. However, the ΔLUC plasmid yields essentially no luciferase activity (Fig. 3.5A) indicating that this spuriously initiated RNA is inactive. The abundances of the correctly initiated transcripts and the corresponding luciferase activities correlate well. In transfected JEG-3 cells not stimulated with forskolin, the abundances of correctly initiated transcripts from p-79LUC and p-108LUC are about the same, but RNA produced by the p-152LUC plasmid is vastly greater, while that produced by p-177LUC is intermediate between p-152LUC and the shorter plasmids. This pattern corresponds with the pattern of LUC activity seen for these plasmids (Fig. 3.5A) and for the related CAT constructions (Fig. 3.3). Similarly, forskolin minimally affects correctly initiated transcripts from p-79LUC

but greatly increases transcripts from p-108LUC, consistent with the localization of a cAMP-response element between -89 and -108 (Fig. 3.5B). Thus the RNase protection data confirm the locations of the regulatory elements identified by the CAT and LUC functional assays in Figs. 3.3 and 3.5 and confirm that the LUC constructions are directing accurately initiated transcription from the P450scc promoter.

Effects of inhibiting protein synthesis with cycloheximide.

Northern blots of P450scc mRNA from JEG-3 cells treated with and without cycloheximide and cAMP indicate that cycloheximide reduces the accumulation of both basal and forskolin-induced P450scc mRNA (Fig. 3.7A). This result is equivalent to those seen earlier (Brentano and Miller, 1992; Waterman and Simpson, 1989). To determine if this is due to a direct effect of cAMP on transcription of the human P450scc promoter, we used the RNase protection assay employed in Fig. 6 to measure accurately initiated transcripts from the cAMP-responsive p-108LUC construction in transfected cells treated with cycloheximide, forskolin, or both (Fig. 3.7C). Similarly to the results of Northern blotting of endogenous JEG-3 cell P450scc mRNA, forskolin alone substantially increased the abundance of the hybrid P450scc/LUC mRNA. However, in contrast to the Northern blotting results, cycloheximide alone also slightly increased the P450scc/LUC mRNA. Furthermore, blocking protein synthesis with cycloheximide did not block (and may have facilitated) the action of forskolin to stimulate transcription of p-108LUC. Thus the effects of forskolin or cAMP are mediated directly by pre-existing protein. Both the transcription of the promoter/reporter constructions (Fig. 3.3 and 3.5) and RNA polymerase run-on assays (Brentano and Miller, 1992) indicate that this action of forskolin or cAMP is to increase transcription.

DNA:Protein interactions.

The various deletion reporter constructions identified a number of *cis*-acting DNA sequences that regulate expression of the human P450scc promoter in JEG-3 cells. To begin characterizing the *trans*-acting proteins interacting with these *cis*-elements, we performed gel mobility shift assays using overlapping DNA fragments from the human P450scc promoter as probes and competitors. JEG-3 cell nuclear proteins shifted these fragments in patterns revealing six independent protein:DNA complexes termed I to VI (Fig 3.8 and 3.9). When labeled double-stranded DNA encompassing bases -276/-211 was incubated as a probe with nuclear proteins it formed complex I (Fig. 3.8A). Specific competition by a 100-fold molar excess of unlabelled probe (-276/-211) inhibited its formation, confirming the specificity of this complex (Fig. 3.8A). By contrast, competition with DNA fragments (-207/-148 and -153/-64) from regions adjacent to -276/-211 did not inhibit formation of complex I. Additional gel shift patterns of minor intensity can also be seen in Fig. 3.8A. However, these complexes are non-specific, as they are not inhibited by excess unlabelled probe.

Incubation of a probe encompassing bases -207 to -148 with JEG-3 nuclear proteins gave rise to two specific complexes (II and III). Formation of complexes II and III is inhibited only by excess unlabelled probe and not by unrelated DNA fragments (Fig. 3.8B). An additional complex that migrated between complexes II and III is non-specific as it was inhibited by excess amounts of fragments -276/-211 and -153/-64 but not by the probe (-207/-148) (Fig. 3.8B). Furthermore, formation of complexes II and III was not affected by the presence or absence of the additional complex.

Probe -153/-114 gave rise to complex IV, again inhibited by competition with a 100-fold molar excess of unlabelled probe, but not by competition with equivalent amounts of unrelated DNA fragments (Fig. 3.8C). As shown in Fig. 3.8D, gel shift analyses with a larger probe (-153/-64) yielded an additional specific complex (V) which migrated with slower mobility than complex IV. Due to its lower abundance, complex IV is not readily

seen in Fig. 3.8D, but is easily identified on over-exposure of the autogradiograph. Complex IV is also inhibited by competition with DNA fragments -154/-85 and -177/-114 without affecting complex V, thus demonstrating that formation of complex V is independent of complex IV (data not shown). Additional experiments using probes -276/-148 and -177/-114, which overlap the ends of the above-mentioned DNA fragments, did not reveal formation of additional complexes.

To define the locations of these protein:DNA complexes more precisely we used a series of smaller probes that localized the interactions to regions of only 27 to 39bp (Fig. 3.9). The protein binding sites leading to complexes I, IV and V were defined to within fragments -276/-247, -153/-114 and -91/-64 respectively. The relative differences in band intensities for complexes II and III seen in Fig. 3.8B and 3.9B probably reflect the use of different batches of nuclear extract. Formation of each complex was inhibited by competition with 100-fold molar excess of unlabelled probe. Similar competition experiments localized complex II within fragment -207/-177 and complex III within fragment -177/-148 (Fig. 3.9B). An excess of unlabelled probe (-207/-148) effectively competed both complexes II and III. However, fragment -177/-148 only inhibited complex III. Thus, formation of complexes II is independent of complex III. Fig. 3.9C shows the detection of another DNA/protein complex not initially detected in Fig. 3.8D. Using a probe encompassing bases -117 to -85, we detected Complex VI. Formation of this complex was inhibited by a 200-fold molar excess of unlabeled probe but not by a 400-fold molar excess of "Oct-Hep" oligonucleotide, which contains the sequence CTCTCATGAATATGCAAATCAG for the Octamer class of DNA binding proteins (Poellinger and Roeder, 1989).

The locations of complexes II, III, IV, V and VI correlate well with regions identified as functionally important in the promoter/reporter assays. Complex I, formed by sequences between -276 to -247, is in a region that did not confer either basal expression or cAMP regulation. It is possible this interaction is functionally redundant, or has a function

that we were not testing with the reporter assays. Complexes II (-207 to -178) and III (-177 to -148) correlate with the repression seen with p-177LUC and p-206LUC. Complex IV (-153 to -114) binds to a region that confers basal activity, whether this complex is responsible for the increased basal activity seen with p-127LUC or that with p-152LUC, or both is unknown. Complex V (-91 to -64) may represent an interaction responsible for some of the basal activity of the shortest deletion plasmid we used, p-79LUC, or for the slight increase in basal activity seen with p-89LUC over p-79LUC. Finally, Complex VI (-117 to -85) correlates well with the region conferring cAMP-responsiveness identified in Fig. 3.5B.

DNase I footprinting of the 206 bases upstream from the transcriptional start site shows footprints at -42 to -54, -86 to -102, and three large, poorly-resolved footprints between -118 and -194 (Fig. 3.10). The locations of these footprints correlate well with both the functional data from deletional mutagenesis (Fig. 3.5) and the band shifting data (Figs. 3.8 and 3.9), indicating that we have identified candidates for the *trans*-acting proteins involved in the transcription of the human P450scc promoter in placental JEG-3 cells.

Discussion

Analyses of the various deletion mutants reveal six potential functional elements in the P450scc promoter in JEG-3 cells. Some basal promoter activity is found in the first 79 bases, which contain a TATA box and a possible CAAT motif (CATT at -63) and yields a footprint at bases -42 to -54. Adding 10 bp increased basal activity slightly; this region contains the sequence $^{-86}$AGCAGGAGGAAGGACG^{-71} (mismatches underlined), which is similar to the "basal transcription element" (BTE) in the human liver P450c gene (Yanagida, et al., 1990). A portion of this region, -91 to -64, formed DNA:protein complex V (Fig. 3.8). But we do not know if this complex is related to protein binding to the BTE of the P450c gene. Adding the sequences between -89 and -108 increased basal

transcription about 2 fold (Fig 3.5A) but this region was more important for cAMP-induced transcription (see below).

Adding the sequences from -110 to -127 increased basal transcription further (Fig. 3.5A). This region contains the sequence, $^{-117}$GGGGAGGAGC^{-108}, which closely matches the consensus SP1 site, $^{G}/_{T}$GGGCGG$^{G}/_{A}{}^{G}/_{A}{}^{C}/_{T}$ (Dynan, et al., 1986). As Sp1 is expressed ubiquitiously, and it increases the basal transcription of many promoters, it is a good candidate for the basal element between -110 to -127. However, this region did not form a detectable DNA:protein complex in the mobility shift assays or a detectable footprint under the conditions we used.

The greatest increase in basal transcription is conferred by the sequences between -142 and -152. The possibility that this powerful element is artificially created by linking -152 to the pUC polylinker in the CAT constructions was eliminated by the internal deletion construction pAnsccΔ252/153 CAT, which retains the high basal activity seen in the -152 deletion mutants but lacks a fusion between -152 and the pUC polylinker. The DNA sequence from -114 to -152 produced protein:DNA complex IV in the gel shift assays. Thus the protein forming complex IV is a good candidate for a basal transcription factor. This region is similar to a portion of a poorly defined progesterone-responsive region in the chicken lysozyme gene (Hecht, et al., 1988), and to the transcription factor site 2 in the *Drosophila* HSP 70 gene (Topol, et al., 1985). The region between -89 to -152 also increased basal transcription of the P450scc promoter in Y1 cells (Moore, et al., 1990), but this increase was only about 500%, and was no greater than that seen with longer constructions. Thus the large increase in basal transcription shown in Figures 3A and 5A is specific to JEG-3 cells.

Adding DNA sequences beyond -152 eliminated most of the basal activity of the -152 constructions (Fig. 3.3A and Fig. 3.5A), indicating the presence of a powerful basal repressor element between -152 and -177. As the -206 plasmid reduced the basal activity below that seen with -177LUC, there may be a second, weaker repressor element between

-177 and -206. Both of these regions form unique DNA:protein complexes. Complex III, formed by sequences between -148 and -177, may identify the protein conferring the powerful repression between -152 and -177; complex II, formed by sequences between -178 and -207, may identify the potential second repressor. This compound repressor system might suppress RNA polymerase II activity directly; however, two lines of evidence suggest it may work through a negative interaction with the nearby powerful basal element at -142 to -152. First, the negative elements only eliminate the increased transcription provided by -142 to -152; plasmids containing promoter sequences of -206 or longer have the same activity as -142. Second, the activity of internal deletion plasmid pAnsccΔ148/86 CAT is not less than that expected for a P450scc promoter lacking the proximal basal elements. The repressor activities of -152 to -206 were not seen when these constructions were transfected into mouse adrenal Y1 cells (Moore, et al., 1990), providing further strong evidence for tissue-specific regulation of basal transcription in JEG-3 cells.

TPA and A23187, a phorbol ester and a calcium ionophore, did not not affect the abundance of P450scc mRNA in JEG-3 cells, hence we did not test these agents on the promoter constructions. As agents that stimulate the protein kinase C pathway, they mimic the actions of angiotensin II on adrenal and Y-1 cell P450scc gene transcription (Moore, et al., 1990). However no known regulator of placental steroidogenesis works through protein kinase C, hence the insensitivity of P450scc mRNA to these agents in JEG-3 cells is not surprising.

Placental steroidogenesis is regulated by LH and hCG via cAMP, although other agents such as EGF (Ritvos, 1988), TPA (Ritvos, 1988), A23187 (Kasugai, et al., 1987), and IGF-I (Nestler and Williams, 1987) may also be important. cAMP can regulate gene transcription through *cis*-acting cAMP-response elements (CRE, consensus binding site TGACGTCA) that bind a family of related CRE-binding proteins (CREB) (Gonzalez, et al., 1989; Hoeffler, et al., 1988) as well as a number of other proteins (discussed in reference (Gonzalez, et al., 1989; Hoeffler, et al., 1988)). cAMP can also increase

transcription through *cis*-acting TPA response elements (TREs), (consensus binding sites CCCCAGGC or TGA$^{C}/_{G}$TCA) (Hoeffler, et al., 1989; Imagawa, et al., 1987), for review see (Karin, 1989). The P450scc promoter construction containing 605 bp of 5' flanking DNA responded to forskolin in JEG-3 cells, showing that cAMP increases P450scc mRNA in the placenta by increasing gene transcription. Our luciferase reporter constructions clearly defined a cAMP-responsive element between -89 and -108. This location is substantially different from the cAMP responsive sequences that function in Y1 cells (Inoue, et al., 1991; Moore, et al., 1990). Progressive deletions of the human P450scc promoter expressed in Y-1 cells initially located the cAMP responsive sequences between -2327 to -605 (Moore, et al., 1990) and later localized them between -1733 to -1621 (Inoue, et al., 1991). Site-directed mutagenesis defined three similar elements within this region that apparently interact to generate a response to cAMP (Inoue, et al., 1991). Although the common motifs between these elements are somewhat similar to a CRE sequence, mobility shift experiments showed that the somatostatin CRE did not compete for the Y-1 protein that binds to these elements (Inoue, et al., 1991). This -1621 to -1733 region does not confer cAMP-inducibility to the P450scc promoter in JEG-3 cells (Fig. 3.3D), but the region between -108 to -89 does. Thus cAMP-induced transcription of this promoter is determined by tissue-specific use of different cAMP regulatory elements. Species-specific use of different elements appears unlikely, as this would require the fortuitious formation of new basal and cAMP-responsive sequences in the human promoter that respond to cAMP in murine Y-1 cells. Thus, our data indicate tissue-specific use of different cAMP response elements.

The region between -108 to -89 does not contain sequences resembling a consensus CRE (TGACGTCA). CRE-like sequences in cAMP responsive regions have been identified for the human P450c17 promoter (Brentano, et al., 1990) and the murine P450c11 promoter (Rice, et al., 1989), but not in other cAMP-responsive genes for steroidogenic enzymes (Hum and Miller, 1992; Moore and Miller, 1992). However, the

cAMP responsive region between -89 and -108 contains significant similarity to the NFAT-I sites in the HIV LTR (TTTGAGCCTGCA and TTGAGCCTG) (Crabtree, 1989) and with an AP-2 site in the SV40 enhancer (GAGCCTG) (Imagawa, et al., 1987). The region containing these similarities yielded a clear DNase I footprint encompassing bases -102 to -86 (GCGGGTTTGAGCCTGCA) (Fig. 3.10). Similarity to both AP-1 and AP-2 binding sites is significant as both may confer cAMP regulation, as well as regulation by PKC (Karin, 1989), and thus are likely candidates for cAMP regulation of the P450scc promoter in JEG-3 cells.

cAMP appears to act on the human P450scc promoter in JEG-3 cells through pre-existing proteins. Forskolin rapidly induced CAT activity (Fig. 3.3C), and inhibiting protein synthesis with cycloheximide does not block cAMP induction of P450scc mRNA (Fig. 3.7 and reference 8) or P450scc gene transcription in JEG-3 cells measured by nuclear run-off assays (Brentano and Miller, 1992). Studies in primary cultures of bovine adrenal cells suggested that cAMP induction of steroidogenic enzymes is indirect, requiring the synthesis of a "steroid hormone inducing protein" (Waterman and Simpson, 1989). That hypothesis is not consistent with transcriptional assays of the P450scc gene in mouse testicular MA-10 cells (Mellon and Vaisse, 1989) or with analyses of the human (Moore, et al., 1990) or bovine (Ahlgren, et al., 1990) P450scc promoter expressed in Y-1 cells.

