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Dopamine-1A (D1A) Receptor Gene Transcription in Renal Cells: Evidence for Novel CRE-like and 5'-UTR Intronic Elements.

by

Dawn A. O'Rourke

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Chair of Examining Committee

Nov. 27, 1996
Date

Executive Officer

Dr. Dennis Healy

Dr. Tong Joh

Dr. Paul Klotman

Dr. Jonathan Licht

Dr. James Roberts
Supervisory Committee

The City University of New York

Abstract

Dopamine-1A (D1A) Receptor Gene Transcription in Renal Cells: Evidence for Novel CRE-like and 5'-UTR Intronic Elements.

by

Dawn A. O'Rourke

Advisor: Dr. Dennis P. Healy

The purpose of this study was to characterize the dopamine-1A (D_{1A}) receptor gene promoter in renal epithelial cells. D_{1A} receptor gene transcription was elevated in LLC-PK_1 cells following short term exposure to dopamine, as D_{1A} receptor mRNA levels were increased D_{1A} receptor mRNA stability was decreased. Transcriptional regulation of the D_{1A} receptor gene was further examined using constructs containing progressive deletions in the D_{1A} 5'-flanking region (-3kb to +53) placed upstream of a promoter-less chloramphenicol acetyltransferase reporter gene (pCAT). The highest activity (>50 fold) in LLC-PK_1 cells was isolated to a construct which extended to -303. DNase I analysis and gel mobility shift assays indicated that several consensus sites (AP2, CRE-like, E-box, GC-box, Sp1/Egr-1) within -240 to -40 of the D_{1A} receptor gene bound LLC-PK_1 nuclear extracts.

The CAT activity of pCAT-303 increased 2-fold in response to increased intracellular cAMP. The -303 to +962 sequence contains two cAMP response-like elements (CRE) separated by 3 bases: 5'-CRE, AGACGTCA, and 3'-CRE; GGACGTCC. The 3'-CRE is better conserved across species and a single base substitution within the 3'-CRE reduced the CAT activity of the pCAT-303 construct to twice basal, indicating the 3'-CRE plays a role in basal and inducible expression of the

D_{1A} receptor gene. Placement of the 5'- and 3'-CRE sequences in front of a heterologous promoter stimulated CAT activity (nearly 3-fold) independent of orientation.

Attempts to further delineate the porcine D_{1A} receptor activating sequence to regions upstream (-303/+38) or downstream (+53/+962) of the transcription start site proved neither region was able to independently promote reporter activity. DNase I analysis and gel mobility shift assays demonstrated that LLC-PK_1 nuclear proteins could bind to the 3'-intron sequence. Detailed analysis of the 5'-UTR indicated that depending on context the intron can be either a positive or negative element and that an interaction between the 5'-UTR intron and that upstream elements are required for transcriptional activation of the D_{1A} receptor gene promoter in renal cells. This is the first evidence that a G-protein coupled receptor gene is regulated by interactions between elements within a 5'-UTR intron and a promoter.

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Table of Contents

List of Tables

List of Figures.

VI. Chapter 1: Introduction

VI. A. Background

Essential hypertension is a complex polygenic disease which is thought to result from a combination of genetic and environmental factors that interact to increase blood pressure. Hypertension has been implicated in the development of congestive heart failure and myocardial infarction (Benjamin et al., 1994; Goldberg, 1972; Lokhandwala and Amenta, 1991; Wang et al., 1993; Lee, 1982) and is directly related to the acceleration of arteriosclerosis, stroke and end stage renal disease (Klag et al., 1996; Kannel et al., 1970; Lassen, 1996; Campese, 1994). Several forms of hypertension are aggravated by sodium loading and are termed salt-sensitive. Multiple hormonal systems exert control in blood pressure regulation, including the renin-angiotensin, vasopressin, atrial natriuretic factor peptide and kallikrenin/kinin (Jose et al., 1993), and abnormalities in the regulation of these systems, principally in the kidney, have been linked to these essential systems (Woolfson and de Wardener, 1996). One such system that has received attention is the renal dopaminergic system.

Intravenous administration of dopamine leads to an increase in sodium and water excretion and an increase in renal blood flow in most mammals (McDonald et al., 1964; Lokhandwala and Amenta, 1991; Jose et al., 1992; Hedge et al., 1989a). In addition, there is strong evidence that the kidney produces dopamine in response to sodium loading (Suzuki et al., 1984; Hayashi et al., 1990). Dopamine is synthesized from decarboxylation of L-dopa by aromatic L-amino acid decarboxylase. The renal source of L-dopa appears to be from the circulation and not overflow from the sympathetic nerves (Baines et al., 1985). L-Dopa is removed from the renal filtrate (Baines and Chan, 1980) in a sodium dependent manner (Lee, 1982). Aromatic L-amino acid decarboxylase levels are high in the proximal tubules as demonstrated by histochemical and immunofluorescence techniques (Hagege and Richet, 1985;

Goldstein et al., 1972; Meister et al., 1992). Benserazide, an aromatic L-amino acid decarboxylase inhibitor, attenuates the natriuretic response to sodium loading, providing evidence that locally formed dopamine is involved in mediating the natriuretic response (Aperia et al., 1987). Similarly, inhibition of aromatic L-amino acid decarboxylase by carbidopa blocked dopamine production and attenuated adenylyl cyclase activity in a proximal tubule-like cell line, LLC-PK_1 (Grenader and Healy, 1991). Thus, locally synthesized dopamine may act in an autocrine or paracrine manner at dopamine receptors in the kidney (Grenader and Healy, 1991).

Renal dopamine receptors like their CNS counterparts are classified as either D_1-like or D_2-like. Renal D_1-like receptors are linked to cAMP production through G_s, (stimulatory guanine nucleotide binding protein) (Cheng et al, 1990, Grenader and Healy, 1991) and renal D_2 receptors are coupled to adenylyl cyclase through G_i (inhibitory guanine nucleotide binding protein) (Felder, 1984). In addition, renal D_2 receptors are present in the medulla which are linked to accumulation of prostaglandin E_2 (Huo and Healy, 1991). The role of D_2 receptors in the natriuretic response to dopamine is not clear. Of the five mammalian dopamine receptor genes which have been cloned, evidence has been provided that indicates that all five dopamine receptors are present in the kidney: D_{1A}, D_{1B}, D_2, D_3, D_4 (Grenader et al., 1995; Nash et al., 1993; Solokoff et al., 1993; Huo et al., 1991; Sun and Schafer, 1996). Cloning of D_{1A} receptor s expressed in the kidney indicated that they are identical to D_{1A} receptors expressed in brain (Nash et al., 1993; Grenader et al., 1995). D_1 and D_2 receptors are located in the renal artery, proximal tubules, cortical collecting duct and the medullary thick ascending limb (Jose et al., 1992). However, radioligand binding experiments and autoradiography indicated that D_1 receptors are primarily localized to proximal tubules (Huo and Healy, 1989; Felder et al., 1989a; Jose et al., 1992; Ricci et al., 1992). The relative density of D_{1A} receptors in the proximal tubules is significantly

higher than the D_{1B} expression (Nash et al., 1993; Albrecht et al., 1996). Autoradiography experiments demonstrated that D_2 receptors are concentrated in the inner medulla of the kidney (Huo and Healy, 1989). D_3 receptor mRNA was detected in the kidney by solution hybridization experiments. Pharmacological evidence of D_4 receptors in the kidney has recently been reported (Sun and Schafer, 1996).

Dopamine induces cardiovascular and renal effects via its actions at α-adrenergic, β-adrenergic and dopaminergic receptors (Lee, 1982; Goldberg, 1972). At high doses, α-adrenergic vasoconstriction increases total peripheral resistance and elevates blood pressure. At intermediate doses, dopamine decreases total peripheral resistance and blood pressure due to renal and mesenteric vasodilatation (Goldberg, 1972). Dopamine may also increase cardiac contractility and cardiac output without significantly altering heart rate or blood pressure through β_1-adrenergic receptors in the heart (Goldberg, 1972, Lee, 1982). At low doses, dopamine increases renal blood flow, glomerular filtration and sodium excretion (Hedge et al., 1989). The unique renal and hymodynamic effects of dopamine have made it useful in treating shock, congestive heart failure and oliguric renal failure (Goldberg, 1972).

The natriuretic actions of dopamine are attenuated by D_1 selective antagonists in a dose-dependent manner (Fredrickson, 1987; Hedge et al., 1989; Bass and Murphy, 1990; Pelayo et al., 1983; Hansell and Fasching, 1991). Siragy et al. (1989) demonstrated that D_1 antagonist, Sch 23390, decreased urine flow and sodium excretion without changing renal plasma flow, glomerular filtration rate, systemic arterial pressure, plasma aldosterone and was reversed by D_1 agonist, fenoldopam. These results provide evidence that natriuretic actions of dopamine are mediated by a D_1 receptor mechanism in the proximal tubule cells in the kidney. A direct consequence of

D_1 receptor activation in proximal tubule cells is dopamine-mediated inhibition of the Na^+/K^+ ATPase (Aperia et al., 1987; Bertorello et al., 1988) and inhibition of Na^+/H^+ exchanger (Felder et al., 1990; Gesek and Schoolwerth, 1990) activity. Increased sodium and water excretion is due to the actions of dopamine at D_1 receptors in the proximal tubules and distal segments of the nephron (Lee,1982; Felder et al., 1989a; Grenader and Healy, 1991, Huo and Healy, 1989).

One strategy that has proven to be useful in identifying genes that are important in a diseased state (i.e. hypertension) is to focus on genes likely to be involved in the regulation of blood pressure (Jose et al., 1992). Since dopamine produced by the renal tubules is an important intrarenal modulator of sodium excretion, it has been proposed that a dysfunctional renal dopaminergic system may contribute to the development of genetic hypertension (Kuchel et al., 1987). Recent experiments using transgenic mice in which the dopamine-1A receptor (D_{1A}) gene was knocked out indicated that mice lacking one or both D_{1A} alleles develop diastolic hypertension (Albrecht et al., 1996). Although a mutation in the coding region of the D_{1A} receptor has not been found and the molecular mechanism of the D_1 receptor defect involved in the etiology of hypertension remains elusive, a causal relationship has been established between the dopamine-1A receptor gene and hypertension (Albrecht et al., 1996).

Accumulating evidence supports the hypothesis that decreased renal dopamine production contributes to the etiology of sodium dependent hypertension. In clinical studies, normotensive subjects typically exhibit increased renal dopamine production as observed by high levels of dopamine and its metabolites in their urine following ingestion of a high sodium meal (Gill et al., 1988). This normal physiological response was not observed in salt-sensitive hypertensive patients (Gill et al., 1988). Abnormalities in dopamine excretion persist in hypertensive patients after increased

dietary precursors for dopamine were provided (via protein meal) (Clark et al., 1992) and is consistent with a renal defect in conversion of L-dopa to dopamine. A defect in the ability of aromatic L-amino acid decarboxylase to convert L-dopa to dopamine in the renal tubules has been further implicated as a contributing factor to hypertension since patients with stable essential hypertension exhibit high plasma levels of L-dopa yet excretion rates of dopamine and its metabolites remained comparable to control subjects (Kuchel and Shigetomi, 1992).

Defects in D_1-like receptor coupling have also been associated with the development of hypertension. Alterations in the renal dopaminergic system are considered to be contributing factors of genetic hypertension in two commonly used animal models, Dahl salt-sensitive (Ohbu et al., 1995) and spontaneously hypertensive rats (Felder et al., 1993; Gurich and Beach, 1994). The dopamine-1 (D_1) receptor subtype expressed in the proximal tubules of these animals is normal but the signaling cascade responsible for coupling the D_1 receptor to adenylyl cyclase is defective. The defect appears to be receptor specific (Kinoshita et al., 1990) and restricted to proximal tubules (Ohbu and Felder, 1993). The major consequence of defective D_1 receptor coupling is the decreased ability of D_1 agonists to inhibit the Na+/H+ exchanger and Na+/K+ ATPase activity (Jose et al., 1993; Gurich and Beach, 1994; Felder et al., 1989; Lokhandwala, 1991).

It is clear that many factors influence the development of hypertension. Sufficient evidence has established a relationship exists between the D_{1A} receptor gene and salt-sensitive forms of hypertension. The molecular mechanisms which regulate the expression of the renal D_{1A} receptor gene are unknown. Therefore, in order to understand the pathological consequences that may arise as a result of defects in the

renal dopaminergic system and design therapeutic approaches to modulate this system in the future, it is important to study the regulation of the D_{1A} receptor gene expression.

VI. B.1. Pharmacological Properties of Dopamine Receptors

As members of the G-protein coupled receptor superfamily, dopamine receptors (Civelli et al., 1993) are presumed to possess seven transmembrane domains. Comparison of the primary amino acid sequence of the dopamine receptor to the rhodopsin and β-adrenergic receptor sequences predicts they share a similar three-dimensional structure (Suryanarayana et al., 1992). Five mammalian dopamine receptors have been identified in the CNS and cloned. Based on their pharmacological and biochemical properties, they have been subdivided into two families: D_1-like and D_2-like (Kebabian and Calne, 1979). Presently, the mammalian D_1-like subfamily contains only the D_{1A} and D_{1B} (formerly D_5) receptors (Sunahara et al., 1990; Sunahara et al. 1991). Two additional D_1 receptors, D_{1C} and D_{1D}, were cloned from non-mammalian vertebrates (Sugamori et al., 1994; Demchyshyn et al., 1995), suggesting that a greater heterogeneity may exist among the mammalian D_1-like receptor family than previously anticipated.

D_1-like receptors have a high affinity for benazepine ligands including the D_1 antagonist, Sch-23390 (Civelli et al., 1993). They are coupled to the stimulatory guanine nucleotide binding protein, G_s (Gingrich and Caron, 1993). Agonist-induced activation of the D_1-like receptors is followed by the subsequent activation of adenylyl cyclase (Kebabian and Greengard, 1971; Monsama et al., 1990). There is also some evidence that the renal D_{1A} receptor may couple to the phospholipase C signaling pathway (Lokhandwala and Amanta, 1991; Jose, 1993; Mahan et al., 1990). The D_2-

like family includes the D_2, D_3, and D_4 receptors and show selectivity for butyrophenones and substituted benzamides (Civelli et al., 1993). Structurally D_2-like receptors differ from D_1-like receptors in that the third cytoplasmic loop of the D_2-like receptors is relatively longer (Selbie et al., 1989; Grandy et al., 1989; Monsama et al., 1989). D_2-like receptors primarily couple to the inhibitory guanine nucleotide binding protein, G_i, and consequently inhibit adenylyl cyclase activity (Onali et al., 1985).

VI. B.2. Localization of Dopamine Receptor mRNA and Protein

The presence of dopamine receptors in the central nervous system has been described based on ligand binding and receptor autoradiography experiments (Civelli et al., 1993). In situ hybridization indicates heterogeneity of dopamine receptor expression in the CNS (Fremeau et al., 1991; Meador-Woodruff, 1991 et al.; Mansour et al., 1990; Meador-Woodruff et al., 1989; Mengod et al., 1989). Although dopamine receptors are expressed in a tissue-restrictive manner, pharmacological and biochemical techniques indicated that dopamine receptors were located in the periphery as well (Sunhara et al., 1990; Felder et al., 1989a; Huo and Healy, 1989). Cloning of peripheral dopamine receptor genes proved to be challenging since detection of dopamine receptor mRNA in the periphery was more difficult than in the brain. Northern hybridization experiments utilizing the D_1 receptor from brain as a probe to screen RNA from peripheral tissues consistently yielded negative results. Use of more sensitive methods such as reverse transcription-polymerase chain reaction (RT-PCR) and solution hybridization indicated that the D_1 and D_3 receptor mRNAs were present in the kidney (Yamaguchi et al., 1993; Solkoloff et al., 1990). Although it has been suggested that dopamine receptors in the central nervous system exhibit higher binding affinities for D_1 ligands than do the peripheral receptors, the discrepancy may be due to

different levels of receptor density (Gingrich and Caron, 1993; Felder and Jose , 1988; Felder et al., 1990; Dearry et al., 1990).

The difficulty in cloning peripheral dopamine receptor genes lead to the speculation that an additional dopamine receptor subtype(s) may be expressed in the kidney. However, the D_{1A} receptors from the opossum kidney (OK) cell line (Nash et al., 1993) and the LLC-PK_1 renal cell line (Grenader et al., 1995) have been cloned and are identical to the D_{1A} receptors expressed in the brain. Opossum kidney expresses both the D_{1A} and D_{1B} receptor subtypes as demonstrated by RNase protection studies (Nash et al., 1993). Mammalian dopamine receptors may be differentially expressed as they are in *Xenopus laevis* (Sugamori et al., 1994). *Xenopus* kidney only expresses D_{1B} and D_{1C} receptors whereas all three D_1-like receptors (D_{1A}, D_{1B}, D_{1C}) are expressed in *Xenopus* brain. The size of the D_1 transcripts in the striatum and kidney are essentially the same (4.2 vs. 4.4 kb). Interestingly, northern hybridization of poly (A^+) RNA from renal cells (LLC-PK_1, porcine renal epithelial cell-line) indicated that there is a weak signal at 6.3 kb (Grenader et al., 1995). Whether this signal represents a functional transcript is not known. It is possible that this weak signal at 6.3 kb represents another dopamine receptor subtype since new subtypes have been isolated in vertebrates (Sugamori et al., 1994; Demchyshyn et al., 1995). Thus, although the existence of additional D_1-like receptors in nonmammalian vertebrates indicates that mammalian counterparts may yet be isolated, currently there is no evidence for a novel peripheral dopamine receptor.

The D_{1A} receptor gene expressed in the proximal tubule-like porcine renal epithelial cell line, LLC-PK_1, has been cloned (Grenader et al., 1995). The porcine D_{1A} receptor protein is 95% identical to the human D_{1A} receptor (Grenader et al., 1995)

at the amino acid level (Figure 1). The porcine D_{1A} receptor may be post-translationally modified since it contains sequence motifs for two N-linked glycosylation sites, two protein kinase C (PKC) sites (in contrast to the human receptor which has only one PKC site), and three cAMP-dependent protein kinase (PKA) consensus sites. In addition, a highly conserved cysteine residue in the carboxyl-terminus may be palmitoylated in a manner analogous to the human D_1 receptor (Ng et al., 1995). The porcine D_{1A} receptor contains several serine and threonine residues in the carboxyl-terminus which are putative substrates for phosphorylation by G-protein coupled receptor kinases (GRK) (Grenader et al., 1995). The renal effects of dopamine are mediated primarily by D_1 receptors in the proximal tubules (Aperia et al., 1987). For this reason, the LLC-PK_1 cell line provides a useful model system to study D_1 receptor mechanisms (Yamaguchi et al., 1992; Grenader and Healy, 1991; Huo and Healy, 1989; Hagege et al., 1985; Lokhandwala and Amenta, 1991).

VI. C.1. G-Protein Coupled Receptor Desensitization

Desensitization of G-protein coupled receptors has been investigated extensively. The β_2-adrenergic receptor is the prototype for G-protein coupled receptors and the molecular mechanisms of β_2-adrenergic receptor desensitization (Inglese et al., 1993; Pei et al., 1994; Kong et al., 1994; Kim et al., 1993) are well established. Thus, the β_2-adrenergic receptor (β_2AR) serves as a paradigm for desensitization of G_s coupled receptors. Desensitization of G-protein coupled receptors is classified as either homologous or heterologous (See Benovic, 1988 for review). Homologous desensitization of receptors occurs after short term agonist exposure and results in post-translational modifications of the activated receptor (Gingrich and Caron, 1993). It has been suggested that homologous desensitization is regulated by G-protein

receptor kinases (GRKs) and PKA which (Benovic, 1988; Lefkowitz, 1993) phosphorylate and subsequently uncouple the receptor from its regulatory G-protein. Phosphorylation of the activated β_2AR by β-adrenergic receptor kinase, βARK, promotes association with β-arrestin, a protein which is recruited to the β_2-adrenergic receptor by association with β γ G-protein subunits. β-arrestin sterically hinders further interactions between the activated receptors and G-proteins, temporarily inactivating the signal transduction pathway (Lefkowitz, 1993). β-arrestin acts as an intermediate in agonist promoted internalization of the β_2-adrenergic receptor (Ferguson et al., 1996; Goodman et al., 1996). Similarly, the rapid agonist-induced desensitization of the β_1-adrenergic receptor (β_1AR) involves phosphorylation of the receptor by both PKA and βARK in intact cells. Like the β_2AR, the β_1AR binds β-arrestin resulting in its subsequent desensitization (Freedman et al., 1995).

Heterologous desensitization of G-protein coupled receptors (e.g. vasopressin receptors) occurs when co-localized receptors (e.g. β_2AR) are activated and secondary messengers (e.g. cAMP) are over-produced. Since the accumulation of second messengers results in activation of protein kinases, it is thought that heterologous desensitization is mediated by cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) (Lefkowitz, 1993). Activation of PKA and PKC leads to phosphorylation of the activated receptor (e.g. β_2AR) and the co-localized heterologous receptor (e.g. vasopressin receptors). Consequently, there is a decrease in agonist-induced responsiveness of heterologous receptors (e.g. vasopressin receptors) when they are exposed to agonist for the first time. Experiments in which PKA and PKC phosphorylation sites of β_2-adrenergic receptors were mutated confirmed this hypothesis, since the mutations only allowed homologous desensitization to occur (Yuan et al., 1994). Conversely, mutating β-adrenergic receptor kinase (β-ARK)

phosphorylation sites allows agonist induced long term desensitization (Proll et al., 1993). Recently PKC and PKA have been shown to be important regulators of the functional desensitization and down regulation of receptors that are not positively coupled to G_s. M_2 muscarinic receptors are coupled to G_i, yet heterologous regulation of m_2 receptor gene expression occurs through gene transcription and results in m_2 receptor uncoupling (Rousell et al., 1995).

VI. C.2. D_1 Receptor Desensitization

Desensitization of D_1 receptors has been observed in rat striatum, bovine retinas and neuroblastoma cells (Ofori et al., 1993; Barton and Sibley, 1990). Human D_1 receptors undergo rapid agonist induced desensitization (Barton and Sibley, 1990; Ng et al., 1995). Decreased adenylyl cyclase activity does not directly correlate with changes in receptor density implying that uncoupling from the regulatory G-protein precedes down regulation (Barton and Sibley, 1990, Ng et al., 1995). Although receptor numbers returned to basal levels within 24 hours, recovery could be blocked by protein synthesis inhibitors, indicating that de novo synthesis is necessary for the recovery process. Evidence that two biochemically distinct mechanisms are operating comes from studies in which inhibition of agonist-induced receptor internalization does not prevent desensitization (Ng et al., 1995). D_1 receptor desensitization is accompanied by an increase in the number of phosphorylated receptors (Ng et al., 1994), supporting the view that rapid agonist-induced D_1 receptor desensitization is regulated by kinases.

Renal D_{1A} receptors are capable of agonist induced desensitization in opossum kidney (OK) cells. The desensitization of D_{1A} receptor stimulated adenylyl cyclase was

rapid and appeared to be independent of down regulation (Bates et al., 1991). Elevated levels of cAMP are not necessary for dopamine induced rapid desensitization in OK cells but are required for D_{1A} receptor down regulation (Bates et al., 1993). Dopamine induced desensitization could be mediated by protein kinase A and an unidentified member of the GRK family. The fact that dopamine induced down regulation is modulated by elevated cAMP levels and closely parallels PKA activity, suggests that transcription of the D_{1A} receptor gene may be regulated in part by cAMP. Although the physiological properties of the D_{1A} receptor have been studied extensively, very little is actually known about the transcriptional regulation of the D_{1A} receptor gene during desensitization.

The porcine renal epithelial cell line, LLC-PK_1 , expresses a D_1-like receptor that is coupled to adenylyl cyclase (Grenader and Healy, 1991). The molecular identity of the porcine D_1 receptor gene was unknown until it was cloned from the LLC-PK_1cell line (Grenader et al., 1995). The D_{1A} receptor in LLC-PK_1 cells undergoes rapid agonist induced desensitization that is dose and time dependent (Grenader, unpublished observations). Since the LLC-PK_1 cell line is a useful in vitro model of proximal tubule properties, it also appears to be an appropriate model to study gene expression of the D_{1A} receptor gene in renal cells.

VI. D.1. Agonist-Induced Down regulation of G-Protein Coupled Receptors

Long term exposure to agonist leads to receptor down regulation. The decrease in receptor density is a more gradual process compared to desensitization. Excessive stimulation is harmful to cells and down regulation appears to be a protective mechanism which occurs in response to chronic exposure to agonist (Lefkowitz, 1993, Gersghorn, 1994). Down regulation may involve increased receptor degradation as well as decreased biosynthesis (Hosoda et al., 1994). Although the underlying mechanisms responsible for down regulation of D_1 receptors are not entirely known, it is likely that they are similar to those of other G-protein coupled receptors. Putative phosphorylation sites in the carboxyl-terminus of G-protein coupled receptors are essential for their down regulation. Mutating tyrosine or threonine residues in the carboxyl-terminus of the β_2-adrenergic (Valiqutte et al., 1990) or the m_3 muscarinic (Yang et al., 1995) receptors, respectively, diminished the capacity of mutant receptors to down regulate and reduced the extent of desensitization (Valiqutte et al., 1990). Aside from their importance in agonist-induced down regulation, phosphorylation sites provide a means by which cross-regulation can occur during cellular stimulation. Specifically, activated β_2-adrenergic receptors induce down regulation of m_2 muscarinic receptors through PKA and PKC in human embryonic lung cells (Roussel et al., 1995). This is consistent with the belief that continuous stimulation is harmful and, therefore, necessitates multiple mechanisms of attenuation.

Many G-protein coupled receptors contain a highly conserved cysteine residue in their carboxyl-terminus, which is palmitoylated. In the β_2-adrenergic receptor palmitoylation of this cysteine residue is important for G_s coupling and agonist

promoted desensitization and for the α_{2A}-adrenergic receptor (Eason et al., 1994) it confers agonist-induced receptor down regulation. Similar agonist-induced post-translational modifications have been shown for D_1 receptors (Eason et al., 1994; Ng et al., 1994). Site directed mutagenesis of Cys^{347} and Cys^{351} in the D_1 receptor indicated that Cys^{347} in the carboxyl terminus is important for antagonist binding, activating adenylyl cyclase, and essential to agonist-induced desensitization (Jensen, 1995). It remains to be seen if the Cys^{347} residue is the same residue which is palmitoylated in the D_1 receptor. The role of palmitoylation in the down regulation and desensitization of D_1 receptors requires further investigation.

VI. D.2. Atypical Agonist-Induced Up Regulation of G-Protein Coupled Receptors

Atypical up regulation of β_3 adrenergic, m_3 muscarinic and vasopressin receptors occurs after long-term agonist exposure (Thomas et al., 1992; Colson et al., 1992; Murasawa et al., 1995; Fukamauchi et al., 1993). Following long-term exposure to agonist, m_3 muscarinic and vasopressin receptors are up regulated due to increased mRNA stability while the increases in β_3 adrenergic receptors are due to increased transcription. Temporal up regulation of D_1 receptors occurs in response to cocaine binges (repeated short doses), presumably through cocaine's ability to increase extracellular dopamine by inhibiting dopamine transporters (Untwald et al., 1994; Thomas et al., 1996). The up regulation is dependent on the dose regimen (binging) since other reports have indicated down regulation or no change in receptor numbers in response to chronic exposure to cocaine (Untwald et al., 1994). The molecular mechanisms responsible for up- and down- regulation of the D_{1A} receptors have not to my knowledge been defined.

VI. E. Agonist-regulation of G-Protein Coupled Receptor mRNA

Agonist-induced changes in G-protein coupled receptor (GPCR) mRNA appear to be receptor specific. Alterations in GPCR mRNA levels may result from agonist-induced changes in mRNA stability, changes in the transcription rate or induction of transcription factors which regulate transcription of the receptor genes (repressive or activating). Alterations in transcription rates following agonist exposure have been detected by nuclear run-on analysis for β_1- and β_2-adrenergic and m_3-muscarinic receptor mRNAs (Hosada et al., 1994; Hosada et al. 1995; Fukamauchi et al., 1993). Some GPCR mRNA levels are down regulated in response to agonist (Fukamauchi, 1993, Hosada, 1995) whereas others exhibit a bi-phasic response (Hosada et al., 1994, 1993, Collins et al., 1989). β_1-mRNA is an example of receptor mRNA that undergoes a biphasic response to agonist, initially there is a 50% increase in receptor transcription following treatment with agonist and is followed by a decrease in transcription with longer agonist exposure. Finally, agonist induced up- or down-regulation of transcription factors may alter mRNA levels. For instance, the β_2-adrenergic receptor gene was determined to be autoregulated by cAMP through a cAMP response element (CRE) (GTACGTCA) (Collins et al., 1990). Activation of β_2-receptor stimulates adenylyl cyclase, resulting in the accumulation of cAMP. cAMP activates PKA, which translocates to the nucleus and phosphorylates the CREB transcription factor bound to a CRE. Once phosphorylated, CREB may associate with CREB binding protein (CBP) which recruits the transcription initiation complex (Kwok et al., 1994; Nordheim, 1994). Therefore, it appears that the β_2-adrenergic receptor participates in a positive feedback loop, in order to regulate its own expression. In contrast, the β_1-adrenergic receptor gene contains a CRE element which binds one of two complexes, each of which contain an ICER (inducible cAMP early repressor) (Fitzgerald, 1996). The up

regulation of ICER mRNA correlates with the down-regulation of β_1-mRNA, suggesting that the decreased rate of transcription is due to induction of this inhibitory transcription factor (Fitzgerald, 1996, Collins et al., 1993, Hosada et al., 1994).

In addition to changes in GPCR mRNA levels due to transcriptional control, alterations in GPCR mRNA levels may be affected by mRNA stability. Increased mRNA stability after agonist exposure up-regulates vasopressin receptor expression in vascular smooth muscle cells while the rate of transcription is unchanged (Murasawa et al., 1995). Interestingly, the stability of the m_3 muscarinic receptor increases with agonist exposure but the mRNA is down-regulated (Fukamauchi et al., 1993). Stability of mRNA is influenced by regulatory elements in the 3'-UTR (i.e. polyadenylation sites, AUUUA destabilization sequences and/or stem loop structures). The AUUUA destabilization sequence (ARE) binds mRNA binding proteins which activate the destabilization of the mRNA. Polyadenylation tails are added to new mRNA transcripts to protect the mRNA from rapid degradation. Agonist-induced increase in β_2-adrenergic receptor gene transcription is accompanied by an increase in agonist-induced destabilization of the β_2-receptor mRNA (Collins et al., 1989). The 3'-UTR of the β -adrenergic receptor mRNA contains several AREs. Destabilization of β_2-AR and thrombin receptor mRNA occurs through interactions between ARE sequences and mRNA binding proteins, AUF1 and β-ARB (Tholanikunnel et al., 1995; Huang et al., 1993; Pende et al., 1996). In fact, up-regulation of the AUF1 mRNA was inversely proportional to expression of β_1 and β_2-mRNA in heart failure patients (Pende et al., 1996) which have increased levels of norepinephrine. Agonist induced destabilization of the m_1 muscarinic acetylcholinergic receptor, however, is due to the ability of the 3'-UTR to form stable stem-loop structures which contain the element responsible for mRNA destabilization (Lee, N.H., et al., 1994). The D_1 receptor gene contains several polyadenylation elements (AAUAAA) and AREs in the 3'-UTR (Caron

et al., 1990). To my knowledge, the effect of dopamine on the D_{1A} receptor mRNA half-life has not been determined.

VI. F. Organization of the Dopamine-1A Receptor (D_{1A}) Gene

Initial characterization of the porcine D_{1A} receptor gene revealed an organization similar to the rat and human D_{1A} receptor genes (Figure 2) (Zhou et al., 1992; Minowa et al., 1992; Grenader et al., 1995). The coding regions of D_{1A} receptor genes are analogous to the β-adrenergic receptor gene in that they are intronless (Kobilka et al., 1987). The pig, rat and human D_{1A} receptor genes have unusually large 5'-noncoding regions (> 1kb) which are interrupted by a small intron (Zhou et al., 1992; Minowa et al., 1993; Grenader et al., 1995) . The D_{1A} receptor gene is similar to "housekeeping" genes in that it is devoid of the canonical TATA and CAAT boxes in and around the transcription initiation site (Figure 3). The D_{1A} receptor gene is high in GC content and has multiple stimulatory protein 1 (Sp1) binding sites, consistent with genes lacking TATA and CAAT boxes (Tugores et al., 1994; Ishii et al., 1985; Azizkhan et al., 1993). While the organization of the D_{1A} receptor gene appears to be analogous to that of housekeeping genes, the fact that the D_{1A} receptor gene has only one transcription start site suggests that unlike housekeeping genes, it is regulated.

The human and rat D_{1A} receptor genes expressed in neuroblastoma cells were characterized by Minowa et al. (1993) and Zhou et al. (1992), respectively. The transcription start site for the human D_{1A} receptor gene was determined to be -1040 relative to the translation start codon. An activation domain was identified 95 bp upstream from the core promoter and could be separated into two smaller adjacent domains, Act I and Act II. Protein-DNA interactions were found to occur primarily

within this region. Analysis of nuclear protein interactions with the human D_1 promoter suggested that stimulatory protein 1 (Sp1) and a novel nuclear transcription factor bound to the activator region. Zhou et al. (1992) determined that the rat D_1 receptor gene transcription start site was -864 relative to the translation start site. Identification of the rat D_1 promoter indicated that it confers cell specificity since it was active in D_1-receptor expressing neuroblastoma NS20Y cells but not in D_1 receptor deficient cells (Zhou et al., 1992). The information contained within the 735 bp of the 5'-flanking region of the rat D_1 receptor gene appears to encode the regulatory elements which impart cell-specific expression. Although primer extension identified the transcription start site of the porcine D_{1A} receptor gene (-1033) to be at a similar location as in the human D_{1A} receptor gene (-1040) relative to the translation start codon, regulation of the porcine D_{1A} receptor gene in renal cells is completely unknown.

Various pharmacological agents have been implicated in the transcriptional regulation of the D_{1A} receptor gene. Transcription of the rat D_1 CAT reporter construct was stimulated by 8-Br cAMP (Zhou et al., 1992). Dexamethasone and phorbol ester, PMA, potentiated the effect of 8-Br cAMP on gene transcription but had no effect in the absence of 8-Br cAMP. Since the rat D_1 promoter is inducible by cAMP, a positive feedback loop may exist in order to allow the D_{1A} receptor gene to control its own gene transcription. This mechanism could prevent the complete loss of receptors during the desensitization process. The ability of dexamethasone to increase the number of maximum D_{1A} receptor binding sites in vascular smooth muscle cells indirectly suggested that dexamethasone may increase transcription of the D_{1A} receptor gene (Yasunari et al., 1994). The evidence to support this hypothesis stems from the fact that dexamethasone did not alter the dissociation constant and dexamethasone-mediated effects were inhibited by cycloheximide, further suggesting that dexamethasone's

ability to increase D_{1A} receptor binding sites involves de novo synthesis. However, this evidence is not conclusive since the rate of transcription was not measured. The transcriptional regulation of the D_{1A} receptor gene in renal cells is completely unknown but may be modulated by various pharmacological reagents.

VI. G. Analysis of the Porcine Dopamine-1A Receptor Gene

Computer scanning analysis of the 5'-flanking and 5'-untranslated region (Figure 3) of the porcine D_{1A} receptor gene with the transcription factor data base (Ghosh, 1993) disclosed numerous potential binding sites for known transcription factors. The GC rich promoter region contains several Sp1 binding sites (Dynan, 1986) and GC boxes, as mentioned previously. Although the porcine D_{1A} receptor gene is devoid of the canonical cAMP-response element, TGACGTCA, (Roesler et al., 1988; Lalli and Sassone-Corsi, 1994), there are two potential cAMP-response-like elements (CRE) (-223/-216, -212/-205) approximately 200 bp upstream of the transcription start site. Flanking the putative CRE elements are an overlapping activating protein 2 (AP-2) (-236/-229) and Sp1 consensus site (-232/-226) and an AP-2 consensus site (-191/-184). An E-box (Ayer et al., 1993) is located downstream of the AP-2 (Medcalf et al., 1990; Roesler et al., 1988) sites at -168 to -163. Two additional AP-2 sites (-112/-104 and -80/-73) surround an inverted GC box sequence and overlapping Sp1 and EGR-1 (early growth response factor) consensus site (-101/-90). Multiple transcription factors often physically compete with each other for binding sites at overlapping sites to control transcription. Competition of activating or repressive factors at overlapping sites may prove to be an important regulatory mechanism for the D_{1A}receptor gene (Diamond et al., 1990).

As previously mentioned, the 5'-untranslated region (5'-UTR) is unusually large. The 5'-UTR also contains several consensus sites for transcription factors. There are several AP-2 (CCCGCGGC) and E-box sites (CACGTG) throughout the 5'-UTR, in addition to an inverted CCAAT box (Dorn et al., 1987) which lies within the intron (+454/+458). Finally, the 5'-UTR contains three potential activator protein 1 (AP-1) sites located at +209/+216, +548/+555, and +921/+928. Each site differs from the AP-1 consensus site TGA(G/C)TCA by one nucleotide (Mitchell and Tijan, 1989). The importance of potential cis-regulatory elements residing within the 5'-UTR is unknown. However, it is possible, given the size of the 5'-UTR, that some of these elements are necessary to regulate expression of the porcine D_{1A} receptor gene.

VI. H. CREB/ATF Transcription Factor Family

The cAMP response element (CRE) is inducible by cAMP (cyclic 3',5'-adenosine monophosphate) and its presence in a promoter region is suggestive that cAMP is involved in mediating gene transcription. The canonical CRE is a palindromic sequence (TGACGTCA) and may mediate cAMP-induced activation or repression of transcription. Numerous variations of the consensus sequence have been reported which are functional (Tamura et al., 1994; Pestell et al., 1994; Kanasaki et al., 1994; Boutillier et al., 1992). In general, the production of cAMP stimulates cAMP-dependent protein kinase (PKA) activity. Once activated PKA translocates to the nucleus and phosphorylates transcription factors of the cAMP response element binding protein (CREB) and activating transcription factor (ATF) families. The CREB/ATF family contains more than 10 distinct mammalian proteins (Habener, 1990) and is part of the basic leucine zipper (bZip) transcription factor family. Members of the bZip transcription factor family include fos, jun and the CREB/ATF family. A distinguishing feature of the leucine zipper is its amphipathic α-helical dimerization

domain which contains a heptad repeat of leucine residues (Richards et al., 1996). The bZip factors form hetero- or homo- dimers which bind preferentially to either CRE or AP-1 sites. AP-1 sites bind either jun-jun or fos-jun dimers and are alternatively referred to as TPA- responsive elements (TRE). Although CREB/ATF dimers recognize the CRE consensus site as previously described, cross-regulation may occur as a result of dimerization with other bZip transcription factors (fos, jun) enabling factors to bind to alternate sites, TRE (Pestell et al., 1994; Sassone-Corsi, 1994; Habener, 1990). The CRE consensus site (TGCATGCA) and the TRE consensus site (TGA(C/G)TCA) are very similar and half-site recognition by factors may be responsible for maintaining specificity in response to various stimuli (Kim and Struhl, 1995).

