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UMI
Naip (THE MURINE HOMOLOGUE OF NAIP) EXPRESSION DURING MOUSE EMBRYOGENESIS

A thesis submitted to the School of Graduate Studies and Research
In partial fulfillment of the requirement for the degree of Master of Science
Department of Biochemistry, Faculty of Medicine, University of Ottawa

by Jennifer Ingram-Crooks

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ABSTRACT

The childhood spinal muscular atrophies (SMAs) are autosomal recessive neurodegenerative conditions characterized by degeneration of lower motor neurons classified based on the age of onset and clinical severity. Type I is the most common and severe form of SMA with clinical presentation either in utero or immediately after birth. The gene encoding NAIP (Neuronal Apoptosis Inhibitory Protein) has been proposed to be a modulator of the severity of SMA and is frequently deleted in type I SMA. In this study I have assessed Naip (murine homologue of NAIP) transcript levels during mouse embryogenesis. Naip mRNA is present in the developing brain and spinal cord of E9.5 to E14.5 mouse embryos as detected by various in situ hybridization techniques. It is also found in the embryonic liver, the branchial arches, the nasal epithelium and in the future digits. At E16.5 Naip transcripts were found in the marginal zone of the lateral ventricle, the follicles of the vibrissae, in the retina and in the intestinal villi. These results are the first report of Naip gene transcript levels in embryogenesis. One model of SMA pathogenesis involves motor neuron attrition in the second and possibly third trimester of gestation. Our observation of Naip transcripts in the spinal cord between E9.5 and E14.5 (equivalent to the second trimester) is consistent with a role for Naip in modifying SMA severity.
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<td>AP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>Apaf-1</td>
<td>apoptosis protease activating factor 1</td>
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<tr>
<td>aw</td>
<td>abdominal wall</td>
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<tr>
<td>Bak</td>
<td>bcl-2 homologous antagonist/killer</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate</td>
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<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
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<td>BIR</td>
<td>baculovirus iap repeat</td>
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<td>bp</td>
<td>base pair</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>°C</td>
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<td>cc</td>
<td>central canal</td>
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<td>complementary DNA</td>
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<td>cpm</td>
<td>counts per minute</td>
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<td>central nervous system</td>
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<td>d</td>
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<td>dNTP</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>E9.5</td>
<td>embryonic day 9.5</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid disodium salt</td>
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<td>4thv</td>
<td>fourth ventricle</td>
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<td>fd</td>
<td>future digit</td>
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<td>forelimb</td>
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<td>intestine</td>
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<td>inhibitor of apoptosis protein</td>
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<td>kilobase</td>
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<td>lat</td>
<td>lateral ventricle</td>
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<td>limb</td>
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<td>liver</td>
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<td>ls</td>
<td>lens</td>
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<td>m</td>
<td>muscle</td>
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<td>molar</td>
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<td>mba</td>
<td>mandibular branchial arch</td>
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<td>MeOH</td>
<td>methanol</td>
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<td>mes</td>
<td>mesencephalon</td>
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<td>milliliter</td>
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<td>medulla oblongata</td>
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<td>msc</td>
<td>mantle layer of the spinal cord</td>
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<tr>
<td>my</td>
<td>myelencephalon</td>
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<td>mz</td>
<td>marginal zone</td>
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<tr>
<td>n</td>
<td>nasal epithelium</td>
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<tr>
<td>NAIP</td>
<td>neuronal apoptosis inhibitor protein</td>
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<tr>
<td>NBT</td>
<td>nitroblue tetrazolium chloride</td>
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<tr>
<td>nc</td>
<td>neopallial cortex</td>
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<td>ne</td>
<td>neural epithelium</td>
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<td>neural lumen</td>
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<td>neural layer of the retina</td>
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<td>np</td>
<td>nasal process</td>
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<td>notochord</td>
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<td>nte</td>
<td>neural tube epithelium</td>
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<tr>
<td>oc</td>
<td>optic chiasma</td>
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<tr>
<td>O.D</td>
<td>optical density</td>
</tr>
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<td>Oe</td>
<td>olfactory epithelium</td>
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<td>os</td>
<td>optic stalk</td>
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<td>ov</td>
<td>otic vesicle</td>
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<td>p</td>
<td>pons</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PBT</td>
<td>1X PBS with 0.1% Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>R</td>
<td>Rathke’s pouch</td>
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<td>rhb</td>
<td>roof of the hindbrain</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
<td>2mba</td>
<td>second branchial arch</td>
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<tr>
<td>S</td>
<td>somite</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>SMA</td>
<td>spinal muscular atrophy</td>
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<td>SMN</td>
<td>survival motor neuron</td>
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<td>snRNP</td>
<td>small nuclear riboprotein</td>
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<td>SSC</td>
<td>standard saline citrate buffer</td>
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<td>3v</td>
<td>third ventricle</td>
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<td>tel</td>
<td>telencephalon</td>
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<tr>
<td>tRNA</td>
<td>transfer RNA</td>
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<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
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<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<td>µm</td>
<td>micrometer</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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vibrissae

wall of the midbrain
CHAPTER I:
INTRODUCTION

1.1 CELL DEATH IN EMBRYONIC DEVELOPMENT

Embryonic development is still a poorly understood series of events and elucidation of the genetic controls of this process is underway. Development involves maturation of the biochemical pathways as well as formation and shaping of physiological functions of organs and tissues. Despite the progressive and constructive nature of development, a great deal of cell death is also taking place (Naruse and Keino, 1995). Cell death in normal development was first observed during frog metamorphosis and was then detected in both invertebrates and vertebrates (Jacobson et al., 1997). The general conservation of the mechanisms, which control cell death, suggests that this phenomenon is a fundamental feature of animal cells. Thus cell death is now being accepted as a widespread feature of normal development. The ability of RNA and protein synthesis inhibitors to suppress this cell death demonstrates the requirement of de novo gene expression for activation of cell death, suggesting a programmed mechanism (Oppenheim, 1991). This naturally occurring cell death is termed apoptosis (Kerr et al., 1972) and we now know that it results from the activation of a chain of metabolic events and specific genes (Nicotera et al., 1999; Fadeel et al., 1999). Morphologically, condensation of the chromatin, blebbing of the plasma membrane containing cytoplasmic organelles and internucleosomal DNA fragmentation are some of the characteristics of apoptosis (Wyllie et al., 1980, 1984). The triggers are as varied as the effector and inhibitor pathways. The idea that cellular death is required for an organism’s survival is
counterintuitive. However, this event has now been well documented and is postulated to serve many purposes. It aids in the sculpting of structures such as witnessed with the removal of interdigital tissue and it is an active process in the hollowing out of structures to create lumina, as seen in the intestine. Programmed cell death also removes vestigial structures and eliminates abnormal, nonfunctional, harmful or misplaced cells. Finally, apoptosis has an important role in controlling cell numbers in systems such as the CNS, where more than 50% of the neurons produced are eliminated by apoptosis during normal development (Oppenheim, 1991). Apoptosis during development has been extensively studied both in invertebrates and in vertebrates.

1.1.1 Cell death in the developing worm

The model organism for the study of development in invertebrates is the soil nematode, *Caenorhabditis elegans*. A pivotal point in programmed cell death studies came with the identification of *ced-3* and *ced-4* in *C. elegans*. In this invertebrate, 131 of the 1090 produced neurons always die before they can establish proper connections (Ellis et al., 1991). Disabling mutations in either *ced-3* or *ced-4* inhibit this cell death, thereby underlining the requirement for the proteins encoded by both genes for cell death to occur (Yuan and Horvitz, 1992). Another gene, *ced-9*, counters the effects of *ced-3* and *ced-4*, and ablation of *ced-9* results in the death of neurons that normally do not die. This suggests that neuronal survival in *C. elegans* is dependent on permanent suppression, by CED-9, of an intrinsic program of cell death (Hengartner et al., 1992). Many of these fundamental events in programmed cellular death have been conserved through evolution, as demonstrated by homologies in structures and conservation of function between genes
from the nematode and those of vertebrates. However, cell death in vertebrates involves many more mechanisms encoded by a greater number of genes, reflecting a complex and diverse control of the process of cell suicide.

