2000-02-03

Sequences Required for Neurotensin Receptor-1 Gene Expression in N1E-115 Neurosblastoma Cells: Critical Importance of a CACCC Element for Activation During DMSO-Induced Neuronal Differentiation: a Dissertation

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SEQUENCES REQUIRED FOR NEUROTENSIN RECEPTOR-1 GENE EXPRESSION

IN N1E-115 NEUROBLASTOMA CELLS: CRITICAL IMPORTANCE OF A CACCC

ELEMENT FOR ACTIVATION DURING DMSO-INDUCED NEURONAL

DIFFERENTIATION

A Dissertation Presented

By

Daniel Jorge Tavares

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 3, 2000

Molecular Genetics

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*This thesis is dedicated to my wife Kelly Tavares and
my parents Fernando and Lucinda Tavares*

Acknowledgments

During the time I spent here working on my thesis, I was fortunate to be surrounded with people who have helped me through every step of the process. First I would like to thank Dr. Paul Dobner for his guidance and support over the years. Despite being my polar opposite on nearly every issue (politics, computer platforms, etc), I believe there is no one else who could have guided me through this thesis better. I would also like to thank the members of my committee who have offered me the advice and guidance that has gotten me to this point in my thesis.

Over the years, the many people who have come and gone through the Dobner lab have all helped out in one way or another. I would first like to thank Dr. Gerry McNeil, who patiently guided me through many of the laboratory techniques I learned in the first couple years in the Dobner lab. Also, the "never-boring" laboratory dynamics that he, Dr. Dobner, and Dr. Bob Harrison created was a deciding factor in choosing to do my thesis in this lab to begin with. I would also like to thank our lab technicians Nancy Deitemeyer and Sheryl Dooley. I can not imagine what the Dobner lab would be like without Nancy who is always more than willing to help out whenever possible and while Sheryl is new to our lab, she was my own personal psychologist through the most stressful part of my thesis.

Most importantly, I have to thank my wife Kelly and my parents, without whom, I could not have made it through this thesis. I met Kelly only a month after starting classes here and she has stuck with me through all the hard times during my thesis. She gives me a reason to want to succeed. My parents have always been a constant source of support

and encouragement, without which, I would never have the self-confidence to make it

through this monumental task.

Abstract

The promoter sequence of the mouse high affinity neurotensin receptor, *Ntr-1*, gene was cloned and characterized, sequences required for positive regulation in N1E-115 cells were localized, and at least two different peptides from these cells were shown to make specific contacts within the most potent positive regulatory element. A mouse neuroblastoma cell line, N1E-115, treated with 1.5% DMSO for 72 hours induces gene expression of both endogenous *Ntr-1*, and reporter constructs driven by the NTR-1 promoter, by 3 - 4 fold. The sequence of the NTR-1 promoter has no canonical TATA box, but is GC rich and contains consensus SP1, CACCC, CRE, and initiator elements. These elements are located within a 193 base positive regulatory region required for DMSO responsive activity and contains the transcriptional start site. Detailed mutational analysis of this region revealed that a CACCC box and the central region of a large GC-rich palindrome are crucial *cis*-regulatory elements for DMSO induction. The SP1 element, an NGFI-A-related element, and the 5' end of the positive regulatory region are required for maintaining basal expression in N1E-115 cells. Cell type differences in the *cis*-regulatory elements that mediate both DMSO induction and maintenance of basal expression are observed. Characterization of proteins in N1E-115 cells that make specific contacts within the CACCC element identified at least two peptides with predicted sizes of 57 kd and 97 kd. Two dimensional UV crosslinking indicates that these proteins might contribute to inducible gel shift complexes that require the CACCC element. Several previously characterized CACCC binding proteins, belonging to the Kruppel-like family of transcription factors, were tested by supershift analysis for their

ability to contribute to NTR-1 CACCC complexes. In fact, a protein closely related to SP1 does bind the CACCC element in N1E-115 cells, but of the other Kruppel-like protein tested, only BKLF contributes to a minor complex in N1E-115 cells. These results provide evidence for the complex regulation of *Ntr-1* gene expression mediated by the cooperation of several *cis*-regulatory elements including a CACCC Kruppel-like binding element.

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Collaborators

I would like to thank the following people for their help and contribution to the completion of this thesis:

Keith Tulley for the EMBL4 screen and cloning of the NTR-1 5' flanking sequence

Dr. Paul Dobner for the DNAse1 nuclease protection assays

John Heneghan for the degenerate Krüppel-like primer RT-PCR screen

Dr. Nirenberg for the N1E-115 cells

Dr. Merlin Crossley for the BKLF antibody

Drs. Conkright and Lingrel for the IKLF antibody and the LKLF antibody and expression construct

Dr. Russel Widom for the cKrox antibody and positive control nuclear extracts

List of Abbreviations

NT	neurotensin
NTR-1	high affinity neurotensin receptor-1
DA	dopamine
DMSO	dimethyl sulphoxide
CNS	central nervous system
VTA	ventral tegmental area
SN	substantia nigra
ARC	arcuate neurons of the hypothalamus
NA	nucleus accumbens
TH	tyrosine hydroxylase
TdT	terminal deoxynucleotidyl transferase
MEF	mouse embryonic fibroblasts
EKLF	erythroid Krüppel-like factor
BKLF	basic Krüppel-like factor

Chapter I

Introduction

Neurotensin (NT) is a thirteen amino acid neuropeptide expressed in the limbic regions of the brain and in the gastrointestinal tract. NT is expressed as part of a precursor protein NT/neuromedin N (Dobner et al, 1986) and is likely to have a widespread role as a neuromodulator (Nemeroff et al., 1982). In the gastrointestinal tract, NT has putative roles in stimulation of pancreatic secretion, colon motility, and facilitation of the translocation of fatty acids (Kitabgi 1982; Gullo et al. 1992). Due to the broad array of putative roles for NT signaling, the study of NT signaling can lead to a better understanding of a wide range of biological systems.

Three different neurotensin receptors have been characterized to date. The first receptor cloned was the high affinity NT receptor (NTR-1) which is a seven transmembrane G-protein coupled receptor (Tanaka et al. 1990), and is expressed in the majority of midbrain dopamine (DA) neurons. Activation of NTR-1 is coupled to phospholipase C and is marked by increases in intracellular Ca^{2+} and cGMP (Gilbert and Richelson, 1984), followed by internalization of activated receptors (Chabry et al. 1994). Two low-affinity receptors have been identified, NTR-2 (Chalon 1996) and NTR-3 (Mazella et al. 1998). Similar to NTR-1, NTR-2 is also a G-protein coupled receptor, but it elicits only a weak response to NT in frog oocytes (Vincent et al. 1999), and no response in CHO cells (Vita et al. 1998). Recent evidence suggests that NTR-2 may actually be activated by an, as yet, unidentified signal that is antagonized by NT (Vita et al. 1998). NTR-3 had previously been cloned in humans as a protein called sortilin, which is synthesized as a precurser protein and processed into a 5 kd peptide and a 95 kd membrane protein (Mazella et al. 1998; Vincent et al. 1999). Recent evidence indicates

that sortilin acts as an endocytic receptor on the cell surface; however the protein has no known intracellular signaling function (Nielsen et al. 1999). While the role of the two low affinity NT receptors in NT signaling is not well understood, there is a growing amount of evidence that NTR-1 mediates the majority of NT signaling in the brain (reviewed in Vincent et al. 1999).

NT has been implicated as a modulator of dopamine (DA) signaling in the brain. NT has an excitatory effect on the midbrain DA neurons (Pinnock 1985; Jiang et al 1994), and the subsequent release of DA activates DA receptors in target cells, resulting in increased locomoter activity (Kalivas et al. 1981; Kalivas et al. 1982). In the ventral tegmental area (VTA), NT stimulates DA neurons both directly (Pinnock 1985; Jiang et al. 1994), and indirectly through attenuation of the inhibitory effects of DA on the D2 autoreceptor (Shi and Bunney, 1991). The NT stimulation of VTA neurons results in an increase in DA release into the nucleus accumbens (Scotty et al. 1998). Unlike in the VTA, NT injection into the nucleus accumbens results in a neuroleptic-like response in that it has a strong negative regulatory effect on DA release and DA-mediated behavioral responses (Nemeroff 1980, Nemeroff and Cain 1985). These responses may result from an NT-mediated reduction in the affinity of D2 autoreceptors for DA (Shi and Bunney 1991; Rimindini et al. 1999) or a counteracting effect of NT on the cooperative responses of D1- and D2-like receptors that intensify DA signaling (Alonso et al. 1999). This is consistent with observations that NT inhibition of DA signaling, through D2-like receptors attenuates amphetamine-stimulated locomoter activity (Nemeroff et al. 1977;

Boudin et al 1996; Herve et al. 1986). These results indicate that NT plays an important role in modulating DA signaling.

The catecholamine, DA, is a chemical messenger involved in a broad range of brain functions. DA is synthesized from tyrosine and is produced in several regions of the brain including midbrain neurons of the ventral tegmental area (VTA) and the substantia nigra (SN), and in arcuate neurons of the hypothalamus (ARC) (Bjorklund and Lindvall 1984). VTA DA neuronal axons extend to the nucleus accumbens (NA), olfactory tubercle, frontal cortex, and the amygdala, while SN neurons project to the striatum (Koob '92, Torres '99). Loss of SN DA neurons is the underlying cause of Parkinson's disease (Hirsch et al. 1988), while altered DA signaling is thought to be involved in schizophrenia and drug addiction (reviewed in Torres and Horowitz 1999). The understanding of how genes are regulated in midbrain DA neurons will likely provide insights into disorders involving imbalances in the DA circuits.

The *Ntr-1* gene provides an excellent model for studying the regulation of a gene that is temporally and spatially regulated throughout the CNS, including midbrain DA neurons. The spatial distribution of NTR-1 in the brain changes throughout development. In the SN and VTA, where midbrain DA neurons develop, there is little or no NTR-1 expression in neonates, but by adulthood these regions have the highest NTR-1 levels in the CNS (Palacios et al. 1988;Sato et al. 1992; Lepee-Lorgeoux et al. 1999;Mendez et al 1997). In the adult rat, NTR-1 is expressed at high levels in the majority of DA neurons in the midbrain (Nicot et al. 1995; Szigethy and Beaudet 1989). Conversely, NT binding sites (Palacios et al. 1988) and NTR-1 mRNA (Sato et al. 1992; Lepee-Lorgeoux et al.

1999) are present at birth in many regions of the brain including the cortex, hippocampus, and striatal neuroepithelium, but expression in these regions is extinguished in the adult. These observations indicate that both positive and negative regulation of the *Ntr-1* gene is important for correct temporal and spatial NTR-1 expression in the CNS, and maturing DA neurons are capable of positively regulating *Ntr-1* gene expression.

The development of midbrain DA neurons requires environmental cues that signal determination steps along a developmental pathway that will ultimately set up the complex patterns of gene expression required in the mature midbrain DA neuron that express NTR-1. Signaling peptides secreted at the midbrain-hindbrain barrier initiate the development of midbrain DA neurons. After the neuroectoderm folds in to form the neural tube, secretion of Sonic hedgehog (Shh) induces the formation of the floor plate (Roelink et. al. 1994, Chiang et. al. 1996). The floor plate then releases Shh into the midbrain-hindbrain barrier to initiate the development of midbrain DA neurons. Shh is thought to be required for the development of most if not all the DA neurons along the anterior/posterior axis of the midbrain (Ye et al. 1998). Another signaling peptide from the midbrain-hindbrain barrier and the anterior neural ridge that induces midbrain DA neuron development is fibroblast growth factor 8 (FGF-8) (Ye et al. 1998). FGF-8 is responsible for the dorsal/ventral positioning of DA neuron development along the anterior/posterior axis of the neural tube. The integration of the Shh and FGF-8 signals can induce the development of DA neurons along both the anterior/posterior and dorsal/ventral axis of the midbrain (Hynes and Rosenthal 1999).

Members of the Gli family of zinc finger transcription factors mediate the intracellular response to Shh signaling in the floorplate and midbrain precursors. Gli1, Gli2, and Gli3 are all closely related, but they have different roles in mediating responses to Shh (Hui et al. 1994). In the absence of Shh, Gli3 is phosphorylated by cAMP-dependent protein kinase (PKA) and represses Gli1, but in the presence of Shh, Gli3 instead binds its co-activator, the cAMP response element binding protein coactivator, CBP, and activates Gli1 (Dai et al. 1999). Gli2 is also required for midbrain development and appears to mediate Gli1 activation in response to Shh (Matise et al. 1998). Gli1 itself acts only as a transcriptional activator and is responsible for transactivating *Hnf-3β* expression in response to Shh (Dai et al.1999, Sasaki et al. 1997). Hnf-3β itself is thought to have a broad role as a regulator of floor plate development (Sasaki and Hogan, 1994; Ruiz I Altaba et al. 1995a; Sasaki et al. 1997) including interactions with enhancer elements in the *Shh* gene (Chaing et al. 1997; Ericson et al. 1997). This cascading effect of environmental signals activating transcription factors that in turn activate more environmental signals is an important part of neuronal development.

During the final stages of midbrain DA neuron development, a new set of environmental signals are required to promote cell survival and differntiation. As maturation begins, neurons extend processes to the various regions of the brain that they must interact with. At this stage, environmental signals aid in axon guidance by promoting growth and survival. Factors that have been shown to provide this function *in vitro* include basic fibroblast growth factor (bFGF) (Knusel et al. 1990), epidermal growth factor (EGF) (Hyman et al., 1994), brain-derived neurotrophic factor (BDNF), and

NT-4/5 (Birlig and Price 1995; Perrone-Capano and Porzio, 1996). In addition, transforming growth factor (TGF) family members, glial cell line-derived neurotrophic factor (GDNF), TGF β2, and TGF β3 have been shown to promote neurite outgrowth *in vitro* as well as promote survival of rat embryonic DA neurons in culture (Perrone-Capano and Porzio, 1996). While it appears that none of these factors is critical to DA neuron survival, these and other signaling molecules may have redundant functionality that contributes to cell survival.

The fully developed DA neuron is marked by the expression of a unique complement of genes. A characteristic marker of a DA neuron is the presence of tyrosine hydroxylase (TH) which is the rate-limiting enzyme for DA synthesis. The DA transporter (DAT) appears later during development, requiring at least one further direct cell-cell interaction between the DA neuronal axons and their striatal targets (Perrone-Capano et al. 1996). The majority of adult midbrain DA neurons express NTR-1 at high levels (Nicot et al. 1995), and in this thesis I have explored the regulating mechanisms controlling *Ntr-1* gene expression during neuronal differentiation. By studying the common mechanisms for regulating the expression of genes present in mature DA neurons, we can better understand how the maturation of these neurons is controlled.

One common characteristic between the NTR-1 promoter region (discussed in chapter 3) and several other genes expressed in DA neurons, is the lack of a canonical TATA box. It was once thought that only ubiquitously expressed "housekeeping" genes did not have TATA boxes which are usually located about -27 bases relative to the transcription start site of most selectively expressed genes (Vote and Vote 1990). It is

now known that tissue specific genes with promoters devoid of a TATA box are expressed in a variety of tissues including the liver (Steffen et al.1999), kidney (Whyte et al. 1999), erythroid cells (O'Leary et al. 1996; Karacay and Chang 1999), and the CNS (Minowa et al. 1992). Several genes involved in DA signaling have TATA-less promoters including the dopamine D1, D2, D4, and D5 receptors (Sehga et al. 1988; Tsukamoto et al. 1991; Minowa et al. 1992; Kamakura et al. 1997; Beischlag et al. 1995), the catecholamine synaptic vesicle pump, vesicular monoamine transporter (VMAT2) (Takahashi and Uhl 1997), DA transporter (DAT) (Kawarai et al. 1997; Donovan et al. 1995), L-amino acid decarboxylase, an enzyme required for catecholamine biosynthesis (Sumi-Ichinose et al. 1992), and the NTR-1 (discussed in chapter 3). The study of these TATA-less promoters could lead to an understanding of common mechanisms required for neuronal-specific gene expression in promoters lacking a TATA box.

Many common characteristics of TATA-less promoters are also seen in neuronal specific genes of this class. One prevalent feature of these promoters is that a majority are GC rich (Takahashi and Uhl 1997; Kawarai et al. 1997, Donovan et al. 1992). The GC rich SP1 consensus element (GGGCGGGG) is present in nearly all of the neuronal specific TATA-less promoters (Takahashi and Uhl 1997; Kawarai et al. 1997; Donovan et al. 1992; Yajima et al. 1998; Zhou et al. 1992; Beischlag et al. 1995), and SP1 family members can either positively or negatively regulate DA D2 receptor promoter driven reporter plasmids (Yajima et al 1998). The transcription start sites of many TATA-less promoters (Li and Altieri 1999), including the DA D4 receptor promoter (Kamakura et al. 1997), are embedded in CpG islands which include over 500 bases with at least 50% G +

C content and an incidence of CpG roughly equal to GpC (Bird 1986). The function of CpG islands is not well characterized, but SP1 mediated blockage of methylation in these regions either through physical interactions (Worrad and Schultz 1997), or through its regulation of methyl-CpG-binding protein (MeCP2) (Philipso and Suske 1999) has been implicated as a switch for developmentally regulated genes. Many TATA-less promoters also contain sequences related to the consensus initiator element, Py-Py- A_{+1}-N-(T/A)-Py-Py (Jovahery et al. 1994), first identified in the terminal deoxynucleotidyl transferase (TdT) gene (Smale and Baltimore 1989). This element is thought to substitute for the TATA box and direct RNA polymerase II to the transcription start site (O'Shea-Greenfied and Smale 1992). TATA-less genes that do not have a TdT initiator, such as the dopamine D1 receptor, frequently have multiple transcription start sites (Sehga et al. 1988; Tsukamoto et al. 1991), while those with TdT initiators, such as the dopamine D2 receptor, typically have well defined transcription start sites (Minowa et al. 1992). The presence of GC rich sequences, SP1 elements, CpG islands, and TdT initiators in the TATA-less promoters of neuronal specific genes suggest that they share common mechanisms for transcriptional regulation with a broad range of TATA-less genes; however, there also must be key differences that drive neuron-specific expression and that correctly pattern expression in the CNS.

Many of the same enhancer elements that regulate the expression of genes with TATA elements are also likely involved in the regulation of TATA-less promoters. In addition to the GC rich elements discussed above, the promoters of the DA receptors contain combinations of consensus *cis*-regulatory elements including AP-1, AP-2 and

CRE sequences (Yajima et al. 1998; Wang et al. 1997; Zhou et al. 1992; Beischlag et al. 1995). Transfection studies indicate that an AP-1 element in the DA D2 receptor promoter contributes to cell-type-specific expression of the D2 receptor (Wang et al. 1997). AP-1 elements and the family of transcription factors collectively referred to as AP-1 are thought to play critical roles in regulating gene expression during development as well as controlling phenotypic plasticity in adult neurons (Kaminska et al. 1995; Couceyro and Douglass 1995). Sequence analysis of the NTR-1 promoter reported in this thesis identified SP1, CACCC, CRE and AP-2 *cis*-regulatory sites, suggesting that multiple signaling pathways and transcriptional regulatory proteins could be required for the correct spatial and temporal regulation of *Ntr-1* gene expression. The identification of similar *cis*-regulatory elements in a variety of neuron-specific TATA-less genes suggests that combinatorial mechanisms are generally involved in regulating this class of genes.

It is particularly interesting that both NTR-1 and the DA receptors are regulated by TATA-less promoters since DA signaling has direct effects on NT signaling that are dependent on the receptor subtype mediating the signal. NT producing neurons and their axons make numerous direct contacts with DA neurons in the midbrain (Szigethy and Beaudet 1989; Woulfe and Beaudet 1989). In the striatum, D1 receptor activation, by the D1 selective agonist SKF 82958, results in increases in NT mRNA (Hanson and Keefe 1999). In the nucleus accumbens shell, the stimulation of D2 class receptors, D2 and D3, results in either decreased or increased NT levels, respectively (Schwartz et al 1995; Griffen et al. 1995). These effects of D2 activity on NT expression are consistent with previous observations that NT expression is stimulated in the dorsal striatum by D2

antagonists and in the ventral striatum by the non-selective DA agonists, cocaine and methamphetamine (Merchant et al. 1994, Betancur et al. 1997). Also, treatment with antisense D3 receptor oligonucleotides in the nucleus accumbens shell results in decreased NT mRNA indicating a possible role for the D3 receptor in maintaining basal levels of NT in these cells. Finally, D4 receptor specific antagonists have implicated this receptor subtype in the amphetamine sensitization which results in the decreased ability of the nucleus accumbens to increase NT levels in response to amphetamines (Feldpausch et al. 1998; Meng et al. 1998). Thus, DA signaling has direct effects on NT signaling, but it is clear that the effect on NT signaling is dependent on which DA receptor subtype is activated.

There is also increasing evidence for the autoregulation of *Ntr-1* gene expression in adult neurons. Increased DA signaling results in the activation of *Ntr-1* gene expression, possibly indirectly due to increased NT levels (Bolden-Watson et al. 1993; Boudin et al. 1996; Herve et al. 1986). Seemingly paradoxical, both long-term NT infusion (Azzi et al 1994) and long-term NT blockade using NT antagonists (Azzi et al. 1996; Yamada et al. 1995; Gulley et al. 1997) results in increased NTR-1 mRNA in midbrain DA neurons. *In vitro* studies indicate that the increased *Ntr-1* gene expression resulting from treatment with either NT or the NT agonist, JMV449, is required for cells to maintain NT sensitivity (Najimi et al 1998; Souaze et al. 1997). These experiments indicate that autoregulation of *Ntr-1* expression is required for neurons to maintain full NT responsiveness.

N1E-115 cells as a model for NTR-1 expression in midbrain DA neurons

The mouse neuroblastoma cell line, N1E-115, provides a useful model for studying NTR-1 gene expression in catecholaminergic neurons. The line was isolated in a screen for catecholamine-producing neuronal cell lines from a mouse brain neuroblastoma (Amano et al. 1972). The N1E-115 line stood out in the screen as the highest producer of acetylcholinesterase and tyrosine hydroxylase and was thought to be the best candidate as a model line for catecholaminergic neurons (Richelson 1973). In addition, N1E-115 cells begin to send out neurites and take on a neuron-like morphology as they enter stationary phase (Amano 1972; Richelson 1973). This maturation is associated with among other things, increased cellular levels of tyrosine hydroxylase (Richelson 1973), an increased sensitivity to acetylcholine mediated by muscarinic receptors (Fakahany and Richelson 1980), and the appearance of high affinity NT binding sites (Poustis et al. 1984). In fact, even after the appearance of the high affinity sites, full NT responsiveness requires further maturation of the N1E-115 cells and becomes maximal after 17-20 days in culture (Cusak et al. 1991). Extensive neurite extension and induction of NT receptor expression is also observed after treatment of N1E-115 cells with 1.5% DMSO for several days (Poustis 1984). Thus, N1E-115 cells provide a model for understanding the mechanisms regulating NTR-1 expression during neuronal differentiation of catecholamine-producing neurons.

NT appears to activate similar intracellular signaling mechanisms in N1E-115 cells, and in a variety of neuronal preparations and cultured cell lines. The major intracellular effector activated by NT is phospholipase C which in turn hydrolyses

phosphatidylinositol 4,5-bisphosphate (PIP$_2$) to diacylglycerol and inositol triphosphate (IP$_3$) (Hermans and Maleteaux 1998). NT induced increases in intracellular IP$_3$ have been demonstrated in rat brain slices (Goedort et al. 1984), rat striatal cells (Weiss et al. 1988), and N1E-115 cells (Snider et al, 1986). The NT-stimulated increase in IP$_3$ results in elevated intracellular calcium in both cultured rat cortical neurons (Sato et al. 1991) and differentiated N1E-115 cells (Snider et al. 1986). Finally, in comparison with other cell lines expressing high affinity NT binding sites, the N1E-115 cell line is the only one in which NT treatment results in increased cGMP levels as is observed in neurons (Vincent 1995). Other cell lines that express high affinity NT binding sites include non-neuronal cell lines such as HT29 and HL-60 which were derived from: human colon carcinoma and leukemia cells, respectively (Amar et al. 1986;Choi et al. 1999). Another neuronal cell line that expresses high affinity NT bnding sites, NG-108, is a mouse neuroblastoma X rat glioma hybrid (Nakagawa et al. 1984). NG-108 cells are a useful model for studying the positive expression of NTR-1 in a neuronal line that constitutively expresses high affinity NT binding sites, but N1E-115 cells are a better model for studying the regulation of *Ntr-1* gene expression in catecholamine-producing neuronal cells. The aim of this thesis is to gain an understanding of the underlying mechanisms that regulate *Ntr-1* gene expression, particularly at the level of transcription. Of all the cell lines that express high affinity NT binding sites, N1E-115 is the best candidate to give us insight into how *Ntr-1* gene expression might be transcriptionally regulated in catecholamine neurons.

DMSO induced cell maturation

DMSO can induce several cell lines, including N1E-115 cells, to enter a cell cycle arrested state that is associated with cell differentiontion. While DMSO is not a physiological agent, it induces cell differentiation in diverse cell lines including chinese hamster ovary (CHO), human leukemia (HL-60), mouse erythroid leukemia (MEL), and N1E-115 cells (Fiore-Degrass et al 1999; Pizzeminti et al. 1999; Moon et al. 1997; Poustis 1984). The differentiation of these cells results in morphological changes and/or altered gene expression associated with maturation and cell cycle arrest, including decreased expression of cell cycle progression genes such as c-myc and B-myb (Trubiani et al. 1999; Rachella et al. 1999) and increased expression of genes that promote quiescence such as the cyclin dependant kinase inhibitor, p27 (Fiore-Degrass et al. 1999). DMSO induced maturation results in the expression of the erythroid-specific β-globin gene in MEL cells (Strauss and Orkin 1992), and the neuronal-specific *Ntr-1* gene in N1E-115 cells (Poustis et al. 1984). These results indicate that, in response to DMSO treatment, several different cell lines can be induced to enter a cell cycle arrested state and undergo the altered gene expression associated with cell differentiation.