Materials and Methods

Tissue Culture.

JEG-3 cells from the ATCC were grown at 37° C in 5% CO_2 and DME H21 medium with 50 μg/ml gentamycin supplemented with 5% fetal calf serum and 5% horse serum; cells treated with drugs were supplemented with only 0.5% fetal calf serum. Forskolin, A23187, 12-Q-tetradecanoylphorbol-13-acetate (TPA), 8-Br-cAMP and luciferin were from Sigma. The protease inhibitors antipain, aprotinin, chymostatin,

cystatin, pepstatin and the adenosine 5'-triphosphate disodium salt were from Boehringer Mannheim.

Transfections.

The P450scc/CAT deletion plasmids (Moore, et al., 1990) and CAT control vectors (Brentano, et al., 1990) have been described. The internal deletion constructions pAnsccΔ252/153CAT and pAnsccΔ148/86CAT were generated using convenient restriction enzyme sites. The deletion plasmids, -222LUC, -142LUC, -136LUC, -127LUC, -110LUC, -108LUC, and -79LUC were created using Bal 31 digests. -206LUC and -177LUC were made by PCR with the oligonucleotides 5'-TGGCACTGAGGGCAG-3' and 5'- CTGTCCTTTGAACC-3', respectively. All constructions were confirmed by restriction enzyme mapping and by dideoxynucleotide DNA sequencing. The JEG-3 transfections were performed similarly to the Y-1 cell transfections (Moore, et al., 1990) except that JEG-3 cells were incubated with the calcium phosphate:DNA precipitate for six hours, aspirated and then treated for one minute with 2.5 ml of 8% glycerol in DME H21. After washing gently with 2.5 ml of DME H21 the cells were incubated 12 hours in regular medium. The medium was then changed to DME H21 supplemented with 0.5% fetal calf serum , gentamycin, and forskolin or vehicle. We harvested total RNA or cell extracts for CAT assays after the cells were incubated with forskolin for 24 hours unless indicated otherwise. For the luciferase plasmids, JEG-3 cells were grown to confluence on 6.0 cm tissue culture dishes and transfected using 150 μl of $CaCl_2$ solution, 150 μl of HEPES-buffered saline (pH 7.4), 9 μg of luciferase plasmid and 0.2 μg of pAnRSVCAT. After 12 hours the media were exchanged for fresh media with or without forskolin (6 μM) and incubated for 12 hours more.

CAT Assays and RNase Protections.

The promoterless control plasmid never generated CAT activity greater than the background for the assay and therefore was not included. Our two-phase kinetic CAT assay and Northern blotting were as described (Moore, et al., 1990). RNA for RNase protections was prepared by acid phenol extraction (Chomczynski and Sacchi, 1987). To synthesize a probe for RNase protection assays of luciferase constructions, the *Sac I/Eco* RI fragment of p-79LUC was subcloned into the *Sma* I site of pBluescript; the plasmid was then linearized with *Not* I and transcribed from the T7 promoter. When transcription is accurately initiated from the P450scc cap site this 896-base probe protects a 684-base fragment.

Luciferase Assay.

The cells were harvested using 1.0 ml of phosphate buffered saline with 3 mM EDTA and without Ca^{++} or Mg^{++}, (PBS/CMF), pelleted in a microfuge and lysed in 200 µl of optimized luciferase lysis buffer (1% Triton X-100, 25 mM glycylglycine pH 7.8, 4 mM EGTA, 15 mM $MgSO_4$ and 1 mM dithiothreitol added just before use). The luciferase assay was done as described (Brasier, et al., 1989) except 50 µl of cellular extract was added to 200 µl of assay buffer (25 mM glycylglycine pH 7.8, 25 mM potassium phosphate pH 7.8, 4 mM EGTA, 15 mM $MgSO_4$, 1 mM dithiothreitol) and the reaction initiated with 100 µl of luciferin (final concentration 0.2 mM) dissolved in lysis buffer without Triton X-100. The light produced was measured in a Monolight Model 1500B luminometer. CAT assays were done as described above on another 50 µl aliquot of the extract to normalize the relative light units.

Nuclear Extracts.

Nuclear proteins were isolated from placental JEG-3 cells as described (Shapiro, et al., 1988) except that nuclei were partially purified by sucrose density gradient centrifugation (Gorski, et al., 1986). Cells from four nearly confluent 15 cm plates were harvested by incubating with ice-cold PBS/CMF, centrifuged at 500 x g for 10 minutes at 4° C, washed in 10 ml of PBS containing both Ca^{++} and Mg^{++}. The cells were pelleted and resuspended in 1.5 ml of homogenization buffer (10 mM HEPES pH 7.9, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 0.25 M sucrose, 10 % glycerol, 1 mM PMSF, 1.0 mM DTT, 0.5 µg/ml antipain, 2 µg/ml aprotinin, 5 µg/ml chymostatin, 10 µg/ml cystatin, 2 µg/ml pepstatin; the DTT and protease inhibitors were added immediately before use). The cells were broken by 50 strokes with the A pestle of a Dounce homogenizer. After combining with 4.5 ml of homogenization buffer containing 1.7 M sucrose, the homogenate was layered over 0.5 ml of homogenization buffer/1.7 M sucrose in polycarbonate ultracentrifuge tubes. The nuclei were pelleted at 200,000 x g for 20 minutes in a Beckman TLS55 rotor, and resuspended in 2.0 ml of nuclear lysis buffer (20 mM HEPES pH 7.9, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 2 mM EGTA, 25% glycerol, 2mM DTT to which 0.1 volumes of saturated $(NH_4)_2SO_4$ were added just before use). The chromatin was precipitated by shaking gently for 30 minutes at 4° and pelleted at 200,000 x g for 15 minutes. Finely powdered $(NH_4)_2SO_4$ (0.33g/ml) was added slowly to the supernatant and the proteins were precipitated by gentle stirring at 4° C. The precipitated protein was pelleted at 200,000 x g for 15 minutes and resuspended in 400 µl of nuclear dialysis buffer (20 mM Hepes pH 7.9, 100 mM KCl, 0.2 mM EDTA, 2 mM EGTA, 20 % glycerol, 2 mM DTT). After dialyzing twice for 90 minutes against 250 volumes of nuclear dialysis buffer the resulting solution was clarified at 12,000 x g for 10 minutes at 4° C and the protein concentration of the supernatant determined by Bradford

assay. This protocol usually yielded about 400 µl of 5 µg/µl nuclear proteins. The extracts were aliquoted and stored at -70° C.

Gel Mobility Shift Assay.

The probes for the mobility shift assays were created using restriction endonuclease sites, PCR, or both. Probes -276/-211 and -276/-247 were *Pst* I/*Rma* I and *Pst* I/*Dde* I fragments, respectively. Following digestion with *Rma* I or *Dde* I the DNA was blunt-ended with Klenow polymerase, cut with *Pst* I, gel-purified, and cloned into the *Pst* I to*Sma* I sites of pBSK+. The 5' ends of the probes -153/-114, -153/-64, and -91/-64 were *Pst* I sites and the 3' ends were determined by PCR primers. Probes -207/-148 and -177/-148 used *Pst* I sites at the 3' ends and PCR at the 5' ends. These fragments were also cloned into the *Pst* I to *Sma* I sites of pBSK+. Probes -207/-64 and -177/-114 were both derived using primers for both ends and PCR, and cloned into the *Sma* I site of pBSK+. Probe -117/-85 was created by hybridizing sense and anti-sense oligonucleotides for this region, each containing a 5' GATC extension for cloning. All probe templates were confirmed by sequencing.

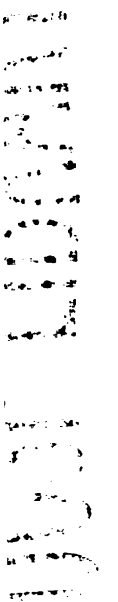
DNA probes for the gel shift assays were excised from pBSK+ with *Bam* HI and *Eco* RI, and end-labeled with Klenow polymerase and α^{32}P-dATP. For the assays 0.5-1.5fmol (5000 -10,000 cpm) of probe was added to 9 µg of JEG-3 cell nuclear extract protein, 4 µg of dI:dC and/or 4 µg of dA:dT, and 10 µl of 2X shift buffer (40 mM HEPES pH 7.9, 120 mM KCl, 2 mM EDTA, 16 % glycerol, 1.0 mM DTT) and water to a volume of 20 µl. The reactions were incubated at 20° C for 20 min., and then loaded onto a 4% polyacrylamide gel which was prerun at 175 V, 4° C for 30 min. Samples were loaded while the gels were running at 50 V, and the voltage was increased to 100 V, then 175 V 5 min later.

DNase I Footprinting.

For DNase I footprinting (Mangalam, et al., 1989) the probe was generated by PCR using the ^{32}P end-labeled anti-sense primer 5'-GTAGCCCTGGGCCACCAG-3' corresponding to bases +3 to -15, and the sense primer 5'-TCGCACTGAGGGCAGA-3', corresponding to bases -191 to -206. About 50,000 cpm of gel-purified probe was incubated with 30 μg nuclear extract or bovine serum albumin (BSA) in a 20 μl reaction volume containing 2 μg of dI:dC, 1 μg salmon sperm DNA, 0.25% non-fat milk, 1 mM DTT, 12 mM HEPES pH 7.9, 4 mM Tris pH 8.0, 60 mM KCl, 0.6 mM EDTA, 0.6 mM EGTA, and 12% glycerol. After 20 min at room temperature, the sample was chilled and combined with 50 μl of 5 mM $CaCl_2$, 1.5 mM EDTA and 2 ng of DNase I (Worthington). The reaction proceeded at room temperature for 3 min and was stopped with 50 μl of 20 mM EDTA, 200 mM NaCl, 1% $NaDodSO_4$. After adding 30 μg of glycogen carrier the sample was extracted with phenol/chloroform, precipitated with EtOH and electrophoresed on a sequencing gel.

Acknowledgements

We thank Dr. Holly Ingraham for advice about the footprinting assays. Supported by March of Dimes predoctoral fellowship #18-88-25 to CCDM, FRSQ (Fonds de la Resherche en Santé du Quebec) postdoctoral fellowship # 900268 to DWH and March of Dimes Grant 6-396 and NIH Grants DK 37922 and DK 42154 to WLM.



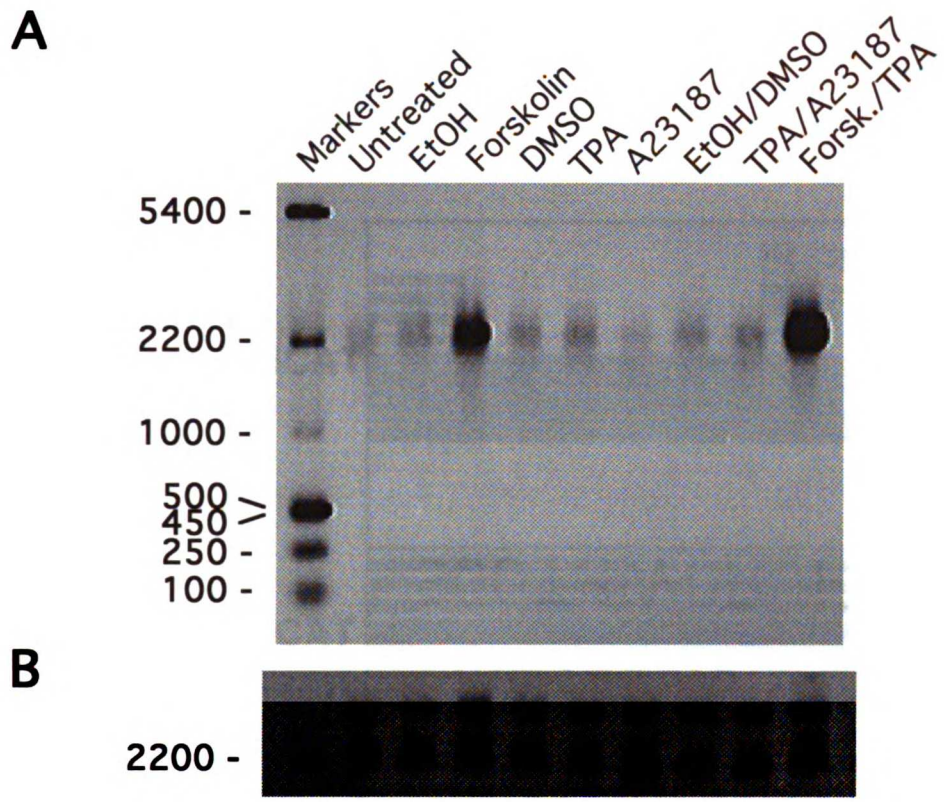


Figure 3.1. Northern blot. JEG-3 cells were treated with the indicated drugs or the appropriate solvent for 12 hours (Forskolin 2.0 x 10^{-5}M, TPA 3.3 x 10^{-6}M, A23187 5.0 x 10^{-7}M). Each lane contains 30 µg of cytoplasmic RNA. (A) Probing with the full-length cDNA for human P450scc. (B) After stripping the P450scc signal from the blot it was reprobed with a 750 bp *Hin*d III fragment of cDNA for human nuclear lamin A. Markers are end-labeled *Hin*d III cut bacteriophage PM2. Lanes labelled EtOH, DMSO and EtOH/DMSO are solvent control samples, (EtOH for forskolin and A23187; DMSO for TPA).

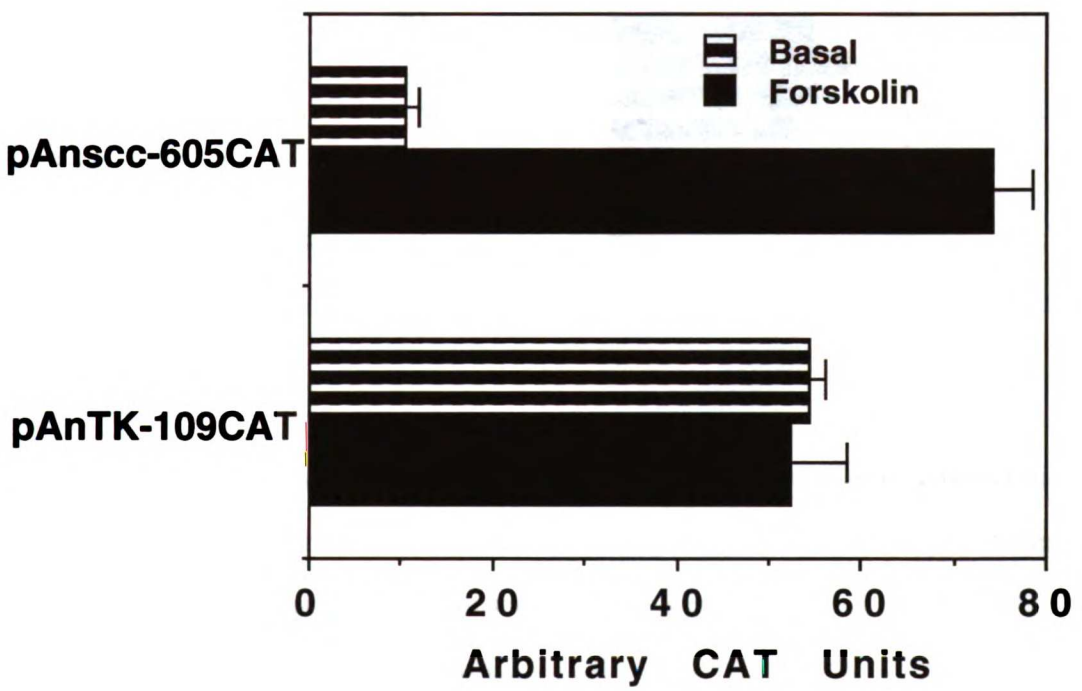


Figure 3.2. CAT assays. Promoter construction containing 605 bases of the human P450scc promoter (pAnscc-605CAT) or the control plasmid, pAnTK-109CAT, were transiently transfected into JEG-3 cells and treated with or without 2.0 x 10^{-5} M forskolin. The results are from one experiment done in triplicate and the averages presented as arbitrary CAT units +/- SEM.