Previously, CREB/ATF factors were divided into two categories, repressors and activators. The activators (CREB, ATF) of cAMP inducible transcription could dimerize with each other prior to binding to the CRE. The repressors (CREM-cAMP response element modulator, ICER-inducible cAMP early repressor) were believed to either bind to the CRE, blocking its activation or dimerize with the activators, preventing transcriptional activation. Several experiments have since proven that this view is oversimplified. CREB knockout experiments demonstrated that CREB -/- mice were healthy, exhibited a normal phenotype and did not experience any impairment of growth (Hummler et al., 1994). However, CREM (τ,α and β) isoforms were overexpressed in all tissues examined, suggesing that CREM isoforms can substitute for CREB in cAMP-responsiveness. Transcriptional activation of CREB-CREMα heterodimers is dependent on the ability of CREMα to be phosphorylated by PKA (Loriaux et al., 1994). Recently, it was established that the transcriptional effects of

CREMα isoforms are dependent on the specific gene, cell type and promoter context of the CRE site (Goraya et al., 1995).

Alternative splicing of CREB mRNA during germ cell development transforms activator isoforms of CREB into repressors (Walker et al., 1996; Delmas et al., 1992). One isoform of CREM, CREMΔC-G, has been described that acts as an inhibitor of PKA induced transcription (Walker et al., 1994). This CREM isoform lacks four exons encoding the PKA phosphorylation domain. In spite of the missing exons, it recognizes the CRE and binds as a homodimer or a heterodimer with CREB or CREM activators (Walker et al., 1994). While this particular isoform is expressed in elongated spermatids, similar isoforms may be expressed in other tissues. The most efficient repressor of CRE mediated transcription is the ICER, inducible cAMP early repressor. ICER consists primarily of the bZip dimerization domain and is sufficient for full antagonism (Lalli and Sassone-Corsi, 1994). Induction of ICER correlates with early down regulation of follicle-stimulating hormone receptor gene expression in Sertoli cells and thyroid-stimulating hormone in the thyroid gland. Since ICER binds to the CRE and represses expression, it has been proposed that this represents a prototype of the molecular mechanism by which hormones elicit homologous long-term desensitization in G-protein coupled receptors in neuroendocrine tissues (Lalli et al., 1995; Monaco et al., 1995). This model may have implications for other G-protein coupled receptors as well.

VI. I.1. cAMP-Mediated Gene Expression

CRE elements are important regulators of transcription for many genes (Kim et al., 1994; Roessler et al., 1995; Collins et al., 1990; Best et al., 1995; Boutiller et al., 1992). They serve to communicate activation of the cAMP signal transduction pathway to the nucleus. CREs are not only present in genes controlled by the cAMP pathway but also serve as integration points in which cellular cross talk can occur at the nuclear level. The rat D_1 receptor gene is regulated by cAMP, presumably through a CRE-like element (Zhou et al., 1992). Zhou et al. (1992) demonstrated the ability of 8-Br cAMP to stimulate transcription of the rat D_1 promoter, however, they did not demonstrate whether or not the proposed CRE was functional. The presence of 2 putative CRE-like sites (-223/-216 and -212/-205) in the porcine D_{1A} receptor gene suggests that these CRE sites in the D_{1A} receptor gene may be functionally significant in regulating D_{1A} receptor gene expression in renal cells. Comparison of the porcine, rat and human D_1 promoters (Figure 4) revealed that the 3'-CRE of the porcine D_{1A} receptor gene appears to be better conserved across species (Healy and O'Rourke, 1996).

VI. I.1.a. CRE and Basal Expression

Many hormonally regulated promoters consist of cis-elements that are involved in constitutive and inducible expression. CREs were implicated in basal transcription of several genes including tyrosine hydroxylase, phosphoenolpyruvate carboxykinase genes and hexose kinase (Kim et al., 1993; Roessler et al., 1988; Osawa et al., 1996). CREB is capable of interacting with the basal transcription factors TFIIB and TFIID independent of PKA activity and other factors. The fact that CREB and TFIIB associate in the absence of PKA phosphorylation is consistent with the ability of CREB to promote basal expression through constitutive activity. The constitutive activation

domain of CREB is sufficient to promote interactions with TFIIB and TFIID (Xing et al., 1995). In addition, CREB binding protein, CBP, a co-activator of CREB, complexes with TFIIB (Kwok et al., 1994). This is particularly interesting since a viral protein Tax has been identified that recruits CBP to the viral HTLV-1 CRE (human T-cell lymphocytic virus) and enhances CREB binding independent of CREB phosphorylation (Kwok et al., 1996). Tax's involvement in maintaining basal expression of the viral CRE implies that a eukaryotic homologue of Tax may exist which mediates basal stimulation in eukaryotic CRE containing genes.

VI. I.1.b. CRE and Inducible Expression

Induction of transcription by cAMP requires multiple PKA mediated events. Inducible expression of genes containing CREs involves a mechanism that is dependent upon phosphorylation of CREB (P-CREB). Phosphorylation of CREB at Ser-133 (Arias et al., 1994) does not alter the affinity of CREB for the CRE site (Richards et al., 1996; Parker et al.,1996; Kwok et al., 1996). CREB phosphorylation does, however, provide a direct binding site for CBP and promotes high-affinity binding between these factors (Parker et al., 1996). Transcriptional activation occurs when the P-CREB and CBP complex binds to the CRE (Brindle et al., 1995) but the CREB-CBP complexes are not assembled unless suboptimal doses of cAMP are present, implying that a second phosphorylation event is necessary for transcription (Brindle et al., 1995). It was proposed that an inhibitory protein may be present that associates with CBP after the second phosphorylation event. Since the viral protein, Tax acts through CBP in a phosphorylation dependent manner to activate cellular CREs (Kwok et al., 1996) it is possible that a mammalian homologue of Tax is involved. However, at this time there is no evidence of such a homologue in mammals. In vitro phosphorylation demonstrated that a second phosphorylation event at Ser-129 of CREB is necessary for

cAMP mediated gene expression (Fiol et al., 1994) but the details regarding the additional kinases and proteins that may be involved in vivo are still unknown.

CRE elements modulate transcription of many hormonally regulated genes such as somatostatin, rat insulin I, dopamine β-hydroxylase and β_2-adrenergic receptor genes (Montminy et al., 1988; Oetjen et al., 1994; Kim et al., 1994; Collins et al., 1990). Variations of the canonical CRE have been shown to be present in the promoters of many of these genes including the renin, human chorionic gonadatropin (HCG) β-, α_1-adrenergic receptor, c-fos and rat D_{1A} receptor genes (Tamura et al., 1994; Pestell et al., 1994; Kanasaki et al., 1994; Boutillier et al., 1992; Collins et al., 1993; Zou et al., 1992). Each of these CRE-like elements were capable of mediating cAMP-responsiveness for each their respective genes (Tamura et al., 1994; Pestell et al., 1994; Kanasaki et al., 1994; Boutillier et al., 1992). The CRE-like element of the rat D_{1A} receptor gene is an 8 bp palindromic sequence (TGGCGCCA) that is very similar to the canonical CRE (TGACGTCA) (Zhou et al., 1992). Examination of the porcine D_{1A} receptor gene revealed that it contains two distinct CRE-like elements at -223/-217 (AGACGTCA) and -212/-205 (GGACGTCC). The 5'-porcine CRE-like element differs from the consensus site in the first nucleotide whereas the 3'-site is an 8 bp palindrome which varies from the canonical CRE in the first and last nucleotides (mismatched bases are underlined). Comparison of the rat (AGGCGTCC), human (GGGCGTCC) and porcine sequences in this area suggests that the 3'-CRE site is better conserved across species (Healy and O'Rourke, 1996) (Figure 4). The β_2-adrenergic receptor contains a CRE-like element (GTACGTCA) which confers cAMP responsiveness in an orientation independent manner and plays an important role in transcriptional autoregulation (Collins et al.,1990). The porcine 3'-CRE-like element is similar to the β_2-adrenergic receptor CRE (GTACGTCA), the first CRE of the pro-

enkephalin gene (GCTGGCGTAGGG) and the c-fos dyad element (GGATGTCCATATTAGGACATC) (Boutillier et al., 1992). Interestingly, these elements vary considerably from the consensus site, yet are still responsive to cAMP. The number of genes regulated by CRE-like elements have demonstrated that the canonical site should not be considered a strict determinant of cAMP-responsiveness. The diversity of the CRE-like elements necessitates testing putative elements for functionality. Apparently variation of the CRE results in differences in CREB binding affinity for various consensus sites. A one nucleotide difference between the somatostatin (TGACGTCA) and enkephalin (TG_CGTCA) CREs results in an order of magnitude difference in binding affinities (Williams et al., 1993). Similarly, the flanking sequence may influence CREB binding affinity (Benbrook and Jones, 1994). X-ray crystallography has indicated that the CREB primary contact sites include both guanine residues on the sense strand and a cytosine on the antisense strand (Keller et al., 1995). The ability of these distinct CREs to mediate cAMP responsiveness supports the hypothesis that the porcine CRE-like elements participate in the transcriptional regulation of the D_{1A} receptor gene.

The presence of multiple CREs in promoters results in maximal cAMP-induced transcriptional activation. Agonist-induced increases in transcription of the β_3-adrenergic receptor gene are mediated through 3 CRE-like elements (Thomas et al., 1992). Similarly, both the tyrosine hydroxylase (Best et al., 1995) and dopamine β-hydoxylase (Shaskus, 1992) genes require two CRE sites to regulate expression of these genes in neuroendocrine cells. Induction of cAMP regulation through 2 precisely arranged CRE sites has been demonstrated for the glycoprotein hormone α-subunit (Silver et al., 1987), vasoactive intestinal peptide (VIP) (Fink et al., 1988) and proenkephalin genes (Spiro et al., 1995). Mutating or deleting either CRE often alters

the response to second messengers and transcription factors (Spiro et al., 1995; Fink et al., 1988). The porcine D_{1A} receptor gene contains 2 CRE-like elements spaced 3 bp apart that may be important to basal and cAMP-mediated transcription in renal cells.

VI. J. CREB Independent cAMP-Responsiveness

cAMP responsiveness may be mediated through elements other than CREs which do not involve CREB/ATF factors. Most commonly, this occurs through activator protein 2 (AP-2) sites which are distinct from CRE sites. The AP-2 transcription factor family is distinct from other known transcription factor families and behaves as a basal or inducible transcription factor (Medcalf et al., 1990). The AP-2 transcription factor differs from CREB in that it is inducible by both cAMP and phorbol esters. Each of the AP-2 consensus sites ($CCC^{A}/_{C}\ N^{G}/_{C}\ ^{G}/_{C}\ ^{G}/_{C}$) of the porcine D_{1A} receptor gene are identical to the human metallothein II_A gene which is known to bind purified AP-2 protein (Mitchell and Tijan, 1989). It is uncertain if these AP-2 sites are necessary for the expression of the D_{1A} receptor gene in renal cells.

CREB independent cAMP-responsiveness was demonstrated for transcription of the enzyme involved in the rate limiting step of steroid biosynthesis, CYP450 (Venepally et al., 1995). CYP450, steroid hydroxylase cholesterol side chain cleavage cytochrome P450, requires two Sp1 sites to mediate basal and cAMP responsiveness (Venepally et al., 1995). The underlying mechanism by which Sp1 mediates cAMP-responsiveness is unknown. Similarly, muscle-specific cis-regulatory elements are recognized by myogenic factors, MyoD and or TEF-1, and mediate cAMP-responsiveness of muscle-specific genes (Gupta et al., 1994). Basal and cAMP induced expression of rat cardiac α myosin heavy chain gene occurs through

overlapping E-box and M-CAT (GGCACGTGGAATG) binding sites and not traditional cAMP response elements (e.g. CRE, AP-2) (Gupta et al., 1994). Interestingly, the c-fos promoter contains a CRE (TGACGTTT) and a dyad symmetry element. Although both of these elements were able to mediate cAMP-responsiveness independently, the dyad symmetry element is interesting in that the cAMP response is CREB-independent in corticotrope cells. The dyad element is dominant relative to the CRE in c-fos promoter since it is not sensitive to CREB antagonism (Boutillier et al., 1992). The molecular mechanisms required to mediate CREB independent cAMP-responsiveness are not known but may be relevant to D_{1A} receptor gene regulation.

VI. K. Co-operative Mechanisms of Gene Transcription

Combinatorial control of gene transcription in eukaryotes by multiple transcription factors may result in powerful activation via synergistic interactions. Synergy between cis-elements is dependent on the context and spacing of those elements and ability of trans-activating factors to bind cooperatively. Cooperative interactions between specific trans-activating factors control the up-regulation of MHCII associated invariant chain gene expression (Wright et al., 1995) and the cell-specific regulation of calcitonin/calcitonin gene related peptide gene (Tverberg et al., 1993). Consequently, several CRE-like sequences have been reported which are not in and of themselves capable of mediating cAMP response (Montminy et al., 1986; Hozawa et al., 1996). It has been proposed that other sequences (upstream or downstream) may participate in these interactions. Most often AP-2 is implicated as a likely participant. Basal activation of the human tissue-type plasminogen activator gene promoter occurs through cooperative interactions between a CRE and AP-2 site which independently mediate activation by PMA and cAMP (Medcalf et al., 1990). It is possible that some of these CRE-like elements require other "non-traditional CRE" sites

such as Sp1, CAAT or a tissue specific factor binding site. Synergistic interactions between CRE and CAAT elements were defined for the tyrosine aminotransferase, the human fibronectin gene, and PEPCK (phosphoenol pyruvate carboxykinase) promoters (Roesler et al., 1995; Muro et al., 1992; Roesler et al., 1996). Spatial restrictions between adjacent cis elements commonly cause of synergistic effects (Diamond et al., 1990). Perhaps the close proximity and exact spatial arrangement of the putative cis-regulatory elements within the 5'-flanking region of the D_{1A} receptor gene promoter could allow cooperative interactions to occur between them.

VI. L. Objective

The D_1 promoter has been extensively investigated in neuronal cells and determined to be expressed in a cell-specific manner (Minowa et al., 1993; Zhou et al., 1992). Preferential gene expression is often established by tissue specific expression of trans-activating factors (Tugores et al., 1994; Tamura et al., 1994; Tamura et al., 1994a; Knepel et al., 1990) and preferential expression of cis-regulatory elements (Pecorino et al., 1991; Shoshani et al., 1990; Lee et al, 1994; Shapiro et al., 1991). Non-neuronal cells may regulate expression of the D_{1A} receptor gene in a manner distinct from its regulation in neuronal cells. The D_{1A} receptor is expressed in renal cells (Grenader and Healy, 1991) but the molecular mechanisms regulating its gene expression are not known.

In order to identify the molecular mechanisms regulating D_{1A} receptor gene expression, I sought to analyze in detail the 5'-flanking and 5'-untranslated region of the porcine D_{1A} receptor gene and to define the cis-regulatory domains of the renal D_{1A} receptor gene promoter. Deletion analysis identified potential cis-elements in the 5'-flanking and 5'-untranslated region which were necessary for transcriptional activation of the D_{1A} receptor gene CAT constructs. In particular, I examined the role of a potential upstream activating region (-303/-165) containing two CRE-like elements (-223/-205) and the intron (+430/+527) in regulating D_{1A} receptor gene transcription. I further determined whether these areas bound LLC-PK_1 nuclear proteins and known transcription factors using DNase I analysis and electrophoretic gel mobility shift assays. The results of these experiments allowed me to construct a novel model of transcriptional regulation in which the 5'-UTR intron and promoter of the D_{1A} receptor gene interact to permit maximal transcriptional activity.

Figure 1: Porcine D_{1A} receptor. Darkened circles are amino acids conserved between pig, human and rat D_{1A} dopamine receptors. Open circles are not conserved. (+) N-linked glycosylation sites; (*) PKA phosphorylation sites; (#) PKC phosphorylation sites.

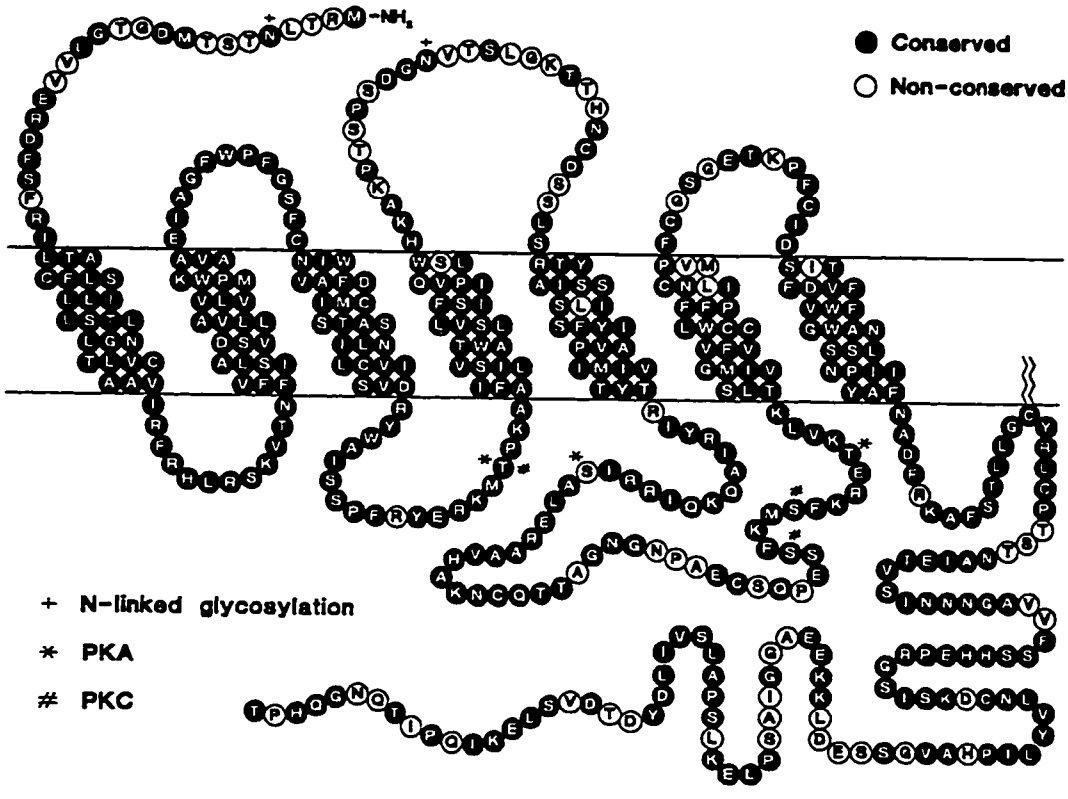


Figure 2: Schematic representation of the D_{1A} receptor gene. The 5' and 3' flanking regions and the intronic segment are shown by the solid line, the 5' and 3' untranslated regions are shown by the hatched bar and the coding region is shown by the solid area. Exons one and two are indicated by E1 and E2, respectively. Restriction sites are designated by the following abbreviation: B, BamHI; E; EcoRI; H, HindIII; Ps, PstI; Pv, PvuII; X, XbaI; Xh; XhoI.

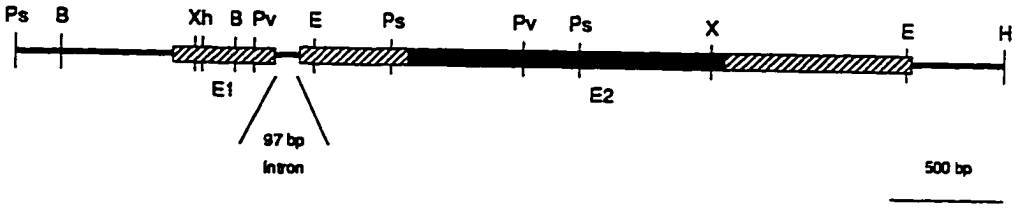


Figure 3: Nucleotide sequence of the 5'-flanking and 5'-untranslated regions of the porcine D_{1A} receptor gene. Nucleotides are numbered relative to the transcription start site, +1, as noted by the downward arrow. The 5'-flanking nucleotide sequence is shown in small case letters as is the 97 bp intron in the 5'-untranslated region which is double underlined. Potential translation start sites are underlined. Nucleotide sequence corresponding to transcription factor binding sites are underlined and designated above the sequence. Restriction sites used for the construction of pCAT chimeras are doubleunderlined.

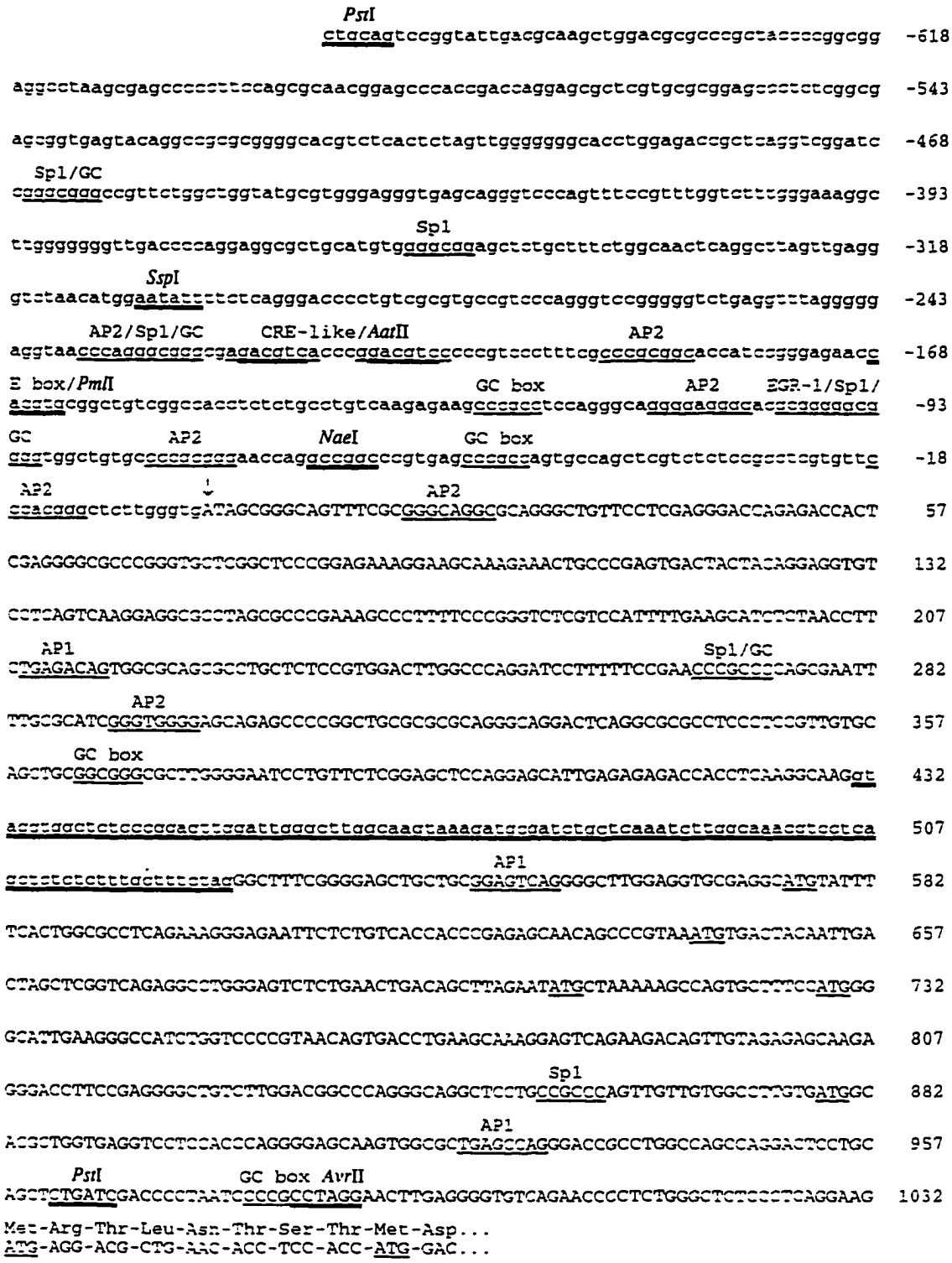
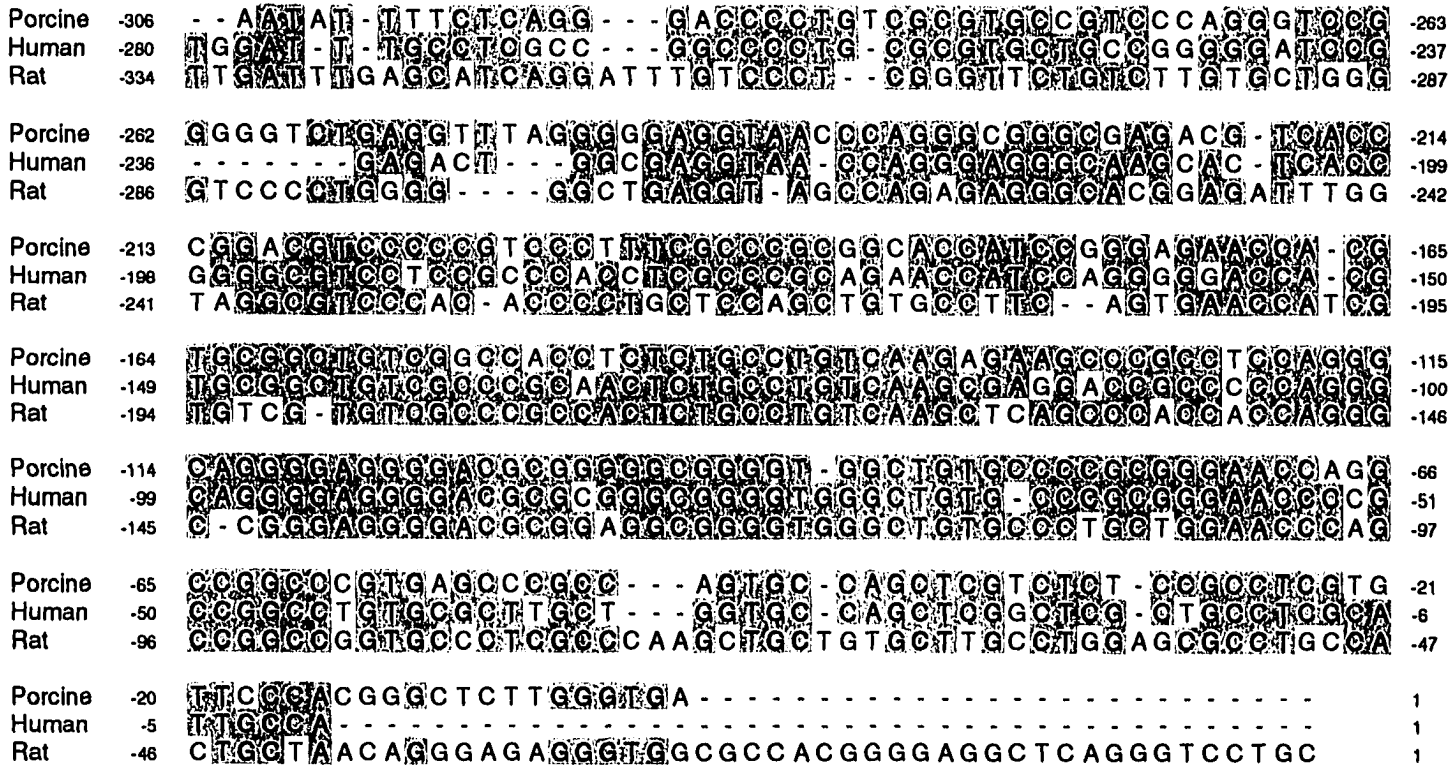


Figure 4: Comparison of the D_{1A} Receptor Gene 5'-Flanking Sequences from the Pig, Rat and Human.

D_{1A} Receptor Gene 5'-Flanking Sequences



VII. Chapter 2: Materials and Methods

VII. A. Cell Culture

LLC-PK_1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/L glutamine, 100 U/ml penicillin, 100 mg/ml Streptomycin and 0.25 mg/ml amphotericin B (GIBCO). Cells were passaged by trypsinization with 0.25% trypsin and 1.0 mM EDTA. Cells were grown in 80-cm^2 plastic flasks at 37°C under an atmosphere of 95% air/ 5% CO_2 and passaged twice a week.

VII.B. RNA-isolation

Poly A^+ mRNA was isolated from LLC-PK_1 cells which had been maintained at confluency for one week using Invitrogen Fast Track 2.0. Cells were harvested and passed through a 20 gauge needle four times and incubated for 1 hr at 50°C. NaCl concentration was adjusted to 0.5 M and again the lysate was passed again through a 20 gauge syringe (4x). Oligo dT cellulose was then added and rotated for 18 hr. After eluting RNA, the concentration was determined by optical density measurement at OD 260.

VII. C. RT-PCR

Reverse transcription (RT) was performed using Gibco/BRL Superscript pre-amplification kit according to the manufacturer's protocol. All RNA samples were pretreated with 30 U of RNase free-DNase (Bohringer-Mannheim) for 15 min at room temperature. Upon the addition of 1 µl of 25 mM EDTA, the RNA was heated to 70 °C for 10 min. The concentration of the samples was then determined by optical density

measurements at OD 260. The reverse transcription step was performed at 50°C for 50 min, with 100 U of Superscript II and a gene specific antisense primer (5'-TTCAAGGAGGAATTAGCCCACCCAAACCAC-3') designed from the coding region (+711/+682). After heat inactivation of the reverse transcriptase, the samples were treated with RNase H for 20 min at 37 °C. PCR was performed using only 10% of the newly synthesized cDNA. Polymerase chain reaction (PCR) was performed with Amplitaq (Perkin-Elmer). One microliter of α^{32}P-dGTP was included in the PCR reaction mixture and 0.2 μM sense (+377/+394 ; 5'-CTTTATCCCAGTGCAGCTCA-3') and antisense (+631/+614; 5'-ATGATCACGGACAGCGTCTT-3') primers. The conditions were as follows: 1 cycle: 5' at 94°C- 1.5' at 55°C- 2' at 72°C, 30cycles: 1' at 94°C- 1.5' at 55°C- 2' at 72°C, 1 cycle: 10' at 72°C. The PCR products were separated on a 2% agarose gel, visualized under UV-light and excised. Excised gel slices were added to scintillation tubes with 5 ml scintillation fluid and counted in a Beckman LS 5000TD scintillation counter.

VII. D. Effect of Dopamine on D_{1A} Receptor mRNA

LLC-PK_1 cells were maintained at confluency for one week. Control cells were not treated with dopamine and were harvested at t = 0. Cells pretreated with 10 μM dopamine were harvested from 30 min-18 h after the addition of dopamine. An antioxidant, sodium metabisulfite 200 μM, was added prior to dopamine to prevent oxidation of dopamine. Poly A^+ mRNA was isolated and analyzed by RT-PCR as described above.

VII. E. Effect of Dopamine on D_{1A} Receptor mRNA Stability

LLC-PK_1 cells were maintained at confluency for one week . To determine the half-life of the D_{1A} receptor mRNA, the cells were incubated with actinomycin D to block transcription (Rodgers et al., 1985). Cells were incubated in the absence or presence of dopamine (10 μM, 30 min) prior to the addition of actinomycin D (5 μg/ml). Cells were harvested at various time intervals (0-360 min). Poly A^+ mRNA was extracted at each of the time points indicated and analyzed by RT-PCR as described above.

VII. F. Transfection

LLC-PK_1 cells were transfected with pCAT-D_{1A} constructs using Lipofectamine reagent (GIBCO BRL) methods (Hawley-Nelson et al., 1993). Lipofectamine transfections were performed in serum-free and antibiotic-free medium according to the manufacturer's protocol. One day prior to transfection confluent LLC-PK_1 cells were split 1:3 and plated 1x 10^5 cells/ 35-mm dish and grown overnight. For each transfection, 0.1 ml of 1.5 μg of DNA diluted in 94 μl of DMEM and 0.1 ml of 5 μl of Lipofectamine diluted in 91 μl of DMEM were combined, mixed gently, and incubated at room temperature for 10-15 min. After the incubation, the mixture was diluted with 0.8 ml DMEM to a final volume of 1.0 ml. Cells were prewashed with DMEM and overlayed with 1.0 ml of DNA- Lipofectamine mixture. Cells were administered 0.1 ml of fetal bovine serum five hours after the start of the transfection and incubated overnight. The next day the DNA containing medium was removed and replaced with

medium containing 10% fetal bovine serum and antibiotics. The cells were harvested 24 hours later.

VII. G. Plasmid Construction

The initial series of pCAT- D_{1A} plasmids (-3000/+983 to -63/+983) were constructed by Dr. A. Grenader and Faiza Zafar (Figure 11). Two additional plasmids [pCAT+53 and pCAT-303 (Δ-165/-63)] were constructed by Dr. Q. Jiang (Figures 11,13). All other constructs were constructed by the author.

Briefly, the XbaI digest of the porcine D_{1A} receptor gene contains the entire untranslated and greater than 2 kb of 5' flanking sequence. The XbaI clone was digested with XbaI (-3000) and AvrII (+983) and inserted into the XbaI site of pCAT-Basic. The resulting construct is referred to as the pCAT-3000 construct. The smaller fragments (pCAT-206, pCAT-165 and pCAT-63) were constructed by digesting the pCAT-3000 construct with the appropriate enzyme, and ligating under appropriate conditions. The pCAT-663 and pCAT-303 were designed by digesting pCAT-3000 with PstI (-663/+962). The resulting fragment was ligated into the PstI site of the pBasic vector, generating pCAT-663. pCAT-303 was designed similarly by digesting the pCAT-663 with SspI (-303) and PstI (+962) and ligating into HindIII/PstI site of pBasic.

The majority of the 5'-UTR was removed from the pCAT-303 construct to create pCAT-303/+38. The D_{1A} receptor gene has an XhoI site at +39 and the pBasic vector contains an XbaI site at the 3'-end of the multiple cloning site. The plasmid, pCAT–303/+38, was constructed by digesting the pCAT-303 construct with XhoI and

XbaI. After removing +39 to +962 sequence, the overhangs were filled in with dNTPs and Klenow enzyme and the blunt ends were ligated.

The intron region (+431/+527) was deleted from pCAT-303 using an Ex-site PCR mutagenesis approach (Stratagene). The antisense (5'-CTTGCCTTGAGGTGGTCT-3'; +430 to +413) and the sense (5'-GGCTTTCGGGGAGCTGCT-3' +528 to +545) primers were phosphorylated and used to amplify the pCAT-303 construct. After 18 cycles at 56°C, the PCR product was digested with DpnI, treated with Pfu polymerase, and ligated according to the manufacturer's recommendations. The resultant plasmid was termed pCAT-303ΔIntron. The 5'-UTR distal to the intron was deleted from pCAT-303 by PCR with a sense primer containing a AatII restriction site (5'-CCGGACGTCCCCCGTCCCTT-3'; -210 to -190) and an antisense primer containing a XbaI site (5'-GACCTCTAGACTCCGCAGCAGCTCC-3'; +527 to +550). The PCR product was digested with AatII and XbaI and inserted into the AatII and XbaI sites of pCAT-303. The resultant plasmid was termed pCAT-303/+542. A similar approach was used to construct pCAT-303/+425, a construct lacking both the intron and the distal 5'-UTR. The same sense primer containing the AatII site was used for PCR with an antisense primer that terminated at the 5' end of the intron (5'-GACCTCTAGACTTGCCTTGAGGTGGTC-3'; +430 to +414).

The ability of the D_{1A} receptor gene intron to influence transcription of a heterologous promoter was tested by placing the intron upstream of a CAT reporter construct containing the core promoter for the thymidine kinase gene (Boschart et al., 1992), termed here pTKCAT. The +430/+527 insert was generated using PCR; sense primer (containing a BamHI restriction site) 5'-

TAACAGGATCCATTTTCTCAGGGAC-3' and the antisense primer 5'-CCCCGGGATCCCTAGAAAGC-3'. PCR conditions were: 30 cycles 1' at 94 °C, 1.5' at 50°C and 2' at 72 °C. The PCR product was ligated into a TA cloning vector (Invitrogen), digested from the vector with BamHI and ligated into the BamHI site of the pTKCAT. Constructs containing the insert in both sense and antisense orientations were isolated and termed pTKCAT+Intron and pTKCAT-Intron, respectively. Similarly, the ability of the -303/-165 fragment of the D_{1A} receptor promoter region to influence activity of the heterologous thymidine kinase promoter, in the absence and presence of the intron, was tested. The -303/-165 fragment was generated by PCR with a sense primer that contained a HindIII site 5'-TAACAAAGCTTATTTTCTCAGGGAC-3' and an antisense primer 5'-GACAGGGATCCGTGGTTCTCCC-3'. The PCR product was ligated into a TA-cloning vector and digested out with HindIII and XbaI and ligated into the HindIII and XbaI site of the pTKCAT+Intron vector, resulting in pTKCAT-303/-165+Intron. Digestion of pTKCAT-303/-165+Intron with BamHI and religation removed the intron and resulted in pTKCAT-303/-165. The antisense orientated -303/-165 insert in pTKCAT (pTKCAT-165/-303) was generated by PCR (sense primer 5'-TAACAGGATCCATTTTCTCAGGGAC-3' containing a BamHI site and antisense primer 5'-GACAGAAGCTTGTGGTTCTCCC-3' containing a HindIII site) and inserted into the BamHI and HindIII sites of pTKCAT.

The pTKCAT-Δ3'CRE was prepared by digesting the pTKCAT-303/-165 plasmid with AatII, blunting with T4 DNA Polymerase, and religating. Once the 3'-CRE deletion was confirmed by sequencing, it was transferred to the HindIII/XbaI site of the pTKCAT and termed pTKCAT-Δ3'CRE. The 39 bp single stranded sense and antisense oligonucleotides (wt-CRE) containing the two CRE-like sites with SalI ends

were hybridized and ligated into the SalI site of the pTKCAT vector. Clones were identified in which the CRE had been inserted in the sense (pTKCAT-5'CRE/3'CRE) and antisense (pTKCAT-3'CRE/5'CRE) orientations. pCAT-303(-211G->C) was constructed using Ex-site mutagenesis approach (Stratagene). The sense primer 5'-CCCGTCCCTTTCGCCCGCGGCACCAT-3' and the antisense primer 5'-GGGTGAGCTCTCGCCCGCCCTGGGTTA-3' were not overlapping. The conditions used were the same as those previously describe.