1.1.2 Cell death genes in mammalian embryogenesis

The mammalian homologue to CED-3 is the cysteine protease identified as ICE (interleukin-1 beta-converting enzyme, 1) (Yuan et al., 1993). Many CED-3/ICE family members have since been identified and have been linked to programmed cell death. They have been named caspases for their capacity to cleave at specific aspartic acids. Caspases mediate the final steps of apoptosis by activating pro-apoptotic enzymes (including themselves) and by inactivating enzymes and proteins required in vital cellular mechanisms (Porter et al., 1997; Kumar, 1999).

Another family of genes that is critical to the control of apoptosis is the CED-9 mammalian homologue BCL-2 family (Tsujimoto and Croce, 1986; Hengartner and Horvitz, 1994). Bcl-2 has been localized to the outer membranes of the mitochondria, prevents cytochrome c release, caspase activation and cell death (Newton and Strasser, 1998). Family members of Bcl-2 such as Bcl-XL (Boise et al., 1993) inhibit cell death, whereas Bax (Oltvai et al., 1993) and Bak (Chittenden et al., 1995) counter the anti-apoptotic effect of Bcl-2. Bcl-2 and Bcl-X are expressed in complimentary and only partially overlapping spatio-temporal patterns in the CNS (Boise et al., 1993; Krajewski et al., 1994). Bcl-2 expression during murine embryogenesis is not only found in developing, proliferating neurons where it confers a critical cytoprotection, but also is expressed in the lung bud, intestinal epithelium, the kidney and the developing limb
(LeBrun et al., 1993; Novack and Korsmeyer, 1994). In the human adult, BCL-2 neuronal expression in the spinal cord drops drastically in comparison to the levels seen ante and perinatally (Yachnis et al., 1998). On the other hand, BCL-XL expression has been documented in both the adult and developing brain (Sohma et al., 1994). In the human, the greatest decline of motorneuron occurs between 12 and 16 weeks (Forger and Breedlove, 1987) which is also the period when BCL-2 down regulation has been documented. The exact function of these proteins during development has yet to be determined. While ablation of bcl-2 causes a pleiotropy of abnormalities in various organs and results in death postnatally, CNS development is unaffected (Kamada et al., 1995). bcl-x null mice die as embryos because of a surplus in cell death in various organs (Jacobson et al., 1997). The absence of a role for Bcl-2 in the developing nervous system might be justified by a supplemental role by other members of the Bcl-2 family or even other anti-apoptotic proteins.

IAPs (apoptosis inhibitory proteins) are another family of genes that have been shown to inhibit apoptosis in vitro and in vivo (Liston et al., 1996; Xu et al., 1997; Lacasse et al, 1998) by direct inhibition of caspases activity (Deveraux et al., 1997; Roy et al., 1997), but their involvement in development has yet to be revealed. To date, only Survivin expression has been studied during human and mouse development. In the mouse, Survivin expression is ubiquitous early in development and becomes more restricted to apoptosis regulated tissues such as the dorsal root ganglia, hypophysis, lung, spinal cord and choroid plexus (Adida et al., 1998). In the adult, SURVIVIN is undetectable in normal tissues (Ambrosini et al., 1997). This IAP is expressed in a developmentally regulated fashion and plays a role in the balance between cell survival
and cell proliferation (Ambrosini et al., 1998). The exact mechanism of action and triggers of the other IAPs remains unknown.

1.1.3 Cell death in developing nervous system

The death of cells in the developing nervous system is puzzling. It has been proposed that cell death in peripheral neurons ensure that the surviving cells match the available targets, resulting in the proper establishment of connections with trophic factor producing cells (Naruse and Keino, 1995). However, cell death prior to synapse formation has also been documented in the retina, spinal cord telencephalon and in sensory ganglia (Lance-Jones, 1982; Homma et al., 1994; Blaschke et al., 1996; Galli-Resta and Ensini, 1996). These observations suggest additional roles for cell death in neurogenesis.

Neuronal death in development is common, occurring in many different types of neurons (motor, autonomic, sensory) and begins very early in development and continues postnatally (Oppenheim, 1991). Cell death in distinct neuronal populations appears to be confined to a defined period that may differ between populations (Cowan et al., 1984). In the rat cortex for example, there is no cell death seen at E10, most is seen after 12 days of embryonic age (E12), with a peak in programmed cell death occurring at embryonic day 14 (E14) and little death observed post-natally (Blaschke et al., 1996).

The studies of mice models lacking genes involved in cell suicide have been of paramount significance in delineating their importance in modulating the development of the CNS. For example, Apaf-1 (the mammalian homologue of Ced-4, apoptosis protease-activating factor 1) and Caspase-9 null mice die at embryonic day 16.5 and perinatally
respectively, exhibiting reduced apoptosis in the brain resulting in severe cranio-facial abnormalities with hyperproliferation of neuronal cells (Cecconi et al., 1998; Kuida et al., 1998; Yoshida et al., 1998). These results show that these genes are critical for normal neuronal development. The multi-factorial control of the cell suicide events is underlined when contemplating the CNS of Bax deficient mice, in which the pro-apoptotic gene has been ablated, and where neuronal apoptosis was still observed (Shindler et al., 1997; White et al., 1998). It is now believed that the intracellular balance between pro and anti-apoptotic members of the Bcl-2 family may serve as a rheostat to regulate whether a cell lives or dies in response to a given signal (Oltvai et al., 1993; Oltvai and Korsmeyer, 1994; Gillardon et al., 1996).

The importance of the proper genetic control of programmed cell death is demonstrated in the discovery of cell death defining genes and the numerous disorders where repression or stimulation of cell death is the underlying pathological cause. It is now apparent that dysfunction of the normal control machinery of apoptosis is likely to have serious pathological consequences such as cancer, autoimmune and neurodegenerative diseases (Reed, 1999; Hetts, 1998; Stefanis et al., 1997). One such example of the disregulation of apoptosis causing abnormal loss of motor neurons possibly because of the deletion of an apoptosis inhibitory gene is spinal muscle atrophy.

1.2. SPINAL MUSCULAR ATROPHY

1.2.1 Spinal muscular atrophy: clinical features

The childhood spinal muscular atrophies (SMA) are characterized by degeneration of the anterior horn cells of the spinal cord causing symmetrical limb muscle atrophy and
weakness (Brooke, 1985). The SMAs are autosomal recessive neurodegenerative conditions classified as type I (Werdnig-Hoffmann), type II and type III (Kugelberg-Welander) forms, based on the age of onset and clinical severity (Dubowitz, 1995). Type I is the most common and severe form of SMA with an onset either in utero or immediately after birth. Infants with this acute and fatal condition are unable to sit unaided and are at risk of recurrent chest infections, with death usually occurring before the first birthday (Hausmanowa-Petrusewicz et al., 1980). Type II (intermediate form) SMA and type III (mild form) SMA are more benign conditions and the type III affected children frequently walk (Hausmanowa-Petrusewicz et al., 1980). The combined frequencies for all three types of SMA is one in 10000, making this disorder one of the most common pediatric autosomal recessive disorder (Crawford, 1996).