The direct targets of DMSO induced differentiation of N1E-115 cells are still unclear, but some of the intracellular responses have been described. When N1E-115 cells are treated with DMSO, a marked increase in activated PLC is observed, followed by increases in DAG, IP_3, and intracellular Ca^{2+} (Clejan et al. 1996). At the same time an increase in ceramide levels mediates the inactivation of phospholipase D which is active in N1E-115 cells not treated with DMSO (Clejan et al. 1996). DMSO treatment also

results in decreased levels of c-myc and N-myc (Larcher et al 1991) as well as increases in expression of c-Jun, JunB, and JunD, as well as AP1 activity in these cells (deGroot and Kruijer 1991). Ultimately, the DMSO induced maturation of N1E-115 cells results in an increase in high affinity NTR-1 binding sites (Poustis 1984). Interestingly, when untreated, the HL-60 granulocyte-like cell line expresses relatively high levels of NTR-1 mRNA, and DMSO treatment greatly decreases NTR-1 mRNA levels (Choi et al. 1999). This indicates that cell type differences in response to DMSO mediate increased NTR-1 expression in N1E-115 cells compared to other NTR-1 expressing lines.

DMSO has been shown to regulate several promoters that have features similar to those of the NTR-1 promoter. The DMSO induced expression of the erythroid-specific genes, aquaporin and β-globin in MEL cells requires CACCC elements in their promoters (Moon et al. 1990; Hartzog and Meyers 1993). Nuclear extracts from Hu11 erythroid-like cell line treated with DMSO produce an increased DNAse1 protection of CACCC elements in the β-globin promoter compared to untreated cells (Stauss and Orkin 1992). Finally, the expression of the TATA-less *CR1* gene is induced in DMSO treated HL-60 cells (Kim et al. 1999). These results suggest that DMSO treatment can stimulate transcription of TATA-less promoters and can act through CACCC elements, and the results presented here indicate that similar mechanisms may be involved in NTR-1 gene activation during DMSO-induced neuronal differentiation of N1E-115 cells.

Krüppel like transcription factors

A cDNA encoding an erythroid-specific factor that specifically binds the β-globin gene CACCC site was first identified using a subtractive hybridization cloning protocol and sequence comparisons revealed a close similarity with the *Drosophila* Krüppel protein (Miller and Bieker, 1993). This erythroid Krüppel-like factor (EKLF) has subsequently been shown to be essential for high level β-globin gene expression in mice (Nuez et al. 1995; Perkins et al. 1995). The Krüppel type zinc finger transcription factors comprise a growing family of proteins that bind GC rich sequences. Members of this family have zinc finger domains similar to the *Drosophila* regulatory protein Krüppel (Wieschaus et al. 1984). The first mammalian Krüppel-like protein to be cloned was SP1 (Kadonaga et al. 1987), but the family has now grown to include at least 16 members. The structural characteristic that is common to members of the Krüppel-like family of transcription factors is the presence of three highly conserved zinc finger DNA binding motifs linked by a conserved Krüppel-like sequence. The peptide sequence of the conserved zinc finger motif is Phe/Tyr-X-**Cys**-$X_{2\text{-}4}$-**Cys**-X_3-Phe-X_5-Leu-X_2-**His**-X-Arg/Lys-X-**His**, and the conserved linking sequence is Thr/Ser-Gly-Glu-Arg/Lys-Pro (Ruppert et al. 1988). Based on this sequence, the predicted DNA binding sequence for this family would have the general structure of CCN CNC CCN (Klevit 1991), and in fact the experimentally determined binding sites for known Krüppel-like transcription factors hold very closely to this predicted sequence (Shields et al. 1998). The *cis*-regulatory elements that have been shown to mediate the transactivating or repressing activities of Krüppel-like transcription factors include GC/GT and CACCC boxes (reviewed in Turner

and Crossley 1999). The tight conservation of the zinc finger domains and the similarity in their DNA binding sequences are what defines the Krüppel-like transcription factors separate from other Krüppel-type zinc finger proteins that simply contain Krüppel-type linking sequences (Phillipsen and Suske 1999).

Krüppel-like transcription factor family members are thought to be involved in the regulation of a variety of housekeeping, tissue specific, and developmental genes. Some members, such as SP1 and SP3 (Turner and Crossley 1999), ubiquitous Krüppel-like factor (UKLF) (Matsumota et al. 1998), and basic Krüppel-like factor (BKLF) (Crossley et al 1996), are expressed in most tissues, while others, including erythroid Krüppel-like factor (EKLF) (Miller and Bieker 1993), SP4 (Philipsen and Suske 1999), gut-enriched Krüppel-like factor (GKLF) (Shields et al. 1996), intestinal Krüppel-like factor (IKLF) (Conkright et al. 1999) and lung Krüppel-like factor (LKLF) (Anderson et al.1995) have tissue-specific patterns of expression. Several of these factors have critical roles in cell/tissue development including EKLF for the fetal γ-globin to adult β-globin switch in erythroid cells (Donze et al 1995; Nuez et al. 1995; Perkins et al. 1995), LKLF in arterial development and maintenance of T-cell quiescence (Kuo et al. 1997a; Kuo et al. 1997b), GKLF in epithelial quiescence (Shields et al. 1996), and SP4 which appears to be required for certain aspects of neuronal development required to elicit reproductive behaviour (Philipsen and Suske 1999). Critical roles in the regulation of tissue specific genes have been suggested even for ubiquitously expressed Krüppel-like proteins such as SP1 and SP3 which are involved in DA D2 receptor gene expression (Yajima et al. 1998). While a role for BKLF in neuronal development has not been reported, it is most highly

expressed in the midbrain during development, and only takes on a more ubiquitous expression pattern as mice reach adulthood (Crossley et al. 1996). These results indicate that several members of the Krüppel-like family of transcription factors are involved in tissue-specific regulation of gene expression, including neuronal regulation.

Some Krüppel-type zinc finger proteins, not considered part of the Krüppel-like transcription factor family due to sequence divergence, also act as transcription factors that bind CACCC elements. One zinc finger protein, cKrox, contains three Krüppel-type zinc fingers (Christy et al 1988) and acts as a transcriptional repressor of the collagen gene (Widom et al. 1997) through binding a CACCC element in the collagen promoter (Ravazzolo et al. 1991). The Gli family of transcription factors, discussed above for its role in DA neuron development, contain five Krüppel-type zinc fingers (Kinzler et al. 1988) and bind a CACCC-related element (Kinzler and Vogelstein 1990). The Gli family is particularly interesting because they function as transcriptional activators and repressors in many of the regions of the brain that express *Ntr-1* (Hui et al. 1994). They appear to share many of the functional characteristics as other Krüppel-type transcription factors, even though they display somewhat different structural features.

The experiments reported in this thesis indicate that high level expression of NTR-1 in developing catecholamine-like N1E-115 cells requires a CACCC-box and may involve regulatory mechanisms that are similar to those involved in the expression of other neuronal TATA-less genes. The 5' flanking sequence of the mouse *Ntr-1* gene is devoid of a canonical TATA box, but contains structural features common to other TATA-less promoters, including SP1 elements and the presence of a CpG island. The

increase in high affinity NT binding sites in N1E-115 cells treated with DMSO is mediated by an increase in *Ntr-1* gene expression that requires a CACCC element and a 40 base palindromic sequence containing the transcription start site and a TdT initiator-related element. At least two proteins expressed in N1E-115 cells bind the CACCC element and while several Krüppel-like proteins are expressed in the N1E-115 line, only BKLF is immunologically related to a minor NTR-1 CACCC binding complex. Structural similarities between the NTR-1 promoter and the promoters of other neuronal genes expressed in DA neurons, such as the DA D2 receptor, suggests that common mechanisms exist for regulation of these genes in catecholamine neurons. The characterization of the NTR-1 promoter reported in this thesis could lead to a better understanding of how gene expression is regulated in developing catecholamine neurons.

Experimental approach

The major objective of this thesis is to understand the molecular mechanisms controlling NTR-1 gene expression during the terminal differentiation of catecholamine-producing neurons. NTR-1 is expressed in a complex pattern in the limbic regions of the central nervous system, but is particularly abundant in midbrain DA neurons. NTR-1 mRNA appears relatively late during the development of DA neurons and several lines of evidence indicate that NT modulates the activity of these neurons, indicating that NTR-1 gene expression is an important determinant of the adult DA neuronal phenotype. The NTR-1 gene, therefore, represents an important model for delineating the mechanisms controlling the terminal differentiation of DA neurons. The tyrosine hydroxylase gene

and several others have been extensively investigated but are expressed at a much earlier

stage in DA neuronal development, most likely in response to distinct developmental

cues. The NTR-1 gene is expressed in a complex pattern in the limbic regions of the

adult brain and is transiently expressed during the development of several brain regions,

including the cerebral cortex. Thus, the NTR-1 gene should be generally useful for

investigating the mechanisms controlling the patterning of gene expression in the nervous

system. The experiments presented here concern NTR-1 gene activation during DMSO-

induced neuronal differentiation of the catecholamine-producing N1E-115 neuroblastoma

cells.

The mouse neuroblastoma N1E-115 cell line produces high levels of

catecholamines and differentiates neuronally in response to DMSO. This line has been

used extensively to investigate NT receptor signaling mechanisms since growth to high

density and DMSO both result in the appearance of high affinity NT binding sites in these

cells. These features suggested that the N1E-115 cell line could be used to model the

terminal differentiation of catecholamine-producing neurons, including midbrain DA

neurons. Furthermore, the conditions for culturing these cells, inducing neuronal

differentiation, and inducing cell surface expression of high affinity NT binding sites had

been well-characterized. The experiments presented here also demonstrate that these

cells can be readily transfected and allowed the identification of *cis*-regulatory sequences

and potential transcriptional regulators required for NTR-1 gene expression.

The basic experimental design of this project includes the cloning and sequencing of the

mouse NTR-1 5' flanking sequences followed by the characterization of its promoter by

transfection studies and biochemical techniques. Segments of the NTR-1 5' flanking sequence were cloned into luciferase reporter constructs for transfection studies in the N1E-115 cells. Activity of these reporters was compared between cells treated with and without 1.5% DMSO to identify broad positive regulatory regions within the NTR-1 promoter. Mutational analysis of these reporters was then used to localize specific *cis*-regulatory regions. These reporters identified sequences required for either basal or DMSO inducible expression. The reporters were also compared in the neuron-like NG108 cell line and the mouse fibroblast line (MEF) to explore cell specific sites of action within the NTR-1 promoter. In N1E-115 cells, the most critical positive regulatory region was found to be a CACCC element closely related to a Kruppel-like binding site so this site was characterized further by additional biochemical techniques. These experiments provided evidence for the existence of at least two proteins in N1E-115 cells that make specific base contacts within the CACCC element. The results reported in this thesis provide evidence for the cell specific regulation of *Ntr-1* expression in N1E-115 cells that involves the cooperation of multiple *cis*-regulatory elements including a CACCC element bound by at least two proteins, and required for DMSO-induced expression.

Chapter II

Experimental Procedures

Isolation of mouse NTR-1 genomic clones

A mouse genomic library was constructed by ligating partially digested high molecular weight DNA isolated from D3 embryonic stem cells (15-20 kb fragments) into Bam HI digested λEMBL4 using standard methods (Sambrook et al. 1989). The library was screened using a ^{32}P-labeled Sma I fragment of the rat NTR-1 cDNA (Tanaka et al. 1990) that contains the 5' 1.3 kb of the cloned sequences, and standard filter lift procedures (Sambrook et al. 1989). Positives were plaque purified and used to prepare phage DNA. Two positives were further characterized by restriction mapping (λmNTR2-2 and λmNTR10-1). Promoter fragments from λmNTR2-2 were subcloned into pGEM4 (Promega) and the promoter region was sequenced using the chain terminator method.

Cell culture

N1E-115 cells were passaged in Dulbecco's modified Eagle's medium containing 4.5 g glucose/liter and supplemented with 10% fetal bovine serum (FBS, Sigma), and 2 mM L-glutamine. NG108 cells were passaged in the medium described above for N1E-115 cells except supplemented with hypoxanthine, aminopterin, and thymidine (GIBCO) as described (Nakagawa et al. 1984). Mouse embryonic fibroblast (MEF) cells were passaged in Dulbecco's modified Eagle's medium containing 1.0 g glucose/liter and supplemented with 10% FBS and 2 mM L-glutamine.

Transfections

N1E-115 and MEF cells were subcultured from confluent dishes by diluting the cells 1:4 with fresh medium 3 days prior to transfection. All lines were subcultured the day before transfection at a density that resulted in 1×10^6 cells per 10 cm dish at the time of transfection. CsCl purified plasmid DNA was transfected by calcium phosphate precipitation using 10 cm dishes. Briefly, for each reporter plasmid, two 10 cm dishes were fed with 8 ml of fresh medium just prior to transfection and 25 µg of plasmid DNA as a calcium phosphate precipitate were added to each dish. DNA precipitates were prepared by diluting 50 µg of plasmid DNA (luciferase reporter and pPGKβ-gal standardization plasmids) into 700 µl NTE (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA), 100 µl of 2 M $CaCl_2$ and 400 µl of transfection buffer (50 mM HEPES, pH 7.1, 180 mM NaCl, 2 mM $NaPO_4$) were added dropwise to duplicate 400 µl aliquots of the DNA, and the mixtures were incubated at room temperature for 20-30 min. The calcium phosphate precipitate (800 µl) was added dropwise to the cells and after 16 hr the medium was replaced and the cells from one 10 cm dish were resuspended by trituration and plated into two 60 mm dishes. The cells were allowed to adhere to the dish for 1 hr, 1.5% DMSO was added to one dish of each pair, and the cells were incubated for an additional 72 hr. Cells were incubated for 72 hr and cell extracts were prepared after washing the cells with ice-cold phosphate buffered saline by lysis in buffer containing 1%

Triton X-100 as described (Brasier et al. 1989). Luciferase and β-galactosidase activities

were determined as described previously (Harrison et al. 1995).

Luciferase reporter constructs

The initial NTR luciferase reporter construct was created by ligating a mouse

NTR-1 gene 1.2 kb Bam HI fragment, containing sequences -1425 to -185 relative to the

NTR methionine initiator codon, into a Bgl II site just upstream of the luciferase gene in

the pXP2 reporter plasmid (Nordeen 1988). Convenient restriction sites (Sma I, Sac I)

and exonuclease III digestion were used to create a series of 5' deletion mutants in pXP-2.

Additional 5' and 3' deletion constructs were created by PCR using appropriate primers.

The PCR primers contained 17 nucleotide regions that were complementary to deletion

end points and resulted in the introduction of a Bam HI site at the 5' end and a Sac I site at

the 3' end. The PCR fragments were digested with Bam HI and Sac I, and cloned into the

Sac I 5' deletion construct described above digested with Bam HI and Sac I. This

minimal promoter fragment was initially selected based on the positions of the

transcriptional start sites that had been determined in rat and human (Le et al. 1997;

Maeno et al. 1996); however, the start point in the mouse actually lies 5' to the Sac I site

used to create the deletion clones. Clustered point mutations were created using the PCR

overlap extension protocol (Ho et al. 1989). Briefly, two overlapping PCR fragments

were generated using either a 5' primer that ends at the promoter Sma I site and introduces

a Bam HI site and a 3' primer containing the clustered point mutations flanked by 10 and

6 nucleotide stretches of complementary sequence on the 3' and 5' sides, respectively, or a 5' primer containing clustered point mutations as described above and a 3' primer with the promoter Sac I site as an endpoint. The two fragments were isolated by electrophoresis on a low melting temperature gel, the gel slices were melted, mixed, and a third PCR reaction was performed using the outer 5' and 3' primers described above. The resulting fragment was digested with Bam HI and Sac I, gel isolated, and ligated into the Sac I deletion clone in pXP-2 described above digested with Bam HI and Sac I. The promoter regions of the mutant constructs were completely sequenced to verify that only the intended changes had been introduced.

RNase protection assay

Riboprobes were synthesized using either mouse NTR-1 gene promoter fragments or a fragment of the rat NTR-1 cDNA subcloned into pGEM4 (Promega Biotech). The mouse NTR-1 gene plasmids were constructed by ligation of either a 293 bp Sac I fragment (Sac 293, derived from a 2.4 kb Pst I fragment subclone) or a 497 bp Eco RI/Bam HI fragment (B/E 497, derived from a 1.2 kb Bam HI subclone) into either Sac I- or Bam HI- and Eco RI-digested pGEM4. A 300 bp Pst I fragment derived from pBSNTR2-2 (Tanaka et al. 1990) was subcloned into pGEM4 digested with Pst I and treated with calf intestinal phosphatase to generate a probe for the quantitation of NTR-1 mRNA (Pst I 300). To synthesize ^{32}P-labeled riboprobes, plasmids were linearized with either Eco RI (B/E 497) or Hind III (Sac I 293, Pst I 300) and transcribed with either T7

(B/E 497) or SP6 (Sac I 293, Pst I 300) RNA polymerase as described (Bullock et al. 1994).

RNase protection assays were performed as described (Bullock et al. 1994). Briefly, 10 μg of total RNA was mixed with 5 X 10^5 cpm of ^{32}P-labeled riboprobe, dried, and dissolved in 30 μl hybridization buffer (80% formamide, 40 mM PIPES, pH 6.7, 0.4 NaCl, 1 mM EDTA). Reactions were denatured by heating to 95 °C for 5 min and hybridized overnight (~16 hr) at 45 °C. Following hybridization, 300 μl of RNase digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 300 mM NaCl, 20 μg/ml nuclease P1, 2 μg/ml RNase T1) was added and the reactions were incubated at room temperature for 1 hr. Reactions were terminated by the addition of 20 μl 10% SDS, 5 μl proteinase K (10 mg/ml), incubation at 37 °C for 15 min, followed by phenol extraction and ethanol precipitation. Protection products were analyzed on a sequencing gel and visualized either by autoradiography or on a phosphorimager (Molecular Dynamics). ^{32}P-labeled RNA size markers were synthesized from a pGEM4 rat NT/N gene subclone (pGEM4-NT1.8) using a mixture of templates that were linearized with different restriction enzymes to yield transcripts of 113 (Rsa I), 158 (Hae III), 196 (Pvu II), 242 (Ava II), 305 (Hinf I), and 496 (Dra I) nucleotides.

RT-PCR

Mouse brain Poly (A)$^+$ RNA was treated with DNase I to remove trace amounts of contaminating genomic DNA and RT-PCR reactions using AMV reverse transcriptase (Promega) and Taq polymerase (Boehringer Mannheim) using the conditions specified by the manufacturers, except that RT reactions were performed at 50°C and betaine was added to the PCR reactions. The reverse transcription reaction was initiated after heat inactivation of the DNase I (70 °C for 5 min) by the addition of 5 pmol of a gene specific primer (PEX-411c 5'-TGGCGCAGAGAGGAGCGCACGCACGGCTGCCCAGC-3') complementary to nucleotides -411 to -376 of the mouse NTR-1 gene and 25 U AMV reverse transcriptase (Boehringer Mannheim). PCR reactions were performed using either mouse brain cDNA or embryonic stem cell genomic DNA templates mixed with 10 pmole of the PEX-411c primer, 10 pmole of one of three different 5' primers (-513, 5'-GTGGAAGCGCGAGGAGCCCG-3'; -548, 5'-TTTTGGATCCACTGCTGGGCGCGCC-3'; -566, 5'-CTCCAACACCCACCCTCCTCCACTG-3', the numbers correspond to the position of the 5' end of the oligonucleotide on the mouse NTR-1 promoter). PCR reactions were supplemented with 2.5 M betaine (Sigma) due to the high GC content of the promoter region and 30 amplification cycles (94°C, 15 sec; 62°C, 1 min; 72°C, 1 min; the extension time was increased to 4 min on the last cycle) were performed after an initial 1 min denaturation step at 94°C (Henke et al. 1997). RT-PCR reactions for the amplification of the Krüppel-like mRNA were done as described above except betaine was not required.

DNase I footprinting and methylation interference assays

^{32}P-end-labeled probes spanning the promoter region were prepared by digestion of a 1.2 kb Bam HI gene fragment subcloned into pGEM4 with either Eco RI or Bam HI followed by Klenow fill-in with ^{32}P-dATP. The plasmids were subsequently digested with either Bam HI or Eco RI, respectively, the resulting fragments were separated on a 1% agarose gel and the 496 bp labeled Eco RI/Bam HI fragments labeled at either the Eco RI or Bam HI sites were recovered by centrifugation through a Gen Elute column (Sulpeco) and ethanol precipitation. Nuclear extracts were prepared from N1E-115 cells that had either been treated with 1.5% DMSO for 48 hr or grown under control conditions as described previously (Shapiro et al. 1988) with minor modifications (Evers et al. 1995) using approximately 20 dishes of cells grown to confluency for each preparation. Protein concentrations were determined using the Bradford method. DNase I footprint reactions were performed as described (Harrison et al. 1995) by mixing 20,000 cpm probe, 160 µg nuclear extract, and treatment with various amounts of DNase I (DPRF, Worthington). Reactions were also performed without the addition of nuclear extract to identify protected regions. The DNase I treated reactions were phenol-chloroform extracted, ethanol precipitated, analyzed on sequencing gels, and visualized by autoradiography with an intensifying screen. Chemical sequencing reactions (G, G+A) were also run so that the footprinted regions could be precisely aligned with the DNA sequence.

Methylation interference reactions were performed as described (Ausubel et al. 1992) using a ^{32}P-labeled probe spanning nucleotides -589 to -507 generated by PCR and labeled by Klenow fill-in of a Bam HI site at the 5' end. The probe (10 µl, 5 x 10^5 cpm) was partially methylated by incubation in 200 µl dimethyl sulfate (DMS) reaction buffer (sodium cacodylate, 1 mM EDTA, pH 8.0) containing 1 µl DMS for 5 min at room temperature followed by the addition of DMS stop buffer (1.5 M sodium acetate, 1 M β-mercaptoethanol, pH 7.0) and ethanol precipitation. The methylated probe was then reacted with 50 µg nuclear extract from N1E-115 cells that had been induced with 1.5% DMSO for 72 hr and bound complexes were separated from free probe by electrophoresis on a non-denaturing gel. The complexes were visualized by autoradiography and the region of the gel containing complexes 1 and 2 and unbound probe were excised, the DNA was electroeluted, phenol and chloroform extracted, and recovered by ethanol precipitation. The DNA was resuspended in 1 M piperidine, incubated at 95 °C for 30 min, lyopholized several times, and analyzed on a sequencing gel.

Gel shift analysis

N1E-115 cells were propagated as described for transfection analysis and treated with 1.5% DMSO or grown under control conditions in 10 cm dishes. The cells were washed twice with ice-cold PBS, resuspended in 400 µl buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 mM aprotinin, 2 mM pepstatin, and 2 mM leupeptin) and incubated on ice for 15 minutes.

The cells were lysed by the addition of 25 µl of 10% NP-40 and vigorous vortexing for 10

seconds followed by centrifugation for 30 sec. The supernatant was discarded, the pellet

was washed once with buffer A on ice, centrifuged and resuspended in 30 µl buffer C (20

mM HEPES 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2

mM aprotinin, 2 mM pepstatin, and 2 mM leupeptin) on ice by vortexing for 10 sec every

5 min for 30 min. Nuclear extracts were stored at -80 °C. Binding reactions were

performed by mixing (10 µg, 1-2 µl) N1E-115 nuclear extract in binding buffer (12.5 mM

HEPES pH 7.9, 100 mM KCl, 10% glycerol, 0.1 mM EDTA pH 8.0, 1.5 mM DTT)

supplemented with 3 µg poly dIdC, 1 µg acetylated BSA, and in some cases unlabeled

competitor DNA (16 µl final volume). The reactions were incubated on ice for 10

minutes, and 1×10^5 cpm probe was added followed by incubation for 15 to 20 minutes at

room temperature. The probe was the same as that used for methylation interference.

Binding reactions were analyzed on low ionic strength Tris-acetate acrylamide gels as

described (Ausubel et al. 1992). The BKLF supershift experiments were done under the

conditions given to us by Dr. Merlin Crossley (Crossley et al, 1995). Binding reactions

were done in a 20 µl volume containing 1 X gel shift buffer (10 mM Hepes pH 1.8, 50

mM potassium chloride, 5 mM MgCl$_2$, 1 mM EDTA, and 5% (v/v) glycerol), 0.5 mM

DTT, 50 ng/µl polydIdC, and 2×10^5 cpm probe. This was mixed together on ice,10 µg

of nuclear extract was added, and finally 1-4 µl of antibody was added and the reactions

were incubated on ice for 20 min. Reactions were loaded on a 6% native 19:1

bis:acrylamide 0.5 X TBE gel and run at 240 V for 2 hours at 4 °C. Gels were then dried

and exposed to X-ray film.

Southwestern blots:

Southwestern blots were done essentially as described (Hubscher 1987). Breifly, 50 μg of nuclear extracts from N1E-115 cells were run on a 7.5% polyacrylamide/SDS gel. Gels were semi-dry blotted onto nitrocellulose membranes, then renatured for 24 hours at 4 °C in renaturation buffer without $MgCl_2$ (10 mM Hepes (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM $ZnSO_4$, 10% (v/v) glycerol, and 5% (w/v) dried milk powder). Following renaturation, blots were incubated at 4 °C in binding buffer without $MgCl_2$ (10 mM Hepes (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 0.25% (w/v) dried milk powder, 10 μg calf thymus DNA, and 2 X 10^5 CPM/blot lane of ^{32}P labeled probe). Finally blots were washed in 3 volumes of binding buffer (without the thymus DNA or probe) 2 times each for 10 minutes at 4 °C, and then dried and placed between saran wrap for autoradiography.

Western blots.

50 μg of N1E-115 nuclear extracts were run on a 7.5% SDS PAGE, semi dry blotted onto nitrocellulose membranes and renatured as described for the southwestern blots. Following renaturation, blots were probed as described in the NEN HRP probing kit (Western Blot Chemiluminescence Reagent Plus). Blots were washed two times in 10 ml PBST (1 X PBS and 0.05% Tween-20) and then placed in 10 ml 1% BSA/PBST containing 5 μl polyclonal rabbit SP1 antibody (Santa Cruz), and incubated at room

temperature for 1 hour on a rotator. This was followed by one 15 min and four 5 min washes in PBST, and then the blots were incubated for one hour in 10 ml 1% BSA/PBST containing 5 µl of HRP conjugated anti-rabbit antibody (Santa Cruz). The blots were again washed five times in PBST and finally treated with 1 ml chemilucent reagent for 1 minute and chemiluminescent bands were detected with a BioRad Flour-S Multiimager.

Southwestern methylation interference.