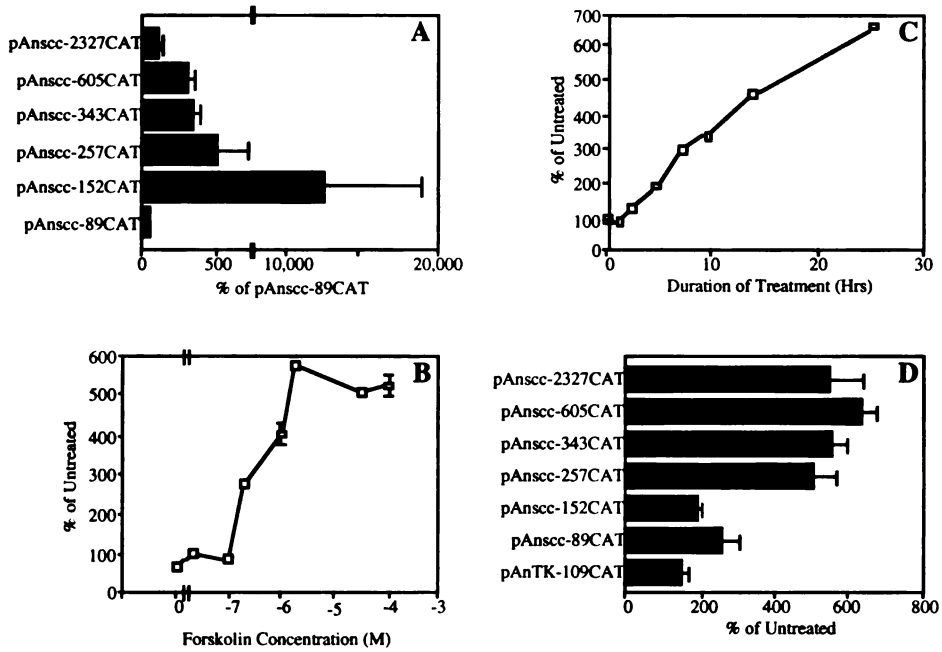


Figure 3.3. Deletional mutagenesis analysis of the P450scc promoter. The constructions are named pAnscc"X"CAT, where "X" refers to the base number of the 5' end of the P450scc promoter sequence relative to the transcription initiation site (Moore, et al., 1990). Each plasmid was transiently transfected into JEG-3 cells and CAT extracts were made 36 hours after the glycerol shock. (A) Basal expression of the progressive deletion plasmids. The values are presented as the % of the shortest construction, pAnscc-89CAT, +/- SEM, from at least three independent transfections using at least three seperate plasmid preparations. (B) Dose response of the transiently transfected pAnscc-605CAT plasmid treated with forskolin. The data are from one experiment done in triplicate with the values presented as the % of untreated +/- SEM. (C) Time course for forskolin induction of pAnscc-605CAT transiently transfected into JEG-3 cells. The data are from one experiment done in triplicate with the values presented as the % of untreated +/- SEM. (D) Forskolin response for the series of P450scc/CAT deletion constructions transiently transfected into

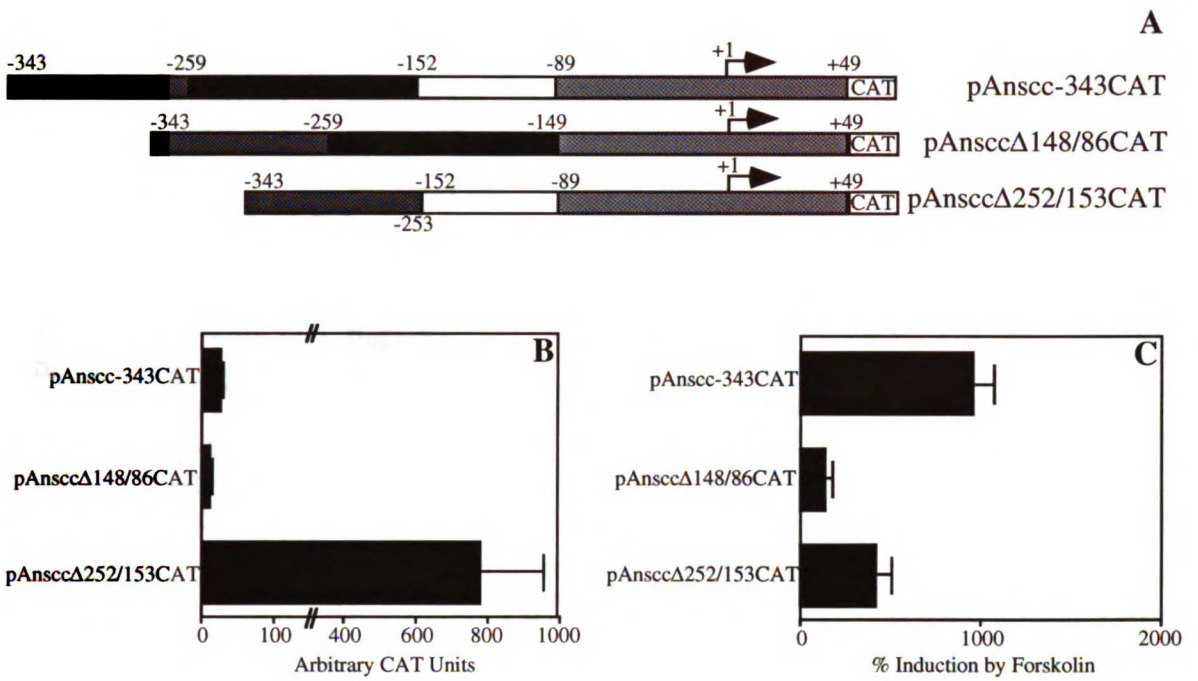
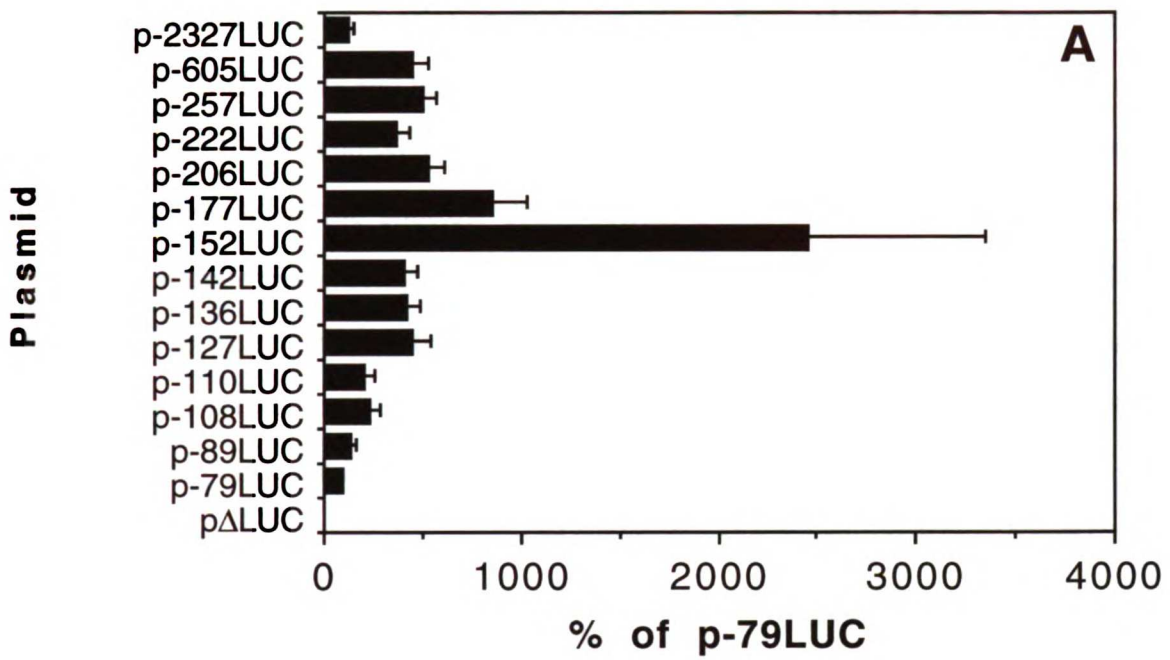


Figure 3.4. Basal expression and forskolin induction of internal deletion plasmids. (A) Diagram of the internal deletions in the human P450scc promoter fused to the CAT gene. (B) Basal expression of these constructions transfected into JEG-3 cells. The values are averages +/- SEM expressed as arbitrary CAT units from at least three seperate transfection experiments using at least three seperate preparations of each plasmid. (C) Forskolin induction (24 h) of the internal deletion plasmids. CAT activity is expressed as the % of the appropriate untreated sample and is the mean +/- SEM of at least three separate transfections using three separate plasmid preparations.


A
Plasmid
p-2327LUC
p-605LUC
p-257LUC
p-222LUC
p-206LUC
p-177LUC
p-152LUC
p-142LUC
p-136LUC
p-127LUC
p-110LUC
p-108LUC
p-89LUC
p-79LUC
pΔLUC
0
1000
2000
3000
4000
% of p-79LUC

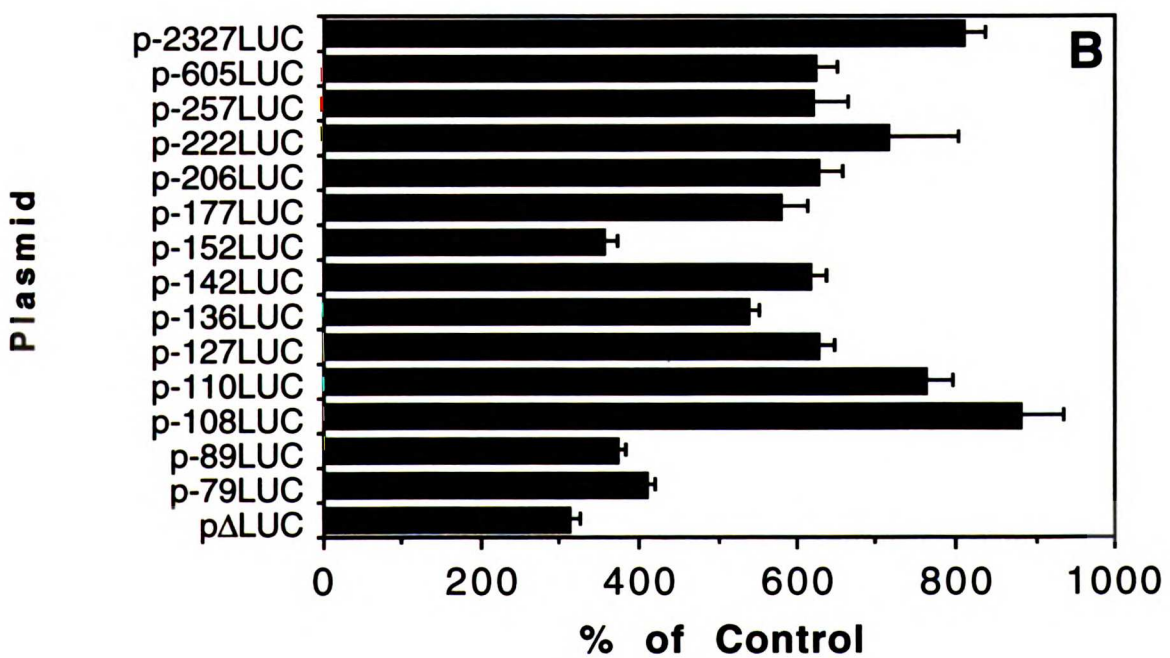

B
Plasmid
p-2327LUC
p-605LUC
p-257LUC
p-222LUC
p-206LUC
p-177LUC
p-152LUC
p-142LUC
p-136LUC
p-127LUC
p-110LUC
p-108LUC
p-89LUC
p-79LUC
pΔLUC
0
200
400
600
800
1000
% of Control

Figure 3.5. Deletional mutagenesis of the P450scc promoter. The constructions are named p"X"LUC, where "X" refers to the base number of the 5' end of the P450scc promoter sequence relative to the transcription initiation site. Each plasmid was transiently transfected into JEG-3 cells and luciferase extracts were made 24 hours after starting the transfection. (A) Basal expression of the progressive deletion plasmids. The values are presented as the % of the shortest construction, p-79LUC, +/- SEM, from nine independent transfections using three separate plasmid purifications. (B) Forskolin responses of these constructions transiently transfected into JEG-3 cells. The values are presented as the % of untreated +/- SEM from nine independent transfections using three separate plasmid purifications.

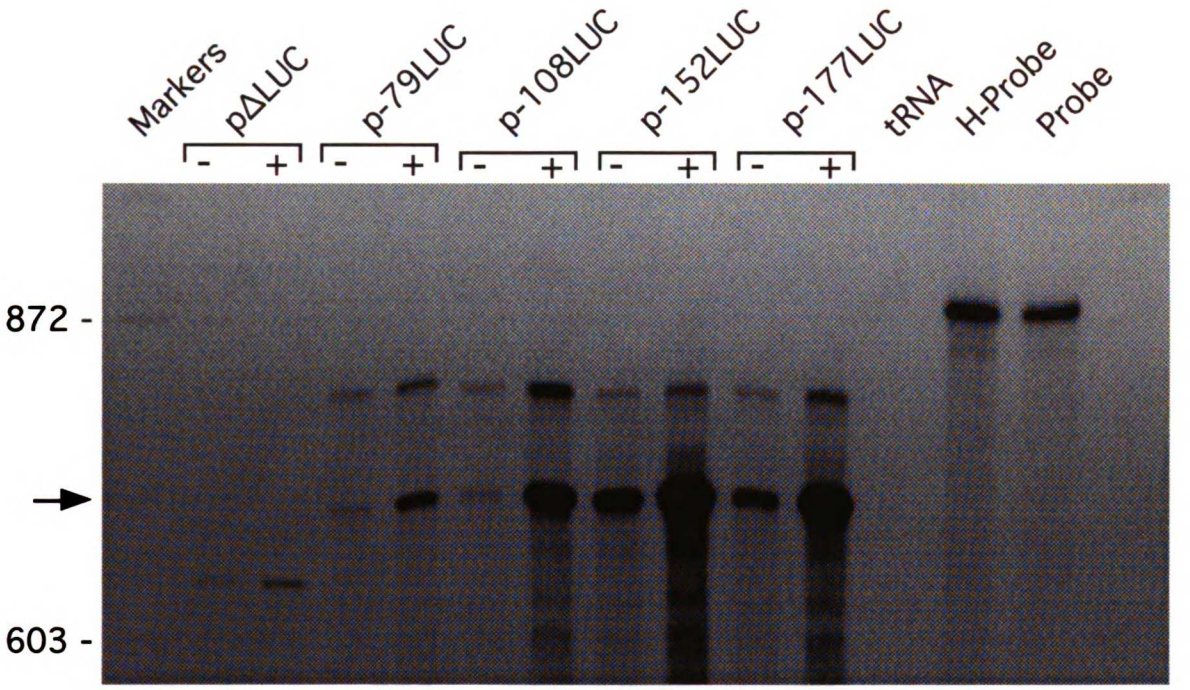
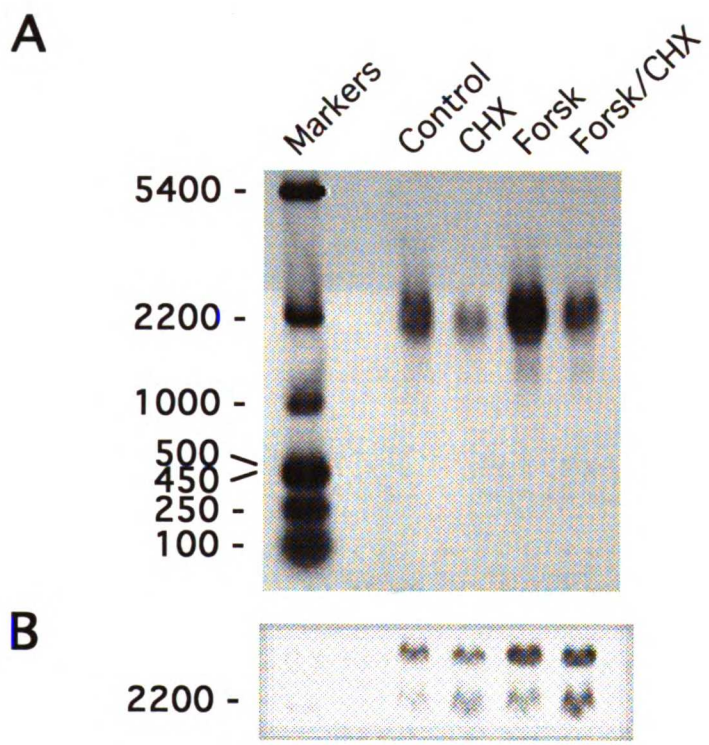


