Transcriptional Regulation of the Endothelin A Receptor: Possible Developmental and Pathophysiological Implications

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A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Institute of Medical Science University of Toronto

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Our laboratory has described two regulatory elements in the ET\_ promoter. Gel shift analysis revealed that a component of P19 cell nuclear extract binds to an ET\_ promoter fragment containing a putative AP-2 binding site. Using a firefly luciferase reporter gene system I have shown that AP-2 upregulates ET\_ promoter activity in a concentration-dependent manner. Decoy transfection of ET\_ (+86 \to +215) into A10 cells led to a 5-fold decrease in ET\_ mRNA expression. I also found that angiotensin II (AII) treatment of A10 cells led to a 1.9-fold increase in ET\_ mRNA expression. Furthermore, AII treatment of A10 cells transfected with several ET\_-firefly luciferase constructs led to a 1.3-1.8-fold increase in luciferase activity. These experiments demonstrate that both AP-2 and AII are able to upregulate ET\_ mRNA levels by activating one or several ET\_ regulatory elements.
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List of Abbreviations

ACE- Angiotensin converting enzyme
AII - Angiotensin II
ANP- atrial natriuretic peptide
AT-1- angiotensin receptor 1
AT-2- angiotensin receptor 2
CAT- chloramphenicol acetyl transferase
cGMP- cyclic guananyl monophosphate
CHF- congestive heart failure
CIAP- calf intestinal alkaline phosphatase
CMV- cytomegalovirus
DAG- diacylglycerol
DDT- dichlorodiphenyltrichloroethane
DMEM- Dulbecco's modified eagles medium
DMSO- dimethyl sulfoxide
ECE- Endothelin converting enzyme
EDTA- ethylene diamine tetraacetic acid
ET-1- endothelin 1
ET-2- endothelin 2
ET-3- endothelin 3
ET\textsubscript{A}- Endothelin A receptor
ET\textsubscript{B}- Endothelin B receptor
FBS- fetal bovine serum
GAPDH-glyceraldehyde-3-phosphate dehydrogenase

HBSS- hepes buffered saline solution

IP₃- inositol-1,4,5-triphosphate

MAPK- mitogen activated protein kinase

MMP-2- matrix metalloproteinase-2

NBS- newborn bovine serum

NO- nitric oxide

NOS- nitric oxide synthase

PBS- phosphate buffered saline

PIP₂- phosphatidylinositol 4,5-biphosphate

PKC- protein kinase C

PLA₂- phospholipase A₂

PLC- phospholipase C

PLD- phospholipase D

PMA- phorbol 12-myristate 13-acetate

PMSF- phenylmethylsulfonyl fluoride

P/S- penicillin/streptomycin

ppET- preproendothelin

RA- retinoic acid

RT-PCR- reverse transcription polymerase chain reaction

STS- staurosporine

SV40- simian viral promoter

TIMP- 2 matrix metalloproteinase-2 tissue inhibitor
TIS - transcription initiation site

UTR- untranslated region

VIC- vasoactive intestinal constrictor

VSMC- vascular smooth muscle cells
**Introduction**

**Endothelin Peptides**

*Background*

In 1984 it was observed that bovine pulmonary artery strips contracted when treated with conditioned medium from bovine aortic endothelial cell culture (O'Brien and McMurry, 1984). It was later shown that a long-lasting increase in tension of isolated coronary arteries upon exposure to endothelial cell conditioned-medium could be blocked by treatment with peptidases, suggesting that the causative agent was peptide derived (Hickey et al., 1985). Yanagisawa and colleagues (1988) isolated a peptide from porcine aortic endothelial cell conditioned-medium responsible for the vasoconstrictor response which was termed, endothelin (ET) (Yanagisawa et al., 1988). Porcine endothelin is a 21 amino acid peptide with a molecular weight of 2942 daltons, a free N- and C terminus and 2 cysteine intrachain disulphide bridges (Yanagisawa et al., 1988). A helix-like region dominates the central portion of the molecule between residues lysine(9) and cysteine(15) which encompass both disulfide bonds. Screening of human, rat and porcine genomic libraries with a portion of the porcine endothelin gene revealed three endothelin isoforms (ET-1, ET-2 and ET-3) all derived from separate gene products and localized to separate chromosomal loci (Bloch et al., 1989; Inoue et al., 1989; Arinami et al., 1991). Furthermore, the mature endothelin peptides were shown to have a high degree of sequence similarity between species. (porcine and human ET-1 are identical), with the venom of a poisonous Israeli burrowing asp *Atractaspis engadensis* (Wollberg et al., 1988), and a vasoactive intestinal constrictor (VIC) found in the mouse (Saida et al., 1989) (Figure 1.).
Endothelin Biosynthesis

The biosynthesis of all three endothelins are extremely similar to each other. They are derived from separate, single-copy genes which encode for a prepropeptide, preproendothelin (ppET) (Yanagisawa and Masaki, 1989; Inoue et al., 1989; Kimura et al., 1990). Preproendothelin contains a hydrophobic secretory sequence at its N-terminus suggesting that it might be transported across the endoplasmic reticular membrane for processing (Fabbrini et al., 1991). The 203/212 peptide (porcine and human respectively) is cleaved at the Lys\textsuperscript{51}-Arg\textsuperscript{52} and Lys\textsuperscript{50}-Arg\textsuperscript{51} residues by furin convertase, a prohormone processing enzyme which cycles in transport vesicles between the cell surface and the trans-Golgi network, cleaving proendothelin in transit and at the cell surface (Denault et al., 1995; Barnes et al., 1998). This results in a 39/38 amino acid intermediate product (porcine and human respectively) named big ET (1,2 and 3). The big ETs are cleaved by specific amino acid metalloprotease, endothelin converting enzymes (ECE, of which there are several isoforms), at Trp\textsuperscript{21}-Val\textsuperscript{22} or Trp\textsuperscript{21}-Ile\textsuperscript{22} to form the mature 21 amino acid peptides ET-1. 2 or 3 (Opgenorth et al., 1992) (Figure 1). In plasma, big ET-1 is found in higher concentrations than the mature peptide, suggesting that big ET-1 is secreted before its conversion to mature ET-1 (Suzuki et al., 1990; Yamaji et al., 1990). Nonetheless, ET-1 is a 140-fold more potent vasoconstrictor than the intermediate peptide.

All three endothelin isoforms exhibit a high degree of conservation in the region encoding the mature 21 amino acid endothelin, with little conservation in the region encoding the prepropeptides (Inoue et al., 1989). The mRNA of all three isoforms is expressed in different proportions in several cell types although only the ET-1 isoform is expressed by endothelial cells (Inoue et al., 1989). Preproendothelin-1 mRNA can be found in many tissues including the heart, kidney, liver, adrenals, posterior pituitary and central nervous system (Simonson and Dunn,
1990; Yanagisawa and Masaki, 1989; Haynes and Webb, 1998). Preproenothelin-1 mRNA has been identified in cardiac myocytes, renal tubular cells, epithelium, glomerular mesangium, glia, the pituitary, macrophages, and mast cells (Miyauchi and Masaki, 1999). Furthermore, ppET-1 mRNA is expressed in vascular smooth muscle cells (VSMC) at 100-fold less expression than in endothelial cells. ET-1 is produced de novo and not stored intracellularly, as it could only be detected 30 minutes after stimulation with thrombin, and the protein synthesis inhibitor cycloheximide prevented the release of ET by endothelial cells and aortic strips (Yanagisawa et al., 1988). It is secreted abluminally (from the endothelium to the vascular media), and observed circulating plasma ET is most likely attributable to spillover into the lumen of the vasculature.

**Endothelin Converting Enzyme**

Rat ECE was originally described in endothelial cells as a 758 amino acid, highly glycosylated type II, integral membrane neutral metalloprotease with a single transmembrane domain near the N-terminus (Wu-Wong, et al., 1990; Takahashi et al., 1993; Shimada et al., 1995). There are several isoforms of ECE which are responsible for cleaving big endothelin into the mature 21 amino acid peptide. ECE-1 mRNA is expressed in many tissues and consists of several isoforms. ECE-1 has both an α and β isoform which differ primarily in the last 28 amino acids of their N-terminus possibly as a result of alternate RNA splicing (Yorimitsu et al., 1995; Shimada et al., 1995). ECE-1α mRNA which is highly conserved among bovine, human and rat species, is abundantly expressed in the lung and adrenals. Overall it is more abundant than ECE-1β (Shimada et al., 1995). Nonetheless, both isoforms exhibit identical specific activity, substrate specificity and subcellular localization. Structurally, ECE-1α and β have identical C-terminal regions which contain the catalytic domain. This would explain the identical, non-selective
activity of ECE-1 α and β. ECE-1 has been localized to endothelial cells, is clustered along the luminal surface of the plasma membrane, and is internalized by endosomes and redistributed to the Golgi (Barnes et al., 1996). Furthermore, in porcine lung, ECE and big ET-1 have been co-localized to the endothelial cell membrane by immunogold labelling (Barnes et al., 1998). A significant amount of ECE-1 is located inside the cell, suggesting that a proportion of big ET-1 is processed intracellularly and then delivered to the cell surface by a constitutive secretory pathway. ECE-1 is regulated in a negative feedback manner, as treatment with 10^{-7}M ET-1 has been shown to downregulate ECE-1 in rat vascular endothelial cells (Naomi, 1998).

The absence of ECE-1 in several ET secreting cells led to the discovery of ECE-2 (Emoto and Yanagisawa, 1995). ECE-2, a 787 amino-acid peptide, is derived from a different gene than ECE-1, but is 59% identical to ECE-1. It was cloned from bovine adrenal cortex and endothelial cell cDNA libraries and studied by transfection in CHO-K1 cells (which do not express ECE). ECE-2 is primarily expressed in the neural tissues such as the cerebral cortex, cerebellum and adrenal medulla, while smaller amounts are found in many other tissues. Nonetheless, ECE-2 mRNA expression was found to be several fold lower than ECE-1 in all tissues studied. Similar to ECE-1, ECE-2 is more efficient in processing big ET-1 than ET-2 or 3. Both enzymes are highly dependent on intracellular pH for their activity; ECE-1 is active at a pH of 6.8, while ECE-2 is functional at 5.5 (Emoto and Yanagisawa, 1995). It has therefore been postulated that ECE-2 is responsible for converting big ET on the lumen of the trans-Golgi network whose pH ranges from 5.5-5.7. Furthermore, ECE-2 is only able to convert endogenously produced big ET-1 as opposed to an exogenous source of big ET-1.
**Endothelin Receptors**

**Expression**

The ET peptides bind to at least two distinct ET receptors with differing affinity. The ET<sub>a</sub> receptor binds ET<sub>1-2>ET<sub>2>ET<sub>3</sub>, while the ET<sub>b</sub> receptor binds all three isopeptides with equal affinity (Masaki et al., 1994). ET<sub>a</sub> has a large extracellular N terminus which recognizes both the carboxyl and amino termini of its ET ligand (Arai et al., 1990). ET<sub>b</sub> however, recognizes only its ligand’s carboxyl terminus (Tamirisa et al., 1995).

The receptors are differentially expressed. Northern analysis in human tissues shows strongest expression of ET<sub>a</sub> mRNA in the aorta and cerebellum and strong expression in the lung, atrium, and cerebral cortex. Little transcript was found in the placenta, kidney, adrenal gland, duodenum, colon, ventricle and liver (Miyamoto et al., 1996). On a cellular level, ET<sub>a</sub> mRNA is abundantly expressed in vascular smooth muscle cells, and mesangial cells (Takeda et al., 1994; Hiraoka et al., 1995). Although ET<sub>a</sub> expression has been demonstrated in some endothelial cell types in-vivo (Nishimura et al., 1995), ET<sub>a</sub> expression has not been identified in human umbilical vein endothelial cells (HUVECs). ET<sub>b</sub> on the other hand, is expressed in the stomach, uterus, brain, adrenals, and kidney, and is the predominant (if not only) endothelin receptor subtype expressed in vascular endothelial cells. It is not, however, expressed in the spleen, testis, aorta or A10 cells (Sakurai et al., 1990). Nonetheless, ET<sub>b</sub> has also been described on VSMCs in some vascular territories and has been shown to mediate contraction (Owe-Young et al., 1999). This suggests that although the receptors are ligand specific, the downstream effects of endothelin ligand-receptor interaction are primarily dependent on the subsequent signal transduction mechanism and availability of secondary messengers following ligand binding.

Studies have shown that pluripotential embryonal carcinoma cells (P19) undergo distinct
differentiation pathways when exposed to given concentrations of either dimethyl sulfoxide (DMSO) or retinoic acid (RA). Exposure of P19 aggregates to 1% DMSO resulted in a phenotypical change to cells that morphologically resemble fibroblast-like cells and contractile myocytes. (McBurney et al., 1982). Furthermore, electron microscopy revealed cells with thin and thick filaments organized into mature myofibrils, numerous mitochondria and glycogen granules. Molecular analysis of these cells also demonstrated the expression of muscle specific myosin and α-actin, clearly suggesting a DMSO induced change in P19 cell aggregates to a myocyte lineage (McBurney et al., 1983). On the other hand, P19 aggregates exposed to \( \geq 10^{-7} \text{M} \) RA morphologically resembled nerve cells, and expressed tetanus toxin binding sites, nerve filaments in the neurons and elevated levels of choline acetyltransferase and choline acetylcholinesterase. Consequently, others have demonstrated that DMSO-treated P19 cells predominantly express ET\(_4\) receptors as is typical of myocytes, whereas RA-differentiated P19 cells express predominantly ET\(_2\), as is the case with neural cells (Monge et al., 1995).

\textit{Cloning}

The ET\(_4\) receptor gene was obtained through expression-cloning of a bovine lung cDNA library in \textit{Xenopus} oocytes which exhibited a strong electrophysiological response to ET-1 and 2, and a moderate response to ET-3 (Arai et al., 1990). The cloned transcript was approximately 3.2 kb coding for a 48.5 kd, 427 amino-acid peptide with seven hydrophobic segments, similar to other G-protein coupled receptors. Northern analysis also revealed a 4.2 kb band, suggesting the existence of multiple ET\(_4\) transcripts (Arai et al., 1990). Later studies confirmed these results and revealed two alternate ET\(_4\) transcripts through RNase protection analysis (Miyamoto et al., 1996). These included transcripts with deletions of the fourth exon, and the third and fourth
exons respectively. The transcript lacking the fourth exon did not translate into a functional ET<sub>\lambda</sub> receptor since the deletion of exon 4 lead to a frameshift which results in the premature termination of ET<sub>\lambda</sub> synthesis. The transcript with a deletion of the third and fourth exons, however, resulted in a translated 318 amino acid-long ET<sub>\lambda</sub> receptor with five membrane spanning domains, two cytoplasmic loops, two extracellular loops and a cytoplasmic C-terminal tail. Nonetheless, no specific binding activity was attributed to the receptor generated from this transcript (Miyamoto <i>et al.</i>, 1996).

<i>Organization of the ET<sub>\lambda</sub> Gene</i>

The ET<sub>\lambda</sub> gene is a 40 kb, single copy gene, containing eight exons and seven introns (Figure 2). Analysis of the human ET<sub>\lambda</sub> gene revealed that exon 1 encodes the 5' untranslated region (UTR). Exon 2 extends 71 bp upstream from the initiation codon and encodes the amino terminus, the first intracellular loop, the first and second membrane-spanning domains and part of the first extracellular loop. Exon 3 encodes the remainder of the first extracellular loop, the third membrane-spanning domain, and part of the second intracellular loop. Exon 4 encodes the remainder of the second intracellular loop, the fourth membrane-spanning domain and the second extracellular loop. The fifth exon encodes the fifth membrane-spanning domain and the third intracellular loop. The sixth exon encodes the sixth membrane-spanning domain and the third extracellular loop. Exon seven encodes the seventh membrane-spanning domain and, and exon eight encodes the cytoplasmic carboxyl terminal domain and the 3' non-coding region (Hosoda <i>et al.</i>, 1991).