1.2.2 Spinal muscular atrophy: morphology of motor neurons and muscle

The predominant loss of anterior horn cells of the spinal cord seen in SMA is at the cervical and lumbar levels (Fidzanska and Hausmanova-Petrusewicz, 1984). Many of the remaining neurons are shrunken and angular (Chou and Fakadej, 1971). A more diffuse CNS degeneration has been proposed where neurons of the Clarke’s column and dorsal root ganglia would be affected (Peress et al., 1986). Towfighi et al., (1985) and Devriendt et al., (1996) have also documented involvement of the thalamus, mesencephalon, pallidum, brainstem and spinal ganglia of type I SMA patients. The factor or factors underlying this neuronal susceptibility have yet to be identified (Devriendt et al., 1996).
Two populations of muscle fibers are present in the muscle biopsies of SMA patients: normal or hypertrophied fibers and shrunken, denervated muscle fibers (Fidzianska et al., 1984; Fidzianska et al., 1990). The muscles of individuals with type I SMA have muscle fibers with reduced diameter, single distributed nuclei and small muscle cells that resemble myotubes (Hausmanowa-Petrusewicz et al., 1980). These features are characteristic of fetal muscle suggesting that the failure of the muscle development was the result of a problem with the fusion rather than an atrophy of mature muscle fibers (Hausmanowa-Petrusewicz and Fidzianska, 1974; Fidzanska et al., 1990). It has been thought that the lack of fusion event is followed by muscle degeneration because of a lack of innervation. A number of studies have now proposed that the opposite may also be true i.e. that the primary loss of the muscle by apoptosis causes loss of motor neurons due to target removal (Fidzianska et al., 1990; Guettier-Sigrist et al., 1998). The presence of immature muscle cells and motor neurons in type I SMA suggests that the death signals transpires early in development (Hausmanowa-Petrusewicz et al., 1980). An interesting aspect of SMA is that the greatest decline in function occurs at the outset of the disease, suggesting a defined and limited loss of motor neurons followed by an increased stability of the surviving neurons, as opposed to a progressive loss of function over time (Crawford and Pardo, 1996). This is consistent with clinical studies of the disease, which shows a fixed, non-progressive disability (Russman et al., 1992). This pattern of cell loss resembles developmental apoptosis, where loss of neurons is followed by reinforcement of the surviving neurons and hence SMA has been postulated to be a disease of apoptosis resulting from faulty genetic control (Sarnat, 1984; Oppenheim, 1991).
1.2.3 Spinal muscular atrophy: gene mapping

In 1990, types I, II and III of SMA were linked to region 5q13 of human chromosome 5 (Brzustowicz et al., 1990; Gilliam et al., 1990; Melki et al., 1990a, 1990b). The heterogeneity of SMA is mirrored in the nature of this region that is characterized by genetic instability and DNA duplication, leading to the presence of several functional copies of various genes, as well as pseudogenes, placed in two inverted elements (Lefebvre et al., 1995; Roy et al., 1995; Scharf et al., 1998). The search for the SMA gene culminated in 1995 with the simultaneous identification of two candidate genes: SMN and NAIP.

1.2.4 Spinal muscular atrophy: candidate genes

1.2.4.1 The SMA causative gene: SMN

1.2.4.1.1 SMN: general information

One of the SMA candidate genes, termed SMN for survival motor neuron, was cloned by Lefebvre et al. (1995). SMN is comprised of a telomeric SMN1 (SMNtel) and a centromeric SMN2 (SMNcen, cBCD541) copy that are both transcriptionally active (Lefebvre et al., 1995). The two genes span 20 kb of genomic DNA and their mRNA is 1.7 kb (Lefebvre et al., 1995). The centromeric and telomeric copies of the gene can be differentiated by the presence of five different nucleotides, none of which alter the protein sequence. Both copies contain nine exons and encode a 38-kDa protein but have different roles in SMA pathogenesis (Lefebvre et al., 1995; Burglen et al., 1996). Exon 7 of SMN1 is deleted in 95% of SMA patients regardless of clinical severity (Lefebvre et al., 1995).
Campbell et al. (1997) have shown that a higher number of SMN2 copies are present in type II and III SMA patients compared to type I patients (Campbell et al., 1997). Valesco et al. (1996) concluded that deletion of SMN1 causes SMA of type I whereas the mutations seen in type II and III SMA are due to the conversion of SMN1 in SMN2 (Velasco et al., 1996).

1.2.4.1.2 SMN: tissue expression

SMN is present in both tissues that are affected in SMA (motor neurons, cerebellar neurons etc...) (Steiman et al., 1980; Towfighi et al., 1985; Murayama et al., 1991) as well as in tissues unaffected by the disease. The hippocampal and cerebellar neurons of the adult mouse brain, neurons of the medulla oblongata, pyramidal cells of the cortex, the Purkinje cells of the cerebellum of the human adult and the motor neurons of the adult human, monkey and rat spinal cord express the highest level of SMN transcript and protein (Lefebvre et al., 1997; Battaglia, et al., 1997; La Bella et al, 1998; Tizzano, et al., 1998). Low levels but generally ubiquitous expression is observed in all other tissues examined (Lefebvre et al., 1997).

1.2.4.1.3 SMN: function

SMN has no homologies to any known protein (Lefebvre et al., 1995). SMN is found in nuclear gems (Gemini of coiled bodies), and interacts with SIP1 (SMN interacting protein 1) (Liu et al., 1997). This SMN-SIP1 complex is directly involved in the biogenesis and trafficking of splicesomal snRNPs (small nuclear ribonucleoproteins) (Fischer et al., 1997). SMN has also been shown to stimulate pre-mRNA splicing.
(Pellizzoni et al., 1998). This surprising role for SMN in RNA metabolism would make it appear as though a disregulation of mRNA generation is the pathogenic cause of SMA (Pellizzoni et al., 1998). However SMN’s role in the specific degeneration of motor neurons seen in SMA has yet to be delineated.

SMN is strongly expressed in the fetus and is essential for murine embryogenesis, as knocking out the single copy of Smn in the mouse results in embryonic death at the morula stage (Bergin et al., 1997; DiDonato et al., 1997; Schrank et al., 1997; Viollet et al., 1997). Studies of SMN protein expression in various human tissues during normal fetal and postnatal development have shown a general reduction of SMN levels in the postnatal period (Burlet et al., 1998). This is consistent with the hypothesis that SMA is a developmental disease.

1.2.4.2 NAIP

1.2.4.2.1 NAIP: general information

The second SMA candidate gene NAIP (neuronal apoptosis inhibitory protein) was cloned in 1995 and is found on human chromosome 5q13 (Roy et al., 1995). The NAIP gene contains 17 exons comprising 6.1 kb of mRNA and spans about 50 kb of genomic DNA (Roy et al., 1995; Chen et al., 1998). The 5' UTR spans the first 3 exons and part of the 4th while exons 4 to 17 code for the protein (Roy et al., 1995; Chen et al., 1998). NAIP consists of 1403 amino acids with a 156 kDa molecular weight (Roy et al., 1995; Chen et al., 1998). 5q13 contains a variable number of copies of deleted and truncated NAIP as well as an intact copy next to SMNI (Roy et al., 1995; Barnes,
NAIP was named because it contains domains with homology to baculoviral apoptosis inhibitory proteins (IAP) (Roy et al., 1995).

IAP homology in the SMA region combined with the possible role of neuronal cell death in SMA suggested that mutations in the NAIP locus might affect normal inhibition of motor neuron apoptosis and the loss of which may contribute to the SMA phenotype (Roy et al., 1995). NAIP exon 4-5 is homozygously deleted in 68% of type I SMA and 15% of type II and III (Roy et al., 1995; Burlet et al., 1996; Rodrigues et al., 1996). Thus SMN1 is deleted with a high frequency in all forms of SMA whereas NAIP deletion occurs most frequently in the severe forms of SMA. This observation has lead to the development of a model in which SMN1 is the main SMA gene and NAIP is acting as a modifier gene (Morrison, 1996; Crawford and Pardo, 1996).