Figure 3.6. RNase protection assay. Each experimental lane contains 20 μg of RNA from JEG-3 cells treated with (+) or without (-) 2.0 x 10^{-5} M forskolin after being transiently transfected with the indicated plasmid. The lane designated tRNA contained 20 μg of yeast transfer RNA. The lane designated Probe contains the untreated 896 probe. The lane designated H. Probe contains the probe hybridized overnight with the samples but not subjected to RNase digestion. The arrow designates correctly initiated mRNA originating from the initiation site in the P450scc promoter protecting a 684 bp product. "Read-through" transcripts that arise within the vector protect the higher band at 766 bp. Molecular size markers are a mixture of end-labeled, *Hae* III-cut, ϕX174, and *Hpa* II-cut pUC18.


A
Markers
Control
CHX
Forsk
Forsk/CHX
5400 -
2200 -
1000 -
500
450
250 -
100 -
B
2200 -

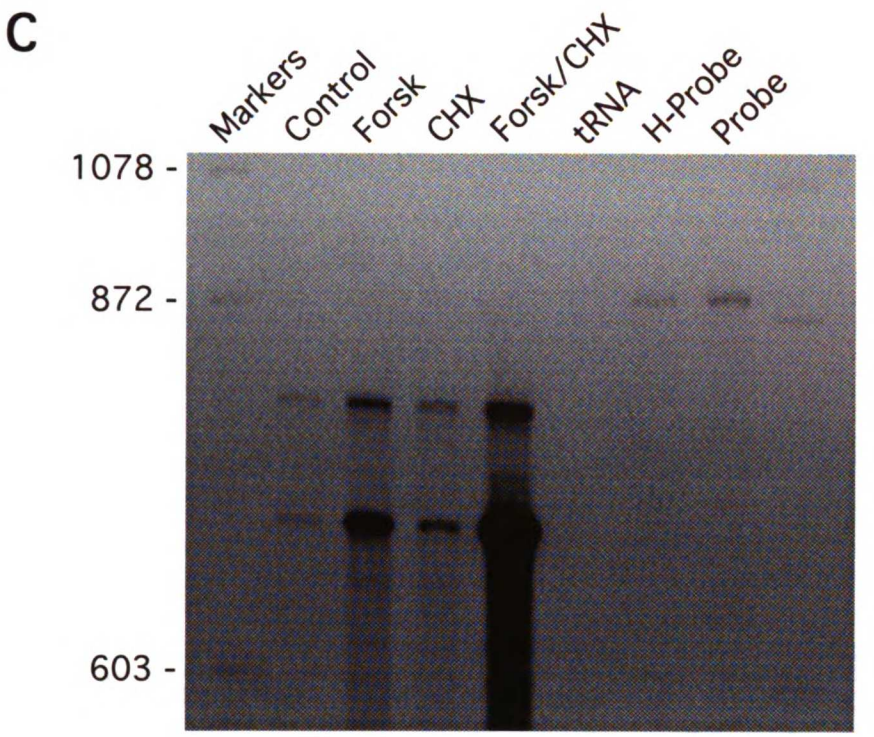
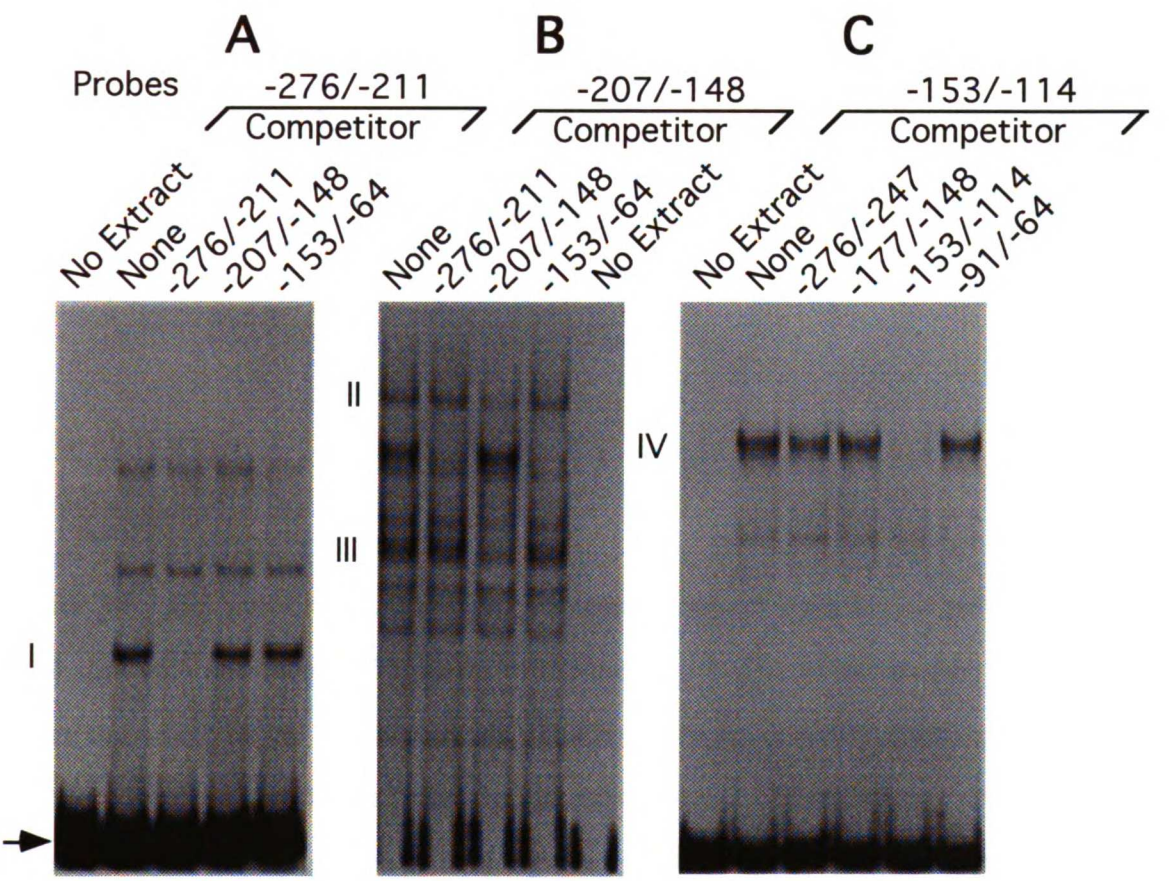


Figure 3.7. Effects of cycloheximide. A) Northern analysis of the endogenous P450scc mRNA in JEG-3 cells treated with 2.0 x 10^{-5} M forskolin, 40 μg/ml cycloheximide or both. Cycloheximide was added 30 minutes prior to the addition of forskolin, and the cells were incubated for 6 hours more before total RNA was prepared. Each lane contains 20 mg of RNA. B) Same blot reprobed with a 750 bp *Him*d III fragment of human nuclear lamin A as a control showing equivalent amount of RNA was loaded in each lane. C) RNase protection assay of 20 μg of RNA from JEG-3 cells transiently transfected with p-108LUC. Treatments were as described in panel A and the RNase protection assay was performed as in Fig. 6. Molecular size markers are combined end-labeled *Hae* III-cut, ϕX174 and *Hpa* II-cut pUC18.


A
B
C
Probes
-276/-211
Competitor
-207/-148
Competitor
-153/-114
Competitor
No Extract
None
-276/-211
-207/-148
-153/-64
None
-276/-211
-207/-148
-153/-64
No Extract
No Extract
None
-276/-247
-177/-148
-153/-114
-91/-64
I
II
III
IV

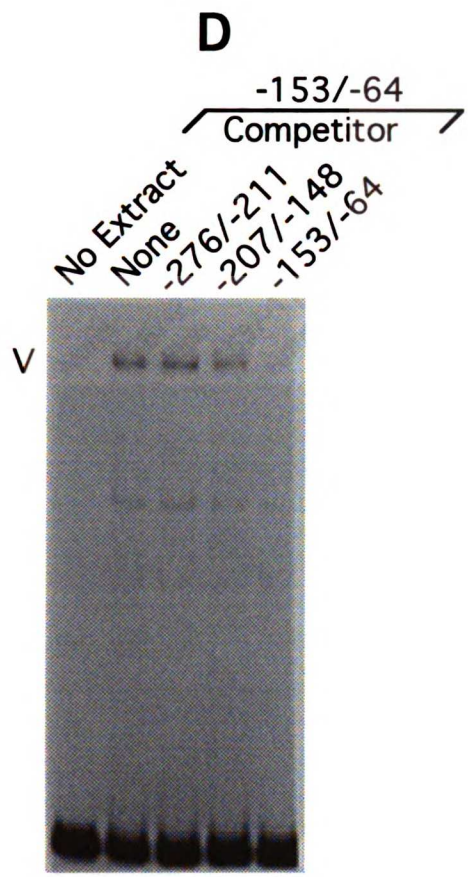


Figure 3.8. Gel mobility shift assays. Proteins extracted from JEG-3 cell nuclei were incubated with end-labeled fragments of the human P450scc promoter and protein:DNA interactions seen by gel electrophoresis. The numbers designate the flanking bases of the fragment used relative to the transcription initiation site of the P450scc promoter. The numbers across the top of each panel designate the labeled probe used and the designations below the bracket identify the competitor fragments present in a 100-fold molar excess. The specific DNA protein complexes are designated I-V and the front of labeled uncomplexed probe is designated by the arrow at the bottom of panel A.

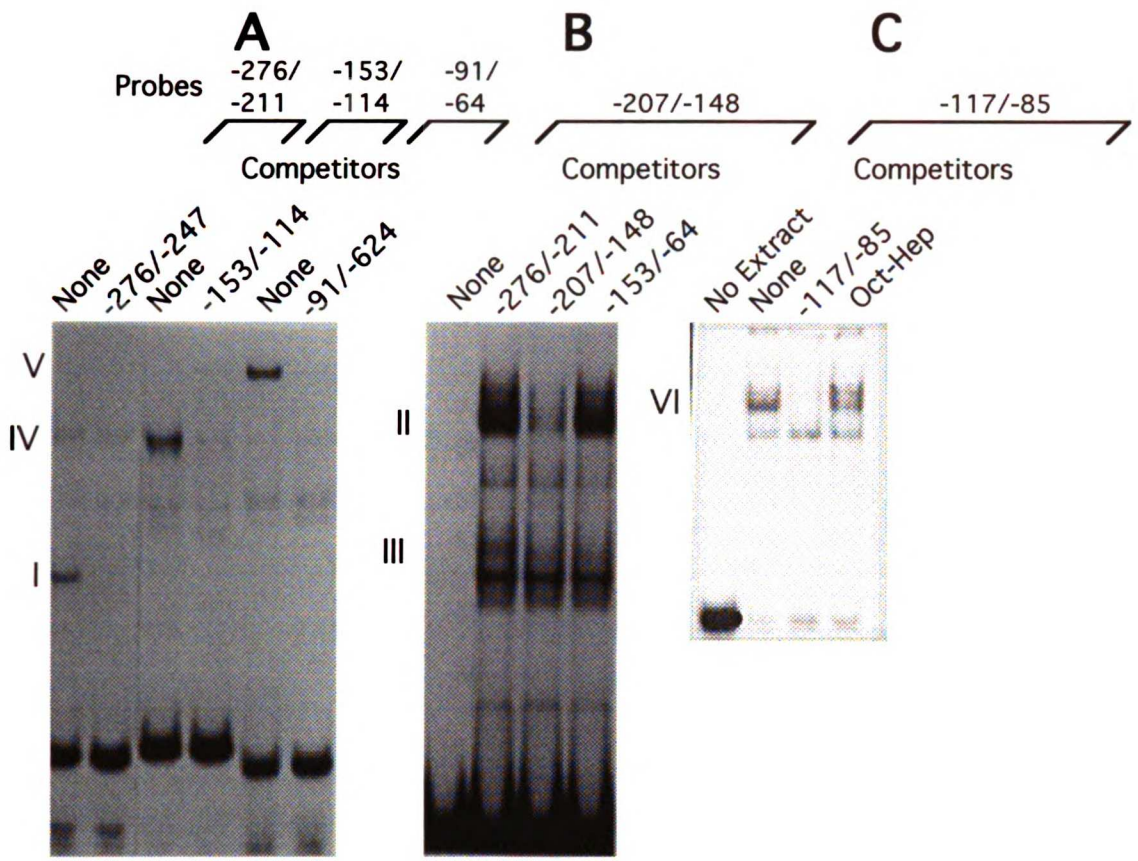


Figure 3.9. Higher resolution gel shift assays. The probes, competitors, and DNA:protein complexes are designated as in Fig. 8. Note that complex III is the upper band near the numeral III that is completed by both -207/-148 and -177/-148; the lower band present in all three lanes is non-specific. Panel C shows an additional complex (VI) not identified in Fig. 8. The Oct-Hep competitor was a 400-fold molar excess of the double-stranded Oct-Hep oligonucleotide (Poellinger and Roeder, 1989)

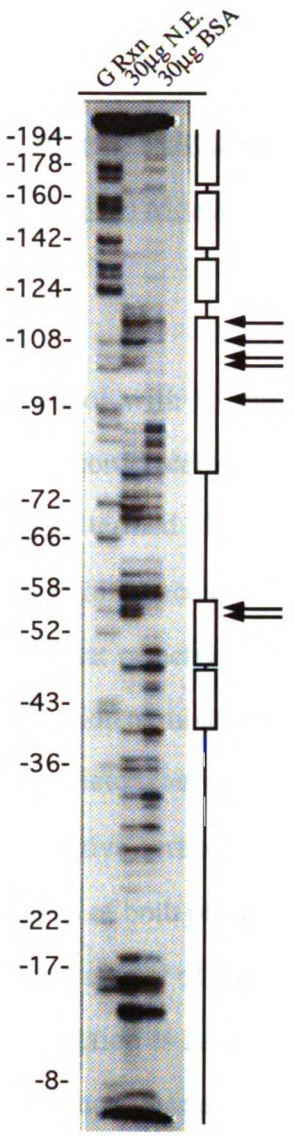


Figure 3.10. DNase I footprint. An end-labeled probe from +3 to -206 of the human P450scc promoter was incubated with JEG-3 cell nuclear extract (N.E.) or with bovine serum albumin as control (BSA) and digested with DNase I. Markers are the guanine-specific Maxam-Gilbert sequencing reaction of the same probe (G Rxn) with the base numbers of selected G-residues indicated. The box diagram to the right indicates the footprinted regions and the arrows indicate DNase I hypersensitive sites.