The ET<sub>\lambda</sub> transcription initiation site (TIS), as determined by primer extension experiments, is 502 bp upstream of the translational start site. The 5' flanking region lacks
TATA and CAAT boxes, but contains a putative SP-1 binding site 27 bp upstream of the TIS. (Hosoda et al., 1991).

Regulatory Elements in the 5' Region of the ET, Gene

Transcriptional efficiency is dependent on several key factors. These include the tertiary structure of the genomic template, the presence of regulatory elements within the genome, and the availability of specific transcription factors and co-activators utilized in the transcription process. Each of these conditions determines when and how transcription will occur, playing a role in assembling the overall transcription complex. (Silver, 1991; Kornberg and Lorch, 1992; Workman and Buchman, 1993; Buratowski, 1994; Gehring et al., 1994; Kohtz et al., 1994)

Consensus sequences within the gene are referred to as putative cis elements. Cis elements are typically located within several hundred bases of the transcription initiation site. Nonetheless, cis elements located at much greater distances may be transcriptionally functional as well. Cis elements bind trans-acting factors which act as a tether to sequester other factors needed for transcription (Gehring et al., 1994). The area surrounding the transcription initiation site is referred to as the promoter, whereas elements located in the introns, 5' flanking and untranslated region that influence transcription are called enhancer elements. Enhancer elements typically serve to enhance transcription, and may not be critical for basal level gene reproduction (Kushida et al., 1997). They may be located several hundred to thousands of base pair from the transcription initiation site and are usually functional independent of their orientation. Nonetheless, there are instances when the loss of an enhancer element has led to total loss of transcriptional ability (Sun and Duckworth, 1999). Another type of consensus element found on many genes are repressor elements. Similar to enhancers, repressors may be several hundred or
even thousands of bases from the transcription initiation site, however, as their name suggests their function is to repress transcription (Ogbourne and Antalis, 1998). In some instances a repressor sequence may overlap with that of an enhancer, thereby, competitively regulating the rate of transcription. Therefore, changing ratios of competing enhancer/repressor element binding factors enables tight regulation of gene expression.

Through deletional analysis of the 5' region of the ET\textsubscript{\lambda} gene, our laboratory has demonstrated several regions which regulate transcriptional activity. When a region between -848 to +92 of the human ET\textsubscript{\lambda} gene, (O being the TIS), was placed in front of a firefly luciferase reporter gene, it resulted in a more than 200 fold increase in luciferase activity especially in ET\textsubscript{\lambda} expressing cells. Furthermore, a deletion of -848 to -1 16 resulted in a slight increase in luciferase activity, indicating that there is weak transcriptional silencing in this region. However, when the gene was further deleted from -1 16 to -38 \([ET\textsubscript{\lambda} (-1 16 \rightarrow -38)]\) there was a 75% decrease in luciferase activity, indicating that a putative regulatory element exists within that region of the gene (Figure 3). These results confirm recently published data which identify a 9 bp c-rich region (bases -9 1 to -83 CCCCACCTT) to be crucial mediators of ET\textsubscript{\lambda} transcription (Yamashita \textit{et al.}, 1998).

Upon sequencing the mouse ET\textsubscript{\lambda} gene, a region with a high degree of sequence similarity between the rat, mouse and human was found at +92 to +215 \([ET\textsubscript{\lambda} (+92 \rightarrow +215)]\). Inclusion of this region in a construct containing the upstream element (i.e. the ET\textsubscript{\lambda} promoter fragment from -253 to +215) in front of the luciferase reporter gene led to a 13.5 fold increase in luciferase activity over ET\textsubscript{\lambda} (-1 16 \rightarrow -38) alone (Figure 4). Furthermore, the ability of the ET\textsubscript{\lambda} regulatory regions to modify simian viral promoter (SV40) activity was examined. \(ET\textsubscript{\lambda} (-1 16 \rightarrow -38)\) and \(ET\textsubscript{\lambda} (+86 \rightarrow +215)\) were ligated into a vector in front of the SV40
promoter and firefly luciferase. These constructs were transfected into A10 rat aortic smooth muscle cells, P-19 teratocarcinoma cells differentiated to a myocyte lineage, and primary cultures of porcine aortic VSMC. Transfection of the ET\(_\alpha\) \((-116 \rightarrow -38)\)-SV40-luciferase construct in A10 cells led to an average 500\% increase in luciferase activity versus the SV40-luciferase construct alone. Transfection of the ET\(_\alpha\) \((+86 \rightarrow +215)\)-SV40-luciferase construct lead to an average 450\% increase (Figure 5). The ability of these elements to regulate SV40 promoter activity was specific to ET\(_\alpha\) expressing cells such as A10 cells and was not seen in cells which do not express ET\(_\alpha\) such as COS green monkey kidney epithelial cells (Monge, unpublished observations, 1995).

**Putative Transcription Factors**

Sequence analysis of ET\(_\alpha\) \((+86 \rightarrow +215)\) revealed several putative transcription factor binding sites for zeste and AP-2 (DNAsis). Zeste was originally discovered in the fly *Drosophila* and is a member of the trithorax group of the *Drosophila* fruit fly that activates the ultrabithorax promoter *in vitro* (Pirrotta *et al.*, 1987; Biggin *et al.*, 1988; Laney and Biggin, 1992). AP-2 is a RA-inducible transcription factor, expressed in epithelial and neural crest cell lineages during murine development. Originally discovered in 1988, AP-2 was described as a 52 kDa binding protein isolated from the human HeLa cervical carcinoma cell line (Williams *et al.*, 1988). It has a helix-span-helix motif with an N terminal activation domain, and a C terminal homodimerization and DNA binding domain (Williams and Tjian, 1991). AP-2 has been investigated primarily for its role in RA-mediated differentiation. *In-situ* hybridization has demonstrated its expression in embryonic tissue stemming from neural crest-derived cell lineages, and limb bud mesenchyme, both of which are sensitive to RA in their development.
A number of AP-2 isoforms transcribed from different genes have been discovered. These include human AP-2α, AP-2β, AP-2γ which differ in their N-terminal region but are highly conserved in their DNA binding and dimerization domains and exhibit homology among the mouse and the human (Williamson et al., 1996; Moser et al., 1997). Similarly, a murine counterpart for the originally described human AP-2 and AP-2.2 is 65% identical to the human AP-2. Moreover, murine AP-2α and β contain a 96 amino acid-long sequence which is nearly identical to the human AP-2 DNA binding domain (Oulad-Abdelghani et al., 1996). AP-2.2 was discovered in mouse P19 cells and was shown to be upregulated by RA induced differentiation. This is in contrast to the observed downregulation of AP-2α by RA treatment. Alternately spliced AP-2 mRNA transcripts have also been described (Buettner et al., 1993; Meier et al., 1995; Moser et al., 1995). One of these differentially spliced mRNA transcripts is lacking part of the DNA binding dimerization domain. Although its activation domain remains fully intact, this shortened form of AP-2α, termed AP-2B, is unable to bind the AP-2 consensus sequence and inhibits AP-2α regulated transcription possibly by sequestering co-activators necessary for AP-2 DNA binding (Buettner et al., 1993).

A "Knockout" of the AP-2 gene was performed by replacing exon 5 with a pGK-neo gene cassette and using this construct for homologous recombination (exon 5 was chosen for knockout because it is common to all known murine AP-2 isoforms). Homozygotes for the AP-2 knockout died in-utero, and presented severe deformation of the face, skull, sensory organs, and cranial ganglia (Schorle et al., 1996; Zhang et al., 1996). There are phenotypical similarities between the ET-1 and the AP-2 knockouts, although the AP-2 knockout abnormalities are more severe. Such observations suggest that AP-2 may play a role in endothelin-mediated development. One possibility is that AP-2 produced in neural crest-derived cells is crucial for ETα transcription.
Endothelin Mediated Signal Transduction

The effects of endothelin ligand binding are mediated through a complex signaling cascade which includes the activation of phospholipase C (PLC), protein kinases, phospholipase D (PLD), phospholipase A₂ (PLA₂), and guanylate cyclase. The ET receptors are coupled to a Gq protein and contain seven transmembrane spanning domains, a characteristic shared by all guanine nucleotide regulatory protein (G-protein) coupled receptors. G-proteins consist of α, β, and γ subunits. There are several isoforms of the α, β, and γ subunits, and their contribution to the G-protein complex determines the downstream signal transduction response to ligand-receptor interaction. Hence, binding of an endothelin peptide to its receptor can stimulate several secondary messengers and signal transduction responses, resulting in various effects.

Phospholipase C

Upon ligand binding, GDP bound to the α subunit is replaced with GTP, resulting in the dissociation of the α subunit from the αβγ complex. The α-GTP subunit is then responsible for the activation of PLC which hydrolyses biphosphate (PIP₂) to inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) (Douglas and Ohlstein, 1997). Both ET₁ and ET₂ mediate PIP₂ hydrolysis which is dependent on the concentration of the endothelin peptide involved (Aramori and Nakanishi, 1992). PLC activation may be categorized into two groups: pertussis toxin sensitive or insensitive. Pertussis toxin causes ADP-ribosylation of the α G-protein subunit and inactivates the G₁ inhibitor of adenylate cyclase. The end result is the production of cAMP, which is normally turned off in the cell. Endothelin stimulated PKC activation has been shown to be both pertussis toxin sensitive [i.e. in VSMC and mesangial cells (Reynolds et al., 1989; Nambi et al., 1990)] and insensitive [i.e. in neurons and fibroblasts (Lysko et al., 1991)]. However, even
the pertussis toxin sensitive PLC response has been shown to only be partially sensitive to the toxin, emphasizing the heterogeneous nature of receptor-G-protein interactions.

IP₃ is also responsible for the release of calcium from the sarcoplasmic reticulum which acts upon voltage gated channels and leads to a subsequent influx of extracellular calcium. In turn, the extracellular calcium further stimulates a small but sustained increase in intracellular calcium (Simonson and Dunn, 1990; Simonson et al., 1989). The combination of both intracellular and extracellular calcium associate with the myofibrils and leads to a potent, long-lasting contraction. Furthermore, the IP₃ induced change in intracellular calcium levels is responsible for coronary vasoconstriction, systemic vascular resistance, and lower cardiac output (Love and McMurray, 1996). ET-1 also induces a change in cellular pH rendering the cell more sensitive to calcium-induced contraction (Tamirisa et al., 1995).

Protein Kinases

The production of DAG following PIP₂ hydrolysis is responsible for activating protein kinase C (PKC), a serine/threonine kinase responsible for phosphorylating many other proteins. Endothelin induced PKC activity has many different roles, including, the stimulation of calcium independent vasoconstriction (Nambi et al., 1995), inhibition of mesangial cell responses to endothelin (Simonson and Dunn, 1990) and ET-1 induced PLC activation in VSMC (Reynolds et al., 1989).

Aside from stimulating PKC, ET-1 has been shown to induce mitogen activated protein kinase (MAPK), a serine/threonine kinase commonly activated by growth factors. MAPK is involved in cellular mitogenesis, regulates fos and jun expression and ET-1 gene transcription (Simonson, 1992). Stimulation of MAPK occurs in a cell specific manner, and may involve
activation of various protein tyrosine kinases. ET-1 activates several isoforms of MAPK through a number of pathways. In neonatal rat myocytes, ET-1 activates p38 MAPK in a PKC dependent manner (Clerk et al., 1998). In rat mesangial cells, ET-1 leads to a biphasic activation of p21 ras (Foschi et al., 1997). The activation of p21 ras leads to an association of phosphorylated shc with sos1 via the Grb2 adapter. This in turn activates the extracellular regulated kinase 2 which in turn has a negative feedback effect on p21 ras. ET-1 also leads to tyrosine phosphorylation via the ET<sub>A</sub> receptor, resulting in calcium dependent transactivation of the epidermal growth factor receptor (Iwasaki et al., 1999). The activation of MAPK by ET-1 occurs in a pertussis toxin insensitive manner and therefore involves a pathway which is independent of G<sub>i</sub> or G<sub>o</sub> stimulation (Bogoyevitch et al., 1995).

**Phospholipase D**

Phospholipase D (PLD) is responsible for hydrolysing phospholipids such as phosphatidylcholine and phosphatidylethanolamine into phosphatidic acid and either choline or ethanolamine. ET-1 has been shown to stimulate PLD activity in mesangial cells (Kester et al., 1992), VSMC (Resink et al., 1990) and fibroblasts (Macnulty et al., 1990; Pai et al., 1991). It is activated in both a PKC dependent and independent manner (Macnulty et al., 1990) contributing to the multiplicity of pathways that mediate the effects of ET signal transduction.

**Phospholipase A<sub>2</sub>**

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is activated directly by endothelin association with either the ET<sub>A</sub> or ET<sub>B</sub> receptors or indirectly by ET mediated changes in intracellular calcium concentrations (Aramori and Nakanishi, 1992). It forms arachidonic acid which is metabolized to
leukotriene, prostaglandins or thromboxanes in a species and tissue dependent manner.

**Guanylate Cyclase**

Endothelin mediated vasodilatory responses are thought to occur in an $\text{ET}_B$ dependent manner via activation of the L-arginine/nitric oxide (NO) pathway in endothelial cells. This likely occurs via an ET-1 induced increase in cGMP levels following endothelin-ET$_B$ binding. The ET-1 induced increase in cGMP occurs in a calcium dependent manner and may be blocked by nitric oxide synthase (NOS) inhibitors or L-arginine analogs (Reiser, 1990; Edwards et al., 1992).

**ET-1-ET$_A$ Binding**

Endothelin is slow to bind to its receptor and mediate vasoconstriction. ET-1-ET$_A$ binding remains 40% reversible within the first five minutes of ligand receptor interaction, within which time only a moderate transient contraction of aortic strips is seen (comparable to AII) (Marsault et al., 1991). Nonetheless, once bound, the ligand receptor complex is virtually irreversible and produces a sustained vasoconstrictor response (Rosendorff, 1996). There are reports, however, that when ET is given in large doses membrane binding sites are rapidly occupied and a fast intracellular calcium transient is produced (Marsault et al., 1991). These observations suggest that the contractile properties of ET-1 are not a direct consequence of ligand receptor interaction, but rather a property of a late intracellular event. The PKC inhibitor staurosporine (STS) has been shown to transform sustained ET-1 contractions into reversible ones (Marsault et al., 1991). It is therefore possible that PKC is involved in maintaining the irreversibility of the ET-1-ET$_A$ complex.
Novel ET Peptides and Their Biological Processing

Recent studies have confirmed the existence of several mature ET peptides which differ from the classical 21 amino acid peptides in several respects. Human chymase was found to cleave big ET at the Try(31)-Gly(32) bond to produce a mature 31 amino acid peptide [ET-1(1-31)] (Kido et al., 1998). Furthermore, chymase is able to cleave all three ET isoforms with equal affinity. ET-1(1-31) leads to a dose dependent increase in intracellular calcium, contraction of rat trachea (Kido et al., 1998), porcine coronary arteries and rat aorta (Kishi et al., 1998). Although it is slower to mediate contraction, its effects are longer lasting than ET-1(1-21). It may be blocked by both an ET₄ or ET₈ antagonist, and therefore some have suggested that it acts upon a third, yet undescribed ET receptor (Kishi et al., 1998). ET-1(1-31) has been described as a mature ET peptide as it is able to induce contraction without being converted to ET-1(1-21) (Kido et al., 1998).

Others have described a third mature ET peptide, ET (1-32) (Fernandez-Patron et al., 1999). Like ET-1(1-21) and ET-1(1-31), ET-1(1-32) is processed from the same big-ET isoform, however, it is processed by the vascular remodeling enzyme matrix metalloproteinase-2 (MMP-2) and not ECE or chymase. It is cleaved at the Gly(32)-Leu(33) bond and therefore forms a 32 amino acid peptide. Its processing occurs primarily in the smooth muscle layer of the vessel, as the matrix metalloprotease-2 tissue inhibitor (TIMP-2) is able to significantly decrease ET-1(1-32) induced contraction in denuded rat mesenteric arteries.