All constructs were confirmed by digestion and dideoxy sequencing at the junctions. All constructs were also sequenced in the CAT coding region to ensure that the observed results were due only to the intended mutations in the D_{1A} receptor promoter.

VII. H. CAT Assay

CAT (chloramphenicol acetyl transferase) enzyme activity was measured using standard procedures (Sambrook et al., 1989). Activity of the promoterless pCAT-Basic vector (Promega) alone established basal CAT activity. The activity of the pCAT-Control vector (which contains SV-40 promoter) was used to establish the maximal efficiency of the transfections. The CAT activity of pCAT- D_{1A} receptor promoter constructs were determined in order to establish which regulatory domains of the D_{1A} promoter were able to direct transcription. Crude cell extracts were tested for CAT activity 48 hours after transfection. Transiently transfected LLC-PK_1 cells prepared as described above were washed with calcium and magnesium free PBS and suspended by incubation with 200 µl 1x lysis buffer (Promega). After incubating at room temperature for 15 minutes, plates were scraped with a rubber policeman. The

cells were resuspended by vortexing and split in two portions prior to heating. Lysates to be used in the CAT assay are heated at 60°C for 10 minutes in order to inactivate endogenous deacetylase activity. Cells were pelleted by centrifugation for 3 minutes. To 55 µl of cell lysate, 5 µl of [^{3}H]-chloramphenicol (sp act 250 Ci/mmol, Du Pont-New England Nuclear, Boston, MA) (diluted 1:20 with 0.25M Tris-HCl, pH 8.0) and 2.5 µl of n-Butyryl Coenzyme A were added. Reaction mixtures were incubated for 1 hour at 37°C. For the liquid scintillation assay, reactions were terminated by adding 300 µl mixed xylenes, and washed 2x with 100 µl 0.25M Tris-HCl, pH 8.0. The xylene upper phase (200 µl) was transferred to a scintillation tube containing 5 ml scintillation fluid and counted (Beckman LS 5000TD). Counts per minute were 10% or less of total radioactivity added. For the thin layer chromatography, [^{14}C]-chloramphenicol (sp act 0.05 Ci/mmol, Du Pont-New England Nuclear, Boston, MA) was included in place of the tritiated compound and reactions were stopped with 250 µl of ethyl acetate, evaporated and redissolved in 30 µl of ethyl acetate. The reaction products were spotted on thin layer chromatograms (J.T. Baker, silica gel TLC plates SG/IB2, 20x 20cm) and resolved in 97:3 chloroform: methanol, dried and autoradiographed.

VII. I. β-Galactosidase Assay

All CAT constructs were co-transfected with a pSV-β-galactosidase expression vector (Promega) as a positive control for transfection efficiency (Sambrook et al., 1989). Extracts were prepared according to CAT assay protocol omitting the heating step. The cell lysates (50 µl) were diluted with 25 µl of 1x lysis buffer. Addition of

75 µl of 2x assay Buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml o-nitrophenyl- β -D-galactopyranoside) to diluted extracts was followed by a 30 minute incubation at 37°C (or until a faint yellow color developed). Reactions were quenched with 250 µl 1.0 M Na_2CO_3. Optical density was read at 420 nm on Bauch & Lomb Spectrophotometric 2000 spectrophotometer. β-Galactosidase activity was used to normalize CAT activities for the different pCAT-D_{1A} constructs.

VII. J. Bradford Protein Assay

Protein concentrations were determined using Bio-Rad protein assay dye according to manufacturer's protocol. Protein extracts (5 µl) are added to 795 µl Tris (10 mM) and 200 µl of Bio-Rad dye. Optical density at 595 nm was determined on a Milton Roy Genesys 2.0 spectrophotometer. Final protein concentrations were determined from a standard curve.

VII. K. Forskolin Effect on pCAT-D_{1A} Reporter Activity

Cells transfected with pCAT(-3000 to -63) constructs were incubated in the absence and presence of 10 µM forskolin 36 hr after transfection. Eighteen hours later cells were harvested and assayed for CAT, and β-gal activities as described above.

VII. L. Nuclear extracts

Nuclear extracts were prepared by a micropreparation technique (Andrews and Faller, 1991) that is derived from the large scale procedure of Dignam et al. (1983). LLC-PK_1 cells (5 x 10^5 cells/ml) were washed 2x with PBS (phosphate buffered saline) and scraped from plates with a rubber policeman in 1.5 ml phosphate buffered saline. The cells were centrifuged at 2000 rpm for 5 min at room temperature, resuspended in 1.5 ml phosphate buffered saline, removed to and eppendorf tube and repelleted. The cells are resuspended in 400 µl of buffer A [10 mM HEPES (pH 7.9, 4°C), 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF (phenylmethylsulfonyl fluoride)]. The cells were allowed to swell on ice for 10 minutes before centrifuging for 10 seconds. The supernatant was discarded and the pellet resuspended in 100 µl of buffer B [20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF] and incubated on ice for 20 minutes. Cellular debris is removed by centrifuging for 5 minutes at 4°C and the supernatant is stored at -80°C in 10 µl aliquots. The yield for 1 x 10^6 cells is 50-75 µg protein.

VII. M. DNase I Footprinting Assay

The DNase I footprinting will be performed according to the method of Hennighausen and Lubon (1987). Probes were synthesized by PCR with either the sense or antisense probes labeled at the 5' end with T4 polynucleotide kinase and [γ-^{32}P]ATP and the other primer is non-radioactive. One µg of poly (dI-dC) and increasing amounts of the crude nuclear extract from LLC-PK_1 cells or recombinant

purified protein (Sp1, AP-2, or CREB) were combined in a total volume of 50 µl buffer (50 mM NaCl, 0.1 mM EDTA, 20 mM HEPES, pH 7.5, 0.5 mM DTT and 10% gycerol). Labeled probe was added (10,000 cpms) and samples were incubated for 15-20 min on ice followed by 2 min at room temperature. Pancreatic DNase I (0.15- 1.0 units) were added to a final concentration of 5 mM $MgCl_2$ and 1mM $CaCl_2$. After 1-2 minutes, 100 µl of stop buffer (0.375% SDS, 15 mM EDTA, 100 mM NaCl, 100 mM Tris-HCl, pH 7.6) E. coli DNA (50 mg/ml) and proteinase K (100 mg/ml) was added and incubated for 15 min at 37 °C and 2 min at 90 °C followed by phenol-chloroform extraction and ethanol precipitation. A dideoxy sequencing reaction using the appropriate primers and the XbaI genomic clone as template was run in parallel to provide a sequence ladder for analysis. The reaction products were separated on a 10% polyacrylamide-7 M urea sequencing gel and exposed to x-ray film.

VII. N. Gel Mobility Shift Assay

The gel mobility shift assay was performed according to the method of Hennighausen and Lubon (1987). Synthetic complementary oligonucleotides with 5'-TCGAC overhangs were annealed and labeled by a filling-in reaction using [α-^{32}P]-dGTP and Klenow enzyme. Crude nuclear extracts (4 µg) from LLC-PK_1 cells, or extracts plus competitors, were preincubated with 1 µg homogenous poly (dI-dC) to reduce the accumulation of nonspecific high molecular weight aggregates. Nuclear extracts were incubated in 10 µl of 10 mM Tris-HCl (pH 7.5), 50-100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 12.5% glycerol, and 0.1% Triton X-100 for 30 min at room temperature. One-tenth nanogram of labeled probe was added and the samples

incubated for an additional 20-40 min. The samples were separated by 5% PAGE gel containing 0.5xTBE. Electrophoresis was performed for 2 h at 250 V at room temperature. Densitometry of autoradiograms was performed on LKB 2222-020 Ultrascan XL Enhanced Laser Densitometer.

VIII. Chapter 3: Results

VIII. A. Dopamine-regulation of D_{1A} receptor mRNA

VIII. A.1. Effect of Dopamine on D_{1A} receptor mRNA Levels

The D_{1A} receptor in LLC-PK_1 cells is known to rapidly desensitize in a time and dose dependent manner (Grenader, unpublished results). The effect of dopamine stimulation on steady state levels of D_{1A} receptor mRNA in LLC-PK_1 cells was investigated. LLC-PK_1 cells were treated with dopamine at various time points (30min, 2h, 6h, 20h) (Figure 5). Poly A+ mRNA was isolated and reverse transcribed with a D_{1A} receptor gene-specific primer. The resulting cDNA was amplified by polymerase chain reaction in the presence of ^{32}P-dGTP. The PCR products were separated on a 2% agarose gel and the bands excised and counted. A maximal increase (approximately 2 fold) in D_{1A} receptor mRNA levels was observed for cells treated for 30 min with dopamine and returned to basal levels after 1 hour of dopamine treatment. A similar profile was observed with 10 μM forskolin stimulation (data not shown).

VIII. A.2. Effect of Dopamine on D_{1A} receptor mRNA Stability

Since an increase in mRNA levels may occur as a result of either an increase in transcription or an increase in the mRNA half-life, it was important to examine the stability of the D_{1A} receptor message in response to dopamine. LLC-PK_1 cells were treated with actinomycin D to inhibit further transcription and collected at various time intervals for measurement of D_{1A} receptor mRNA (Figure 6). Under these conditions,

the half-life of the D_{1A} receptor mRNA was determined to be approximately 4.5 h in control cells (Figure 7). Pretreatment of cells with 10 μM dopamine for 30 min did not increase mRNA stability as would be expected if increased mRNA stability was responsible for the observed increase in D_{1A} receptor mRNA. Instead, the half-life of the D_{1A} receptor mRNA appeared to be decreased. Pretreatment of control cells with cycloheximide and dopamine increased mRNA half-life indicating that de novo protein synthesis was required for the degradation of D_{1A} receptor mRNA (Izzo et al., 1994). Since the cells pretreated with cycloheximide and dopamine experienced essentially no change in the D_{1A} receptor mRNA levels, it appears that dopamine increases the D_{1A} receptor mRNA gene transcription and simultaneously decreases D_{1A} receptor mRNA stability.

VIII. B. Characterization of D_{1A} Receptor 5'-Flanking Region

VIII. B.1. Identification of D_{1A} Receptor Activation Region

Since nothing is known regarding the transcriptional regulation of the D_{1A} receptor gene in renal cells, a series of pCAT- D_{1A} chimeras were designed with progressive deletions in the D_{1A} receptor gene 5'-flanking region in order to characterize the regulatory domains of the D_{1A} receptor gene. Each of these chimeras were ligated upstream from a promoterless chloramphenicol acetyltransferase (CAT) reporter gene. The deletions were designed such that the proposed promoter region (>-3000 to +53) was successively truncated (Figure 8, left). In addition, a vector containing a 3'-deletion in pCAT -303 was constructed, -303/+38. The eight

constructs were transiently expressed in LLC-PK_1 cells and tested for their ability to stimulate transcription of the CAT reporter gene relative to the pCAT-Basic vector, a promoterless CAT expression vector.

Transient transfections of pCAT-3000, pCAT-663, pCAT-303, pCAT-206, pCAT-165, pCAT-63, pCAT+53 and pCAT-303/+38 constructs in LLC-PK_1 cells were performed and CAT activity was determined after 48 h (Figure 8, right). Expression of the pCAT-303 construct resulted in the most prominent activity, nearly 60-fold higher than the negative control plasmid. Porcine D_{1A} receptor promoter constructs which extended upstream to -663 and -3000 exhibited 2.5-fold and 6-fold reduction in CAT activity, respectively, relative to pCAT-303, indicating the presence of negative regulatory elements in these upstream sequences. Progressive deletions downstream of -303 (pCAT-206, pCAT-165, pCAT-63), resulted in a gradual diminishment of CAT activity. The approximate four-fold increase in CAT activity from -63 to -303 suggests that an activating sequence is contained within this region but the boundaries are not obvious from these data. The CAT activity of the pCAT-303 construct was three fold greater than the activity of the pCAT-165 construct and taken to be indicative of the presence of an additional activating domain upstream of -165. The pCAT+53 construct contains the majority of the 5'-UTR with the 5'-flanking region deleted, including the transcription start site. The complete loss of CAT activity observed with pCAT+53 is consistent with the core promoter being located near -63. Suprisingly, however, deletion of more than 95% of the 5'-UTR (pCAT-303/+38) dramatically reduced the level of CAT activity. These results demonstrate that neither pCAT-303/+38 nor pCAT+53 were sufficient to independently promote transcription. Thus, interactions between upstream (-303/+38) and downstream (+53/+962) elements appear to be required to achieve efficient transcription of the D_{1A} receptor gene in renal cells.

VIII. B.2. DNase I Footprint Assay of D_{1A} receptor 5'-Flanking Region

The ability of the proposed activator region (-303 to -63) to bind nuclear proteins and known transcription factors was initially analyzed by DNase I footprinting assays (Figure 9, 10). LLC-PK_1 nuclear extracts were incubated with a ^{32}P-end-labeled DNA probe containing the upstream nucleotide sequence from -240 to -140 (figure 9A). The results are demonstrated schematically in Figure 11 and indicated that eight sites of the proposed activating region were protected (F1-7) and one hypersensitive site was identified (H1). F_1 (see summary in Figure 11) represents protection of -240 to -233 and corresponds to an overlapping AP-2/Sp1 site. While F_2 and F_3 contain the two CRE-like elements (-222/-216, -209/-205). H_1 corresponds to a hypersensitive area upstream of an AP-2 site (-182/-177). F_4 protects a region which contains an E-box consensus site (-169 to -163). F_6 and F_7, respectively (Figure 10, 11), correspond to protection of a radiolabeled antisense probe at -160 to -170 and -147 to -158 with LLC-PK_1 extracts. The F_6 protected region contains an E-box whereas the F_7 area (-144 to -139) does not represent a known consensus and may serve as a binding site for a novel or tissue-specific factor. Taken together these results indicate that the nuclear proteins are bound to the sense and antisense strand of -250 to -134 of the D_{1A} receptor gene promoter. The antisense probe (-149/-220) was weakly protected from -192 to -179 with purified AP-2 protein (data not shown). These results were confirmed by incubating the upstream probe (-240 to -40) with human recombinant proteins (Sp1, AP-2, CREB-1) as shown in Figure 9B. Interestingly, a Sp1 site at position -232/-226 was not protected by recombinant Sp1. A similar observation was made by Minowa et al. (1993) for the Act-2 region of the human D_1 promoter. F_1, H_1 and a portion of F_5 were protected by purified AP-2 and each of these

regions contain putative AP-2 sites (-236/-229, -212/-205, -182/-177; respectively). These results suggest that AP-2 may be an important regulator of the D_{1A} receptor gene.

LLC-PK_1 nuclear extracts were incubated with a ^{32}P-end-labeled DNA probe to the downstream nucleotide sequence -171 to -40 (Figure 9C, lanes 1-5). The downstream probe contains the region of the porcine D_{1A} receptor gene that corresponds to the Act-1 and Act-2 region of the human D_1 receptor gene. Extensive protection was observed and labeled F_5 (Figure 11). The F_5 protected region includes the F_4, F_6 and F_7 protected areas as well as GC box and AP-2 consensus sites. Footprinting assays with human recombinant purified proteins and downstream labeled probe containing nucleotide sequence from -171 to -40 (Figure 9C, lanes 7-15) were performed. F_5 contains a GC box (-127/-122) and the overlapping Egr-1/Sp1 site (-101/-90) which were protected by purified Sp1 protein. These results indicate that Sp1 protein is able to bind to the porcine D_{1A} receptor gene activating region at two of the three putative consensus sites contrary to the findings of Minowa et al (1993) for the human D_{1A} receptor gene. H_1 and F_5 each contain prospective AP-2 sites (-190/-180, -112/-104; respectively) which were protected by recombinant AP-2.

A comparison of the DNase I protection of -250/-120 was determined using either LLC-PK_1 or SK-N-MC (human neuroblastoma cells) extracts (Figure 12). The LLC-PK_1 extracts exhibited protection similar to that described earlier (Figure 9A). The SK-N-MC cell extracts were more effective in protecting the radiolabeled probe from DNase I digestion. Protection was observed for essentially the entire region encompassing -250 to -120. This region did not activate transcription of the human D_1

CAT reporter in NS20Y cells (Minowa et al., 1993) as well as the Act-I and Act-II sequence and therefore suggests this area may suppress activity in neuronal cells.

VIII. B.3. Mutational Analysis of pCAT-303

Deletion analysis indicated that pCAT-303 had the highest CAT activity (Figure 8) and DNase I protection assay indicated that LLC-PK_1 nuclear extracts extensively protected the region from -303 to -63 (Figure 9). Minowa et al. (1993) previously identified an activating region of the human D_{1A} receptor promoter which is conserved in the porcine homologue and corresponds to -171 to -90 of the porcine sequence. Since the region from -165 to -63 corresponds to Act-I and Act-II of the human D_{1A} receptor gene, an additional construct was created [pCAT-303 (Δ-165/-63)] to examine the activity of -303 to -165 in the absence of -165 to -63 (Figure 13). The pCAT-303 (Δ-165/-63) construct displayed a 70% decrease in CAT activity, relative to pCAT-303. These results indicate that -165 to -63 is necessary for optimal activity of pCAT-303.

DNase I protection analysis indicated that LLC-PK_1 nuclear extracts protected the two CRE-like elements. Since the 3'-CRE site is better conserved than the 5'-CRE across species, a construct containing a point mutation in the 3'-CRE site (GCACGTCC) was created, pCAT-303 (-211G->C), to examine the significance of the 3'-CRE in pCAT-303 (Figure 13). Surprisingly, the point mutation in the 3'-CRE greatly decreased the CAT activity 90% compared to the pCAT-303 construct. This level of activity is approximately two fold greater than pBasic. These results indicate that the 3'-CRE-like element is necessary to maintain basal activity for the pCAT-303 construct.

VIII. B.4. Regulation of a Heterologous Promoter by -303 to -165

In order to determine if the -303 to -165 region contained an activating domain, it was inserted in front of the thymidine kinase promoter upstream of a CAT reporter gene (TKCAT) in sense and antisense orientations. Placement of the -303 to -165 sequence upstream of the TK-promoter did not enhance the level of activation (Figure 14). The antisense orientation resulted in a 70% decrease in TKCAT activity indicating that activity of -303 to -165 is orientation dependent. Although a TKCAT construct containing 2 copies of the sequence from -303 to -165 in the sense orientation or 6 copies of (-303 to -165) allowed transcription of the TKCAT reporter gene, the activity was not enhanced by the presence of multiple copies of this sequence. These results indicate that other cis-elements are required to interact with -303 to -165 in order to achieve a high level of expression. The previous results from the 5'-deletion analysis (Figure 8) indicated a two-fold decrease in CAT activity of pCAT-206 relative to pCAT-303. Since pCAT-206 disrupts the 3'-CRE-like site, a construct was created in which the 3'-CRE-like element (GGACGTCC)was deleted from pTKCAT-303/-165. Deletion of the 3'-CRE demonstrated the importance of this site in transcription since disrupting it inhibited transcription of the TKCAT reporter gene by 85%. Interestingly, inserting a 22 bp oligonucleotide containing both CRE-like elements in front of the TKCAT promoter, pTKCAT-5'CRE/3'CRE, resulted in a moderate (2.7x) orientation independent increase in CAT activity. These results indicate that the 5'-CRE/3'-CRE-like elements have properties consistent with that of an enhancer but their effects are silenced within the larger context of the -303/-165 segment when placed upstream of a heterologous promoter.

VIII. B.5. Effect of Forskolin on D_{1A} receptor gene transcription

Agonist-induced desensitization and down-regulation of D_1 receptors is an extensively studied phenomenon. It is unknown, however, whether transcriptional mechanisms are involved in this process. Basal activity of the pCAT- D_{1A} constructs required the 3'-CRE (Figure 13) which was also shown to be a moderate enhancer of a heterologous promoter (Figure 14). In order to determine if the CRE-like sites within the porcine D_{1A} promoter were inducible, cells were transfected with pCAT-D_{1A} constructs and incubated in the presence and absence of forskolin and assayed for CAT activity.

Transiently transfected LLC-PK_1 cells were incubated, 30 hours after transfection in the absence and presence of 10 μM forskolin and assayed for CAT activity 18 hours after application. The greatest stimulation in transcription was observed for the pCAT-303 construct (Figure 15 and 16B, upper panel) which exhibited a 2-fold increase in CAT activity. Forskolin stimulated transcription of the pCAT-206 construct was significantly less than the pCAT-303 construct. Interestingly, pCAT-206 does not contain either of the CRE-like elements, indicating that these CRE-like elements may be essential for cAMP-responsiveness. In order to further examine the function of the 3'-CRE-like element, the pCAT-303(-211G->C) CAT construct which was previously determined to be essential for basal transcription was tested for forskolin responsiveness. Transient expression of this point mutant in LLC-PK_1 cells revealed that not only was the CRE-like element responsible for basal expression but it was necessary for the forskolin response as well. However, LLC-PK_1 cells transiently expressing the TK-CAT constructs (pTKCAT-303/-165, pTKCAT-Δ3'CRE or pTKCAT-5'CRE/3'CRE) did not exhibit any significant in change the level of CAT

expression in response to forskolin treatment (Figure 16B, lower panel). Although the 5'/3'-CRE element and the D_{1A} receptor gene sequence -303 to -165 did not confer forskolin response in the TK-CAT vector it is conceivable that the CRE-like element acts synergistically with downstream elements to mediate cAMP responsiveness.

VIII. B.6. Gel Mobility Shift Assay

In order to further characterize the interactions between the proposed D_{1A} receptor activator region (-303/-165) and LLC-PK_1 nuclear proteins, gel mobility shift assays were performed with double stranded oligonucleotides for areas of interest.

VIII. B.6.a. CRE-like

Double stranded oligonucleotides containing the two wild type CRE-like sites (-223/-205) from the porcine D_{1A} receptor promoter were used as a radiolabeled probe (Figure 17 A, B). The wild type oligonucleotide and oligonucleotides with the 5'- or 3'- CRE site mutated, singly or together, were added as competitors in gel mobility shift competition assays. Three major protein-DNA complexes (labeled A, B and C) were observed to form between the LLC-PK_1 nuclear extracts and DNA containing the intact 5'- and 3'- CRE sequences (Figure 17B). The addition of 50- or 500-fold molar excess concentrations of unlabeled wild type CRE-like competitor efficiently competed for these complexes (lanes 3, 4). The oligonucleotide containing the mutated 5'-CRE competed more effectively for the top (A) and lower (C) bands, indicating that the 3'-CRE bound proteins to form this complex (lanes 5, 6). The oligonucleotide with the 3'-CRE mutated competed effectively for the middle (B) and lower (C) bands (lanes 7, 8). The CRE double mutant oligonucleotide was an ineffective competitor (lanes 9, 10) while the CRE consensus competed in a manner similar to but more efficiently than the

3'-mutated CRE for the second and third band (B and C) (lanes 11, 12). These results suggest that the 5'-CRE site which is more similar to the CRE consensus site competes similarly whereas the 3'-CRE site competes for different protein-DNA complex suggesting that a different protein may bind to the 3'-CRE site.

VIII. B.6.b. 3'-CRE-like

Since the results of site-directed mutagenesis indicated the 3'-CRE-like site was important for basal transcription and the above gel mobility shift assay suggested that the 5' and 3'- CRE sites may bind different proteins, the 3'-CRE site was examined separately. A double stranded oligonucleotide containing the 3' CRE-like sequence alone was radiolabeled (Figure 18 A, B). Incubation of the 3'CRE probe with LLC-PK_1 nuclear extracts resulted in the formation of three protein-DNA complexes (Figure 18 B; lane 2). Competition with cold wild type 3' CRE competed effectively for protein-DNA complexes (lanes 3,4,5). Quantitation of the protein-DNA complexes by densitometry indicated that an oligonucleotide containing the G->C point mutation (lanes 6,7,8) in the 3'-CRE competed less efficiently (20%) compared to the wild type CRE. A small difference (5%) was detected in the abilities of the consensus CRE (lanes 9,10, 11) and the wild type CRE to compete. It is likely that a different CREB/ATF isoform is bound to the 3'-CRE in the absence of the 5'-CRE compared to the wild type CRE.

VIII. B.6.c. AP-2

A gel shift assay using a radiolabled AP-2 probe (202/-174) containing the consensus sequence CCCGCGGC was performed. Incubation of an AP-2 probe with LLC-PK_1 nuclear proteins generated 2 protein-DNA complexes (Figure 19). The

unlabeled wild type AP-2 double stranded oligonucleotide effectively competes for each of these complexes and at higher concentration (500-fold molar excess) all of the bands were competed. Interestingly, competition with an AP-2 consensus oligonucleotide indicated that (lanes 5,6) it did not compete as well as the wild type sequence. A non-specific competitor (lane 7) did not compete at concentrations as high as 500-fold molar excess. These results indicate that LLC-PK_1 nuclear proteins interact specifically with AP-2 sites in the D_{1A} receptor promoter.

VIII. B.6.d. E-box

A synthetic probe containing the CACGTG consensus sequence from -171 to -154 of the D_{1A} receptor promoter was radiolabeled and incubated with LLC-PK_1 nuclear extracts (Figure 20). Although two retarded bands were detected, only the top band represents a specific protein-DNA interaction as evidenced by the ability of the unlabeled probe to compete for this band. These results confirm that the interactions between the E-box of the D_{1A} receptor gene and LLC-PK_1 nuclear extracts are specific.

VIII. B.6.e. Egr-1/ Sp1

Using the overlapping Egr-1/Sp1 site (-124/-81) as a probe, one major protein-DNA complex was formed (Figure 21 A, B). The wild type Egr-1 probe competed effectively when added in concentrations at 50-fold and 500-fold molar excess. Sp1 and Egr-1 consensus oligonucleotides competed for the band. A mutated Egr-1 oligonucleotide did not compete for the protein-DNA complexes. Similarly, an AP-2 consensus site could not compete in a similar experiment even though the wild type Egr-1 probe contains the upstream AP-2 site (data not shown). These data suggest that Sp1 and Egr-1 may interact at the overlapping Egr-1/Sp1 site in the D_{1A} receptor gene.

VIII. C. Role of 5'-UTR in D_{1A} Receptor Gene Transcription

VIII. C.1. Analysis of the D_{1A} Receptor Gene 5'-UTR

In order to establish if the intron and the 5' untranslated region are involved in regulating the D_{1A} receptor gene transcription, four additional pCAT constructs with various deletions of the 5'-untranslated region were prepared and evaluated in LLC-PK_1 cells (Figure 22). The pCAT-303 ΔIntron construct contains a deletion only in the region containing the intron (+430/+ 527). The pCAT-303/+542 construct was created by deleting the distal 5'-untranslated region (+542/+983) of the pCAT-303 construct. The pCAT-303/+425 construct contains a deletion of both the distal 5'-untranslated region and the intronic sequences (+425/+983) of the pCAT-303 construct. The pCAT-303 Δintron and pCAT-303/+542 constructs were both found to decrease reporter gene activity nearly 60-fold to levels comparable to the negative control plasmid, pBasic. These results suggest the presence of cis-elements in the intron and 5'-UTR which are functional enhancers. In contrast, pCAT-303/+430 increased the observed reporter CAT activity 22-fold over pBasic. Comparison of the pCAT-303/+425 to the pCAT-303/+38 would suggest that the area between +38 to +425 contains an activating sequence. Although full activity is not restored with pCAT-303/+425, it appears that removal of the intron from the pCAT-303/+542 construct resulted in a dramatic increase in CAT activity, suggesting that the intron behaves as a repressor.

VIII. C.2. Regulation of a Heterologous Promoter by D_{1A} Intronic Sequence

In order to establish if the intron acts as an enhancer or repressor in a context independent manner, the intron was inserted into the pBLCAT5 vector upstream of the TK-CAT reporter gene in the forward (+) or reverse (-) orientation (Figure 23). Transient expression of the pTKCAT+intron or pTKCAT-intron in LLC-PK_1 cells indicated the intron repressed transcription from the TK-promoter independently of its orientation. The intron reduced transcriptional activity of the TK-promoter by 80% compared to the wild-type TK-promoter or pTKCAT-303/-165. In an effort to restore the transcriptional activity to the TK-promoter, a second construct was designed in which the -303/-165 region of the D_{1A} receptor gene was inserted 150 bp upstream of the intronic sequence (+430/+527) in pTK+intron. The pTKCAT-303/-165+Intron construct exhibited reporter gene activity that was 70% less than the wild-type TK-CAT promoter, in spite of the presence of the -303/-165 sequence. It appears in this context that -303/-165 can not independently overcome the repression imposed by the intron.

VIII. C.3. DNase I Footprint Analysis of D_{1A} Receptor Gene Intronic Sequence

In order to test for the presence of regulatory sequences in the intron, DNase I footprint assays were performed. One of two ^{32}P-end-labeled DNA probes containing nucleotide sequence from +377 to +527 (Figure 24, left) or +430 to +527 (Figure 24, right) were incubated with nuclear extracts from LLC-PK_1 cells and then digested with DNase I. The 5'-probe (+377 to +527) was protected from +420 to +460 while the 3'-probe (+430 to +527) was protected in two regions +452 to +480 and +485 to +515

(presented diagramatically in Figure 25). Both assays confirmed that LLC-PK_1 nuclear proteins bound to the intronic sequence. Interestingly, the protected sequence at +452 to +460 (GGAATTGGG) contains an inverted CAAT box and the TGGCA binding site is located at +493 to +497. The remainder of the intronic sequence does not contain known consensus sequences (Figure 25).

VIII. C.4. Competition Assay of D_{1A} Receptor Gene Intronic Sequence

Using the intronic sequence as a probe in a gel mobility shift assay verified that the intron is capable of binding nuclear proteins from LLC-PK_1 extracts (Figure 26). Two protein-DNA complexes were retarded on a polyacrylamide gel. The binding primarily occurred in the 3'-distal portion of the intron (+448/+527) as determined by competition experiments (Figure 26, lanes 7-10). This is particularly interesting since the sequence does not contain any consensus sites and would suggest that a novel and perhaps even tissue specific factor may be involved. The 3'-distal portion of the intron (+478/+527) competed effectively for the 2 protein-DNA complexes with 50-fold molar excess of competitor. The 5'-proximal region of the intron (+430/+477) was a less effective competitor, beginning to compete weakly when 100-fold molar excess of competitor (lane 6). These results confirm that the LLC-PK_1 nuclear proteins are able to recognize regions in the 3'-distal region of the intron and bind to them.

Figure 5: Effect of dopamine stimulation on D_{1A} receptor mRNA in LLC-PK_1 cells. Cells were incubated with dopamine for various lengths of time as indicated. The cells were harvested, poly A^+ mRNA isolated and analyzed by RT-PCR as described in the methods. PCR products, with ^{32}P incorporated, were separated by gel electrophoresis, bands were excised and counted in a liquid scintillation counter. Values are the mean $\pm$ SEM of two experiments.

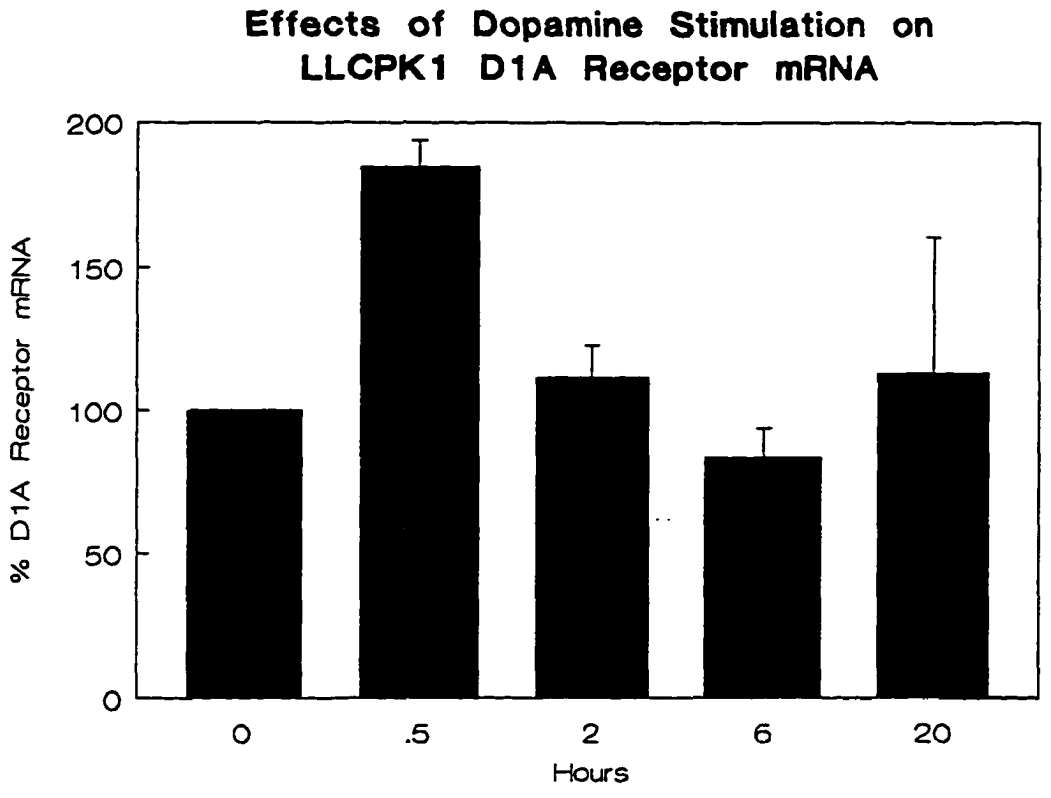


Figure 6: Representative RT-PCR examining effect of dopamine on D_{1A} receptor mRNA stability. Ethidium bromide staining of PCR products following reverse transcription of LLC-PK_1 poly A^+ mRNA with a D_{1A} receptor gene specific primer (+711/+682). Products were separated on a 2% agarose gel. Prior to RT-PCR, cells were treated with actinomycin D for the various periods of time as indicated. The cells were harvested, poly A+ mRNA isolated and analyzed by RT-PCR as described in the methods. Lane1: DNA marker; MspI digest of pBR322; lanes 2: no cDNA-negative control; lane 3: no RT-negative control; lanes 4-7: results from cells pretreated with cycloheximide (2h) followed by dopamine (30 min); lanes 8-11: results from control LLC-PK_1 cells; lanes 12-15: results from LLC-PK_1 cells pretreated with 10 μM dopamine for 30 min.; lane 16: positive control using porcine caudate total RNA.

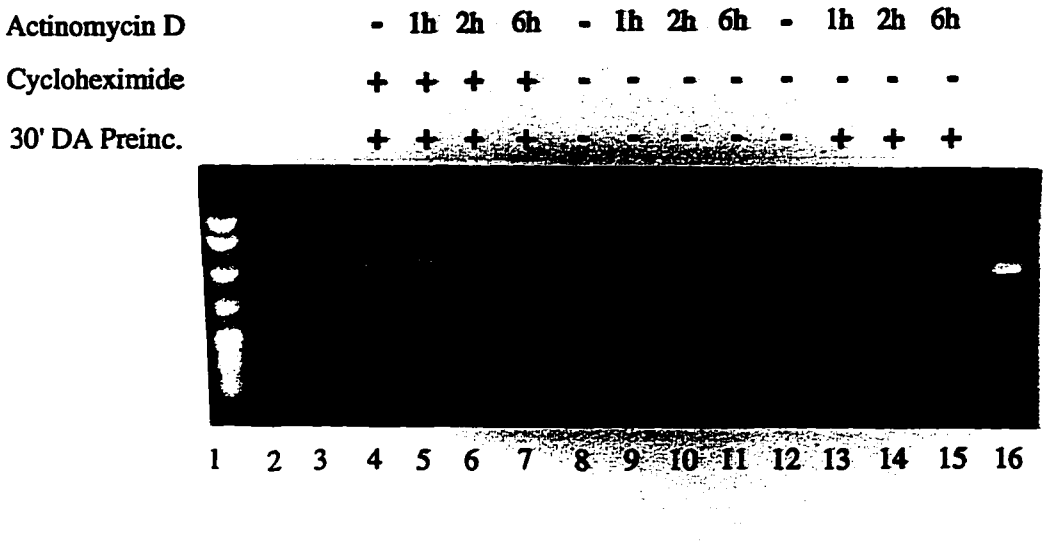


Figure 7: Effect of dopamine stimulation on the stability of LLC-PK$_1$ D$_{1A}$ receptor mRNA. Prior to RT-PCR, cells were treated with actinomycin D for the various periods of time as indicated. The cells were harvested, poly A+ mRNA isolated and analyzed by RT-PCR as described in the methods. Values shown are the mean $\pm$ SEM of three experiments.

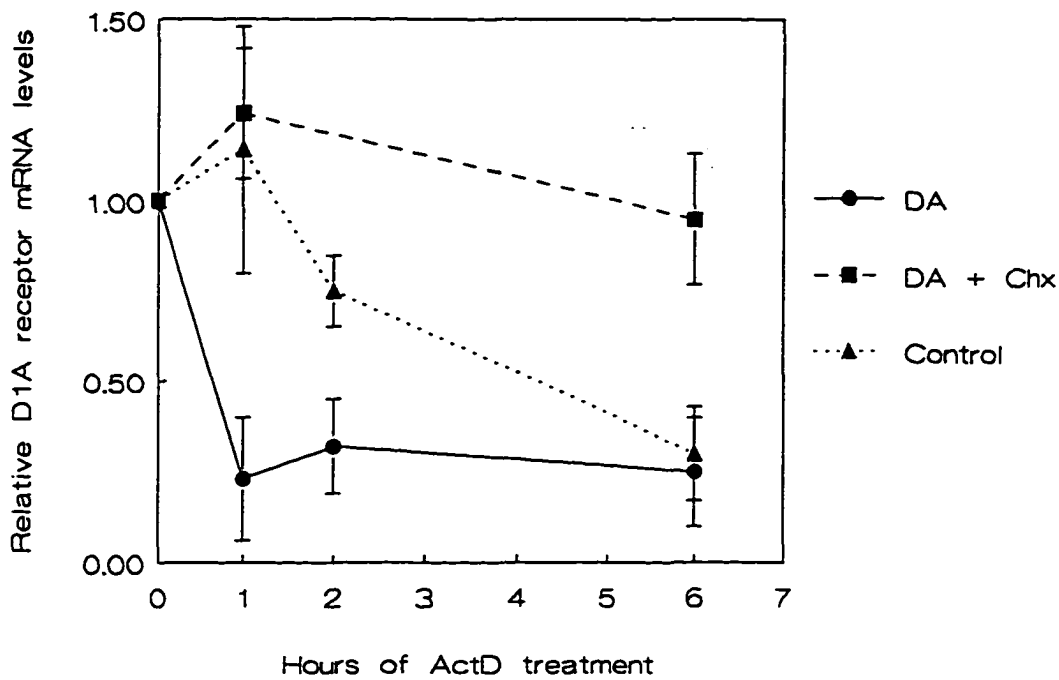


Figure 8: Transcriptional activity of the D_{1A} receptor gene in LLC-PK_1 cells. *A*, schematic representation of the D_{1A} receptor gene. The 5' and 3' flanking regions and the intron segment are shown by the solid line, the 5' and 3' untranslated regions are shown by the thicker bar and the coding region is shown by the shaded area. The transcription start site is indicated by the arrow. *B*, on the left is a schematic of the pCAT-D_{1A} reporter gene constructs. Progressive deletions of the D_{1A} receptor gene 5'-flanking and 5'-untranslated regions (from -3000 to +53 bp) were fused upstream of CAT reporter gene as described in the methods. *C*, on the right is the transcriptional activity of each construct following transient expression in LLC-PK_1 cells. Transcriptional activity is expressed relative to promoterless pCAT-Basic construct. Values represent the mean $\pm$ S.E.M. of 9 separate experiments, each point determined in triplicate.