Part II

Role of the Hepatic 11β-Hydroxysteroid Dehydrogenase in End-Organ Modulation of Cortisol in the Squirrel Monkey (*Saimiri sciurea*)

Overview

The interconversion of cortisol with its biologically inactive keto derivative cortisone (or corticosterone to dehydrocorticosterone in rodents) is catalyzed by a non-cytochrome-P450 microsomal enzyme termed 11β-hydroxysteroid dehydrogenase (11β-HSD). The proposed physiological significance of this reaction has evolved over the last decade to include an end-organ rheostat of active glucocorticoids, and a mechanism for providing specificity of aldosterone action at mineralocorticoid target tissues. As a rheostat 11β-HSD would maintain the appropriate level of active hormone, cortisol, in the target tissue by either converting it to the inactive cortisone, or converting circulating cortisone to the active cortisol. The latter reaction is of both physiological and medicinal significance as free, circulating cortisone is a substantial pool of potentially active steroid (Hammami and Siiteri, 1991) and this conversion reaction is necessary to activate hydrocortisone. The second possible function is to defend the mineralocorticoid or Type I receptor (MR) from responding to glucocorticoids. As cortisol binds to and activates the MR and its circulating concentration exceeds that of aldosterone 11β-HSD is needed to inactivate the cortisol and thereby allow appropriate signalling by aldosterone.

The first clues for the unique physiological role of 11β-HSD in MR specificity came from clinical observations. The first observation is an inborn error of metabolism, Apparent Mineralocorticoid Excess (AME) (New, et al., 1977), a rare, probably autosomal recessive genetic disease (Dimartino-Nardi, et al., 1987). Patients with AME present as children or adults (Stewart, et al., 1988) suffering from hypernatremia, hypertension, and potentially fatal hypokalemia - symptoms expected and seen in patients

suffering from aldsoterone excess, yet AME patients have normal or low circulating levels of both renin and aldosterone. These patients improve with spironolactone treatment (Dimartino-Nardi, et al., 1987) (an inhibitor of the MR), and suppression of the glucocorticoid pathway (Stewart, et al., 1988), while treatment with ACTH or cortisol exacerbates their disease (Dimartino-Nardi, et al., 1987; Stewart, et al., 1988). These observations led to the hypothesis that an unknown adrenal steroid with mineralocorticoid action was overproduced in AME patients (New, et al., 1977; New, et al., 1976).

That the unknown adrenal steroid is cortisol was suggested by experiments in patients with AME. Infusion of hydrocortisone worsened their condition, implying that cortisol has a mineralocorticoid effect (New, et al., 1982; Oberfield, et al., 1983). Furthermore, this effect was reversed by treatment with the MR specific antagonist spironolactone (Oberfield, et al., 1983). Thus, New *et al* proposed that the cause for AME was a defect in 11β-HSD resulting in disordered conversion of cortisol to cortisone and improper signalling through a defective MR (New, et al., 1982).

This model could be simplified once the mineralocorticoid, Type I receptor (MR) and glucocorticoid, Type II receptor (GR) were cloned. In vitro the MR lacks steroid specificity and binds glucocorticoids with an affinity equal to that of aldosterone and concomittantly activates gene promoters containing mineralocorticoid response elements (Arriza, et al., 1987). This specificity problem is further compounded because circulating levels of free cortisol are between 10 to 100 times that of aldosterone (Funder, et al., 1988). Under these conditions the MR should be saturated by cortisol and unable to respond to the appropriate mineralocorticoid, aldosterone, however *in vivo* mineralocorticoid target tissues do selectively bind and respond only to aldosterone (Funder, et al., 1988). As the normal MR binds cortisol Stewart *et al* proposed that a lesion of 11β-HSD was therefore the sole cause of AME (Stewart, et al., 1988), preventing mineralocorticoid target tissues from inactivating cortisol and defending their MR for signalling by aldosterone.

Consistent with this model were clinical observations of licorice toxicity in patients who consumed large quantities of licorice (Conn, et al., 1968) or licorice containing chewing tobacco (Blachley and Knochel, 1980). These patients present with life-threatening hypokalemia, and a suppresed renin-angiotensin-aldosterone system - identical symptoms to that in the inborn form of AME. Their symptoms resolve if they stop consuming licorice. The pharmacological ingredient responsible for this disease is glycyrrhizinic acid and its hydrolytic derivative glycyrrhetinic acid, both steroid "look-alikes". Early experiments suggested these pharmacological agents acted via the MR (Ulmann, 1975;Armanini, 1983;Armanini, 1985, Takada, 1987) but they, and their medicinally important cousin carbenoxalone, are now recognized as potent inhibitors of 11β-HSD (Monder, et al., 1989). Thus, these patients suffer from AME due to a pharmacological block in their ability to convert the active glucocorticoid cortisol to the inactive cortisone in mineralocorticoid target tissues.

More recent experiments suggest a further subtlety in patients with AME. Studying patients with genetic defects in their MR causing pseudohypoaldosteronism Funder *et al* demonstrated that glucocorticoids have mineralocorticoid effects through the glucocorticoid Type II receptor as well as by activating the MR (Funder, et al., 1990). Treating these patients with carbenoxolone, to inhibit 11β-HSD, causes marked mineralocorticoid effects despite the non-functional MR, furthermore in rats the GR specific antagonist RU48386 causes antinatruiresis (Funder, et al., 1990). These results reconciled the clinical observations that treating AME patients with dexamethasone, a potent glucocorticoid with minimal mineralocorticoid activity, fails to resolve their disease despite low cortisol and aldosterone levels. In addition these patients respond more strongly to cortisol treatment than aldosterone treatment suggesting that cortisol was causing mineralocorticoid effects through more than just the MR. At a molecular level this can be explained by binding of the activated GR to the mineralocorticoid response element, whose sequence closely resembles that for the glucocorticoid response element, further underlining the significance of having

an end-organ enzymatic mechanism for steroid specificity (Funder, et al., 1990). Thus, the action of 11β-HSD in the kidney is probably twofold, to confer receptor specificity for the MR and allow signalling by aldosterone, and to limit the mineralocorticoid actions possible through the glucocorticoid receptor.

To explore this model for a unique mechanism for conferring hormonal specificity required cloning 11β-HSD. This was recently accomplished by Agarwal *et al* who isolated the rat hepatic cDNA using antibodies raised against the purified hepatic enzyme and a liver cDNA expression library (Agarwal, et al., 1989). The enzyme is a 34 kD glycoprotein that, when expressed in Chinese hamster CHO cells, manifests both dehydrogenase and reductase activities (Agarwal, et al., 1989). The expressed enzyme is indistinguishable from the rat hepatic enzyme, having similiar K_m's for both dehydrogenase and reductase, similar pH optima for each reaction, and the reductase activity is very unstable (Agarwal, et al., 1990). That both activities were in one peptide was suprising as biochemical data and clinical observations suggested that the dehydrogenase and reductase reactions were actually catalyzed by separate enzymes, possibly an enzyme complex. Purified 11β-HSD had undetectable reductase activity (Lakshmi and Monder, 1988), while patients suffering from AME are still able to reduce cortisone to cortisol (Monder, et al., 1986), while patients lacking reductase activity can convert cortisol to cortisone (Phillipou and Higgins, 1985). Such misleading information is reminiscent of other steroidogenic enzymes such as P450scc, P450c11, and P450c17, enzymes that catalyze multiple reactions - in keeping with their heritage as multi-function oxidases (discussed in Miller, 1988). The sequence of 11β-HSD is homologous to two other eukaryotic steroidogenic enzymes, estradiol 17β-hydroxysteroid dehydrogenase and 3β-hydroxysteroid dehydrogenase, and has limited homology to other dehydrogenase enzymes including and as evolutionarily distant as the nodG protein in the nitrogen fixing bacterium *Rhizobium meliloti* (Agarwal, et al., 1989; Tannin, et al., 1991), thus defining a new family of evolutionarily conserved enzymes.

Since the isolation and cloning of 11β-HSD many have tried to confirm the proposed role of 11β-HSD in conferring steriod specificity in mineralocorticoid tissues by localizing 11β-HSD in mineralocorticoid responsive tissues. Unfortunately the results are inconclusive. 11β-HSD activity is found in mineralocorticoid responsive tissues such as the kidney, parotid gland and colon (Naray-Fejes-Toth, et al., 1991), however the amount of activity is relatively low in the colon (Funder, et al., 1988). In addition, this activity is apparently not from the cloned, hepatic form of the enzyme. Although *in situ* analysis by several groups found mRNA's with homology to the hepatic enzyme in both the proximal and distal portions of the nephron (Moisan, et al., 1992; Stewart, et al., 1991; Yau, et al., 1991) others could not co-localize 11β-HSD protein with the MR in rat kidney distal tubules and cortical collecting ducts, although abundant immunoreactive material was found in the proximal tubules (Rundle, et al., 1989). A similar distribution was seen using a different antibody raised against a renal form of the enzyme (Castello, et al., 1989). This led to a refined hypothesis where 11β-HSD worked by a paracrine mechanism (Rundle, et al., 1989), converting cortisol to cortisone in the blood as it passed through the proximal tubules so that when it reached the distal tubules it was mostly inactive cortisone.

The paracrine model, however was inconsistent with the localization of activity in the rabbit kidney nephron where microdissected sections of the distal portions of the nephron had much higher activity than the proximal portions (Bonvalent, et al., 1990). A direct test of the model showed that after one pass through the kidney less than 5% of the corticosterone was converted to dehydrocorticosterone.

Similar problems arose in other mineralocorticoid target tissues. By northern analysis the abundance of the mRNA for the hepatic form of 11β-HSD was very low in the colon (Krozowski, et al., 1990) and low or absent in the parotid glands (Funder, 1990; Krozowski, et al., 1990), nor was the protein detected by immunocytochemistry in the parotid gland (Funder, 1990). Both tissues have high levels of 11β-HSD activity, thus

there must be another enzyme with 11β-HSD activity, or the hepatic form must have significantly greater biological activity in these tissues than in the liver.

Additional evidence against the hepatic 11β-HSD as the steroid specificity mechanism in mineralocorticoid target tissues is a missing experiment. Given the trivial task of cloning and sequencing a gene as small as 11β-HSD (Tannin, et al., 1991), no genetic defect in the human 11β-HSD locus that can account for the defect in patients with AME has been found. The implication is that people have looked, but have not found a defect. Admittedly the absence of such a negative experiment is not proof that the hepatic 11β-HSD is not used in steroid specificity, however it is highly suspicious.

One explanation that accounts for these inconsistencies is that more than one 11β-HSD enzyme exists (Funder, 1990; Krozowski, et al., 1990). There are alternatively spliced versions of the known 11β-HSD in the rat kidney as four, different sized bands are seen on northern blots (Krozowski, et al., 1990). However, only a single species is detected in humans (Tannin, et al., 1991). In addition bands of about 1.4 kb and 3.4 kb are seen on northerns of RNA from rat colon (Whorwood, et al., 1992). Enzyme kinetic data shows that about 90 % of the 11β-HSD enzymatic activity in the rat kidney has a K_m of about 9.2 mM, similar to that of the hepatic enzyme, while the remaining 10 % has a much lower K_m of about 0.06 mM (Monder and Lakshmi, 1989). When enzymatic kinetics were done with 11β-HSD from isolated kidney collecting ducts only a low K_m form was found, strongly implying that the relevant kidney enzyme is different from the hepatic form. The isolated kidney form also strongly favored almost complete dehydrogenation of cortisol to cortisone up to substrate concentrations of 10^{-7} M, and no detectable conversion of physiological concentrations of cortisone to cortisol was seen (Naray-Fejes-Toth, et al., 1991). Further evidence for an enzyme unrelated to the hepatic form is a recently isolated enzyme that is NADH dependent as opposed to the hepatic form that is NADPH dependent. There is precedence for this in the world of steroidogenic enzymes as a non-P450c21

enzyme exists that catalyzes in the liver the same reaction mediated by P450c21 in the adrenal (Mellon and Miller, 1989).

Although most of the attention on the physiology of 11β-HSD has focused on it's role in defending the MR in mineralocorticoid responsive tissues such as the kidney, colon and possibly the brain (Lakshmi, et al., 1991) it may also be important in other physiological settings. Studies have shown that the ratio of dehydrogenase and reductase activities may differ between tissues and may also be under hormonal control, thus it may control the availability of cortisol in glucocorticoid target tissues (Hammami and Siiteri, 1991) or defend non-glucocorticoid target tissues from the deleterious effects of cortisol (Phillips, et al., 1989). An example for this is the significant 11β-HSD activity of the placenta that is regulated during gestation by estrogen. This may be important in defending the fetus from maternal cortisol during fetal development, modulating the development of the fetal adrenal (Pepe, et al., 1988), and the declining 11β-HSD activity towards the end of gestation in the lung may be important in allowing local cortisol levels to rise and cause lung maturation before parturition.

Nature has provided us with her own experiment to test current models for 11β-HSD action and the role of the cloned, hepatic 11β-HSD enzyme. A New World primate, the squirrel monkey (*Saimiri sciurea*), has circulating levels of free cortisol much greater than our own, levels that would cause Cushings disease (Chrousos, et al., 1984; Chrousos, et al., 1983; Chrousos, et al., 1984; Chrousos, et al., 1982). To avoid this the squirrel monkey is relatively glucocorticoid insensitive, possibly due to elevated 11β-HSD activity (Hammami and Siiteri, 1991) which inactivates the elevated cortisol at the target tissue. Such a mechanism is consistent with 11β-HSD conferring steroid specificity in mineralocorticoid target tissues, and modulating cortisol in glucocorticoid responsive tissues.

If the model proposed for 11β-HSD is correct then we can make the following prediction for the squirrel monkey. To compensate for the increased levels of cortisol they

must have increased 11β-HSD activity, especially in the mineralocorticoid target tissues. Early experiments by Hammami (unpublished) demonstrate that tissues from the squirrel monkey do have relatively greater 11β-HSD activity than their human counterparts due to either greater specific activity, greater abundance, or possibly both. In the former case knowing the structure of the squirrel monkey 11β-HSD could provide insights into the structure-function relationship of this enzyme. If, however, there is increased abundance of the enzyme it would be interesting to know if this arises from increased transcription of the gene, or if the protein is more stable than the human enzyme. To address these questions, as well as the more global issue of what role the hepatic 11β-HSD plays in glucocorticoid metabolism and signalling of the MR, we decided to clone the cDNA and gene for the SM 11β-HSD and also compare the activities of the SM protein with that for the human protein.