Endothelin in Heart Failure

Endothelin plays a significant role in the development and pathophysiology of heart failure. Left ventricular ET-1 and ET receptor (ET₄ and ET₈) mRNA levels were increased in a
rat model of left ventricular heart failure, which was reversed by the ET\(_{\alpha}\) receptor antagonist LU135252 (Picard et al., 1998). Likewise, plasma ET-1 and ppET-1 gene expression were significantly higher in rats with congestive heart failure (CHF) following left coronary artery ligation, and infusion of the ET\(_{\alpha}\) antagonist BQ123 significantly decreased both heart rate, left ventricular end-diastolic pressure and mortality (Sakai et al., 1996). In a similar study involving pacing-induced CHF in pigs, plasma ET-1 was increased, and myocyte contractility was decreased in response to endothelin treatment (Thomas et al., 1996). Furthermore, in the rabbit, the ET\(_{\alpha}\) receptor antagonist FR139317 was reported to reduce myocardial infarct size when administered before coronary artery occlusion, suggesting that endothelin may be involved in the responses to the early stages of myocardial infarction (Burke and Nelson, 1997).

**Role of ET During Physico-Chemical Stress**

The endothelin system is also activated by various stress stimuli. In hypoxia induced hypertrophy of neonatal rat cardiomyocytes, BQ123 partially inhibited mitogenesis, (measured by \(^{1}\)H-leucine incorporation), and skeletal alpha-actin mRNA, implicating ET-1 as an autocrine/paracrine mediator of the effects of mild hypoxia (10%O\(_2\), 85%N\(_2\), 5%CO\(_2\)) (Ito et al., 1996). Furthermore, production of atrial naturetic peptide (ANP) (a marker of cardiac hypertrophy) in response to anoxia can be abolished with ET\(_{\alpha}\) antagonists (Skvorak and Dietz, 1997).

Endothelin production is also upregulated during atrial stretch (Skvorak and Dietz, 1997.). ET-1 in the medium of cultured cardiomyocytes and ET-1 mRNA levels were increased after 24 hrs of stretching, and observed MAP kinase activation and concomitant increase in protein synthesis was blocked with administration of the ET\(_{\alpha}\) antagonist BQ123 (Yamazaki et
al., 1996).

Endothelin Stimulants

Endothelin production is increased by numerous stimuli including: exposure to adrenaline, thrombin (Sakamoto et al., 1992; Zoja et al., 1991; Fukunaga et al., 1991), Ca^{2+} ionophore A13187, angiotensin II (Kohno et al., 1992), arginine vasopressin (Bakris et al., 1991; Kohno et al., 1993), cyclosporin, oxidized low density lipoprotein (Martin-Nizard et al., 1991; Boulanger, 1992), cytokines (Ohta et al., 1990; Kohan, 1992), and growth factors (Sakamoto et al., 1992; Zoja et al., 1991; Horie et al., 1991), as well as physical stimuli such as hypoxia, shear stress (Yanagisawa et al., 1988), exposure to free radicals and endotoxins (Nakamura et al., 1991; Pernow et al., 1989; Tsukada et al., 1993; Morise et al., 1994; Takahashi et al., 1990; Vemulapalli et al., 1991). ET production is most likely controlled on a transcriptional or translational level, as no storage vesicles for ET have been discovered. ET-1 is quickly cleared from the plasma and has a half-life of less than one minute. ET-1 is cleared by ligand-receptor internalization and has a high tissue uptake in the lungs and kidneys because of the large number of 'clearance receptor' binding sites (ET_{b}) in these organs.

Endothelin and the Renin-Angiotensin Systems

Angiotensin II -ET-1 Interaction

Until the discovery of endothelin, angiotensin II (AII) was recognized as the most potent vasoconstrictor yet described. It is now known that ET-1 is approximately 10-fold more potent than AII on an equimolar basis (Yanagisawa et al., 1988). Aside from replacing AII in its role as vasoconstrictor par excellence, ET-1 and AII share many physiological and pathological
functions, and appear to act synergistically in mediating several signaling pathways and disease states. They both act as potent mitogens or comitogens (Dubin et al., 1989; Hirata et al., 1989) and play a significant role in the development of cardiac hypertrophy (Stula et al., 1998).

**AII Stimulates ET-1 production**

Several studies have shown AII to stimulate the release of ET-1 from various cell types (Rosendorff et al., 1996; Sung et al., 1994; Love and McMurray, 1996; Tamirisa et al., 1995; Ito et al., 1991; Ito et al., 1993; Kawaguchi et al., 1991; Hatekeyama et al., 1994). This includes rat VSMC, cultured endothelial cells, neonatal rat cardiomyocytes, bovine endothelial cells, and human pulmonary artery smooth muscle cells. Furthermore, a time and dose dependent upregulation of ET-1 mRNA has been demonstrated in AII stimulated neonatal rat cardiomyocytes (Ito et al., 1993; Kanno et al., 1993) and cultured VSMC (Love and McMurray, 1996).

**Mechanisms of AII Stimulated ET-1 Expression**

AII induced ET-1 production likely occurs via the angiotensin II type-1 receptor (AT-1) since the AT-1 antagonist SK&F 108566 was able to block AII induced ET-1 expression (Sung et al., 1994). Furthermore, studies utilizing ET₃ antagonists have shown that AII stimulates ET-1 production in an ET₃ dependent manner.

Administration of angiotensin converting enzyme (ACE) inhibitors to endothelial cells reduces bradykinin degradation, thereby leading to an increase in NO levels and a consequential downregulation of ET (Tamirisa et al., 1995). Additionally, the inability of the calcium channel blocker nicardipine to block ET-1 release from endothelial cells following AII treatment suggests
that the influx of extracellular calcium into the cell by L-type calcium channels does not play a role in AII induced ET-1 synthesis (Emori et al., 1991).

The involvement of PKC in ET and AII-induced muscle contraction in rat aortic rings has been demonstrated (Oriji and Keiser, 1997). Rat aortic rings were isolated and subjected to several different conditions after which the contractile response to AII and the concomitant change in ET secretion into the surrounding medium were measured. Upon exposure of intact (not denuded) aortic rings to AII, a dose dependent contraction and a concomitant release of ET into the surrounding media was observed. After treating the rings with PKC inhibitors, AII induced contraction was reduced and ET release was inhibited. Furthermore, upon pretreatment with the ET1 specific antagonist BQ123, a 47% reduction in AII induced contraction, and a 55% reduction in AII induced ET release was observed. These results suggest that AII affects ET release through a PKC involved mechanism.

PKC also plays an important role in AII induced ppET-1 expression (Ito et al., 1991). AII has been observed to upregulate ppET-1 mRNA by as much as three-fold within 30 minutes after AII treatment. Treatment with the phorbol ester, TPA, which is a PKC activator, led to a similar increase in transcription of the ppET-1 gene independently of AII treatment. Furthermore, AII induced ppET-1 gene expression was blocked by the PKC inhibitor H-7 (Ito et al., 1993) which also inhibits immunoreactive ET-1 release from bovine endothelial cells (Emori et al., 1991). These experiments suggest that AII stimulates ET production in a PKC dependent manner.

In addition, there is debate in the literature regarding the regulation of AII on ET receptor transcription and translation. Several studies have shown that AII administration leads to a downregulation of ET-1 binding sites in a concentration-dependent manner. AT-1 inhibitors have been shown to attenuate this downregulation (Roubert et al., 1989; Sung et al., 1994).
Furthermore, preincubation of the cells with a PKC activator led to a similar downregulation of ETₐ expression. These findings suggest that AII regulates ETₐ through the actions of PKC. These experiments were done through ligand-receptor binding experiments, measuring the levels of ¹²⁵I labeled ET-1 that bound to the receptors. An evaluation by Northern blotting of ETₐ mRNA after AII treatment in human pulmonary artery VSMC showed, in contrast, that the ETₐ message was upregulated in a dose-dependent manner (Hatekeyama et al., 1994).

**Mitogenic Properties of ET-1 and AII**

In addition to their acute vasoconstrictor properties, chronically expressed ET-1 and AII are mitogenic and hypertrophic factors. Both ET-1 and AII activate transcription factors such as Egr-1 and c-fos independently of calcium, and stimulate DNA and protein synthesis. Of particular interest is the dependence of AII on the DAG-PKC pathway in order to induce ppET-1. Furthermore, the dependence of AII-induced protein synthesis on the ET-1-ETₐ interactions is indicative of ET's role in AII induced hypertrophy.

A common mechanism for induction of DNA synthesis and cell growth by G-coupled protein receptors, is through the activation of MAPK. The activation of MAPK is a downstream result of the DAG-PKC pathway (Douglas and Ohlstein, 1997). MAPK acts through a series of complex kinase signaling transduction mechanisms. It involves the activation of the extracellular signal regulated kinase (erk) which results in the formation of a guanine nucleotide exchange complex. This complex facilitates the exchange of GDP for GTP on a small G-protein which then proceeds to take part in an intricate phosphorylation cascade. The outcome of this cascade is the activation of transcription factors which regulate gene expression and leads to protein synthesis and cell growth. Since MAPK is activated by PKC, an increase in calcium is associated
with DNA and protein synthesis.

A study by Grohe et al. in 1994 looked at the effects of calcium inhibition on the actions of c-fos and immediate/early gene (Egr-1) expression and protein synthesis in adult rat ventricular cardiomyocytes. Although under normal cellular conditions ET-1 increases c-fos mRNA by fifteen fold and AII increases c-fos by ten to twelve fold, upon exposure to 1 mM Nisoldipine (a dihydropyridine calcium channel blocker) the ET-1 and AII induced upregulation of gene expression is inhibited. Furthermore, the observed eight and ten fold increase in Egr-1 in ET-1 and AII- induced gene regulation respectively, was similarly inhibited by Nisoldipine administration. These effects did not occur as a result of Nisoldipine administration alone, as gene transcription was unaffected with Nisoldipine administration in the absence of ET-1 and AII.

The same study looked at the effects of calcium regulation by ET-1 and AII on protein synthesis by rat ventricular cardiomyocytes (Grohe et al., 1994). Under normal conditions, 1 µM ET-1 increases cardiomyocyte protein synthesis by 135%, and 1 µM AII increases protein synthesis by 108%. Pre-treatment with Nisoldipine inhibited ET-1 and AII-induced protein synthesis in a concentration dependent manner. Furthermore, antisense oligodeoxynucleotides complimentary to Egr-1 mRNA abolish the ET-1/AII induction of protein synthesis. This suggests that ET-1/AII induced protein synthesis is dependent on activation of the Egr-1 transcription factor via a calcium dependent pathway.

Studies that evaluated ET-1 and AII's abilities to induce cellular protein production, established that both peptides stimulated the uptake of tritiated leucine by twofold over untreated
control cells, and that this uptake was inhibited by the \( \text{ET}_\lambda \) specific antagonist BQ123 (Ito et al., 1991). However, only AII induced protein synthesis was inhibited by the angiotensin receptor antagonist DuP753. Moreover, the addition of an antisense oligonucleotide against the coding region of ppET-1 mRNA in rat cardiomyocytes blocked the transcription of ppET-1 mRNA, and inhibited AII-induced incorporation of tritiated leucine. Consequently, it may be postulated that the ability of AII to regulate cellular protein production is dependent upon ET-1 production and its subsequent binding to \( \text{ET}_\lambda \).
**Hypothesis**

*Part I*

Transcriptional Regulation of an ET, Regulatory Element

Our laboratory’s previous data demonstrating several regulatory elements within the 5' UTR and flanking region of the ET, gene [ET, (+86 → +215) and ET, (-116 → -38)] prompted us to examine the role of putative transcription factors in ET, transcriptional regulation.

BLAST comparison of the rat and human ET, (-116 → -38) sequence (the sequence of the mouse 5' flanking region is not currently available) revealed a region of 19 bp which is 100% conserved between these two species. Cross-species comparison of ET, (+86 → +215) revealed a 39 bp region that is 95% conserved between the mouse and the rat and 87% conserved between the mouse, rat and human (Figure 6). A search for putative transcription factor consensus sites (DNAsis) uncovered two AP-2 sites within ET, (+86 → +215). One AP-2 site is located 68 or 11 bases downstream from the highly conserved region (on the human or rat and mouse genes respectively), and the other AP-2 consensus sequence was identified within the highly conserved region on the ET, (+86 → +215) fragment. Although the BLAST program found other potential transcription factor binding sites, the existence of an AP-2 site seemed the most significant as both ET, and AP-2 are expressed in a similar temporal and spacial manner and play important roles in embryonic development of structures derived from the cranial neural crest.

In view of these findings I hypothesized that the AP-2 nuclear transcription factor binds the ET, (+86 → +215) fragment and serves as an essential component of ET, transcription.
Part II

Impact of All on ET, Transcriptional Regulation

Cells respond to various stimuli by turning on or off gene transcription in order to control mRNA production. Regulation of vascular receptor expression is a key component in vascular response mechanisms which may be controlled through transcriptional regulation (Lin et al., 2000, Horiuchi et al., 2000). It is common for receptor mRNA expression to be regulated in either a positive or negative feedback manner through changes in ligand expression (Camp and Dudley, 1995; Nickenig and Murphy, 1996). As discussed previously, several investigators have demonstrated that All increases ET-1 expression (Rosendorff, 1996; Sung et al., 1994; Love and McMurray, 1996; Tamirisa et al., 1995; Ito et al., 1991; Ito et al., 1993; Kawaguchi et al., 1990; Hatekeyama et al., 1994), and that ET-1-ETA binding likely contributes to several observed pathophysiological responses of All (Ishiye et al., 1995; Galatius-Jensen et al., 1996).

Furthermore, our lab and others have identified two elements that control ET, driven transcription in a firefly luciferase reporter gene system (Yamashita et al., 1998; Monge, unpublished results). I therefore hypothesized that All controls ET, mRNA expression in either a direct or indirect manner through regulation of ET, (-116 → -38), ET, (+86 → +215) or a combination of both elements.
**Materials**

Angiotensin II was purchased from Sigma-Aldrich Chemicals, Oakville ON, Canada.

All tissue culture reagents were purchased from Gibco BRL, Burlington ON, Canada.

ET-1 was purchased from Peninsula Laboratories, Belmont CA, USA.

QIAprep Spin Miniprep Kit, Qiagen Plasmid Maxi Kit and SuperFect was purchased from Qiagen, Mississauga ON, Canada.

All restriction enzymes, radioactive compounds and the BandShift kit were purchased from Amersham Pharmacia Biotech, Baie d’Urfe QUE, Canada.

AP-2 plasmids were generously provided by Dr. M. Tainsky, Karmanos Cancer Institute, Detroit MI, USA.

pGL2, pRL-TK plasmids, the Dual-Luciferase Reporter Assay System and the β-galactosidase kit were purchased from Promega, Madison, WI, USA.

The ET-1 enzyme immunoassay kit (ELISA) was purchased from Biomedica, (distributed by American Research Products, Inc.) Belmont MA, USA.

A10 cells, P19 cells, and HepG2 cells were purchased from the American Type Culture Collection (ATCC), Rockville MD, USA.

The Berthold Lumat LB9501 Luminometer was distributed by Fisher Scientific, Nepean ON, Canada.

The Spectramax 250 spectrophotometer was purchased from Molecular Devices, Sunnyvale CA, USA.

The GS-700 densitometer was purchased from BioRad, Mississauga, ON, Canada.
Experimental Methods

Cell Maintenance

Rat aortic vascular smooth muscle cells (A10).

A10 cells were grown in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% penicillin/streptomycin (P/S), at 37°C, in a humidified 5% CO₂ atmosphere. Confluent cells were washed twice with 1x phosphate buffered saline (PBS), trypsinized in 0.05% (w/v) trypsin and passaged in a ratio of 1:10.