Southwestern blots were performed as described above. Instead of probing these blots with the 32 base wild type probe, the 80 base URmP probe described in chapter 3 methylation interference experiments was used. 6.2×10^6 CPM of this probe was methylated as described for gel shift methylation interference while an equal amount was not methylated. Three lanes of a blot were probed with methylated probe while three lanes were incubated with unmethylated probe. Blots were probed for 24 hours and then washed, and exposed for four hours to X-ray film. Nitrocellulose membranes were then aligned with the X-ray film and the positive signals were cut out of the membranes. Probe was stripped from the membranes by incubating them in 200 µl TE at 65 °C for 30 min. Each tube was then ethanol precipitated and tubes corresponding to signals from unmethylated probe were resuspended in methylation reactions as described in chapter 3. Following methylation, all of the tubes were treated with piperidine, lyophilized, and analyzed on a 10% polyacrylamide/8 M Urea gel.

<u>Two dimensional UV crosslinking</u>.

The coding strand of the wildtype or mutant probe was annealed to a 12 base pair

oligo corresponding to the 3' ends of these probes. Klenow fill in reactions were done in

the presence of 10 mM BrdU in place of 10 mM dTTP, and 6000 Ci/mmole ^{32}P α labeled

dATP. Binding reactions were done as described for tris-acetate gel shift reactions except

with 1 X 10^6 cpm of either wildtype or mutant probe (five fold the usual amount) and 20

µg of nuclear extract (twice the usual amount). After the 20 min room temperature

incubation, binding reactions were place on ice and exposed to 7.2 X 10^6 J/cm^2 UV light

over the course of 30 min. Reactions were then run on a 4% non-denaturing

polyacrylamide gel as described for tris-acetate gel shifts. Undried gels were exposed to

X-ray film overnight at 4 °C and the film was developed for alignment with the second

dimension gel. The lanes corresponding to wildtype and mutant probe were soaked in 1

X tris/SDS stacking gel buffer (pH 6.8) for 5 min, then cast into the stacking gel of a

7.5% SDS polyacrylamide gel and run along with BioRad prestained low molecular

weight markers. The gel was then dried and exposed to X-ray film.

<u>Zinc chelation and rescue of nuclear extracts</u>.

Chelation of zinc cations from N1E-115 extracts was done essentially as

described by (Kadonaga et al. 1987). Nuclear extracts were treated with 3 mM EDTA on

ice for 45 min, followed by further treatment with either 3 or 6 mM ZnCl$_2$, MgCl$_2$, CaCl$_2$

or under control conditions for an additional 30 min on ice. These extracts were then

analyzed by tris-acetate gel shift analysis as described.

Chapter III

Sequences required for induction of neurotensin receptor gene expression during neuronal differentiation of N1E-115 neuroblastoma cells

Summary

The promoter region of the mouse high affinity neurotensin receptor (NTR-1) gene was characterized and sequences required for expression in neuroblastoma cell lines that express high affinity NT binding sites were characterized. DMSO-induced neuronal differentiation of N1E-115 neuroblastoma cells increased both the expression of the endogenous NTR-1 gene and reporter genes driven by NTR-1 promoter sequences by 3- to 4-fold. Deletion analysis revealed that an 83 bp promoter region containing the transcriptional start site is required for DMSO activation. Detailed mutational analysis of this region revealed that a CACCC box and the central region of a large GC-rich palindrome are the crucial *cis*-regulatory elements required for DMSO induction. The CACCC box is bound by at least one factor that is induced upon DMSO treatment of N1E-115 cells. The DMSO effect was found to be both selective and cell type restricted. Basal expression in the neuroblastoma cell lines required a distinct set of sequences, including an Sp1-like sequence, and a sequence resembling an NGFI-A binding site; however, a more distal 5' sequence was found to repress basal activity in N1E-115 cells. These results provide evidence that NTR-1 gene regulation involves both positive and negative regulatory elements located in the 5' flanking region, and that NTR-1 gene activation involves the coordinate activation or induction of several factors, including a CACCC box binding complex.

Introduction

Neurotensin (NT) is a thirteen amino acid peptide (Carraway and Leeman 1975) that is expressed in a complex pattern in the limbic regions of the brain and in the gastrointestinal tract (Reinecke 1985). NT and the related peptide neuromedin N are generated from a common precursor protein and are thought to have overlapping signaling functions (Dobner et al. 1987; Kislauskis et al. 1988). There is considerable anatomical and functional evidence indicating that NT functions as a neuromodulator in the dopamine (DA) pathways in the CNS. The majority of midbrain DA neurons express the cloned high affinity NT receptor (NTR-1) and there is evidence that at least some DA neurons are directly contacted by NT-positive axons (Szigethy and Beaudet 1989; Woulfe and Beaudet 1989). NT appears to excite midbrain DA neurons both directly (Jiang et al. 1994; Pinnock 1985) and through inhibition of DA D_2 autoreceptors (Shi and Bunney 1991) resulting in locomotor activation (Kalivas et al. 1981; Kalivas et al. 1982). However, NT also attenuates d-amphetamine locomotor activation after i.c.v. administration or after direct application in the ventral striatum possibly through the inhibition of post-synaptic D_2 signaling (Ervin et al. 1981; Kalivas et al. 1984; Nemeroff et al. 1977). These results suggest that the expression of NTR-1 in midbrain DA neurons is important for appropriate regulation of DA-mediated behaviors.

NTR-1 was cloned using an expression assay in frog oocytes and sequence analysis revealed that it is a member of the G protein coupled receptor superfamily

(Tanaka et al. 1990). A lower affinity levocobastine-sensitive receptor (NTR-2) was subsequently cloned by low stringency hybridization using an NTR-1 cDNA probe (Chalon et al. 1996; Mazella et al. 1996). Recent evidence suggests that NT does not stimulate signaling through NTR-2, suggesting that NT acts mainly through NTR-1 (Botto et al. 1997). NTR-1 is expressed at high levels in midbrain DA neurons and dopamine regulates NTR expression in corticolimbic structures in the rat brain (Bolden-Watson et al. 1993; Boudin et al. 1996; Herve et al. 1986). The ability of DA to modulate NTR expression suggests that changes in DA signaling results in plastic changes in NT signaling. This hypothesis is further supported by the observations that the indirect DA agonists cocaine and methamphetamine and D_2 antagonists stimulate NT gene expression in the dorsal and ventral striatum (Betancur et al. 1997; Merchant et al. 1994). Long-term NTR-1 blockade and continuous infusion of NT also result in alterations in NTR-1 expression (Azzi et al. 1996; Azzi et al. 1994). These results collectively indicate that DA, NT, and perhaps other signals can result in plastic changes in NTR-1 expression; however, the underlying mechanisms controlling NTR-1 expression are poorly understood.

Tissue culture cell lines that express NTR-1 in a regulated or constitutive manner potentially provide model systems for understanding the mechanisms controlling NTR-1 gene expression. The N1E-115 neuroblastoma cell line was isolated as part of a screen for catecholamine-producing neuronal cell lines from a mouse brain neuroblastoma (Amano et al. 1972), and DMSO-induced neuronal differentiation of these cells (Kimhi et

al. 1976) is accompanied by increased expression of high affinity NT binding sites

(Poustis et al. 1984). The transition of these cells to a post-mitotic stationary phase also

results in the induction of NTR expression (Cusack et al. 1991). A neuroblastoma x

glioma cell line has also been described that expresses high constitutive levels of high

affinity neurotensin binding sites (Nakagawa et al. 1984). The N1E-115 cells are a

particularly attractive model to probe NTR-1 gene regulation since the NTR-1 gene is

expressed in catecholminergic neurons *in vivo* (Nicot et al. 1994). They should also be

useful for identifying signaling mechanisms that activate the NTR-1 gene during neuronal

differentiation (Poustis et al. 1984).

To investigate the pathways controlling NTR-1 gene expression in these and other

cell types, we have cloned the mouse NTR-1 gene and sequenced the promoter region.

Detailed mutational analysis of the NTR-1 promoter has revealed sequence elements that

are crucial for DMSO induction and basal expression in N1E-115 cells that are conserved

in the rat and human promoters. A CACCC sequence appears to be the most critical

sequence element for DMSO responsiveness, and gel shift and DNase I footprinting

experiments indicate that a DMSO-inducible complex binds to this site. Several

sequence elements contribute to basal expression, including an Sp1-related site and a

sequence that is similar to the initiator element first identified in the terminal

deoxynucleotidyl transferase gene (Smale and Baltimore 1989). We also present

evidence that a transcriptional silencer controls the activity of this positive regulatory

region. These results provide evidence that the NTR-1 gene is transcriptionally activated

during DMSO-induced neuronal differentiation of N1E-115 cells most likely through a

mechanism involving the induction of a complex that binds to a sequence that includes a

CACCC motif.

Results

Cloning and characterization of the mouse NTR-1 gene

Mouse NTR-1 clones were isolated by screening a genomic library constructed from D3 mouse embryonic stem cell DNA with a ^{32}P-labeled 1.3 kb Sma I 5' fragment of NTR-2 cDNA (Tanaka et al. 1990), and two were further analyzed (2-2, 10-1) by restriction mapping and Southern blotting. Exon one was localized to a ~7 kb Hind III fragment that contained sequences extending ~2.0 kb 5' to the initiator methionine codon. A portion of the first exon and the 5' flanking region were sequenced (Fig. 3-1A) and comparison with the corresponding regions of the rat (Maeno et al. 1996) and human (Le et al. 1997) NTR-1 genes revealed a region that is highly conserved between all three species (Fig. 3-1B). This region contains Sp1-, CRE-, CACCC box-, and initiator element-related sequences as well as a large 40 bp palindromic sequence (mPAL) that includes the initiator element-related sequences (Fig. 3-1B). This conserved sequence is located within a 200 bp region that is required for expression.

RNase protection experiments were performed to map the approximate location of the transcriptional start site. Two ^{32}P-labeled antisense riboprobes (depicted schematically in Fig. 3-2A) were hybridized to total brain, kidney, spleen or yeast RNA, followed by digestion with a mixture of nuclease P1 and ribonuclease T1, and the

A

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AAGCTTTGGA  GACACAAGGC  TGGCTTAGCC  AAAAGGTCAG  GACTAGAAAA  CTAAACATGA  ACTATGCTGC  CTTTGAGATG  GCCTCTCCAC  GTGGCTGCAG -1957
CTCCTGTTGC  CAAACGGATA  TACTGCTGTG  GTCACCGTCT  AGGACCCTCC  TCTGGGGAAT  CCCCTGATGG  AGGAGGCCAG  ACGGCACTGT  TGGTGGTTGA -1857
GTTTGGGTGA  GACCAAGGAC  ACCACAGGGG  AGTGAGACAT  GGGATCATAT  ATAGCCTAGT  GCTACTACTT  CCTAATCATG  CAGGCCCTCC  CCAGCCCTCC -1757
CCATGCTCAT  TAAGTTGGTG  CACAGAATCT  GTCGGAGAAC  AAATCTAGTC  TCAAGCCCAC  CTTACCCGCA  GTCAATGGGC  AAGCAGCCAC  AACCCATCAG -1657
GGCAGGTTTC  AAGTTCCCTT  CCACAATGGA  AGAAGCTGAG  GCTTGCCCAA  ATATTAGAAG  CCTGGTCAAC  CCCTACACCC  TCAGCCTGCT  GTACTGGCTT -1557
CTCTTAAGGG  GACACAGTAG  TTTCCTTTGA  AACCAAGTGT  TTAAGCTCTG  CTTTTCATGT  GTTAACATTC  TGTGGAGGGC  ACCAGCTTTT  GTGTGAGTAG -1457
GCACTGAAGC  CTGAGGGTCG  CTGCTGCGCA  GGGATCCAGG  GAGGAGTTTT  CTGGAGGAAG  AGGGCAGGAG  AAAGGCTGAG  AGAGCCAAGT  GGGGAAATAG -1357
AGGCAAGGGC  CCTGGGGCAA  GCAAGATGGC  AGCAAGCCTG  GAAGCTCTGG  CCAGGGCCCC  TGCCAAAGAG  GACAAGATGA  AAAGACCTTT  GTTCCCCGGG -1257
TCCCTGACTG  GGGCAGGGTC  CCCTGAGAAA  AGCTGTGCTG  GGCTGAGTGC  ACAGGCTTCA  GGGTTTCCTG  CAAGGACAGA  CGCAGCTCTG  TGTCGCCCAC -1157
AAACCTGCCC  TTCCCCTCTG  AAACGACAGG  GCACCAGCTC  TGAGAGTCAC  TGCTAGTGAC  CAAATCCAGG  TGGCTACAGG  AGGGCAGAAG  GTCCCATCAT -1057
ACCCAAAGGC  AGCTAAGACC  GTGAGACTGC  GTGGAGAGAT  GGGCTGCTCT  AACTTCCTGC  TCTATGCTTT  CCTTCACCAG  ACCCTTCACA  GGCTAGAGGG  -957
CCCCCTGGTG  CTTCTGGCAC  CCCACAGGCA  CCCAGAAGGG  AGGAGGTCGA  GGCAGTTGTA  CAAAGGCCCT  GACTTATCAG  CAGGGTGCCG  CCTCCTTCTC  -857
CGCCTTCCCC  TCGAGTACAC  CCTCGTGTCA  CTAAGGGAGC  TGAAGACGGC  TACGGGGAGC  TTTCTTTTTG  GGGTGGGAGA  AGTTGGGACC  AACGATCGTG  -757
AAAGAAAGAT  CGTGCTGCCC  AGGAGAGGTT  GAAAGGAAAG  ATCCAATATC  CCCTCATTCT  CCCTCGCCTG  GTAGGGAATT  CTGCTTCCTG  GGGTACTCAT  -657
CCTAGCCCAG  GTGGTCCCCG  GGAGCGTGCA  GGAGCAACCG  TCAGCCAGTA  TCCGGGTGGG  GAGGGGACAG  TGGCTGTGGC  GTCTCTGACT  CTCCAACACC  -557
CACCCTCCTC  CACTGCTGGG  CGCGCCGCGC  AGCCGCCAGC  TCAGTGGAAG  CGCGAGGAGC  CCGGCAGGCT  CCGGCGCAGC  AGGAGGTGTG  CGAAGTCGGG  -457
TGCGGGAAGA  GCTCCAAGCA  GAAGAGGGAG  AACGAGGGTG  AGCACGCTGG  GCAGCCGTGC  GTGCGCTCCT  CTCTGCGCCA  CAGGCTCGCC  CAACGAAGCA  -357
CAAGCCAGCA  GGGTCGCGGT  CCTGAGGACA  TCGCTGCCAC  CCAGGAGTGC  AGAGAACCAA  CCACAGAATC  TGAGGTCGGT  AGCAGCCTCG  ATCTAGGCAG  -257
GCTCGGAGCC  AGGGGGGGTG  CCGAGGAAGA  ACCGGAAAGC  AGATCCCAGA  CTCTGAAGGA  GAAAGAAGAG  TGGATCCCTG  AGCCGGCACG  TCTCGAGCCC  -157
CAGAGCTCCC  AGGATCTCCT  GGATAGCTGG  CTTCGCCGCC  GGACGGCACG  CCCCCCTTAG  CTTCCACACC  CTCTCCTCTG  AGCGCCTCCT  CATCTGCCCC   -57
TGGAGGGACT  TTCACCTTCA  AAGGTGCAGG  ACAAAGCCGC  ATATCTGGCG  CCCACCATGC  ACCTCAACAG  CTCCGTGCAG  CAGGGAGCCC  CAAGTGAACC   +44
                                                           M  H       L  N  S    S  V  Q    Q  G  A  P    S  E  P

CGGTGCCCAG  CCCTTCCCAC  ATCCACAGTT  CGGACTGGAG  ACGATGCTCC  TG  +92
G  A  Q    P  F  P  H    P  Q  F    G  L  E    T  M  L  L
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B

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                 Sma1  tt k/g hd ————          CRE ———            SP1 ——————
mouse   5'- CCCGGG-AGCGTGCAGGAGCAAC-CGTCAGCCAGTAT------CCGGGTGGGGAG
rat                 –                AG         –              ------      A
human               G      C  AG  G        C   C       G T C CCCGGG A    C    T
                                                                      AP2 ———
                                                           Initiator  mPal
                  SP1 ——                         CACCC box ———————      +
mouse   GGGACAGTGGCTGTGTGGCGTCTCTGACTCTCCAACACCCACCCTCCTCCACTGCT
rat
human        C----- G G    TG –  CC    GT CG CG          - --

        AP2 ——   NGFI-A ———————————
        SP1 ————— Initiator ———————————
        mPal ——————————————
mouse   GGGCGCGCCGCGCAGCCGCCAGCTCAGTGGAAGCGCGAGGAGCCCGGCAGGCTC
rat
human            G         G    C    GA   CA      G   AG   G