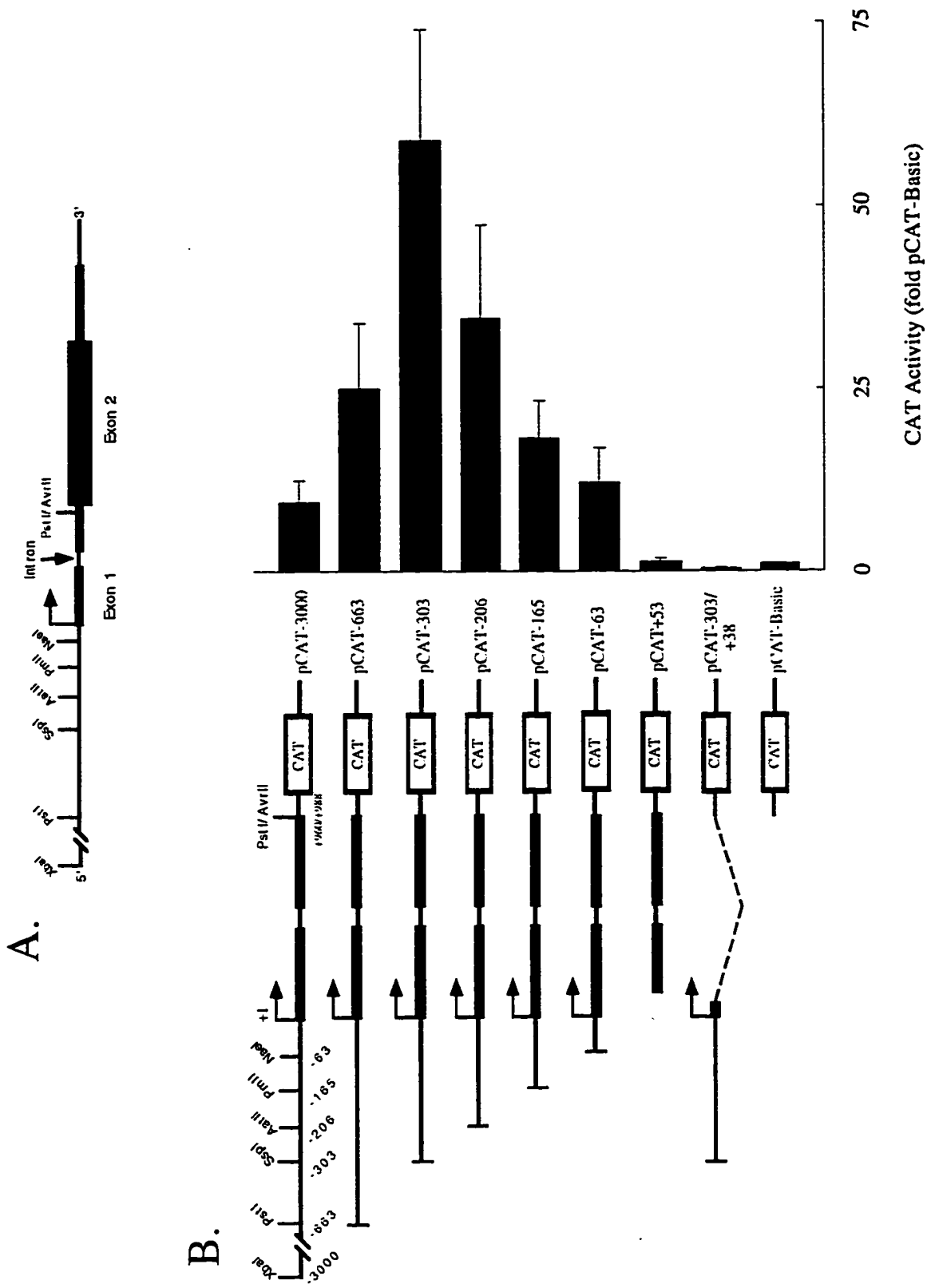
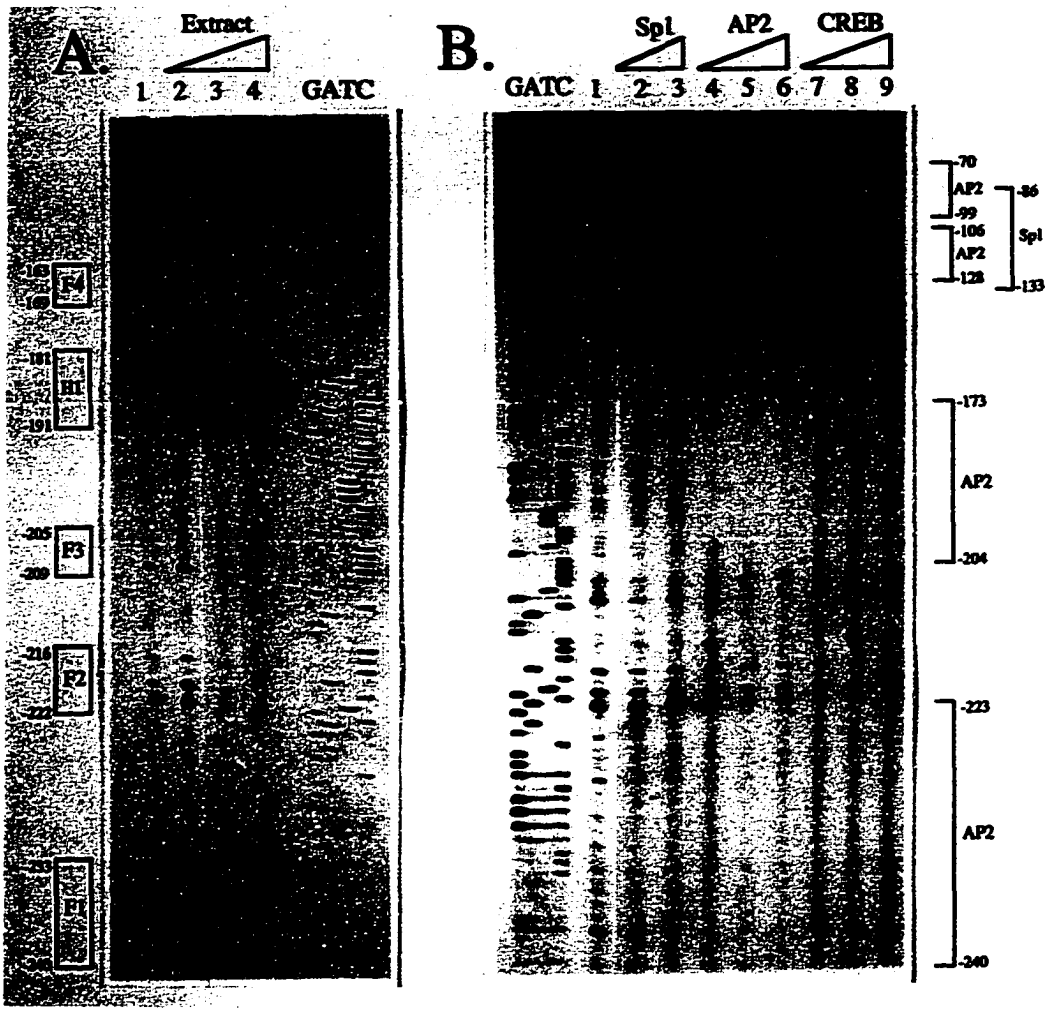


Figure 9: DNase I protection analysis of the D_{1A} receptor gene 5'-flanking region. *A*, a sense ^{32}P-labeled probe (-240/-40) was incubated with increasing concentration of LLC-PK_1 nuclear extracts (10, 15, 25 μg, lanes 2-4, respectively) or *B*, with increasing concentrations of Sp1 (150,300 ng, lanes 2-3, respectively), AP-2 (20, 60, 120 ng, lanes 4-6, respectively) or CREB (50, 150, 250 ng, lanes 7-9, respectively), and treated with DNase I . *C*, a sense labeled probe (-184/-40) was incubated with increasing concentration of LLC-PK_1 nuclear extracts (5, 10, 20, 25 μg, lanes 2-5, respectively) and increasing concentration of purified recombinant AP-2 (20, 60, 120 ng, lanes 7-9, respectively), Sp1 (50, 150,300 ng, lanes 10-12, respectively) and CREB (50, 150, 250 ng, lanes 13-15, respectively) and treated with DNase I. The resulting products were resolved on a 10% PAGE denaturing gel. Lane 1 contains probe digested with DNase I without the addition of nuclear extracts. Dideoxy sequencing reactions using the ^{32}P-labeled sense primer were electrophoresed simultaneously.



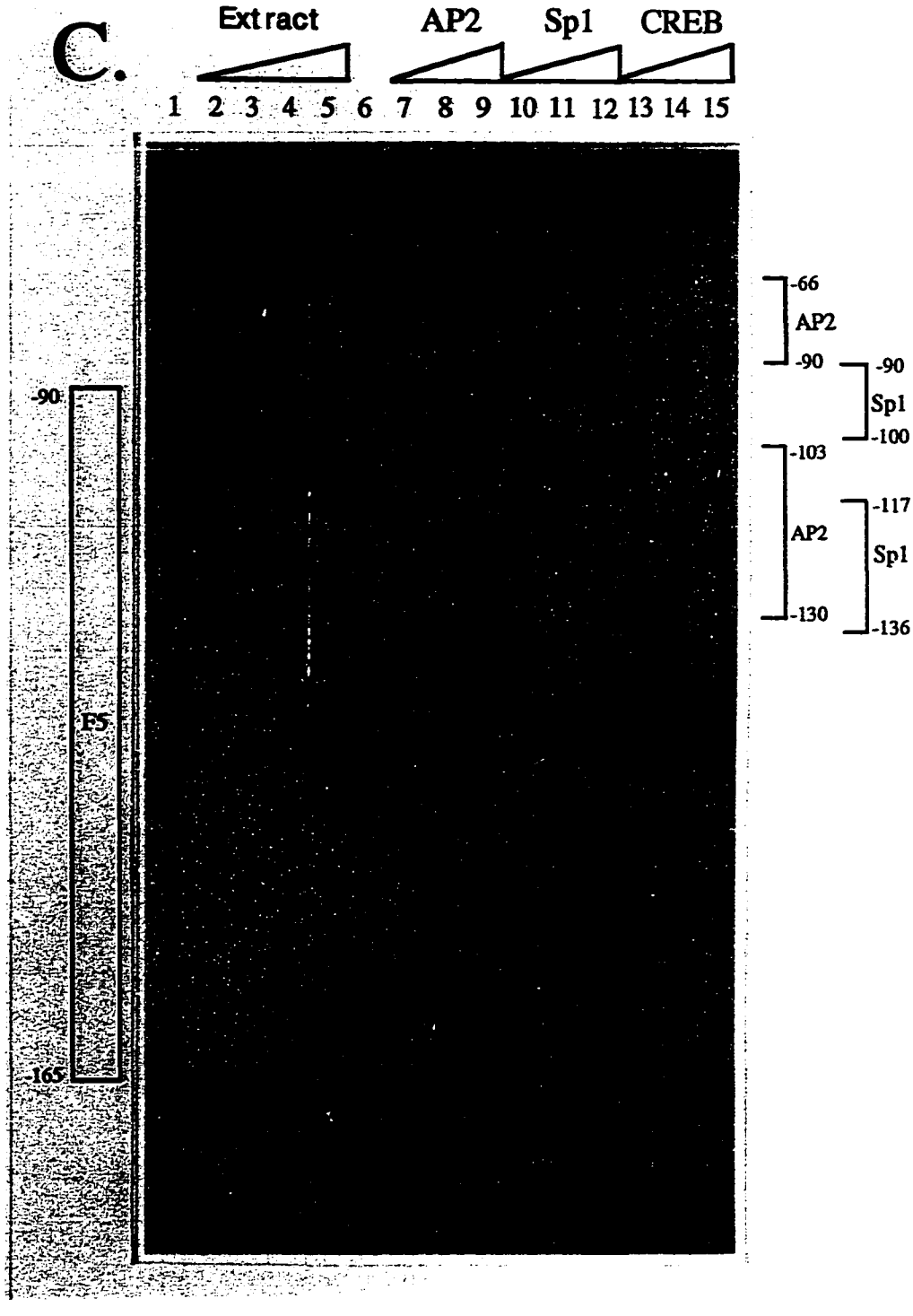


Figure 10: DNase I protection analysis of the D_{1A} receptor gene 5'-flanking region. An antisense ^{32}P-labeled probe (-134/-266) was incubated with increasing concentration of LLC-PK_1 nuclear extracts (5,10, 15, 20, 25 , 50μg, lanes 2-7, respectively), treated with DNase I and resolved on a 10% PAGE denaturing gel. Lane 1 contains probe digested with DNase I without the addition of nuclear extracts. Dideoxy sequencing reactions using the ^{32}P-labeled antisense primer were electrophoresed simultaneously.

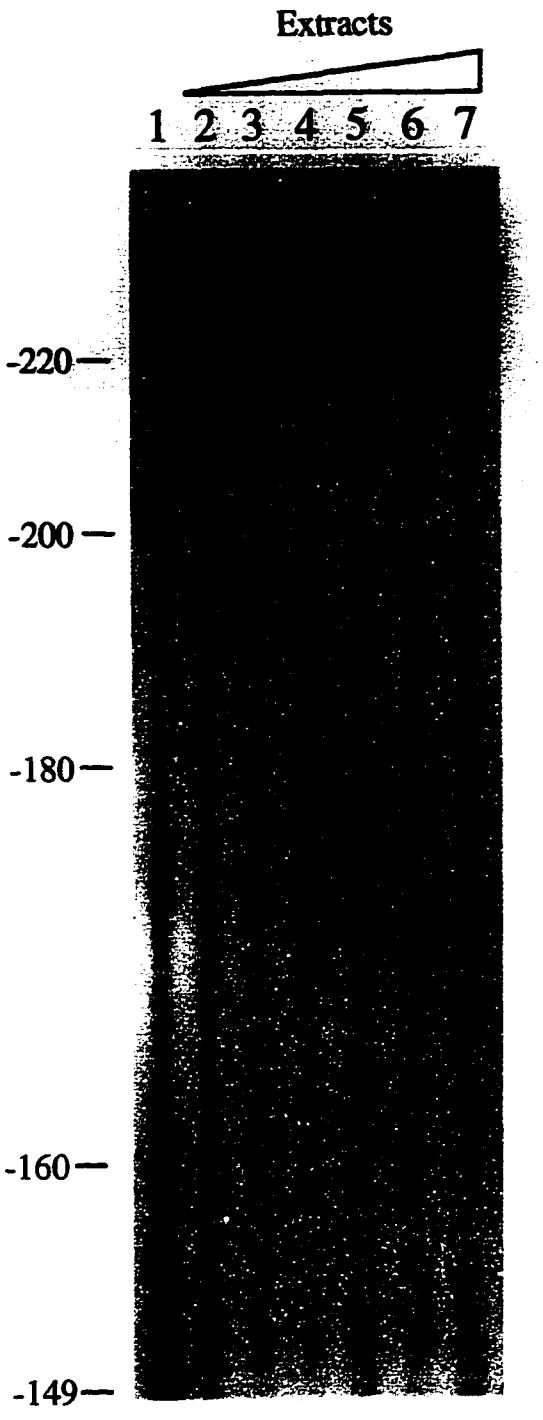


Figure 11: Schematic representation of DNase I protection analysis of the D_{1A} receptor gene 5'-flanking region (-245/-65). Solid bars indicate areas protected by LLC-PK_1 nuclear extracts. Stippled bars indicate protection with AP-2 recombinant protein. Hatched regions indicate sites that were hypersensitive to DNase I and open bars indicate areas protected by Sp1 recombinant protein.
Bottom is a schematic representation of putative transcription factors which have consensus sites in the protected areas. Abbreviations: AP-2, activator protein 2; CRE, cAMP responsive element binding protein, E, E-box; EGR, early growth response factor; Sp1, stimulatory protein 1.

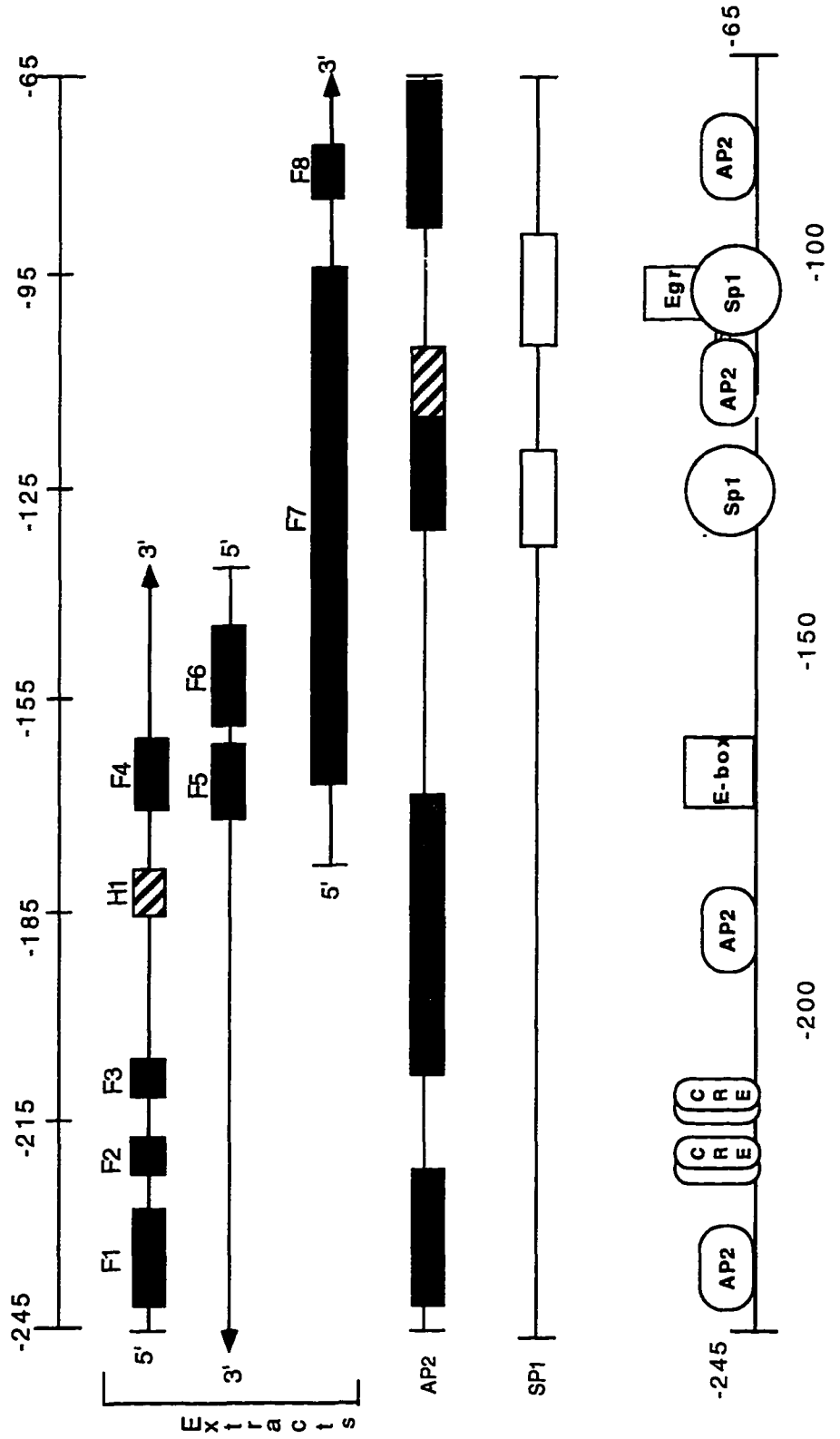


Figure 12: DNase I protection analysis of the D_{1A} receptor gene 5'-flanking region using LLC-PK_1 or SK-N-MC (human neuroblastoma cells) nuclear extracts. A sense ^{32}P-labeled probe (-266/-18) was incubated with increasing concentration of LLC-PK_1 nuclear extracts (5,10, 15,20, 25 μg, lanes 2-6, respectively) or SK-N-MC nuclear extracts (5,10, 15,20, 25 μg, lanes 7-11, respectively) treated with DNase I and resolved on a 10% PAGE denaturing gel. Lane 1 contains probe digested with DNase I without the addition of nuclear extracts. Dideoxy sequencing reactions using the ^{32}P-labeled antisense primer were electrophoresed simultaneously.

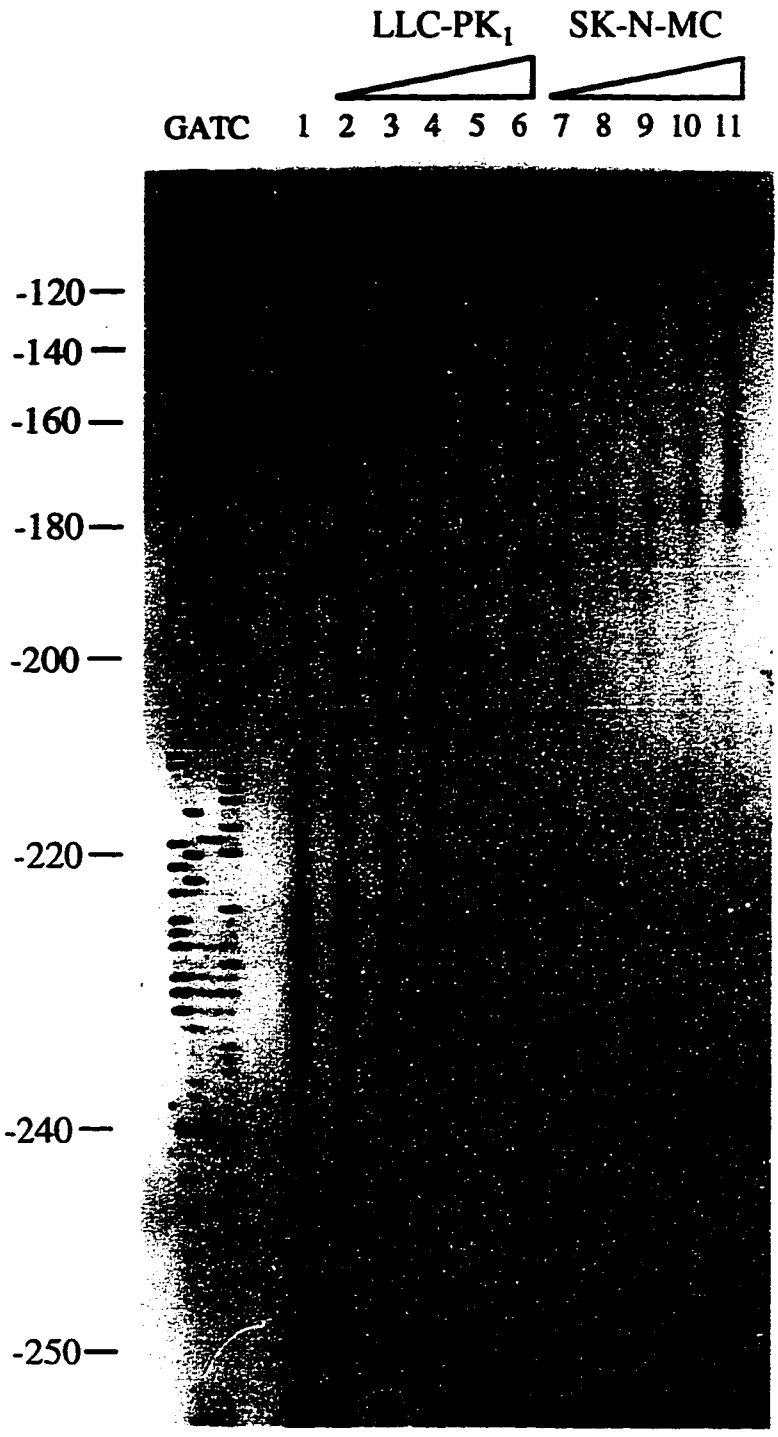


Figure 13: Transcriptional activity of mutational analysis of pCAT-303 . Mutations were introduced into the pCAT-303 construct, either a deletion of (-303 to -165) or a point mutation (-211 G->C). On the left is a schematic representation of the CAT constructs which were tested for CAT activity as previously described. On the right is the transcriptional activity of each construct following transient expression in LLC-PK_1 cells, with activity expressed as %pCAT-303. Values represent the mean ± S.E.M. of 5 separate experiments, each point determined in triplicate.

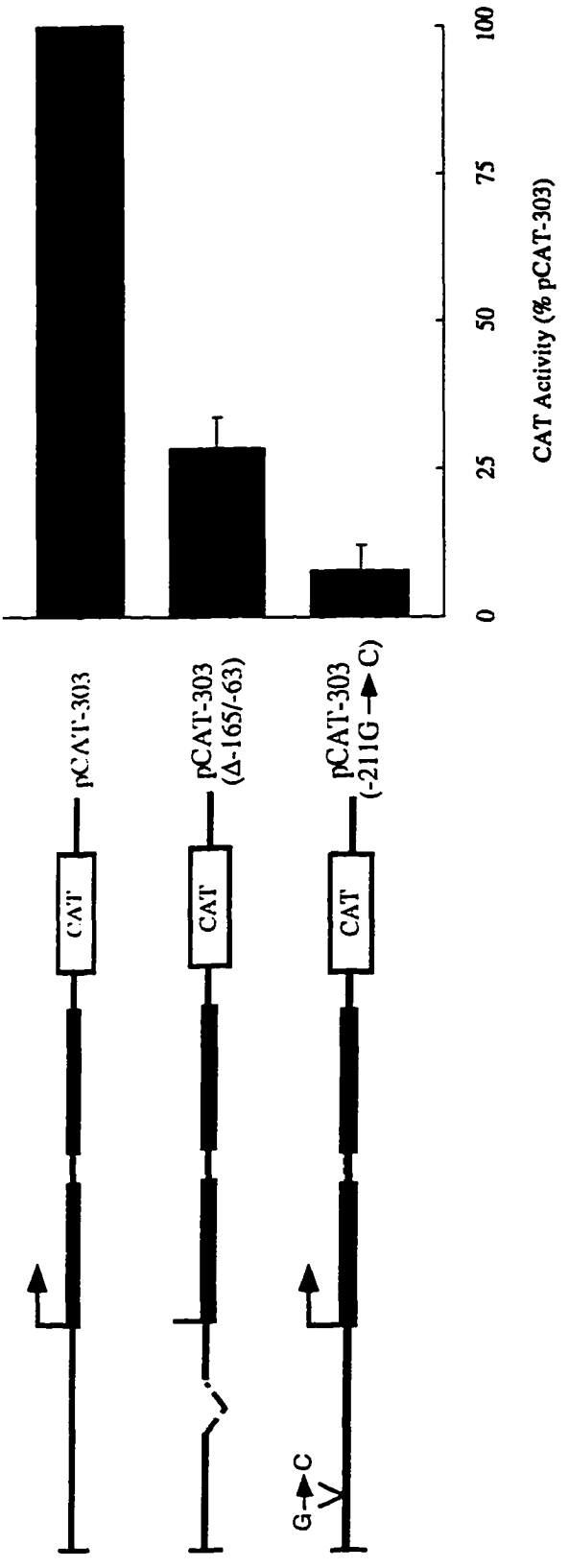


Figure 14: Transcriptional activity of a heterologous promoter/reporter construct with the D_{1A} receptor 5'-UTR intron placed upstream. On the left is a schematic representation of the heterologous thymidine kinase (TK) promoter/CAT construct and the various sequences placed upstream and tested for enhancer or suppresser activity as previously described. The intron sequence was tested alone or in combination with a portion of the D_{1A} receptor gene 5'-flanking region (-303 to -165). On the right is the transcriptional activity of each construct following transient expression in LLC-PK_1 cells, with activity expressed as fold pTKCAT. Values represent the mean $\pm$ S.E.M. of 8 separate experiments, each point determined in triplicate.

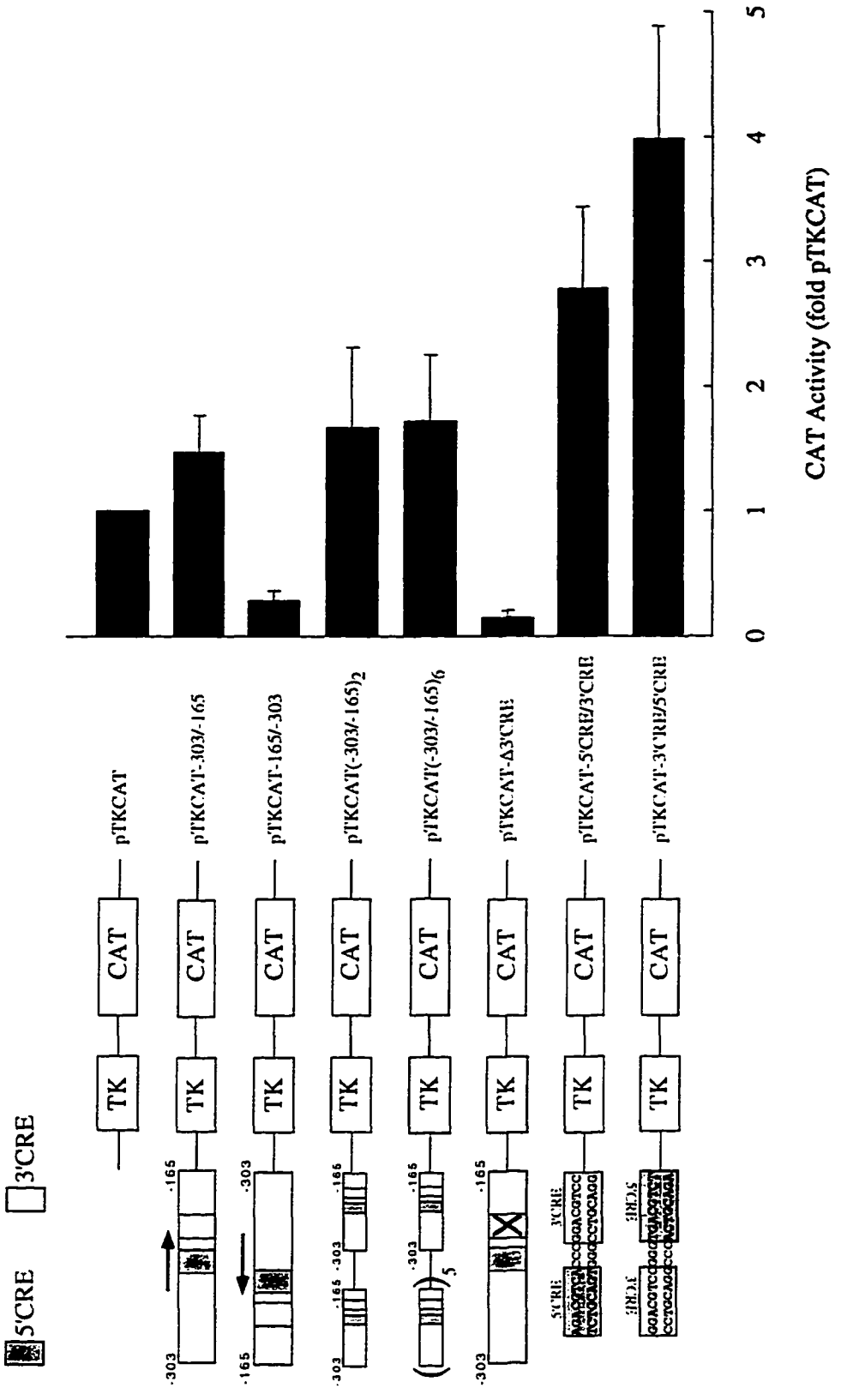


Figure 15: Representative TLC of the effect of forskolin on the transcriptional activity of the pCAT-D_{1A} chimeric constructs. Thin arrow at the bottom shows unreacted chloramphenicol. Thick arrows indicate mono- (middle) and diaceylated (top) products. Lanes 1,3,5,7,9,11; without forskolin, lanes 2,4,6,8,10,12: with 10 μM forskolin treatment, lane 13; pBasic, negative control, lane 14; pControl, positive control, lane 15; negative control, non-transfected cells.

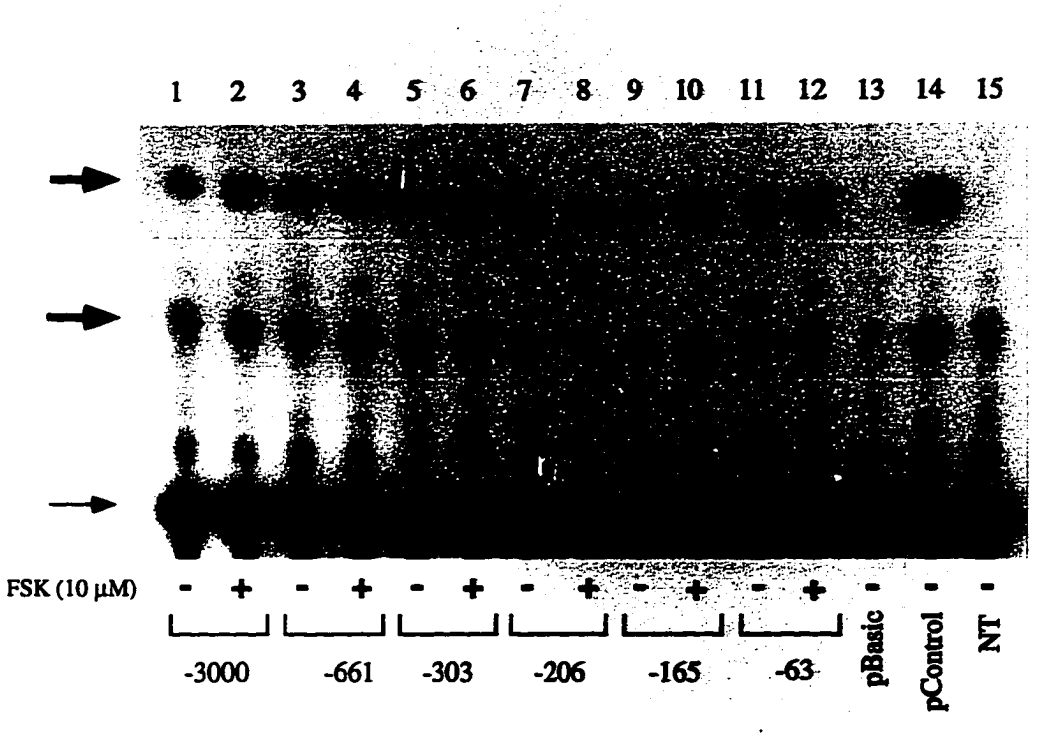


Figure 16: Effect of Forskolin on Expression of pCAT-D_{1A} fusion genes in transfected LLC-PK_1 cells. *A*, schematic representation of the D_{1A} receptor gene. The 5' and 3' flanking regions and the intron segment are shown by the solid line, the 5' and 3' untranslated regions are shown by the thicker bar and the coding region is shown by the shaded area. The transcription start site is indicated by the arrow. *B*, on the left is a schematic of the pCAT-D_{1A} or pTK-CAT reporter gene constructs. On the right is the CAT reporter gene expression in control cultures is shown by solid bars. Hatched bars indicate cultures treated with 10 μM forskolin for 18 h. CAT activity is expressed as fold over pCAT-Basic or fold over pTK-CAT. Values represent the mean ± S.E.M. of 6 separate experiments, each point determined in triplicate.