Murine teratocarcinoma embryonal cells (P19)

P19 cells were grown in α Modified Eagles Medium (αMEM) supplemented with 5% fetal bovine serum, 5% newborn bovine serum (NBS), and 2% penicillin/streptomycin, at 37°C, 5% CO₂. When the cells reached confluence, they were washed twice in PBS, trypsinized in 0.025% trypsin and passaged in a ratio of 1:10.

Differentiation of Murine teratocarcinoma embryonal cells to a Myocyte Lineage

P19 cells were resuspended at 7.5 x 10³ cells in 15 ml of αMEM supplemented with 10% serum and 1% DMSO and incubated in a 100mm bacterial grade petridish at 37°C for 48hrs. The aggregate culture was then transferred into a 50 ml conical-bottom Falcon tube and allowed to settle at 37°C. The supernatant was removed and the aggregates were resuspended in 40 ml of fresh αMEM supplemented with serum and 1% DMSO in 150mm bacterial grade dishes for 48hrs. The aggregates were again transferred to a 50 ml tube allowed to settle and the medium
was replaced as described previously. Following a 24hr incubation, the medium was replaced with αMEM supplemented with serum but not DMSO and the aggregates were transferred to a tissue culture grade dish and allowed to attach and differentiate (Rudnicki and McBurney, 1987, Monge et al., 1995).

After 48 hrs a spindle like morphology characteristic of smooth muscle cells was clearly visible in 10-20% of the culture plate.

*Freeze Preparation of Cells*

After trypsinization, cells were centrifuged at 900 xg for 4 minutes and the supernatant was removed. The pellet was resuspended in medium supplemented with serum, antibiotics and 10% DMSO, aliquoted to 1 ml cryo-vials and frozen at -80°C overnight. The vials were then transferred to liquid nitrogen for long-term storage.

*Gel Mobility Assay*

The gel mobility assay is commonly used to detect protein-DNA interaction. It involves incubating a radioactively labelled DNA fragment less than 300bp long with a protein extract, and allowing them to bind at the appropriate binding conditions. The complex is run on a gel and exposed to radioactive film for analysis. A protein-DNA complex will show up as a retarded band, as it runs with slower mobility than the free DNA.
Preparation of Nuclear Extract

All glassware was pre-chilled, and the entire procedure was performed at 4°C. DMSO-differentiated P19 cells were washed with PBS 1mM PMSF and harvested in 10 ml PBS by cell scraping. The lysates were centrifuged at 2000 rpm in an IEC minicentrifuge for 5 min at 4°C and resuspended in 4 ml PBS/pellet. The pellets were pooled and re-centrifuged at 2000 rpm for 5 min. Following centrifugation the pellets were resuspended in buffer I pH 7.6 (15mM HEPES pH 7.6, 10mM KCl, 5mM MgCl₂, 0.1mM EDTA pH 7.6, 1mM DDT, 10mM Na₂S₂O₅, 2μg/ml leupeptin, 1μg/ml pepstatin, 2μg/ml aprotinin, 1μg/ml chymostatin, 1μg/ml antipain, 1μg/ml PMSF) and cycled 5 times through a 22 gauge needle. The extract was spun at 2000 rpm for 10 minutes, and the pellet was gently resuspended in 10 ml buffer A (10mM HEPES pH 7.6, 115mM KCl, 5mM MgCl₂, 0.1mM EDTA, 1mM DTT, 10mM Na₂S₂O₅, 1mM PMSF, 2μg/ml leupeptin, 1μg/ml pepstatin, 2μg/ml aprotinin, 1μg/ml chymostatin, 1μg/ml antipain). The volume of the extract was recorded, with the supernatant representing the cytoplasmic proteins. The suspension was adjusted to 0.5M (NH₄)₂SO₄ pH 7.8 and gently rocked for 30 minutes. The viscous cell extract was centrifuged in a Beckman Ti 70.1 rotor at 35 000 rpm for 70 minutes. The supernatant was collected and 0.3g (NH₄)₂SO₄ per ml for half the supernatant was added gradually with inversion. The supernatant was then shaken for 15 minutes and spun at 10,000 rpm for 20 minutes in a SS34 rotor. The pellet was drained, re-suspended in 10ml Buffer II (25mM HEPES pH 7.6, 50mM KCl, 12.5mM MgCl₂, 0.1mM EDTA, 1mM DTT, 10% Glycerol, 1μg/ml chymostatin, 2μg/ml leupeptin, 1mM PMSF, 2μg/ml aprotinin, 1μg/ml pepstatin, 1μg/ml antipain) and dialyzed against 500ml buffer II for 1hr. The dialyzing buffer was changed and the
pellet was dialyzed for an additional hour. The extract was centrifuged for 5 minutes in a microcentrifuge, and the supernatant was aliquoted and stored at -80°C.

The protein concentration was determined by the Bradford method. The protein integrity was determined by electrophoresis in a polyacrylamide gel and coomassie blue staining.

**Target DNA Preparation**

ET\(_{\Delta} (-116 \rightarrow -38)\) and ET\(_{\Delta} (+86 \rightarrow +215)\) fragments were removed from their vectors by a Kpn I, Xho I digestion, separated by 2% agarose gel electrophoresis, isolated from the agarose gel by using Qiaex II (Qiagen) and resuspended in TE buffer. Ten ng/µl target DNA [either ET\(_{\Delta} (-116 \rightarrow -38)\) or ET\(_{\Delta} (+86 \rightarrow +215)\)], 10µl dATP labelling mix (0.1mM dGTP, dCTP, dTTP, 50mM Tris-HCl pH7.5, 50mM MgCl\(_2\), 250mM NaCl, 25mM β- mercaptoethanol), 1µl Klenow fragment, and 20µCi [α\(^{32}\)P]dATP in a final volume of 50µl was incubated for 1hr at 37°C. In order to remove the free radioactive nucleotides, the reaction was equilibrated with 70µl STE buffer(10mM Tris-Cl pH 7.5, 10mM NaCl, 1mM EDTA pH 8.0) and passed through a previously primed Stratagene push-column. The labelled DNA was eluted from the column with another 70µl of STE and the specific activity was determined in a scintillation counter.

**Protein-DNA Binding and Visualization**

The following binding conditions were included in the gel mobility assay experiment:

- labelled target DNA without nuclear extract as a control
- labelled target DNA with 0.5µg P19 nuclear extract
-labelled target DNA with 0.5µg P19 nuclear extract + 20, 60 or 200x molar excess of unlabelled target DNA as a specific competitor

-labelelled target DNA with 0.5µg P19 nuclear extract + 20, 60 or 200x unrelated DNA, a 200 bp fragment of the chloramphenicol acetyl transferase coding region (CAT coding region) as a nonspecific competitor.

The binding reaction included 10 000 cpm target DNA (approximately 1 ng), 0.5 µg nuclear extract, 4 µg poly dIdC, 4.5 µg bovine serum albumin (BSA), 2 µl buffer A (see nuclear extract protocol), 4 µl 50% glycerol, varying amount of specific or non-specific competitors and sterile distilled de-ionized water (SDDW) to a final volume of 20 µl. The reaction was incubated at room temperature for 15 minutes, and run on a polyacrylamide gel to separate the DNA-protein complexes from the free target DNA. The gel was then dried under vacuum, exposed to autoradiograph film and after 24-48 hrs the film was developed.

Quantitative Competitive Reverse Transcription Polymerase Chain Reaction (qcRT-PCR) for Detection of ET₅ mRNA

In order to quantify the amount of ET₅ mRNA in various cell culture samples, we used a qcRT-PCR technique. qcRT-PCR was performed using methods previously described (Smith et al., 1999). This method utilizes a dilution series comprised of known molar quantities of a mutated ET₅ cDNA fragment which is co-amplified with equal amounts of wild-type total RNA and competes for primer binding and amplification. The ratio of wildtype RNA to mutant RNA can then be compared and the concentration of wildtype RNA determined.
Preparation of Total RNA

A10 cells were grown to confluence in DMEM supplemented with 10% FBS, 2% penicillin/streptomycin (p/s) on a 10 cm² dish. The cells were washed twice with 4 ml phosphate buffered saline (PBS) and treated with 10⁻⁷M AII, or the appropriate volume of vehicle in 4 ml DMEM 1% FBS and incubated for 24hrs. Following incubation, the cells were washed with PBS and total RNA was extracted using the TRIZOL reagent (Gibco BRL). RNA integrity was assessed on a 1.2% agarose-formaldehyde gel by looking at the 28s:18s ratio, and the RNA was quantified by spectrophotometry. The RNA was aliquoted and stored at -80°C.

Design of Mutant Competitor (Smith et al., 1999)

Nucleotide sequences for the ET₁ receptor were obtained from the Genbank data base and used in designing the mutant cDNA and PCR primers. Using the megaprimer method of site-directed mutagenesis (Sarkar and Sommer, 1990), the mutant was engineered to contain a single-base mutation which substituted a thymidine nucleotide for a guanine at 101 bp downstream from the 5' end (GAGATC was changed to GATATC) thereby incorporating an EcoRV site not present in the wild type. EcoRV (Pharmacia Biotech Inc. Que. Canada), digestion of the mutant therefore resulted in two distinct mutant bands when electrophoresed in a polyacrylamide gel (101bp and 131bp), as opposed to the undigested 232 bp wild-type band. The DNA mutant template was then linearized with Sac I (Pharmacia Biotech) (1U/µg DNA), run on an agarose gel to confirm complete linearization and purified from the gel using a QIAquick gel extraction kit (QIAGEN Inc., Chatsworth, CA, USA). The 3' overhang was blunted using the Klenow
Fragment of DNA Polymerase I (Pharmacia Biotech) at a concentration of 5U/µg DNA for 15 min at 22°C. The DNA template was then converted to RNA by *in vitro transcription* using a RiboMAX Large scale RNA production system-T7 (Promega Corporation, Madison, WI, USA).

**Design of PCR primers**

Oligonucleotide primers were designed to be compatible for both mutant and wildtype DNA using the OLIGO software program (National Biosciences), and synthesized on a PerSeptive Expedite nucleic acid synthesizer (Millipore Canada LTD., Mississauga, ON, Canada). The sense and antisense primers were as follows:

5'-TTTTTATCGTGGGAATGGTGGG-3' (bases 275 to 296 human mRNA; GenBank L06622) and 5'-GACTTCTGCAAAGGGAACA-3' (bases 506 to 458 human mRNA; GenBank L06622) respectively. The primers were homologous between species and designed to span an intron in order to avoid synthesis of genomic DNA-template PCR products.

**RT-PCR**

Total wild-type RNA (2µg) was combined with a dilution series of competitor ET₄ mRNA (10⁻¹⁶M to 10⁻¹²M), 200U Moloney Murine Leukemia Virus (M-MLV reverse transcriptase-Gibco BRL), 0.5 mM dNTPs, 1x First Strand Buffer (final concentration-50 mM Tris-HCl pH 8.3 at 37°C, 75 mM KCl, 3 mM MgCl₂) and 100 pM Random Primer oligodeoxyribonucleotides in a final volume of 20 µl. Reverse transcription was performed by incubation at 37°C for 90 min. The reverse transcriptase was inactivated by heating at 95°C for 4
min before proceeding to the PCR step. The entire volume was then used for PCR amplification using a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA, USA). The cDNA was combined with 100pMol sense and antisense primer, 200 µM dNTP, 1x Taq buffer (final concentration, 10 mM Tris-HCl, pH 9.0 at 25°C, 50 mM KCl and 1.5 mM MgCl₂) and 5U Taq polymerase in a total volume of 100 µl and subjected to 35 rounds of PCR (1 min denaturation at 95°C, 1 min primer annealing at 57°C, 2 min extension at 72°C and a final primer extension at 72°C for 10 min).

**Quantitative Analysis**

The PCR products were then digested with 12 U EcoRV for 3 hrs at 37°C, separated by electrophoresis on a 6% polyacrylamide gel, and stained with ethidium bromide. The wild-type mRNA was represented by a 232 base-pair band, while the mutant appeared as two bands; 101 and 131 base-pair. The intensity of the bands were quantified by densitometry, normalized relative to the density of competitor, and plotted as log (wild-type/competitor) ratio vs log [competitor cRNA]. The exact amount of ET₄ mRNA was then calculated by extrapolating from the intersection of the curves at the point of molar equivalence.

**Transfections**

_Engineering of Plasmid Constructs:_

The following plasmids were used for transfection experiments (Figures 7 and 8):

pETA (-848 → +215), pETA (-116 → +215), pSETA (-116 → -38), pSETA (+86 → +215),
The ET\(_\lambda\) (-848 \(\rightarrow\) +215) cassette was generated by PCR from human genomic DNA extracted from HepG2 cells. The identity of the fragment and the absence of mutations induced by PCR were confirmed by sequencing. Subsequent ET\(_\lambda\) cassettes were generated by PCR using ET\(_\lambda\) (-848 \(\rightarrow\) +215) as a template. ET\(_\lambda\) (-848 \(\rightarrow\) +215) and ET\(_\lambda\) (-116 \(\rightarrow\) +215) were inserted into the PGL2B vectors which contain a firefly luciferase reporter gene but no internal promoter or enhancer elements.

ET\(_\lambda\) (-116 \(\rightarrow\) -38) and ET\(_\lambda\) (+86 \(\rightarrow\) +215) were inserted into the PGL2P vector which contains both the firefly luciferase reporter gene and the SV40 viral promoter (generating pSETA (+86 \(\rightarrow\) +215) and pSETA (-116 \(\rightarrow\) -38) respectively).

pSAP-2\(\alpha\) and pSAP-2B were graciously donated by Dr. M. Tainsky. They consist of the AP-2\(\alpha\) and AP-2B coding regions (respectively), under the control of the SV40 promoter inserted into the pSG5 plasmid in between 2 EcoRV restriction sites.

pRL-TK and pCMV-\(\beta\)-gal are both products of Promega Corporation. pRL-TK contains the renilla luciferase coding region under the control of the thymidine kinase promoter. pCMV-\(\beta\)-gal consists of the \(\beta\)-galactosidase coding region, under the control of the cytomegalovirus promoter.

**Preparation of Genomic DNA from HepG2 Cells:**

HepG2 cells were removed from liquid nitrogen and centrifuged in a microcentrifuge.

The pellet was washed in 1x Phosphate Buffered Saline (PBS) and lysed by incubation in 300 \(\mu\)l
1% SDS at room temperature for 10 minutes. Genomic DNA was extracted from cell lysates in 300 µl phenol-chloroform-isoamyl alcohol (24:25:1 vol:vol:vol), vortexed for 10 seconds and centrifuged 10 seconds at room temperature. The aqueous layer was removed and its volume recorded. Three M sodium acetate was added to the aqueous layer at 1/10 the volume of the aqueous layer. The DNA was then washed in 2.5x vol. 100% ethanol at -80°C, and centrifuged at 13 000 RPM in an IEC minicentrifuge. The pellet was washed in 70% ethanol and resuspended in 30 µl 10 mM Tris pH 8.0. The DNA was then allowed to redissolve overnight at 4°C.

**Primer Design for ETγ constructs**

The primers were designed to minimize self-annealing and annealing between primers, and to keep an ideal G/C content (less than 50%). The sequences were based on the published GenBank sequence (GenBank L06622). The sense primers included a Kpn I site and the antisense primers included an Xho I site in their 5' end in order to facilitate incorporation into the appropriate vector.

The sequences for the primers were as follows (Kpn I and Xho I sites are underlined):

ETγ -848 sense 5'-GTCGGTACCGGATCCTCCAGCCCCTGCTAC-3'
ETγ -116 sense 5'-GTCGGTACCAATTCTGAGTCTTGTCTGTCAAA-3'
ETγ +86 sense 5'-GACGGTACCGAGGACAGACTGGAGGCGTGTTC-3'
ETγ -38 antisense 5'-CCTCTCGAGCmAACTATTTCCTTCGAGCAGAGGCGTGTTC-3'
ETγ +215 antisense 5'-CCTCTCGAGACCAGGATGGATGGATGAAGCCT-3'
**PCR**

The PCR reaction was carried out in a 100 µl volume and included 1 µg of template, 100 pmol of primers 10 mM each dNTP, 3 units of Taq polymerase (Bohreinger Mannheim) and 1x Taq buffer (final concentration, 10 mM Tris-HCl, pH 9.0 at 25°C, 50 mM KCl and 1.5 mM MgCl₂).