1.2.4.2.2 NAIP: tissue expression and function

Hybridization of a Northern blot containing adult tissue mRNA with NAIP cDNA detected a 7 kb band in hepatic and placental RNA (Roy et al., 1995). No visible bands were seen in the CNS tissue, however reverse transcriptase PCR (RT-PCR) amplification of the NAIP transcript using spinal cord suggests transcriptional activity in this tissue (Roy et al., 1995). Experiments with adult mouse RNA has revealed expression in brain, spinal cord, liver, lungs, kidney and spleen by RT-PCR (Roy et al., 1995).

Cell lines overexpressing part of the NAIP transcript (exon 4-15) show resistance to apoptosis when compared to control cell lines (Liston et al., 1996). In vivo studies on rats show reduced ischemic damage in NAIP expressing neurons of the rat hippocampus (Xu et al., 1997a). An extensive immunohistochemical study of the distribution of NAIP
in rat CNS has documented NAIP expression in the CNS in structures affected by SMA (motor neurons, thalamic neurons) (Xu et al., 1997b). NAIP is also been shown to be an inhibitor of Caspase-3 and Caspase-7 (J. Maier, personal communication). NAIP’s cellular distribution as well as its anti-apoptotic function suggests a role for NAIP in the prevention of the apoptosis of CNS cells. The loss of NAIP, an apoptotic inhibitor, in infants with the most severe form of SMA is in accordance with the pathological loss of motor neurons by apoptosis seen in SMA patients.

1.2.4.2.3 NAIP: *Naip* the murine homologue of *NAIP*

*Naip*, the murine homologue of *NAIP*, is localized on mouse chromosome 13 (D1-D3). This region is syntenic to the human chromosome 5 q11-q23 region harboring *NAIP* (Scharf et al., 1996). *Naip* also contains three BIR domains and an ATP/GTP binding site as recognized in its human counterpart. Six copies of *Naip* (*Naip1- Naip6*) have been identified; however only three of these loci have the required 5' UTR to be translationally active in the CNS (Yarahgi et al., 1998). An adult mouse mRNA tissue Northern blot revealed low expression levels of *Naip* mostly in the lung, spleen, liver and heart. No expression was seen in skeletal muscle (Yarahgi et al., 1998). *Naip1* is expressed in the CNS while *Naip2* is expressed in the spleen (Yarahgi, et al., 1999). *Naip2* differs from *Naip1* in that it contains an additional exon (exon 9a), which does not interrupt the open reading frame and shows no homology to any known motifs. Furthermore, multiple 5'UTRs have been found for *Naip2* whereas *Naip1* only has one (Yarahgi, et al., 1999). The purpose of these alternatively spliced 5'UTRs has not yet been established but suggests differences in the regulation of translation of these genes (Yarahgi, et al., 1999).
1.2.4.3 H4F5

Recently a third potential SMA modifying gene, termed 4F5, has been identified (Scharf et al., 1998). Human 4F5 has 5 exons and exists in two copies, both of which generate 1.8 kb and 0.7 kb transcripts. H4F5 shows homology to a protein known to colocalize with snRNPs, which suggests a role in the same pathway as SMN. 90% of type I SMA patients are deleted for the H4F5 copy adjacent to SMN1 identified (Scharf et al., 1998). More study into the roles of both copies will help elucidate the role of 4F5, if any, in the pathology of SMA (Gendron and MacKenzie, 1999).

1.3 THESIS OBJECTIVE AND OUTLINE

NAIP deletions occur most frequently in the severe forms of SMA (type I) which has a onset ranging from in utero up to six months of age. This suggests that the involvement of NAIP in SMA pathogenesis may occur during development. Furthermore, NAIP expression in adult human and mouse tissue is low, as seen by RT-PCR and Northern blots (Roy et al., 1995; Yaraghi and MacKenzie, 1998). This information suggests that there may be developmental regulation of Naip. To explore this possibility the analysis of the spatial and temporal expression patterns of Naip during murine embryogenesis has been undertaken using whole mount in situ hybridization and 33P in situ hybridization. Naip was present in the developing spinal cord, brain and liver from E9.5 to E14.5. At E16.5 Naip transcripts were found only in the forebrain, retina and in the villi of the intestine. While our data do not determine whether SMA is exacerbated
from impaired *NAIP* expression in the spinal cord and skeletal muscle, they are consistent with a role for *NAIP* during embryonic development.
2. MATERIALS AND METHODS

2.1 Embryos

Female CD-1 mice at Days 9.5, 10.5, 11.5, 12.5, 13.5, 14.5, 15.5 and 16.5 of timed pregnancy (where noon of the day the vaginal plug is found is designated 0.5 dpc (days post-coitum)) were received from Charles River. The uteri were collected and placed in cold PBS, and the embryos were dissected free of extra-embryonic membranes in cold PBS to prevent proteolysis. Embryos were then either frozen at -80°C (for RNA extraction), fixed for several days in formalin (for sectioning) or fixed in paraformaldehyde overnight at 4°C (for whole mount in situ hybridization).

2.2 Reverse Transcription and PCR of embryonic mouse RNA

2.2.1 Isolation of total RNA

Total RNA was extracted from frozen whole embryos at different developmental stages and from various adult tissues using a modified guanidine thiocynate RNA isolation kit from Clontech (ATLAS Pure RNA Isolation Kit). In brief, 100 mg of frozen tissue was homogenized with a PT 1200C polytron (Kinematica) for 1 minute on ice in 1 ml of Denaturing solution (2.7 M guanidine thiocyanate, 1.3 M ammonium thiocynate, 0.1 M NaOAc (pH 4.0)). After a 10 minute incubation on ice, samples were vortexed and centrifuged at 15,000 x g for 5 minutes at 4°C in a J2-MC centrifuge (Beckman). The supernatants were transferred to new tubes and 2 ml of buffered phenol was added. Samples were vortexed for 1 minute and then placed on ice for 5 minutes prior to the addition of 0.6 ml of chloroform. The samples were vortexed for 2 minutes and iced for 5 minutes. The homogenates were centrifuged at 15,000 x g for 10 minutes at 4°C. The
upper aqueous phase was transferred to a fresh tube where the phenol-chloroform step was repeated with only 1.6 ml of phenol. After the centrifugation step and the transfer of the upper phase to a fresh tube, 2 ml of isopropanol were added slowly with occasional mixing. The solution was placed on ice for 10 minutes and then centrifuged at 15,000 x g for 10 minutes at 4°C. The RNA pellet was washed with 80% ethanol and allowed to air dry after the ethanol was discarded. The pellets were resuspended in 100 µl of RNase-free water and DNase treated with 10 units of DNase I (Clontech) at 37°C for one hour. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the mixture. The samples were vortexed and spun in a 5415C microcentrifuge (Eppendorf) at 10,000 rpm for 10 minutes. This step was repeated and then followed by a chloroform treatment under the same conditions. 1/10 of the volume of 2M Sodium Acetate pH 4.5 and 2.5 volumes of 96% ethanol were added to the sample. After being vortexed the samples were centrifuged for 20 minutes. The supernatant was carefully removed and the pellets were washed with 80% ethanol, spun for 10 minutes and then allowed to air dry. The pellets were resuspended in 30 µl of water and the concentration was adjusted to 1µg/µl after determining the concentration by O.D. The RNA samples were analyzed for the expression of Naip by RT-PCR using primers for different copies of Naip.