Chapter 5

The Hepatic Form of Squirrel Monkey 11β-Hydroxysteroid Dehydrogenase Does Not Protect the Renal Mineralocorticoid Receptor

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Abstract

Both cortisol and aldosterone bind to and activate the mineralocorticoid receptor. Cortisol concentrations are generally 100 to 200-fold higher than aldosterone concentrations, yet mineralocorticoids clearly exert effects different from glucocorticoids. A current hypothesis holds that the mineralocorticoid receptor is "protected" from cortisol by 11β-hydroxysteroid dehydrogenase (11β-HSD), which converts cortisol to biologically inactive cortisone. The circulating concentrations of cortisol in the squirrel monkey are 20-50 fold higher than human cortisol concentrations, yet this animal has no evidence of glucocorticoid or mineralocorticoid excess. We used this experiment of nature to test the hypotheses that the known (hepatic) form of 11β-HSD protects renal mineralocorticoid receptors from the action of cortisol and that it modulates glucocorticoid concentrations in target tissues. Using a long oligonucleotide based on the rat sequence, we cloned the squirrel monkey 11β-HSD cDNA and gene. The encoded monkey amino acid sequence is 75% and 91% identical to the corresponding rat and human sequences, respectively. The tissue abundance of the mRNA for the monkey enzyme was similar to or less than that seen for the rat and human enzymes. Both the monkey and human 11β-HSD cDNAs were cloned into an expression vector and used to transfect cultures of CHO cells. Both vectors were transcribed and translated into equivalent amounts of 11β-HSD enzyme. The monkey enzyme was slightly more efficient than the human enzyme in converting ^{3}H cortisol to cortisone, and estimates of the Km and Vmax of both enzymes are similar. These data indicate that the abundance and activity of the hepatic form of 11β-HSD are insufficient to inactivate the very high concentrations of cortisol in the squirrel monkey, suggesting that this form of 11β-HSD does not defend the mineralocorticoid receptor, or protect tissues from high cortisol concentrations. Rather this enzyme appears to favor conversion of cortisone to cortisol, thus maximizing tissue concentrations of cortisol to overcome glucocorticoid resistance associated with a 50% reduction in glucocorticoid receptors.

Introduction

The interconversion of cortisol or corticosterone with their inactive 11-keto derivatives cortisone and dehydrocorticosterone by 11β-hydroxysteroid dehydrogenase (11β-HSD) has important physiological consequences (reviewed in Monder, 1991). Disruption of this enzymatic activity in the syndrome of Apparent Mineralocorticoid Excess (AME) (Stewart, et al., 1988; Ulick, et al., 1979) and in those suffering from licorice toxicity (Monder, et al., 1989; Stewart, et al., 1987) results in decreased oxidation of cortisol to cortisone. These patients have hypernatremia, hypertension and hypokalemia, suggesting hyperaldosteronism, yet the renin-angiotensin-aldosterone axis is supressed (New, et al., 1977; Stewart, et al., 1988). Cortisol binds to the mineralocorticoid (Type I) receptor with an affinity equivalent to that of aldosterone (Arriza, et al., 1987), and concentrations of free cortisol are 100 to 1000 times greater than those of aldosterone. These observations fostered the proposal that 11β-HSD functions as an end-organ inactivator of cortisol in mineralocorticoid target tissues such as the kidney (Funder, et al., 1988; Stewart, et al., 1988). In this model, 11β-HSD defends the mineralocorticoid receptor by converting cortisol to cortisone, and thus allowing proper signaling by aldosterone, so that renal cortisol concentrations do not overwhelm the action of aldosterone, leading to a syndrome similar to AME or licorice toxicity. However not all studies are consistent with this hypothesis (Ulick, et al., 1990).

11β-HSD may also function as an end-organ modulator of glucocorticoid action in tissues containing the Type II (glucocorticoid) receptor: the balance between 11β-HSD oxidation and reduction would determine the effective concentration of the biologically active cortisol in the target tissue. For example, 11β-HSD might protect the testis from glucocorticoid inhibition of testosterone production (Monder, et al., 1991). Similarly, placental 11β-HSD appears to protect the fetus from maternal glucocorticoids. Near parturition, however, lung maturation appears to proceed using cortisol produced in the fetal lung by increasing pulmonary 11β-HSD dehydrogenase activity (Monder, et al.,

1991). 11β-HSD may also contribute to receptor specificity, explaining the colocalization of the mineralocorticoid and glucocorticoid receptors in specific regions of the brain (Monder, et al., 1991). Finally 11β-HSD may also play a major role in regulating glucocorticoid effects in target cells. The preferred direction for the reaction is cortisol formation in target tissues such as skin fibroblasts and liver, in which concentrations of NADPH are high, as demonstrated by induction of aromatase activity by cortisone; furthermore glucocorticoids induce and insulin supresses both activities of 11β-HSD in cultured human skin fibroblasts, indicating that the action of 11β-HSD is hormonally regulated (Hammami and Siiteri, 1991).

The purification (Lakshmi and Monder, 1988) and cloning (Agarwal, et al., 1989) of this enzyme from rat liver provided the reagents needed to test these hypotheses, but not all results have been consistent. Both immuno-cytochemistry (Castello, et al., 1989) and *in situ* hybridization histochemistry (Stewart, et al., 1991; Yau, et al., 1991), co-localized 11β-HSD and the Type I receptor in the distal portion of the renal collecting duct, but others detected 11β-HSD in the proximal tubules but not in collecting ducts, suggesting a paracrine mechanism that cleared cortisol from blood before it reached the distal collecting duct (Rundle, et al., 1989). There may also be multiple forms of 11β-HSD, as there are several sizes of 11β-HSD mRNA in the rat kidney (Krozowski, et al., 1990). An isozyme in the distal collecting duct having a different K_m (Naray-Fejes-Toth, et al., 1991) and an isozyme dependent on NAD^+, rather than $NADP^+$ (Mercer and Krozowski, 1992), have been reported. Thus, although there are several candidate physiological roles for 11β-HSD, the one played by the isolated and cloned hepatic form remains unknown.

Glucocorticoid-resistance syndromes may provide further insight into the roles of 11β-HSD. Human glucocorticoid resistance is rare, but New World primates such as the squirrel monkey (*Saimari scireus*) are severely resistant, having cortisol concentrations up to 10 μM (360 μg/dl, normal human values 5-20 μg/dl), yet they do not suffer from Cushing syndrome or AME (Chrousos, et al., 1984; Chrousos, et al., 1983; Chrousos, et

al., 1982). Genital skin fibroblasts from these animals have less than half the number of glucocorticoid receptors found in human genital skin fibroblasts, and these receptors have a slightly higher K_D of 5-8 x 10^9 M compared to 3 x 10^9 M for the human receptor (Chrousos, et al., 1982; Siiteri, 1986). Thus squirrel monkeys have extreme insensitivity to cortisol due to a reduction in cortisol receptor number, which is compensated for by high cortisol secretory rate, a low metabolic clearance rate and diminished binding of cortisol to corticosteroid binding globulin. These seemingly paradoxical changes produce the high intracellular cortisol concentrations needed to saturate receptors completely, achieving functional responses equivalent to those seen when receptor numbers are normal and are only 50% saturated. These considerations predict that 11β-HSD expression should be nearly maximal in both glucocorticoid and mineralocorticoid target cells to either promote or negate the effects of cortisol. Indeed, both activities of 11β-HSD are 100-fold higher in squirrel monkey than in human skin fibroblasts (M. Hammami and P. K. Siiteri, unpublished observations). To study the 11β-HSD activity in this experiment of nature, we cloned the squirrel monkey cDNA and gene for 11β-HSD. The abundance of 11β-HSD mRNA in tissues other than liver and fibroblasts was low, and could only be detected by RNase protection experiments. As generated from equivalent cDNA expression vectors transfected into CHO cells, human and squirrel monkey 11β-HSD had similar values for K_m and V_{max} for converting cortisol to cortisone. The relatively low mRNA abundance and enzymatic activity equivalent to the human enzyme are inconsistent with a role for the cloned hepatic enzyme in counteracting the high levels of cortisol in the squirrel monkey, or in defending the mineralocorticoid receptor in target tissues such as the kidney. The enzyme may instead maximize cortisol concentrations in target tissues to overcome the squirrel monkey's relative glucocorticoid resistance associated with decreased glucocorticoid receptor number.

Methods

cDNA cloning. A cDNA library was constructed using polyadenylated RNA from cultured squirrel monkey fibroblasts. The cDNA was synthesized using both random primers and oligo dT, and was cloned into the *Eco* RI site of λgt10. In the absence of a primate cDNA sequence for 11β-HSD we identified a portion of the rat 11β-HSD cDNA sequence (Agarwal, et al., 1989) that was fairly well conserved among numerous dehydrogenases, then used known human codon preferences to design the best-guess 76- base oligonucleotide GGTGGAGAAGAAGCCGTCCAGGGCGAACTTGCTGGCAGAG TAGGAGGCGATCAGGGGCTGGGTCATCTTGCCGGCC corresponding to amino acids 168 to 192 of the rat enzyme. This oligonucleotide was end-labeled using ^{32}P-γATP and T4 DNA kinase to a specific activity of about 1 x 10^8cpm/μg and used to screen about 750,000 cDNA clones propagated in *E coli* Y1080 at about 50,000 plaques per 15 cm plate. The filters were hybridized overnight with about 500,000 cpm/ml in 6X SSPE (1X SSPE: 150 mM NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA) at 50°C then washed at progressively higher temperatures in 6X SSPE, 1% $NaDodSO_4$ until possible positives were distinguished from background. One of the initial positives, termed clone 16E, had a sequence substantially similar to rat 11β-HSD cDNA.

Southern blotting.

Genomic DNA was prepared from squirrel monkey fibroblasts, digested overnight with restriction enzymes and electrophoresed through 0.8% agarose gel as described (Morel, et al., 1989). After staining the gel with ethidium bromide, the DNA was depurinated with 0.25 M HCl for 15 min before transfer to nylon membrane (Amersham, Hybond) overnight in 0.4 M NaOH, 1.5 M NaCl. After neutralization in 0.5 M Tris, pH 7.0, 1.5 M NaCl for 2 min, the DNA was crosslinked to the filter with 1.2x10^5 μjoules using a UV Stratalinker 1800 (Stratagene). This filter was probed with the 16E cDNA clone ^{32}P-labeled with random primers.

Gene cloning.

Genomic DNA was isolated from squirrel monkey fibroblasts, partially digested with *Mbo* I and cloned into the *Bam* HI site of λEMBL-3 SP6/T7. The library was plated onto NM538 cells and about 750,000 plaques were screened using the 1040 bp *Eco* RI fragment of the 16E cDNA ^{32}P-labeled to 10^9cpm/μg. Following hybridization overnight the filters were washed under high stringency conditions, 0.1X SSC (1X SSC: 150 mM NaCl, 15 mM NaCitrate), 0.1% $NaDodSO_4$ at 65°C for 30 min and auto-radiographed for 48 h with intensifying screens. Two positive clones were detected, plaque purified and grown overnight in liquid culture in LB media with 5 mM $CaCl_2$. Intact phage were isolated by our modifications of the procedure of Helms et al (Helms, et al., 1985). Phage were incubated for 60 min at 37°C in 5 mM $MgSO_4$ 0.4 μg/ml RNase A and 8.0 mg/ml DNase I; the bacterial cells and debris was adsorbed onto an equal volume of DEAE-cellulose (DE52, Whatman, suspended in 50 mM HCl and then titrated to pH 6.8 with 1 M NaOH) and, after centrifugation in a microfuge at 13,000 x g, the intact phage in the supernatant were lysed by adding phenol and vortexing. The aqueous phase was re-extracted with 1:1 phenol:chloroform and then chloroform, followed by ethanol precipitation. Restriction fragments that were detected by labeled fragments of the squirrel monkey 11β-HSD cDNA were subcloned into Bluescript SK+ (Stratagene) and sequenced.

RNA analysis.

Tissue samples were removed from healthy squirrel monkeys and immediately frozen in liquid nitrogen. RNA was prepared from 0.5 g samples of tissue as described (Moore, et al., 1992). Northern analysis was done with 20 μg samples of RNA as described (Moore, et al., 1990) using a 870 bp *Eco* RI to *Sac* I fragment of the squirrel monkey 11β-HSD cDNA as a probe. RNase protections were done essentially as described (Mellon and Miller, 1989; Moore, et al., 1990) using a 180 bp *Eco* RI/*Hha* I fragment of

the squirrel monkey 11β-HSD cDNA cloned into the *Sma* I site of Bluescript SK+ (Stratagene) so that T3 polymerase generated a 252 bp antisense RNA. The hybridizations were done at 58°C overnight with 20 μg of RNA, followed by treatment for 60 min with RNase A and RNase T1 at 37°C.

To map the transcriptional initiation site we used a 263 bp *Bam* HI to *Eco* RI fragment encompassing part of exon I, and a region of the putative 5' flanking DNA. This fragment was cloned into the *Bam* HI and *Eco* RI sites of BSK+, and a 380 bp cRNA synthesized using T3 RNA polymerase and used in a protection assay as described above.

Expression of the squirrel monkey and human11β-HSD cDNAs.

To express 11β-HSD cDNAs, we constructed a hybrid expression vector from the Bluescript cloning vector and the SV40-based expression vector pECE (Ellis, et al., 1986). Bluescript SK+ was cut with *Sac* I and *Kpn* I, then blunt-ended with T4 DNA polymerase. The 618 bp *Pvu* II/*Bam* HI fragment of pECE, containing the SV40 promoter/enhancer, polylinker cloning site, and SV40 polyadenylation site was blunt-ended with Klenow polymerase and cloned into the blunt-ended Bluescript. The resulting vector, termed pBSKECE, is a high-copy-number, pUC-based plasmid which eliminates the need for large-volume chloramphenicol-amplified cultures that characterize pECE, and facilitates sequencing of cloned inserts from standard Bluescript primers. The squirrel monkey cDNA including the 3' untranslated DNA to the *Xho* I site 60 bp downstream from the stop codon was inserted into the *Xba* I to *Sma* I sites in pBSKECE. Similarly the entire coding region for the human 11β-HSD cDNA (generously provided by Dr. P. C. White), (Tannin, et al., 1991) was excised with *Xba* I and *Hin*d III and cloned into the *Xba*I and *Hin*d II sites of pBSKECE. All these constructions were confirmed by dideoxy sequencing.

Plasmids were transfected into CHO cells grown on 6 cm culture dishes, as described (Moore, et al., 1990). CHO cells were grown in Ham's F12 media supplemented with 10% fetal calf serum and 50 μg/ml gentamycin; for transfections, the

medium was changed to DME H21 with the same supplements. Stable cell lines expressing the squirrel monkey or human 11β-HSD were created by transfecting the cells with 20 μg of the appropriate plasmid plus 4 μg of pSVNeo. Selection with 50 μg/ml G418 was started 48 hours after transfection, and continued for 10 days with the media changed every day.

11β-HSD enzyme assays.

11β-HSD activity was assayed as described (Hammami and Siiteri, 1991). Cell monolayers in 6-well plates were incubated with radioactive substrates in DME at 37°C in 95% air, 5% CO_2 for various times. Conditioned medium was extracted with 10 volumes of ethyl acetate, evaporated under N_2 and reconstituted with 100 μl of ethyl acetate containing 1 mg/ml each nonradioactive cortisol and cortisone. Duplicate aliquots were applied to TLC plates and another aliquot counted to monitor recovery. Chromatography was in chloroform:ethanol:t-butanol (9:1:1) to separate cortisol from cortisone, and the spots were developed with glacial acetic acid:sulfuric acid:p-anisaldehyde (50:1:1) and heating at 100°C under vacuum for 5-10 minutes. The steroid spots were cut out, eluted in 0.3 ml of ethanol and counted. Total cellular protein was determined by Bradford assay. Kinetic data were linearized according to Eadie-Hofstee analysis (V/S=Vmax/Km-V/Km, where V is the rate in pmoles/mg/hr and S is the substrate concentration in nM). The Km and Vmax were calculated using a least squares fit.