For ET₄ (-848 → +215) the HepG2 genomic DNA template was denatured at 95°C for 10 minutes at which point Taq was added and 31 cycles of PCR begun.

For all other PCR reactions ET₄ (-848 → +215) was used as a template and the previous step was skipped. The PCR profile was as follows: one minute of 95°C denaturation, one minute 57°C primer annealing, and 2 minutes of primer extension at 72°C. Following 31 cycles, a further 10 minutes of primer extension was included to ensure the completion of any shortened PCR products. The PCR product was extracted using the Quickspin columns (QIAGEN Inc., Chatsworth, CA, USA), and quantified via spectrophotometric analysis, and agarose gel electrophoresis.

**Ligation & Transformation**

All cassettes and vectors were prepared for ligation by generating “sticky” ends via digestion with Kpn I and Xho I. The terminal phosphates of the vectors were removed by incubation with calf intestinal alkaline phosphatase (CIAP) (vector DNA, 0.1 U CIAP, 1 mM ZnCl₂ was incubated at 37°C for 30 min.) in order to prevent self ligation. The vectors were cleaned using the Quickspin column (QIAGEN Inc.) and the inserts were electrophoresed on an
2% agarose gel and extracted using Qiaex II (QIAGEN Inc.). The cassettes were ligated into the vector by combining the DNA (3:1 insert:vector) in a total volume of 20 μl in the presence of 0.1 unit T4 DNA ligase (Gibco BRL) and 1x ligase buffer (final concentration, 50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 1mM ATP, 1 mM dithiothreitol and 5%(w/v) polyethylene glycol 8000) and incubating at room temperature for 3 hours. The reaction was then heat inactivated at 75°C for 10 minutes and the ligated product was transformed via heat shock into competent E. Coli JM109 (Promega). The heat shock was as follows: the ligation product was mixed with 1 μl JM109 and incubated 30 min on ice, heat shocked at 42°C for 1 min, and cooled on ice for 2 min. SOC Medium (450 μl) (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10mM MgCl2, 10mM MgSO4, 20mM glucose) was added, and the heat-shocked bacteria were incubated at 37°C for 1 hr. The cells were then plated on LB ampicillin containing plates (10 g tryptone, 5 g yeast extract, 5g NaCl, 1 ml 1 N NaOH, 15 g agar filled to a final volume of 1L with distilled deionized water, adjusted to pH 7.6 and autoclaved. After cooling to 55°C, 1 mg/ml ampicillin is added, and the LB agar is poured onto petri dishes and allowed to set), and incubated at 37°C overnight. Only bacteria that contain the plasmid with the ampicillin resistant gene should grow on ampicillin LB plates. Following the overnight incubation at 37°C, resulting colonies were picked and grown at 37°C overnight in 5 ml LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 1 N NaOH final volume 1 L pH 7.6) with 1 mg/ml ampicillin. DNA was isolated via Qiagen’s miniprep spin columns. This method of DNA isolation includes lysing the bacteria, binding the DNA to a spin column, and eluting the DNA in 10mM Tris pH 8.0. The DNA was then tested for the presence of the desired insert by digestion with Kpn I and Xho I.
Large Scale Plasmid Purification (Maxi Prep)

Following identification of positive colonies (those containing the desired clone), the DNA was amplified using the Qiagen plasmid maxi kit. Briefly, 250 ml LB with 100 μg/ml ampicillin is inoculated with a colony containing the desired plasmid and grown overnight at 37°C while being shaken at 250 RPM. The bacteria are then pelleted by centrifugation and lysed by alkaline lysis. The lysate is then applied to an anion-exchange resin which facilitates DNA-resin binding, and removal of contaminating RNA, proteins, dyes and low molecular weight impurities by a medium-salt wash. The plasmid DNA is then eluted in a high-salt buffer and concentrated and desalted by isopropanol precipitation. An ethanol wash is then done to replace isopropanol with the more volatile ethanol in order to make the DNA easier to redisolve. Finally, the DNA is redisolved in 10 mM Tris.Cl, pH. 8.5, and the DNA is quantified by reading its absorbance at 260 nm.

Transfections

Once confluent, A10 cells were trypsinized and plated at 1.5 x 10⁴ cells/well in 6 well dishes, and incubated overnight in DMEM 10% FBS, 2% P/S, 37°C, 5%CO₂. Following this incubation, cells were co-transfected with the appropriate constructs using SuperFect (Qiagen) transfection reagent. SuperFect is a polycation of defined shape and diameter which assembles DNA into compact structures. The DNA-SuperFect complex possesses a net positive charge thereby facilitating binding to negatively charged cell surface receptors and uptake by the cells. Once inside the cells, SuperFect ensures the stability of the DNA-SuperFect complex by
inhibiting lysosomal nucleases. The transfection protocol consisted of combining the plasmid DNA and SuperFect in a 1:5 ratio in a total volume of 100 µl serum free DMEM. The mixture was vortexed for 10 seconds and incubated at room temperature for 10 minutes. DMEM (600 µl) with 10% FBS, and 2% P/S was added to the SuperFect-DNA solution and added to the cells which had been washed once with HBSS. The cells were incubated at 37°C, 5%CO₂ for 2 hours. The medium was removed, the cells were washed with PBS and 2 ml DMEM 10% FBS, 2% P/S was added. The cells were incubated overnight before being either treated with ET-1 or AII or assayed for firefly luciferase, renilla luciferase or β-gal activity.

**AII or ET-1 treatment of cells**

A10 cells were washed twice with PBS and treated with either 10⁻⁷ M AII or 10⁻⁷ M ET-1 in DMEM supplemented with 1% FBS for 24 hrs.

**Analysis of Firefly and Renilla Luciferase and β-galactosidase Activity**

Transfected cells were washed once with PBS, lysed in 300 µl passive lysis buffer (Promega) and scraped. The lysates were freeze-thawed once at -80°C, spun at 13,000 rpm and the supernatant was removed and kept on ice. Twenty µl of lysate was added to a glass tube containing 100 µl luciferase assay reagent II and mixed. Firefly luciferase light units were then measured for 10 seconds in a luminometer and recorded. The firefly luciferase signal was then quenched by adding 100 µl of Stop & Glo buffer and vortexing the tube. Renilla luciferase light units were then measured for 10 seconds in the luminometer.
For β-galactosidase analysis, a standard curve was prepared (0-6 mU) using the β-galactosidase enzyme provided in the Promega β-galactosidase kit. Fifty μl of the standards or samples were mixed with 50 μl of 2x buffer in a 96-well plate. The plate was covered and incubated from 30 min. to 1 hr (depending on progress of the color development) at 37°C. The reaction was stopped by adding 150 μl of 1 M sodium carbonate and mixing with a pipette. The enzyme activity was then determined by spectrophotometry at 420 nm.

**Quantitation of AP-2α and AP-2.2 mRNA in P19 and A10 Cells**

Total RNA was extracted from undifferentiated, DMSO-differentiated or RA-differentiated P19 using TRIZOL. One μg of RNA was reverse transcribed as described earlier. One μl of the RT reaction was then subjected to PCR using the primers and PCR profile as described (Oulad-Abdelghani et al., 1996). In brief, the AP-2α primers used were:

AP-2α antisense 5'-GAGGGCCTCGGTGAGATAGT-3'
AP-2α sense 5'-ACCCGAGTGTCTCAACGCGW'-3'

AP-2.2 primers used were:

AP-2.2 antisense 5'-CCTCCAGCCCTGAAATATGG-3'
AP-2.2 sense 5'-CCTGGATTTAACTGGCGACT-3'

The DNA was subjected to 30 PCR cycles with an annealing temperature of 55°C.

One μl of the RT reaction was used to amplify a fragment of GAPDH to be used as an internal control. The primers were as follows:

GAPDH sense 5'-CTCTAAGGCTGTGGGCAAGGTCAT-3'
GAPDH antisense 5'-GAGATCCACCACCGCTGTTGCTGTA-3'

The DNA was subjected to 25 rounds of PCR with an annealing temperature of 60°C.

Statistics

All statistics were done using the program GraphPAD InStat version 1.15, GraphPad Software, USA.

Statistics were performed using the nonparametric Mann-Whitney method, with the exception of the AP-2 dose response which was calculated by an Analysis of Variance using an adjusted t test with the p values corrected by the Bonferroni method. All error bars are represented as the standard error of the mean, and p<0.05 is considered significant.
Results

Gel Mobility Assay

Our laboratory has previously demonstrated the existence of two positive regulatory elements within the 5' region of the ET_\alpha receptor gene, one of which has also been reported by Yamashita et al., 1998). These elements upregulate the activity of the SV40 promoter and enhance the expression of the firefly luciferase reporter gene in a tissue specific manner. One element is located between -116 to -38 bp upstream of the transcription initiation site, and the other at +86 to +215 downstream. In order to evaluate potential interactions between nuclear transcription factors and the ET_\alpha promoter regulatory elements, we co-incubated aliquots of a nuclear extract from P19 cells differentiated to a myocyte lineage with radiolabelled ET_\alpha (+86 \to +215) and analysed its subsequent mobility through a polyacrylamide gel.

ET_\alpha (+86 \to +215) migrated through the gel with slower mobility when incubated with 0.5\mu g of nuclear extract compared to the "free" radiolabelled ET_\alpha fragments (Figure 9). The retarded mobility of the DNA-nuclear extract co-incubation is attributed to the formation of DNA-protein complexes, which, because of their larger molecular weight, migrate through the gel slower than unbound radiolabelled DNA. A dilution series of 20 to 200-fold molar excess of unlabelled, unrelated DNA sequence (the CAT coding region) was included in the reaction in order to test for possible non-specific competition. Our results showed that even a 200-fold molar excess of unrelated sequence was unable to diminish the ET_\alpha-nuclear extract interaction. Furthermore, a 200 M excess of the same ET_\alpha fragment eliminated the retarded ET_\alpha protein complex, indicating that the observed gel shift was specific in nature.
These results suggest that the ET\(\alpha\) (+86 \(\rightarrow\) +215) regulatory element specifically binds to an unknown component of a myocyte-differentiated P19 nuclear extract.

**AP-2 ET\(\alpha\) Interactions**

**AP-2 Expression**

Results from gel shift experiments indicated that a potential nuclear transcription factor was binding to the ET\(\alpha\) regulatory elements. Further analysis of these regulatory sequences revealed putative transcription factor binding sites on ET\(\alpha\) (+86 \(\rightarrow\) +215) for Zeste and AP-2. The mouse knockout model of AP-2 has many developmental similarities to the previously described ET-1 and ET\(\alpha\) knockout mice. Consequently, we evaluated potential interactions between AP-2 and the ET\(\alpha\) (+86 \(\rightarrow\) +215) regulatory region.

RT-PCR analysis of DMSO differentiated P19 cells (whose nuclear extract led to the observed gel shift), revealed expression of both murine AP-2.2 (Figure 10) and AP-2\(\alpha\) (Figure 11) mRNA. P19 cells differentiated to a neural cell lineage express 3.6-fold more AP-2\(\alpha\) and 4-fold less AP-2.2 mRNA than undifferentiated cells. P19 cells differentiated to a myocyte lineage express 1.4-fold more AP-2\(\alpha\) and 1.5-fold more AP-2.2 than undifferentiated cells. All AP-2 mRNA values were normalized by GAPDH mRNA expression.

Further analysis of potential AP-2 ET\(\alpha\) promoter interactions was performed in rat A10 SMCs which also express ET\(\alpha\) and can be readily transfected. RT-PCR analysis revealed that both AP-2.2 and AP-2\(\alpha\) are expressed in A10 cells and that AP-2.2 levels are 4-fold greater than AP-2\(\alpha\) (Figure 12).
*AP-2α Increases ET₁ (-116 → +215) Activity in a Dose Dependent Manner*

The pSAP-2α plasmid which contains the AP-2α coding region in front of the SV40 promoter, was cotransfected with ET₁ (-116 → +215)-luciferase into A10 SMCs in order to analyse the effects of AP-2 on ET₁ transcription. AP-2α expression was compared between cells transfected with either PSAP-2α or the empty PSG5 vector (which was obtained by removing the AP-2α coding region via an Eco RI digestion and subsequent ligation of the vector), using RT-PCR followed by agarose gel electrophoresis, ethidium bromide staining and densitometric analysis. Band densities were normalized by GAPDH expression which was also determined through RT-PCR analysis. Cells transfected with 1μg PSAP-2α exhibited a 5-fold increase in AP-2α expression over cells transfected with PSG5 (Figure 13). Interpretation of this data should take into account that the increase in mRNA expression may be greater than indicated as previous findings in our lab have shown that approximately 20% of A10 cells are transfected using the SuperFect method (Campbell, unpublished results, 1999). Initial experiments required the titration of PSAP-2α in order to determine the optimal concentration of AP-2α necessary to enhance the transcriptional activity of ET₁. AP-2 has been shown to interact with several cofactors and it may be essential that the correct stoichiometric amounts of AP-2, AP-2 cofactors and AP-2 binding sites are present to regulate transcription. The response of increasing doses of AP-2α on a constant amount of ET₁ (-116 → +215) plasmid was examined. One μg of ET₁ (-116 → +215) was cotransfected with 0 to 4μg of pSAP-2α and the luciferase activity was determined 24 hrs later. The empty vector pSG5 was included in each transfection to bring the
total DNA transfected to 6μg. The β-galactosidase coding region under the control of the cytomegalovirus promoter (CMV-βgal) was included as an internal control for transfection efficiency. AP-2α transfection resulted in a dose dependent increase in luciferase expression driven by the ET, (-116 → +215) promoter element. A 5-fold increase was observed with 4μg of pSAP-2α (p<0.001) versus ET, pSG5 cotransfection controls (Figure 14). This however may not represent the highest dose of PSAP-2α needed to obtain a maximum response of ET, (-116 → +215) as a plateau had not yet been reached with 4μg of pSAP-2α. We were unable however to test a higher dose of pSAP-2α due to limitations in our transfection model, (transfection of a total of more than 6μg of DNA proved to be toxic to the A10 SMCs as it caused cell detachment). Furthermore, controls to ensure that AP-2 does not affect luciferase transcript or protein stability need to be performed.

**ET, Regulatory-Element Decoy Inhibits ET, Transcription**

An effective strategy for testing the functional activity of putative transcription factor binding sites is competitive inhibition by the decoy method (Yamashita *et al.*, 1998). In a preliminary experiment, 6μg of ET, (+86 → +215) was transfected into A10 cells, (6μg of total DNA was determined to be the upper limit of DNA for transfection without compromising cell viability), and 6μg of the empty PGL2P vector was transfected into A10 controls. Total RNA was extracted and ET, and GAPDH mRNA levels were assessed using RT-PCR. Densitometric analysis revealed ET, mRNA to be decreased by 5-fold in ET, (+86 → +215) transfected A10s;
when compared to PGL2P transfected cells (Figure 15).

**AII ET**<sub>α</sub> **Interactions**

* **AII Increases ET**<sub>α</sub> **mRNA Expression**

Although there are conflicting results in the literature with regards to the impact of AII on the expression of the ET<sub>α</sub> receptor, many investigators have shown that AII is able to activate the endothelin system [Stula *et al.*, 1998; Barton *et al.*, 1997; Emori *et al.*, 1991; Hatakeyama *et al.*, 1994; Imai *et al.*, 1992; Kohno *et al.*, 1992]. After treating A10 SMCs with 10<sup>−7</sup>M AII for 24hrs in 1% FBS DMEM cells were harvested and total RNA was extracted and assayed for ET<sub>α</sub> mRNA expression using a competitive quantitative RT-PCR technique. A10 cells treated with AII exhibited a 1.9-fold increase in ET<sub>α</sub> mRNA expression versus control cells treated with an equal amount of vehicle (n=4, p=0.012)(Figure 16).