2.2.2 Reverse Transcription

The RNA was reversed transcribed and PCR amplified to ascertain the presence of the mRNA of interest. Five micrograms of total RNA isolated from adult mouse spleen and brain and from mouse embryos at different stages of development, (i.e. embryos at E10.5, E11.5, E12.5, E13.5, E14.5, E15.5 and E16.5) were reversed transcribed and the desired sequences were amplified. The templates used in PCR were generated by reverse
transcription of total RNA in the following conditions: the RNA was incubated with 0.05 
µg of a specific primer and water for a total volume of 7.5 µl at 85°C for 5 minutes and 
then cooled on ice for an additional 5 minutes. Reverse transcription was primed off with 
an exon 10 primer conserved in all Naip copies (primer A) as well as a β-actin reverse 
primer that was used as a control. All Naip specific primers were obtained from Dr. Z. 
Yaraghi. The β-actin primers were designed according to Silva et al. (1996). Table 1 lists 
the Naip primer sequences and Figure 1 shows the relative positions of all Naip primers 
used. Table 2 lists the β-actin primers used. The 20 µl reaction consisted of the 
RNA/primer mixture as well as 1st strand buffer (5X) (250 mM Tris-HCl pH 8.3, 375 
mM KCl, 15 mM MgCl₂)(Gibco), 0.01 M DTT, 2 mM dNTPs, 5 units Ribonuclease 
inhibitor (Gibco) and 400 units of SuperScript II Reverse Transcriptase (Gibco). After 60 
minutes at 37°C the reaction was terminated by heating it to 65°C for 10 minutes.

2.2.3 Polymerase Chain Reaction amplification of RT products

A first round of PCR amplification was performed in a Perkin-Elmer Cetus DNA 
Thermal Cycler 480. For the first round of thirty cycles, a Naip exon 10 reverse primer 
(primer B) and a Naip exon 1 forward primer (primer B') were used (Table 1). Both 
primers recognize all Naip copies. The β-actin forward and reverse primers are compiled 
in Table 2. The 25 µl reaction contained: 5 µl of cDNA mixture, 1 unit of Taq DNA 
polymerase (Gibco), 0.4 mM of each dNTP, 1X of 10 X PCR buffer (200 mM Tris-HCl 
(pH 8.4), 500 mM KCl, 25 mM MgCl₂ (Roche)) and 0.05 µg of each primer as indicated. 
The following program was used: 5 minutes at 94°C, then 30 cycles of denaturing for 1 
min at 94°C, annealing for 1 min at 58°C and extension for 1 min at 72°C. An additional
<table>
<thead>
<tr>
<th>Primer Name</th>
<th><em>Naip</em> Exon</th>
<th>Strand</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>reverse</td>
<td>5' AGCGATCAATAAGCAGGTCCG 3'</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>reverse</td>
<td>5' GCAGCATCCTGATGCCCAGAG 3'</td>
</tr>
<tr>
<td>B'</td>
<td>1</td>
<td>forward</td>
<td>5' CTCGTGCCCTGTCACCCTG 3'</td>
</tr>
<tr>
<td>C</td>
<td>9a</td>
<td>reverse</td>
<td>5' GAAGATAGGTCCACTGGATG 3'</td>
</tr>
<tr>
<td>C'</td>
<td>9a</td>
<td>forward</td>
<td>5' TTTCTCAAAGTGAAGCCCA 3'</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>reverse</td>
<td>5' TTGTAGTGTGCTTTGTATTGGG 3'</td>
</tr>
<tr>
<td>D'</td>
<td>2</td>
<td>forward</td>
<td>5' GGGACATCACCACGTGTACTC 3'</td>
</tr>
<tr>
<td>E'</td>
<td>1</td>
<td>forward</td>
<td>5' GAAGCAGGAGCCCTGACTGAAC 3'</td>
</tr>
</tbody>
</table>

**Table 1:** Exon location, sequence and strand location of *Naip* specific primers used in the reverse transcription and polymerase chain reaction of total RNA from mouse embryos and from adult mouse tissues.
Figure 1: Schematic representation of the first exons of *Naip*. A) Schematic representation of the first 10 exons of consensus *Naip* depicting the relative position of primers used for the reverse transcription and PCR of total mouse embryo RNA as well as the probes used for *in situ* hybridization. B) Schematic of the first eleven exons of *Naip2*. The expected size of the amplified fragments is indicated in brackets above their relative positions in the sequence.
A)

B' E' D'  

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

D' \( (487 \text{ bp}) \)  

D

E' \( (1038 \text{ bp}) \)  

D

B)

B' E' D' C'  

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 9a | 10 |

D' \( (487 \text{ bp}) \)  

D

E' \( (1038 \text{ bp}) \)  

D

C' \( (119 \text{ bp}) \)  

C
<table>
<thead>
<tr>
<th>Primer</th>
<th>Strand</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-β-actin</td>
<td>reverse</td>
<td>5’ AGCCATGTACGTagCCATCC 3’</td>
</tr>
<tr>
<td>F-β-actin</td>
<td>forward</td>
<td>5’ TGTGGGTGGTGAAGCTGTAGC 3’</td>
</tr>
<tr>
<td>R-myogenin</td>
<td>reverse</td>
<td>5’ ATAGCAACCAGTCTTTATTCA 3’</td>
</tr>
<tr>
<td>F-myogenin</td>
<td>forward</td>
<td>5’ ATAGCAACCAGTCTTTATTCA 3’</td>
</tr>
</tbody>
</table>

**Table 2:** Primers used for the synthesis of β-actin and myogenin probes. β-Actin was used as a loading control in RT-PCR while myogenin was used as a positive control for *in situ* hybridization. The expected product size of β-actin is 220 bp while myogenin will yield a 415 bp product.
extension at 72°C for 10 minutes was performed and the samples were kept at 4°C thereafter.

A round of nested PCR was then performed on the amplified DNA fragments. This time 1 µl of a 1:10 dilution of the product from the first round of PCR was incubated in the same conditions as above with primers specific to various copies of Naip as indicated in Table 3. Twenty microliters of the nested PCR reaction was migrated on a 2% agarose gel containing ethidium bromide (0.5 µg/ml) and then photographed under UV illumination. The expected product size as well as the copy of Naip that is recognized by the primers used are indicated in Table 3. The sequences were verified by DNA sequencing in house (Performed by C. Neville) on a ABI 373A automated sequencer according to Applied Biosystem's instructions.

2.3 Whole Mount in situ hybridization

2.3.1 Synthesis of DIG labeled probes

2.3.1.1 Preparation of DIG-dNTPs

A 100 X concentrated dNTP mix was prepared as follows: 20 mM dATP, 20 mM dCTP, 20 mM dGTP and 17 mM dTTP. A 10x DIG-dNTP mix was made in a reaction volume of 50 µl as follows: 5 µl of the 100X dNTP mixture was added to 0.3 mM of DIG-11-dUTP 1mM (Digoxigenin-11-2’-deoxy-uridine-5’-triphosphate, alkali-labile (Roche)). This gives a 1:7 ratio of DIG-dUTP: dTTP.

2.3.1.2 Probe synthesis by unidirectional PCR

Naip cDNAs were provided by Dr. Zari Yaraghi and probes complimentary to Naip were synthesized by using unidirectional PCR. Four hundred nanograms of DNA
<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Product size (base pairs)</th>
<th>Copy of Naip recognized</th>
</tr>
</thead>
<tbody>
<tr>
<td>B- B’</td>
<td>1672</td>
<td>All*</td>
</tr>
<tr>
<td>C- C’</td>
<td>119</td>
<td>Naip 2</td>
</tr>
<tr>
<td>D- D’</td>
<td>487</td>
<td>All*</td>
</tr>
<tr>
<td>D- E’</td>
<td>1038</td>
<td>All*</td>
</tr>
</tbody>
</table>

**Table 3:** Expected product size of PCR amplified Naip templates using various primer pairs to different regions of the Naip gene. See figure 1 for relative positions of primers. Primers B/B’ were used for the first round of PCR then the other primer pairs were used to perform nested PCR.