Western blot.

Stably transfected cells were rinsed with PBS three times, scraped off with a rubber policeman, pelleted by centrifugation, resuspended in an equal volume of lysis buffer (25 mM Tris pH 7.4, 10% glycerol, 1 mM EDTA, 0.5 μg/ml Leupeptin, 1 μg/ml Aprotinin, 0.2 mM PMSF, 2 mM DTT and 1% Triton X-100) and frozen on dry ice. The lysate was thawed on ice, cleared by centrifugation at 12000 xg and the protein concentration of the

supernatant measured using the Bradford assay. 25 or 50 μg of lysate was electrophoresed on an $NaDodSO_4$/10% polyacrylamide gel for 45 minutes at 200V. The separated proteins were transferred overnight at 40V, 4°C to a polyvinylidine diflouride membrane (Millipore). After transfer the membrane was incubated 1 hour in TBS (Tris 10 mM, pH 7.5, 0.15 M NaCl) containing 3% non-fat dry milk, then rinsed with TBS and incubated another 2 hours in TBS with 3% milk, 0.2% Tween 20 and rabbit antibody to rat hepatic 11β-HSD (a gift of Dr. C. Monder). The membrane was then washed with TBS and TBS plus 0.2% Tween 20 and developed using an Amersham ECL kit.

Results

cDNA Cloning.

To probe for squirrel monkey 11β-HSD sequences, we synthesized a 76-base "best guess" oligonucleotide coresponding to amino acids 168-192 of the rat 11β-HSD sequence. This region was considered to be less prone to evolutionary drift, as it is quite similar in the sequences for rat 11β-HSD (Agarwal, et al., 1989) and human 17β-HSD (Luu-The, et al., 1989). Using this probe, we identified a nearly full-length 11β-HSD cDNA from the squirrel monkey kidney cDNA library we constructed in λgt10. Initial subcloning of the cDNA from the phage into pBSK revealed a sequence beginning at amino acid 27 due to the presence of an *Eco* RI cleavage site at this location in the cDNA, so that the proximal 5' 150 bp of the cDNA had to be subcloned and sequenced separately. The entire cDNA is 1150 bp long and encodes 292 amino acids, 70 bp of 5' untranslated DNA and 204 bp of 3' untranslated DNA. Shortly after we completed the cDNA sequence, the sequence of the human 11β-HSD cDNA and gene became available (Tannin, et al., 1991). The squirrel monkey amino acid sequence is 91% identical to the human sequence but only 75% idential to rat 11β-HSD. Like the human sequence, the amino-terminal end of the monkey sequence has two in-frame methionine codons which could represent potential translational

initiation codons; as in the human sequence the first of these is a better fit to the consensus sequences for translational initiation (Kozak, 1984).

Squirrel monkey 11β-HSD gene.

Genomic DNA from squirrel monkey fibroblasts was digested with five restriction endonucleases and examined by Southern blot probed with the full-length cDNA. This analysis yielded a simple pattern: *Bam* HI gave only a single band, and three of the four enzymes that gave two bands (*Eco* RI, *Xba*I, and *Sac*I) cut cDNA (exon) sequences. This suggests that the squirrel monkey genome has a single gene for 11β-HSD, and that the gene is fairly small. Reprobing the blot under low stringency conditions did not reveal additional bands (not shown) suggesting the squirrel monkey genome does not contain other genes with substantial sequence similarity to the 11β-HSD gene.

We constructed a library of squirrel monkey genomic DNA in λ EMBL3 and screened about 750,000 phage, identifying two positive clones. After plaque purification, their identity as clones of 11β-HSD was confirmed by Southern blots showing some fragments of the same size as seen in Fig. 5.2. Appropriate fragments of both phage were subcloned and sequenced, identifying exons 1, 2, 5, and 6; neither phage contained exons 3 and 4. The intron-exon junctions identified by sequencing were identical to those found in the human gene (Tannin, et al., 1991).

To identify the transcriptional initiation site of the squirrel monkey 11β-HSD gene we sequenced about 90 bp of 5' flanking DNA upstream from exon 1 (Fig. 5.3A). This sequence contains the potential CAAT promoter sequence CCAATC, but no obvious TATAA sequence. We subcloned the 236 bp *Eco* RI/*Bam* HI fragment extending through this region to the codon for amino acid 27, and prepared a 380-base ^{32}P-labeled riboprobe. An RNase protection assay showed two major protected bands of about 173 and 179 bp (Fig 5.3B), identifying the transcriptional start sites shown in Fig 5.3A. The presence of

the CCAATC sequence, the lack of an apparent TATAA box, and the two transcriptional start sites all correspond exactly with the findings in the human gene (Tannin, et al., 1991).

Tissue distribution of 11β-HSD mRNA .

Northern blot analysis detected a single species of 11β-HSD mRNA of about 1.8 kb only in the liver (Fig. 5.4A) and in the fibroblast RNA from which the cDNA library was constructed (data not shown). The autoradiograph shown is a 14 d exposure of a blot hybridized to a probe with a specific activity of > 10^9cpm/μg DNA. To increase the limits of detection we re-analyzed the same RNA samples using a much more sensitive RNase protection assay (Fig. 5.4B). Using this 11β-HSD assay, mRNA was most abundant in the liver, followed closely by fibroblasts. Substantial amounts of 11β-HSD mRNA were also detected in the brain, lung, heart and ovary, and smaller amounts were found in kidney, skin and spleen. After a very long autoradiographic exposure a very weak signal was seen in the adrenal, but we did not detect any 11β-HSD mRNA in the testis (data not shown). The sample of pancreatic RNA was degraded, hence pancreatic content of 11β-HSD cannot be determined.

Activity of squirrel monkey and human 11β-HSD.

To examine the relative activities for the squirrel monkey and human hepatic forms of 11β-HSD we stably transfected CHO cells with vectors expressing the squirrel monkey or human cDNAs. Stably transfected, rather than transiently transfected cells were used so that experiments with long-term incubation of substrate could be performed and so that the transformed cell lines could be standardized and compared. The conversion of cortisol to cortisone, and of cortisone to cortisol were monitored by incubating transfected CHO cells with increasing concentrations of labeled substrates and then quantifying the substrates and products by thin layer chromatography. Kinetic parameters were calculated from Eadie-Hofstee plots by linear regression (Fig. 5.5). The K_m values (slope) for both the

monkey and the human enzyme were similar to each other for the two reactions (Table 5.1). The Vmax estimates (Y-intercept) for the conversion of cortisone to cortisol were also similar for both enzymes while that for the reverse reaction was five-fold greater for the monkey enzyme than the human (Table 5.I). There was no detectable conversion with CHO cells transfected with the parent expression vector pBSKECE. The relative abundance of the human and squirrel monkey enzymes in the transfected CHO cells was indistinguishable by Western blotting (Fig. 5.6). The smaller, 29kD form of 11β-HSD seen in the CHO cells transfected with vectors expressing monkey (but not human) 11β-HSD cDNA, is also seen in squirrel monkey tissue samples (Hammami and Siiteri, unpublished data), and is not an artifact of the transfection system.

Discussion.

The model of 11β-HSD as an end-organ modulator of endocrine function depends fundamentally on proper tissue-specific expression and abundance, and on the redox state of the pyridine nucleotide cofactor concentrations in target tissues. Changes in the redox state appear crucial. The hypothesis that 11β-HSD protects renal mineralocorticoid receptors requires a ratio of NADPH:NADP+ that is considerably less than 1, a situation not encountered in normal cells. By enzymological assays 11β-HSD activities are found in many tissues (Murphy, 1981; Weidenfeld, et al., 1982), although this activity is most abundant in the liver and kidney. Inconsistent results from analyzing the tissue-specific expression of the recently cloned hepatic 11β-HSD (Agarwal, et al., 1989) using either RNA analysis or antibodies specific for this form suggests that there may be more than one pyridine nucleotide-dependent 11β-HSD. Four lines of evidence suggest that these are not the same as the cloned enzyme. First, Northern blotting reveals multiple sizes of rat 11β-HSD mRNA (Krozowski, et al., 1990; Whorwood, et al., 1992). Second, Western immunoblotting identifies several different sizes of rat 11β-HSD in liver and kidney (Monder and Lakshmi, 1990). Third, antisera raised against the cloned hepatic form of rat

11β-HSD do not detect this enzyme in the distal nephron, even though 11β-HSD mRNA is detected by *in situ* hybridization histochemistry (Monder and Lakshmi, 1990) and by Northern blotting (Krozowski, et al., 1990). Fourth, a newly identified form of rat 11β-HSD activity requires NAD+ rather than on NADP+, as used by the "conventional" enzyme (Mercer and Krozowski, 1992; Walker, et al., 1992). Thus the present data suggest that there are at least two different 11β-HSD enzymes, encoded by more than one gene.

In general, the abundance of the mRNA for the cloned 11β-HSD corresponds to the level of enzymological activity found in the rat (Whorwood, et al., 1992). However there are exceptions (Krozowski, et al., 1990). In order of decreasing abundance, we detected squirrel monkey 11β-HSD mRNA in the liver, fibroblasts, brain, lung, kidney, skin, spleen and adrenal, and we detected no 11β-HSD mRNA in the testis (Whorwood, et al., 1992). By contrast, the survey of rat tissues did not detect 11β-HSD activity in the heart or spleen but did find considerable activity in the testis. In other tissues our findings in the squirrel monkey are generally comparable to the findings in the rat (Krozowski, et al., 1990; Walker, et al., 1992; Whorwood, et al., 1992) although the greater sensitivity of our RNase protection assay permitted detection in more tissues.

A truncated form of 11β-HSD, termed 11β-HSD2, is found in rat kidneys and might be an isoform responsible for defending the Type I receptor (Krozowski, et al., 1990). There is no direct evidence that this truncated version is functional. This isoform arises from a kidney-specific promoter in intron I (Moisan, et al., 1992). Although we see a 29kD band of 11β–HSD protein by Western blotting, this cannot arise in the same fashion as rat 11β–HSD, as the RNase protection probe we used (Fig. 5.4B) spans this region and would generate a fragment 7 bp shorter than the one we see. No shorter fragment is seen in the kidney, or any other tissue (Fig. 5.4B), even after an over-exposure of the autoradiogram (data not shown). Thus, if there is a role for this isoform in cortisol metabolism in the rat kidney it is not conserved in the squirrel monkey.

The abundance of squirrel monkey 11β-HSD mRNA we found was considerably lower than expected given this animal's profoundly elevated circulating concentrations of cortisol. This form of 11β-HSD is detectable in most rat tissues by Northern analysis (Krozowski, et al., 1990; Moisan, et al., 1992; Whorwood, et al., 1992) but squirrel monkey 11β-HSD mRNA could be detected by Northern analysis only in the liver and fibroblasts; its presence in other tissues was only seen by RNase protection assay. This is inconsistent with this form of 11β-HSD being responsible for the conversion of cortisol to cortisone, especially in the kidney where high 11β-HSD activity would presumably be needed to defend the mineralocorticoid receptor from the extremely high concentrations of cortisol found in the squirrel monkey.

However, the possibilities remained that the enzyme was extremely stable, or extremely active, (or both), thus allowing low levels of mRNA to generate sufficient enzyme activity. To determine if squirrel monkey 11β-HSD has a much greater specific activity, or is much more stable than the human enzyme, we expressed the squirrel monkey and human 11β-HSD cDNAs in CHO cells. Cells expressing the squirrel monkey 11β-HSD had equivalent activity to cells expressing the human enzyme, except for a five-fold greater Vmax for converting cortisol to cortisone. Western blotting indicated the CHO cells transfected with either the human or monkey 11β–HSD cDNA expressed similar amounts of 11β-HSD protein (Fig. 5.6). However, the role of the 29 kd form of monkey 11β-HSD is unknown; if it has enzymatic activity, it would mean there is twice as much enzyme in the cells transfected with the monkey construction; this might partially explain the higher Vmax seen with these cells. The approximately equal amounts of 11β-HSD protein seen in Fig. 6 also suggest that the monkey enzyme is not vastly more stable than the human enzyme, and that therefore the abundance of 11β–HSD mRNA is a reasonable index of 11β-HSD enzyme abundance and activity. Thus the squirrel monkey does not compensate for its high cortisol concentrations either by making large quantities of 11β-HSD, or by making an especially active 11β-HSD.

The low abundance of the hepatic form of squirrel monkey 11β-HSD without a compensating increase in activity strongly indicates that this enzyme cannot be responsible for this animal's glucocorticoid resistance, or for defending its mineralocorticoid receptors. Similarly, these data suggest it is unlikely that the hepatic form of 11β-HSD could perform its proposed roles in the testis and brain (Monder, 1991). However, this enzyme may play an important role in maintaining high cortisol activity in the liver and fibroblasts, in order to overcome the squirrel monkey's extreme cortisol insensitivity due to fewer glucocorticoid receptors. The reasons why New World primates have fewer receptors for glucocorticoids (Chrousos, et al., 1984), progesterone (Chrousos, et al., 1984), and androgens (Siiteri, 1986) remain unknown. However it appears clear that multiple endocrine mechanisms, including increased 11β-HSD activity, are involved in compensating for these receptor deficiencies.

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Figure 5.1. Sequence of the squirrel monkey 11β-HSD cDNA. (A) cDNA sequence and translation of the open reading frame starting at the best consensus Kozak sequence. Underlined amino acids are identical in the squirrel monkey, human, and rat sequences. Starred amino acids are the same in the squirrel monkey and human proteins. (B) Sequencing strategy used for the longest cDNA clone. The wide solid bar indicates the coding regions and the thinner bars represent 5' and 3' untranslated regions. The selected restriction sites shown are *Xho* I (X), *Sac* I (S), *Eco* RI (E), *Hin*c III (H) and *Neo* I (N).