In view of reports by other investigators that ET-1 expression is significantly increased by AII treatment, we tested to see whether ET-1 treatment alone was sufficient to increase ET<sub>α</sub> mRNA expression. Treatment of A10s with 10<sup>−7</sup>M of ET-1 for 24 hrs did not affect the expression of ET<sub>α</sub> mRNA (Figure 17). This would suggest that ET-1 alone has no affect on the transcriptional rate of ET<sub>α</sub> mRNA over a period of 24hrs. This may be further applied to AII’s ability to stimulate ET<sub>α</sub> expression suggesting that AII activates ET<sub>α</sub> transcription through a more complex mechanism including direct action rather than only through modulation of the secretion of ET-1. Although both the AII and ET are known to stimulate similar G proteins, our results suggest that another pathway is involved.
ET\_4 Regulatory Elements are Activated by AII Treatment

We further investigated whether the aforementioned positive regulatory elements, ET\_4 (-116 → -38) and ET\_4 (+86 → +215), were affected by AII treatment. A10s were transfected with 2\(\mu\)g of a firefly luciferase reporter gene under the control of the SV40 promoter and either ET\_4 (-116 → -38) or ET\_4 (+86 → +215), and treated with 10^{-7}M AII in 1%FBS DMEM. A renilla luciferase vector was included as a control for transfection efficiency. Following a 24hr incubation in the presence of 10^{-7}M of AII, a 1.8 and 1.3-fold increase in luciferase activity for the ET\_4 (-116 → -38) (Figure 18) and ET\_4 (+86 → +215) (Figure 19) elements, respectively, versus transfected cells treated with vehicle alone (p<0.05) was noted. The effect of AII on the regulatory activity of these elements is not additive, as ET\_4 (-848 → +215) a firefly luciferase reporter gene vector including both these elements and a larger portion of the 5' end of the ET\_4 gene, only exhibited a 1.54-fold increase in luciferase activity when compared to transfected cells treated with vehicle alone (p=0.004) (Figure 20).
Discussion

AP-2 and ET₄

Numerous determinants are involved in the developmental fate of a cell. These determinants include the genetic contents of the cell cytoplasm, cell-cell contact and diffusion of signalling molecules between cells (Gurdon, 1992; Gurdon et al., 1992; Gurdon, 1999). Nonetheless, the most influential component of cell differentiation is gene expression. Temporal and spatial gene regulation is necessary for cells to undergo normal differentiation.

The success of genetic engineering has eased the process of determining the developmental, physiological, and pathophysiological significance of numerous genes. Mice can now be generated with specific genes deleted in order to study the biological importance of the missing gene. These “knockout” mice are created by mutating or removing the gene of interest from stem cells which are then injected into blastocyst and allowed to recombine with the genomic DNA in a process known as homologous recombination.

The knockout technique has been applied to the endothelin system and has revealed many interesting results. Aside from being a lethal mutation (ET-1 prenatally and ET₄ perinatally), inactivation of ET-1 or ET₄ lead to aberrant embryonal development including severe malformations in neural crest-derived structures such as the craniofacial region, the great vessels and the cardiac outflow tract. Such deformities have been attributed to the failure of neural crest cells to differentiate following migration to the branchial/pharyngeal arches (Kunhara et al., 1994; Clouthier et al., 1998; Yanagisawa et al., 1998; Kempf et al., 1998; Thomas et al., 1998).

In-situ hybridization analysis has revealed specific ET-1 and ET₄ expression in several
areas of the developing mouse embryo. ET₁ is predominantly expressed in migrating cephalic neural crest cells, in the mesenchyme of several branchial/pharyngeal arches, in the myocardium of the ventricles and atrium and in the aortic arch mesenchyme of the developing mouse (Clouthier et al., 1998; Yanagisawa et al., 1998). ET-1 expression was localized to the ectodermal epithelium of the corresponding branchial/pharyngeal arches, and the endocardium of the heart chambers and endothelial lining of the arch arteries (Thomas et al., 1998). These findings reveal the importance of carefully regulated gene expression for the normal development of the mouse embryo.

In addition to its potential role in development, ET₁ is important in vascular pathobiology as well. Increases in ET₁ mRNA expression have been shown to influence vascular responses to conditions such as arterial injury (Picard et al., 1998) and myocardial hypertrophy (Brown et al., 1995). These increases in ET₁ mRNA levels are the result of either a change in transcript stability or the regulation of ET₁ transcription. Transcriptional activation is highly dependent upon the cell surroundings, the structural conformation of the genomic DNA and the availability of the necessary transcriptional machinery such as transcription factors and polymerases.

Deletional analysis of the ET₁ promoter has revealed two elements which are important for tissue specific transcriptional regulation. One element is located in the 5' flanking region between 116 to 38 bp upstream of the transcription initiation site. This element has been previously identified through gel shift assay and Dnase footprinting and its promoter activity may be silenced through the decoy strategy (Yamashita et al., 1998). The other element, to the best of our knowledge identified only in our laboratory, is located in the 5' untranslated region between
86 to 215 bp downstream from the transcription initiation site. Both elements are able to modify SV40 promoter activity in a tissue specific manner, as indicated by reporter gene experiments with firefly luciferase (Monge, unpublished results). Our analysis of ETₐ (+86 → +215) using gel mobility assay, indicates that ETₐ (+86 → +215) binds specifically to factors from a DMSO-differentiated P19 nuclear extract. These cells have been shown to morphologically resemble a cardiac and smooth muscle cell lineage, and express more ETₐ than their undifferentiated counterpart (Monge et al., 1995). Hence it may be inferred from these results that there is a potential DNA-protein interaction between these regulatory elements and one or more nuclear transcription factors. Analysis of the ETₐ (-116 → +215) sequence in a transcription factor database (DNAxis), indicates the presence of several putative transcription factor binding sites within the regulatory element. One potential binding site present twice in this region is that of the transcription factor AP-2. AP-2 is a RA inducible transcription factor which binds primarily to GC rich sequences (Oulad-Abdelghani et al., 1996).

The putative binding sequences thus far revealed are:

TCCCCANGCG (Imagawa et al., 1987)

CCCCAGGGC (Mitchell et al., 1987)

CCSCRGGC (Mitchell et al., 1987)

YCSCCMNSSS (Imagawa et al., 1987)

GSSWGSCC (Imagawa et al., 1987)

CCCMNSSS (Faisst and Meyer, 1992)

ET\textsubscript{\Lambda} and AP-2 knockout animals share many similar properties (Table 1). The AP-2 knockout mice die perinatally (Schorle \textit{et al.}, 1996; Zhang \textit{et al.}, 1996) and the ET\textsubscript{\Lambda} knockout animals die shortly after birth (Clouthier \textit{et al.}, 1998). Both exhibit aberrant development of branchial/pharyngeal arch-derived structures, as well as the craniofacial and respiratory regions (Schorle \textit{et al.}, 1996; Zhang \textit{et al.}, 1996; Clouthier \textit{et al.}, 1998; Yanagisawa \textit{et al.}, 1998). This resembles the human condition known as CATCH 22 (cardiac anomaly, abnormal face, thymic hypoplasia, cleft palate, hypocalcemia, and chromosome 22 deletions) which is the result of dysfunctional differentiation of neural crest cells (Clouthier \textit{et al.}, 1996).

One striking similarity between AP-2 and ET\textsubscript{\Lambda} is the spatio-temporal expression pattern of the two genes. Both ET\textsubscript{\Lambda} and AP-2 are initially expressed in migrating neural crest cells between the 8\textsuperscript{th} and 12\textsuperscript{th} day of embryo development and influence neural crest cell differentiation but not migration (Zhang \textit{et al.}, 1996; Clouthier \textit{et al.}, 1998; Yanagisawa \textit{et al.}, 1998). The expression of ET\textsubscript{\Lambda} lags a little behind that of AP-2 with AP-2 expression beginning at 8.5dpc and peaking at 11.5 dpc (Mitchell \textit{et al.}, 1991) and ET\textsubscript{\Lambda} expression beginning at 9.5dpc and peaking at 12dpc (Clouthier \textit{et al.}, 1998; Yanagisawa \textit{et al.}, 1998). AP-2 mRNA is detected in the lateral head mesenchyme as early as 8dpc and continues to be expressed in the head and the surface ectoderm at 9dpc (Mitchell \textit{et al.}, 1991; Meier \textit{et al.}, 1995; Moser \textit{et al.}, 1995; Chazaud \textit{et al.}, 1996; Moser \textit{et al.}, 1996). Similarly, ET\textsubscript{\Lambda} is expressed in migrating neural crest cells located in the head and body mesenchyme at 9.5 dpc—a slightly later time point than AP-2 expression in that area (Clouthier \textit{et al.}, 1998; Yanagisawa \textit{et al.}, 1998).

AP-2 mRNA expression has been localized to neural crest cells and their derivatives
between 8.5 to 12.5 dpc. These include the cranial and spinal sensory ganglia, the facial mesenchyme, surface ectoderm, longitudinal column of the spinal cord and hindbrain in contact with neural crest-derived sensory ganglia, limb bud mesenchyme, and the meso-metanephric region (Mitchell et al., 1991).

Developmentally, ETₐ and AP-2 play a role in the formation of many similar structures. Most of the abnormalities observed in the ETₐ and AP-2 knockout mice are derived from events that occur in the craniofacial region at 10-12dpc and result from a lack of fusion between the neural crest mesenchyme and the encasing epithelium. These abnormalities include: mandibular dysmorphology, facial clefting, abnormal formation of the skull, eyes, and ears, and hypoplasia of the jaw and tongue (Moser et al., 1995; Zhang et al., 1996; Schorle et al., 1996; Clouthier et al., 1998). These structures are derived from the branchial/pharyngeal arch-derivatives of the migrating neural crest. It should be noted, however, that while the Meckel’s cartilage and hyoid of the AP-2 knockouts appeared normal (Zhang et al., 1996; Schorle et al., 1996), they were both either deformed or missing in the ETₐ knockouts (Clouthier et al., 1998).

In the heart, ETₐ knockouts experienced interruption of the aorta, tubular hypoplasia, a missing right subclavian artery, extra arteries branching from the carotid, and right sided ductus arteriosus. Such defects suggest that ETₐ is important for proper aortic arch vessel patterning (Yanagisawa et al., 1998). Furthermore, the expression pattern of ETₐ first appears around the branchial/pharyngeal arches in human embryo at somatic stage 10-11 (Yanagisawa et al., 1998). The branchial/pharyngeal arches are derived from neural crest cells in the neural tube and lead to the formation of the embryonic circulatory system including the subclavian, the capillaries and
the aorta. Despite demonstrating AP-2 expression in the branchial/pharyngeal arches of the developing mouse embryo at 10dpc (Moser et al., 1995) and low levels of AP-2 expression in the heart at 13.5 dpc (Mitchell et al., 1991), its direct developmental effect on the heart and great vessels has not been described.

Pharmacological inactivation of ET, in the avian embryo results in similar defects of the branchial/pharyngeal arch derivatives as observed in the mouse knockout models. The advantage of using a pharmacological inactivation technique over a gene knockout model is that it allowed the investigator to inhibit ET, expression at various time-points and analyse its role during different stages of avian embryo development (Kemf et al., 1998). Their investigation demonstrated that the contribution of ET, to the normal development of neural crest-derived structures in the branchial/pharyngeal arches is dependent on a narrow window of time. Hence, not only is ET, 's role in neural crest cell differentiation limited to the post-migratory stage but its developmental importance is limited to a very specific period of time in a manner similar to AP-2.

Despite the many similarities between the ET, and AP-2 knockout mice, the malformations observed in the AP-2 knockouts are often more severe and may include defects not seen in the ET, knockout mice. These includes limb pattern formation defects, a missing eye or eye cavity, and body wall closure defects behind the ears, in the neck or midline of the mouse (Nottoli et al., 1998). When considered in conjunction with the fact that AP-2 expression occurs independently of ET, (Clouthier et al., 2000 demonstrated AP-2 expression in ET, knockout mice), and that the temporal and spacial expression of ET, lags slightly behind that of AP-2, it is
likely that if AP-2 and ET\(_\lambda\) expression are indeed connected, AP-2 expression lies upstream of ET\(_\lambda\). In order to test this hypothesis, the expression levels of ET\(_\lambda\) in the AP-2 knockout mice needs to be characterized. One would expect that if AP-2 is a critical mediator of ET\(_\lambda\) transcription, ET\(_\lambda\) expression would either be absent or severely down regulated in these animals.

The phenotypic similarities observed between the AP-2 and ET\(_\lambda\) knockout mice implies that they may overlap in their roles in development. This, coupled with our discovery of two AP-2 sites in the ET\(_\lambda\) 5' region, prompted us to examine the possible role of AP-2 in ET\(_\lambda\) (-116 → +215) driven transcription. ET\(_\lambda\) (-116 → +215) was obtained through PCR and cloned into the enhancerless and promoterless firefly-luciferase expression vector PGL2B. Cotransfection of A10 rat thoracic aortic smooth muscle cells with an ET\(_\lambda\) (-116 → +215) driven firefly luciferase expression gene, increasing doses of PSAP-2\(\alpha\) (the AP-2\(\alpha\) coding region under the control of the SV40 promoter) and pCMV-\(\beta\)gal revealed a dose dependent increase in luciferase activity over cells cotransfected with ET\(_\lambda\) (-116 → +215) and the PSG5 empty vector controls. These results implicate AP-2\(\alpha\) in the regulation of ET\(_\lambda\) (-116 → +215) driven transcription.

Although others have reported DNA-protein interaction within the 5' region of the ET\(_\lambda\) gene (Yamashita et al., 1998), this study represents the first time a potential AP-2 transcription factor binding site has been observed to influence the regulatory ability of ET\(_\lambda\) promoter, more specifically the ET\(_\lambda\) (-116 → +215) region. Furthermore, the preliminary finding that ET\(_\lambda\) mRNA levels can be down regulated 6-fold by decoy transfection of ET\(_\lambda\) (+86 → +215), (which contains both putative AP-2 binding sites), suggests that ET\(_\lambda\) (+86 → +215) alone is a critical
component of ETₐ transcription. However, the effect of AP-2 administration on general transcription would have to be assessed in order to determine that our observations were not a consequence of overall transcriptional upregulation. This could be accomplished by comparing the overall mRNA content before and after AP-2 administration. Furthermore, in order to conclude that ETₐ (+86 → +215) inhibits ETₐ transcription by acting as a decoy for AP-2, a direct interaction of ETₐ (+86 → +215) and AP-2 would have to be demonstrated. This could be accomplished by the “supershift” technique whereby labelled ETₐ (+86 → +215) is incubated with nuclear extract and AP-2 antibodies. AP-2 bound DNA would produce a DNA-AP-2/AP-2-antibody complex which should exhibit slower mobility in a polyacrylamide gel than the nuclear extract-DNA complex alone. The precise bases within ETₐ (+86 → +215) necessary for AP-2 binding may then be determined by Dnase footprint analysis. This assay takes advantage of Dnase ability to digest double stranded DNA while leaving “protected fragments” (ie those bound by a component of nuclear extract) intact. The intact fragment may then be elecrophoresed on a sequencing gel, and the sequence of the protected fragment determined. Gel mobility assays incorporating nuclear extract and sequential bp mutations of the AP-2 consensus sequence should then be performed, and the minimal nucleotide sequence required for AP-2 binding could then be determined. Trans-activation decoy experiments utilizing the putative binding sites in the ETₐ promoter or the determined mutants would be helpful in confirming these results in a cell culture model.