*By all, we mean Naip1, Naip2 and Naip3; this remains unclear for Naip4, Naip5 and Naip6*
were incubated with 5 µl of 10X DIG-dNTP, 2.5 µl of 10 x PCR buffer (Roche), 1 Unit of Taq DNA polymerase (Gibco) and 150 ng of the reverse primer (antisense probe) or 150 ng of the forward primer (sense probe). The mixture was incubated for 35 cycles in the PCR thermal cycler under the following conditions: 94°C for 1 minute, 58°C for 1 min 30 sec, 72°C for 1 min 30 sec. Following the PCR run, the reaction was purified using QIAquick PCR purification columns (QIAGEN) according to manufacturer’s instructions. Five volumes of Buffer PB were added to the PCR mix and the new mixture was placed on a QIAquick spin column to allow the DNA to bind by centrifuging the column for 30 seconds. The flow-through was discarded and the column was washed with 0.75 mL of Buffer PE (containing ethanol) and centrifuged for 30 seconds. All of the residual Buffer PE was removed by an additional 30 second spin. The columns were then placed in fresh microcentrifuge tubes and 30 µl of water was placed on the column in order to elute the sample. After allowing the columns to stand for 1 minute, they were centrifuged for 30 seconds to collect the probe. Fragment size was verified by gel electrophoresis on a 1% agarose gel containing ethidium bromide (0.5 µg/ml), and visualization was performed under UV light. While several probes were tried, only the results from the 1038 bp probe are presented. The Naip antisense and sense probes are 24% homologous at the nucleotide level.

2.3.1.3 Evaluation of the probe incorporation

DIG-dUTP incorporation into the single-stranded probe was evaluated using DIG quantification and DIG control test-strips as directed by the manufacturer (Boehringer Mannheim). Briefly, a series of dilutions of the DIG-labeled probe are applied to a DIG quantification strip. DIG control test-strips are already loaded with defined dilutions of a
control DNA and are used as standards. The test-strips are then subjected to immunological detection with Anti-Digoxigenin-AP (Boehringer Mannheim) and the colour substrates NBT/BCIP (Boehringer Mannheim). DIG-labeling efficiency can be determined by comparing the signal intensity of the spots on the test-strip with those of the control test-strip. Probes are used at 0.5 µg of labeled probe per milliliter of hybridization solution.

2.3.2 Preparation of embryos

In situ hybridization was performed using a modified Wilkinson protocol (Wilkinson, 1992). E9.5-E13.5 embryos were fixed overnight in 4% paraformaldehyde/ PBS-0.2% gluteraldehyde at 4°C (PBS: phosphate buffered saline). The following day they were dehydrated by being placed twice for 10 minutes in 30%, 50%, 75%, 85%, 95% MeOH/ PBT (methanol/ PBS with Tween) and finally in 100% methanol. Embryos were then either stored in methanol at −20°C or used for whole mount in situ hybridization.

2.3.3 Hybridization of E9.5 to E13.5 embryos

E9.5-E11.5 embryos were left intact whereas E11.5 and older embryos were punctured in the hindbrain region with a fine needle in order to allow free exchange of reagents and probe. E12.5 and E13.5 embryos were also hemi-sectioned along the median for the same reason. All solutions and plastic-ware were RNase free and all tubes were rocked gently in a hybridization incubator to allow thorough exchange of solutions. The embryos were rehydrated at room temperature in 75% MeOH/PBT, 50% MeOH/PBT and 25% MeOH/PBT for 5 minutes in each solution. Following a 10-minute wash in PBT the embryos were bleached in 6% hydrogen peroxide for 1 hour at room temperature to
inhibit some of the endogenous phosphatase activity. They were then washed in PBT and digested in 10 µg/ml of proteinase K/PBT solution. The incubation time of the embryos in this solution was dependent on the size of the embryos and had to be optimized. E9.5 embryos were treated for 10 minutes at room temperature and for each additional day of development 5 minutes of incubation were added such that E14.5 embryos were treated for 35 minutes. Embryos were then washed in PBT and fixed in 4% paraformaldehyde/PBT-0.2% gluteraldehyde for 20 minutes at room temperature. Embryos were subsequently placed in pre-hybridization buffer (50% deionized formamide, 5X SSC pH 4.5, 1X SDS, 50 µg/ml heparin) for 1 hour at 55°C. Fifty micrograms per milliliter of yeast tRNA was added to the pre-hybridization mix and the embryos were incubated an additional hour at 55°C. Finally 0.5 µg/ml of DIG-DNA-labeled probe was added to the tRNA/pre-hybridization solution and the embryos were left overnight at 55°C.

2.3.3.1 Washing and immunological detection of hybridized embryos

Embryos were washed twice for 30 minutes at 55°C in 50% deionized formamide, 5X SSC pH 4.5 and 1% SDS (Solution 1) after which time the un-annealed probe was removed by ribonuclease treatment of the embryos. The washes involved incubating the embryos three times for 5 minutes in 0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 0.1% Tween-20 (solution 2) followed by a incubation at 37°C for 30 minutes in Solution 2. The embryos were then washed twice for 30 minutes in 50% deionized formamide, 2X SSC pH 4.5 (Solution 3) at 55°C and rinsed twice in PBT for 10 minutes. The embryos were pre-blocked with 10% sheep serum, 2% BSA in PBT for 3 hours at room temperature. The sheep serum was heated to 70°C for 30 minutes prior to use in order to inactivate any
endogenous phosphatases. During this time the anti-DIG antibody was pre-absorbed (see section 2.3.3.1.1) and following pre-absorption the embryos were incubated overnight at 4°C in a antibody solution comprised of 1:2000 dilution of alkaline phosphatase conjugated anti-digoxigenin antibody (Boehringer Mannheim) and 10% sheep serum, 2% BSA in PBT.

**2.3.3.1.1 Preparation of the anti-DIG antibody**

The mouse embryo powder was prepared as follows: E12.5 to E14.5 mouse embryos were homogenized in a minimum volume of PBS using a PT 1200C polytron (Kinematica). Four volumes of ice-cold acetone were then added. The mixture was incubated on ice for 30 minutes and then spun at 10000 rpm for 10 minutes. The pellet was washed with ice cold acetone and spun again under the same conditions. The pellet was then ground into a fine powder on a piece of filter paper, allowed to air dry and the powder was stored at 4°C.

The anti-DIG antibody was then pre-absorbed using the prepared embryo powder. For each 3 mg of embryo powder added to 0.5 ml of 10% sheep serum, 2% BSA in PBT, 1µL of anti-DIG-AP Fab antibody (Boehringer Mannheim) was added. The solution was rocked gently for at least 3 hours at 4°C and then spun down for 10 minutes at 14000 rpm at 4°C. The supernatant was diluted to 2 ml using 10% sheep serum, 2% BSA in PBT giving a 1:2000 antibody dilution.

**2.3.3.2 Post antibody washes**

The embryos were washed three times for 5 minutes at room temperature in PBT before being transferred to 15 ml tubes. The PBT was changed every hour for 4 hours and the samples were then incubated overnight in PBT with gentle rocking.
2.3.3.3 Colour detection

The embryos were washed for 30 minutes in a fresh solution of NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM MgCl₂, 0.1% Tween-20 and 2 mM levamisole). They were then placed in 0.175 mg/mL BCIP and 0.150 mg/mL NBT in NTMT for 6 hours in the dark. Colour development was monitored by microscope and stopped by washing the embryos in PBT. Embryos were then photographed using a SC35 Olympus camera mounted on an Olympus SZH10 dissecting microscope.