                                              Sac1
mouse   CGGCGCAGCACGAGGTGTGCGAAG-TCGGGTGCGGGAAGAGCTC-3'
rat       A                      –
human   G       G  G          GG C G       C  G       G
```

C

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                      AP2 ———
              Initiator  SP1 ———           NGFI-A ——————————
        CACCC ———— mPal ———————                Initiator ——————————
mNTR-1  CACC-CACCCTCCTCCACTGGTGGGCGCG-G---CCGCGGAGCGCCCAG-CTCAGTGGA
rDAD2 R gcc-gcgCCCTCCTCCTGCGCAGCGCTGTGATTCCGCGGAGCTGTCCAG-CTCAGTGCC
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Fig. 3-1 <u>Sequence of the promoter region of the mouse NTR-1 gene and comparison with other NTR-1 sequences and the dopamine D2 receptor gene.</u> (A) A Hind III restriction fragment of the mouse NTR-1 gene was subcloned and the sequence of the 5' flanking region and a portion of exon one was determined on both strands using the chain terminator method. The positions of the transcriptional start site (+) and the 5' end of a mouse NTR-1 cDNA clone (*) are shown. (B) Comparison of the mouse NTR-1 sequence underlined in (A) with the corresponding regions of the rat and human NTR-1 promoters (sequence differences are indicated). Potential transcriptional regulatory sequences (overlined) in the conserved region and the transcriptional start site (+) are indicated. (C) Sequence comparison between the mouse NTR-1 and rat dopamine D2 receptor promoter (Minowa et al. 1992) regions. Sequences that are identical are boxed.

resulting products were analyzed on a sequencing gel. An antisense probe transcribed from a subcloned Sac I fragment (Sac 293, -448 to -149 relative to the AUG start codon) was fully protected after hybridization with mouse brain RNA but not the control RNAs, indicating that the start site is located upstream of the region encompassed by this probe (Fig. 3-2B). Hybridization of mouse brain RNA with an antisense probe that extends 233 nucleotides further in the 5' direction (B/E 497) resulted in three closely spaced protected fragments (Fig. 3-2C). The estimated sizes of these protection products indicate that transcription starts within the mPAL region (in the vicinity of position -523). Identical protection results were obtained with RNA isolated from DMSO-induced N1E-115 cells (data not shown). We attempted to confirm this result using several different oligonucleotides for primer extension experiments using poly $(A)^+$ brain RNA and control RNAs isolated from several different tissues, but no specific products were detected. Therefore, we employed RT-PCR to delineate the 5' boundary of the gene using a common 3' primer and several closely spaced 5' primers located at succesively further 5' positions. Control PCR reactions using the same primer sets to amplify mouse embryonic stem cell genomic DNA were also performed (Fig. 3-2D). Specific products were detected by electroblotting the RT-PCR products and hybridizing with a ^{32}P-labeled rat NTR-1 gene probe from within the amplified region. Primers with 5' endpoints at -513 and -548 produced products of the expected size; however, much less product (at least 10-fold less) was detected after RT-PCR with the -566 primer (Fig. 3-2D). These results indicate that the major transcriptional start site is close to position -548 (most likely at
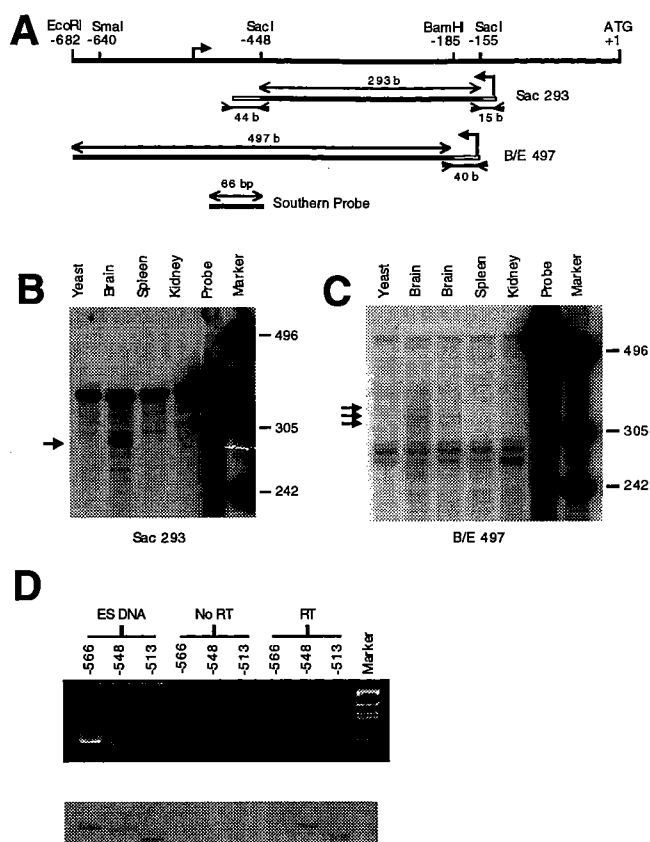
Fig. 3-2 <u>Mapping the transcriptional start site by RNase protection and RT-PCR.</u> RNase protection experiments were performed by hybridizing either ^{32}P-labeled Sac 293 (panel B) or B/E 497 (panel C) antisense riboprobes with total RNA (10 μg) from the indicated tissues, subsequent digestion with nucleases P1 and T1, and analysis of the products on a sequencing gel. ^{32}P-labeled RNA markers were prepared by transcription of a mixture of pGEM4-NT1.8 templates linearized at different sites to produce transcripts of the indicated lengths as described in Experimental Procedures. (A) Schematic diagram of the antisense riboprobes used. Thick black lines represent NTR-1 gene sequences and open lines represent pGEM4 vector sequences. The sizes of the regions of the transcript derived from NTR-1 sequences and vector sequences are indicated. The Sac I 293 probe encompasses a 293 nucleotide region located between the Sac I sites at -448 and -155. The B/E 497 probe spans 497 nucleotides of the NTR-1 gene between the Eco RI site at -682 and the Bam HI site at -185. (B, C) RNase protection products resulting from hybridization to the Sac 293 (B) or B/E 497 (C) probes were analyzed on sequencing gels and visualized by autoradiography. Specific protection products were detected only in reactions containing brain RNA and are indicated by arrows. (D) RT-PCR reactions were performed as described in Experimental Procedures using a common 3' primer and three different 5' primers with 5' endpoints at the indicated positions (-566, -548, -513) to map the transcriptional start site more precisely. RT-PCR reactions in which reverse transcriptase was omitted from the initial reaction (no RT) were performed to ensure that PCR amplified products were derived from reverse transcribed mRNA templates. Control PCR reactions were also performed with the same primers using mouse embryonic stem cell DNA as template. The reaction products were analyzed on a 5% polyacrylamide gel, electroblotted and probed with a ^{32}P-labeled fragment of the mouse NTR-1 gene [-514 to -448, see (A), Southern probe]. The gel was stained with ethidium bromide and photographed prior to electroblotting (top panel) and hybridization signals were detected by film autoradiography (bottom panel). The results indicate that the major transcriptional start site is in the vicinity of -548, since much less RT-PCR product was generated with the -566 primer than with the -548 and -513 primers.

-545) near the 5' end of the mPAL within a sequence that is strikingly similar to the consensus initiator element (Javahery et al. 1994; Smale and Baltimore 1989).

Sequence analysis revealed that an approximately 500 bp region surrounding the transcriptional start site has the structural features of a CpG island (Fig. 3-3). CpG islands have been identified in the promoter regions of a variety of housekeeping and tissue-specific genes from higher eukaryotes (Bird 1987; Gardiner-Garden and Frommer 1987). The CpG island in the mouse NTR-1 promoter region has close to the expected frequency of CpG dinucleotides (CpG/GpC $\geq$ 0.6) in a region that has greater than 50% G+C content and encompasses at least 500 bp (Fig. 3-3). Although the function of CpG islands remains uncertain, they are commonly found in promoter regions, including a large number of promoters that lack TATA elements like the NTR-1 promoter (Bird 1987; Gardiner-Garden and Frommer 1987).

Cell density and DMSO treatment increase NTR-1 gene expression in N1E-115 cells

N1E-115 cells plated at low density are devoid of high affinity NT binding sites; however, large increases in binding activity are observed when the cells are grown to high density (Cusack et al. 1991) or treated with DMSO (Poustis et al. 1984). To examine whether these culture conditions result in increased NTR-1 gene expression, NTR-1 mRNA levels were quantitated by nuclease protection assay using a ^{32}P-labeled antisense riboprobe corresponding to nucleotides 296-632 of the rat NTR-1 cDNA (Pst I 300).
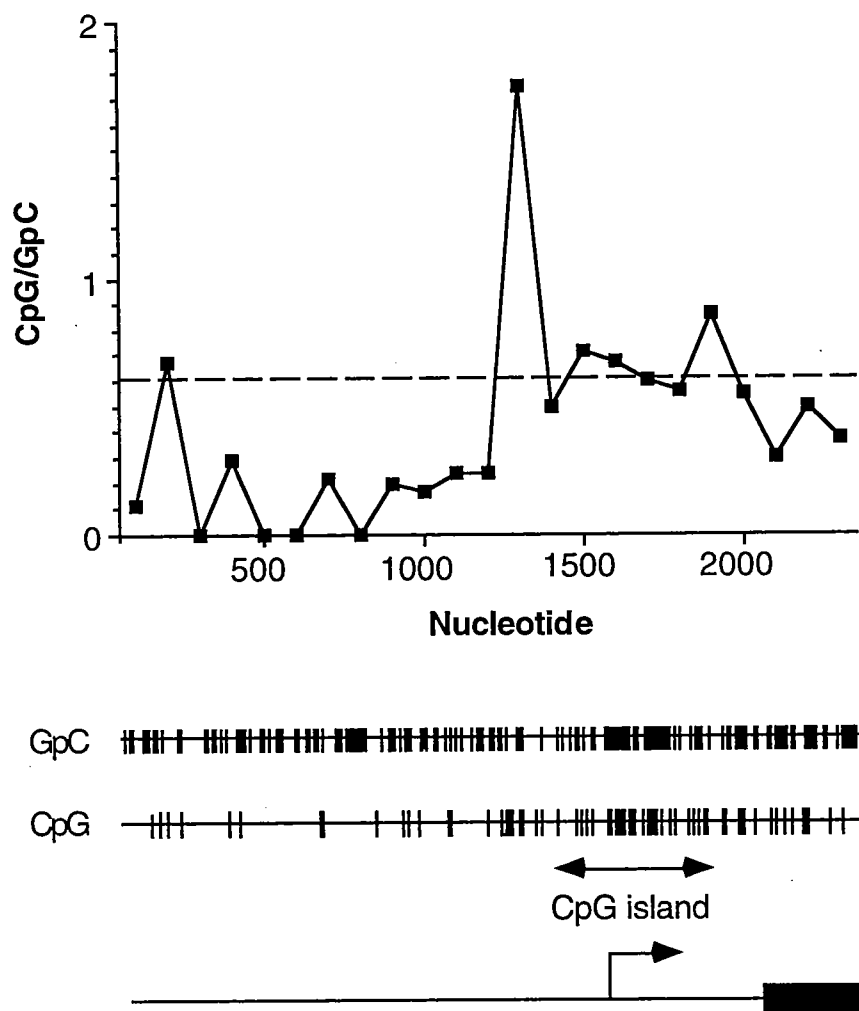
Fig. 3-3 <u>The transcriptional start site of the NTR-1 gene is located in a CpG island.</u> The sequence of the promoter region was analyzed in 100 nucleotide blocks for G/C content and the frequency of CpG and GpC dinucleotides. The ratio of CpG/GpC was calculated and values of $\geq$ 0.6 in regions that had $\geq$ 50% G+C content were defined as meeting the criteria for CpG islands as described (43). A 500 bp region extending from approximately 140 bp upstream of the transcriptional start site to 350 bp into exon one has the characteristics of a CpG island. A graph of the ratio of CpG/GpC is shown and the positions of the GpC and CpG dinucleotides are depicted schematically below along with a schematic of the mouse NTR-1 promoter region. The transcriptional start site is denoted by an arrow and the coding region is depicted as a black box.

N1E-115 cells were plated at low density (5×10^5 cells/15 cm dish) and propagated

without subculture, but with frequent medium changes in the first set of experiments as

described (Cusack et al. 1991). Cells were harvested from duplicate cultures at the

indicated times, RNA was prepared, and NTR-1 mRNA levels were quantified using an

RNase protection assay (Fig. 3-4A). Culturing the cells at low density resulted in an

initial decline in NTR-1 gene expression, followed by a gradual increase during growth to

stationary phase. NTR-1 mRNA levels increased >20-fold compared to day 3 levels after

17 days of culture, but declined precipitously thereafter as the cultures deteriorated (Fig.

3-4). To examine NTR-1 gene expression following DMSO treatment, cells were plated

in duplicate in medium containing reduced serum and treated with 1.5% DMSO for the

indicated times (Fig. 3-4B). DMSO treatment resulted in a 4-fold increase in NTR-1

gene expression over the course of 72 hr. These results indicate that increased NTR-1

gene expression underlies the previously observed increases in NT binding sites in these

cells (Cusack et al. 1991; Poustis et al. 1984).

Promoter sequences required for DMSO induction in N1E-115 neuroblastoma cells

To determine what promoter sequences are required for the response to DMSO, a

series of promoter deletion constructs in which up to 1.4 kb of 5' flanking sequences were

fused to a luciferase reporter gene were transfected into N1E-115 cells, and the cells were

either grown under control conditions or treated with 1.5% DMSO for 72 hr. To control

for variations in transfection efficiency, a PGK-βgal plasmid was co-transfected and β-
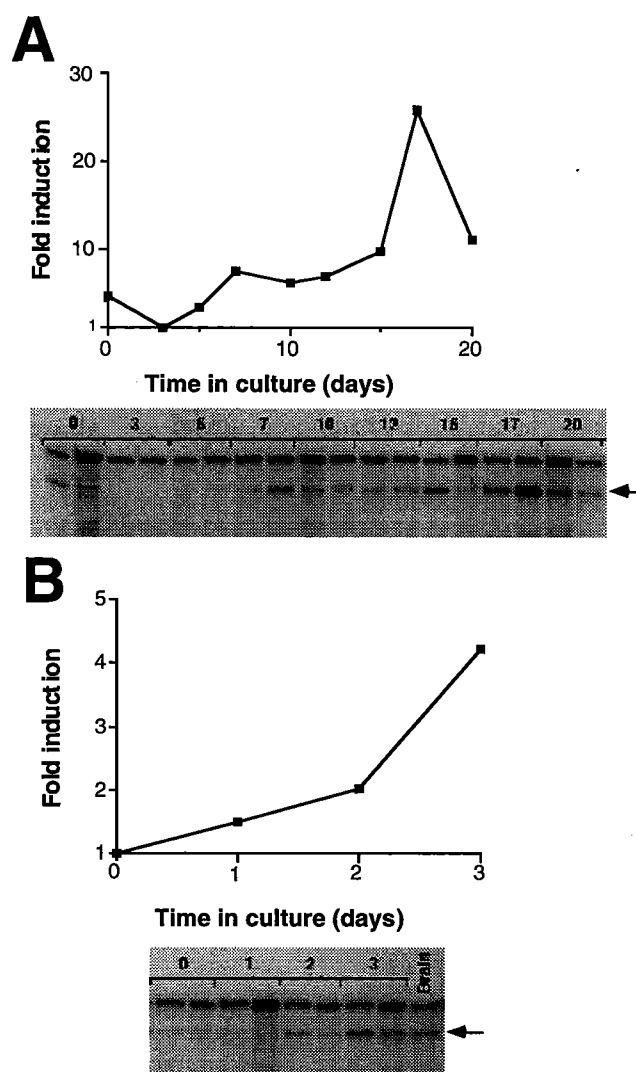
Fig. 3-4 Cell density and DMSO induce NTR-1 gene expression in N1E-115 cells. (A)
Cell density experiments were performed in duplicate by plating N1E-115 cells at low
density and incubation without further subculturing, but with frequent medium changes
as described in Experimental Procedures. Cells were harvested at the indicated times for
RNA preparation. Total RNA isolated from each duplicate culture dish (10 µg) was
hybridized with ^{32}P-labeled Pst I 300 riboprobe, nuclease-treated, and the protected
products were analyzed on sequencing gels. The relative levels of NTR-1 mRNA were
quantitated using a phosphorimager and autoradiographs of the gels are shown below the
graphs. The two lanes under each time point represent the protection products obtained
with RNA preparations from each of the duplicate culture dishes. (B) DMSO induction
was analyzed by treating N1E-115 cells with 1.5% DMSO in duplicate for the indicated
times and quantitation of NTR-1 mRNA as described in (A).

galactosidase activity was used to standardize luciferase activity. The initial series of constructs revealed two interesting features of the regulatory region. First, sequences upstream of -640 [we have used the numbering system previously used for the rat and human NTR-1 genes (Le et al. 1997; Maeno et al. 1996) where +1 is the A of the initiator methionine codon] appear to suppress basal promoter activity (Fig. 3-5A). Second, promoter elements required for DMSO induction are located between a Sma I site at -640 and a Sac I site at -448 (Fig. 3-5A). The -640 deletion construct is induced 3- to 5-fold upon DMSO treatment; however, deletion to the Sac I site at -448 completely abolishes DMSO induction and this construct is actually repressed after DMSO treatment (Fig. 3-5A). The effect of DMSO was selective, since the expression of a reporter gene controlled by the Rous sarcoma virus LTR was not affected by DMSO treatment (data not shown). These experiments define a DMSO responsive region between -640 and -448 and indicate that sequences upstream of -640 suppress basal promoter activity.

To more accurately determine the boundaries of the regulatory region, additional 5' and 3' deletion mutants were constructed and tested in N1E-115 cells (Fig. 3-5B). The response to DMSO was maintained through deletion to -589; however, deletion to -569 nearly abrogated the response. In contrast, basal activity decreased in a graded fashion as sequences were deleted from the 5' end, except for a small increase when sequences between -589 and -569 were deleted, perhaps indicating that a weak repressor element is located in this region. Deletion in the 3' direction from the Sac I site at -448 to -526 had a severe impact on both basal activity and DMSO induction. Further 3' deletions had no
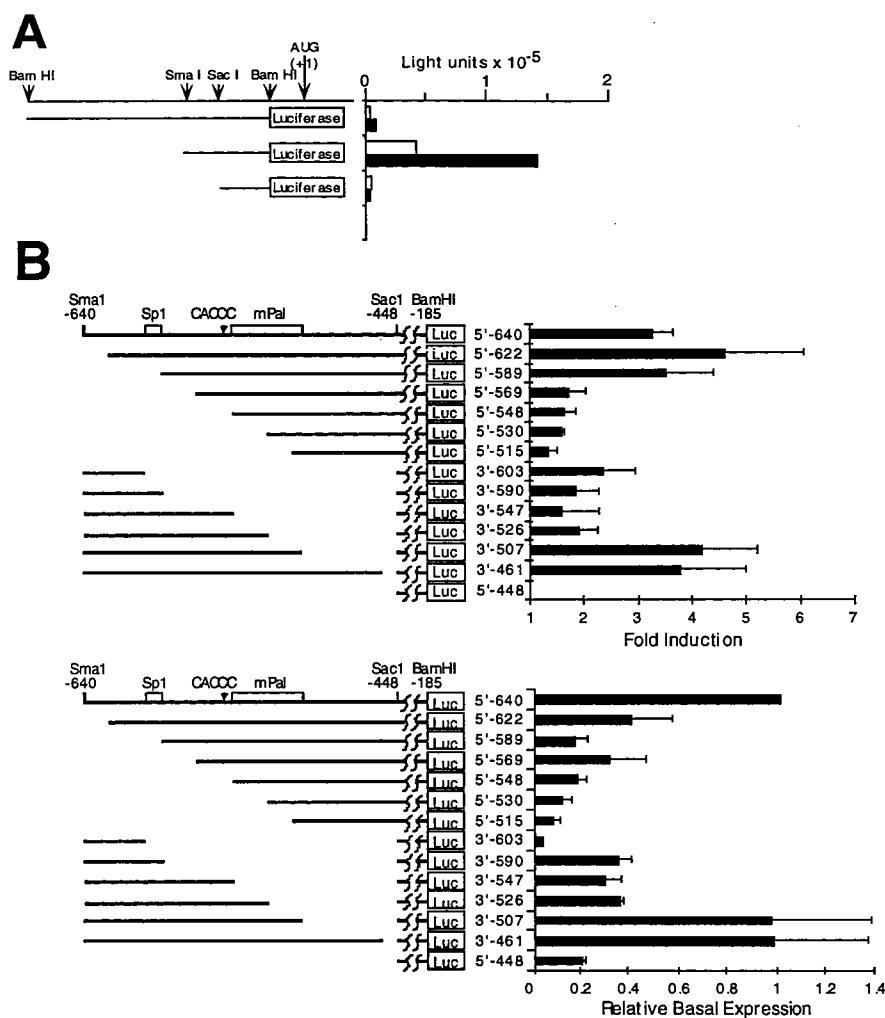
Fig. 3-5 Transfection analysis of NTR-1 gene promoter region deletion constructs in N1E-115 cells. (A) An initial series of deletion constructs was constructed by cloning the indicated restriction fragments upstream of the luciferase reporter gene in pXP-2 (33). The plasmids were co-transfected with a PGK-β-gal standardization plasmid into N1E-115 cells using calcium phosphate precipitation and the cells were either grown under control conditions (open bars) or in the presence of 1.5 % DMSO (black bars) for 72 hr. The cells were harvested, extracts were prepared, luciferase and β-galactosidase activities were determined, and β-gal activity was used to correct for variations in transfection efficiency. The constructs are depicted schematically and their activity in light units is plotted. (B) An extensive series of 5' and 3' deletion constructs was generated using PCR methods and tested as described above. The constructs are depicted schematically and activity is plotted as either fold induction or relative basal expression. Transfections were performed in duplicate and the luciferase and β-galactosidase activities were determined in duplicate for each transfection. To correct for variations in transfection efficiency, luciferase activity was divided by β-galactosidase activity. Fold induction was calculated by dividing the corrected induced value by the corrected control value. Relative basal expression was calculated by dividing the corrected mutant control value by the corrected wild type control value. The mean values are plotted and the error bars indicate the SEM (n=3-8). Promoter fragments generated by PCR were completely sequenced to verify that only the intended changes had been introduced.

effect until removal of an Sp1-like sequence (compare constructs 3'-590 and 3'-603 that have deletion endpoints on either side of the Sp1-like site) which essentially eliminated expression, indicating that this Sp1-like site is important for basal expression. These results indicate that the DMSO regulatory region lies in the 83 bp region located between -589 and -507, and that the entire region between -640 and -507 is required for full basal expression. This regulatory region is highly conserved in the rat and human NTR-1 promoters (Fig. 3-1B).

The mPAL was examined in more detail by evaluating the effects of clustered mutations along the length of the palindrome (Fig. 3-6A, mPal mut-1 to -6). Several mutations reduced basal activity; however, the most mPal mut-6 had the largest effect, reducing activity by ~70%. Removal of this sequence in the 3'-526 deletion construct greatly reduced both basal activity and DMSO induction (Fig. 3-5A and B). Mutations near the 5' end of the mPal all had similar effects, reducing basal activity by about half (Fig. 3-6A, mPal mut-1, -3, and -5). The transcriptional start site lies within the region affected by mPal mut-3. Deletion of the entire mPAL (Fig. 3-6A, dmPal) reduced basal expression by ~60%, consistent with this sequence being important for basal expression; however, the residual basal expression of this construct indicates that transcription can also initiate outside this region. A mutation near the center of the mPAL (mPal mut-2) had little effect on basal activity, but severely curtailed DMSO induction (Fig. 3-6A). Two other mutants that reduced basal expression had no effect on DMSO induction (mPal mut-4 and -5), while the remaining mutations (mPal mut-1, -3, -6) reduced both basal and DMSO-inducible expression. These results indicate that the mPAL is
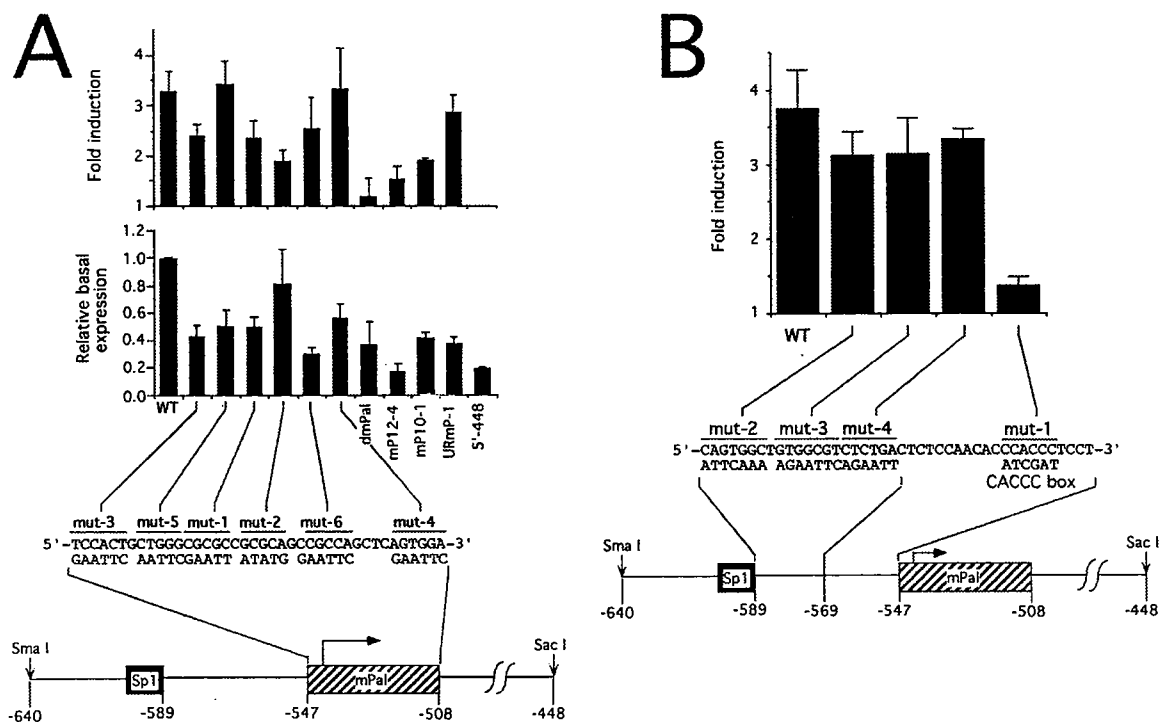
Fig. 3-6 Sequences required for DMSO induction and basal expression of the NTR-1 gene. Clustered point mutations were introduced into the regulatory region using the PCR overlap extension technique (36) and cloned upstream of the luciferase reporter gene. All mutant promoters were completely sequenced to verify the mutation and to confirm that no other substitutions had occurred. The mutant promoters were analyzed as described in Fig. 5. The mean values are plotted with the SEM indicated by error bars (n=5-8). Mutational analysis of the mPAL region (A) and the region immediately upstream (B) are shown. Schematic diagrams of the promoter regions analyzed and the specific substitutions are shown below the graphs.

composed of multiple *cis*-regulatory sequences that contribute to basal and DMSO-induced expression. The mPAL core (affected by mPal mut-2) appears to be most critical region involved in DMSO induction, although sequences at the 5' border of the mPAL are also important.

To examine the regulatory properties of the mPAL in more detail, one (mP12-4) or two (mP10-1) copies of an oligonucleotide spanning the mPAL were cloned upstream of the Sac I deletion construct (5'-448) which displays very low basal expression and is actually inhibited by DMSO treatment (Fig. 3-6A). Both constructs conferred some DMSO responsiveness to the deleted promoter, but two copies were required to increase basal expression, although neither construct had full activity. To determine whether inclusion of sequences just upstream of the mPal could restore full DMSO-responsiveness, a construct containing sequences between -589 and -507 cloned upstream of the Sac I promoter deletion was tested (Fig. 3-6A, URmP-1). Inclusion of these more 5' sequences restored DMSO induction nearly completely, although the basal activity of this construct was increased only marginally (similar to the mP10-1 construct). These results provide additional evidence that sequences immediately upstream of the mPAL and the mPAL itself are required for full DMSO responsiveness.

To define the additional sequences located in the upstream region that are required for full DMSO induction, a series of mutations in this region were analyzed by transfection in N1E-115 cells (Fig. 3-6B, mut-1 to -4). Mutants 2-4 had little affect on

DMSO induction (Fig. 3-6B) and none of the mutants had a large affect on basal expression, each retaining greater than 85% wildtype basal activity (data not shown). In contrast, mut-1, which specifically alters the conserved CACCC box, nearly abolished DMSO-inducibility (Fig. 3-6B), while maintaining more than 80% of the wildtype basal activity. These results indicate that the conserved CACCC box that lies immediately upstream of the mPAL is required for DMSO induction. This result was somewhat surprising in view of the fact that 5' deletion to -569 results in a large drop in DMSO responsiveness (Fig. 3-5, 5'-569) and this deletion removes the sequences mutated in mut-2 to -4. This apparent discrepancy could result from either functional redundancy within the region mutated in mut-2 to -4 (e.g. the sequence GTGGC is directly repeated), the presence of a *cis*-regulatory element within the deleted region that is not inactivated by any of the individual mutations, or the creation of junction sequences during cloning that have an adventitious effect on activity. Thus, although it remains possible that one or more sequences in the region affected by mut-2 to mut-4 contribute to DMSO responsiveness, the CACCC box is clearly an important *cis*-regulatory element required for DMSO induction.

Similar promoter elements are required for expression in NG108 neuroblastoma cells

NG108 neuroblastoma x glioma cells display high constitutive levels of high affinity NT binding sites (Nakagawa et al. 1984). To determine whether similar promoter elements are required for constitutive expression and to examine possible regulation by