A comparison of the rat, mouse and human ETₐ gene reveals a 49 bp region within the ETₐ (+86 → +215) fragment (between +97 → +145) which exhibits a high degree of cross-
species homology (95% conserved between rat and mouse, and 87% conserved with human). Furthermore, a CCCMNSSS putative AP-2 binding site exits within 11 (mouse and rat) to 69 (human) bp downstream of the 40bp conserved region (bases +205 \(\rightarrow\) +212 in the human) and an additional putative AP-2 site found only in the human sequence between bases +130 \(\rightarrow\) +137. The lack of a detectable second AP-2 site within the mouse and rat sequences is only due to one missing base pair, which may or may not indicate their ability to interact with AP-2 (Figure 6). Therefore, further analysis of AP-2-ET \_ interaction should begin with this region.

The study of AP-2's role in transcriptional activity is further complicated by the existence of several AP-2 isoforms. Three human AP-2 isoforms have thus far been identified: AP-2\(\alpha\) (Mitchell, 1987; Williams et al., 1988), \(\beta\) and \(\gamma\), (Williamson et al., 1996). Similarly, three isoforms of AP-2 have been described in the mouse: AP-2\(\alpha\) (Moser et al., 1996), AP-2\(\beta\) (Moser et al., 1995) and AP-2.2 (Oulad-Abdelghani et al., 1996). While all published isoforms have been localized to different chromosomal locations (Williamson et al., 1996) with differing expression patterns (Moser et al., 1995), all three exhibit a high degree of homology in their DNA binding and homodimerization domains, and contain sequences rich in proline and glutamine residues (Oulad-Abdelghani et al., 1996) which have been suggested to play a role in hAP-2 (now referred to as human AP-2\(\alpha\)) transactivation potential (Williams and Tjian, 1991).

We have shown through RT-PCR analysis that A10 cells exhibit a 4-fold greater expression of the AP-2.2 isoform than AP-2\(\alpha\). Furthermore, P19 cells differentiated to a myocyte phenotype exhibit 1.5-fold more AP-2.2 and 1.4-fold more AP-2\(\alpha\) than undifferentiated P19s. This is in
contrast to our findings in RA-differentiated P19 cells (neural lineage), which exhibit 3.6-fold more AP-2\(\alpha\) and 4-fold less AP-2.2 than undifferentiated cells. [Note that others have demonstrated an increase in both AP-2 isoforms in RA treated P19 cells (Oulad-Abdelghani et al., 1996)] The predominance of the AP-2.2 isoform in cells with higher ET\(_A\) expression (A10s and P19 SMCs), might indicate that AP-2.2 plays a more important role in ET\(_A\) transcription, however, our transactivation experiments suggest that the AP-2\(\alpha\) isoform is able to sufficiently upregulate ET\(_A\) transcriptional activity. It is possible that variation in AP-2 expression is a result of tissue specific regulation and that the activation of ET\(_A\) transcription is not specific for one AP-2 isoform over the other. This is supported by the fact that AP-2\(\alpha\), \(\beta\) and AP-2.2 require the same consensus sequence for binding and have similar binding and homodimerization domains (Oulad-Abdelghani et al., 1996). Our results show an overall increase in AP-2 RNA expression in DMSO treated P19 cells (1.4-fold AP-2\(\alpha\) and 1.5-fold AP-2.2), and no overall change in AP-2 RNA levels in RA treated P19s (while there is a 3.6-fold increase in AP-2\(\alpha\) RNA levels, there is a 4-fold decrease in AP-2.2). Hence it may be that overall AP-2 expression is a key mediator of ET\(_A\) expression as it is more prevalent in ET\(_A\) expressing DMSO treated P19s, as opposed to predominantly ET\(_B\) expressing RA treated cells (see Monge et al., 1995).

The AP-2 transcription factors act in conjunction with other co-activators in facilitating ET\(_A\) gene expression. To date the transcriptional activators PC4 (Kannan and Tainsky, 1999) and PolyADP-ribose polymerase (PARP) (Kannan et al., 1999) have been identified as positive coactivators of AP-2 transcriptional activity. These coactivators act independently of one another,
and are responsible for relieving AP-2 self-interference. The potential interaction of such cofactors need to be considered when analysing the role of AP-2 in transcriptional regulation. It is also necessary to determine the proper stoichiometric ratio of AP-2: cofactor: consensus-sequence as too high or low concentrations of either may render the transcriptional factors ineffective by sequestering AP-2 or one of its cofactors. Furthermore, high levels of AP-2 may also silence transcriptional activation since the amino-terminal region of AP-2 has been shown to regulate its own transcription in a negative feedback manner to the extent that an overabundance of AP-2 in PA-1 cells lead to reduced AP-2-mediated transcriptional activation (Kannan et al., 1994; Kannan P and Tainsky, 1999). This may be a potential explanation to the absence of ET receptor expression in COS cells despite the high levels of AP-2 mRNA expression. However, there are several other plausible explanations for these findings. One could also hypothesize that an absence in the cofactors necessary for AP-2-mediated ET\textsubscript{A} transcription, (as opposed to a stoichiometric imbalance), would also to silence ET\textsubscript{A} transcription despite the abundance of AP-2 expression in the COS cells. Alternatively, the tertiary structure of the chromosomal DNA in COS cells may be such that the ET\textsubscript{A} gene or promotor region of the gene does not lie in an area which is accessible to transcription factor binding (i.e. a hypersensitive area). This would result in a lack of AP-2 binding and no subsequent ET\textsubscript{A} mRNA transcription.

The similarities between the AP-2 and ET\textsubscript{A} knockouts suggests that an important link exists between the expression of these two proteins in development. Nevertheless, without further experimentation possible interactions between ET\textsubscript{A} and AP-2 may be attributed to the interruption of parallel mechanisms which contribute to similar developmental patterns. The
results of our transactivation experiments strengthen the postulated interaction of AP-2 and \( ET_\lambda \) expression and directly implicate AP-2 activity in \( ET_\lambda \) transcription. However, in order to chart the precise course of AP-2 involvement in \( ET_\lambda \) transcription much work remains to be done. One possible experiment would be to inhibit AP-2\( \alpha \) activity by transfecting the pSAP-2B plasmid into \( ET_\lambda \) expressing cell lines and evaluating its effect on endogenous \( ET_\lambda \) expression. It would be expected that if AP-2 did play a role in \( ET_\lambda \) transcription, inactivation of AP-2 would lead to the downregulation of \( ET_\lambda \) transcription. Nonetheless, differences between cell types must be taken into account since AP-2's role in transcriptional activation varies from one cell type to another, and from one stage of differentiation to the next (Kannan et al., 1994).

In order to convincingly demonstrate that AP-2 is essential for \( ET_\lambda \) mediated development, thorough in-vivo experiments need to be performed. Possible experiments include \textit{in-situ} hybridization on sections of the \( ET_\lambda \) and AP-2 knockouts using cRNA probes for AP-2 and \( ET_\lambda \) respectively. If AP-2 expression lies upstream of \( ET_\lambda \) transcription, decreased \( ET_\lambda \) mRNA in cranial neural crest-derived structures would be detected in the AP-2 knockouts although AP-2 mRNA might be observed in the \( ET_\lambda \) knockouts. Furthermore, the development of an \( ET_\lambda \) rescue model in the AP-2 knockout mouse would greatly contribute to the understanding of these two proteins in murine development. One possibility would be to place \( ET_\lambda \) expression under the control of the AP-2 promoter and study the expression of \( ET_\lambda \) in the \( ET_\lambda \) knockout mouse. It is conceivable that if \( ET_\lambda \) expression is lacking in the AP-2 knockouts, it may be “rescued” in this transgenic animal.

Alternatively, an \( ET_\lambda \) “knockdown” model placing the \( ET_\lambda \) antisense under the control of
the AP-2 promoter, could be created. This would result in the expression of the ET$_\alpha$ antisense parallelling that of AP-2. If, in fact, ET$_\alpha$ expression and its downstream consequences were dependent on AP-2 activity, they would be countered by the effects of the AP-2 driven ET$_\alpha$ antisense message.

_In-vivo decoy_ experiments utilizing the ET$_\alpha$-AP-2 binding site to compete for AP-2 binding during embryo development would also help to define the role of ET$_\alpha$-AP-2 interactions in development.

Despite the lack of information pertaining to the events upstream of ET$_\alpha$ transcription, several groups have described factors involved in the downstream signalling cascade of endothelin mediated development. Disruption of the ET$_\alpha$ gene lead to similar craniofacial defects as knockouts of the _goosecoid_ gene, which is commonly expressed in the branchial/pharyngeal arches of the developing mouse (Clouthier _et al._ 1998). Moreover, ET$_\alpha$ knockouts stained for _goosecoid_ using whole mount _in-situ_ hybridization failed to detect _goosecoid_ in the first and second branchial/pharyngeal arches suggesting that goosecoid might be a downstream factor in an ET$_\alpha$ signalling cascade. Others have shown that the transcription factors eHAND and dHAND which are normally expressed in the mesenchyme underlying the branchial/pharyngeal arch epithelium, are down regulated in ET-1 null mice resulting in hypoplasia of the branchial/pharyngeal arches and aberrant development of the cranial neural crest (Thomas _et al._, 1998). Furthermore, just like the ET$_\alpha$ knockouts, neural crest cell migration remains normal in dHAND null mice however, instead of differentiating into their appropriate structures, mesenchymal cells enter an apoptotic pathway. It is postulated that ET-1 in the epithelium
activates the ET$_A$ receptor in the underlying neural crest leading to ectomesenchyme differentiation of which dHAND and eHAND play an integral part.

Through the use of knockout models, other genes have been implicated in neural crest development as well. These include: type IB activin receptor which is important for axial patterning and lateral asymmetry in the mouse (Oh and Li, 1997), the mouse homeobox genes hox-1.5 and 1.6 which participate in thyroid, submaxillary, throat, heart, ear, hindbrain and craniofacial development (Chisaka and Capecchi, 1991; Chisaka et al., 1992; Carpenter et al., 1993), the neurofibromatosis (NF1) gene which is responsible for heart and neural crest-derived tissue development (Brannan et al., 1994), and the RA receptor (RAR) which aside from inducing AP-2 activation, contribute to the development of the neck, trunk, abdominal regions, limb and cardiovascular development of the mouse (Mendelson et al., 1994, Sucov et al., 1996, Gruber et al., 1996, Sanford et al., 1997). Development is a complex process involving the coordinated effort of many different genes and biological systems. It is therefore possible, and even likely, that any one of the aforementioned systems together with yet unidentified factors contribute to ET$_A$'s role in the developmental process.

**AII and ET$_A$**

Aside from its potential transcriptional significance in murine development process, we have demonstrated that the 5' region of the ET$_A$ gene plays a role in the transcriptional response of the endothelin system to AII as well.

The Angiotensin and Endothelin systems closely resemble one another, regulate each
other in various pathological states, and share several common signal transduction mechanisms. AII and ET-1 are derived from larger precursors (AI and big-ET-1) and are processed by substrate specific converting enzymes (ACE and ECE). They have each been implicated as key players in the development of hypertension and myocardial hypertrophy, and have been shown to act as potent mitogens and proliferative agents. Furthermore, their actions are carried out via activation of a G-protein coupled receptor which in turn activates a complicated signalling cascade involving mediators such as PKC and MAP kinase.

Recent studies have shed light on the intricate interactions that exists between these two systems. In cell culture, AII has been shown to increase ppET-1 mRNA in cardiac fibroblasts (Fujisaki et al., 1995), VSMC (Sung et al., 1994), and endothelial cells (Imai et al., 1992) via the AT-1 receptor, in an ET, dependent manner. Furthermore, in rat cardiac fibroblasts, ET-1 has been shown to activate a positive feedback loop by stimulating the conversion of AI to AII, which in turn leads to an increase in ET-1 production (Ito et al., 1993). This has also been demonstrated in VSMC where ET-1 treatment resulted in a 1.9 fold increase in ACE activity (Fukazawa et al., 1996), and a similar increase has been seen in pulmonary artery endothelial cells (Kawaguchi et al., 1991).

In-vivo, subcutaneous infusion of AII in rats has led to an increase in ET-1 protein expression in the aorta, femoral artery and kidneys which was attenuated by pretreatment with the ET, antagonist LU13525. Furthermore, the observed increase in ET-1 is not localized to endothelial cells, as denudation of femoral artery and aortic sections exhibit similar staining intensities as intact tissue sections (Barton et al., 1997; Barton et al., 1998). Also, much like the
in-vitro observations, AII stimulates ECE activity in the rat aorta which can be blocked with an ET$_{A}$ antagonist (Barton et al., 1997). It should also be noted that the ACE inhibitor, fosinopril, has also been shown to reduce the circulating ET-1 plasma level in patients with congestive heart failure, presumably by reducing the available amount of AII (Galatius-Jensen et al., 1996).

Such observations, however, are dependent on the tissue, and organ being discussed. Under hypertensive promoting conditions, long term infusion of AII increases blood pressure via AT-1 in an ET$_{A}$ dependent manner (d'Uscio et al., 1997; Rajagopalan et al., 1997; Herizi et al., 1998). The same seems to be true for the observed increase in ppET-1 mRNA in the kidney and the heart following AII induced hypertension. However, under basal conditions, ET-1 levels in the kidney are unaffected by treatment with an ET$_{A}$ antagonist (Barton et al., 1998).

Our study has demonstrated that aside from its ability to regulate the ET-1 ligand, AII is also able to increase the transcriptional activity and hence the mRNA production of the ET-1 specific receptor ET$_{A}$. Rat aortic VSMC treated with $10^{-7}$M AII for 24 hrs exhibited a 1.9-fold increase in ET$_{A}$ mRNA as demonstrated by the extremely sensitive quantitative RT-PCR technique. This corresponds well to previous observations that human pulmonary artery VSMC exhibit an increase in ET$_{A}$ mRNA and receptor expression (Hatakeyama et al., 1994), and that the ACE inhibitor, quinapril, was able to lower ET$_{A}$ mRNA in nephrectomized rats (Ruiz-Ortega et al., 1997). However, the effect of AII administration on general transcription would have to be assessed in order to determine that our observations were not a consequence of overall transcriptional upregulation. This could be accomplished by comparing the overall mRNA content before and after AII administration. Others investigators have suggested the opposite,
namely that AII downregulates the number of ET-1 binding sites in cell culture (Roubert et al., 1989), however, these studies were limited to observations based on binding studies which may not accurately reflect the number of receptors present. Because of the strong interactions between ET-1 and ET₄, the difficulty in discerning accurate information from binding studies is highlighted in several animal studies. Rats infused with AII exhibited an increase in blood pressure, cardiac hypertrophy, and proteinuria all of which were minimized or abolished following ET₄ antagonism. Nonetheless, binding studies revealed no change, or a decrease in receptor number following the AII treatment (Herizi et al., 1998; d’Uscio et al., 1998; Rajagopalan et al., 1997). The authors suggest that perhaps binding studies are not an accurate method of measuring ET₄ expression, since AII stimulation leads to an increase in endogenous ET-1 which may occupy all of the available receptors, making it impossible to detect whether there had been a change in the overall receptor population.

This study further delineated several regions within the 5' region of the ET₄ gene which respond to AII treatment by increasing transcription of a heterologous promoter in a firefly luciferase reporter gene system. Both ET₄ (-116 → -38) and ET₄ (+86 → +215) exhibited a 1.37- and 1.75-fold increase in firefly luciferase expression (respectively) when treated with AII. Furthermore, when both elements were placed together in a firefly luciferase reporter gene plasmid (without the SV40 promoter), their transfection into and the subsequent AII treatment of the A10 VSMC led to a 1.54-fold increase in firefly luciferase activity. These results indicate that AII increases the ability of these separate elements to drive the activity of the heterologous SV40 promoter, and to regulate luciferase expression independent of the SV40 promoter when both are
located within the same plasmid construct. This is likely a tissue specific event, as previous studies have demonstrated these elements to be active exclusively in ET\textsubscript{A} expressing cells. Future experiments should investigate the effects of AII on the empty PGL2 promoter plasmid, in order to demonstrate that our observations are a direct result of increased ET\textsubscript{A} activity and not AII stimulation of the SV40 promoter.