2.3.3.4 Identification of structures

The structures were identified by correlation with those depicted in The Atlas of Mouse Development (Kaufman, 1994).

2.3.3.5 Controls

Various controls were used during the performance of this experiment. Along with the use of the sense probe, additional negative controls used include incubating embryos without probe or antibody, with probe and no antibody and without probe but with antibody. As a positive control myogenin was used as indicated in Sassoon et al. (1989). As a template, a 500 base pair EcoR1 fragment excised from the 3’ end of the myogenin gene cloned into a pcDNA3 expression vector (a gift from Suzanna Drmanic). Primers were designed to amplify a 415 base pair antisense and sense probe in the same conditions as indicated above (Table 2 and section 2.3.1.2). Multiple embryos of each developmental stage were always processed concurrently.

2.4 ³²P Hybridization
2.4.1 Paraffin embedding and sectioning of embryos

After the embryos were removed from the uterus, they were placed in formalin for several days before being processed and embedded in paraffin. Paraffin sections were cut to 6 μm thickness, mounted on silane coated slides (Sigma) and stored at 4°C. Transverse and sagittal sections were collected.

2.4.2 Riboprobe synthesis

Two riboprobes were employed for this method. Firstly, primers containing a 5’ extension corresponding to the promoter sequence of T7 RNA polymerase were synthesized. The 5’ TAATACGACTCACTATAGGGAGG 3’ promoter sequence was added to primers D, D’, and E’ (Table 1 and Table 4). The 487 base pair and 1038 base pair regions of mouse Naip cDNA indicated in Figure 1 were PCR amplified using one of the T7 linked primers and the corresponding “nude” primers to amplify the DNA. These templates with T7 overhangs were used in the synthesis of the riboprobes. Zero point two micrograms (0.2μg) of DNA were incubated with 0.4 mM dNTPs, 5 μl of 10X PCR buffer (Roche), 1.25 units of Taq DNA polymerase (Gibco) and 0.05 μg of each primer. Samples were amplified, after a ten-minute incubation at 94°C, in the following conditions: thirty cycles of 94°C for one minute, 58°C for one minute and 72°C for one minute. For synthesis of the antisense template, the T7 linked reverse primer and the corresponding “nude” primer were used, whereas for the synthesis of the sense template, the T7 linked forward primer and the corresponding “nude” reverse primer were used. Refer to Table 4 for the identification of the primer pairs used. After the PCR
<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Product size (base pairs)</th>
<th>Probe</th>
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<tr>
<td>T7D- E'</td>
<td>1038</td>
<td>antisense</td>
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<tr>
<td>T7E'-D</td>
<td>1038</td>
<td>sense</td>
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<tr>
<td>T7D- D'</td>
<td>487</td>
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<tr>
<td>T7D'- D</td>
<td>487</td>
<td>sense</td>
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**Table 4:** Primer pairs used for the synthesis of templates for riboprobes used for $^{33}$P- *in situ* hybridization. Primers D, D' and E' indicated in Table 1 were linked on the 5' end with the T7 RNA polymerase promoter sequence: (5' TAATACGACTCACTATAGGG GAGG 3').
amplification, the reaction was purified using QIAquick PCR purification columns (QIAGEN) as indicated in section 2.3.1.2. Again the fragment size was verified by electrophoreses on a 1 % agarose gel and visualized, with the help of ethidium bromide, under UV light.

The PCR fragments with T7 overhangs were then used as templates for the synthesis of $^{33}$P- radio-labeled sense and antisense RNA probes. All riboprobes were synthesized by in vitro transcription with a NTP mix containing $^{33}$[P] UTP using a MAXIscript In Vitro Transcription Kit (Ambion) following the manufacturers instructions. In brief, the transcription reaction was assembled in the following order: nuclease-free water to make the total volume 20 µl, 10X transcription buffer (containing DTT), 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 3.125 µM $^{33}$P labeled UTP, 1 µg of the DNA template and 20 units T7 RNA polymerase. The reaction was incubated for 1 hour at 37°C. The DNA template was then removed by adding 4 units of RNase-free DNase I and incubating again at 37°C for 15 minutes. Unincorporated nucleotides were removed by size exclusion chromatography on RNase-free Sephadex G-50 spin columns (Sigma). The total amount of radioactive nucleotide incorporated in the RNA probes was quantified using a 1450 Microbeto PLUS liquid scintillation counter (WALLAC).

2.4.2.1 Control riboprobes

myogenin was used as a positive control. As the fragment was cloned in both the sense and antisense orientation, in the multiple cloning site of pCDNA3, which has the T7 promoter at the 5' end, it was not necessary to use the strategy as outlined in section 2.3.2.5. Rather the proper fragments, linearized with EcoRI were incubated directly in
the conditions indicated in section 2.3.2.5. The *myogenin* sense and antisense radio-
labeled riboprobes were synthesized as described in section 2.4.2.

2.4.3 In Situ hybridization

The hybridization was carried out using a modified Wilkinson protocol
(Wilkinson et al., 1987). Paraffin was removed from the slide-mounted sections
(prepared in section 2.4.1) with xylene, the slides were then re-hydrated in decreasing
amounts of ethanol, immersed in 0.9% NaCl for 5 minutes followed by immersion in 1X
PBS for 5 minutes. They were then fixed with 4% paraformaldehyde in PBS for 20
minutes and washed twice for 5 minutes in 1X PBS. Samples were digested for 5 minutes
in 20 μg/ml Proteinase K (Gibco-BRL) in 50 mM Tris-HCl pH 7.2, and 5 mM EDTA pH
7.2. After washing again for 5 minutes in 1X PBS, sections were re-immersed in 4%
paraformaldehyde for 20 minutes. Subsequently, slides were dipped in water and
acetylated with 0.1 M triethanolamine containing 0.25% acetic anhydride in 0.9% NaCl
for 10 minutes. Finally the sections were rinsed in PBS, dehydrated in increasing
concentrations of ethanol, air dried and hybridized at 58°C overnight in a humidified
chamber, with 40 x 10^6 cpm of each probe in 1 ml of hybridization buffer (50%
fomalide, 0.3M NaCl, 20 mM Tris-HCl pH 7.4, 5 mM EDTA pH 7.4, 10 mM NaH₂PO₄
pH 8.0, 1X Denhardt’s, 10% Dextran sulfate).

After hybridization, coverslips were removed by incubating the sections in pre-
warmed 5X SSC, 10 mM DTT at 50°C for 30 minutes. Sections were then washed at
high stringency at 58°C for 30 minutes in 50% formamide, 2X SSC, 100 mM DTT. A
rinse in NTE (0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0) was followed
by a 30 minute incubation at 37°C in 20μg/ml of RNase A (Gibco) in NTE. The sections were then washed for 15 minutes at room temperature in 2X SSC and then in 0.1X SSC. After the washes the sections were dehydrated in a series of ethanol washes containing 0.3M ammonium acetate, air-dried and exposed to X-OMAT AR Kodak film for 4 days. In order to obtain cellular resolution, the sections were coated at 42°C with Kodak NTB-2 liquid autoradiographic emulsion (Inter Science) and kept in light-tight boxes with desiccant at 4°C for 25 days. Photographic development was carried out in Kodak D-19 developer for 5 minutes at 16°C. The slides were then fixed in Kodak fixer and counterstained with a 1:100 solution of toluidine blue. The pattern observed for both the 1038 base pair and the 480 base pair anti-sense probes was identical, therefore only the results for the 1038 base pair probe are presented. Analysis was performed in light and dark field using a Zeiss microscope. As only intense signals can be visualized under bright-field conditions, a more sensitive means of visualizing the silver grains is to use dark field. Images were obtained by direct capture with a Sony PowerHAND video camera mounted on the Zeiss microscope and using Northern Eclipse software.