DMSO, these cells were transfected with a series of promoter constructs using the same methods as were used for N1E-115 cells. DMSO treatment reproducibly increased expression of the wild type promoter construct (5'-640) by 2- to 3-fold (Fig. 3-7A). The results obtained in these cells were similar to those obtained in N1E-115 cells. The 5'- and 3'-deletion mutants define a region between -589 and -507 that is required for full induction and the analysis of selected clustered point mutations indicate that the CACCC box (URmut-1) and the center of the mPAL (mPalmut-2) are critical sequence elements required for induction (Fig. 3-7A). The only real difference was that mPal mut-6 had a modest negative effect on DMSO induction in N1E-115 cells, but slightly enhanced the response in NG108 cells. The region required for constitutive expression (-622 to -507) was also similar to that found in N1E-115 cells. The major differences being that 5' deletion to -622 had no effect on constitutive expression in NG108 cells but decreased basal expression in N1E-115 cells by about one half, and several mPal mutants that reduce basal expression in N1E-115 cells had no effect on expression in NG108 cells (mPalmut-1, 3, and 5). These results indicate that similar promoter elements are required for expression in these these two neuroblastoma cell lines, and provide further evidence that the CACCC box and the core of the mPAL are critical elements required for DMSO induction.
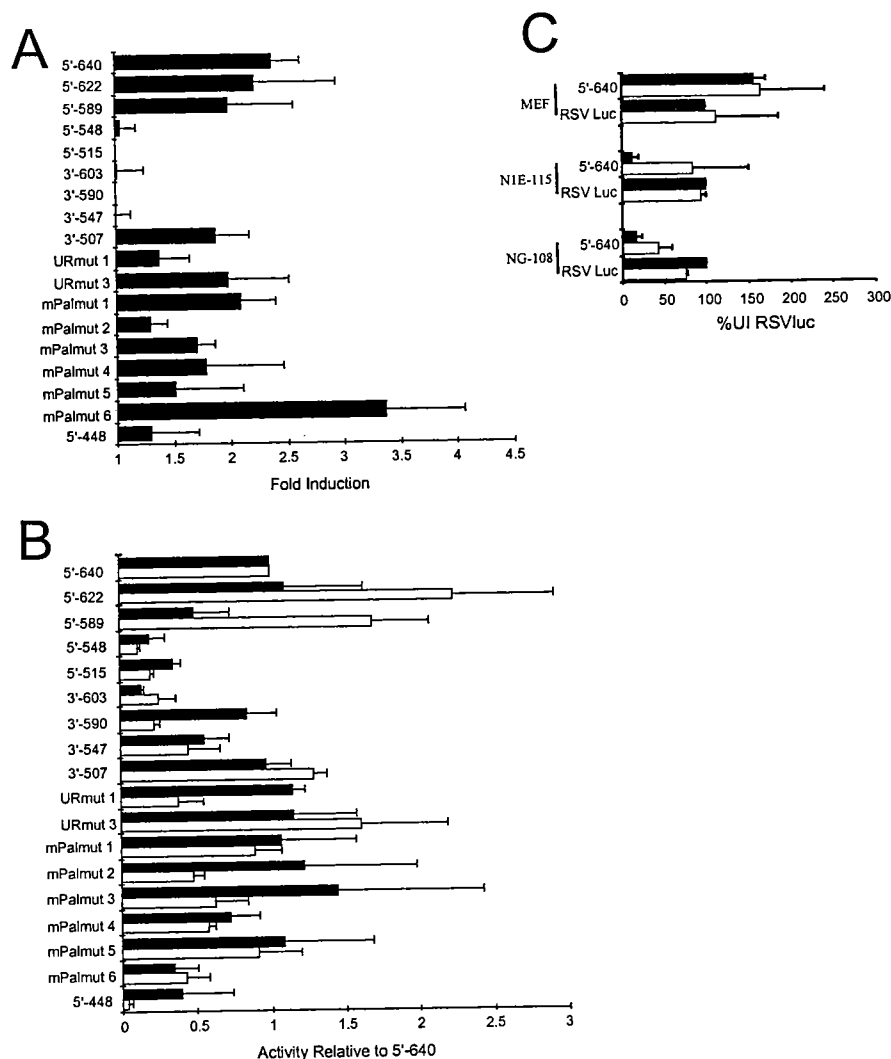
Fig. 3-7 <u>Transfection analysis of NTR-1 gene promoter constructs in NG108 and MEF cells.</u> A subset of the reporter constructs diagramed in Figs. 5 and 6 were transfected into either NG108 cells or MEF cells together with a PGK-β-gal standardization plasmid as described for N1E-115 cells. Initial experiments indicated that the full-length NTR-1 gene promoter construct was induced by DMSO in NG108 cells, but not in fibroblasts. Therefore, reporter constructs were transfected into NG108 cells and assayed exactly as described for N1E-115 cells in the presence or absence of 1.5% DMSO, but DMSO induction was not further analyzed in MEF cells. (A) NG108 cells were transfected with the indicated constructs and grown either under control conditions or in the presence of 1.5% DMSO for 72 hr. The data are plotted as fold-induction as described for N1E-115 cells (n=3-4). (B) The basal activity of the indicated NTR-1 promoter constructs in either NG108 (solid bars) or MEF (open bars) cells is plotted relative to the activity of the -640 wild type promoter construct (n=3-4). (C) The relative activity of the -640 wild type NTR-1 gene promoter construct in the different cell lines was calculated by dividing luciferase activity by β-gal activity. In each case, cells were transfected with 10 μg of the -640 NTR-1 promoter luciferase and 2.5 μg of the PGKβ-gal reporter constructs. Filled bars represent activity in untreated cells while open bars represent activity in cells treated with 1.5% DMSO for 72 hours. The data are plotted as a percentage of the relative activity of an RSV promoter driven reporter in untreated cells and the standard error is indicated (n=3).

Distinct promoter requirements for expression in mouse embryo fibroblasts

To determine whether the NTR-1 promoter is expressed in a cell type-specific

manner, the same series of constructs that were tested in NG108 cells were also

transfected into mouse embryo fibroblasts which do not express the endogenous NTR-1

gene (data not shown). Preliminary experiments revealed that DMSO treatment of these

cells had no effect on the expression of the wild type promoter, indicating that DMSO

induction is cell type specific. The wild type promoter (5'-640) was active in these cells ;

however, this basal expression was dependent on largely different promoter elements

compared to the neuroblastoma cell lines. There were several major differences. First,

the Sp1-like sequence that is removed in the 5'-589 promoter construct appears to be

unimportant for expression in MEF cells (Fig. 3-7B). Second, deletion of the region

between the Sp1-like site and the mPAL (5'-548) essentially abolished expression in MEF

cells, but had only a modest effect in NG108 cells (Fig. 3-7B) and essentially no effect in

N1E-115 cells (Fig. 3-5B). The CACCC box appears to be critical for basal expression in

these cells (Fig. 3-7B), in contrast to the situation in the neuroblastoma cell lines where

the CACCC box is required for DMSO induction and makes at most a modest

contribution to basal expression (data not shown). The mPAL was also found to be

important for basal expression in MEF cells; however, the core of the mPAL appears to

be extremely important for basal expression in these cells (Fig. 3-7B, mPal mut-2), but

again is mainly involved in DMSO induction in the neuroblastoma cell lines. These

results indicate that substantially different sequence elements are responsible for basal

expression in MEFs as compared to neuroblastoma cells. The endogenous NTR-1 gene

must normally be repressed in fibroblasts so that the sequence elements identified in

transient transfection experiments are masked in the endogenous gene.

Factors binding to the regulatory region

DNase I footprint analysis was used to identify protein binding sites in the NTR-1

gene regulatory region. Nuclear extracts were prepared from N1E-115 cells that were

either grown under control conditions or treated with 1.5% DMSO for 48 hr. ^{32}P end-

labeled promoter fragments were mixed with protein from either control or induced cells,

incubated on ice, treated with different concentrations of DNase I, phenol extracted, and

analyzed on a sequencing gel (Fig. 3-8A). A probe labeled at the Bam HI site at -175 that

extends to an Eco RI site at -680 revealed that the functional promoter, defined by the

mutational analysis described above, is extensively protected by nuclear extracts from

DMSO-induced cells (Fig. 3-8A). The specific protections are indicated by circles and

fall into six footprinted regions (FP1-6). Inducible or partially inducible protections are

represented by open and gray circles, respectively, and constitutively protected sites are

represented by black circles. Comparison of the patterns obtained with DMSO-induced

and control N1E-115 nuclear extracts indicates that FP1-3 and FP6 are induced by

DMSO, FP4 is better protected by DMSO-induced cell extracts, and FP5 is constitutive.

PC12 cell nuclear extract resulted in a protection pattern that was nearly identical to that

obtained with control N1E-115 cell extracts (data not shown), indicating that the pattern

obtained with uninduced control cell extracts is not cell-type-specific. PC12 cells express no detectable NTR-1 mRNA (data not shown). A cluster of constitutive hypersensitive sites (indicated by arrows in Fig. 3-8A) was observed just downstream of a putative Sp1 site, indicating that this region is occupied in both DMSO-induced and control cells. The significance of FP5 and FP6 is uncertain since deletion of this region has no effect on either basal expression or DMSO induction. However, the DMSO-inducible footprints FP3 and FP4 lie over functionally important sequences.

To examine FP-1 in more detail, the Eco RI/Bam HI promoter fragment was labeled at the Eco RI site and subjected to footprint analysis using DMSO-induced and control N1E-115 cell nuclear extracts (Fig. 3-8B). The footprint was found to consist of a region of DMSO-induced protections (FP1A) and an adjacent region that was constitutively protected (FP1B). The constitutive footprint at least partially overlaps a half CRE site (CGTCA), and many CREs bind CREB-family proteins constitutively (Weih et al. 1990). The DMSO-inducible FP1A is within a region that the 5'-622 deletion construct indicates is required for full basal expression in N1E-115 cells but does not affect DMSO induction (Fig. 3-5B); thus, the significance of this site is uncertain. Since there is a relative paucity of strong DNase I cleavages 5' of FP1A it remains possible that additional proteins may bind to this region.

To further characterize the factors binding to the promoter region, gel shift experiments were performed using a probe that spans the mPAL and CACCC element
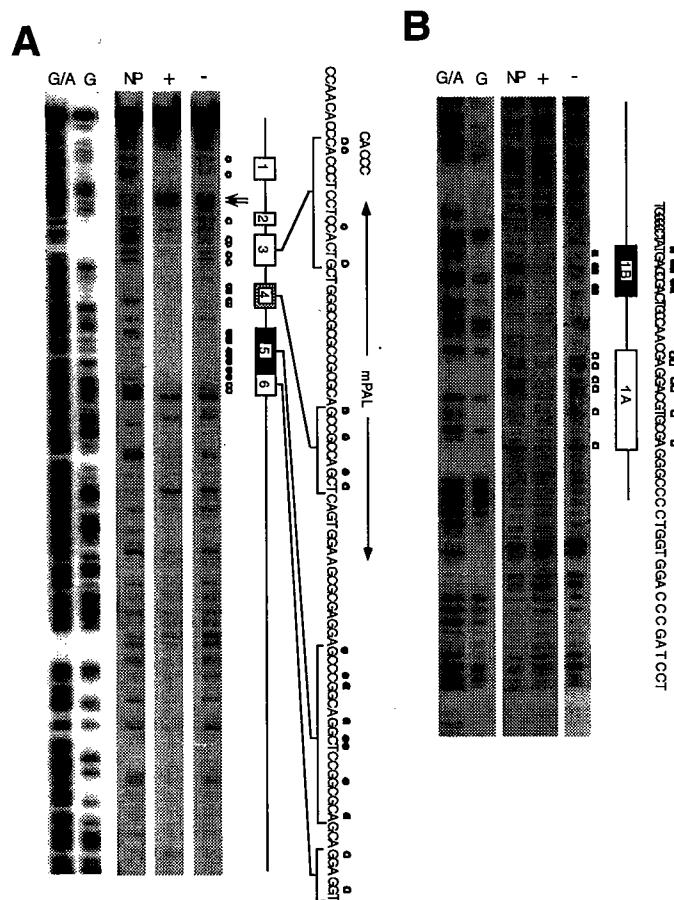
Fig. 3-8 <u>DNase I footprint analysis of the mouse NTR-1 gene regulatory region.</u> DNase I footprinting assays were performed using a Eco RI/Bam HI mouse NTR-1 promoter fragment [32]P-end-labeled at either the Bam HI (panel A) or Eco RI (panel B) site and nuclear extracts (160 μg) from either control (-) or DMSO-induced (48 hr, +) N1E-115 cells. A series of reactions containing increasing amounts of DNase I were performed and equal counts (2,000 cpm) were loaded on a sequencing gel for analysis. The products of a reaction in which the probe was treated with DNase I in the absence of protein (NP) were also analyzed. Lanes in which the extent of probe digestion was similar were compared and are depicted. Chemical sequencing reactions (lanes G/A, G) were used to generate markers for the alignment of footprinted regions on the promoter sequence. DNase I protections are indicated by either open circles (DMSO-inducible), gray circles (partially inducible), or black circles (constitutive). Footprinted regions are indicated schematically by boxes shaded like the circles and are numbered 1-6. The mouse NTR-1 promoter sequence in the region analyzed is depicted to the right of the autoradiographs and promoter schematics and the positions of the mPAL and CACCC box are indicated. In panel B, the antisense strand was labeled but the coding sequence is shown for clarity.

(-589 to -507). Nuclear extracts were prepared from N1E-115 cells that were either treated with 1.5 % DMSO for 48 hr or grown under control conditions, incubated with the ^{32}P-labeled promoter fragment, and the resulting complexes were analyzed on non-denaturing acrylamide gels (Fig. 3-9A). At least four complexes (Fig. 3-9A, complexes 1-4) were identified that were specifically competed by cold wild type probe fragment (longer exposure times were required to detect complexes 3 and 4, Fig. 3-9A, bottom panel). To examine whether the functional sequence elements defined in the transfection experiments described above were required for the formation of these complexes, competition experiments were performed with wild type and mutant promoter fragments. An oligonucleotide corresponding to the collagenase TRE (Angel et al. 1987) was used as a non-specific competitor. Competition with a cold mutant promoter fragment containing clustered point mutations in the CACCC element (mut-1 in Fig. 3-6B) identified two complexes that require this sequence for binding (Fig. 3-9A, lane 4, complexes 1 and 2). Comparison between the induced and control lanes indicates that DMSO treatment results in the specific induction of complex 1 (Fig. 3-9A, compare lanes 2 and 6). Most of the gel shift experiments performed in this study included both uninduced and induced N1E-115 nuclear extracts for comparison and the observed induction of complex 1 in these experiments was similar to the gel presented in Fig. 3-9A. Close DNA contacts in the inducible complex were analyzed using a methylation interference assay (Fig. 3-9B). Complexes were formed by mixing partially methylated ^{32}P-end-labeled probe (the same probe used in the experiment depicted in Fig. 3-8A) with nuclear extract from DMSO-induced N1E-115 cells, separated on a native acrylamide gel, and the regions
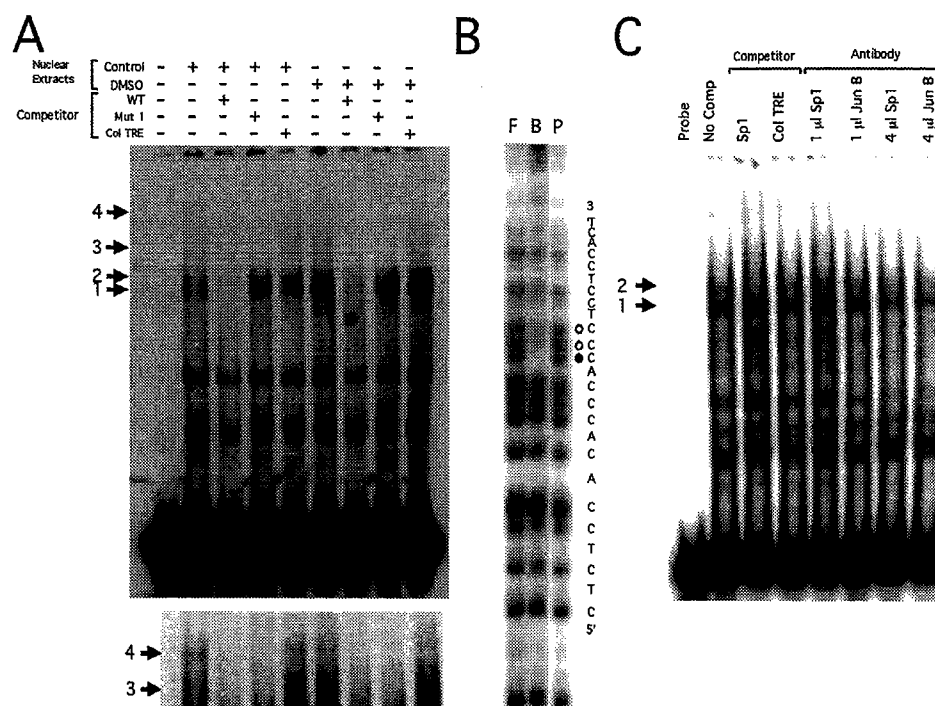
Fig. 3-9 A DMSO-inducible complex binds to the CACCC element in the NTR-1 regulatory region. (A) A ^{32}P-labeled probe spanning the mPAL and immediate 5' flanking sequences (-589 to -507) was incubated with nuclear extracts prepared from N1E-115 cells that had either been treated with 1.5% DMSO for 48 hr or grown under control conditions as indicated and the resulting complexes were analyzed on a non-denaturing acrylamide gel and visualized by autoradiography with an intensifying screen for 16 hr. A longer exposure (72 hr) of the upper part of the gel is shown below to visualize complexes 3 and 4. Binding reactions were performed either in the absence of unlabeled competitor DNA (lanes 2 and 6), or in the presence of a 100-fold molar excess of either wild type (WT), CACCC mutant (Mut 1), or collagenase TRE (Col TRE) DNA. Complexes 1 and 2 require an intact CACCC element for binding. (B) Nucleotide contacts required for binding of complexes 1 and 2 were analyzed by methylation interference. The probe described above was partially methylated with dimethyl sulfate, mixed with nuclear extract from DMSO-induced cells, and the resulting complexes were separated on a low ionic strength polyacrylamide gel. The regions of the gel containing complexes 1 and 2 (lane B, bound) and free probe (lane F, free) were excised, the DNA was purified, cleaved with piperidine, and the products were analyzed on a sequencing gel. The input probe was also analyzed (lane P, probe). (C) Complex 2 is closely related to Sp1. The probe described above was incubated with nuclear extracts of cells treated with 1.5% DMSO for 72 hours and the resulting complexes were analyzed on a non-denaturing acrylamide gel. Binding reactions were performed either in the absence of competitor (No comp), or in the presence of a 100-fold molar excess of oligonucleotides containing either a consensus Sp1 binding site (SP1) or the collagenase TRE (Col TRE). To examine whether complexes 1 and 2 were immunologically related to Sp1, two different amounts (1 µl, 4 µl) of either an Sp1-specific or control Jun B-specific antibody were added to the binding reactions as indicated. The free probe was also loaded (Probe).

corresponding to complex 1/2 and free probe were excised for analysis of methylated G residues on sequencing gels. Several bands were underrepresented in complex 1/2 compared to free probe, specifically the Gs that are complementary to last three Cs in the CACCC sequence (Fig. 3-9B). Analysis of the other strand was not informative most likely due to the absence of G residues in the region containing the CACCC sequence. These results indicate that the DMSO-inducible complex makes specific DNA contacts in the CACCC element, since complex 1 is the major component of the complex 1/2 band in DMSO-induced cell extracts (see Fig. 3-9A).

The CACCC box is a binding site for a number of zinc finger transcription factors, including Sp1-related and Krüppel-like proteins (Anderson et al. 1995; Miller and Bieker 1993; Shields et al. 1996). To examine whether complexes 1 and 2 were Sp1-related factors, competition and antibody detection experiments were performed using the same probe (Fig. 3-9C). A competitor oligonucleotide containing a consensus Sp1 site effectively competed for binding of complex 2 but not complex 1, indicating that complex 2 has a binding specificity similar to Sp1. To determine whether complex 2 contains Sp1, a specific Sp1 antibody was added to the binding reaction at two different concentrations (1-4 µl) and for comparison a Jun B-specific antiserum was added to control reactions. Complex 2 was abolished by addition of 4 µl of Sp1 antiserum, but was not affected by the same amount of Jun B antiserum, indicating that complex 2 contains Sp1 (Fig. 3-9C). These results provide evidence that complex 2 is due to binding of Sp1 to the CACCC box; however, complex 1 appears to be due to a distinct

factor(s), perhaps a Krüppel-like protein, that is specifically induced during DMSO-induced neuronal differentiation of N1E-115 cells.

Discussion

<u>Several sequences, including a CACCC box, are required for DMSO induction of NTR-1</u>

<u>gene expression</u>

DNA transfection experiments were used to define promoter elements required for

NTR-1 gene induction in neuroblastoma cells. Previous work has shown that DMSO-

stimulated neuronal differentiation of N1E-115 neuroblastoma cells is accompanied by

increased expression of high affinity neurotensin binding sites (Poustis et al. 1984). We

demonstrate here that DMSO treatment induces the expression of the endogenous NTR-1

gene from 3- to 5-fold and has a similar effect on the expression of a luciferase reporter

gene driven by NTR-1 promoter sequences. DMSO also increased NTR-1 gene

expression in NG108 neuroblastoma x glioma hybrid cells that have previously been

shown to express high affinity NT binding sites (Nakagawa et al. 1984). This was a

selective effect since the expression of a luciferase reporter gene controlled by the RSV-

LTR was not affected by DMSO treatment of either neuroblastoma cell line. In contrast,

DMSO had no effect on NTR-1 reporter constructs transfected into fibroblasts.

Mutational analysis of the NTR-1 promoter has defined an 80 bp region encompassing a

20 base region just downstream of an SP1 element, a CACCC box and large GC-rich

palindrome as critical for DMSO induction. The CACCC box is bound by a complex in

N1E-115 cells induced induced 2-4 fold by DMSO. Several regions within the mPAL are

required for full DMSO induction; however, the central core appears to be the most

critical DMSO responsive sequence. The transcriptional start site was mapped to the 5'

end of the mPAL to a region that closely matches the initiator element that was first

identified in the TdT gene (Smale and Baltimore 1989). The regulatory sequences

identified through transfection analysis here are highly conserved between mouse, rat, and

human (Fig. 3-1B), indicating that they are functionally important *in vivo*. These results

provide evidence that a conserved region surrounding the transcriptional start site is

critical for NTR-1 gene activation during a program of neuronal differentiation initiated

by DMSO treatment in N1E-115 cells.

The CACCC box was first identified through sequence comparisons of β-globin

genes and has been shown to be important for expression in erythroid cells. This site

binds a number of related zinc-finger transcription factors, including Sp1- and Krüppel-

related proteins (Crossley et al. 1996; Miller and Bieker 1993). The majority of the

characterized Krüppel-related genes are expressed in tissue-restricted patterns. Gene

targeting approaches have revealed that the erythroid Krüppel-like factor (EKLF) is

essential for high level erythroid-specific β-globin gene expression (Nuez et al. 1995;

Perkins et al. 1995) and that lung Krüppel-like factor (LKLF) is essential for T-cell

development (Kuo et al. 1997), indicating that specific Krüppel-like proteins play key

roles in transcriptional control and terminal differentiation. The β-globin CACCC boxes

are required for activation during DMSO-induced differentiation of mouse erythro

leukemia (MEL) cells (Strauss and Orkin 1992), possibly through the phosphorylation of

EKLF (Ouyang et al. 1998). The CACCC box in the NTR-1 promoter is clearly required

for DMSO induction and is bound by both constitutive and DMSO-inducible complexes in N1E-115 neuroblastoma cells. The major constitutively expressed complex is closely related to Sp1 (Fig. 3-9C, complex 2). The inducible complex clearly has a binding specificity different from that of the Sp1-related proteins and makes close contacts within the CACCC box, similar to Krüppel-like proteins [see for example ref. (Crossley et al. 1996)]. These data suggest a model in which the induction of one or more Krüppel-like factors is required for NTR-1 gene activation during N1E-115 cell differentiation. Intriguingly, both gut-enriched (Shields et al. 1996) and neuron-enriched (Chowdhury et al. 1988; Hasan et al. 1995; Payen et al. 1998) Krüppel-like factors have been described and these are the major sites of NTR-1 gene expression *in vivo* (Nicot et al. 1994; Tanaka et al. 1990).

The mutational analysis of the mPAL revealed that it most likely consists of several independent *cis*-active elements that influence DMSO-induced and basal expression. Several mutations along the length of the mPAL reduced DMSO-inducibility in both N1E-115 and NG108 cells (Figs. 3-6A and 3-7A). Alteration of sequences near the center of the mPAL greatly reduced DMSO responsiveness (Figs. 3-6A and 3-7A, mPal mut-2). This sequence is a perfect palindrome consisting of alternating G and C residues and is conserved in both the rat and human genes; however, comparisons with known transcription factor binding sites did not reveal any close similarities, indicating that either it is a novel binding site or functions as a DNA structural element. Mutation of an additional sequence located at the 5' end of the palindrome (mPal mut-3) also

decreased DMSO activation in both N1E-115 and NG108 cells, but appears to affect a

distinct functional element. This sequence is nearly identical to the consensus initiator

element and mPal mut-3 also reduced basal activity in N1E-115 and MEF cell, but