The common theme in many AII induced pathological states seems to be the downstream increase in ET-1 expression, and the ability to block the detrimental effects of AII treatment using ET\textsubscript{A} antagonists. This would suggest that AII is situated upstream of many ET controlled events, and mediates its effects via ET. Not only is this evident in whole animal studies, but it has been demonstrated in an \textit{in-vitro} experiment as well. Experiments indicate that once activated by either AII or ET-1, PKC is subsequently desensitized to further activation by the same stimulant. Similarly, once the translocation of PKC to the cell surface is initiated by ET-1, it becomes desensitized to subsequent stimulation by AII. However, stimulation of PKC translocation by AII does not render it insensitive to further stimulation by ET-1 (Feng \textit{et al.}, 1998). Such observations suggest that ET-1 is somehow able to override AII induced stimulation of PKC, while AII may not override ET-1 induced stimulation indicating a stronger ability of ET-1 to stimulate PKC. Although one may inhibit an AII mediated event using an ACE inhibitor or an AT-1 antagonist, such antagonism may lose some of its efficiency if circumvented with ET-1 stimulation. Furthermore, since ET-1 is such a potent ligand, even a small increase in amount of circulating ligand or receptor number could lead to a potent downstream effect. Based on this reasoning, ET\textsubscript{A} antagonism may prove to be the superior to AII receptor antagonists in
controlling AII mediated events, such as hypertension or myocardial hypertrophy.

The present study demonstrates that stimulation of A10s with $10^{-7}$ M of ET-1 fails to evoke a change in the levels of ET$_{\Lambda}$ mRNA over a period of 24 hrs. Such a result suggests that the observed increase in ET$_{\Lambda}$ mRNA following stimulation with AII occurs as a direct consequence of an AII signalling cascade and not due to the AII-induced secretion of ET-1. Nonetheless, it must be considered that several investigators have shown an increase in ET-1 mRNA and protein levels upon stimulation with AII. One possible model for the AII induced increase of ET$_{\Lambda}$ in A10 SMC is that AII binds to its AT-1 receptor and activates a signalling cascade that leads to the observed change in ET$_{\Lambda}$ mRNA. AII is likely acting through the AT-1 and not the AT-2 receptor since most of the known the common pathophysiological effects of endothelin and angiotensin stimulation occur through the ET$_{\Lambda}$ and AT-1 receptors respectively. However, this would have to be verified through AT-1 inhibitor studies which could be formed using one of the many AT-1 antagonists available.

Despite numerous studies showing that increases in ET$_{\Lambda}$ receptor expression occur concomitantly with changes in ET-1, few have attributed this increase to be downstream of ET-1 regulation. However, as is the case with most gene expression studies, many factors need to be taken into consideration before a meaningful conclusion can be reached. Regulation of ET and the endothelin receptors may vary depending on the experimental conditions. The functional effects of endothelin and the regulation of the ET$_{\Lambda}$ receptor have been shown to differ in the failing and nonfailing human and rat heart (Pieske et al., 1999; Kobayashi et al., 1999). Failing human hearts exhibited a 3-fold increase in ET-1 and upregulated ET$_{\Lambda}$ receptor density versus
nonfailing myocardium, (although the significance of such a finding needs to be examined since nonfailing hearts were found to be more sensitive to the ionotropic effects of ET-1 than failing hearts) (Pieske et al., 1999). Furthermore, the same authors found ET receptor expression to be differentially regulated in dilated versus ischemic cardiomyopathy. Similar results were also seen in CHF rats which exhibited an increase in ET-1 expression in the cardiomyocytes independent of ECE activity, and an increase in both ET receptor mRNA and [125I]ET-1 binding in left ventricular membrane preparations (Kobayashi et al., 1999). Others have shown an increase in ET-1 and ET₄ mRNA levels in the hypertrophied myocardium of the aorto-venacaval fistula rat (Brown et al., 1995). It cannot be elucidated from this study whether the change in the levels of ET-1 may have led to an increase in ET₄ or vice-versa. The AII induced increase in ET₄ mRNA may be due to a change in endogenous ET-1 levels which may mediate a different response than exogenously added ET-1. Pretreatment of A10s with an ET₄ antagonist such as BQ123 prior to addition of AII would help clarify this issue.
**Conclusion**

ET-1 plays a role in many pathophysiological events and is a crucial mediator of embryonic development. It preferentially binds to the ET$_A$ receptor and as such, delineating ET$_A$ transcriptional and translational regulation is essential for defining the role of the ET system in these processes. We have identified two regions in the ET$_A$ promoter which are capable of regulating a heterologous promoter in a tissue-specific fashion. One of these sequence elements contains putative binding sites for the transcription factor AP-2 which interacts with a component of DMSO treated P19 cell nuclear extract. Both the AP-2 and ET$_A$ knockout mice exhibit a similar phenotype characterized by abnormal development of the craniofacial region and other neural crest derived structures. Through co-transfection experiments, we have shown that AP-2$\alpha$ upregulates ET$_A$ (-848 $\rightarrow$ +215) driven luciferase activity in a concentration-dependent manner. Furthermore, decoy transfection of an element containing two putative AP-2 binding sites [ET$_A$ (+86 $\rightarrow$ +215)] led to a 5-fold reduction in ET$_A$ mRNA expression. These results suggest that AP-2 plays a role in ET$_A$ transcriptional activity, and may be an important link in ET$_A$ mediated development.

The ET system has also been shown to interact with the renin-angiotensin system under numerous pathophysiological conditions. Several investigators have shown that AII mediated events can be blocked by administration of ET$_A$ antagonists. As such, we hypothesized that AII activity may control the expression of ET$_A$ mRNA expression. Through quantitative, competitive RT-PCR analysis, we have shown that ET$_A$ mRNA is increased by 1.9-fold in AII treated A10 cells. Furthermore, we investigated the role of the previously identified ET$_A$ regulatory elements
in AII-induced ET₄ expression. AII treatment of A10 cells transfected with ET₄ (-116 → +215), ET₄ (+86 → +215) or ET₄ (-848 → +215) led to a 1.8-fold, 1.3-fold or 1.54-fold increase in luciferase activity respectively, indicating that these elements regulate transcriptional activity in response to AII stimulation.

These experiments highlight the importance of the ET₄ promoter elements which maybe regulated on a transcriptional level through interactions with the AP-2 transcription factor and are functionally important in AII's modulation of ET₄ expression.
Table 1

AP-2 VS ETₐ Knockouts

<table>
<thead>
<tr>
<th></th>
<th>AP-2</th>
<th>ETₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lethal</td>
<td>Yes</td>
<td>Die of respiratory failure within 30 minutes after birth</td>
</tr>
<tr>
<td>Neural crest cells</td>
<td>Doesn’t affect migration</td>
<td>Doesn’t affect migration</td>
</tr>
<tr>
<td></td>
<td>Expression high at 11.5dpc</td>
<td>Expression high at 12dpc</td>
</tr>
</tbody>
</table>
| Craniofacial abnormalities mainly due to lack of fusion between neural crest mesenchyme and epithelium | Mandible poorly formed
Facial clefting
Eyes, ears abnormal/missing
Hypoplastic tongue and jaw
Meckel’s cartilage and hyoid missing
Peripheral nervous system abnormal | Similar to AP-2 (defects in cephalic neural crest derivatives) but less severe
Meckel’s cartilage deformed by 14 dpc |
| Vasculature            | Expressed in branchial arches at 10dpc    | In branchial arches at 10dpc              |
|                        | Otherwise not well described              | Key role in vessel arch patterning       |
| Body wall & others     | Closure defects                           | Sunken ventral neck                      |
|                        | Limb pattern formation defects            | Doesn’t affect limbs                      |
|                        |                                          | Not as severe                             |

AP-2 and ETₐ knockout mice experience many similar developmental defects. Listed are the major similarities between the two mice. Note the slight delay in ETₐ mediated development, suggesting that its expression is downstream of AP-2.
Preproendothelin is converted to big Endothelin by furin convertase. Big Endothelin is further cleaved by one of several endothelin converting enzyme isoforms to form the three mature endothelin peptides.
Shown here is a graphical representation of the endothelin A receptor gene and its resulting transcript. Exon and intervening intron sizes are indicated.
**ET<sub>A</sub> Promoter Activity in DMSO-Differentiated P19 Cells**

<table>
<thead>
<tr>
<th>TIS [848]</th>
<th>Lucif</th>
<th>LUCIFERASE ACTIVITY %</th>
</tr>
</thead>
<tbody>
<tr>
<td>+1</td>
<td>+92</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TIS [-116]</th>
<th>Lucif</th>
<th>LUCIFERASE ACTIVITY %</th>
</tr>
</thead>
<tbody>
<tr>
<td>+92</td>
<td></td>
<td>206 (p&lt;0.05)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TIS [-38]</th>
<th>Lucif</th>
<th>LUCIFERASE ACTIVITY %</th>
</tr>
</thead>
<tbody>
<tr>
<td>+92</td>
<td></td>
<td>56 (p&lt;0.01)</td>
</tr>
</tbody>
</table>

Constructs containing serial deletions of the ET<sub>A</sub> promoter in a firefly luciferase reporter gene system were transfected into A10 cells and luciferase activity was measured.
Human \( \text{ET}_A \): Effect on Promoter Activity of a Highly Conserved Domain in the 5’ UTR

<table>
<thead>
<tr>
<th>LUCIFERASE ACTIVITY %</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>-253</td>
<td></td>
</tr>
<tr>
<td>-116 -38 +92</td>
<td>Lucif</td>
</tr>
</tbody>
</table>

-253 conserved -116 -38 +92 +215

\( \text{ET}_A (-253>92) \) and \( \text{ET}_A (-253>215) \) were placed in front of a firefly luciferase reporter gene and transfected into A10 cells. Following an overnight incubation, luciferase activity was determined.
ET\textsubscript{A} 5’ Flanking Region: Regulation of a Heterologous Promoter

\begin{center}
\begin{figure}
\centering
\includegraphics[width=0.5\textwidth]{figure.png}
\caption{Bar graph showing luciferase activity comparison between variouspromoters.}
\end{figure}
\end{center}

ET\textsubscript{A} (-116>−38) and ET\textsubscript{A} (+86 >+215) were ligated into the PGL2 promoter vector (in front of the SV40 promoter and firefly luciferase reporter gene) and transfected into A10 cells. Luciferase activity was then determined. (*p<0.04 compared to SV40 promoter.)
A Putative AP-2 Binding Site is Conserved Between Species

Rat ET<sub>λ</sub>  g g a g g c g t g t t c c t c c a g a g t t t g c t t t t c t t g g a g c c t c g c g c c +3nt  c c c a t c c c
Mouse ET<sub>λ</sub>  g g a g g c g t g t t c t c t g g a g t t t g c t t t t c t t g g a g c c t c g c g c c +3nt  c c c a t c c c
Human ET<sub>λ</sub>  g g a g g c g t g t t c t c c g g a g t t t t t c t t t t c t g c g a g e e c c e c t c g c g c c +60nt  c c c a e c c c

95% conserved between rat and mouse
87% conserved between rat, mouse and human

The rat, mouse and human ET<sub>λ</sub> gene sequences were compared (NCBI BLAST analysis) and analyzed for putative transcription factor binding sites (Dnasis). Bases differing from the rat sequence are shown in bold. AP-2 consensus sequences are underlined.
**ET\textsubscript{\textalpha}** Promoter Constructs

Three plasmid constructs were created for use in transfection experiments. \(\text{ET}\textsubscript{\textalpha} (-848>+215)\) was ligated into the PGL2B vector between the Kpnl and XhoI sites. The PGL2 basic vector does not include any promoter or enhancer elements. \(\text{ET}_\text{\alpha} (+86>+215)\) and \(\text{ET}_\text{\alpha} (-116>-38)\) was ligated into the PGL2P vector in front of the SV40 promoter between a Kpnl and XhoI site. All three vectors contain a firefly luciferase construct downstream of the multiple cloning region.
A10 cells were cotransfected with ETₜ₋₁₁₆ → +215 which had been ligated into the PGL2B vector as well as pSAP-2α and pCMV-β-galactosidase. The AP-2α expression vector included the AP-2α coding region under direction of the SV40 promoter ligated into the pSG5 vector. A CMV driven β-galactosidase expression vector was included in all transfections as a control for transfection efficiency.
**ETₐ (+86→+215) Band Mobility Shift with P19 Nuclear Extract**

Protein-DNA binding between ETₐ (+86→+215) and components of a P19 nuclear extract was tested by gel electrophoresis mobility assay. ETₐ (+86→+215) was incubated with 0.5 μg of DMSO-differentiated P19 nuclear extract. Separate reactions included a 200-fold molar excess of unlabeled ETₐ (+86→+215) or the CAT coding region (non-specific competitor).
Expression of AP2.2 in P19 Cells

AP-2.2 mRNA levels in control, retinoic acid and DMSO treated P19 cells were measured by RT-PCR. PCR products were electrophoresed on a 2% agarose gel and the bands were quantified by densitometry (n=1).
Expression of AP2α in P19 Cells

AP-2α mRNA levels in control, RA and DMSO treated P19 cells were measured by RT-PCR. PCR products were electrophoresed on a 2% agarose gel and the bands were quantified by densitometry.
Expression of AP-2 in A10 Cells

GAPDH

AP-2

AP-2α AP-2.2

AP-2α and AP-2.2 mRNA levels in A10 cells were measured by RT-PCR. PCR products were electrophoresed on a 2% agarose gel and the bands were quantified by densitometry.
Expression of AP-2α in Transfected A10 SMC

AP-2α mRNA in A10 cells transfected with either pSG5 or AP-2α was measured by RT-PCR. The PCR product was electrophoresed on a 2% agarose gel and quantified by densitometry.
AP-2α Upregulates ETA (-116 → +215)
Driven Luciferase Activity in a Dose Dependent Manner

Response of ELA(-116>+215) driven luciferase activity to increasing doses of pSAP-2α. (n=4, *p<0.05 compared to 0μg AP-2α, †p<0.05 compared to 1μg AP-2α, ‡p<0.001 compared to 2μg AP-2α, ††p<0.01 compared to 3μg AP-2α, error bars are SEM.)
**ETₐ (+86 → +215) Decoy Suppresses ETₐ Transcription**

Response of ETₐ mRNA levels in cells transfected with 6µg of ETₐ (+86 to +215). ETₐ mRNA levels were determined by RT-PCR analysis and normalized by GAPDH. Note: these results represent only one experiment and are therefore preliminary.
Effect of AII on $ET_A$ mRNA in A10 Cells

Quantitative, competitive RT-PCR analysis of $ET_A$ mRNA levels in A10 cells treated with $10^{-7}$M AII for 24 hrs. Error bars are SEM. (n=4, p=0.012)
Effect of ET-1 on $E_{TA}$ mRNA in A10 Cells

Response of $E_{TA}$ mRNA levels in cells treated with 10-7M ET-1 for 24 hrs. ETA mRNA levels were3 determined by competitive RT-PCR analysis. Note: this experiment only represents an n of 1 and is therefore preliminary.
Regulation of the ET_A(-116 → -38) Regulatory Element by AII

Response of ET\_\_ promoter fragment (-116>-38) to angiotensin II treatment in A-10 cells. Luciferase activity in untreated cells is expressed as 100% after normalization for β-galactosidase activity. (n=5, p=0.05.)
Regulation of the ET$_A$(+86 → +215) Regulatory Element by AII

Response of ETA promoter fragment (+86>+215) to angiotensin II treatment in A-10 cells. Luciferase activity in untreated cells is expressed as 100% after normalization for β-galactosidase activity. (n=5, p=0.034)
Regulation of ET\(_A\) (-848 → +215) by Angiotensin II

Response of ET\(_A\) promoter fragment (-116>+215) to angiotensin II treatment in A-10 cells. Luciferase activity in untreated cells is expressed as 100% after normalization for β-galactosidase activity. (n=4, p=0.0004)


Ref Type: Abstract