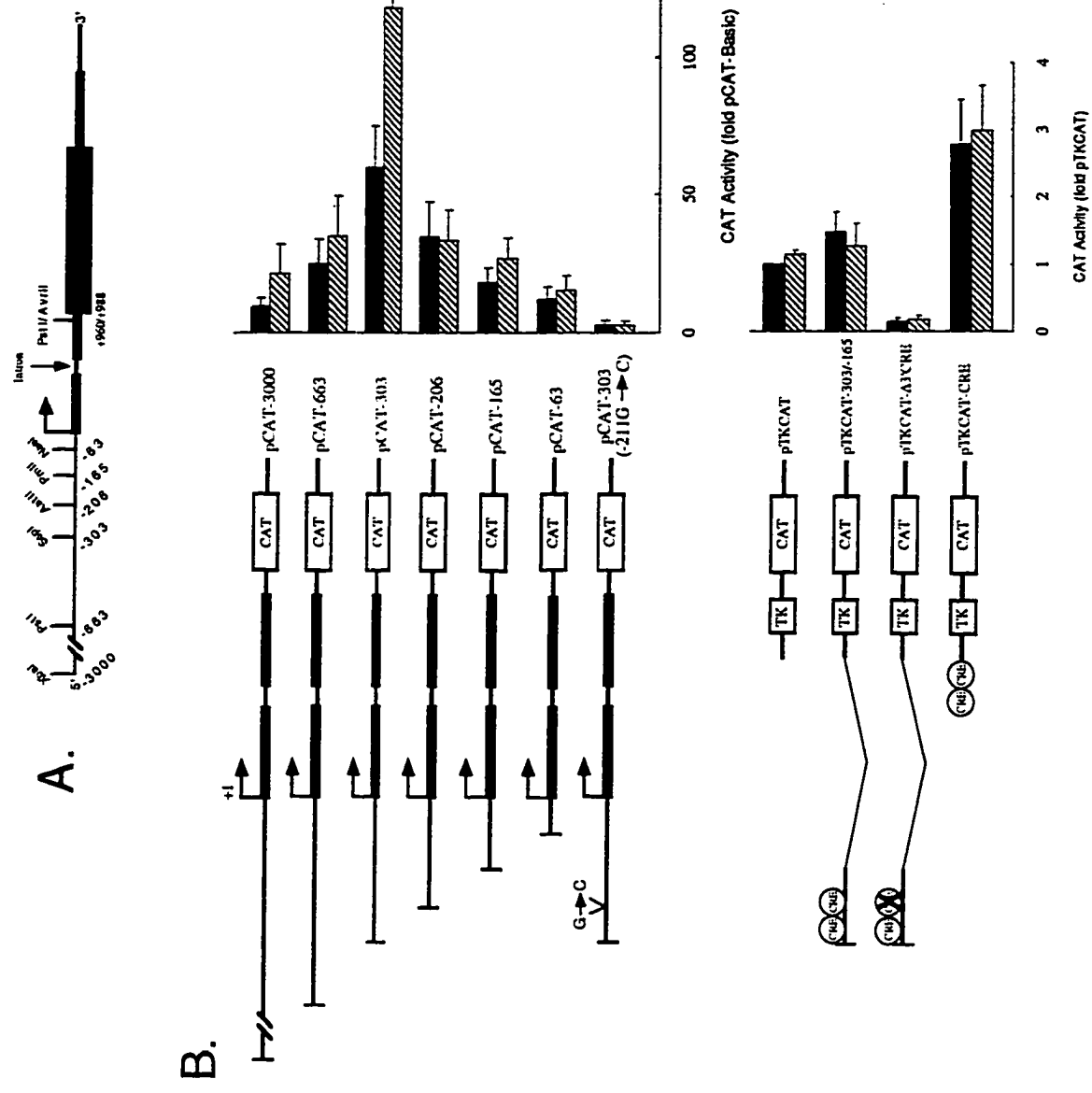


Figure 17: Gel mobility shift assay using a probe containing 5'- and 3'-CRE-like elements. *A*, comparison of double stranded oligonucleotides used in CRE Gel mobility shift assay. *B*, gel mobility shift assay was performed using a probe containing the 5- and 3'-CRE sites of the D_{1A} receptor gene (-223/-205) and LLC-PK_1 cell nuclear extracts. The WT CRE probe for the gel mobility shift assay was generated by hybridizing single stranded oligonucleotides containing SalI overhangs and filling in the ends with ^{32}P-dGTP, dNTPs and Klenow fragments. Excess probe was incubated in the absence (lane 1) or presence (lanes 2-12) of LLC-PK_1 nuclear extracts. The DNA-protein complexes seen in lane 2 (probe + extract, without competitor) were competed for with increasing concentrations of WT cold probe (50-, 500-molar excess, lanes 3,4), 5'CRE mutant (50-, 500-molar excess, lanes 5,6) or 3'CRE mutant (50-, 500-molar excess, lanes 7,8), double CRE mutant (50-, 500-molar excess, lanes 9, 10) or consensus sequence (50-, 500-molar excess, lanes 11,12). A, protein-DNA complex due to 3' site; B,C protein-DNA complexes due to 5' site; NS, non-specific competitor.

CRE GMSA Oligonucleotides

wt-CRE	5′-AGACGTCACCCGGACGTCCCCC-3′
consensus-CRE	5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′
5′-CRE Mut.	5′-AGAATTCACCCGGACGTCCCCC-3′
3′-CRE Mut.	5′-AGACGTCACCCGGAATTCCCCC-3′
Dbl CRE Mut.	5′-AGAATTCACCCGGAATTCCCCC-3′

Underlined nucleotides identify cis-elements, double underline indicates mutations.

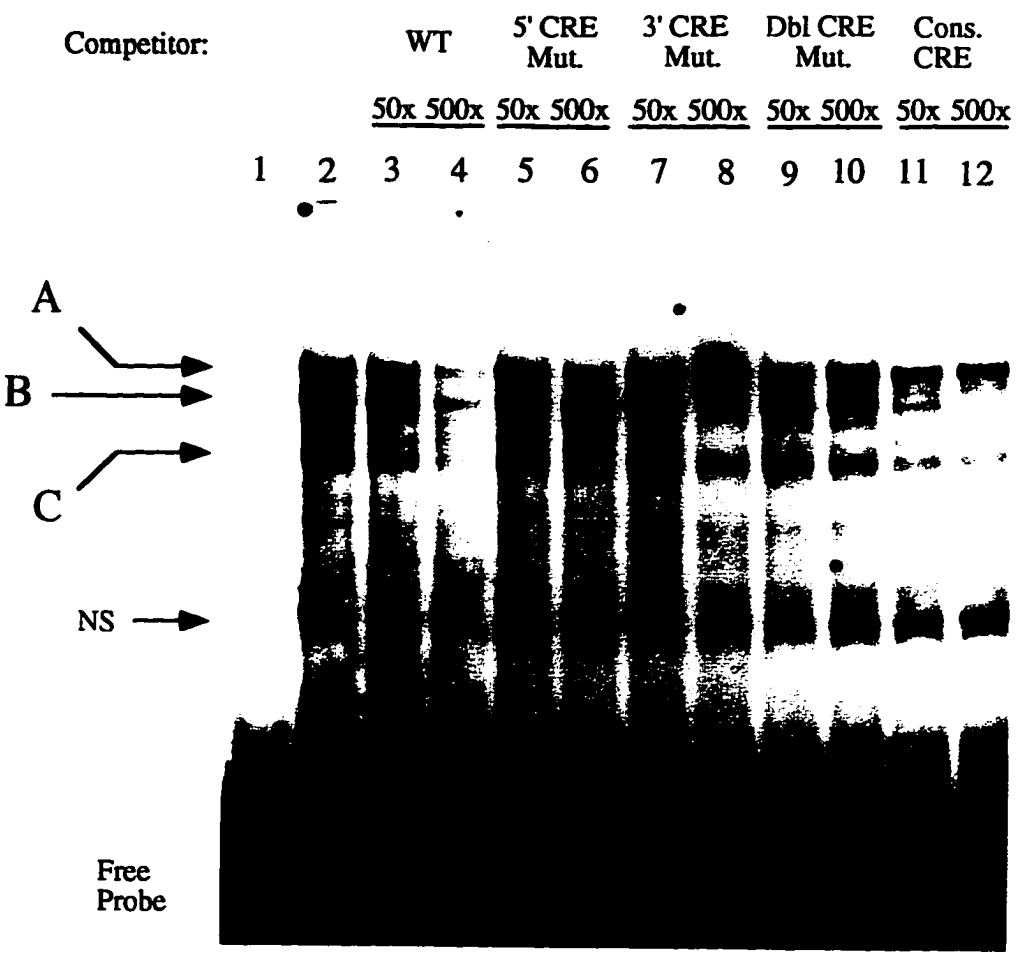


Figure 18: Gel mobility shift assay using 3'-CRE-like element as a probe. *A*, list of competitors used in 3'-CRE competition assay. *B*, gel mobility shift assay was performed using a probe containing the 3'-CRE site of the D_{1A} receptor gene (-212/-205) and LLC-PK_1 cell nuclear extracts. The CRE probe for the gel mobility shift assay was generated by hybridizing single stranded oligonucleotides containing SalI overhangs and filling in the ends with ^{32}P-dGTP, dNTPs and Klenow fragments. Excess probe was incubated in the absence (lane 1) or presence (lanes 2-12) of LLC-PK_1 nuclear extracts. The DNA-protein complexes seen in lane 2 (probe + extract, without competitor) were competed for with increasing concentrations of WT cold probe (10-, 50-, 500-molar excess, lanes 3,4,5), 3'-pt mutant (10-, 50-, 500-molar excess, lanes 6,7,8) or CREB consensus (10-,50-, 500-molar excess, lanes 9,10,11). A, B protein-DNA complexes due to 3' site.

3'-CRE GMSA Oligonucleotides

wt-CRE	5'-AGACGTCACCCGGACGTCCCCC-3'
3' CRE	5'-GCTCACCCGGACGTCCCCCGTCCCT-3'
3'CRE-pt.mut	5'-GCTCACCCGCACGTCCCCCGTCCCT-3'
consensus-CRE	5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3'

Underlined nucleotides identify cis-elements, double underline indicates mutations.

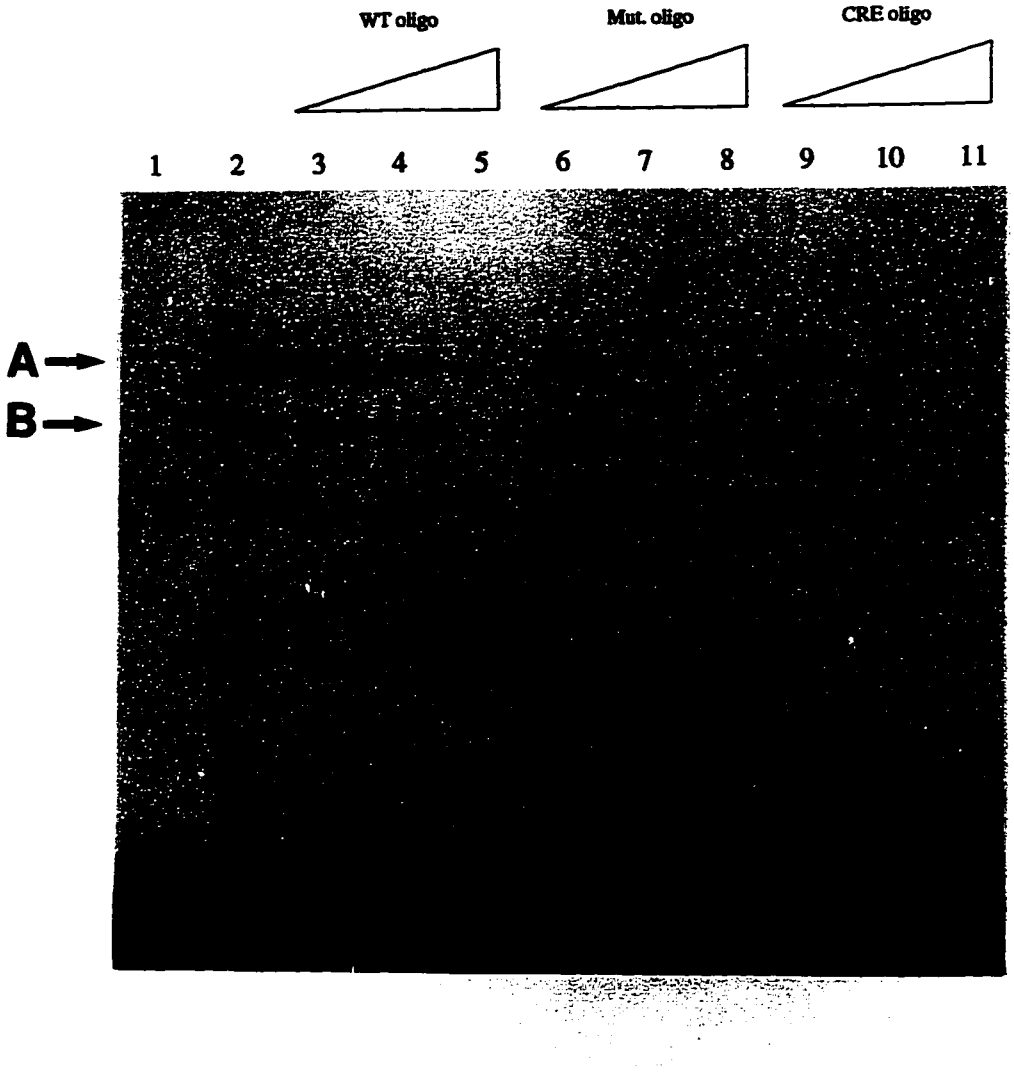


Figure 19: Gel mobility shift assay using a probe to an AP-2 site of the D_{1A} receptor gene (-191/-184) and LLC-PK_1 cell nuclear extracts. The WT (wild type) AP-2 probe for the gel mobility shift assay was generated by hybridizing single stranded oligonucleotides containing SalI overhangs and filling in the ends with ^{32}P-dGTP, dNTPs and Klenow fragments. Excess AP-2 probe was incubated in the absence (lane 1) or presence (lanes 2-7) of LLC-PK_1 nuclear extracts. The DNA-protein complexes seen in lane 2 (probe + extract, without competitor) were competed for with increasing concentrations of WT AP-2 (50-, 500-molar excess, lanes 3,4) or AP-2 consensus sequence (50-, 500-molar excess, lanes 5,6) or a non-specific competitor (500-molar excess, lane 7). The probe contained the following sequence: 5'-TCGACCGTCCCTTTCGCCCGCGGCACCATCCGGGG-3'.

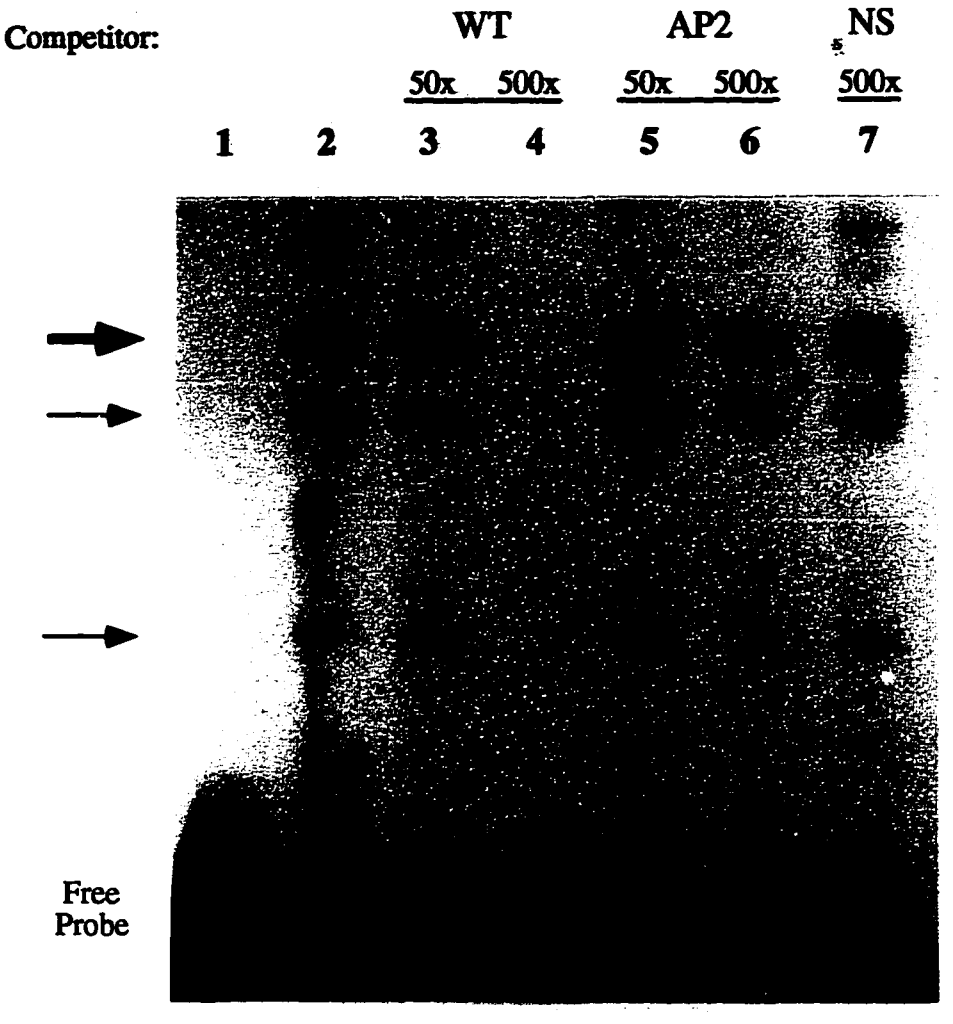


Figure 20: Gel mobility shift assay using a probe to an E-box site of the D_{1A} receptor gene (-168/-163) and LLC-PK_1 cell nuclear extracts. The E-box probe for the gel mobility shift assay was generated by hybridizing single stranded oligonucleotides containing SalI overhangs and filling in the ends with ^{32}P-dGTP, dNTPs and Klenow fragments. The E-box probe was incubated in the absence (lane 1) or presence (lanes 2-5) of LLC-PK_1 nuclear extracts. The DNA-protein complexes seen in lane 2 (probe + extract, without competitor) were competed for with increasing concentrations of cold E-box (50-, 500-molar excess, lanes 3,4) or (NS) a non-specific competitor (500-molar excess, lane 5).
The probe contained the following sequence:
5'-TCGACACCATCCGGGAGAACCACGTGCGGCTGTCGG-3'.

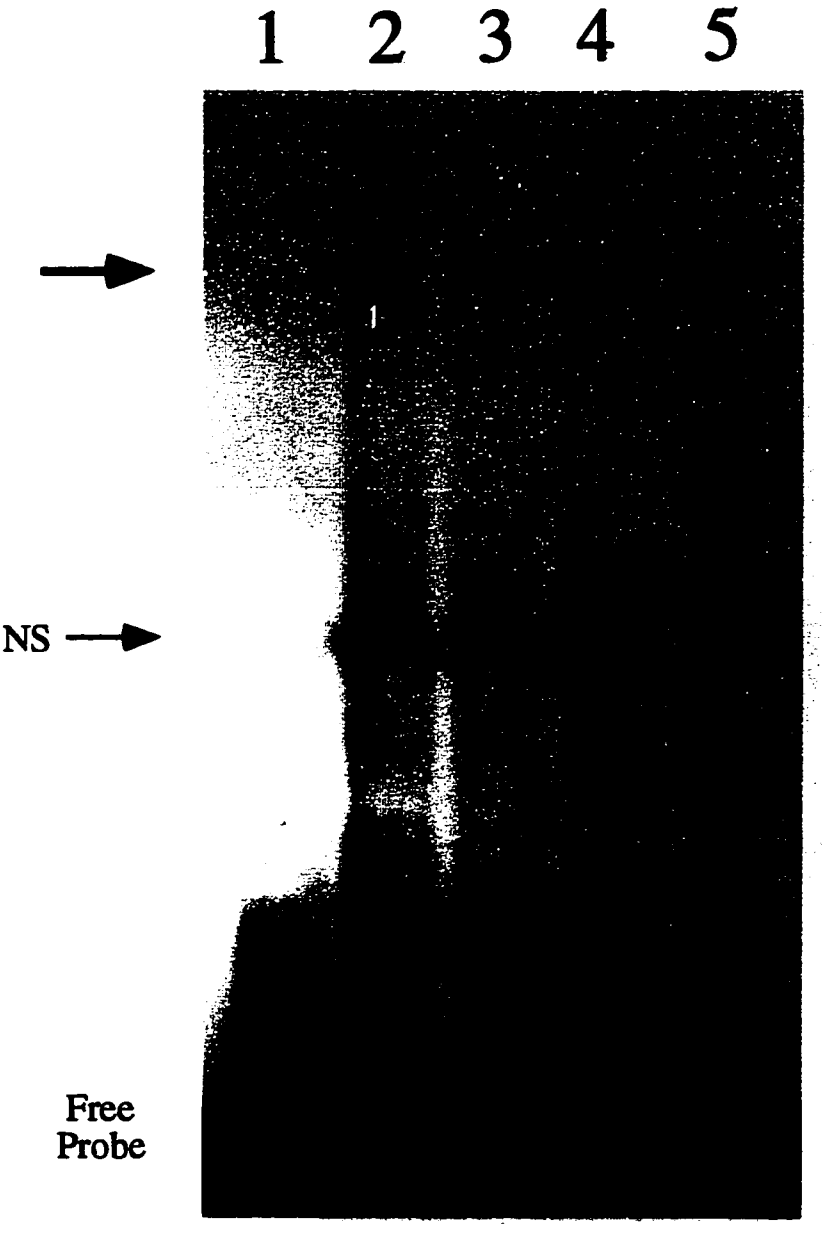


Figure 21: Gel mobility shift assay using Egr-1/Sp1 probe. *A*, list of oligonucleotides used in competition assay. *B*, gel mobility shift assay was performed using a probe containing the overlapping Egr-1/Sp1 site of the D_{1A} receptor gene (-101/-90) and LLC-PK_1 cell nuclear extracts. The WT Egr-1/Sp1 probe for the gel mobility shift assay was generated by hybridizing single stranded oligonucleotides containing SalI overhangs and filling in the ends with ^{32}P-dGTP, dNTPs and Klenow fragments. Excess probe was incubated in the absence (lane 1) or presence (lanes 2-10) of LLC-PK_1 nuclear extracts. The DNA-protein complexes seen in lane 2 (probe + extract, without competitor) were competed for with increasing concentrations of WT cold probe (50-, 500-molar excess, lanes 3,4) or Sp1 consensus sequence (50-, 500-molar excess, lanes 5,6) or consensus sequence (50-, 500-molar excess, lanes 7,8) or a mutated Egr-1 competitor (50-, 500-molar excess, lane 9, 10). Bold arrow indicates the major protein-DNA complex while smaller arrows indicated weaker protein-DNA complexes.

Oligonucleotides for Egr-1 GMSA Competition

wt-Egr-1	5′-TCGACGCCTCCAGGGCAGGGGAGGGGACGCGGGGGCGGGGTGGCTGTGCG-3′
Sp1	5′-ATTCGATCGGGGCGGGGCGAGC-3′
Egr-1 consensus	5′-GGATCCAGCGGGGGCGAGCGGGGGCGA-3′;
Egr-1mut.	5′-GGATCCAGCTAGGGCGAGCTAGGGCGA-3′

Underlined nucleotides identify cis-elements, double underline indicates mutations.

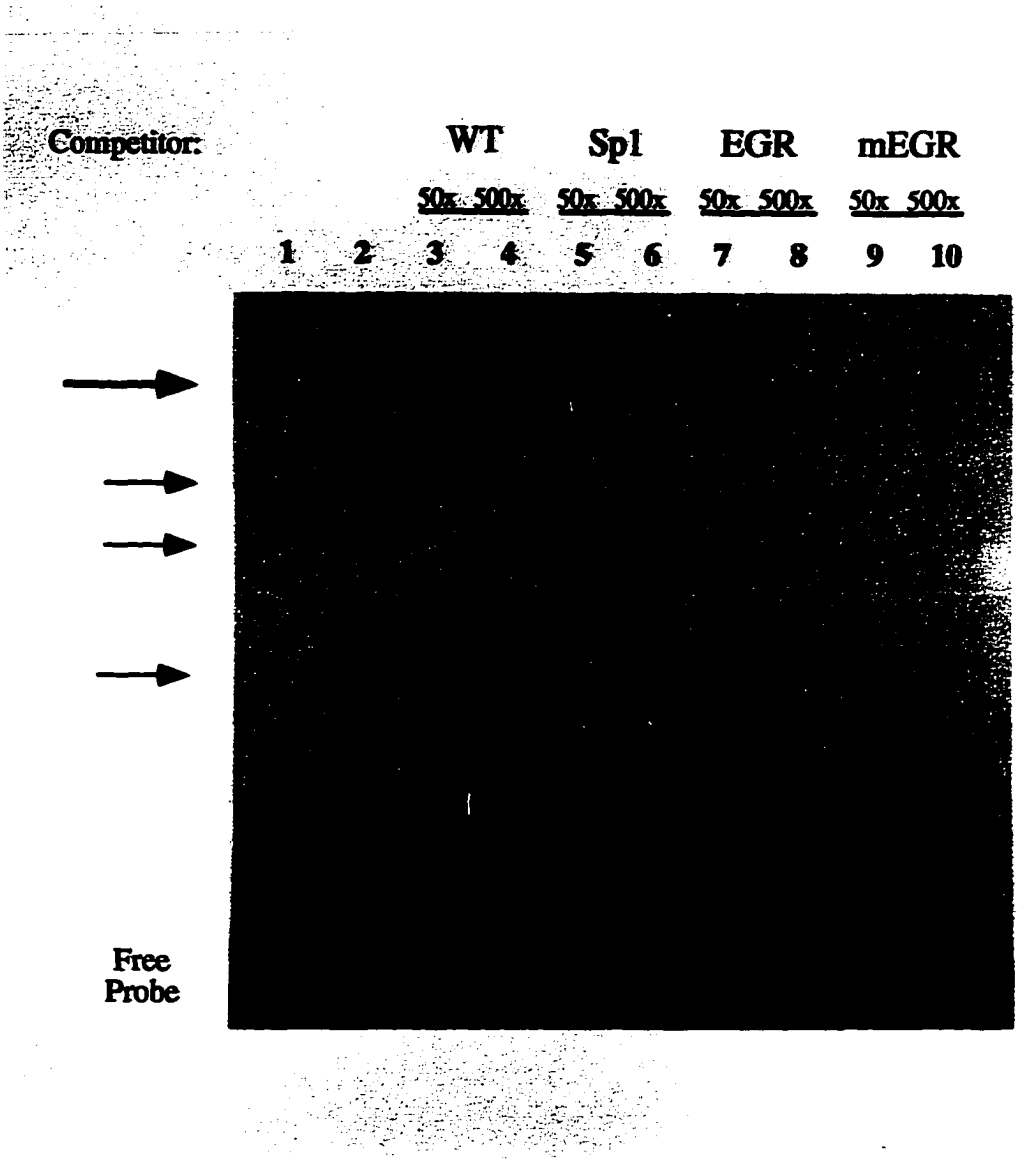


Figure 22: Transcriptional activity of the pCAT-303 following deletion analysis of the 5'-untranslated region and the 5'-UTR intron. On the left is a schematic representation of the various deletions of the D_{1A} receptor 5'-UTR from the previously tested pCAT-303/+960 construct. On the right is the transcriptional activity of each construct following transient transfection into LLC-PK_1 cells, with activity expressed relative to the promoterless pCAT-Basic construct. Values represent the mean $\pm$ S.E.M. of 4-6 separate experiments, each point determined in triplicate.

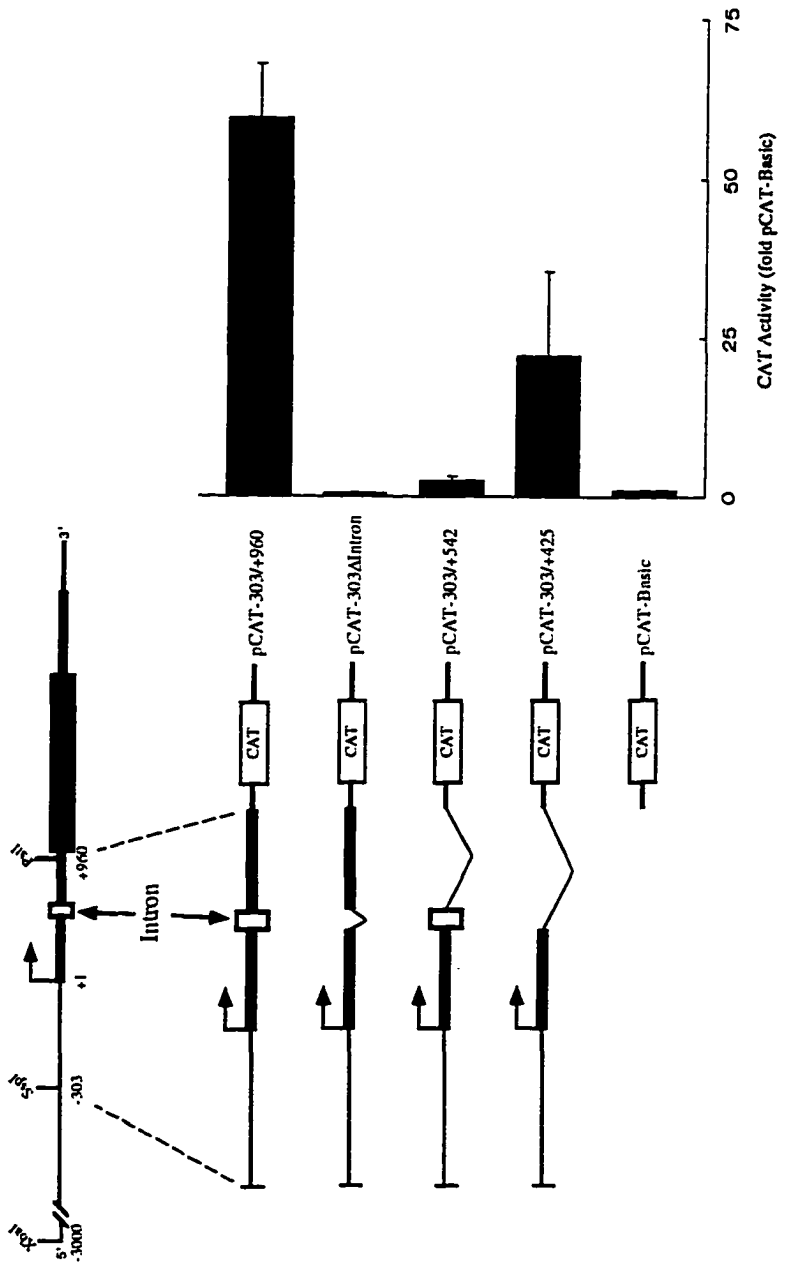


Figure 23: Transcriptional activity of a heterologous promoter/reporter construct with the D_{1A} receptor 5'-UTR intron placed upstream. On the left is a schematic representation of the heterologous thymidine kinase (TK) promoter/CAT construct and the various sequences placed upstream and tested for enhancer or suppresser activity as previously described. The intron sequence was tested alone or in combination with a portion of the D_{1A} receptor gene 5'-flanking region (-303 to -165). On the right is the transcriptional activity of each construct following transient expression in LLC-PK_1 cells, with activity expressed as fold pTKCAT. Values represent the mean $\pm$ S.E.M. of 8 separate experiments, each point determined in triplicate.

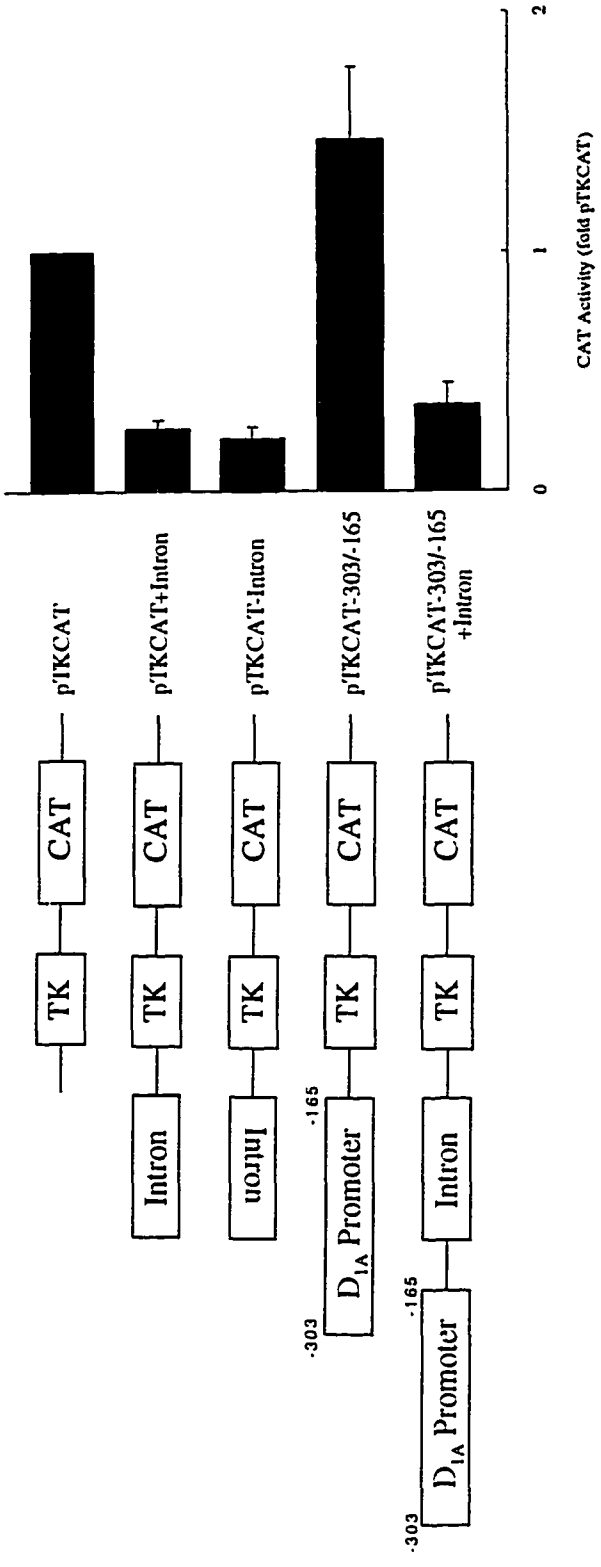


Figure 24: DNase I protection analysis of the D_{1A} receptor gene 5'-UTR intron with LLC-PK_1 cell nuclear extracts. Two sense ^{32}P-labeled probes (+377/+527 on the left and +430/+527 on the right) were incubated with increasing concentration of LLC-PK_1 nuclear extracts (5, 10, 15, 20, 25, 50 μg, lanes 2-7, respectively) treated with DNase I and the resulting DNA fragments were separated on a 10% PAGE denaturing gel. Lane 1 contains probe digested with DNase I without the addition of nuclear extracts. Dideoxy sequencing reactions using the ^{32}P-labeled sense primer were electrophoresed simultaneously.

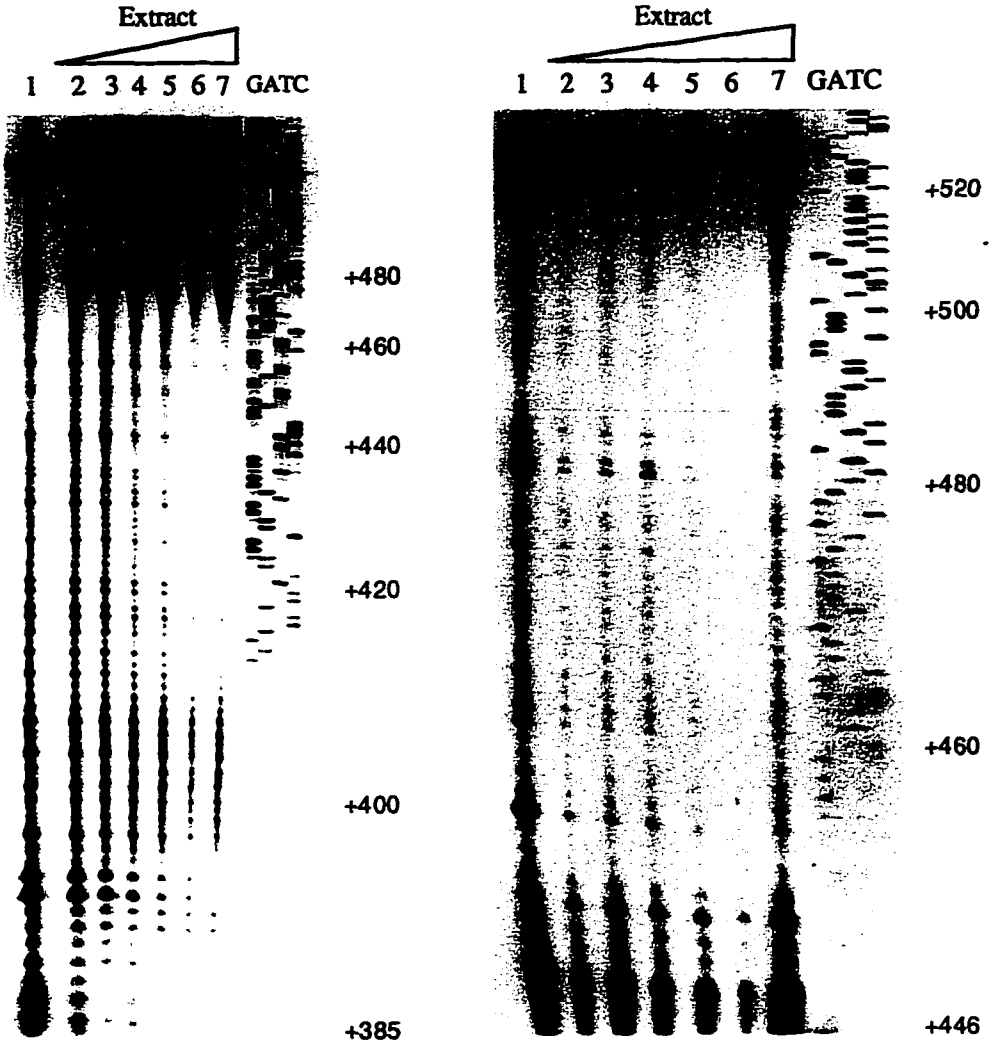


Figure 25: Schematic representation of DNase I protection analysis of the D_{1A} receptor gene intronic sequence. Nucleotide sequence is shown above with putative cis-elements boxed or underlined. Stippled bars indicate protection with LLC-PK_1 nuclear extracts.