surprisingly not NG108 cells (Figs. 3-6B and 3-7B). The major transcriptional start site

was also mapped to this location making it likely that this is a functional initiator. DNase

I footprint analysis indicates that factor binding to this site is regulated by DMSO in N1E-

115 cells (Fig. 3-8A). This could either be due to either increased expression of a factor

that binds to this site, or cooperative interactions with the DMSO-inducible CACCC box-

binding factor. Thus, the mutational analysis indicates that there are at least two distinct

cis-regulatory elements within the mPAL that are required for full DMSO-

responsiveness, and DNase I footprint analysis indicates that DMSO-inducible complexes

bind in the mPAL region (Fig. 3-8A and B). The central portion of the palindrome

contains few DNase I sensitive sites; thus, further analysis will be required to determine

whether DMSO-inducible complexes bind to the mPAL core region. These results

coupled with the results discussed above for the CACCC box indicate that DMSO

induction of NTR-1 gene expression requires cooperative interactions between multiple

sites, including the CACCC box, the mPAL core, and the initiator element.

<u>Different promoter sequences required for basal expression in neuroblastoma and MEF</u>

<u>cells</u>

There were several differences in the sequences required for basal expression in the three cell lines, although the results obtained in N1E-115 and NG108 cells were similar. A notable difference was that the CACCC box and the central region of the mPAL are required for high level basal expression in MEF cells, but are principally involved in DMSO responsiveness in the neuroblastoma cell lines. The contribution of the CACCC box to basal expression in MEFs is most likely due to the expression of one or more CACCC-box binding proteins in these cells, for instance BKLF is expressed in fibroblasts (Turner and Crossley 1998). A CACCC box has been shown to be important for β-globin gene expression in transient expression assays in non-erythroid cell lines (Myers et al. 1986), although this element mediates cell-specific expression through the binding of EKLF in erythroid cells (Nuez et al. 1995; Perkins et al. 1995; Strauss and Orkin 1992). The endogenous NTR-1 gene is most likely repressed in most cell types and only accessible to Krüppel-like proteins and perhaps other CACCC-box binding proteins in neurons and a restricted set of other cell types. The induction of these proteins during neuronal differentiation or in response to environmental cues could underlie NTR-1 gene activation in specific neuronal populations.

There were also differences in the requirement for the Sp1-like sequence located between -590 and -603 and a sequence near the 5' end of the positive regulatory region for basal expression. The Sp1-related sequence was clearly important for basal activity in the neuroblastoma cell lines, but not in MEF cells (compare 3'-590 and 3'-603 in Figs. 3-5B and 3-7B). Sp1 is constitutively expressed in N1E-115 cells (see Fig. 3-9A, complex 2)

and nuclear extracts from both control and DMSO-induced cells create DNase I

hypersensitive sites just downstream of the Sp1-related sequence indicating that this site

is constitutively occupied. Sequences near the 5' end of the positive control region were

found to be important for basal expression in N1E-115 cells (Fig. 3-5B, compare 5'-640

and 5'-622), but not in NG108 or MEF cells (Fig. 3-7B). DNase I footprinting

experiments indicate that this region binds both constitutive and DMSO-inducible factors

(Fig. 3-8B), and sequence comparisons indicate that this region is similar to a neural-

specific regulatory element identified in the *Drosophila* dopadecarboxylase (*Ddc*) gene

(Bray and Kafatos 1991). The NTR-1 gene is expressed at high levels in midbrain

dopamine neurons (Nicot et al. 1994), and the homology to the *Drosophila* neural

element raises the intriguing possibility that this region may be required for expression in

catecholamine-producing neurons (N1E-115 cells produce catecholamines).

Although there were significant differences in the promoter elements required for

basal expression, certain regions of the mPAL are important in all the lines examined.

The most crticial sequence is defined by mPal mut-6 which had a severe impact on basal

expression in all lines, but had only a modest effect on DMSO induction in N1E-115 cells

(Figs 3-6A and 3-7A and B). The sequence affected by this mutation is similar to the

consensus NGFI-A/Egr-1/Krox 24 binding site (Swirnoff and Milbrandt 1995) and

DNase I footprint analysis indicates that it is bound by a factor that is regulated by DMSO

but also expressed in uninduced N1E-115 cells (Fig. 3-8A). Neurotrophic factors and

other stimuli induce NGFI-A gene expression (Swirnoff and Milbrandt 1995); however,

further work will be required to determine whether NGFI-A plays a role in NTR-1 gene expression.

Transcription initiates within the mPAL

A combination of RNase protection experiments and RT-PCR were used to map the transcription initiation site of the NTR-1 gene to a position that is either at or near a consensus initiator sequence at position -545 (see Fig. 3-1A). This position is well upstream of the sites previously described for the rat and human NTR-1 genes which were mapped solely by primer extension analysis (Le et al. 1997; Maeno et al. 1996). The RNase protection data presented in Fig. 3-2B strongly indicate that the sites mapped in the rat and human are not utilized in the mouse, since a probe spanning these sites was fully protected. The sequences of the mouse and rat promoter regions are closely similar (Fig. 3-1B) making it unlikely that the transcriptional start site would differ substantially. Primer extension within the GC-rich promoter region is problematic and the rat start sites were viewed as tentative for this reason (Maeno et al. 1996). Our own attempts to map the transcription start site by primer extension using multiple primers were unsuccessful. The RT-PCR analysis (Fig. 3-2D) that was performed to more accurately map the transcriptional start site required the use of high concentrations of betaine (Henke et al. 1997) to allow PCR to proceed through the GC-rich promoter region. The recent characterization of a mouse NTR-1 cDNA (Genbank accession number AB017127) with a 5' end corresponding to position -451 of the mouse gene sequence (Fig. 3-1A) provides

additional support that transcription starts considerably upstream of the sites proposed for the rat, although this is most-likely not a full-length cDNA. There is considerably more sequence divergence between the mouse and human NTR-1 gene promoters, although the regulatory region identified here is closely conserved (Fig. 3-1B). Transcription could initiate further downstream in the human as indicated by primer extension experiments (Le et al. 1997). We conclude that the major transcriptional start site of the mouse gene is located at or near the initiator consensus sequence near the 5' end of the mPAL, a sequence that is closely conserved in the rat and human genes.

The NTR-1 and DA D_2 receptor regulatory regions share considerable sequence homology

Previous studies have demonstrated that the majority of midbrain DA neurons express NTR-1 (Szigethy and Beaudet 1989) and these neurons also express DA D_2 autoreceptors (Meador-Woodruff et al. 1989). Comparison of the promoter regions from the DA D_2 receptor and NTR-1 genes (Fig. 3-1C) revealed a striking similarity [66% sequence identity over a 60 bp region of the D_2 receptor (-53 to +4) that includes the transcription start site (Minowa et al. 1992)] that extends through the mPAL and into the CACCC box of the NTR-1 gene (-559 to -507). These sequences are required for DA D_2 receptor gene expression in a neuroblastoma cell line (NB41A3) and are negatively regulated by upstream sequences (Minowa et al. 1992; Wang et al. 1997). This homology suggests that transcriptional initiation and perhaps other aspects of the regulation of these

two genes are similarly controlled. The D_2 receptor promoter also contains a sequence composed of three TGGG repeats that is similar to the upstream Sp1-related sequence in the NTR-1 promoter (-602 to -590) required for full basal promoter activity. The TGGG repeat region of the D_2 receptor is paired with a consensus Sp1 site in a negative modulatory region of the D_2 receptor gene promoter (Minowa et al. 1992; Minowa et al. 1994). The negative regulatory action of these sites may be conferred by a protein that binds these sites but is not Sp1 by several criteria (Minowa et al. 1994). These sequence similarities suggest that these two genes that are co-expressed in DA neurons could rely on similar regulatory strategies; however, additional regulatory elements are also likely to be required to generate the specific complex patterns of expression characteristic of these two genes.

Chapter IV

Characterization of NTR-1 CACCC binding proteins

The experiments in chapter 3 identified the NTR-1 CACCC element as a critical site required for DMSO induction in N1E-115 cells. Deletion or site-directed mutants affecting the CACCC element dramatically reduced the DMSO-inducible luciferase activity of reporter constructs transiently transfected into N1E-115 cells. Both DNase I footprint analysis and electrophoretic mobility shift assays provided evidence that the CACCC site is recognized by at least one DMSO-inducible factor in N1E-115 nuclear extracts. Methylation interference analyses of this inducible complex indicate several base contacts within the CACCC element are required for complex formation. This chapter expands upon these results and further characterizes the proteins that specifically bind the NTR-1 CACCC element.

Most proteins found to bind CACCC elements in other promoters have DNA binding domains that contain Krüppel-type zinc fingers, including a majority of the members of the Krüppel-like family of transcription factors (reviewed in Turner and Crossley 1999). The first Krüppel-like transcription factor cloned was the widely expresssed SP1 (Kinzler et al. 1988). In recent years a number of novel Krüppel-like factors have been identified including the cell-type specific erythroid Krüppel-like factor (EKLF) (Miller and Bieker 1993), intestinal Krüppel-like factor (IKLF) (Conkright et al. 1999), and lung Krüppel-like factor (LKLF) (Anderson et al.1995), as well as the more broadly expressed basic Krüppel-like factor (BKLF) (Crossley et al. 1996) and core promoter binding protein (CPBP) (Koritschoner et al. 1996). Each of the Krüppel-like transcription factors contains a highly homologous DNA binding domain with zinc finger regions that recognize and bind GC rich sequences. Supershift experiments presented in

chapter 3 indicate that the major constitutive complex that specifically binds the NTR-1 CACCC element is immunologically related to SP1. While SP1 is broadly expressed in most tissues, several Krüppel-like proteins including the Gli family (Kinzler et al. 1988; Matise et al. 1998) and BKLF (Crossley et al. 1996) are expressed in the midbrain during development. It is possible that in addition to SP1, other Krüppel-type zinc finger proteins bind to the NTR-1 CACCC element in developing and mature midbrain dopamine neurons.

The experiments presented in this chapter address the possibility that the major inducible CACCC complex might also be related to the Krüppel-like transcription factors. Two CACCC binding proteins were identified by southwestern blottting, and have estimated sizes of 57 and 97 kD. Methylation interference of these southwestern proteins, together with a mutational analysis of the NTR-1 CACCC element, indicate that both proteins make crucial contacts within the NTR-1 CACCC element. EDTA chelation experiments suggest that both of these proteins require divalent cations (possibly zinc) for gel shift complex formation, providing evidence that the NTR-1 CACCC binding proteins may contain zinc finger domains. Finally, while RT-PCR experiments indicate that several previously characterized Krüppel-like proteins are expressed in N1E-115 cells, supershift analyses with antibodies for six Krüppel-like proteins suggest that the major complexes that bind the NTR-1 CACCC element are not closely related to any of these proteins. These results suggest that at least two proteins expressed in N1E-115 cells bind the NTR-1 CACCC element, and may be previously unidentified members of the Krüppel-type family of zinc finger proteins.

Results:

<u>Sequence comparisons</u>

The NTR-1 CACCC element is closely related to CACCC elements that have

been identified in numerous other genes. The full NTR-1 CACCC binding sequence has

only one base divergence from the predicted general binding site for Krüppel-like

transcription factors, CCN CNC CCN (Klevit 1991). Figure 4-1 compares this predicted

sequence to the NTR-1 CACCC element and to CACCC elements that have been shown

to bind BKLF with high affinity (Crossley et al. 1996). The NTR-1 CACCC element has

only one or two base differences from each of the sites listed. The human β-globin

promoter DNase I hypersensitive site 2 (HS2) also contains a CACCC sequence with the

identical single base divergence from the predicted Krüppel-like general binding site as

the NTR-1 CACCC element (Crossley et al. 1996). These comparisons provide evidence

that the NTR-1 CACCC element is closely related to previously characterized CACCC

elements that can be bound by members of the Krüppel-like family.

<u>RT-PCR identification of Krüppel-like genes expressed in N1E-115 cells</u>

A number of Krüppel-like proteins are expressed in the brain (Turner and

Crossley 1999) and seemed likely candidates for controlling *Ntr-1* expression in the

neuronal N1E-115 cell line. RT-PCR was used to determine whether the mRNA's for

these proteins are expressed in these cells. Specific primers were designed to amplify

mouse cDNA sequences corresponding to the N terminal regions of BKLF, mKr2, zfp37,

and Gli2, that are not conserved among these proteins. Each of these Krüppel-type

Site	Sequence
Predicted Kruppel-like binding site.......	CCNCNCCCN
	-563 -546
NTR1 CACCC element.......................	caacACCCACCCTcctcc
Strong BKLF binding sites	
β-Globin promoter..................	agagCCACACCCTggtaa
βh1-Globin..........................	agagCCCCACCCCggtaa
GATA-1 (proximal site).............	actgCCCCACCCActggg
Human HS3 (site i).................	ctgaCCCCACCCCaccc
Human HS2...........................	gggcACACACCCTaagcc
PBGD (distal site)................	taggCCCCACCCTccagc
Probe sequences	
Wild type (WT).....................	tccaacACCCACCCTcctccactgctgggcgc
Mutant (Mut).......................	tccaacACATCGATTcctccactgctgggcgc
555t...............................	tccaacACCCTCCCTcctccactgctgggcgc
554t...............................	tccaacACCCATCCTcctccactgctgggcgc
553g...............................	tccaacACCCACGCTcctccactgctgggcgc
552g...............................	tccaacACCCACCGTcctccactgctgggcgc
	-565 -534

Fig. 4-1 Sequence comparison of the NTR-1 CACCC-element to Krüppel-like binding sites and the probes used in this study. The predicted Krüppel-like binding site is aligned with the NTR-1 CACCC element (Klevit 1999), where N can be A, T, G, or C. The predicted zinc-finger base contacts are in capitals. This sequence is aligned with several CACCC-elements that are bound *in vitro* by previously characterized Krüppel-like transcription factors (Crossley et al. 1996). The oligonucleotide sequences for the wild type and mutant probes used in this study are also aligned. The base changes for each mutant oligonucleotide is underlined. The marked base numbers correspond to the NTR-1 5' flanking sequences numbered in figure 3-1.

factors is expressed in distinct patterns in the developing and adult brain (Crosley et al. 1996; Matise et al. 1998; Payen et al. 1998; Chowdhurry et al. 1988). First strand synthesis from polyadenylated N1E-115 RNA was performed with M-MLV reverse transcriptase and random hexamer primers. This was followed by a PCR reaction with the respective primers for each protein mRNA and the PCR products were analyzed on either a 2% agarose or 6% polyacrylamide gel (Fig. 4-2). Positive bands are seen only in RT PCR reactions for BKLF, mKr2, and zfp37 (Fig. 4-2 A), indicating that the mRNA's encoding these Krüppel-type proteins are expressed in N1E-115 cells. RT-PCR reactions using RNA from DMSO-stimulated and control N1E-115 cells indicate that none of these Krüpple-type mRNA's are induced in N1E-115 cells. Even though a positive band for Gli2 is observed in RT PCR reactions with brain mRNA, no Gli2 mRNA is detected in N1E-115 mRNA (Fig. 4-2 B). The results of these experiments provide evidence that transcripts for Krüppel-type proteins BKLF, zfp37, and mKr2, but not Gli 2, are expressed in N1E-115 cells.

To further identify Krüppel-type zinc finger proteins that are expressed in N1E-115 cells and to possibly characterize novel members of this family, degenerate primers were designed for an RT-PCR screen of N1E-115 mRNA. The amino acid and corresponding DNA sequences of the Krüppel-like zinc finger domains are highly conserved among different family members (Fig. 4-3 A). CODEHOP software (Rose et al. 1998) was used to design degenerate primers (Fig. 4-3 A) for RT-PCR, based on the highly conserved DNA binding domains of seven Krüppel-type proteins (EKLF, BKLF, GKLF, LKLF, UKLF, BTEB2, and CPBP). These degenerate primers should be able to

A

	BKLF	ZFP37	mKr2
RT	- + -	- + -	- + -
ES DNA	- - +	- - +	- - +
N1E-115 RNA	+ + -	+ + -	+ + -

B

	BKLF	Gli2	
RT	- + -	- - + + +
N1E-115 RNA	+ + +	- - + - -
Brain RNA	- - -	+ + - + +

BKLF →
Gli2 →

M
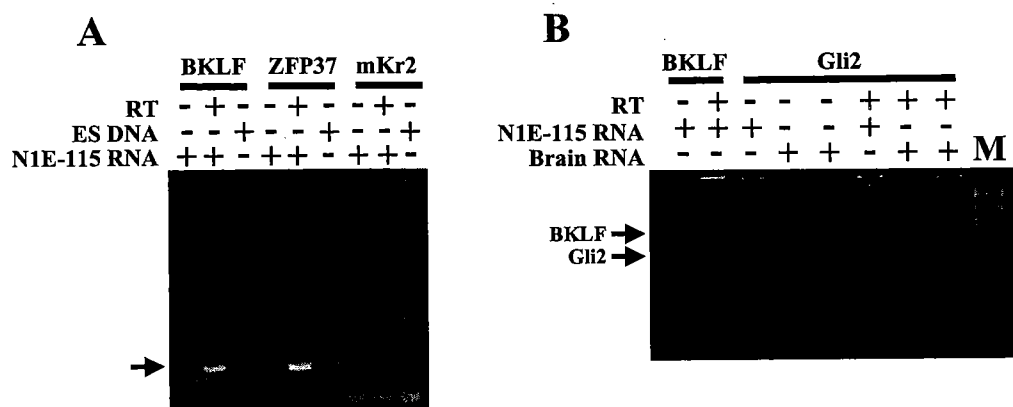
Fig. 4-2 Kruppel-like RTPCR. RT-PCR reactions were performed as described in "Materials and Methods" using primers specific to the N-terminal seqences of BKLF, ZFP37, mKr2 (A), or Gli2 (B)(Crosley et al. 1996; Matise et al. 1998; Payen et al. 1998; Chowdhurry et al. 1988) and RNA from N1E-115 cells treated with 1.5% DMSO. RT-PCR reactions in which reverse transcriptase was omitted from the initial reactions were performed to ensure that PCR-amplified products were derived from reverse-transcribed mRNA templates. Control PCR's were also performed with the same primers using mouse embryonic stem cell DNA (A) or mouse brain RNA (B) as template. The reaction products were analyzed on either 2% agarose (A) or 5% polyacrylamide gels (B) and then stained with ethidium bromide and photographed. Arrows mark the positive RT-PCR products that correspond to the predicted size..

amplify closely related, novel Krüppel-like zinc finger DNA sequences in addition to those used in their design. RT-PCR reactions were performed using these primers as described above, and the resulting amplification products were cloned and sequenced [cDNA cloning and sequencing was carried out by John Hennighan]. Several previously cloned Krüppel-type zinc finger proteins were identified in this screen, but no novel zinc finger proteins were identified (Fig. 4-3 B). Interestingly, of the three Krüppel-type zinc finger proteins initially found to be expressed in N1E-115 cells, only BKLF was detected in this screen. Nearly half of the sequenced Krüppel-like clones were identical to CPBP, possibly indicating that this transcription factor is the most highly Krüppel-like protein in N1E-115 cells. This would be in contrast to *in vivo* tissue distribution observations that suggest CPBP is expressed in most tissues, but is expressed at comparatively low levels in the brain (Koritschoner et al. 1996). At the same time, BKLF is preferentially expressed in the brain (Crossley et al. 1996; Turner and Crossley 1999), but was represented by only 2 of 22 sequenced clones. Alternatively, the high number of CPBP clones could simply indicate that the degenerate primers most efficiently amplify the CPBP zinc finger domain DNA sequence, although sequence comparisons indicate this is probably not the case. The results of this degenerate primer RT-PCR screen, coupled with the previous RT-PCR analysis, indicate that at least 8 Krüppel-type factors are expressed in N1E-115 cells, however, no novel Krüppel-type factors were identified.

Supershift assays with available Krüppel-type antibodies

The 80 bp probe (URmP1) used previously in gel shift experiments (fig. 3-9) contains the mPa1 region and detects complexes that do not bind the NTR-1 CACCC

A

Degenerate Oligonucleotides for RT-PCR

Kruppel-type Zinc Finger Domains