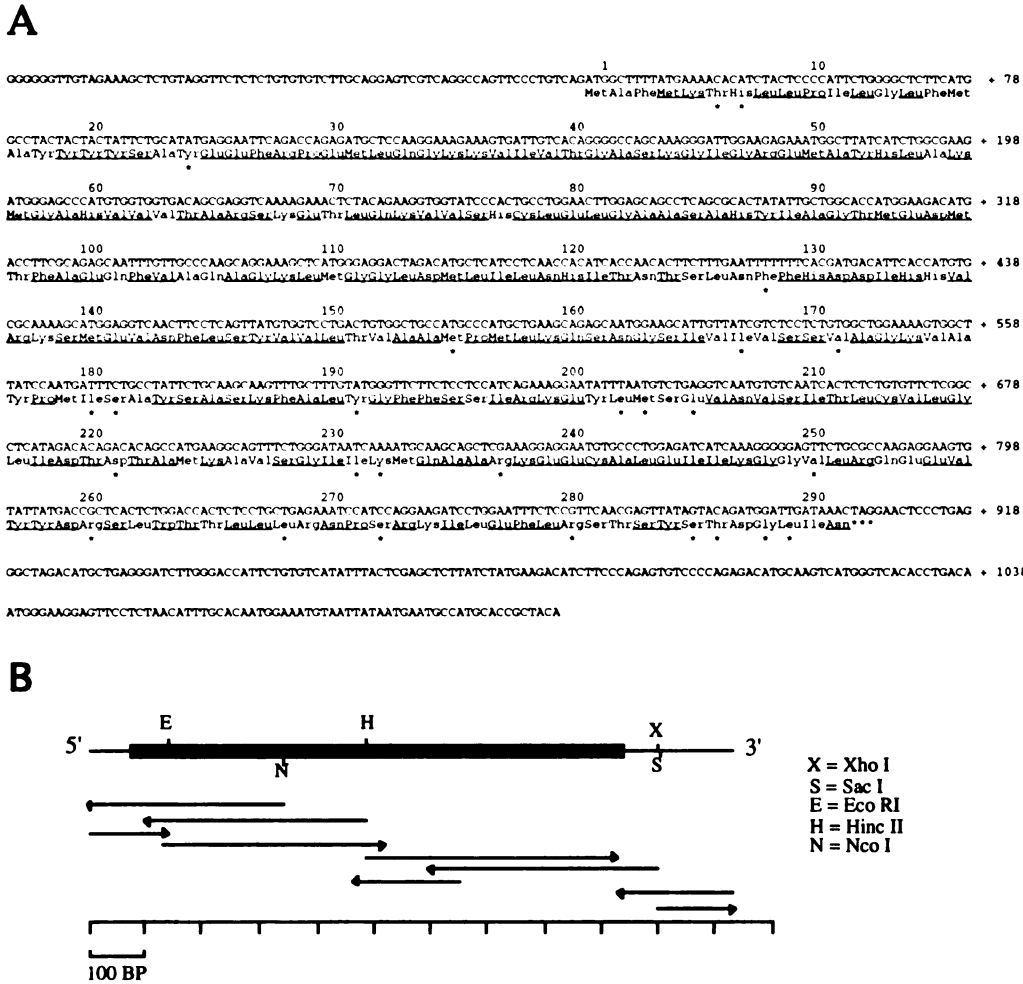

Figure 4.1. Sequence of the squirrel monkey 11β-HSD cDNA. (A) cDNA sequence and translation of the open reading frame starting at the best consensus Kozak sequence. Underlined amino acids are identical in the squirrel monkey, human, and rat sequences. Starred amino acids are the same in the squirrel monkey and human proteins. (B) Sequencing strategy used for the longest cDNA clone. The wide solid bar indicates the coding regions and the thinner bars represent 5' and 3' untranslated regions. The selected restriction sites shown are *Xho* I (X), *Sac* I (S), *Eco* RI (E), *Hinc* III (H) and *Neo* I (N).

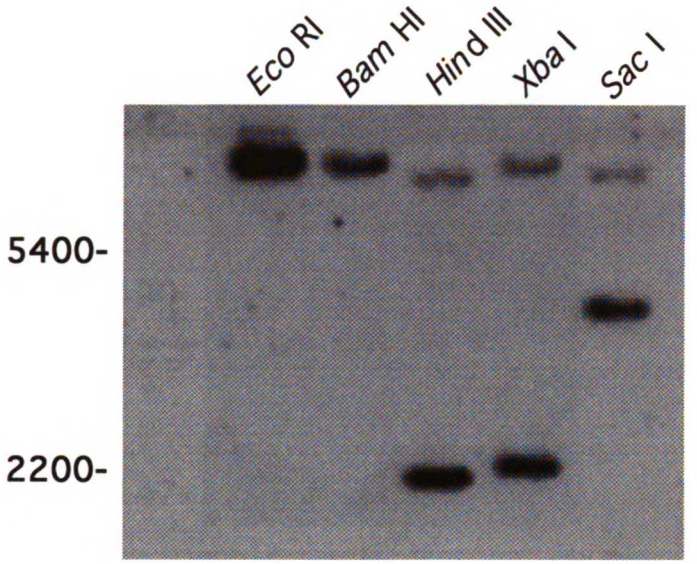

Figure 4.2. Southern blot of squirrel monkey genomic DNA. Fibroblast DNA (10 μg) was digested with the enzymes indicated, displayed by Southern blotting probed with squirrel monkey 11β-HSD cDNA. Molecular sizes are from phage PM-2 DNA cut with *Hin*dIII, run in another lane, and seen by staining with ethidium bromide.

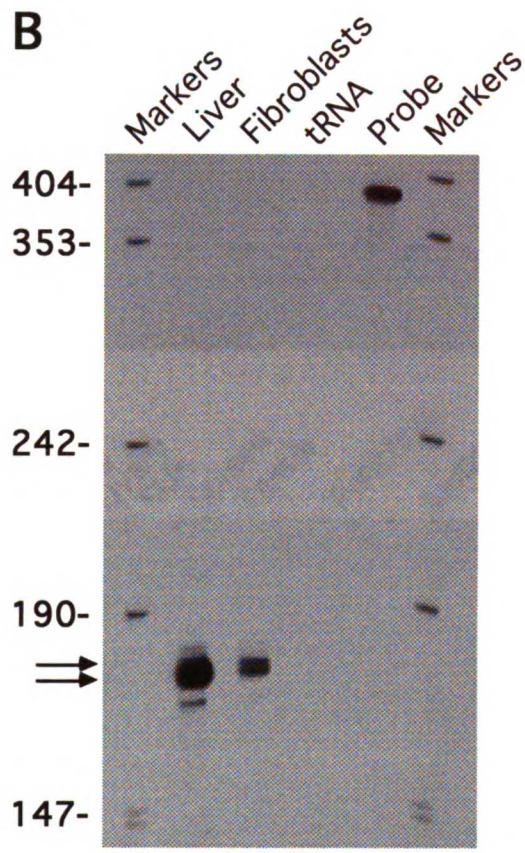

Figure 4.3. Analysis of the transcriptional start site of the squirrel monkey 11β-HSD gene. (A) Sequence of the 5' flanking DNA; the mRNA initiation sites are indicated with arrows, and the possible CAAT box is underlined. (B) RNase protection analysis of 10 μg of squirrel monkey liver and fibroblast RNA. The probe encompasses part of exon I, the 5' untranslated region and part of the putative 5' flanking DNA. The bases corresponding to the initiation sites revealed by the protected fragments of 179 and 173 bases are designated by arrows over the sequence in panel A.

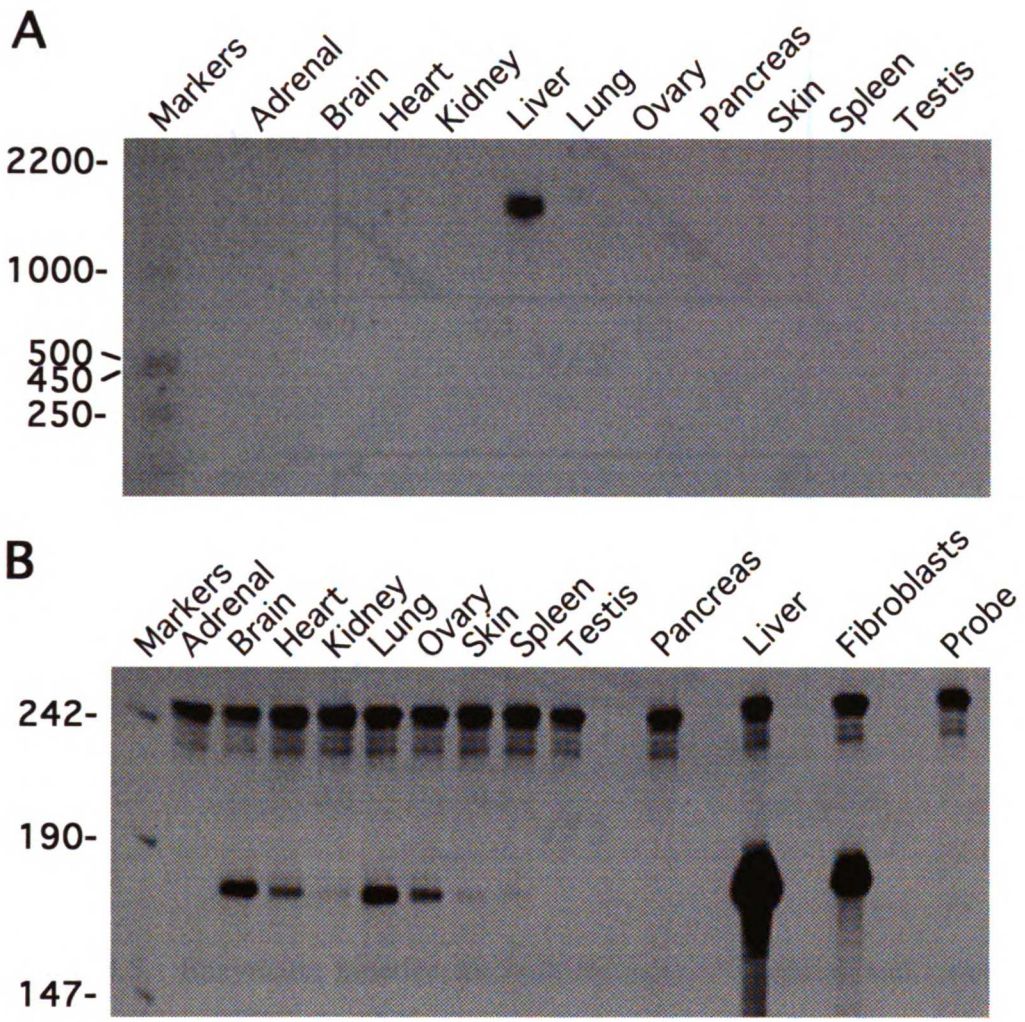

Figure 4.4. Tissue distribution of squirrel monkey 11β-HSD expression. (A) Northern blot probed with an 870 bp *Eco* RI to *Sac* I fragment of the squirrel monkey cDNA and exposed to film for 2 weeks. Each lane contains 20 μg of total RNA from the tissues indicated. 11β-HSD mRNA is detected only in liver. (B) RNase protection of 20 μg samples of total RNA using a 252 bp antisense probe that protects 180 bp of squirrel monkey 11β-HSD mRNA. Markers are end-labeled *Hin*d III cut bacteriophage PM2. 11β-HSD mRNA is detected in all tissues except adrenal, testis, and pancreas.

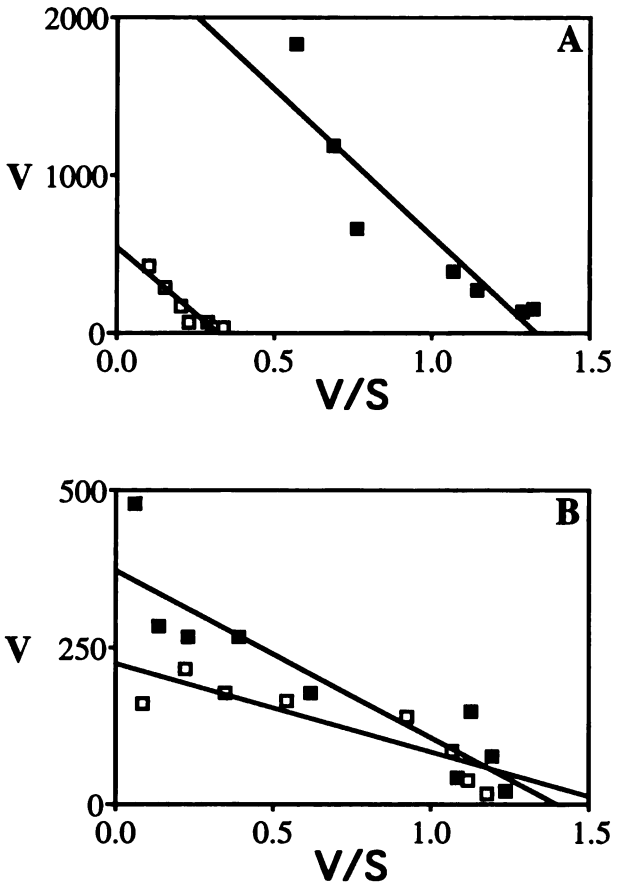

Figure 4.5. Enzymatic kinetics for both the squirrel monkey and human 11β-HSD enzymes. CHO cells were stably transfected with the expression vector pBSKECE containing the cDNAs for either the human or the squirrel monkey 11β-HSD. The conversion of radio-labeled substrates was monitored using thin layered chromatography. Data are presented as Eadie-Hofstie plots (V/S=Vmax/Km-V/Km, where V is the rate in pmoles/mg protein/hr and S is the substrate concentration in nM); the Km (the slope) and Vmax (the Y intercept) values are calculated using linear regression to yield the data shown in Table I. (A) Conversion of cortisol to cortisone. (B) Conversion of cortisone to cortisol. Open symbols are for the human enzyme, closed symbols are for the squirrel monkey enzyme.

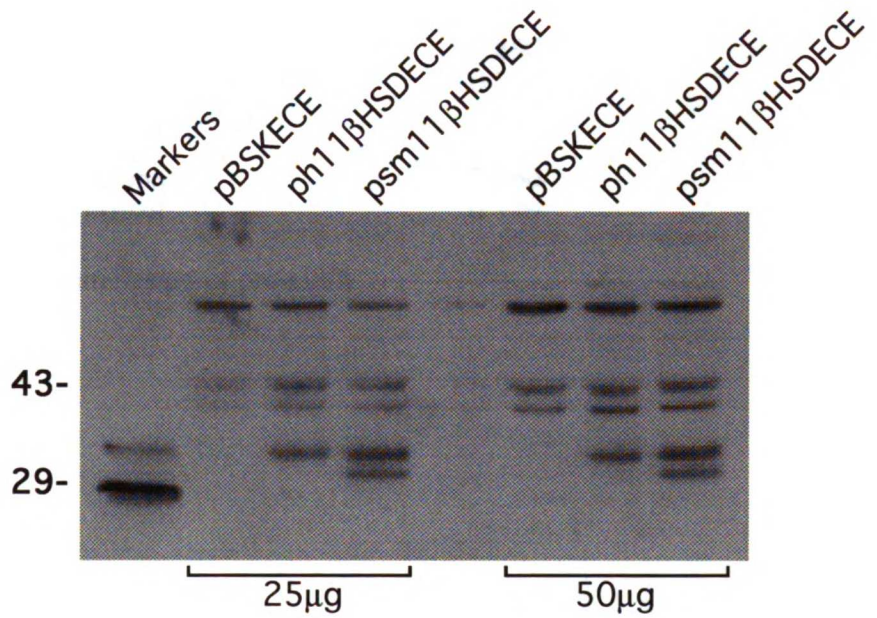

Figure 4.6. Western blot of 11βHSD expressed by stably transfected cell lines. Protein from pools of cells stably transfected with vectors expressing either the human (ph11βHSD-ECE) or the squirrel monkey (psm11βHSD-ECE) 11βHSD cDNAs. Total cellular protein (25 or 50 µg) was displayed by $NaDodSO_4$/10% polyacrylamide gel, electroblotted and probed with antibody to rat hepatic 11βHSD. Plasmid pBSK-ECE is the expression vector without a cDNA insert. Markers are low molecular weight protein markers from BRL.

Table I. Enzymatic Parameters for Human and Monkey 11β-HSD Expressed in COS-1 Cells

Reaction	Km (μM)	Vmax*
Monkey		
Cortisol→Cortisone	1.890	2490
Cortisone→Cortisol	0.272	0.376
Human		
Cortisol→Cortisone	1.660	540
Cortisone→Cortisol	0.140	0.216

*Vmax nmoles/μg of protein/h

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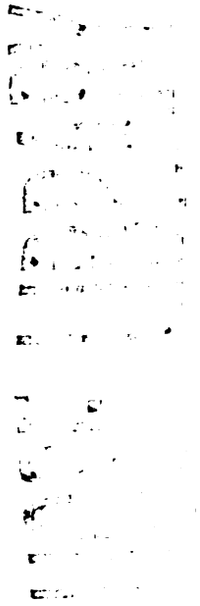

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