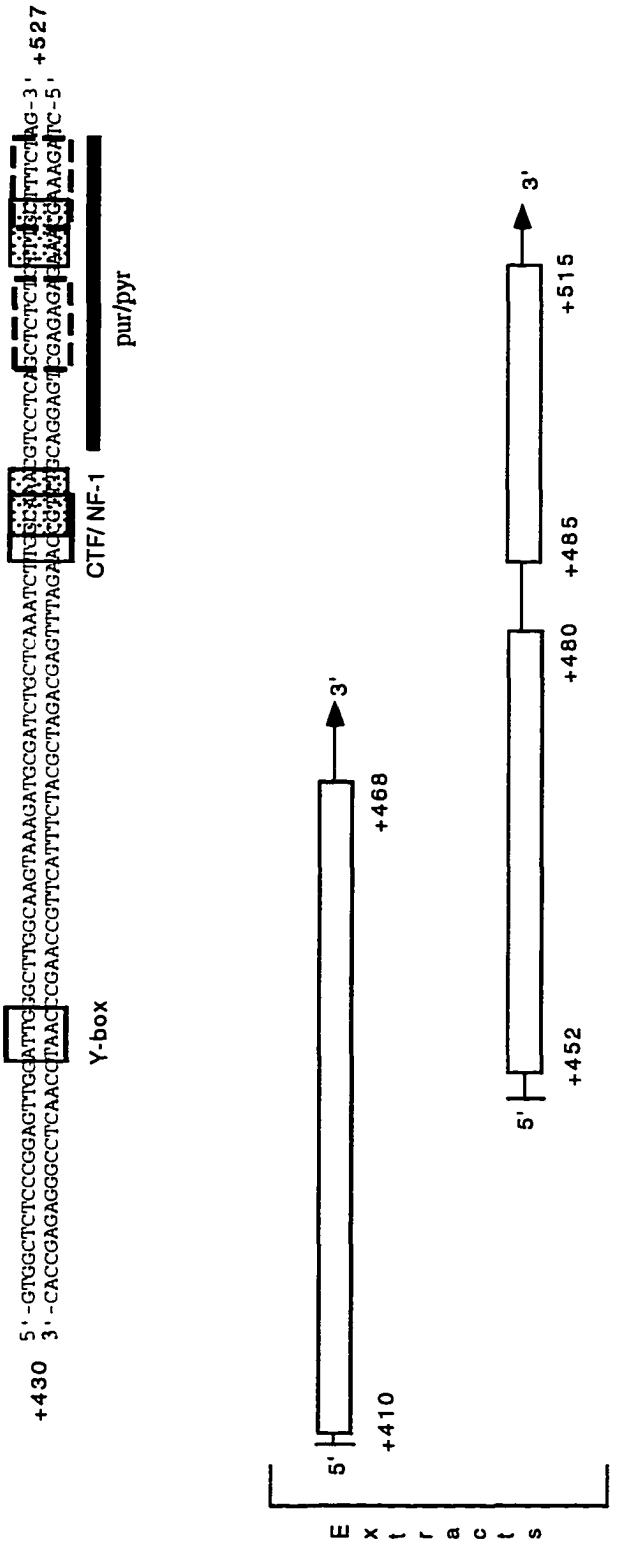
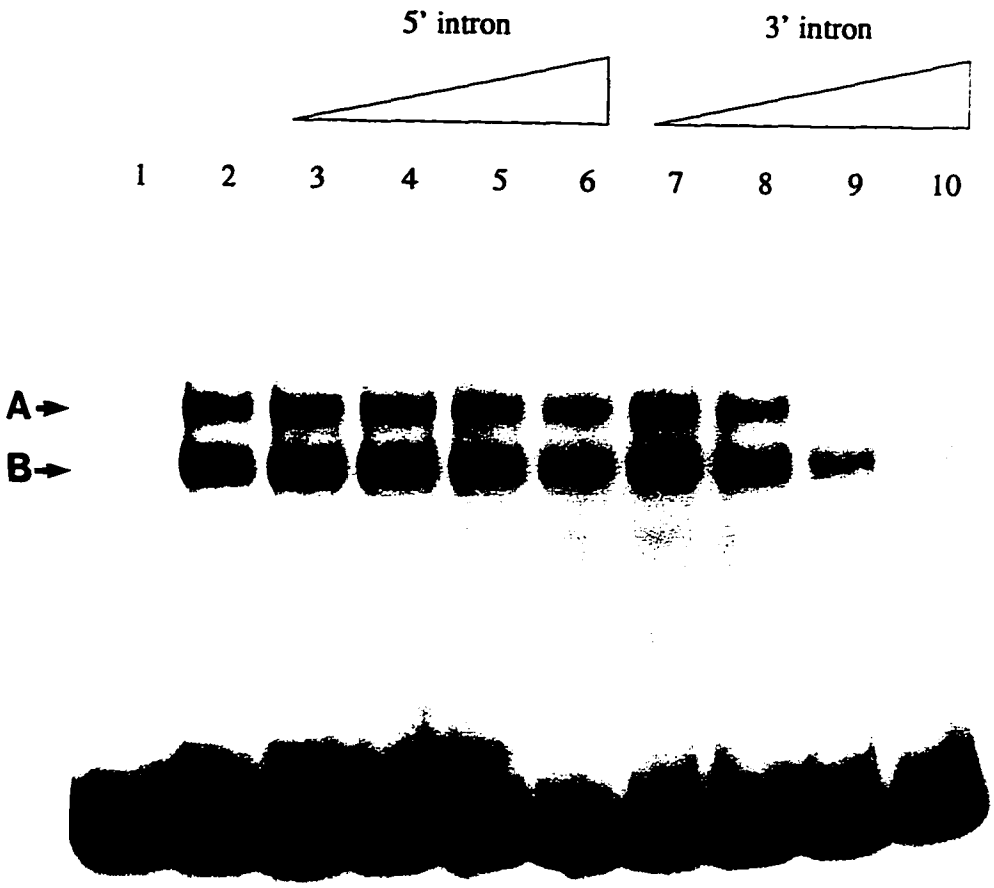


Figure 26: Gel mobility shift assay of the D_{1A} receptor gene 5'-UTR intron with LLC-PK_1 cell nuclear extracts. The D_{1A} receptor intronic probe for the gel mobility shift assay was generated by PCR in the presence of ^{32}P-dGTP and incubated in the absence (lane 1) or presence (lanes 2-10) of LLC-PK_1 nuclear extracts. The DNA-protein complexes seen in lane 2 (probe + extract, without competitor) were competed for with increasing concentrations of unlabeled proximal (+420/+477) or distal (+478/+527) intron double stranded DNA; lanes 3-6: 1-,10-, 50-, 100- fold molar excess of proximal intron; lanes 7-10: 1-, 10-, 50-, 100-fold molar excess of distal intron, respectively.



IX. Chapter 4: Discussion

IX. A. Regulation of D_{1A} Receptor Expression

The molecular mechanisms regulating D_{1A} receptor gene transcription during receptor desensitization are not known. Although Zhou et al. (1992) demonstrated in neuroblastoma cells that the rat D_1 promoter is inducible by cAMP they did not functionally analyze the response elements that may mediate the cAMP-response. Likewise, whether D_{1A} receptor expression is regulated differently in renal vs. neuronal cells has not been addressed. Therefore, the purpose of this study was to elucidate the molecular processes involved in regulating D_{1A} receptor gene expression in renal cells under basal and stimulated conditions. Interestingly, two CRE-like elements are contained between -223 to -205 in the porcine D_{1A} receptor gene. The 3'-CRE-like site is highly conserved in the rat and human genes (Figure 27). In the discussion that follows, it will become evident that these CRE-like sites are essential to both basal and cAMP-inducible transcription of the porcine D_{1A} receptor gene in LLC-PK_1 cells. The activity of these CRE-like sites is context dependent and appears to require assistance from additional trans-acting factors bound to their respective cis-elements in the -303 to -63 region. In addition, evidence of a co-operative interaction between 5'-UTR intronic elements and the 5'-flanking region is described and is necessary for transcriptional activity.

The LLC-PK_1 cell-line is a widely used in vitro model of proximal tubule-like properties and endogenously express the D_{1A} receptor gene (Grenader et al., 1995). D_{1A} receptors in LLC-PK_1 cells undergo a rapid agonist induced desensitization that is time and dose dependent. D_{1A} receptor activation of adenylyl cyclase is decreased by 50% within 15 min exposure to dopamine and D_{1A} receptors are completely desensitized after 30 min dopamine exposure (Grenader, unpublished results). The

expression of many GPCR genes is regulated by the agonists which activate these receptors. Barton and Sibley (1990) demonstrated that de novo protein synthesis is required for recovery of D_1 receptors after long term but not short term agonist exposure, implying that increased transcription (or translation) may participate in the recovery process. Zhou et al. (1992) found that the rat D_1 promoter is inducible by cAMP and proposed that a positive feedback loop may exist, allowing the D_{1A} receptor gene to control its own gene transcription. Examination of the effect of dopamine on D_{1A} receptor mRNA levels in LLC-PK_1 cells revealed that D_{1A} receptor mRNA levels increased 2-fold within 30 min exposure to dopamine and coincided with D_{1A} receptor desensitization. D_{1A} receptor desensitization increased the level of its mRNA. This increase may be the result of increased transcription or increased mRNA stability.

To establish if dopamine affected the stability of the D_{1A} receptor mRNA, LLC-PK_1 cells were treated with a transcriptional inhibitor, actinomycin D, in the absence and presence of dopamine. Dopamine induced a decrease in the apparent half-life of the D_{1A} receptor mRNA. Cycloheximide increased D_{1A} receptor mRNA levels in the presence of dopamine, indicating that the half-life of the D_{1A} receptor mRNA is regulated at least in part by a protein that regulates receptor degradation (Izzo et al., 1994). These results indicate that short term agonist exposure decreases mRNA stability. The increase in D_{1A} receptor mRNA levels during destabilization indicates the increased D_{1A} receptor mRNA levels are due to increased transcription.

The change in D_{1A} mRNA stability is consistent with multiple destabilization sequences in the 3'-UTR (Dearry et al., 1990; Grenader et al., 1995). mRNA stability is associated with mRNA binding proteins that recognize destabilization sequences (AUUUA), termed AREs. These mRNA binding proteins contribute to the mRNA

destabilization process (Stoecklin et al., 1994; Huang et al., 1993; Tholanikunnel et al., 1995). Since destabilization of D_{1A} receptor mRNA requires de novo protein synthesis, this would suggest that perhaps a similar mechanism is involved in regulating the D_{1A} receptor mRNA. Jose et al. (1995) proposed that post-transcriptional regulation of the D_{1A} receptor involves the D_{1A}-BP and the AUUUA binding proteins. They identified a D_{1A} receptor mRNA binding protein (D_{1A}-BP) that binds to a region in the 3'UTR that is 220 bp upstream of the AUUUA region and is therefore distinct from the AUUUA binding protein (Jose et al., 1995). Further characterization of the D_{1A}-BP has not been described.

The apparent half-life of the D_{1A} receptor mRNA can not be accurately obtained from these data, the D_{1A} receptor mRNA half-life appears to decrease following exposure to dopamine. Since the concentration of mRNA depends on the rate of synthesis and the stability, increased transcription must maintain the steady state levels of D_{1A} receptor mRNA. This would imply that the D_{1A} receptors in renal cells participate in autoregulation of their own gene expression in a manner analogous to β_2-AR and the rat D_1 receptor genes. Precise regulation of D_{1A} receptor expression is appears to result from a combination of transcriptional and post-transcriptional control mechanisms.

IX. B. Functional Analysis of the D_{1A} Receptor Gene 5'-Flanking Region

The cloning and characterization of the D_{1A} receptor gene from the LLC-PK_1 cells confirmed that the receptor expressed in this proximal tubule-like cell-line was of

the D_{1A} receptor subtype (Grenader et al., 1995). Comparison of the 5'-flanking sequence of the porcine D_{1A} receptor gene to the rat and human sequences indicated that it is 80% homologous within the first 250 nucleotides upstream of the transcription start site (Grenader et al., 1995) (Figure 4). Closer examination of the porcine D_{1A} receptor gene 5'-flanking and 5'-untranslated regions revealed the presence of multiple putative cis-regulatory elements (Healy and O'Rourke, 1996). Previously, it was shown that the promoter is not bi-directional (Grenader, unpublished observations). Deletion analyses of the porcine D_{1A} receptor gene 5'-flanking sequence indicated that the sequence downstream from -63 (pCAT-63) appears to contain the core promoter since it was sufficient for minimal CAT activity (Figure 8). Progressively extending the 5'-flanking sequence to -303 gradually increased CAT activity (pCAT-165, pCAT-206, pCAT-303) above that of the core promoter. The highest level of transcriptional activity was attained with -303/+962 (pCAT-303), almost 60-fold over the negative control, pBasic. A negative cis-element apparently lies upstream of -303 and suppresses the activity of the -303/+962 sequence since pCAT-3000 and pCAT-663 exhibited CAT activities which were significantly lower than pCAT-303. The transcriptional activity of pCAT-63 CAT was approximately 4-fold lower compared to pCAT-303. These data suggest that an activator region is present between -303 to -63, however, its boundaries may not be precisely determined from these data.

Minowa et al. (1993) previously identified two activating regions within the human D_{1A} receptor promoter expressed in mouse neuroblastoma cells which lie 150 bp upstream of the transcription start site. The corresponding sequence in the porcine D_{1A} receptor gene is contained within -171 to -90 (Healy and O'Rourke, 1996) and is highly conserved (>90%) (Figure 4). Therefore it seemed likely that this domain may participate in regulation of the porcine D_{1A} receptor gene. Deletion of the -165 to -63 sequence (pCAT-303(Δ-165/-63) reduced CAT activity (relative to pCAT-303) to levels

that were similar to the activity of the core promoter (pCAT-63). Results from pCAT-303, pCAT-206, pCAT-165 and pCAT-303(Δ-165/-63) CAT indicate that elements contained within -303 to -63 are all essential for maximal transcriptional activity of the D_{1A} receptor gene in LLC-PK_1 cells. The implication is that interactions may occur between trans-acting factors bound to their respective cis-regulatory elements within -303 to -165 and -165 to -63 in order to achieve high transcriptional activity since neither region can induce transcriptional activity equivalent to pCAT-303 in the absence of the other. These results support the hypothesis that co-operative interactions occur between these elements to induce the observed 4-fold increase in transcription exhibited by pCAT-303 (relative to pCAT-63).

Comparison of the CAT activity of pCAT-303 relative to pCAT-165 indicated that the 2-3 fold increase in CAT activity exhibited by pCAT-303 may be due to the presence of positive elements that reside upstream of -165. The region from -303 to -165 was found to confer renal cell specificity to the pCAT-D_{1A} constructs (Grenader, unpublished observations). Minowa et al. (1993) extensively investigated the -165 to -63 region of the human D_{1A} promoter in neuroblastoma cells and so to further characterize the porcine D_{1A} promoter -303/-165 sequence , it was placed in front of a heterologous promoter. Insertion of -303 to -165 (in the sense orientation) upstream of a heterologous promoter (TK) increased CAT reporter activity 50% (Figure 13). In contrast, the antisense orientation decreased CAT activity 70% relative to pTKCAT. The ability of -303 to -165 to increase transcription, albeit weakly, in the 5'-3' orientation but decrease transcription in the 3'-5' orientation supports the contention that this region contains a positive and negative element which influence transcription. Thus, the -303 to -165 domain is not an enhancer but contains elements which are important for transcriptional activation. Multiple copies (2 or 6) of the -303 to -165

sequence did not greatly increase transcriptional activity (only 70%) of the reporter gene as would be expected if it possessed enhancer-like properties. These findings indicate that additional cis-regulatory elements are required for efficient transcriptional activation of the D_{1A} receptor gene or that a negative element within -303 to -165 is dominant in this context.

Activation of D_{1A} receptors in LLC-PK_1 cells by dopamine leads to an accumulation of cAMP (Grenader and Healy, 1991). Increased cAMP levels trigger the D_{1A} receptor desensitization mechanisms (Grenader, unpublished results) and increases D_{1A} receptor gene transcription within 30 min. The profile exhibited by the porcine D_{1A} receptor mRNA is consistent with cAMP-stimulation of transcriptional events in that cAMP-induced transcription and CREB phosphorylation normally peak at 30 min, and decline gradually (4-6 hr) (Armstrong et al., 1995). The effect of forskolin on the activity of the pCAT deletion constructs (pCAT-3000 to pCAT-63) was examined. A 2-fold increase in CAT activity was observed for pCAT-303 when transfected cells (LLC-PK_1) were exposed to forskolin. The fact that constructs containing sequences downstream of -206 (pCAT-206, pCAT-165, pCAT-63) did not exhibit increased CAT activity when challenged with forskolin suggests that the CRE-like elements mediate cAMP-responsiveness. In fact, pCAT-206 deletes both CRE-like elements and led to the hypothesis that these sites are necessary for cAMP responsiveness. Evidence for this hypothesis was provided by a construct containing single base substitution at -211, G->C, in pCAT-303. The point mutation eliminated the response to forskolin while simultaneously reducing basal levels of CAT activity. This dramatic effect demonstrates that the 3'-CRE is important for both basal and cAMP-mediated expression of the D_{1A} receptor gene. These results are consistent with other genes whose basal activity is similarly regulated through a CRE (Kim et al., 1993, Osawa et

al., 1996). It is interesting to note that the 5'-CRE and 4 AP-2 sites were not sufficient to mediate cAMP-responsiveness.

The speculation that the porcine CRE-like elements were essential to transcriptional activity of the D_{1A} receptor gene originated from the observation that the D_{1A} receptor mRNA is regulated by dopamine. Agonist binding to dopamine receptors stimulates the production of cAMP, subsequently activating protein kinase A. Translocation of protein kinase A to the nucleus enables it to phosporylate CREB transcription factors. Zhou et al. (1992) found transcription of the rat D_1 promoter was regulated by 8-Br-cAMP but the element responsible for the cAMP responsiveness was not identified (Zhou et al., 1992). The element proposed by Zhou et al. (1992) was TGGCGCCA (-29/-22) but the rat D_1 promoter contains an additional element at -240/-233 which is very similar to the porcine 3'-CRE like element (Healy and O'Rourke, 1996). In fact, this element is conserved in the human D_1 promoter as well (-197/-190) (Figure 27). A consensus site was determined, RGRCGTCC (where R is a purine), and used to search the rat and human D_1 receptor gene sequences. The rat promoter contained only the single CRE-like site (-240/-233) described above and the human D_1 promoter contained an additional CRE-like element at position -441/-434 which is identical to the porcine 3'-CRE. Since the functional significance of the CRE-like elements were not previously determined, deletion analyses were performed to examine the possibility that these CRE-like elements are involved in D_{1A} receptor gene transcription. Disruption of the 3'-CRE site in pCAT-206 resulted in a 50% decrease in CAT activity relative to pCAT-303, implying that this site may be important in D_{1A} receptor gene regulation (Figure 8). Similarly, deletion of the 3' CRE from -303 to -165 (pTKCAT-303/-165) in front of a heterologous promoter (TK) suppressed reporter activity (figure 13), confirming the importance of the 3'-CRE site in transcriptional activation of the D_{1A} receptor gene. These results also demonstrate the inability of the

5'-CRE to independently promote transcriptional activation in this context. Introduction of a point mutation in -303/+962 at -211, [pCAT-303(-211 G->C)] confirmed that the 3'-CRE is essential for basal activity of the D_{1A} receptor gene and that the 5'-CRE is not sufficient for transcriptional activation in this context. This is consistent with the observations of others (Fink et al., 1988; Spiro et al., 1995) that mutating or deleting a CRE in a promoter containing multiple CREs alters the response to second messengers and transcriptional activators. Another interpretation of these results is that the two D_{1A} receptor -CRE sites may not act in concert as transcriptional activators. One site may be primarily involved in transcriptional repression while the other mediates activation or the activity of each site may depend on the surrounding context (Goraya et al., 1995; Benbrook and Jones, 1994).

Closer examination of the wild type CRE in front of a heterologous promoter (pTKCAT 5'-CRE/3'-CRE) indicated that it increased CAT activity nearly 3-fold over TKCAT in an orientation independent manner. The activity of the wild type D_{1A} receptor CRE-like element (pTKCAT 5'-CRE/3'-CRE) is higher than -303/-165 (pTKCAT-303/-165) indicating that an element within -303/-165 may suppress the CRE activity. This is consistent with the results of pTKCAT-165/-303 (antisense) which suggested a negative element is present in this region which is dominant in the antisense orientation. A similar observation was reported for the CRE in the calspermin gene in which inversion of 111bp sequence containing the CRE resulted in a loss of CREMτ stimulation (Sun et al., 1995). This may reflect the stereospecificity of the transcription initiation complex (Oelgeshlager et al., 1996).

The inability of pTKCAT -303/-165 and pTKCAT 5'/3'-CRE to increase transcriptional activity in response to forskolin indicates that the CRE-like elements

require additional cis-elements in the context of a heterologous promoter to mediate cAMP-responsiveness. CRE elements often mediate cAMP responsiveness through interactions with additional cis-elements (Medcalf et al., 1990; Roesler et al., 1995; Roesler et al., 1996; Muro et al., 1992). A similar observation was reported for the somatostatin CRE (Montimony et al., 1986) which when placed upstream of a heterologous promoter indicated that this 10 bp CRE alone was not cAMP responsive. However, in contrast to the porcine D_{1A} receptor CRE, the somatostatin CRE became responsive when additional 5'-flanking sequences were added. Presumably, the inability of the somatostatin 10 bp CRE and the D_{1A} receptor CRE to mediate cAMP-responsiveness in front of a heterologous promoter is due to required interactions with other cis-elements. Although a CRE-like element (-29/-22) was assumed to mediate cAMP-responsiveness within the rat D_1 promoter (Zhou et al., 1992) characterization of this CRE-like element was not demonstrated, therefore whether it acts alone or in concert with additional cis-elements is not known.

CREB/ATF factors are members of the bZip transcription factor family and are characterized by a motif more commonly known as the leucine zipper. In general, CREB-ATF factors homodimerize or heterodimerize with other bZip transcription factors and bind to the CRE consensus site. Footprinting assays with LLC-PK_1 extracts indicated that both the 5` and 3`- CRE-like sites (-223/-216, -212/-205) were protected. However, the inability of purified CREB-1 peptide to the DNA binding domain to protect either of these sites suggests that a novel protein may be involved in these interactions. A gel mobility shift assay using a probe containing the 5'/3'-CRE-like sites demonstrated that each of these sites interact with distinct protein complexes. The slowest migrating band appears to be due to specific protein interactions with the 3`-site where as the middle band appears to arise from nuclear protein binding to the 5`-CRE site. The fastest migrating band appears to be sensitive to competition with the 5`

CRE, 3' CRE and the consensus site and suggests that a heterodimer may form this complex. It is possible that this lower band may represent a new site created from sequences from both the 5' and 3' CRE-like sites as was shown for the proenkephalin gene (Spiro et al., 1995). The consensus CRE oligonucleotide could not compete well for the top band, indicating that perhaps a protein distinct from CREB may recognize the 3'-CRE-like site. No band was detected in the GMSA which was sensitive to competition with an oligonucleotide containing a mutation in both CRE sites. Analysis of the 3' CRE element by GMSA demonstrated that the 3' CRE competed effectively for protein-DNA complexes. The affinity of the point mutant oligonucleotide appears to be lower than the wild type. In the absence of the 5'-CRE site, the consensus and wild type exhibited similar binding affinities. This may reflect the different conformations allowed in the absence of the 5'-CRE site as was demonstrated for the proenkephalin gene (Spiro et al., 1995). Although the 3'-CRE can function independently as a binding site, it can not by itself support efficient transcription (Xu, preliminary results). Since transcriptional activation requires the continuous presence of the activating domain in order to maintain transcription (Ho et al., 1996) it is conceivable that any alteration in affinity will destabilize the transcription activation complex (Williams et al., 1993; Keller et al., 1995; Kim et al., 1995). Preliminary supershift experiments (Lu, unpublished results) using CREB-1 and CREM-1 antibodies suggest that CREB-1 binds to the 5'-CRE site and CREM-1 binds to the 3'-CRE site. Goraya et al. (1995) previously demonstrated by that CREM may repress or activate transcription depending on the context of the CRE and the abundance of CREM isoforms. This may explain why the 5'-CRE can not act independently in the absence of the 3'-CRE in the context of the D_{1A} receptor 5'-flanking sequence (pCAT-303 (-211 G->C), pTKCAT Δ3'-CRE) yet is a strong activator of transcription in pTKCAT in the absence of the 3'-CRE and the porcine 5'-flanking sequence (Xu, unpublished observations).

IX. C. Characterization of Additional Cis-Elements in the D_{1A} Promoter

Deletion analysis indicated that the sequence from -303 to -63 is important to D_{1A} receptor gene regulation. This region contains many consensus sites for transcription factors which may potentially regulate D_{1A} receptor gene transcription. Footprinting assays using LLC-PK_1 extracts indicated that the porcine D_{1A} receptor gene was protected extensively. Numerous regulatory factors bound to long and multiple stretches between -266 and -18 (Figure 9, 10, 11). DNase I protection was observed within areas containing several consensus sites including several AP-2, 5'- and 3'-CRE, E-box, Egr1/Sp1, GC box and a novel site at CCTCTCTG (-149/-142). A comparison of DNase I protection with LLC-PK_1 and SK-N-MC extracts indicated a similar protection pattern. The stronger protection pattern observed with the SK-N-MC extracts is probably due to higher concentrations of transcription factors in these extracts compared to the LLC-PK_1 extracts. The novel site (-149/-142) was protected by both neuronal and renal cell extracts and therefore it is not likely to bind a tissue-specific factor. An antisense probe incubated with LLC-PK_1 extracts confirmed that the E-box (-168/-163) and the novel site CCTCTCTG (-149/-142) were protected but neither of the CRE-like sites were protected. Protein interactions with the CRE-like sites on the antisense strand may be sterically hindered by the conformation of the DNA.

The Sp1 factor is required to maintain basal transcription in GC rich promoters lacking a TATA box, like the D_{1A} receptor promoter (Dynan, 1986). Recombinant purified SP1 protein bound to the GC box (-127/-122) and the overlapping Egr-1/SP1 site (-101/-90) in DNase I footprinting analysis of the -240/-40 region. Protection of these sites with LLC-PK_1 extracts suggests that Sp1 interactions at these sites are

involved in D_{1A} receptor gene transcription. The region of the Sp1 protection corresponds to the activating domains (Act I and II) of the human D_{1A} receptor gene. Although Minowa et al. (1993) previously reported this region did not bind purified Sp1 in their footprinting and UV-cross linking assays, they demonstrated that a protein antigenically related to Sp1 bound to these sights in supershift assays.

AP-2 is a transcription factor which is inducible by phorbol esters or cAMP (Medcalf et al., 1990; Roesler et al., 1988). DNase I footprinting analysis of the porcine D_{1A} receptor promoter region using purified recombinant AP-2 protein indicated that AP-2 bound to consensus sites within the -240/-40 region. Four AP-2 sites were protected in this region (-236/-229, -194/-184,-117/-110, -80/-73) and one hypersensitive site occurred at -112/-104. Of the protected AP-2 sites, one included an overlapping Sp1/AP-2 site (-236/-229) which did not bind purified Sp1 protein. GMSA confirmed that LLC-PK_1 extracts interacted specifically with an AP-2 probe (-194/-184). The two specific protein-DNA complexes that formed may be attributed to the existence of two members of the AP-2 family (AP-2 and AP-2B, an alternatively spliced isoform which represses transcription). Competition assays indicated that although the AP-2 protein-DNA complexes were effectively competed for with cold probe, the AP-2 consensus oligonucleotide could not compete as well. The fact that the AP-2 consensus oligonucleotide did not compete as effectively may suggest the importance of the 5'-flanking sequence in binding trans-acting factors.

There appeared to be a discrepancy between the protection exhibited by NS-20Y extracts and AP-2 protein in DNase I analysis of the human D_1 promoter (Minowa, 1993). AP-2 bound to 2 of the 3 proposed sites (-106/-101 and -66/-59) which are conserved in the human D_{1A} receptor gene (Figure 4). The porcine AP-2 sites were protected in a similar manner with LLC-PK_1 extracts and purified AP-2. However, the

third AP-2 site (-86/-77) examined by Minowa et al. (1993) did not bind AP-2 or Sp1 and they proposed a novel factor bound to this site. However, purified Sp1 did bind to the corresponding site (-101/-90) in the porcine D_{1A} receptor promoter. Careful examination of this region (Figure 4) indicated that the site is conserved between species and represents an overlapping Egr-1/Sp1 site (-101/-90). It is possible that Egr-1 is the factor Minowa et al. (1993) observed at this site and not a novel factor.

Egr-1 (early growth response factor) is an important mediator of transcription induced by mitogens and its ability to induce transcription of early response genes is central to regulating cell proliferation (Cao et al., 1993). Increases in D_{1A} receptor mRNA levels were observed following exposure to serum (Grenader, unpublished observations). Serum is known to contain a variety of growth factors that induce transcription of early response genes, including Egr-1. Egr-1 is ubiquitously expressed transcription factor and its presence in the LLC-PK_1 cell line has been established (Kinane et al., 1994). Therefore, it is possible that the serum induced increases in D_{1A} receptor gene transcription may be mediated by Egr-1. The Egr-1 consensus site is also recognized by the Wilm's tumor suppresser gene product, WT_1 (Blackwell and Weinberg, 1990; Madden et al., 1991) which is expressed in a restrictive manner but present in the urogenital system (Pelletier et al., 1991). DNase I protection was observed at the Egr-1 /Sp1 site (-101/-90) with LLC-PK_1 nuclear extracts. A gel mobility shift assay using a probe containing the Egr-1/Sp1 site (-107/-90) generated a complex pattern of protein-DNA complexes. Although immediately upstream of the Egr-1/Sp1 site is an AP-2 consensus site (-111/-104), a previous competition assay (data not shown) demonstrated that an AP-2 consensus oligonucleotide sequence did not compete for any of the observed DNA-protein complexes. Egr-1 and Sp1 oligonucleotides were effective competitors for the protein-DNA complexes but not as efficient as the unlabeled probe suggesting the flanking sequence may contribute to

specificity of protein binding. The Sp1 oligonucleotide competed more effectively than the Egr-1 consensus sequence. This difference may be due to affinity differences or to differences in the relative abundance of Sp1 and Egr-1. Egr-1 is an inducible factor that is temporally expressed in LLC-PK_1 cells (Kinane et al., 1994) whereas Sp1 is constitutively expressed. Although AP-2 bound this area in footprinting assays, the inability for the AP-2 consensus oligonucleotide to compete is somewhat reminiscent of the observations of Minowa et al. (1993). This apparent discrepancy may be explained by the fact that nuclear extracts contain multiple transacting factors which must compete with each other for binding sites compared to the use of recombinant purified protein. Therefore, due to their close proximity, AP-2 may be physically incapable of binding to its site (-111/-104) upstream of the Egr-1/Sp1 site when other transacting factors are previously bound.

Overlapping consensus sites are commonly involved in regulating gene expression (Xu et al., 1993; Shingu et al., 1994; Cao et al., 1993; Mitchell and Tijan, 1989; Ebert and Wong, 1995; Harrington et al., 1993; Diamond et al., 1990). D_{1A} receptor gene regulation through this site appears to be complex. DNase I analysis and competition assays indicated that Sp1 and LLC-PK_1 extracts bound to an overlapping Egr-1/Sp1 site (-101/-90). While the results suggest that Egr-1 and Sp1 may bind to this site, they are by no means conclusive. WT_1 may also contribute to D_{1A} receptor gene regulation at this Egr-1/Sp1 site (-101/-90) since WT1 is expressed in LLC-PK_1 cells (Kinane et al., 1994). Physical competition for binding sites and the relative abundance of nuclear factors may influence (Diamond et al., 1990) which trans-acting factor is bound to this Egr-1/Sp1 site at any given time. Protection of a cis-regulatory element by a transcription factor does not indicate apriori whether transcription is activated or repressed in response to the bound factor. Egr-1, an inducible factor, was shown to inhibit its own gene expression through an overlapping Sp1/EGR-1 site while

Sp1, a basal factor, stimulates its transcription (Cao et al., 1993). It is plausible that the D_{1A} receptor gene expression may be differentially regulated through the overlapping Egr-1/Sp1 site in a manner analogous to phenylethanolamine N-methyltransferase (PNMT) gene (Ebert, 1995) where selective regulation of Sp1, Egr-1 or WT_1 through different stimuli may control D_{1A} receptor gene expression.

The E-box consensus sequence, CACGTG, is recognized by transcription factors from the basic helix-loop-helix (bHLH) transcription factor family such as myc, mad and max (Ayer et al., 1993; Fisher et al., 1993). This site is conserved between the porcine and human D_{1A} receptor genes (Figure 4, Healy and O'Rourke, 1996). DNase I footprinting analysis and gel mobility shift assay indicated that a probe to the porcine D_{1A} receptor gene bound LLC-PK_1 nuclear extracts in the E-box region. A characteristic feature of these factors is the presence of a basic rich domain followed by two amphiphilic α helices connected by a variable loop. (Blackwell and Weintraub, 1990; Anthony-Cahill et al., 1992). The bHLH transcription factors form dimers which preferentially bind DNA sequences. Half-site recognition adds to the complexity of gene regulation by allowing for specific responses to a diverse array of stimuli through the formation of various heterodimers.

IX. D. Role of 5'-UTR in D_{1A} Receptor Gene Expression

Although the coding regions of a considerable number of G-protein coupled receptor genes are uninterrupted by introns, many of these G-protein coupled receptor genes contain a 5'-UTR intron. In addition, a significant number of G-protein coupled receptor genes whose coding regions are encoded by multiple exons and contain 5'-UTR introns as well. Therefore, the presence of an intronic sequence in the 5'-UTR of

a GPCR gene appears to be independent of the gene organization (Table I). Among the G-protein coupled receptor genes possessing a 5'-UTR intron and having coding regions uninterrupted by an intron are the D_{1A}, D_{1B}, platelet activating factor, angiotensin II type I and type 1, and muscarinic receptor genes (Grenader et al., 1995; Beischlag et al., 1995; Chase et al., 1993; Pang et al., 1995; Meida et al., 1996; Bonner et al., 1987; Liao et al., 1989; Zhou et al., 1992; Minowa et al., 1993; Takeuchi et al., 1993; Takayangi et al., 1994; Martin et al., 1995). Examples of receptors with introns in their coding and 5'-UTR are adenosine 1, bradykinin 2, dopamine 2, endothelin 1A, thromboxane A2, oxytocin and neuropeptide Y (Ren et al., 1994; Pesquero et al., 1994; O'Malley et al., 1990; Minowa et al., 1992; Hosada et al., 1992; Nusing et al., 1993; Rozen et al., 1995; Ball et al., 1995). Interestingly, most of these GPCR genes containing a 5'-UTR intron are TATA-less and the presence of the 5'-UTR intron appears to be independent of the second messenger system. The significance of an intronic sequence within the 5' untranslated region of G-protein coupled receptor genes has not been previously demonstrated but intronic sequences have been shown to influence transcription of WT_1, immunoglobin μ, calspermin, endotoxin ribonuclease reductase and thyroid hormone receptor α genes (Malick et al., 1995; Grosscheld and Baltimore, 1985; Sun et al., 1995; Tiffany et al., 1996; Lazar et al., 1995). It is likely that a common regulatory mechanism is responsible for controlling expression of G-protein coupled receptor genes with a 5'-UTR intron.

The first evidence that the 5'-UTR may be relevant to transcription of the D_{1A} receptor gene was provided by 2 constructs (pCAT-303/+38, pCAT+53) designed to further delineate the porcine D_{1A} receptor activating sequence to regions upstream (-303/+38) or downstream (+53/+962) of the transcription start site. Both pCAT-303/+38 and pCAT+53 had low activity indicating that neither region alone can

function as an independent promoter. The inability of this (pCAT-303/+38) construct to independently promote transcription demonstrated that -303 to +38 was necessary but not sufficient for transcriptional activation. It also indicated that elements in the 5'-UTR are required for efficient transcriptional activation (Figure 8). The fact that +53/+962 had low activity indicated that the intron did not contain an alternative promoter downstream of the major transcription start site. The inability of pCAT+53 to direct transcription is consistent with the hypothesis that the core promoter lies near -63. Therefore, a more detailed deletion analysis of the 5'- UTR was performed and provided evidence that the intronic element could be positive or negative. Differences in the transcriptional activity of pCAT-303 ΔIntron relative to pCAT-303 suggested that the intron contains enhancer-like elements. In addition, deletion of the distal 5'-untranslated region (pCAT-303/+542) abolished CAT activity relative the pCAT-303 and suggested that the distal 5'-UTR may contain a positive element. On the other hand, simultaneous deletion of the intron and the distal 5'- UTR (pCAT-303/+425) resulted in a moderate increase in CAT activity, 22-fold over pBasic. Although the reporter activity of pCAT-303/+425 was 3-fold lower than pCAT-303, the activity was higher than pCAT-303/+542. This implies that the intron may contain negative elements which suppress transcriptional activity. However, deletion of almost the entire 5'-untranslated region (pCAT-303/+38) resulted in complete loss of CAT activity which suggests that a positive element within +38 to +425 is necessary for transcription. These results taken together indicate that an interaction between upstream (-303/+38) and downstream (+53/+962) cis-elements are required to activate transcription of the D_{1A} receptor gene in renal cells.

The 5'UTR of the D_{1A} receptor transcript is unusual in that it contains several AUG codons upstream of the receptor cistron (figure 3). Although only 10% of eukaryotic genes possess 5'-uORF, a considerable number of GPCR genes have 5'-

uORF (Bonner et al. 1987) including all of the GPCR listed in table I. When such upstream open reading frames are translated efficiently, the resulting peptides may inhibit translation of the downstream open reading frame (Parola and Kobilka, 1994) and may contribute to mRNA destabilization (Oliveria and McCarthy, 1995). Combining secondary structure (stem loops) with small open reading frames inhibits translation more efficiently than either alone (Kozak, 1990). Although two possible stable hairpin loops exist in D_{1A} receptor gene 5'-non-coding region 14 and 17 nucleotides downstream of the purported translation initiation site, +1 (Grenader et al., 1995), these structures appear to lie too far downstream to allow translation to initiate effectively from the upstream AUG codons . Whether or not the 5'uORFs of the D_{1A} receptor gene are translated is not known but translation may be initiated at non-Kozak sites even in the weakest context if it is the first AUG codon (and an A in position -3) regardless of the remaining downstream AUG codons (Kozak, 1991). The presence or absence of upstream AUG codons can not account for the results obtained from deletion analysis of the 5'-UTR. Deletion of the intron from pCAT-303 (i.e. pCAT-303Δintron) abolished CAT activity. Comparison of the intron deletion to pCAT-303 suggests that the observed differences are due to transcriptional effects since the same AUG codons should be present in mature mRNAs from both constructs. Whether the five remaining AUG codons suppress translation of the downstream receptor cistron relative to the level of translation which may be attained in their absence remains to be seen. Deletion of the distal 5'-untranslated region abolished CAT activity relative the pCAT-303. The intron contains a 5'sORF (short open reading frame) which is presumably spliced out of the mRNA from the construct containing the distal 5'-UTR deletion. Therefore, comparison of the CAT activity from the construct with the distal 5'-UTR deleted (-303/+542) and expression of the construct with the intron and distal 5'-UTR deleted (-303/+425) suggests that the differences in observed CAT activities are due to differences in transcription since the mRNAs would lack all of the AUG

codons. Removal of all the AUG codons did not restore the maximal transcriptional activity (pCAT-303/+425, pCAT-303/+38). It appears that the results of deletion analysis of the 5'-UTR can not be explained by effects on translation since complete removal of these 5'-uORF did not increase transcription, however, further studies are required before translational effects are completely dismissed. It is likely that the destabilization of the D_{1A} receptor mRNA may be mediated by proteins that interact at the 3'-UTR (Jose et al., 1996).

Analysis of the D_{1A} receptor gene intron in front of a heterologous promoter provided a context independent system to clarify whether the intron inherently acts as a positive or negative element. The +430/+527 sequence of the D_{1A} receptor gene effectively suppressed transcription of the heterologous promoter (TK) in an orientation independent manner. Positioning the -303 to -165 sequence 150bp upstream of the intron did not overcome the observed transcriptional repression. This result may be attributed to the fact that -303 to -165 is not an enhancer and the intron is a repressor in this context. Although -303 to -165 contains a positive regulatory element (CRE), it may contain negative elements as well to modulate the level of activity. In addition, it can not be concluded from these experiments whether the intron is a positive or negative regulator of transcription since it is promoter specific. It appears that the spatial arrangement and context of the intron strongly influence whether it is an enhancer or repressor. These results taken together confirm that upstream and downstream cis-elements are interacting to activate D_{1A} receptor gene transcription. Disrupting these interactions through deletions or insertions may affect the level of transcription by forcing new interactions to occur or disturbing necessary interactions.