```
Mouse EKLF    THTGEKPYACSWDGCDWRFARSDELTRHYRKHTGHRPFCCGLCPRAFSRSDHLALHMKRH
Mouse BKLF    THTGEKPYKCTWEGCTWKFARSDELTRHFRKHTGIKPFQCPDCDRSFSRSDHLALHRKRH
Mouse GKLF    THTGEKPYHCDWDGCGWKRARSDELTRHYRKHTGHRPFQCQKCDRAFSRSDHLALHMKRH
Mouse LKLF    THTGEKPYHCNWEGCGWKFARSDELTRHYRKHTGHRPFQCHLCDRAFSRSDHLALHMKRH
Human UKLF    THTGEKPYKCSWEGCEWRFARSDELTRHYRKHTGAKPFKCNHCDRCFSRSDHLALHMKRH
Human BTEB2   THTGEKPYKCTWEGCDWRFARSDELTRHYRKHTGAKPFQCGVCNRSFSRSDHLALHMKRH
Mouse CPBP    THTGEKPYRCSWEGCEWRFARSDELTRHFRKHTGAKPFKCSHCDRCFSRSDHLALHMKRH

Composite     THTGEKPYHCSWEGCDWRFARSDELTRHYRKHTGHKPFQCHHCDRCFSRSDHLALHMKRH
```

mKl1 5'GCGGACCCACACCGGNGARAARCC-3'   mKl2 3'-CTRGTRRANCGGGACGTGTACTTCGC-5'

Degenerate Oligonucleotides

B

Screen results

Krüppel-Type Protein	Number of clones
Core Promoter Binding Protein (CPBP)	10
cKrox	4
Lung Krüppel Like Factor (LKLF)	3
Basic Krüppel Like Factor (BKLF)	2
Intestine Krüppel Like Factor (IKLF)	2
Erythroid Krüppel Like Factor (EKLF)	1

Fig. 4-3 Degenerate oligonucleotide RT-PCR screen primers and results. A) The amino acid sequences for the zinc finger domains of the Krüppel-like proteins that were used to design degnerate oligonucleotides are aligned. CODEHOP software (Rose et al. 1998) was used to determine the composite amino acid sequence and design the mKl1 and mKl2 degenerate primers (N; A, T, G or C, and R; A or G). RT-PCR products from reactions using poly A purified RNA from N1E-115 cells treated with DMSO for 72 hours were cloned and sequenced, and compared to Genbank sequences using BLAST 2.0 software. The number of clones recovered that matched the sequences of previously characterized Krüppel-like sequences is presented in (B). No novel Krüppel-like sequences were identified in this screen. .

element, so to characterize NTR-1 CACCC binding proteins in N1E-115 cells, a 32 bp

probe centered on the NTR-1 CACCC element (Fig. 4-1, WT) was tested in gel shift

assays using nuclear extracts prepared from either DMSO stimulated or control N1E-115

cells (Fig 4A). This probe was expected to detect the CACCC-specific complexes that

were previously observed using the larger 80 bp URmP1 probe, but not additional

complexes that did not require the CACCC sequence for binding (Fig. 3-9). The 32 bp

probe detects four major complexes (Fig. 4-4 A) that are specifically competed by cold

wild-type probe, but not non-specific competitor oligonucleotide (collagenase TRE,

Angel et al. 1987). It is not clear how these complexes relate to the major CACCC

complexes described in chapter 3 (Fig. 3-9), but the slower migrating complexes 1 and 2

appear to migrate similarly to those described in chapter 3. Complexes 3 and 4 migrate

considerably faster and do not appear to correspond to any of the major complexes

described in chapter 3. These two complexes may have been obscured by a comigrating

non-specific complex in the gel shift experiments where the longer URmP probe was

used. Thus, at least 4 major CACCC-specific complexes are detected with the 32 bp

NTR-1 CACCC probe.

To determine whether any of the Krüppel-like factors identified by RT-PCR are

present in these CACCC-specific gel shift complexes, antibody supershift/disruptive

experiments were performed using the 32 bp NTR-1 CACCC probe discribed above and

nuclear extracts from induced N1E-115 cells. Antibodies specific for BKLF (gift from

Dr. Merlin Crossley, University of Sidney, Australia), IKLF , LKLF (gifts from Drs. M.

Conkright and J. Lingrel, University of Chicago, Illinois), CPBP/zf9 (Santa Cruz), and
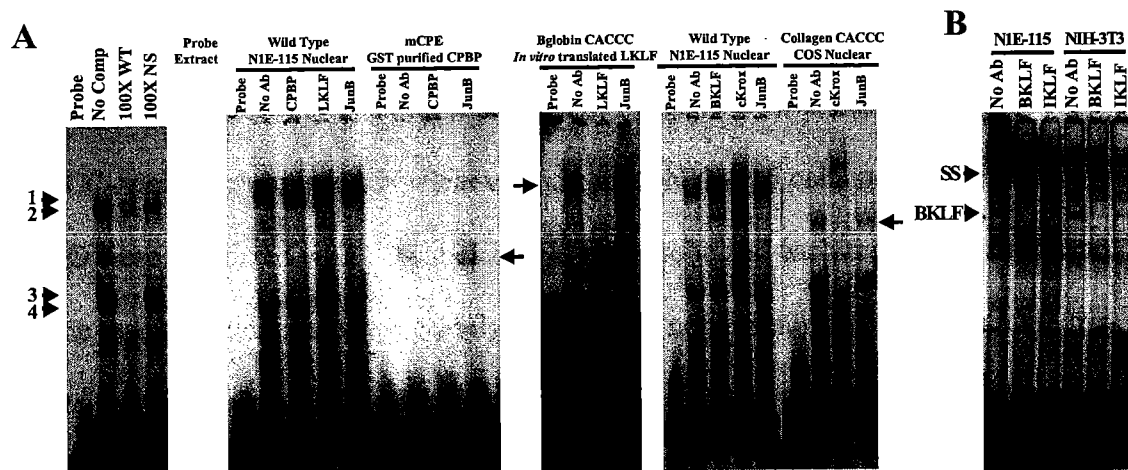
A

	Probe Extract	Wild Type N1E-115 Nuclear	mCPE GST purified CPBP	Bglobin CACCC *In vitro* translated LKLF	Wild Type N1E-115 Nuclear	Collagen CACCC COS Nuclear

B N1E-115 NIH-3T3

Fig. 4-4 Immunological analysis of NTR-1 CACCC-specific gel shift complexes. A) The 32 bp wild type CACCC site oligonucleotide was ^{32}P-labeled and incubated with nuclear extracts prepared from N1E-115 cells that had been treated with 1.5% DMSO for 72 h and the resulting complexes were analyzed at room temperature on a 4% non-denaturing Tris-acetate acrylamide gel, and visualized by autoradiography. Binding reactions were performed in either absence (No Comp) or presence of a 100-fold molar excess of eith unlabeled specific (WT), or non-specific (NS, collagenase TRE) oligonucleotides. To determine whether any of the specific complexes contained previously identified Krüppel-like factors, specific antisera for CPBP, LKLF, IKLF, BKLF, c-Krox, and Jun-B were added to the indicated reactions and the resulting complexes were compared to those formed when no antiserum was added (No Ab). The free probe was also loaded (Probe). Four CACCC-specific complexes are marked. B) The probe described above was incubated with nuclear extracts from either N1E-115 cells treated with 1.5% DMSO for 72 h, or NIH-3T3 cells, and the resulting complexes were analyzed at 4 °C on a 5% non-denaturing 0.5 X TBE acrylamide gel. Binding reactions were performed either in the absence of antibody (No Ab), or in the presence of either BKLF-specific or IKLF-specific antibody. A complex closely related to BKLF is marked, as well as the corresponding supershift complex (SS).

cKrox (gift from Dr. Russel Widom, Boston University, Massachusetts) were obtained

and tested for their ability to either supershift or interfere with the formation of NTR-1

CACCC-specific complexes. Nuclear extracts from N1E-115 cells treated with 1.5%

DMSO were incubated with ^{32}P-labeled wild type probe and each respective antiserum,

and the resulting complexes were analyzed on a 4% nondenaturing polyacrylamide/tris-

acetate gel. For comparison, a Jun B-specific antiserum was added to control reactions.

None of these antibodies was able to supershift any of the CACCC-specific complexes

(Fig. 4-4 A). Control experiments demonstrated that antibodies for CPBP, cKrox, and

LKLF could supershift or disrupt the formation of complexes formed using either

overexpressed, bacterially expressed, or *in vitro* translated proteins, and probes for

previously characterized binding sites. The IKLF antibodies were not well-characterized

and cloned recombinant or overexpressed IKLF was not available, so the appropriate

control could not be performed. BKLF is expressed at relatively high levels in NIH-3T3

cells (Turner and Crossley, 1998); however, in preliminary experiments using nuclear

extracts fom these cells and native Tris-acetate gels, addition of the BKLF antibody had

no effect. Therefore, these experiments were repeated using conditions that had

previously been used for BKLF antibody supershift experiments (Crossley et al, 1995).

Nuclear extracts from either induced N1E-115 cells or NIH-3T3 cells were incubated

with ^{32}P-labeled wild type NTR-1 CACCC probe +/- BKLF antibody on ice, and the

resulting complexes were analyzed on a 5% non-denaturing polyacrylamide 0.5 X TBE

gels at 4 °C (Fig. 4-4 B). BKLF antibody supershifted a minor complex present in the

N1E-115 nuclear extracts that was similar to the complex detected in NIH-3T3 nuclear

extracts. This minor complex may either be obscured by non-specific complexes or be unstable when analyzed on Tris-acetate gels and appears to migrate faster than the major NTR-1 CACCC-specific complexes. These experiments suggest that the major NTR-1 CACCC binding proteins detected in DMSO-induced N1E-115 cell nuclear extracts are not closely related to CPBP, cKrox, LKLF, IKLF, or BKLF.

EDTA chelation

Proteins with zinc finger DNA binding domains chelate zinc molecules to form the tertiary structure required for DNA binding (Creighton 1993). To provide evidence that the NTR-1 CACCC binding proteins may belong to the Krüppel-type family of zinc finger proteins, the zinc dependence of CACCC-specific complexes in gel shift experiments was investigated. N1E-115 nuclear extracts were treated with between 1 and 10 mM EDTA to chelate divalent cations including zinc (Kadonaga et al.1987). Three reactions that were treated with 3 mM EDTA were subsequently incubated with either 1.5 mM, 3 mM or 6 mM $ZnCl_2$, and complex formation was analyzed by EMSA (Fig. 4-5 A). CACCC-specific complexes 1 and 2 are fully inhibited by pretreatment with 3 mM EDTA, and rescued by treatment with 3 mM $ZnCl_2$. Complexes 3 and 4 do not appear to be affected by EDTA chelation or zinc in this experiment. To examine whether other divalent cations could also restore complexes 1 and 2, similar EDTA chelation experiments were performed using different divalent cations (Fig. 4-5 B). Although complex restoration was somewhat variable in this experiment, the addition of either $ZnCl_2$, $MgCl_2$, or $CaCl_2$ appeared to result in the reappearance of complexes 1 and 2.
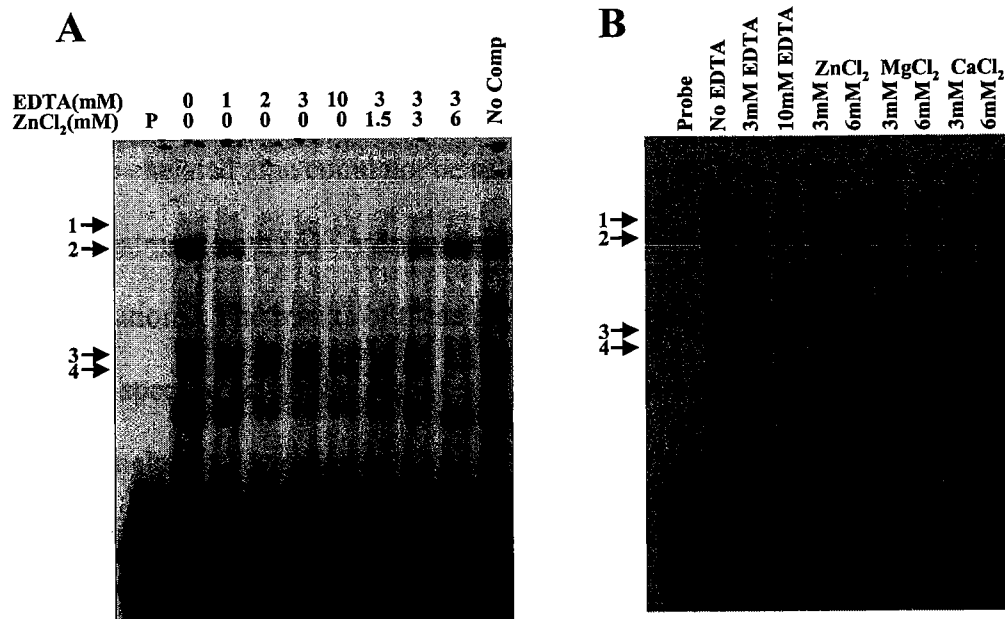
A

| EDTA(mM) | | 0 | 1 | 2 | 3 | 10 | 3 | 3 | 3 | No Comp |
| ZnCl₂(mM) | P | 0 | 0 | 0 | 0 | 0 | 1.5 | 3 | 6 | |

1→
2→

3→
4→

B

Probe | No EDTA | 3mM EDTA | 10mM EDTA | ZnCl₂ 3mM | 6mM | MgCl₂ 3mM | 6mM | CaCl₂ 3mM | 6mM

1→
2→

3→
4→

Fig. 4-5 NTR-1 CACCC complexes require divalent cations. A) Nuclear extracts prepared from N1E-115 cells that had been treated with 1.5% DMSO for 72 h were incubated on ice for 45 min either in the absence of EDTA or in the presence of 1 to 10 mM EDTA. This was followed by a 30 min incubation on ice either in the absence of ZnCl₂ or the presence of 1.5 to 6 mM ZnCl₂. Finally these nuclear extracts were added to binding reactions containing ^{32}P-labeled wild type probe, and the resulting complexes were analyzed on a native acrylamide gel, and visualized by autoradiography. The free probe was also loaded (P). The four CACCC specific complexes are marked. B) Nuclear extracts of cells treated with 1.5% DMSO for 72 h were incubated on ice for 45 min either in the absence of EDTA (No EDTA) or in the presence of 3 or 10 mM EDTA, followed by a 30 min incubation on ice during which 6 reactions that had been treated with 3 mM EDTA were treated with 3 or 6 mM ZnCl₂, MgCl₂, or CaCl₂ as indicated. The nuclear extracts were then added to binding reactions containing ^{32}P-labeled wild type probe and the resulting complexes were analyzed on a native acrylamide gel. The free probe was also loaded (Probe). The four CACCC-specific complexes are marked.

Interestingly, complex 3 formation was also dependent on divalent cations, particularly

$ZnCl_2$, in this experiment. The possibility that $MgCl_2$ and $CaCl_2$ were effective because

they displace chelated zinc could not be ruled out, since dialysis of nuclear extracts

following treatment with EDTA dramatically reduced complex formation in the presence

of divalent cations. The results of these experiments provide evidence that at least two of

the CACCC-specific gel shift complexes require divalent cations for formation, but a

definitive conclusion regarding the requirement for zinc could not be reached.

Size predictions for NTR-1 CACCC binding proteins

Since the sizes of previously characterized Krüppel-like binding proteins are

known, size predictions for the NTR-1 CACCC binding proteins provide a preliminary

basis for comparison. To predict the size of NTR-1 CACCC binding proteins, nuclear

extracts from N1E-115 cells that had been treated with 1.5% DMSO for 72 hours were

analyzed by southwestern blotting. Nuclear extracts were run on a 7.5% denaturing SDS

polyacrylamide gel and proteins were transferred electrophoretically to a nitrocellulose

membrane (Towbin et al. 1979). The membrane was soaked in renaturation buffer and

subsequently probed either with ^{32}P-labeled NTR-1 CACCC wild-type or with mutant

oligonucleotides. Two proteins were detected using the wild-type probe and had

estimated molecular weights of 57 and 97 kD (Fig. 4-6 A). The mutant probe lacking the

CACCC sequence failed to detect the 57 kD band and the labeling of the 97 kD band was

greatly reduced (Fig. 4-6 A), indicating that both proteins are at least partially dependent

on the CACCC sequence for binding. The residual labeling of the 97 kD band could

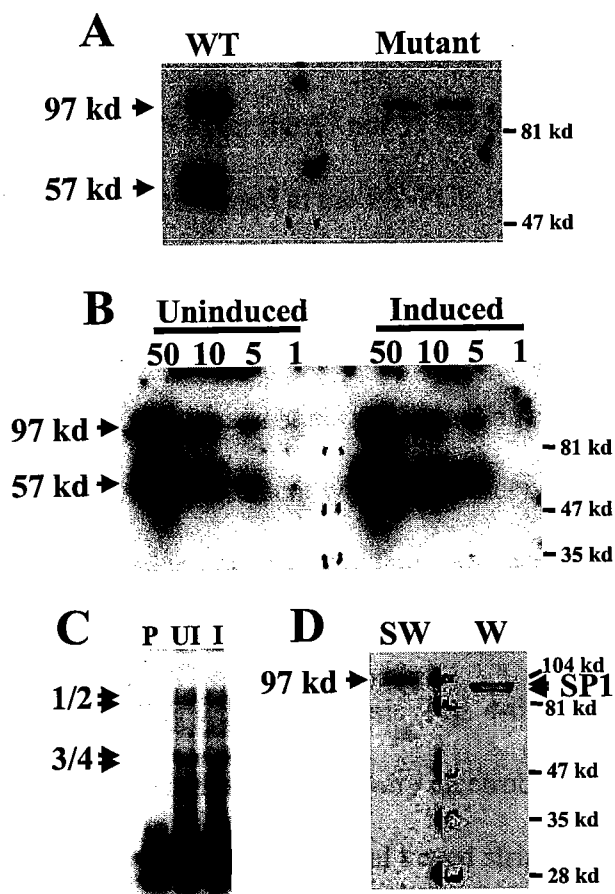either reflect a reduced requirement for the CACCC sequence for binding,

A   WT   Mutant

97 kd ➤   ─ 81 kd

57 kd ➤   ─ 47 kd

B   Uninduced   Induced

50 10 5 1   50 10 5 1

97 kd ➤   ─ 81 kd

57 kd ➤   ─ 47 kd

                             ─ 35 kd

C   P UI I   **D**   SW   W

                          ─104 kd
97 kd ➤   ◄SP1
1/2 ➤   ─81 kd

3/4 ➤   ─ 47 kd

                          ─ 35 kd

                          ─ 28 kd

Fig. 4-6 Southwestern analysis of N1E-115 Nuclear Extracts. A) 50 μg of nuclear extracts from N1E-115 cells treated with 1.5% DMSO for 72 h were analyzed by 7.5% SDS-PAGE and semi dry blotted onto a nitrocellulose membrane. Blots were then renatured for 24 hours (Hubscher 1987) at 4 °C. The blots were probed for an additional 24 hrs with ^{32}P-labeled wild type or mutant probe. Filters were washed two times and the exposed to x-ray film. Arrows mark two CACCC-specific signals with estimated sizes of 57 and 97 kD. B) N1E-115 cells were either incubated with 1.5% DMSO or under control conditions for 72 h, and were harvested for the preparation of nuclear extracts. The indicated amounts (μg) of protein were analyzed by 7.5% SDS-PAGE and southwestern blotting using the 32 bp CACCC site probe. The same nuclear extracts (UI, uninduced; I, induced) were used for gel shift analysis using the ^{32}P-labeled CACCC site probe. The four CACCC-specific complexes formed using this probe are indicated. A reaction containing the probe but no nuclear extract was also analyzed (lane P). D) 50 μg of nuclear extract from N1E-115 cells treated with 1.5% DMSO for 72 h were analyzed by 7.5% SDS/PAGE together with prestained molecular size markers, and semi dry blotted onto a nitrocellulose membrane. The blot was renatured as described above and then cut in half in a keyed fashion. One half was probed with ^{32}P-labeled wild type oligo while the other half was probed with SP1 antibody (Santa Cruz) as described in the NEN HRP probing kit (Western Blot Chemiluminescence Reagent Plus). The southwestern signals were detected by autoradiography while the western signals were detected using a BioRad Flour-S Multi-imager. The blots were aligned using the keyed cuts in the membranes and positions of fluorescent markers corresponding to the position of pre-stained molecular weight markers. Arrows mark the 97 kD southwestern band and both the 95 kD unphosphorylated and 105 kD phosphorylated SP1 western signals.

or the labeling of this band could be due to a mixture of CACCC-specific and non-specific DNA binding proteins. Neither the 57 nor 97 kD band is consistent in size with of any of the Krüppel-like proteins cloned in the RT-PCR screen of N1E-115 poly A RNA, but extended exposures of the southwestern blots reveal multiple weak bands within the size range of these proteins (32 to 50 kD). The 97 kD southwestern band is similar in size to SP1 (95 kD; Kardonaga et al. 1987), and a protein closely related to SP1 (Fig. 3-9) binds the NTR-1 CACCC element with somewhat reduced affinity suggesting that the 97 kD band may correspond to SP1. To determine whether SP1 co-migrates with the 97 kD protein(s), N1E-115 nuclear extracts were analyzed by 7.5% SDS PAGE followed by either southwestern or western analysis (Fig. 4-6 D). Parallel gel lanes were transferred to a nitrocellulose membrane by semi-dry electroblotting, the entire blot was renatured as for southwestern blots, and individual keyed strips of the blot were subsequently probed with either ^{32}P-labeled wild type NTR-1 CACCC probe or an SP1 polyclonal antibody (Santa Cruz). The blots were aligned using the keyed cut of the membranes and fluorescent dyes placed over the positions of prestained molecular weight markers. The SP1 antibody detected two forms of SP1 which have been previously reported to be 95 and 105 kD in size, and both appear to migrate faster than the 97 kD southwestern band, indicating that SP1 is not responsible for the southwestern signal.

DMSO increases at least one gel shift complex that binds to the NTR-1 CACCC site and it seemed possible that increased expression of either the 57 kD, 97 kD or both proteins could account for this increase in complex formation. To test this possibility, nuclear extracts were prepared from either uninduced control or DMSO-stimulated N1E-

115 cells and analyzed by southwestern blotting or gel shift assay using the 32 bp wild type probe described above (Fig. 4-6 B and C). For the southwestern analysis, different amounts of nuclear extract (1, 5, 10, 50 μg) were analyzed to ensure that the results were in the linear range of the assay. The 5 and 10 μg nuclear extract southwestern signals were quantitated with a Bio-Rad Flour S molecular imager and the average fold-increase (induced/uninduced) for the 57 and 97 kD bands was 1.4 and 1.1 fold respectively. In comparison, quantitation of inducible gel shift complexes 2 and 4 (Fig. 4-6 C) indicates these are induced 2.0 and 1.9 fold respectively. The fold induction observed in this gel shift experiment is about half the induction described in chapter 3 gel shifts (Fig. 3-9) and this difference may be in part due to the lack of a complete mPal in the 32 bp probe used in these gel shifts. These results indicate that proteins giving rise to the 57 and 97 kD southwestern signals are not significantly induced in N1E-115 cells treated with DMSO.

To examine whether proteins of comparable size were present in gel shift complexes detected with the 32 bp CACCC site probe, a two-dimensional gel shift/SDS PAGE/UV crosslinking technique (Akopov et al. 1998) was used. For this procedure, wild-type or mutant 32 bp oligonucleotide probes were uniformly labeled with both BrdU (to facilitate UV-cross-linking) and ^{32}P-dATP, incubated with nuclear extracts from DMSO-induced N1E-115 cells to allow complex formation, and UV-irradiated (7.2 X 10^6 J/cm^2). The resulting complexes were separated on a native gel in the first dimension, followed by analysis of crosslinked proteins by SDS-PAGE in the second dimension (as described in Materials and Methods). The gel shift lanes corresponding to the wild-type and mutant probe reactions were excised from the gel, soaked in stacking buffer, cast into

the stacking gel portion of identical SDS polyacrylamide gels, and the gels were

electrophoresed together, followed by visualization of ^{32}P-labeled proteins by film

autoradiography (Fig. 4-7). Comparison of the gel shift gels obtained with the wild type

and mutant probes indicates that there are at least two CACCC-specific complexes that

migrate similarly to the previously described complexes 1 and 2 (Fig. 4- 7). The SDS-

PAGE analysis indicates that there is a labeled protein derived from these complexes that

is absent when the mutant probe is used for similar analysis (Fig. 4-7). The estimated

molecular weight of this protein after subtraction of the probe molecular weight is 104

kD which is consistent with the 97 kD southwestern signal. The molecular weights of the

UV-cross-linked proteins were determined by subtracting the molecular weight of the

double-stranded oligonucleotide probe (23 kD) from the molecular weights that were

estimated based on the molecular weight markers (93 to 104 kD and 127 kD

respectively). Previous UV-cross-linking analysis of BKLF using a similarly sized probe

(32 bp) indicates that the probe results in an approximately 23 kD increase in the

molecular weight of BKLF on SDS-polyacrylamide gels (Crossley et al., 1996).

Gel shift complexes 3 and 4 result in a broad range of specific and non-specific UV-

cross-linked bands. A group of signals that migrate with an estimated size range between

54 and 92 kD, after subtraction of probe molecular weight, are specific to the wild type

probe. These signals contain proteins consistent with both the 57 and 97 kD

southwestern signals. There is also a large amount of non-specific UV-cross-linked

signal in gel shift complexes 3 and 4. Since the formation of complexes 3 and 4 is greatly

reduced when the mutant probe is used in the standard gel shift assay, the non-specific
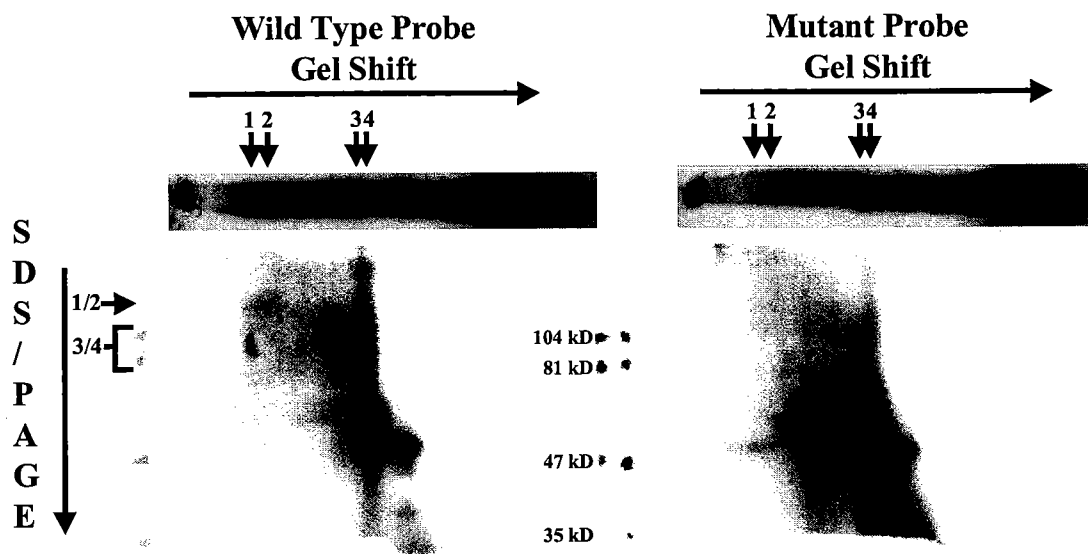
Fig. 4-7 2D UV cross-linking gel shift/SDS PAGE analysis. Nuclear extracts from N1E-115 cells treated with 1.5% DMSO for 72 h were incubated with ^{32}P-labeled wild type or mutant probe incorporated with BrdU for 20 min. Binding reactions were then exposed to 7.2 X 10^6 μJ of UV irradiation and complexes were separated on a 4% native polyacrylamide gel. Individual lanes were then cut out, soaked in stacking gel buffer, cast into the stacking gels and analyzed on 7.5% SDS polyacrylamide gels along with prestained molecular weight markers. The ^{32}P-labeled cross-linked proteins were visualized by autoradiographs and are shown together with autoradiographs of the corresponding gel shift lanes with the position and orientation corresponding to the actual position on the SDS-polyacrylamide gel. CACCC specific gel shift complexes and their respective second dimension signals are indicated.

signals detected by SDS-PAGE in the vicinity of complexes 3 and 4 apparently result

from the UV-crosslinking procedure or the incorporation of BudR into the probe (Fig. 4-

9). These experiments provide evidence that the NTR-1 CACCC specific gel shift

complexes contain proteins that are similar in size to the southwestern bands.

Critical base contacts required for binding of the 57 and 97 kD proteins

Methylation interference analysis was used to determine whether the 57 and 97

kD proteins detected by southwestern blotting are similar to CACCC-specific gel shift

complexes in their requirements for contacting the CACCC site. The URmP1 80 bp

probe that was used previously for methylation interference experiments (Fig. 3-9) was

used to probe southwestern blots so that the results could be directly compared. Blots

were incubated in the presence of either methylated or unmethylated URmP1 probe and

then autoradiographed to visualize the 57 and 97 kD bands. These bands were cut out of

the nitrocellulose filter and the bound probe was eluted by heating to 65□C in TE for 30

minutes. For comparison, the unmethylated probe recovered from an identical blot was

eluted, methylated, and processed identically. The recovered probes were piperidine

treated and analyzed on a 10% polyacrylamide/urea gel (Fig. 4-8). Different methylation

interference patterns were observed for the 57 and 97 kD proteins, but both make critical

contacts within the final three C's of the NTR-1 CACCC element (-554 to -552). This is

similar to the methylation interference pattern of the gel shift complex (Fig. 3-9) which is

included in figure 4-8 for comparison. Methylation of bases throughout the entire
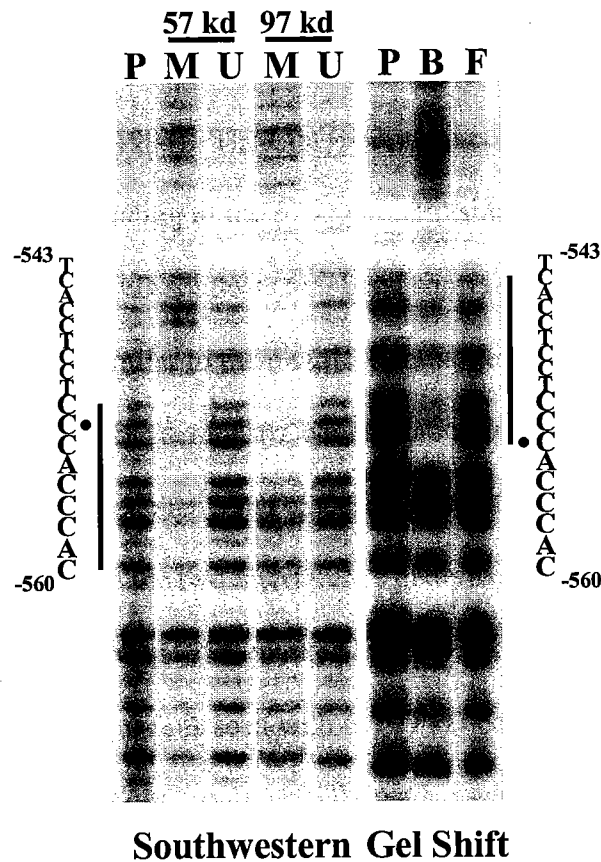
Southwestern Gel Shift

Fig. 4-8 Methylation interference by southwestern and gel shift analysis.
Southwestern blots prepared as described in Fig. 3-6A were probed with either methylated (M) or unmethylated (U) ^{32}P-labeled 80 bp URmP1 (described in chapter 2) oligonucleotides and the 57 and 97 kD bands were visualized by autoradiography. The bands were cut out of the blot, and separately incubated in TE for 15 min at 65 °C to elute the probes. The recovered unmethylated probe was methylated by treatment with DMS and each of the recovered probes, as well as control methylated probe (P), was treated with piperidine, lyophilized, disolved in sequencing dyes, and analyzed on an 8% polyacrylamide/10 M urea gel. A portion of the probe sequence is depicted on either side of the autoradiographs. Bars denote broad regions of methylation interference. The filled circles indicates a bases that were nearly completely absent when the methylated probes were analyzed. The gel shift methylation interference image from Fig. 2-9 is included for comparison but displays slightly larger range than presented in Fig. 2-9. ^{32}P-labeled, methylated URmP1 probe was incubated with nuclear extract from DMSO-stimulated N1E-115 cells; the resulting complexes were separated on a native polyacrylamide gel and bands corresponding to a mixture of complexes 1 and 2 (B) and free (F) probe were sliced from the gel and analyzed. Methylated probe that was not incubated with nuclear extracts was also analyzed (P).

predicted Krüppel-like CACCC element reduced probe binding to the 57 kD band, reminiscent of the pattern obtained in similar experiments with EKLF and the β-globin CACCC element (Feng et al. 1994). The C at -553, marked by the filled circle, appears to be the most critical site for binding of the 57 kD protein since methylation removes this band almost entirely. The 97 kD southwestern band shows a methylation interference pattern that is overall more similar to the methylation inteference pattern of the gel shift complex. Both of these patterns demonstrate methylation interference beginning at position -554 in the CACCC element, and extending into the 5' end of the mPal sequence. The major difference between the two interference patterns is that in the gel shift methylation interference, the C at -554, marked by a filled circle, is completely missing, while no such critical interference is seen with the 97 kD band. These results demonstrate that the 57 and 97 kD southwestern bands each contain a protein or proteins that make critical contacts within NTR-1 CACCC element, but produce somewhat different methylation interference patterns.

To further identify critical base contacts within the NTR-1 CACCC element and to compare the CACCC specific gel shift complexes with the two southwestern signals, a set of mutant CACCC probes (see Fig. 4-1) was analyzed by both gel shift and southwestern bloting. The probe set was designed based on single base changes previously shown to dramatically reduce binding and promoter activity of the β-globin CACCC site (Hartzog and Myers 1993; Feng, et al. 1994). In addition, a single base change (555A to T) that only modestly affected β-globin promoter function was also analyzed. The number of NTR-1 CACCC-specific and DMSO-inducible complexes

detected with the wild type probe (Fig. 4-9) is greater than with the URmP1 probe (Fig. 3-9). Complexes 1 and 2 (Fig. 4-9) appear to correspond to the major constitutive and DMSO inducible complexes described in chapter 3 (Fig. 3-9), although complex 2 is only modestly enhanced compared with the observed 2 to 3 fold induction in previous experiments (eg Fig 3-9). Complexes 3 and 4 (Fig. 4-9) specifically bind the wild type CACCC probe and complex 4 is increased when nuclear extracts from DMSO-induced cells was used. These two CACCC-specific complexes (3,4) are not seen in binding reactions with URmP1 probe (Fig. 3-9) where they may obscured by nonspecific complexes. The NTR-1 CACCC mutant probe (mut) (CCACCC to ATCGAT) is unable to form complexes 1,2, and 4 (Fig. 4-9), and greatly reduces complex 3 formation. Surprisingly however, only site mutant -552g disrupts formation of complexes 1, 2, and 4, while all of the mutants could still form complex 3. Since the NTR-1 site mutant probe forms far less complex 3 than wild type probe, it is possible that bases at the 5' end of the NTR-1 CACCC element, which were not investigated by single base change mutants, are more critical for complex 3 formation. Methylation of the bases at −552, −553, and especially −554 dramatically reduced gel shift complex formation (Fig. 4-9), so it is surprising that mutant −554g does not affect complex formation, and mutant −553 actually appears to enhance complexes 1 and 2. It is possible that the base substitutions in these mutants do not affect complex formation, unlike methylation of the G residues on the other strand. These results indicate that while the −552 C to G base change affects formation of complexes 1, 2, and 4, none of the NTR-1 CACCC gel shift complexes demonstrate a base specificity consistent with EKLF binding in the β-globin promoter.
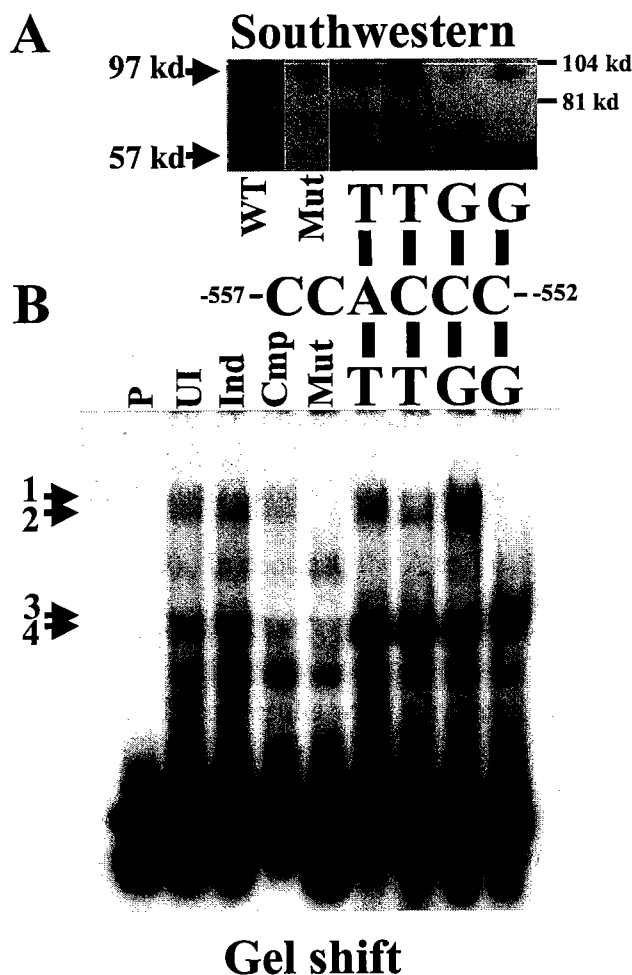
A **Southwestern**

97 kd➤   ━104 kd
           ━81 kd
57 kd➤

WT Mut T T G G

B ₋₅₅₇-CCACCC--₋₅₅₂

P UI Ind Cmp Mut T T G G

1
2 ➤

3
4 ➤

Gel shift

Fig. 4-9 Southwestern and gelshift analysis of CACCC site mutant probes. A) Individual lanes were probed with either wild type (WT) or a series of CACCC-site mutant ^{32}P-labeled oligonucleotides with either the indicated single base substitution, or clustered mutations affecting the entire CACCC sequence (Mut). The 57 and 97 kD bands are indicated by arrows. B) Nuclear extracts from N1E-115 cells treated with 1.5% DMSO for 72 h were incubated with the indicated ^{32}P-labeled probes and analyzed on a 4% native polyacrylamide gel. The binding reactions used the wild type and mutant probes described above as indicated. For comparison, gel shift reactions were performed with either nuclear extracts from control, (UI) or DMSO-induced (Ind) N1E-115 cells and ^{32}P-labeled 32 bp wild type oligonucleotide. To establish specificity, a 100-fold molar excess of unlabeled wild type probe was added to a reaction (Cmp) that was otherwise identical to the reaction analyzed in the adjacent lane (Ind). A reaction containing the wild type probe, but no nuclear extract was also analyzed (P).

This same set of point mutant probes was used for southwestern analysis to determine whether the mutations had similar effects on probe binding to the 57 and 97 kD proteins and complex formation (Fig. 4-9, top panel). The southwestern analysis was performed as described previously except that individual nitrocellulose strips corresponding to identically loaded (25 μg nuclear extract) gel lanes were probed with the indicated ^{32}P-labeled probes. The C to G transversions at postions –552 and -553 dramatically curtailed binding to the 57 and 97 kD southwestern proteins, similar to the full CACCC site mutant (Fig. 4-9). In contrast, the A to T substitution at position –555 had no effect and the C to T substitution at –554 had at most a modest effect on the southwestern results (Fig. 4-9). These results were similar to the gel shift results for complexes 1, 2 and 4 (except for the –553 mutation), but none of the substitutions had a major impact on complex 3 (Fig. 4-9). The C to G substitution at –553 appeared to slightly enhance the formation of complexes 1 and 2, although it greatly decreased the labeling of the 57 and 97 kD southwestern bands (Fig. 4-9). This discrepancy could either mean that neither the 57 nor the 97 kD band are present in these complexes, that protein interactions within the complex stabilize binding to the –553 mutant probe, or that the different assay conditions differentially affect binding to this probe. Collectively, these results indicate that there are some similarities in the CACCC site requirements for the formation of complexes 1, 2 and 4, and the detection of the 57 and 97 kD southwestern proteins. While the discordant effects of the –553 substitution suggest that the two assays detect different proteins, broad differences in experimental conditions

between these assays exist and may result in somewhat different binding requirements, therefore a link between these bands can not be out ruled.

Discussion

The experiments in this chapter provide a preliminary characterization of proteins that bind the NTR-1 CACCC sequence in southwestern blots and gel shift experiments. Southwestern blots identify proteins with estimated sizes of 57 and 97 kd, present in N1E-115 cells that bind the NTR-1 CACCC element. Two-dimensional UV crosslinking experiments indicate that proteins of the same size are present in gel shift complexes detected with the NTR-1 CACCC site probe. These proteins make critical contacts within the NTR-1 CACCC element, but appear to recognize a somewhat different sequence and have different binding specificities compared to the previously characterized Krüppel-like protein, EKLF (Hartzog and Meyers 1993; Feng et al. 1994). These results indicate that at least two proteins in N1E-115 cells bind the NTR-1 CACCC element and suggest these proteins might contribute to complexes required for DMSO indcution of *Ntr-1* gene expression.

RT-PCR and southwestern blotting procedures were used to identify CACCC binding proteins that could potentially mediate DMSO induction of *NTR-1* gene expression. RT-PCR experiments identified eight Krüppel-type genes that are expressed in DMSO-induced N1E-115 cells, although only a distinct minor gel shift complex contains a protein immunologically related to one of these Krüppel-type proteins, BKLF. Since specific antibodies for many Krüppel-type proteins were not available, southwestern blotting was performed to characterize proteins that bind to the NTR-1 CACCC site. Detection of DNA binding proteins by this method requires that they can be successfully renatured after separation on SDS-polyacrylamide gels and that the

blotted proteins can specifically bind DNA, most likely either as monomers or homo-oligomers. This approach identified two bands with predicted molecular weights of 57 and 97 kD, that appeared to specifically require the CACCC sequence for binding, although the binding of the 97 kD band appeared to be somewhat less dependent on the CACCC sequence than the 57 kD band. The majority of the characterized Krüppel-like proteins have predicted molecular weights between 32 and 50 kD (Reviewed in Phillipson and Suske 1999) and the southwestern results indicate that Krüppel-like proteins in this size range either do not bind the NTR-1 CACCC-site probe, or do not renature after SDS-polyacrylamide gel electrophoresis and blotting. An isoform of the midbrain transcription factor, Gli2, has a predicted size of 57 kD (Tanimura et al. 1993), but Gli2 mRNA is not detectable in N1E-115 cells by RT-PCR. Supershift experiments in chapter 3 indicate that the major constitutive gel shift complex is closely related to SP1, but the 97 kD southwestern band appears to migrate slightly slower than SP1 on SDS PAGE. These results indicated that the 57 and 97 kD proteins could account for the gel shift complexes detected with NTR-1 CACCC site probes.

UV cross-linking experiments indicate that proteins similar in size to those detected by southwestern blotting are present in the specific gel shift complexes identified with a probe spanning the CACCC sequence. SDS polyacrylamide gel analysis of cross-linked proteins after initial non-denaturing gel separation of gel shift complexes provided evidence that gel shift complexes 1 and 2 contain at least one protein with an estimated size of 103 kD that requires the CACCC sequence for binding and UV cross-linking. Thise size estimate is determined by subtracting the double stranded probe size (23 kD)

from the estimated size of the probe plus protein determined by the migration on SDS

PAGE compared with molecular weight markers. This method is based on the the effect

of a similar 32 bp probe on the migration of BKLF (Crossley et al. 1996), but the estimate

size is only approximate since the effects of cross-linked oligonucleotide probes on the

migration of proteins in SDS gels has not been systematically investigated. Gel shift

reactions with both wild type and mutant probes resulted in the formation of complexes 3

and 4 and SDS polyacrylamide gel analysis indicates that these complexes are formed by

a mixture of proteins only some of which require the CACCC element for binding.

Comparison of the labeled proteins obtained with the wild type and mutant probes

indicates that the wild type probe specifically labels a smear of proteins covering an

estimated molecular weight between 54 and 92 kD after subtraction of the probe

molecular weight. This result suggests that the 57 kD protein detected by southwestern

blotting may be present in either complex 3 or 4 or perhaps in both complexes. However

complexes 3 and 4 appear to be heterogeneous since a broad non-specific band that has an

estimated molecular weight range between 15 and 42 kD (after probe subtraction) is

apparent on the SDS polyacrylamide gel when either the wild type or CACCC mutant

probe is used. This analysis provides evidence that complex 2 contains an approximately

97 kD protein(s) that specifically recognizes the CACCC sequence, and, although

complexes 3 and 4 are heterogeneous, evidence that one or both of these complexes

contains a CACCC-specific 57 kD protein(s). Thus, this experiment provides a

preliminary linkage between the 57 and 97 kD proteins detected by southwestern blotting

and specific gel shift complexes.

The methylation interference results also support the possibility that the 97 kD southwestern band is required for the slower migrating gel shift complexes 1 and/or 2. The methylation interference pattern obtained after elution of the CACCC oligonucleotide probe from the 97 kD southwestern band was nearly identical to the pattern obtained previously for gel shift complexes 1 and 2 (Fig. 3-9, and Fig. 4-8). Minor differences in these two interference patterns (e.g. methylation of the C at position –554 had a larger impact on the binding of the gel shift complex) could be attributed to either additional proteins in the gel shift binding reaction that are not present in the 97 kD southwestern band, or differences in the binding reaction itself, such as the buffer conditions or incubation times. The southwestern analysis involves several additional steps including denaturation, renaturation, and immobilization of DNA binding proteins, all of which could result in differences in the methylation interference pattern. The extended interference of the 97 kD band reaches into a TCCTCC sequence similar to a site in the collagen gene that has been shown to bind both SP1 and SP3 (Ihn et al. 1997). Both of these proteins are similar in size to the 97 kD protein and are required for regulation of the DA D2 receptor gene (Yajima et al. 1998). The methylation interference pattern of the 57 kD band indicates that methylation of G's base paired with the C's between –552 and –554 severely curtails binding of the 57 kD protein(s), similar to the 97 kD protein(s). However, methylation of G's upstream of these positions also at least partially inhibits binding of the 57 kD protein, but had little or no effect on binding of the 97 kD protein. These results indicate that the 57 kD protein(s) recognizes a sequence that is similar to previously defined Krüppel-like protein binding sites (Klevit 1991). The

interference pattern determined for the 57 kD protein(s) is in fact quite similar to the methylation interference pattern determined for binding of purified EKLF to the β-globin CACCC element (Feng et al. 1994). Based on this evidence, the 57 kD protein(s) may be a stronger candidate for a Krüppel-type DNA binding protein than the 97 kD protein(s). The different methylation patterns obtained with the 57 kD protein(s) argues that this protein is not responsible for gel shift complex 1/2; however, the possibility that the interference patterns result from multiple proteins with distinct DNA binding specificities can not be ruled out. The similarity of the gel shift complex 1/2 and 97 kD protein(s) methylation interference patterns is consistent with the hypothesis that the 97 kD protein(s) is responsible for complex 1 and/or 2.

The set of single base mutant probes provides evidence that the NTR-1 CACCC binding proteins require specific bases within the CACCC element, but these binding characteristics are different from the binding of EKLF to the β-globin CACCC element. When designing these mutant probes, it was thought that because these base changes affect binding of EKLF at the β-globin CACCC element (Harzog and Meyers 1993; Feng et al. 1994), they may also affect other CACCC binding proteins similarly. This base mutation scheme was further validated by the observation that all of the methylation interference experiments indicate that the C's at -554, -553, and -552 are critical for binding. It is therefore surprising that while the CACCC mutant (CCACCC to ATCGAT) can neither form the gel shift complexes, nor the bind southwestern blots, many of the single base change mutants either affect binding only slightly or not at all. In particular, methylation at -544 completely disrupts gel shift complex formation, but the -

554C to T base substitution has little effect on complex formation or southwestern blot signals with N1E-115 nuclear extracts. The corresponding mutant in the β-globin promoter is unable form a gel shift complex with EKLF (Hartzog and Meyers, 1993) and renders reporter constructs unable to be transactivated by EKLF (Feng et al. 1994). This indicates that the NTR-1 CACCC binding proteins do not have the same binding characteristics as EKLF, but it is possible that alternate base changes at -554 and -553 might disrupt complex formation and southwestern detection similar to methylation of the G's based paired to the C's at position –554 and –553. It is difficult to predict that any specific base change will affect binding based on the obstruction of binding by methylation at a specific position. In fact, the methylation interference of EKLF binding to the β-globin CACCC element identifies a critical base at the 5' end of this site (Feng et al. 1994) that can still form a gel shift complex with EKLF when this base is changed from a C to a T (Hartzog and Meyers 1993). The comparison of the gel shift and southwestern binding characteristics make it difficult to link the 57 and 97 kD bands to specific gel shift complexes, but procedural differences in these two experiments could explain some of these seemingly dissimilar base requirements. In addition to binding reactions that are very dissimilar between gel shift analysis and southwestern blots, the contributions by multiple proteins, especially in gel shift binding reactions, could contribute to different base requirements. Because of this, the possibility that the 57 and 97 kD southwestern proteins are required for the gel shift complex formation remains open. The critical requirement for the C at -552 provides one strong link between the results of methylation interference, southwestern blots, and the gel shift complexes. This

mutant further suggests that alternate base changes at -554 and -553 could also result in

mutants that demonstrate a similar disruption in binding that could better link these

results to the methylation interference results. Taken at face value, it appears all four gel

shift complexes have different base requirements from each other, and from the two

southwestern signals.

In conclusion, the results described in this chapter suggest that there are at least

two distinct NTR-1 CACCC binding proteins expressed in N1E-115 cells. RT-PCR and

immunological analysis indicate that several previously characterized Krüppel-type

proteins are expressed in N1E-115 cells, but only one of these, BKLF formed a minor

complex in binding reactions with site probe and N1E-115 nuclear extracts.

Southwestern blots identified both 57 kD and 97 kD proteins which make specific

contacts within the NTR-1 CACCC element. Based on critical base contacts identified in

methylation interference, the 57 kD band binds the full CACCC element that contains the

predicted Krüppel-type binding sequence, while the 97 kD band binds the 3' end of the

CACCC element as well as sequence just downstream of this site. The data collectively

indicate that the 97 kD protein(s) may be involved in formation of complex 2 and the 57

kD protein(s) may be involved in the formation of complexes 3 / 4, although conclusive

evidence for these assignments will required further purification and characterization of

these proteins. Also, analysis of single base mutant probes indicates that differences in

critical bases exist between all three gel shift complexes and the two southwestern

complexes, but the requirement for specific bases within the CACCC element are clear in

both experiments. This binding information provides the basis for the future purification

and identification of these proteins.

Chapter V

Discussion

The experiments presented here provide evidence that the *Ntr-1* gene is transcriptionally activated during DMSO-induced neuronal differentiation of N1E-115 cells and define the promoter elements and possible factors involved. N1E-115 cells can be induced by DMSO treatment to increase expression of the endogenous *NTR-1* gene transcript 3-5 fold. Figure 5-1 diagrams a model for the DMSO induced expression of the *Ntr-1* gene in N1E-115 cells based on the combined results discussed in chapters 3 and 4. The DNA sequence of the NTR-1 5' flanking sequence has no canonical TATA box, but contains many characteristic features of TATA-less promoters including a GC rich sequence that has the characteristic features of a CpG island (this includes the entire region diagramed in Fig. 5-1), a consensus SP1 element, a terminal deoxynucleotidyl transferase initiator element, and a CACCC element. Transfection analysis of NTR-1 promoter driven reporter constructs indicates that basal expression of NTR-1 requires sequences closely related to *ttk*, SP1, and TdT initiator elements, while inducible expression also requires a CACCC element and the core of a 40 base palindrome (mPal).

Southwestern blotting and UV-crosslinking experiments indicate that there are two proteins with estimated molecular weights of 57 and 97 kD that bind the NTR-1 promoter CACCC element, and that the levels of these proteins are not affected by DMSO treatment. In contrast, there are at least two DMSO-inducible CACCC-binding complexes detected in N1E-115 nuclear extracts and the CACCC element is crucial for DMSO induction of *Ntr-1* gene transcription. These results suggest that post-translational modification of the 57 and/or 97 kD proteins may be responsible for both increased complex formation and transcriptional activation, although it remains possible that these
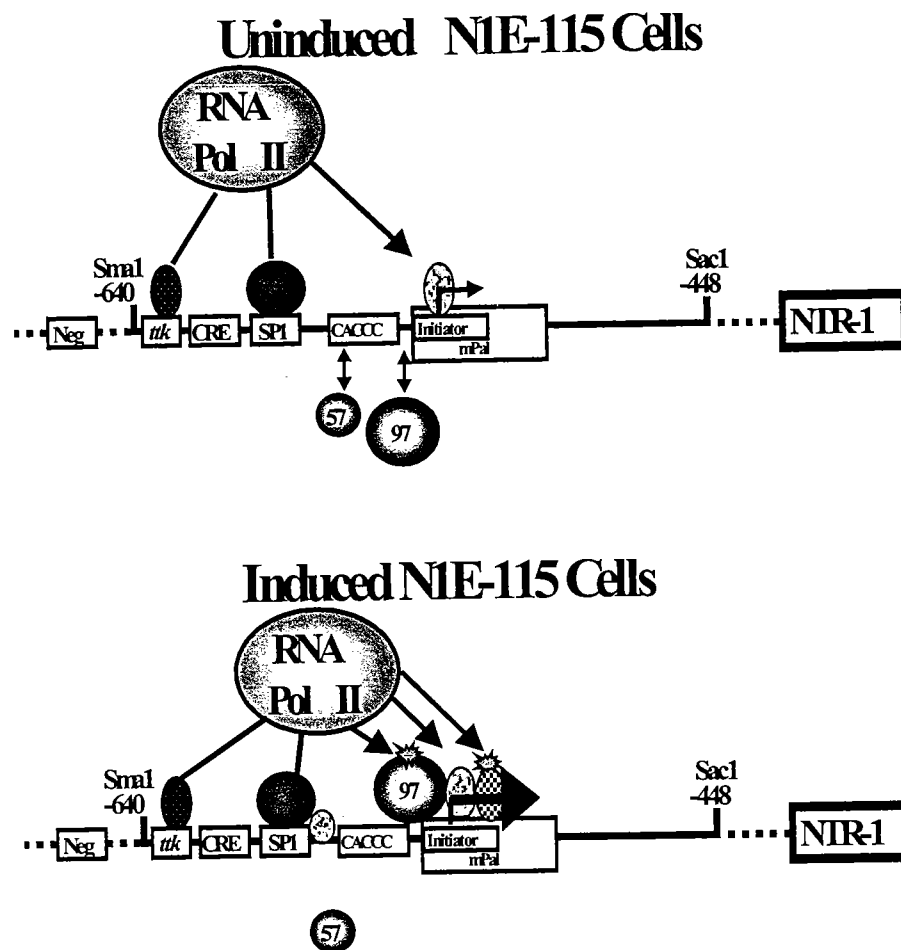
Fig. 5-1 Model for DMSO-induced NTR-1 expression in N1E-115 cells. In uninduced N1E-115 cells, factors that bind the SP1 and *ttk* elements in the NTR-1 promoter are required to recruit the RNA-polymerase II complex to the TdT initiator element at the 5' end of mPal. This drives the basal expression of the *Ntr-1* gene with transcription initiation beginning within the initiator element. The 57 and 97 kD proteins compete for binding at the CACCC element, but do not make any significant contribution to gene expression in uninduced cells. In induced N1E-115 cells, the 57 and/or 97 kD protein(s) become activated by a post-translational modification event, and bind the CACCC element with an increased affinity. The figure uses the 97 kD protein as an example, but it is also possible that the 57 kD protein binds; however, since they require overlapping sequences, it is not likely that they both bind at the same time. The mPal core is also required for the inducible expression, possibly mediated by the binding of an additional complex. The increased binding of the 97 kD CACCC-binding protein in combination with an activation step involving the mPal core synergistically activates *Ntr-1* gene expression, most likely by increased recruitment of RNA polymerase II and increased transcription initiation at the TdT initiator element.

proteins are not involved. The DMSO-induced increase in CACCC complex 2 (e.g. Fig. 3-9) is similar in magnitude to the transcriptional induction of the *Ntr-1* gene, suggesting that transcription is controlled at the level of complex formation. Previous work has demonstrated that DMSO stimulation of N1E-115 cells results in alterations in intracellular signaling, including the activation of the PKC pathway (Clejan et al. 1996). Thus, phosphorylation is one possible mechanism that could control the binding activity of the 57 and/or 97 kD proteins.

The 57 and 97 kD CACCC-binding proteins have overlapping binding sites suggesting that they could bind in a mutually exclusive fashion. Several lines of evidence suggest that the DMSO-inducible complex 2 (Fig. 3-9) may contain the 97 kD protein detected by southwestern blotting. First, the methylation interference patterns of the complex and the 97 kD protein are similar and indicate that the last three CG base pairs of the CACCC element and additional downstream positions are important for binding. Second, UV cross-linking/2D gel analysis indicates that a protein that is similar in size to the 97 kD protein is cross-linked in complex 2. Therefore, in the model depicted in Fig. 5-1, the 97 kD protein is post-translationally modified in response to DMSO and binds to the CACCC element in the NTR-1 promoter. The binding of the 97 kD protein most likely activates *Ntr-1* gene transcription by either recruiting factors that bind to the initiator element which in turn recruit RNA polymerase II, or by recruiting chromatin remodeling factors similar to EKLF (Armstrong et al. 1998). Since the 57 and 97 kD proteins share many binding characteristics, it remains possible that both proteins are involved in transcriptional activation. Mutational analysis also indicates that sequences

near the center of the mPal, and just upstream of the CACCC element are also important for DMSO activation. These sequences were not explored in detail in the experiments presented here, but could either bind additional transcriptional regulatory proteins or function as a structural element required for DMSO activation. This model describes a mechanism whereby DMSO-induced post-translational modification of the 97 kD (and perhaps the 57 kD) protein(s) results in increased binding to the NTR-1 promoter CACCC element and increased NTR-1 gene transcription.

The NTR-1 CACCC element is closely related to a Krüppel-like binding site, which suggests that the 57 and/or 97 kD proteins, identified by southwestern analysis, might belong to this family of transcription factors. The full NTR-1 CACCC element differs at only one position from the predicted Krüppel-like binding site (Fig. 4-1) and is similar to many CACCC elements that can bind BKLF (Crossley et al. 1996). Methylation interference experiments indicate that the 57 kd protein(s) makes critical contacts throughout much of the NTR-1 promoter CACCC element, similar to the contacts made by EKLF in the β-globin CACCC element (Feng et al. 1994). The 97 kD protein(s) makes critical contacts beginning at the final 3 C's of the CACCC element and continuing into a TCCTCC sequence similar to the SP1/SP3 binding site in the human α2(I) collagen promoter (Ihn et al. 1997). The methylation interference pattern of this protein(s) appears to be similar to that obtained for a mixture of gel shift complexes 1 and 2 (Fig. 4-8) suggesting that the 97 kD protein is present in these complexes. Since complex 2 is the predominant complex in this mixture, these results suggest that the 97 kD protein could be the predominant CACCC-binding protein in the DMSO-inducible

complex 2. The UV-cross-linking experiments also indicate that a protein similar in size

to the 97 kD protein(s) is present in complex 2 (Fig. 4-7). Complexes 1 and 2 require

divalent cations (perhaps zinc) for binding to the CACCC element, providing a further

mediation that these complexes contain Krüppel-like zinc finger proteins. These results

are consistent with the possibility that the 97 kD protein(s) may be part of complex 2 and

related to a Krüppel-like zinc finger protein.

The 57 and 97 kD CACCC-binding proteins detected by southwestern blotting in

N1E-115 cells are apparently not related to a number of previously characterized

Krüppel-like proteins. RT-PCR and degenerate RT-PCR were used to identify Krüppel-

like genes that are expressed in N1E-115 cells and there appear to be at least eight

Krüppel-like proteins expressed in these cells. Antibody supershift and blocking

experiments were used to determine whether proteins that were immunologically related

to these proteins were present in the complexes that bind to the NTR-1 CACCC site.

These experiments indicate that complex 1 contains a protein that is immunologically

related to SP1, since SP1 antibodies blocked the formation of this complex (Fig. 3-9). In

addition, using somewhat altered conditions, a minor complex related to BKLF was

detected using a BKLF-specific antiserum. However, none of the five Krüppel-like

proteins analyzed appeared to be present in complex 2. Since complex 1 contained a

protein related to SP1, it seemed possible that the 97 kD southwestern protein might be

SP1; however, comparisons between southwestern and western blots indicate that SP1

does not co-migrate with the 97 kD band. This result indicates that the 97 kD band is not

SP1 and that SP1 is not detected in the southwestern analysis.

Proteins of similar size to the 57 kD southwestern band are contained in gel shift complexes 3 and 4 (Fig. 4-7) which most likely also require divalent cations for binding. There are several isoforms of the Gli 2 transcription factor and one of these has a predicted molecular weight of approximately 57 kD (Tanimura et al. 1993); however, Gli2 mRNA is not expressed in N1E-115 cells, indicating that the 57 kD protein detected by southwestern blotting is not Gli2. The Krüppel-like proteins that are expressed in N1E-115 cells have predicted molecular weights (32 to 50 kD) that are considerably less than 57 kD, and, with the exception of BKLF, do not bind to the NTR-1 promoter. These results indicate that the 57 kD protein detected by southwestern blotting does not correspond to any other previously characterized Krüppel-like proteins that are expressed in N1E-115 cells. This results suggest that both the 57 and 97 kD protein(s) are novel, possibly Krüppel-type zinc finger proteins, and the results presented in this thesis provide the basis for their detection, purification and characterization.

Transactivation and repression of gene expression mediated by Krüppel-like transcription factors involves many of the same types of interactions and modifications utilized by other transcription factors. Krüppel-like transcriptional activators such as EKLF and SP1 compete for the same binding sites as their respective Krüppel-like repressors BKLF and SP3 to regulate their target genes (Turner and Crossley 1998; Imhof et al. 1999; Cook et al. 1998). It is possible that the 57 and 97 kD protein(s) compete for binding at the NTR-1 CACCC sites, and each promotes a different activity. Neither gel shift experiments nor transfection analysis supports this however, since there are no apparent complexes that diminish in induced nuclear extracts, and CACCC mutants have

little effect on basal reporter activity in N1E-115 cells. It seems more likely that at least

one of the NTR-1 CACCC binding proteins undergoes posttranslational modification to

activate transcription. The activation domain of EKLF is acetylated by the CREB binding

protein (CBP) (Zhang and Bieker 1998), relies on a SWI/SNF related chromatin-

remodeling complex, termed the EKLF-coactivator remodeling complex (E-

RC1)(Armstrong et al. 1998), and contains a casein kinase II phophorylation site that

when mutated abolishes transactivation activity (Tanese et al 1996). SP1 itself is

phosphorylated (Jackson et al. 1990), glycosylated (Jackson and Tjan 1988), and interacts

with the coactivator CRSP (Cofactor Required for SP1 activation) (Ryu et al. 1999) and

TBP associated factors (TAF's) (Tanese et al. 1996). Similar mechanisms involving post-

translational modification or cofactors could activate specific N1E-115 Krüppel-like

factor(s) in response to DMSO treatment, and induce *Ntr-1* gene expressioin.

Both widely expressed and cell-type restricted Krüppel-like proteins are required

to regulate tissue specific expression of various genes. Many of the Krüppel-like proteins

that contribute to cell-specific gene expression such as the erythroid factor EKLF and the

neuronal factor Gli2 (Miller and Bieker 1993; Matise et al. 1998) are themselves

expressed in tissue restrictive patterns, while others such as BKLF and SP1 are widely

expressed (Crossley et al. 1996; Kadonaga et al. 1987). Interestingly, transfection

analysis reported in this thesis indicates that the NTR-1 SP1 element is a critical site for

basal expression in N1E-115 cells, but has no effect on basal expression in NIH-3T3

fibroblast cells. Instead, the CACCC element and the mPal core promote high level basal

expression in fibroblasts. Since NTR-1 is not expressed in fibroblasts, it is likely that

negative regulatory regions, observed in preliminary transfection analyses, that are located upstream of the positive regulatory region described here, normally repress basal expression in certain cell types, perhaps including fibroblasts. Additional experiments would be required to determine whether the 57 or 97 kD NTR-1 CACCC binding proteins are tissue specific. If NTR-1 CACCC binding proteins such as the 57 or 97 kD proteins are widely expressed, post translational regulation could control their activity in non-neuronal cells. BKLF can act as either an activator or repressor of gene expression dependent on its association with the C-terminal binding protein, CtBP2 (Turner and Crossley 1998). Similarly the neuronal-specific transcription factor, Gli-3, contains both activation and repression domains (Dai et al. 1999). A transfection analysis in the HL-60 granulocyte cell line, utilizing the reporter constructs described in this thesis could shed some light on how NTR-1 is negatively regulated in non-neuronal lines, since as these cells differentiate in response to DMSO treatment, NTR-1 gene expression is extinguished (Choi et al. 1999). A combination of tissue specific and widely expressed Krüppel-like proteins could be required for cell-type expression of *Ntr-1*.

The mechanisms for regulating NTR-1 diagramed in figure 5-1 could also apply to other TATA-less genes that are expressed in midbrain DA neurons. In addition to the *Ntr-1* gene several neuronal-specific genes that are expressed in midbrain DA neurons have TATA-less promoters. The sequences of the positive regulatory regions of the NTR-1 and DA D2 receptor gene promoters share many similarities (Fig. 3-1C) suggesting that common mechanisms are involved in the expression of these TATA-less genes in midbrain DA neurons. The nine bases determined by methylation interference to

be required for binding by the 97 kD southwestern protein(s) are identical to a

corresponding sequence in the DA D2 receptor promoter, and the CACCC-box, the mPal

core, and two TdT initiator-like elements are nearly identical (Fig 3-1C). In addition, co-

transfection analysis in *Drosophila* SL2 cells indicates that SP1 can activate the DA D2

promoter (Yajima et al. 1998), which is consistent with observations that the SP1 element

in the NTR-1 promoter is required for basal expression. The model presented in figure 5-

1 is similar to models that propose SP1 (Oleary et al. 1996) and/or proteins that bind

initiator elements (O'Shea-Greenfield and Smale 1992) replace the role of the TATA box

for recruiting RNA polymerase II specifying the transcription initiation site. The

activation and increased binding of NTR-1 CACCC-specific complexes might enhance

the recruitment of RNA polymerase II and or it's cofactors, to the transcription initiation

site. Similar mechanisms could potentially be involved in the coordinate activation of

neural-specific genes in DA neurons. The further characterization of the CACCC-

binding proteins identified in this thesis is likely to provide insights into the coordinate

regulation of gene expression during the terminal differentiation of midbrain DA neurons.

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