Analysis of the 5'-flanking and 5'-UTR of the porcine D_{1A} receptor gene (Figure 4) with Transcription Factor Data base (Ghosh, 1993) revealed that the 5'-UTR

contains several consensus sites for known transcription factors (AP-1, AP-2, Sp1/GC box) while only two known consensus sites are present in the intron. The 5' portion of the 97bp intron contains an inverted CAAT box and the 3'-portion contains a CTF/NF-1 site. GMSA competition and footprinting assays indicated that LLC-PK_1 nuclear protein interactions primarily occurred at the 3' portion of the intron. The 3'-portion of the intron contains a TGGCA site (+492/+502) which may be recognized by CAAT-binding protein (CBP) and CTF/NF-1 proteins. Interestingly, these proteins are capable of recognizing the CAAT box as well (Dorn et al., 1987a). Since the LLC-PK_1 cells are known to express a cAMP-inducible CAAT-binding protein (Kinane et al., 1993), it is tempting to speculate that this protein contributes to D_{1A} receptor gene regulation through interactions with the intron and perhaps is required for cAMP responsiveness. In addition, the 3'-portion of the porcine D_{1A} receptor gene intron contains three motifs which may represent functional cis-elements: tandem repeat, inverted repeat and purine/pyrimidine tract (Figure 25). Tandem repeats are involved in the activation of vitamin D receptor response elements whereas inverted repeats are typical of glucocorticoid receptor, estrogen receptor, and thyroid hormone response elements (Evans, 1988). Poly-pyrimidine tracts are important in branch site recognition and binding general transcriptional repressors (Sommerville and Ladomery, 1996; Norton, 1994; Mulligan et al., 1992). Novel transcription factors or tissue specific factors may be involved in interactions with these sites and require further investigation.

Intronic sequences have been identified which repress (Malicki et al., 1995; Lazar et al., 1995) or enhance gene expression (Lazar et al., 1995; Grosscheld and Baltimore, 1985; Sun et al., 1995). Multiple promoters within some introns are alternatively transcribed resulting in different sized transcripts with distinct properties. (Cervini et al., 1995) Differential expression of thyroid hormone receptor α and β

genes is due to the presence of a purine rich element and two octamer binding sites in the intron of the α gene that are not present in the β gene (Lazar et al.,1994).

Communication between the 5'- and 3'-UTR and intronic sequences of the embryonic β-type globin gene demonstrates the complex mechanisms that have evolved to silence enhanced gene expression (Wandersee et al., 1996). Enhanced expression of the eosinophil-derived neurotoxin ribonuclease gene occurs as a result of interactions between an intron within the 5'-untranslated region (5'-UTR) and the promoter (Tiffany et al., 1996). However, to my knowledge, this is the first demonstration of the involvement of a 5'-UTR intron in the transcriptional regulation of a G-protein coupled receptor gene through interactions with the promoter.

IX. E. Model of D_{1A} Receptor Gene Transcription

Activation of transcription in eukaryotes is a complex process which takes place either in multiple steps (Stargell and Struhl, 1996) or in one concerted step (Halle and Meisteremst, 1996). Regardless, TATA-less genes, like the D_{1A} receptor gene, require interactions with basal transcription factors (TFIID and TAF factors) to form the transcription initiation complex (Wiley et al., 1992; Oelgeshlager et al., 1996; Aso et al., 1994). The general transcriptional machinery is recruited to the transcription start site of a TATA-less gene by other transcription factors. Since Sp1 and CREB are known to interact with TFIID (Azizkhan et al., 1993; Xiang et al., 1995; Ferreri et al., 1994), it is plausible that either CREB or Sp1 or both may be involved in recruiting the TFIID complex to the D_{1A} receptor gene transcription start site. A stereospecific initiation complex is formed as a result of the TFIID complex's interactions with downstream elements (Oelgeshlager et al., 1996).

D_{1A} receptor gene expression is complicated and involves several cis-elements. Activation of D_{1A} receptor gene transcription requires nuclear transacting factors (AP-2, CRE-like, Sp1, Egr-1, E-box and intron binding proteins) to bind to their respective consensus sites. Small changes in an activation domain can be discerned by the arrangement of basal elements (Das et al., 1995). This is apparent in the D_{1A} receptor gene since transcriptional activation of the D_{1A} receptor gene relies on the precise spatial arrangement of cis-regulatory elements. A proposed model would involve these transcription factors acting cooperatively to induce conformational changes in the DNA duplex possibly resulting in looping (hairpin) or twisting of the DNA. Thus, permitting interactions between the 5'-flanking and 5'-UTR of the D_{1A} receptor gene by bringing

5' and 3' elements in close proximity to each other (Figure 28). This model allows optimal interactions to occur between the transcription factors bound to these cis-elements. The new conformation allows the general transcription factors and RNA polymerase II to bind to the transcription start site and initiate transcription. While the focus here was on the role of the 5'-UTR intron in D_{1A} receptor gene transcription, the 5'UTR contains many consensus sites which may participate in D_{1A} receptor gene regulation as well. The proposed mechanism may apply to other members of the GPCR-superfamily which are organized similarly.

IX. F. Future Directions

While my results provide some insight into D_{1A} receptor expression in renal cells, there are many questions concerning the regulation of the D_{1A} receptor at the transcriptional and translational level which may be addressed in the future. One concern is whether the receptor protein levels are effected during early stages of desensitization during which transcription is elevated. Recently, antibodies to the human D_{1A} receptor have become commercially available which make addressing this issue feasible. In addition, the identity of the proteins which bind to the CRE-like elements and the 5'-UTR intron should be determined. If they are novel proteins, then characterization of these proteins should be attempted. The 3'-UTR and 3'-flanking regions of many genes participate in gene regulation, therefore, these regions should be studied in order to determine if additional regulatory elements reside in these downstream regions of the D_{1A} receptor gene. Finally translational regulation appears to be an important component of the D_{1A} receptor expression. Issues which should be addressed include the relevance of the upstream AUG condons, whether the 5'-sORFs are translated and if so what effects they have on the translation of the downstream receptor cistron. Studies on the regulation of the D_{1A} receptor synthesis at the level of gene transcription and translation are fundamentally important because understanding how the D_{1A} receptor gene is regulated is essential to the development of novel antihypertensives. Future studies will provide further insight into the regulation of the renal D_{1A} receptor expression at the level of gene transcription and translation.

IX. G. Conclusions

The renal D_{1A} receptor gene is regulated through transcriptional and post-transcriptional mechanisms during desensitization. Dopamine simultaneously decreased the stability of the D_{1A} receptor mRNA and increased transcription in what may be an autoregulatory mechanism to prevent a complete loss of D_{1A} receptors. Functional analysis of the 5'-flanking and 5'-untranslated regions identified an area -303 to -165 as being functionally important. This region is distinct from the activating region of the human D_{1A} receptor gene expressed in neuroblastoma cells. The -303 to -165 sequence contains an element that is responsible for cAMP-responsiveness D_{1A} receptor gene, however, this response is context and orientation dependent. Two CRE-like elements 3 bases apart were identified within this region and were shown to moderately increase transcription of a heterologous promoter in an orientation independent manner. These CRE elements are dependent on additional cis-elements to mediate cAMP response. The 3'-CRE is essential to basal activity of the D_{1A} receptor gene expression and likely to associate with a protein other than CREB. This site is highly conserved across species and may be important in regulating transcription of the human and rat D_{1A} promoters as well. GMSA and footprinting assays indicated AP-2, Sp1, CREB/ATF, Egr-1, E-box sites bound LLC-PK_1 nuclear proteins and purified AP-2 and Sp1 in a specific manner.

Deletion of the majority of the 5'-UTR (+39/+962) or the upstream sequence (-303/+52) suggested that an interaction is required between upstream and downstream cis-elements. Regulation of the D_{1A} receptor gene involves the small 97 bp 5'-UTR intron. Footprinting and GMSA demonstrated that LLC-PK_1 nuclear proteins primarily bound to the 3' portion of the intron. This area of the intron contains a TGGCA

binding site, a tandem repeat, an inverted repeat and a purine/pyrimidine tract which may be involved in the protein interactions. The activity of the intron is context dependent and relies on spatial arrangements and therefore can not be classified as a positive or negative regulatory element. A novel mechanism for regulating expression of the D_{1A} receptor gene in renal cells is proposed in which interactions between the 5'-flanking region (-303 to -63) and the 5'-untranslated intron (+430/+527) are necessary for optimal transcriptional activation. These results are the first to demonstrate the involvement of a 5'-UTR intron in the transcriptional regulation of a G-protein coupled receptor gene through interactions with the promoter.

Figure 27: Comparison of sequence of porcine, rat and human D_{1A} - receptor genes containing CRE-like elements.

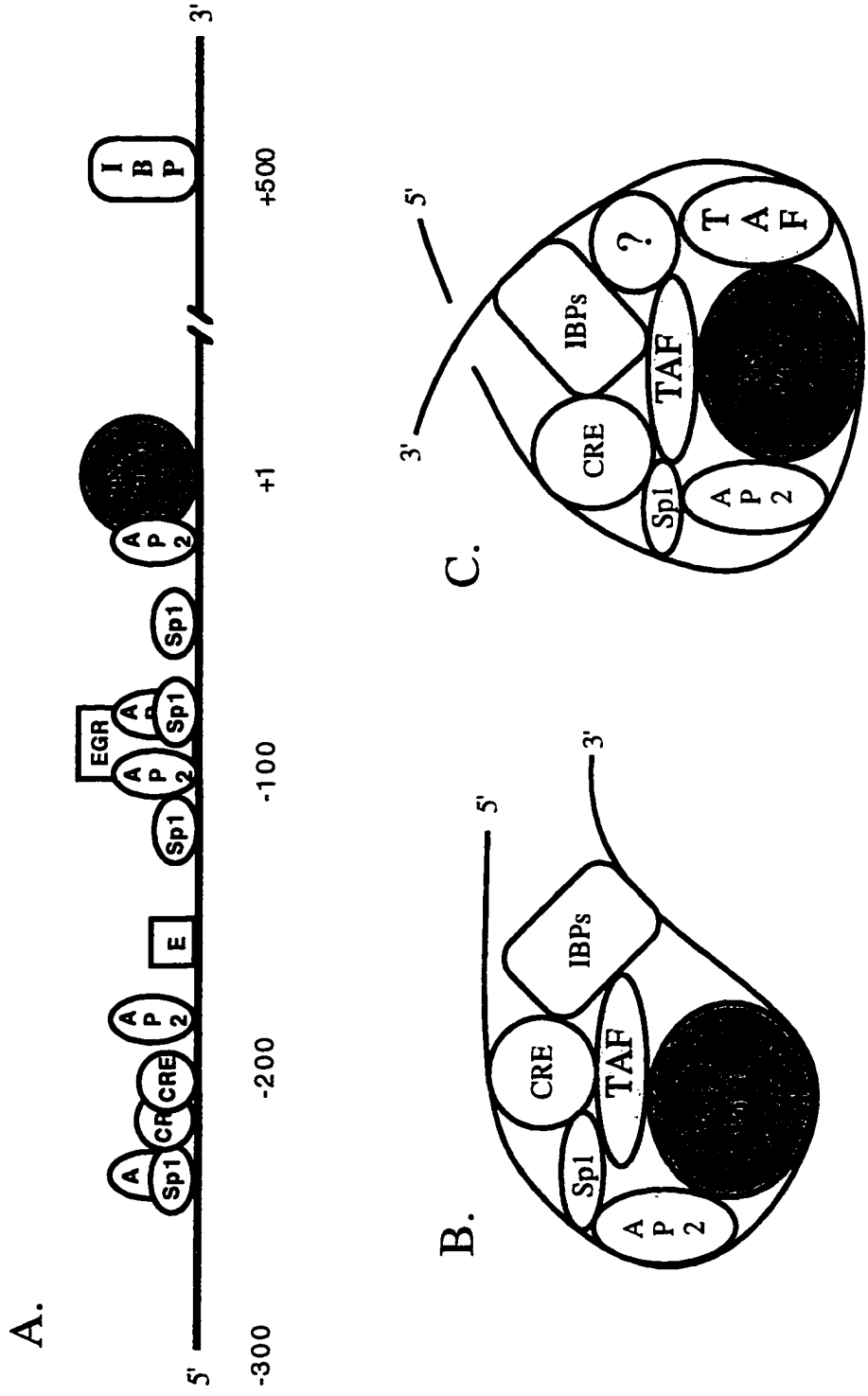
Table I

Genomic organization of G-protein coupled receptors containing 5'-UTR Intron(s).

Receptor	Species	TATA box	5'-UTR Intron	Coding Region Intron	Size 5'UTR (Kb)	Multiple 5'- AUG Codons	Second Messenger System
A1 adenosine	human	no	4	1	<1,>2, 0.354, 0.351	yes	(-) AC
AT1 angiotensin	human	yes	2	-	8.3, >59	yes	(+) PLC
AT2 angiotensin	human	yes	2	-	0.150, 1.2	yes	(+) PLC
BK2 Bradykinin	rat	no	3	1	>21, 1.8, 0.211	yes	(+) PLC
D1A dopamine	human	no	1	-	0.116	yes	(+) AC
D1A dopamine	rat	no	1	-	0.115	yes	(+) AC
D1A dopamine	porcine	no	1	-	0.097	yes	(+) AC
D1B dopamine	human	no	1	-	0.155 or 0.179	yes	(+) AC
D2 dopamine	rat	no	1	6	25	yes	(-) AC
Endothelin-A	human	no	1	6	4	yes	(-) AC
Neuropeptide Y-Y1	human	no	3	1	6.4	yes	(-) AC
Platlet activating factor	human	no	1	-	16	yes	(+) PLC
Thromboxane A2	human	no	1	1	6.3	yes	(+) PLC
Oxytocin	human	yes	2	1	0.639, 0.166	yes	(+) PLC
Oxytocin	rat	no	1	1	0.097	yes	(+) PLC
M1 muscarinic	rat	n.a.	≥1	-	0.073	n.a.	(+) PLC
M2 muscarinic	human	n.a.	≥1	-	0.148	n.a.	(-) AC
M3 muscarinic	rat	n.a.	≥1	-	0.733	n.a.	(+) PLC
M4 muscarinic	human	no	2	-	0.8, 4.4	yes	(-) AC
M5 muscarinic	rat	n.a.	≥1	-	0.374	n.a.	(+) PLC

AC: adenylyl cyclase, PLC: phospholipase C, n.a.: data not available

Figure 28: Model of the porcine D_{1A} gene promoter. A, a schematic representation of the D_{1A} receptor gene 5'-flanking region with the relative position of binding sites for a variety of transcription factors based on analysis of the 5'-flanking porcine sequence (37). Also shown schematically is the presence of unspecified intron binding proteins (IBP) binding to the area of the 5'-UTR intron. B, possible formation of hairpin-like loops within the D_{1A} receptor gene to bring the downstream IBPS in proximity to the upstream transcriptional machinery. c, possible loop formed by twisting of the DNA within the D_{1A} receptor gene to bring the downstream IBPs in proximity to the upstream transcriptional machinery. Abbreviations: AP-2, activator protein 2; CRE, cAMP responsive element binding protein, E, E-box; EGR, early growth response factor; TAF, transcription associated factors.



X. Bibliography

Albrecht, F. E., Drago, J., Felder, R.A., Printz M.P., Eisner, G.M., Robillard, J.E., Sibley, D.R., Westphal, H.J., and Jose, P.A. (1996). "Role of the D_{1A} Dopamine Receptor in the Pathogenesis of Genetic Hypertension." J. Clin. Invest. **97**: 2283-2288.

Anthony-Cahill, S. J., Benefield, P.A., Fairman, R., Wasserman, Z.R., Brenner, S.L., Stafford, W.F., Altenbach,C., Hubbell, w.L., and DeGrado, W.F. (1992). "Molecular Characterization of Helix-Loop-Helix Peptides." Science **255**: 979-983.

Andrews, N. and Faller, D. (1991). "A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells." Proc. Natl. Acad. Sci. **19** (9): 2499.

Aperia, A., Bertorello, A. and Seri, I. (1987). "Dopamine causes inhibition of Na+-K+-ATPase activity in rat proximal convoluted tubule segments." Am. J. Physiol. **252**(21): F39-F45.

Arias, J., Alberta, A.S., Brindle, P., Claret, F.X., Smeal, T., Karin, M., Feramisco, J., and Montminy, M. (1994). "Activation of cAMP and mitogen responsive genes relies on a common nuclear factor." Nature **370**: 226-229.

Armstrong, R., Wen, W., Meinkoth, J. Taylor, s., and Montminy, M. (1995). "A Refractory Phase in Cyclic AMP-Responsive Transcription Requires Down Regulation of Protein Kinase A." Mol. Cell. Biol. **15**(3): 1826-1832.

Aso, T., Conaway, J.W., and Conaway, R.C. (1994). "Role of Core Promoter Structure in Assembly of the RNA Polymerase II Preinitiation Complex." J. Biol. Chem. **269**(42): 26575-26583.

Ayer, D. E., Kretzner, L., and Eisenman, R.N. (1993). "Mad: A Heterodimeric Partner for Max That Antagonizes Myc Transcriptional Activity." Cell **72**: 211-222.

Azizkhan, J. C., Jensen, D.E., Pierce, A.J., and Wade, M. (1993). "Transcription from TATA-less Promoters: Dihydrofolate Reductase as a Model." Critical Reviews in Eukaryotic Gene Expression **3**(4): 229-254.

Baines, A. D., and Chan, W. (1980). "Production of urine free dopamine from DOPA: a micropuncture study." Life Sciences **26**: 253-259.

Baines, A. D., Drangova, R., and Hatcher, C. (1985). "Dopamine production by isolated glomeruli and tubules from rat kidneys." Can. J. Physiol. **63**: 155-158.

Ball, H. J., Shine, J., and Herzog, H. (1995). "Multiple Promoters Regulate Tissue-specific Expression of the Human NPY-Y1 Receptor Gene." J. Biol. Chem. **270**(45): 27272-27276.

Barton, A., and Sibley, D. (1990). "Agonist-Induced Desensitization of D_1-Dopamine Receptors Linked to Adenylyl Cyclase Activity in Cultured NS20Y Neuroblastoma Cells." Mol. Pharm. **38**: 531-541.

Bass, A. S., and Murphy, M.B. (1990). "Role of endogenous dopamine in the natriuresis accompanying various sodium challenges." Am. J. Hypertens. **3**: 90s-92s.

Bates, M., M. Caron, and J. Raymond (1991). "Desensitization of D_{A1} dopamine receptors coupled to adenylyl cyclase in opossum kidney cells." Amer. J. Physiology **260**: F937-F945.

Bates, M., C. Olsen, B. Becker, F. Albers, J. Middleton, J. Mulheron, S. Jin, M. Conti and J. Raymond (1993). "Elevation of cAMP is Required for Down-regulation, but not Agonist-induced Desensitization, of Endogenous Dopamine D_1 Receptors in Opossum Kidney Cells studies in Cells that Stably Express a Rat cAMP Phosphodiesterase (rPDE3) cDNA." J. Biol. Chem. **268**(20): 14757-14763.

Beischlag, T. V., Marchese, A., Meador-Woodruff, J.H., Damask, S.P., O'Dowd, B.F., Tyndale, R.F., Van Tol, H.H.M., Seeman, P., and Niznik, H.B. (1995). " The Human Dopamine D_5 Receptor Gene: Cloning and Characterization of the 5'-Flanking and Promoter Region." Biochemistry **34**: 5960-5970.

Benbrook, D. M., and Jones, N.C. (1994). "Different binding specificities and transactivation of variant CRE's by CREB complexes." Nucl. Acids Res. **22**(8): 1463-1469.

Benjamin, E. J., Levy, D., Vaziri, S.M., D'Agostino, R.B., Belanger, A.J., and Wolf, P.A. (1994). "Independent Risk Factors for Atrial Fibrillation in a Population-Based Cohort The Framingham Heart Study." JAMA **271**: 840-844.

Benovic, J. L., Bouvier, M., Caron, M.G., and Lefkowitz, R.J. (1988). "Regulation of Adenylyl Cyclase-Coupled β-Adrenergic Receptors." Ann. Rev. Cell. Biol. **4**: 405-428.

Bertorello, A., Hokfelt, T., Goldstein, M. and Aperia, A. (1988). "Proximal tubule Na+-K+-ATPase activity is inhibited during high-salt diet: evidence for DA-mediated effect." Am. J. Physiol. **254**(23): F795-F801.

Best, J. A., Chen, Y., Piech, K.M., and Tank, W. (1995). "The Response of the Tyrosine Hydroxylase Gene to Cyclic AMP is Mediated by Two Cyclic AMP-Response Elements." J. Neurochem. **65**: 1934-1943.

Blackwell, T. K., and Weintraub, H. (1990). "Differences and Similarities in DNA-Binding Preferences of MyoD and E2A Protein Complexes Revealed by Binding Site Selection." Science **250**: 1104-1110.

Bonner, T. I., Buckley, N.J., Young, A.C. and Brann, M.R. (1987). "Identification of a family of muscarinic acetylcholine receptor genes." Science **237**: 527-532.

Boshart, M., Klueppel, M., Schmidt, A., Schuetz, G., and Luckow, B.H.R. (1992). "Reporter constructs with low background activity utilizing the cat reporter gene." Gene **110**: 129-130.

Boutillier, A., Berthel, F., Roberts, J.L., and Loeffler, J.P. (1992). "β-Adrenergic Stimulation of cFOS via Protein Kinase A is mediated by cAMP regulatory element binding protein (CREB)-dependent and tissue-specific CREB-independent mechanisms in corticotrope cells." J. Biol. Chem. **267**(33): 23520-23526.

Brindle, P., Nakajima, T., and Montminy, M. (1995). "Multiple protein kinase A-regulated events are required for transcriptional induction by cAMP." Proc. Nat. Acad. Sci. **92**: 10521-10525.

Campese, V. M. (1994). "Salt Sensitivity in Hypertension Renal and Cardiovascular Implications." Hypertension **23**: 531-550.

Cao, X., Mahendran, R., Guy, G., and Tan, Y.H. (1993). "Detection and Characterization of Cellular EGR-1 Binding to Its Recognition site." J. Biol. Chem. **268**(23): 16949-16957.

Cervini, R., Houhou, L., Pradat, P., Bejanin, S., Mallet, J., and Berrard, S. (1995). "Specific Vesicular Acetylcholine Transporter Promoters Lie within the First Intron of the Rat Choline Acetyltransferase Gene." J. Biol. Chem. **270**(42): 24654-24657.

Chase, P. B., Halonen, M. and Regan, J.W. (1993). "Cloning of a Human Platelet-activating Factor Receptor Gene: Evidence for an Intron in the 5'-untranslated Region." Am. J. Respir. Cell Mol. Biol. **8**: 240-244.

Cheng, L., Precht, P., Frank, D., and Liang, C. (1990). "Dopamine stimulation of cAMP production in cultured opossum kidney cells." Amer. J. Physiol. **258**: F877-F882.

Civelli, O., Bunzow, J., and Grandy, D. (1993). "Molecular Diversity of the Dopamine Receptors." Annu. Rev. Pharmacol. Toxicol. **32**: 281-307.

Clark, B., Rosa,R., Epstein,F., Young,J., and Landsberg, L. (1992). "Altered Dopaminergic Responses in Hypertension." Hypertension **19**(6): 589-594.

Collins, S., Bouvier, M., Bolanowski, M.A., Caron, M.G., and Lefkowitz, R.J. (1989). "cAMP stimulates transcription of the β_2-adrenergic receptor gene in response to short-term agonist exposure." Proc. Natl. Acad. Sci. **86**: 4853-4857.

Collins, S., Altschmied, J., Herbsman, O., Caron, M.G., Mellon, P.L., and Lefkowitz, R.J. (1990). "A cAMP Response Element in the β_2-Adrenergic Receptor Gene Confers Transcriptional Autoregulation by cAMP." J. Biol. Chem. **265**(31): 19330-19335.

Collins, S., Ostrowski, J., and Lefkowitz, R.J. (1993). "Cloning and sequence analysis of the human β_1-adrenergic receptor 5'-flanking promoter region." Biochim. Biophys. Acta **1172**: 171-174.

Colson, P., Ibarondo, J., Devilliers, G., Balestre, M.N., Duvoid, A., and Guillon, G. (1992). "Upregulation of V1a vasopressin receptors by glucocorticoids." Am. J. Physiol. **263**: E1054-E1062.

Das, G., Hinkley, C.S., and Herr, W. (1995). "Basal Promoter Elements as a Selective Determinant of Transcriptional Activator Function." Nature **374**: 657-660.

Dearry, A., Gingrich, J.,Falardeau, P., Fremeau, R., Bates, M., and Caron, M. (1990). "Molecular cloning and expression of the gene for a human D_1 dopamine receptor." Nature **347**: 72-75.

Delmas, V., Laoide, B.M., Masquilier, D., De Groot, R.P., Foulkes, N.S., and Sassone-Corsi, P. (1992). "Alternative usage of initiation codons in mRNA encoding the cAMP-responsive-elements modulator generates regulators with opposite functions." Proc. Natl. Acad. Sci. **89**: 4226-4230.

Demchyshyn, L. L., Sugamori, K.S., Lee, F.J.S., Hamadanizadeh, S.A., and Niznik, H.B. (1995). "The Dopamine D_{1D} Receptor cloning and characterization of three pharmacologically distinct D1-like receptors from *Gallus domesticus*." J. Biol. Chem. **270**(8): 4005-4012.

Diamond, M., Miner, J., Yoshinaga, S., and Yamamoto, K. (1990). "Transcription Factor Interactions: Selectors of Positive of Negative Regulation from a Single DNA Element." Science **249**: 1266-1272.

Dignam, J., Lebovitz, R., and Roeder, R. (1983). "Accurate transcription intitiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei." Nucleic Acids Research **11**: 1475-1489.

Dorn, A., Durand, B., Marfing, C., LeMeur, M., Benoist, C., and Mathis, D. (1987). "Conserved major histocmpatibility complex class II boxes-X and Y- are transcriptional control elements and specifically bind nuclear proteins." Proc. Natl. Acad. Sci. **84**: 6249-6253.

Dorn, A., Bollekens, J., Staub, A., Benoist, C., and Mathis, D. (1987a). "A Multiplicity of CCAAT Box-Binding Proteins." Cell **50**: 863-872.

Dynan, W. S. (1986). "Promoters for housekeeping genes." Trends Genet **2**: 196-197.

Eason, M., Jacinto, M., Theiss, C., and Liggett, S. (1994). "The palmitoylated cysteine of the cytoplasmic tail of α_{2A}-adrenergic receptors confers subtype-specific agonist-promoted downregulation." Proc. Natl. Acad. Sci. **91**: 11178-11182.

Ebert, S. N., and Wong, D.L. (1995). "Differential Activation of the Rat Phenylethanolamine N-Methyltransferase Gene by Sp1 and Egr-1." Journal of Biological Chemistry **270**(29): 17299-17305.

Evans, R. M. (1988). "The Steroid and Thyroid Hormone Receptor Superfamily." Science **240**: 889-895.

Felder, C., Blecher, M., and Jose, P. (1989). "Dopamine-1-mediated Stimulation of Phospholipase C Activity in Rat Renal Cortical Membranes." J. Biol. Chem. **264**(15): 8739-8745.

Felder, C., T. Campbell, F. Albrecht, and P. Jose (1990). "Dopamine inhibits Na+-H+ exchanger activity in renal BBMV by stimulation of adenylate cyslase." Amer. J. Physiol. **259**: F297-F303.

Felder, C. C., McKelvey, A.M., Gitler, M.S., Eisner, G.M., and Jose, P.A. (1989). "Dopamine receptor subtypes in renal brush border and basolateral membranes." Kid. Inter. **36**: 183-193.

Felder, R., K. Nakamura, J. Robillard, M. Kanadjian and P. Jose. (1988). "Dopamine receptors in the developing sheep kidney." Pediatric Nephrology **2**: 156-162.

Felder, R., Kinoshita, S., Ohbu, K., Mouradian,M., Sibley, D., Monsma, F., Minowa,T., Minowa, M., Canessa, L., and Jose, P. (1993). "Organ specificity of the dopamine$_1$ receptor/adenylyl cyclase coupling defect in spontaneously hypertensive rats." Am. J. Physiol. **264**: R726-R732.

Felder, R. A., Blecher, M., Eisner, G.M., and Jose, P.A. (1984). "Cortical tubular and glomerular dopamine receptors in the rat kidney." Am. J. Physiol. **246**: F557-F568.

Ferguson, S. S. G., Downey, W.E., Calapietro, A., Barak, L.S., Menard, L., and Caron, M.G. (1996). "Role of β-Arrestin in Mediating Agonist-Promoted G Protein-Coupled Receptor Internaliztion." Science **271**: 363-366.

Ferreri, K., Gill, G., and Montminy, M. (1994). "The cAMP-regulated transcription factor CREB interacts with a component of the TFIID complex." Proc. Natl. Acad. Sci. **91**: 1210-1213.

Fink, J. S., Verhave, M., Kasper, S., Tsukada, T., Mandel, G., and Goodman, R.H. (1988). "The CGTCA sequence motif is essential for biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer." Proc. Natl. Acad. Sci. **85**: 6662-6666.

Fiol, C. J., Williams, J.S., Chou, C., Wang, Q.W., Roach, P.J., Andrisani, O.M. (1994). "A Secondary Phosphorylation of CREB341 at Ser-129 is Required for the cAMP-mediated Control of Gene Expression." J. Biol. Chem. **269**(51): 32187-32193.

Fisher, F., Crouch, D.H., Jayaraman, P.-S., Clark, W., Gillespie, D.A.F., and Goding, C.R. (1993). "Transcription activation by Myc and Max: flanking sequences target activation to a subset of CACGTG motifs in vivo." The EMBO Journal **12**(13): 5075-5082.

Fitzgerald, L., Li, Z., Machida, C.A., Fishman, P.H. and Duman, R.S. (1996). "Adrenergic Regulation of ICER (Inducible Cyclic AMP Early Repressor) and β_1-Adrenergic Receptor Gene Expression in C_6 Glioma Cells." J. Neurochem. **67**: 490-497.

Fredrickson, E. D., Bradley, T., and Goldberg, L.I. (1985). "Blockade of renal effects of dopamine in the dog by the DA_1 antagonist SCH 23390." Am. J. Physiol. **249**: F236-F240.

Freedman, N. J., Liggett, S.B., Drachman, D.E., Pei, G., Caron, M.G., and Lefkowitz, R.J. (1995). "Phosphorylation and Desensitization of the Human β-Adrenergic Receptor." J. Biol. Chem. **270**(30): 17953-17961.

Fremeau, R. J. J., Duncan, G.E., Fornetto, M.-G., Dearry, A., and Gingrich, J.A. (1991). "Localization of D_1 dopamine receptor mRNA in brain supports a role in cognitive, affective and neuroendocrine aspects of dopaminergic transmission." Proc. Natl. Acad. Sci. **88**: 3772-3776.

Fukamauchi, F., Saunders, P.A., Hough, C. and Chuang, D. (1993). "Agonist-Induced Down-Regulation and Antagonist-Induced Up-Regulation of m_2- and m_3-Muscarinic Acetylcholine Receptor mRNA and Protein in Cultured Cerebeller Granule Cells." Mol. Pharm. **44**: 940-949.

Gershengorn, M. C. (1994). "Excessive Stimulation is Bad, So Desensitization is Ubiquitous." Endocrinology **134**(1): 5-6.

Gesek, F., and Schoolwerth, A. (1990). "Hormonal interactions with the proximal Na+-H+ exchanger." Amer. J. Physiol. **258**: F514-F521.

Ghosh, D. (1993). "Status of the transcription factors database (TFD)." Nucleic Acids Res **13**: 3117-3118.

Gill, J., Gullner, H., Lake,C., Lakatua, D., and Lan, G. (1988). "Plasma and Urinary Catecholamines in Salt-Sensitive Idiopathic Hypertension." Hypertension **11**(4): 312-319.

Gingrich, J. A., and Caron, M.G. (1993). "Recent Advances in the Molecular Biology of Dopamine Receptors." Annu. Rev. Neurosci. **16**: 299-321.

Goldberg, L. I. (1972). "Cardiovascular and Renal Actions of Dopamine: Potential Clinical Applications." Pharmacological Reviews **24**(1): 1-29.

Goldstein, M., Fuxe, K., and Hokfelt, T. (1972). "Characterization and Tissue Localization of Cathecolamine Synthesizing Enzymes." Pharmacological Reviews **24**: 293-309.

Goodman, O.B., Krupnick, J.G., Santini, F., Gurevich, V.V., Penn, R.B., Gagnon, A.W., Keen, J.H. and Benovic, J.L. (1996). "β-Arrestin acts as a clathrin adaptor in endocytosis of the β_2-adrenergic receptor." Nature. **383**: 447-450.

Goraya, T. Y., Kessler, S.P., Stanton, P., Hanson, R.W., and Sen, G.C. (1995). "The Cyclic AMP Response Elements of the Genes for Anigotensin-Converting Enzyme and Phosphoenolpyruvate Carboxykinase (GTP) Can Mediate Transcriptional Activation by CREMτ and CREMα." J. Biol. Chem. **270**(32): 19078-19085.

Grandy, D. K., Zhang, Y., Bouvier, c., Zhou, Q.-Y., and Johnson, R.A. (1989). "Cloning of the cDNA and a gene for a human D_2 dopamine receptor." Proc. Natl. Acad. Sci. **86**: 9762-9766.

Grenader, A., and Healy, D. P. (1991). "Locally formed dopamine stimulates cAMP accumulation in LLC-PK_1 cells via a D_{A1} dopamine receptor." Am. J. Physiol. **260**(29): F906-F912.

Grenader, A. C., O'Rourke, D.A., and Healy, D.P. (1995). "Cloning of the porcine D_{1A} dopamine receptor gene expressed in renal epithelial LLC-PK_1 cells." Am. J. Physiol. **268**(37): F 423-F434.

Grosschedl, R., and Baltimore, D. (1985). "Cell-Type Specificity of Immunoglobulin Gene Expression is Regulated by at Least Three DNA Sequence Elements." Cell **41**: 885-897.

Gupta, M. P., Gupta, M., and Zak, R. (1994). "An E-Box/M-CAT Hybrid Motif and Cognate Binding Protein(s) Regulate the Basal Muscle-specific and cAMP-inducible Expression of the Rat Cardiac α-Myosin Heavy Chain Gene." J. Biol. Chem. **269**(47): 29677-29687.

Gurich, R. W., and Beach, R.E. (1994). "Abnormal regulation of renal proximal tubule Na+-K+ATPase by G proteins in spontaneously hypertensive rats." Am. J. Physiol. **267**: F1069-F1075.

Habener, J. F. (1990). "Cyclic AMP Response Element Binding Proteins: A Cornucopia of Transcription Factors." Mol. Endocrin. **4**(8): 1087-1094.

Hagege, J., and Richet, G. (1985). "Proximal tubule dopamine histofluorescence in renal slices incubated with L-dopa." Kidney International **27**: 3-8.

Halle, J.-P., and Meisterernst, M. (1996). "Gene expression: increasing evidence for a transcriptosome." Trends Genet. **12**(5): 161-163.

Hansell, P., and Fasching, A. (1991). "The effect of dopamine receptor blockade on natriuresis is dependent on the degree of hypervolemia." Kidney Int. **39**: 253-258.

Harrington, M., Konicek, B., Song, A., Xia, X., Fredericks, W., and Rauscher, F. (1993). "Inhibition of colony-stimulating factor-1 promoter activity by the product of the Wilm's tumor locus". J. Biol. Chem. **268**(28): 21271-21275.

Hawley-Nelson, P., Ciccarone, V., Gebeyehu, G., Jessee, J., and Felgner, P. (1993). "Lipofectamine Reagent: A New, Higher Efficiency Polycationic Liposome Transfection Reagent." Focus **15**: 73-80.

Hayashi, M., Yamaji, Y., Kitajima, W., and Saruta, T. (1991). "Effects of high salt intake on dopamine production in rat kidney." Am. J. Physiol. **260**: E675-E679.

Healy, D. P. and O'Rourke, D.A. (1996). "Regulation of Dopamine-1A (D_{1A}) Receptor Gene Transcription." J. Clin. Exp. Hypertens. **in press**:

Hedge, H., Ricci, A., Amenta, F., and Lokhandwala, M.F. (1989). "Evidence from Functional and Autoradiographic Studies for the Presence of Tubular Dopamine-1 Receptors and Their involvement in the Renal Effects of Fenoldopam." J. Pharm. Exp. Ther. **251**(3): 1237-1245.

Hegde, S. S., Jadhav, A.L., and Lokhandwala, M.F. (1989). "Role of Kidney Dopamine in the Natriuretic Response to Volume Expansion in Rats." Hypertension **13**: 828-834.

Hennighausen, L., and Lubon, V. (1987). "Interaction of Protein with DNA in Vitro." Methods in Enzymology **152**: 721-735.

Ho, S. N., Biggar, S.R., Spencer, D.M., Schreiber, S.L., and Crabtree, G.R. (1996). "Dimeric ligands define a role for transcriptional activation domains in reinitiation." Nature **382**: 822-826.

Hosoda, K., Nakao, K., Tamura, N., Arai, H., Ogawa, Y., Suga, S., Nakanishi, S., and Imura, H. (1992). "Organization, Structure, Chromosomal Assignment, and Expression of the Gene Encoding the Human Endothelin-A Receptor." J. Biol. Chem. **267**(26): 18797-18804.

Hosoda, K., and Duman, R.S. (1993). "Regulation of β_1-Adrenergic Receptor mRNA and Ligand Binding by Antidepressant Treatments and Norepinephrine Depletion in Rat Frontal Cortex." J. Neurochem. **60**: 1335-1343.

Hosoda, K., Feussner, G., Rydelek-Fitzgerald, L., Fishman, P., and Duman, R. (1994). "Agonist and Cyclic AMP-Mediated Regulation of β_1-Adrenergic Receptor mRNA and Gene Transcription in Rat C_6 Glioma Cells." J. Neurochem. **63**: 1635-1645.

Hosoda, K., Fitzgerald, L.R., Vaidya, V.A., Feussner, G.K., Fishman, P.H., and Duman, R.S. (1995). "Regulation of β_2-Adrenergic Receptor mRNA and Gene Transcription in Rat C6 Glioma cells: Effects of Agonist, Frskolin, and Protein Synthesis Inhibition." Mol. Pharm. **48**: 206-211.

Hozawa, S., Holtzman, E.J. and Ausiello, D.A. (1996). "cAMP motifs regulating transcription in the aquaporin 2 gene." Am. J. Physiol. **270**: C1695-C1702.

Huang, L.-Y., Tholanikunnel, B.G., Vakalopoulou, E., and Malbon, C.C. (1993). "The Mr 35,000 β-Adrenergic Receptor mRNA-binding Protein Induced by Agonists Requires Both an AUUUA Pentamer and U-Rich Domains for RNA Recognition." J. Biol. Chem. **268**(34): 25769-25775.

Hummler, E., Cole, T.J., Blendy, J.A., Ganss, R., Aguzzi, A., Schmid, W., Beerman, F., and Schutz, G. (1994). "Targeted mutation of the CREB gene: Compensation within the CREB/ATF family of transcription factors." Proc. Natl. Acad. Sci. **91**: 5647-5651.

Huo, T., and Healy, D. (1989). "Autoradiographic localization of dopamine DA1 receptors in rat kidney with [^{3}H]Sch 23390." American J. Physiology : F414-F423.

Huo, T., Ye, M.Q., and Healy, D.P. (1991). "Characterization of a dopamine receptor (DA_{2K}) in the kidney inner medulla." Proc. Natl. Acad. Sci. **88**: 3170-3174.

Huo, T., Grenader, A., Blandina, P., and Healy, D.P. (1991). "Prostaglandin E_2 production in rat IMCD cells II. Possible role for locally formed dopamine." Am. J. Physiol. **261**: F655-F662.

Inglese, J., Freedman, N., Koch, W. and Lefkowitz, R. (1993). "Structure and mechanism of the G protein-coupled receptor kinases". J. Biological Chemistry **268**: 23735-23738.

Ishii, S., Xu, Y., Stratton, R., Roe, B, Merlino, G. and Pastan, I. (1985). "Characterization and sequence of the promoter region of the human epidermanl growth factor receptor gene." Proc. Natl. Acad. Sci. **82**: 4920-4924.

Izzo, N. J., and Colucci, W.S. (1994). "Regulation of α_{1B}-adrenergic receptor half-life: protein synthesis dependence and effect of norepinephrine." Am. J. Physiol. **266**: C771-C775.

Jensen, A. A., Pendersen, U.B., Kiemer, A., Din, N., and Anderson, P.H. (1995). "Functional Importance of the Carboxyl Tail Cysteine Residues in the Human D_1 Dopamine Receptor." J. Neurochem. **65**: 1325-1331.

Jose, P. A., Raymond, J.R., Bates, M.D., Aperia, A., Felder, R.A., and Carey, R.M. (1992). "The Renal Dopamine Receptors." J. Am. Soc. Nephrol. **2**: 1265-1278.

Jose, P. A., Eisner, G.M., and Felder, R.A. (1993). "Dopaminergic defect in hypertension." Ped. Nephrol. **7**: 859-864.

Jose, P. A., Wright, A., Slobodyansky, E., Mouradian, M.M., Wang, W., and Clerch, L.B. (1995). "Kidney Extracts from Spontaneously Hypertensive Rats (SHR) Have Greater Dopamine 1A (D_{1A}) Receptor RNA-Binding Activity than Extracts from Normotensive Wistar-Kyoto (WKY) Rats (Abstract)." The FASEB J. **10**(3): A629.

Kanasaki, M., Matsubara, H., Marasawa, S., Masaki, H., Nio, Y., and Inada, M. (1994). "cAMP Responsive Element-mediated Regulation of the Gene Transcription of the a1B Adrenergic Receptor by Thyrotropin." J. Clin. Invest. **94**: 2245-2254.

Kannel, W.B., Wolf, P.A., Verter, J., and McNamara, P.M. (1970). "Epidemiologic Assesment of the Role of Blood Pressure in Stroke. The Framingham Study." JAMA **214**: 301-310.

Kebabian, J. W., and Greengard, P. (1971). "Dopamine-sensitive adenylyl cyclase: possible role in synaptic transmission." Science **174**: 1346-1349.

Kebabian, J. W., and Calne, D.B. (1979). "Multiple receptors for brain dopamine." Nature **277**: 93-96.

Keller, W., Konig, P. and Richmond, T.J. (1995). "Crystal Structure of a bZIP/DNA Complex at 2.2 A: Determinants of DNA Specific Recognition." J. Mol. Biol. **254**: 657-667.

Kim, C., Dion, S., and Benovic, J. (1993). "Mechanism of β-Adrenergic Receptor Kinase Activation by G Proteins." J. Biol. Chem. **268**(21): 15412-15418.

Kim, J., and Struhl, K. (1995). "Determinants of half-site spacing preferences that distinguish AP-1 and ATF/CREB bZIP domains." Nucleic Acids Res. **23**(13): 2531-2537.

Kim, K., Lee, M.K., Carroll, J., and Joh, T.H. (1993). "Both the Basal and Inducible Transcription of the Tyrosine Hydroxylase Gene Are Dependent upon a cAMP Response Element." J. Biol. Chem. **268**(21): 15689-15695.

Kim, K., Ishiguro, H., Tinti, C., Wagner, J., and Joh, T. (1994). "The cAMP-Dependent Protein Kinase Regulates Transcription of the Dopamine β-Hydroxylase Gene." J. Neuroscience **14**(11): 7200-7207.

Kinane, T., Finder, J., Kawashima, A., Brown, D., Abbate, M., Shang, C. , Fredericks, W., Rauscher, F. , Sukhatme, V. and Ercolani,L. (1994). "LLC-PK_1 Cell Growth is Repressed by Wt1 Inhibition of G-protein α_i--2 Protooncogene Transcription." J. Biol. Chem. **269**(44): 27503-27509.

Kinane, T. B., Shang, C., Findre, J.D., and Ercolani, L. (1993). "cAMP Regulates G-protein ai-2 Subunit Gene Transcription in Polarized LLC-PK_1 Cells by Induction of a CCAAT Box Nuclear Binding Factor." J. Biol. Chem. **268**(33): 24669-24676.

Kinane, T. B., Finder, J.D., Kawashima, A., Brown, D., Abbate, M., Shang, C., Fredericks, W.J., Rauscher, F.J., Sukhatme, and Ercolani, L. (1994). "Growth of LLC-PK_1 Renal Cells Is Mediated by EGR-1 Up-regulation of G Protein α_i-2 Protooncogene Transcription." J. Biol. Chem. **269**(44): 27503-27509.

Kinoshita, S., Ohlstein, E.H., and Felder, R.A. (1990). "Dopamine-1 receptors in rat proximal convoluted tubule: regualtion by intrarenal dopamine." Am. J. Physiol. **258**: F1068-F1074.

Klag, M. J., Whelton, P.K., Randall, B.L., Neaton, J.D., Brancati, F.L., Ford, C.E., Shulman, N.B., and Stamler, J. (1996). "Blood Pressure and End-Stage Renal Disease in Men." N. Eng. J. Med. **334**: 13-18.

Knepel, W., Jepeal, L., and Habner, J. (1990). "A Pancreatic Islet Cell-specific Enhancer-like Element in the Glucagon Gene Contains Two Domains Binding distinct Cellular Proteins." J. Biol. Chem. **265**(15): 8725-8735.

Kobilka, B. K., Frielle, T., Dohlman, H.G., Bolanowski, M.A., Dixon, R.A.F., Keller, P., Caron, M.C., and Lefkowitz, R.J. (1987). "Delineation of the Intronless Nature of the Genes for the Human and Hamster β_2-Adrenergic Receptor and Their Putative Promoter Regions." J. Biol. Chem. **262**(15): 7321-7327.

Kong, G., Penn, R. and Benovic, J. (1994). "A β-Adrenergic Receptor Kinase Dominant Negative Mutant Attenuates Desensitization of the β2-Adrenergic Receptor." J. Biol. Chem. **269**(18): 13084-13087.

Kozak, M. (1990). "Downsream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes." Proc. Natl. Acad. Sci. **87**: 8301-8305.

Kozak, M. (1991). "Structural Reatures in Eukaryotic mRNAs That Modulate the Initiation of Translation." ." J. Biol. Chem. **266**(30): 19867-19870.

Kuchel, O., Racz, K., Debinski, W., Falardeau, P., Buu, N.T. (1987). "Contrasting dopaminergic patterns in two forms of genetic hypertension." Clin. Exp. Hypertens. **A9**: 987-1008.

Kuchel, O. and Shigetomi, S. (1992). "Defective Dopamine Generation from Dihydroxyphenylalanine in Stable Essential Hypertensive Patients." Hypertension **19**(6): 634-638.

Kwok, R. P. S., Lundbland, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G.E., Green, M.R., and Goodman, R.H. (1994). "Nuclear protein CBP is a coactivator of the transcription factor CREB." Nature **370**: 223-226.

Kwok, R. P. S., Laurance, M.E., Lundblad, J.R., Goldman, P.S., Shih, H.-M., Connor, L.M., Marriott, S.J., and Goodman, R.H. (1996). "Control of cAMP-regulated enhancers by the viral transactivator Tax through CREB and the co-activator CBP." Nature **380**: 642-646.

Lalli, E., and Sassone-Corsi, P. (1994). "Signal Transduction and Gene Regulation: The Nuclear Response to cAMP." J. Biol. Chem. **269**(26): 17359-17362.

Lalli, E., and Sassone-Corsi, P. (1995). "Thyroid-stimulating hormone (TSH)-directed induction of the CREM gene in the thyroid gland participates in the long-term desensitization of the TSH receptor." Proc. Natl. Acad. Sci. **92**: 9633-9637.

Lassen, N.A. (1996). "Epidemiologic Assesment of the Role of Blood Pressure in Stroke." JAMA **276**: 1279-1280.

Lazar, J., Desvergne, B., Zimmerman, E.C., Zimmer, D.B., Magnuson, M.A., and Nikodem, V.M. (1994). "A Role for Intronic Sequences on Expression of Thyroid Hormone Receptor a Gene." J. Biol. Chem. **269**(32): 20352-20359.

Lee, A. L., Yu, A.S., and Lytton, J.L. (1994). "Tissue-specific Expression of Na+-Ca2+ exchanger Isoforms." J. Biol. Chem. **269**(21): 14849-14852.

Lee, M. R. (1982). "Dopamine and the kidney." Clin. Sci. Lond. **62**: 439-448.

Lee, N. H., Earle-Hughes, J., and Fraser, C.M. (1994). "Agonist-mediated Destabilization of m_1 Muscarinic Acetylcholine Receptor mRNA." J. Biol. Chem. **269**(6): 4291-4298.

Lefkowitz, R. (1993). "G Protein-Coupled Receptor Kinases." Cell **74**: 409-412.

Liao, C. F., Themmen, A.P.N., Joho, R., Barberis, C., Birnbaumer, M. and Birnbaumer, L. (1989). "Molecular cloning and expression of a fifth muscarinic acetylcholine receptor." J. Biol. Chem. **264**: 7328-7337.

Lokhandwala, M., and Amenta, F. (1991). "Anatomical distribution and function of dopamine receptors in the kidney." FASEB **5**: 3023-3030.

Loriaux, M. M., Brennan, R.G., and Goodman, R.H. (1994). "Modulatory Function of CREB-CREMα Heterodimers Depends upon CREMα Phosphorylation." J. Biol. Chem. **269**(18): 28839-28843.

Madden, S., Cook, D., Morris, J., Gashler, A., Sukhatme, V., and Rauscher, F. (1991). "Transcriptional Repression Mediated by the WT1 Wilms Tumor Gene Product." Science **253**: 1550-1553.

Mahan, L., Burch, R., Monsma, F., Sibley, D. (1990). "Expression of striatae D_1 dopamine receptors coupled to inositae phosphate production and Ca^{2+} mobilization in *Xenopus* oocytes." Proc. Natl. Acad. Sci. **87**: 2196-2200.

Malik, K. T. A., Wallace, J.I., Ivins, S.M. and Brown, K.W. (1995). "Identification of an antisensw WT1 promoter in intron 1: implications for WT1 gene regulation." Oncogene **11**(8): 1589-1595.

Mansour, A., Meador-Woodruff, J.H., Bunzow, J.R., Civelli, O., Akol, H., Watson, S. (1990). "Localization of the dopamine D_2 receptor mRNA and D_1 and D_2 receptor binding in the rat and pituitary." J. Neurosci. **10**: 2587-2600.

Martin, M. M., and Elton, T.S. (1995). "The Sequence and Genomic Organization of the Human Type 2 Angiotensin II Receptor." Biochem. Biophys. Resch. Comm. **209**(2): 554-562.

McDonald, R. H., Goldberg, L.I., McNay, J.L., Tuttle, E.P. (1964). "Effect of dopamine in man: augmentation of sodium excretion, glomerular filtration rate and renal plasma flow." J. Clin. Invest. **43**: 1116-1124.

Meador-Woodruff, J. H., Mansour, A., Bunzow, J.R., Van Tol, H.H.M., Watson, S.J.Jr., and Civelli, O. (1989). "Distribution of D_2 dopamine receptro mRNA in rat brain." Proc. Natl. Acad. Sci. **86**: 7625-7628.

Meador-Woodruff, J. H., Mansour, A., Healy, D.J., Kuehn, R., Zhou, Q. Y., Bunzow, J.R., Akil, ., Civelli, O., and Watson, S.J. (1991). "Comparison of the distribution of D_1 and D_2 dopamine receptor mRNAs in rat brain." Neuropsychopharmacology **5**: 231-242.

Medcalf, R. L., Ruegg, M. and Schleuning, W. (1990). "A DNA Motif Related to the cAMP-responsive Element and an Exom-located Activator Protein-2 Binding Site in the Human Tissue-type Plasminogen Activator Gene Promoter Cooperate in Basal Expression and Convey Activation by Phorbol Ester and cAMP." J. Biol. Chem. **265**(24): 14618-14626.

Meister, B., Fried, G., Holgert, H., Aperia, A., and Hokfelt, T. (1992). "Ontogeny of aromatic L-amino acid decarboxylase-containing tubule cells in rat kidney." Kidney International **42**: 617-623.

Mengod, G., Matrinez-Mir, M.I., Vilaro, M.T., Palacios, J.M. (1989). "Localization of the mRNA for the dopamine D_2 receptor in the rat brain by in situ hybridization histochemistry." Proc. Natl. Acad. Sci. **86**: 8560-8564.

Mieda, M., Haga, T., and Saffen, D.W. (1996). "Promoter Region of the Rat m_4 Muscarinic Acetylcholine Receptor Gene Contains a Cell Type-specific Silencer Element." J. Biol. Chem. **271**(9): 5177-5182.

Minowa, M. T., Minowa, T., Monsma, F.J., Sibley, D.R., and Mouradian, M.M. (1992). "Characterization of the 5'-flanking region of the human D_{1A} dopamine receptor gene." Proc. Natl. Acad. Sci. **89**: 3045-3049.

Minowa, M. T., Minowa, T., and Mouradian, M.M. (1993). "Activator Region Analysis of the Human D_{1A} Dopamine Receptor Gene." J. Biol. Chem. **268**(31): 23544-23551.

Minowa, T., Minowa, M.T., and Mouradian, M.M. (1992). "Analysis of the Promoter Region of the Rat D_2 Dopamine Receptor Gene." Biochemistry **31**(36): 8389-8396.

Mitchell, P., and Tjian, R. (1989). "Transcriptional Regulation in Mammalian Cells by Sequence-Specific DNA Binding Proteins." Science **245**: 371-378.

Monaco, L., Foulkes, N.s., and Sassone-Corsi, P. (1995). "Pituitary follicle-stimulating hormone (FSH) induces CREM gene expression in Sertoli cells: Involvement in long-term desensitization of the FSH receptor." Proc. Natl. Acad. Sci. **92**: 10673-10677.

Monsma, F., Mahan, L., McVittie, L., Gerfen, C., and Sibley, D. (1990). "Molecular cloning and expression of a D_1dopamine receptor linked to adenylyl cyclase activation." Proc. Natl. Acad. Sci. **87**: 6723-6727.

Monsma, F. J. J., McVittie, L.D., Gerfen, C.R., Mahan, L.C., and Sibley, D.R. (1989). "Multiple D_2 dopamine receptors produced by alternative RNA splicing." Nature **342**: 926-929.

Montminy, M. R., Sevarino, K.A., Wagner, J.A., Mandel, G., Goodman, R.H. (1986). "Identification of a cyclic-AMP-responsive element within the rat somatostatin gene." Proc. Natl. Acad. Sci. **83**: 6682-6686.

Mulligan, G.J., Guo, W., Wormsley, S., Helfman, D.M. (1992). "Polypyrimidine Tract Binding Protein Interacts with Sequences Involved in Alternative Splicing of β-Tropomycin Pre-mRNA". J. Biol. Chem. **267** (35): 25480-25487.

Murasawa, S., Matsubara, H., Kizima, K., Maruyama, K., Mori, Y., Inada, M. (1995). "Glucocorticoids Regulate V1a Vasopressin Receptor Expression by Increasing mRNA Stability in Vascular Smooth Muscle Cells." Hypertension **26**: 665-669.

Muro, A. F., Bernath, V.A., and Kornblihtt, A.R. (1992). "Interaction of the -170 Cyclic AMP Response Element with the Adjacent CCAAT Box in the Human Fibronectin Gene Promoter." J. Biol. Chem. **267**(18): 12767-12774.

Nash, R., Godinot, N., and Caron, M. (1993). "Cloning and Characterization of the Opossum Kidney Cell D_1 Dopamine Receptor: Expression of Identical D_{1A} and D_{1B} Dopamine Receptor mRNAs in Opossum Kidney and Brain." Mol. Pharm. **44**: 918-925.

Ng, G., Mouillac, B., George, S., Caron, M., Dennis, M., Bouvier, M., and O'Dowd, B. (1994). "Desensitization, phosphorylation and palmitoylation of the human dopamine D_1 receptor." Eur. J. Pharm. **267**: 7-19.

Ng, G., Trogadis, J., Stevens, J., Bouvier, M., O'Dowd, B.F., and George, S.R. (1995). "Agonist-induced desensitization of dopamine D_1 receptor-stimulated adenylyl cyclase activity is temporally and biochemically separated from D_1 receptor internalization." Proc. Natl. Acad. Sci. **92**: 10157-10161.

Nordheim, A. (1994). "CREB takes CBP to tango." Science **370**: 177-178.

Norton, P.A. (1994). "Polypyrimidine tract sequences direct selection of alternative branch sites and influence protein binding." Nucleic Acids Res. **22**(19): 3854-3860.

Nusing, R. M., Hirata, M., Kakizuka, A., Eki, T., Ozawa, K., and Narumiya, S. (1993). "Characterization and Chromosomal Mapping of the Human Thromboxane A2 Receptor Gene." J. Biol. Chem. **268**(23): 25253-25259.

O'Malley, K. L., Mack, K.J., Gandelman, K., and Todd, R.D. (1990). "Organization and Expression fo the Rat D_{2A} Receptor Gene: Identification of Alternative Transcripts and a Variant Donor Splice Site." Biochemistry **29**: 1367-1371.

Oelgeshlager, T., Chiang, C., and Roeder, R.G. (1996). "Topology and reorganization of a human TFIID-promoter complex." Nature **382**: 735-738.

Oetjen, E., Diedrich, T., Eggers, A., Eckert, B., and Knepel, W. (1994). "Distinct Properties of the cAMP-responsive Element of the Rat Insulin I Gene." J. Biol. Chem. **269**(43): 27036-27044.

Ofori, S.,. B, O. and Schorderet, M. (1993). "Agonist-Induced Desensitization of Dopamine D-1 Receptors in Bovine Retina and Rat Striatum." J. Pharm. and Exp. Ther. **266**(1): 350-357.

Ohbu, K., and Felder, R.A. (1993). "Nephron specificity of dopamine receptor-adenylyl cyclase defect in spontaneous hypertension." Am. J. Physiol. **264**: F274-F279.

Ohbu, K., Kaskel,F., Kinoshita, S., and Felder, R. (1995). "Dopamine-1 receptors in the proximal convoluted tubule of Dahl rats: defective coupling to adenylate cyclase." Am. J. Physiol. **268**: R231-R235.

Oliveria, C.C., and McCarthy, J.E.G. (1995). "The Relationship between Eukaryotic Translation and mRNA Stability." ." J. Biol. Chem. **270** (15): 8936-8943.

Onali, P., Olianes, M.C., and Gesa, G.L. (1985). "Characterization of dopamine receptors mediating inhibition of adenylate cyclase activity in rat striatum." Mol. Pharmacol. **28**: 138-145.

Osawa, H., Robey, R.B., Printz, R.L., and Granner, D.K. (1996). "Identification and Characterization of Basal and Cyclic AMP Response Elements in the Promoter of the Rat Hexokinase II Gene." J. Biol. Chem. **271**(29): 17296-17303.

Pang, J. S., Hung, R., Wu, C., Fang, Y. and Chau, L. (1995). "Functional Characterization of the Promoter Region of the Platelt-activating Factor Receptor Gene: Identification of an initiator element essential for gene expression in myeloid cells." J. Biol. Chem. **270**(23): 14123-14129.

Parker, D., Ferreri, K., Nakajima, T., LaMorte, V.J., Evans, R., Koerber, S.C., Hoeger, C., and Montminy, M.R. (1996). "Phosphorylation of CREB at Ser-133 Induces Complex Formation with CREB-Binding Protein via a Direct Mechanism." Mol.Cell Biol. **16**(2): 694-703.

Parola, A.L., and Kobilka, B.K. (1994). "The Peptide Product of a 5' Leader Cistron in the b2 Adrenergic Receptor mRNA Inhibits Receptor Synthesis." ." J. Biol. Chem. **269**(6): 4497-4505.

Pecorino, L. ;. D.. A. and Strickland, S. (1991). "In Vitro Analysis of the Tissue Plasminogen Activator Promoter Reveals a GC Box-Binding Activity Present in Murine Brain but Undetectable in Kidney and Liver." Mol. Cell. Bio. **11**(6): 3139-3147.

Pei, G., Samama, P., Lohse, M., Wang, M., Codina, J., and Lefkowitz, R.J. (1994). "A constitutively active mutant β_2-adrenergic receptor is constitutively desensitized and phosphorylated." Proc. Natl. Acad. Sci. **91**: 2688-2702.

Pelayo, J. C., Fildes, R.D., Eisner, G.M., and Jose, P.A. (1983). "Effects of dopamine blockade on renal sodium excretion." Am. J. Physiol. **245**: F247-F253.

Pelletier, D., Schalling, M., Buckler, A., Rogers, A., Haber, D., and Housman, D. (1991). Genes and Devel. **5**: 1345-1356.

Pende, A., Tremmel, K.D., DeMaria, C.T., Blaxall, B.C., Minobe, W.A., Sherman, J.A., Bisognano, J.D., Bristow, M.R., Brewer, G., and Port, J.D. (1996). "Regulation of the mRNA-binding Protein AUF1 by Activation of the β-Adrenergic Receptor Signal transduction Pathway." J. Biol. Chem. **271**(14): 8493-8501.

Pesquero, J. B., Lindsey, C.J., Zeh, K., Paiva, A.C.M., Ganten, D. and Bader, M. (1994). "Molecular structure and espression of rat bradykinin B_2 receptor gene." J. Biol. Chem. **269**(43): 26920-26925.

Pestell, R., Hollenberg, A., Albanese, C., and Jameson, J. (1994). "c-Jun Represses Transcription of the Human Chorionic Gonadotropin α and β Genes through Distinct Types of CREs." J. Biol. Chem. **269**(49):

Proll, M., Clark, R. and Butcher, R. (1993). "β_2-Adrenergic Receptor Mutants Reveal Structural Requirements for the Desensitization Observed with Long Term Epinephrine Treatment." Mol. Pharm. **44**: 569-574.

Ren, H., and Stiles, G.L. (1994). "Characterization of the Human A_1 Adenosine Receptor Gene." J. Biol. Chem. **269**(4): 3104-3110.

Ricci, A., Escaf, S., Vega, J.A., and Amenta, F. (1992). "Autoradiographic Localization of Dopamine D_1 Receptors in the Human Kidney." J. Pharm. Exp. Ther. **264**(1): 431-437.

Richards, J. P., Bachinger, H.P., Goodman, R.H., and Brennan, R.G. (1996). "Analysis of the Structural Properties of cAMP-responsive Element-binding Protein (CREB) and Phosphorylated CREB." J. Biol. Chem. **271**(23): 13716-13723.

Rodgers, J. R., Johnson, M.L., and Rosen, J.M. (1985). "Measurement of mRNA Concentration and mRNA Half-life as a Function of Hormonal Treatment." Methods in Enzymology **109**: 572-592.

Roesler, W. J., Vandenbark, G.R., and Hanson, R.W. (1988). "Cyclic AMP and the Induction of Eukaryotic Gene Transcription." J. Biol. Chem. **263**(19): 9063-9066.

Roesler, W. J., Graham, J.G., Kolen, R., Klemm, D.J., McFie, P.J. (1995). "The cAMP Response Element Binding Protein Synergizes with other Transcription Factors to Mediate cAMP Responsiveness." Journal of Biological Chemistry **270**(14): 8225-8232.

Roesler, W. J., Crosson, S.M., Vinson, C., and McFie, P.J. (1996). "The a-Isoform of the CCAAT/Enhancer-binding Protein is Required for Mediating cAMP Responsiveness of the Phosphoenolpyruvate Carboxykinase Promoter in Hepatoma Cells." J. Biol. Chem. **271**(14): 8068-8074.

Rousell, J., Haddad, E.B., Mak, J.C.W., and Barnes, P.J. (1995). "Transcriptional Down-regulation of m_2 Muscarinic Receptor Gene Expression in Human Embryonic Lung (HEL 299) Cells by Protein Kinase C." Journal of Biological Chemistry **270**(13): 7213-7218.

Rozen, F., Russo, C., Banville, D., and Zingg, H.H. (1995). "Structure, characterization, and expression of the rat oxytocin receptor gene." Proc. Natl. Acad. Sci. **92**: 200-204.

Sambrook, J., Fritsch, E.R., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual." **2nd.Ed.**(Cold Spring Harbor Laboratory): Cold Spring Harbor, NY.

Sassone-Corsi, P. (1994). "Goals for signal transduction pathways: linking up with transcriptional regulation." EMBO Journal **13**(20): 4717-4728.

Selbie, L. A., Hayes, G., and Shine, J. (1989). "The major dopamine D_2 receptor: Molecular analysis of the human D_{2A} subtype." DNA **8**: 683-689.

Shapiro, L. H., Ashmun, R. A., Roberts, W.M. and A. T. Look (1991). "Separate Promoters Control Transcription of the Human Aminopeptidase N Gene in Myeloid and Intestinal Epitheleial Cells." J. Biol. Chem. **266**(18): 11999-12007.

Shaskus, J., Greco, D., Asnani, L.P., and Lewis, E.J. (1992). "A Bifunctional Genetic Regulatory Element of the Rat Dopamine β-Hydroxylase Gene Influences Cell Type Specificity and Second Messenger-mediated Transcription." J. Biol. Chem. **267**(26): 18821-18830.

Shingu, T., and Bornstein, P. (1994). "Overlapping Egr-1 and Sp1 Sites Function in the Regulation of Transcription of the Mouse Thrombospondin 1 Gene." J. Biol. Chem. **269**(51): 32551-32557.

Shoshani, B., N., Trus,M., and Reshef, L. (1990). "Regulatory elements that confer differential expression upon the rat gene encoding phosphoenolpyruvate carboxykinase in kidney and liver." Gene **101**(2): 279-283.

Silver, B. J., Bokar, J.A., Virgin, J.B., Vallen, E.A., Milsted, A., and Nilson, J.H. (1987). "Cyclic AMP regulation of the human glycoprotein hormone α-subunit gene is mediated by an 18-base-pair element." Proc. Natl. Acad. Sci. **84**: 2198-2202.

Siragy, H. M., Felder, R.A., Howell, N.L., Chavalier, R.L., Peach, M.J., and Carey, R.M. (1989). "Evidence that intrarenal dopamine acts as a paracrine substance at the renal tubule." Am. J. Physiol. **257**: F469-F477.

Sokoloff, P., Giros,B., Matres, M., Bouthenel, M., and Schwartz, J. (1990). "Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics." Nature **347**: 146-150.

Sommerville, J., and Ladomery, M. (1996). "Masking of mRNA by Y-box proteins." FASEB J. **10**: 435-443.

Spiro, C., Bazett-Jones, D.P., Wu, X., and McMurray, C.T. (1995). "DNA Structure Determines Protein Binding and Transcriptional Efficiency of the Proenkephalin cAMP-responsive Enhancer." J. Biol. Chem. **270**(17): 27702-27710.

Stargell, L. A., and Struhl, K. (1996). "Mechanisms of transcriptional activation *in vivo*: two steps forward." TIG **12**(8): 311-315.

Stoecklin, G., Hahn, S., and Moroni, C. (1994). "Functional Hierarchy of AUUUA Motifs in Mediating Rapid Interleukin-3 mRNA Decay." J. Biol. Chem. **269**(46): 28591-28597.

Sugamori, K., Demchyshyn, L., Chung, M., and Niznik, H. (1994). "D_{1A}, D_{1B}, and D_{1C} dopamine receptors from *Xenopus laevis*." PNAS **91**: 10536-10540.

Sun, D., and Schafer, J.A. (1996). "Dopamine inhibits AVP-dependent Na+ transport and water permeability in rat CCD via a D4-like receptor." Am. J. Physiol. **271**: F391-F400.

Sun, Z. a. M., A.R. (1995). "An Intron Facilitates Activation of the Calspermin Gene by Testis-specific Transcription Factor CREMτ." J. Biol. Chem. **270**(36): 20962-20967.

Sunahara, R., Niznik, H., Weiner, D., Stormann, T., Brann, M., Kennedy, J., Gelernter, J., Rozmahel, R., Yang, Y., Isreal, Y., Seeman, P., and O'Dowd, B. (1990). "Human dopamine D_1 receptor encoded by an intronless gene on chromosome 5." Nature **347**: 80-83.

Sunahara, R. K., Guan, H.C., O'Dowd, B.F., Seeman, P., and Laurier, L.G. (1991). "Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1." Nature **350**: 614-619.

Suryanarayana, S., von Zastrow, M., and Kobilika, B. (1992). "Identification of Intramolecular Interactions in Adrenergic Receptors." J. Biol. Chem. **267**(31): 21991-21994.

Suzuki, H., Nakane, H., Kawamura, M., Yoshizawa, M., Takeshita, E., and Saruta, T. (1984). "Excretion and metabolism of dopa and dopamine by isolate perfused rat kidney." Am. J. Physiol. **247**: E285-E290.

Takayanagi, R. O., K., Sakai, Y. Ikuyama, S. and Nawata, H. (1994). "Molecular Cloning and Characterization of the promoter for Human Type-1 Angiotensin II Receptor Gene." Biochm. Biophys. Res. Comm. **200**(3): 1264-1270.

Takeuchi, K., Alexander, R.W., Nakamura, Y., Tsujino, T., and Murphey, T.J. (1993). "Molecular Structure and Transcriptional Function of the Rat Vascular AT_{1a} Angiotensin Receptor Gene." Circ Res. **73**: 612-621.

Tamura, K., Umemura, S., Ishii, M., Tanimoto, K., Murakami, K. and Fukamizu, A. (1994). "Mechanism of cAMP Regulation of Renin Gene Transcription by Proximal Promoter." J. Clin. Invest. **94**: 1959-1967.

Tamura, K., Umemura, S., Ishii, M., Tanimoto, K., Murakami, K., and Fukamizu, A. (1994). "Molecular Mechanism of Transcriptional Activation of Angiotensinogen Gene by Proximal Promoter." J. Clin. Invest. **93**: 1370-1379.

Tholanikunnel, B. G., Granneman, J.G., and Malbon, C.C. (1995). "The Mr 35,000 β-Adrenergic Receptor mRNA-binding Protein Binds Transcripts of G-protein-linked Receptros Which Undergo Agonist-induced Destabilization." J. Biol. Chem. **270**(21): 12787-12793.

Thomas, R. F., Holt, B.D., Schwinn, D.A., and Liggett, S.B. (1992). "Long-term agonist exposure induces upregulation of β_3-adrenergic receptor expression via multiple cAMP response elements." Proc. Natl. Acad. Sci. **89**: 4490-4494.

Thomas, W. L., Cooke, E.S. and Hammer, R.P. (1996). "Cocaine-Induced Sensitization of Metabolic Activity in Extrapyramidal circuits Involves Prior Dopamine D1-Like Receptor Stimulation." J. Pharmacol. Exp. Ther. **278**: 347-353.

Tiffany, H. L., Handen, J.S., and Rosenberg, H.F. (1996). "Enhanced Expression of the Eosinophil-derived Neurotoxin Ribonuclease (RNS2) Gene Requires Interaction between the Promoter and Intron." J. Biol. Chem. **271**(21): 12387-12393.

Tugores, A., Magness, S., and Brenner, D. (1994). "A Single Promoter Directs Both Housekeeping and Erythroid Preferential Expression of the Human Ferrochelatase Gene." J. Biol. Chem. **269**(49): 30789-30797.

Tverberg, L. A., and Russo, A.F. (1993). "Regulation of the Calcitonin/Calcitonin Gene-related Peptide Gene by Cell-specific Synergy between Helix-Loop-Helix and Octamer-binding Transcription Factors." J. Biol. Chem. **268**(21): 15965-15973.

Unterwald, E. M., Ho, A., Rubenfeld, J.M., and Kreek, M. (1994). "Time Course of the Development of Behavioral Sensitization and Dopamine Receptor Up-Regulation During Binge Cocaine Administration." **270**(3): 1387-1397.

Valiqutte, M., Bonin, H., Hnatowich, M., Caron, M., Lefkowitz, R., and Bouvier, M. (1990). "Involvement of tyrosine residues in the carboxyl tail of the human β_2-adrenergic receptor in agonist-induced down-regulation of the receptor." Proc. Natl. Acad. Sci. **87**: 5089-5093.

Venepally, P., and Waterman, M.R. (1995). "Two Sp1-binding Sites Mediate cAMP-induced Transcription of the Bovine CYP11A Gene through the Protein Kinase A Signaling Pathway." J. Biol. Chem. **270**(43): 25402-25410.

Walker, H. W., Girardet, C., and Habener, J.F. (1996). "Alternative Exon splicing Controls a Translational Switch from Activator to Repressor Isoforms of Transcription Factor CREB during Spermatogenesis." J. Biol. Chem. **271**(33): 20145-21050.

Walker, W. H., Sanborn, B.M., and Habener, J.F. (1994). "An isoform of transcription factor CREM expressed during spermatogenesis lacks the phosphorylation domain and represses cAMP-induced transcription." Proc. Natl. Acad. Sci. **91**: 12423-12427.

Wandersee, N. J., Ferris, R.C., and Ginde, G.D. (1996). "Intronic and Flanking Sequences are Required to Silence Enhancement of an Embryonic β-Type Globin Gene." Mol. Cell. Biology **16**(1): 236-246.

Wang, Z., Way, D., Shimizu, K., Fong, F., Trigg, L., and McGrath, B.P. (1993). "Beneficial Acute Effects of Selective Modulation of Renal Dopamine System by γ-L-Glutamyl-L-Dopa in Rabbits with Congestive Heart Failure." J. of Cardiovascular Pharm. **21**: 1004-1011.

Wiley, S. R., Kraus, R.J., and Mertz, J.E. (1992). "Functional binding of the "TATA" box binding component of transcription factor TFIID to the -30 region of TATA-less promoters." Proc. Natl. Acad. Sci. **89**: 5814-5818.

Williams, J. S., Dixon, J.E., and Andrisani, O.M. (1993). "Binding Constant Determination Studies Utilizing Recombinant ΔCREB Protein." DNA and Cell Biol. **12**(2): 183-190.

Woolfson, R. G., and De Wardener, H.E. (1996). "Primary renal abnormalities in hereditary hypertension." Kidney International **50**: 717-731.

Wright, K. L., Moore, T.L., Vilen, B.J., Brown, A.M., and Ting, J.P.Y. (1995). "Major Histocompatability complex class II-associated Invariant Chain Gene Expression is Up-regulated by Cooperative Interactions of SP1 and NF-Y." Journal of Biological Chemistry **270**(36): 20978-20986.

Xiang, L., Gopal, V.K., and Quinn, P.G. (1995). "cAMP Response Element-binding Protein (CREB) Interacts with Transcriptional Factors IIB and IID." J. Biol. Chem. **270**(29): 17488-17493.

Xu, J., Thompson, K., Shephard, L., Hudson, L., and Gill, G. (1993). "T3 Receptor Suppression of SP1-dependent Transcription from Epidermal Growth Factor Receptro Promoter via Overlapping DNA-binding Sites." J. Biol. Chem. **268**(21): 16065-16073.

Yamaguchi, I., Jose, P., Mourdanian, M., Canessa, L., Monsma, F., Sibley, D., Takeyasu, K., and Felder, R. (1993). "Expression of dopamine D_{1A} receptor gene in proximal tubule of rat kidneys." Am. J. Physiol. **264**: F280-F285.

Yang, J. ;. W., J.A.; Yule, D.I. and Logsdon, C.D. (1995). "Mutation of Carboxyl-Terminal Threonine Residues in Human m_3 Muscarinic Acetylcholine Receptor Modulates the Extent of Sequestration and Desensitization." Mol. Pharm. **48**: 477-485.

Yasunari, K., Kohno, M., Yokokawa, K., Horio, T., and Takeda, T. (1994). "Dopamine D_{A1} receptors on vascular smooth muscle cells are regulated by glucocorticoid and sodium chloride." Am. J. Physiol. **267**: R628-R634.

Yuan, N., Friedman, J., Whaley, B., and Clark, R. (1994). "cAMP-dependent Protein Kinase and Protein Kinase C Consinsus Site Mutations of the β-Adrenergic Receptor." J. Biol. Chem. **269**: 23032-23038.

Zhou, Q., Grandy, D., Thambi, L., Kushner, J., Van Tol, H. Cone, R., Pribnow,D., Salon, J., Bunzow, J., and Civelli, O. (1990). "Cloning and expression of human and rat D_1 dopamine receptors." Nature **347**: 76-79.

Zhou, Q., Li,C., and Civelli, O. (1992). "Characterization of Gene Organization and Promoter Region of the Rat Dopamine D_1 Receptor Gene." J. Neurochemistry **59**: 1875-1883.