2.5 In situ hybridization of slides using a DIG riboprobe

2.5.1 Preparation of DIG labeled riboprobe

The PCR fragments with T7 overhangs synthesized in step 2.4.2 were used as the templates with the DIG RNA labeling mix 10X (Roche) to generate DIG labeled riboprobes. The DIG RNA labeling mix contains 10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP. Briefly, 200 ng of the PCR template was incubated with 2 μl of the 10X DIG RNA labeling mix, 2 μl of transcription buffer 10X
(400 mM Tris-HCl, pH 8.0 (20°C), 60 mM MgCl₂, 100 mM dithiotreitol, 20mM spermidine), 2 µl T7 RNA polymerase and water to a final volume of 20 µl. The reaction was incubated at 37°C for 2 hours after which 2 µl of RNase-free DNase I was added and incubated for another 15 minutes. The addition of 2.5 µL of 4M LiCl and 75 µl of ethanol and incubation of the reaction at −70°C for 30 minutes precipitated the RNA transcripts. Following centrifugation at 4°C at 12000 g for 15 minutes, the pellets were washed with cold 70% ethanol, centrifuged again, air dried and resuspended in RNase free water to a final concentration of 0.1 µg/µl. DIG incorporation was assessed as indicated in step 2.3.1.3.

2.5.2 In Situ hybridization

The slides used are prepared identically as indicated in step 2.4.1. The slides were prepared for in situ hybridization as indicated in section 2.4.3. The slides were prepared for hybridization as indicated in section 2.4.3 however, after rehydrating the slides in decreasing amounts of ethanol, the sections were first immersed in PBS (not NaCl) followed by a incubation in 6% H₂O₂ for 30 minutes. Also, the sections were not acetylated or dehydrated prior to hybridization. The hybridization buffer was the same as the one utilized for the whole mount in situ hybridization in section 2.3.3. The final concentration of the riboprobe was 0.5 µg/ml and the slides were hybridized overnight at 55°C in a humid chamber.

2.5.2.1 Washing and immunological detection of hybridized slides

The slides were washed, as were the embryos in step 2.3.3.1 except that the RNase step was omitted. The slides were treated with blocking buffer as indicated in step
2.3.3.1 and antibody binding conditions were identical to those used for the whole mounts.

**2.5.2.2 Post antibody washes and colour detection**

The following day, the slides were first washed three times for 5 minutes in PBT then they were washed three times for 30 minutes in the same solution. The slides were then incubated in NTMT as in indicated in step 2.3.3.3. The colour development was allowed to progress for 6 to 7 hours at 37°C. The reaction was stopped by placing the slides in PBT and then rinsing them in water.
3. RESULTS

3.1) Reverse Transcription and PCR of embryonic mouse RNA

NAIP expression was demonstrated to be very low in all adult tissues examined, with the highest expression being observed in the placenta and liver (Roy et al., 1995). Murine Naip also exhibits low levels of expression although Naip is detectable by Northern analysis in the liver, lung, heart and spleen of adult mice (Yaraghi et al., 1998). Given the role postulated for NAIP in a disease with antenatal onset, we were interested in assessing the distribution of Naip at different stages of embryonic development. Reverse transcription of total mouse embryonic RNA followed by PCR amplification was undertaken to verify the presence of Naip in embryonic tissue. Figure 2 shows the products of RT-PCR of mouse embryo RNA at different ages of development. The primers selected recognize Naip 1, Naip 2 and Naip 3 or Naip 2 only (see tables 1 and 3). Figure 2A shows the presence of Naip 2 in all samples examined including the adult mouse brain (lane 2). Sixty cycles of PCR amplification were required to detect the expected 119 base pair, exon 9a specific product (exon 9a is exclusively found in Naip 2). Figure 2B illustrates the ubiquitous presence of the expected 487 base pair product in all samples analyzed using primers D and D' which amplify all copies of Naip (by all, we mean Naip 1, Naip 2 and Naip 3). Primers D and E' also amplify a 1038 base pair fragment from all Naip copies (Figure 2C). Naip 1 specific primers failed to work (data not shown). β-actin was used as a loading control to verify that relatively equal amounts of cDNA was amplified (Figure 2D). We were unable to distinguish between the different Naip copies at the various developmental stages examined. Naip is present
Figure 2: RT-PCR of total mouse embryo RNA of different developmental stages and adult mouse spleen and brain RNA. A) Using Naip2 specific primers (primers C and C’) to perform nested PCR, cDNAs from adult mouse brain (lane 1), adult mouse spleen (lane 2), E11.5 total embryo (lane 3), E12.5 (lane 4), E13.5 (lane 5), E14.5 (lane 6), E15.5 (lane 7) and E16.5 (lane 8) were amplified after a total of 60 cycles of PCR. Lane L designates the 1 kb ladder used for the molecular weight marker. Aliquots were electrophoresed on a 2% gel to resolve the expected 119 bp fragment. B) and C) The same cDNA samples were amplified, in the same conditions, by using primer pairs D- D’ and D- E’ respectively. The expected fragment sizes of 487 bp and 1038 bp are indicated. D) β- Actin primers were used to amplify the same cDNAs as above. The 220 bp fragment is detectable after only 30 cycles of PCR.
throughout development though its transcript levels are very low, as compared to β-actin transcript levels.

3.2) Whole mount in situ hybridization of mouse embryos with a Naip specific probe

Naip expression patterns were assessed during mouse embryonic development using whole mount in situ hybridization. This technique allows visualization of transcripts without the necessity of sectioning the tissues. This is feasible only in embryos younger than 13.5 dpc as the higher level of organogenesis of the older embryos makes probe penetration into individual organs impossible. Nonetheless, this approach also has the advantage of permitting the simultaneous processing of several embryos which allows for a time course study to be undertaken as well as having numerous samples of a given age.

The hybridization of E9.5 to E11.5 embryos with a Naip antisense probe revealed staining primarily in the CNS. In E9.5 samples, Naip transcripts were detected in the fourth ventricle as well as in the rostral neuroepithelium of the neurotube (Figure 3A). This pattern was the same in E10 embryos with the expression in the neurotube progressing more caudally (Figure 3A) and into various brain structures. The staining in different structures of the brain becomes more prominent in E11 and E11.5 embryos (Figure 3A and 3C). The sense labeled Naip probe, used to assess background staining, was limited to the otic vesicle of E11.5 embryos (Figure 3B). E11.5 embryos revealed Naip distribution in the periphery of the telencephalic vesicle, in the mesencephalic
Figure 3: Whole mount in situ hybridization of E9.5 to E11.5 mouse embryos with the 1038 base pair Naip probe. A) Naip expression was detected in the fourth ventricle (4thv) and neuroepithelium (ne) of the neural tube of E9.5, E10, E11 and E11.5 embryos (x15). Naip is also distributed in the CNS of E11 and E11.5 mouse embryos. B) The staining of the antisense probe was compared to that of the sense probe to estimate the levels of background. The otic vesicle (ov) in E11.5 embryos is labeled with the sense probe. C) E11.5 embryos showed presence of Naip at the margin of the mandibular component of the first branchial arch (mba) as well as that of the second branchial arch (2ba) when compared to the sense labeled embryo (C; left-sense, right-antisense). Naip was also found at the periphery of the telencephalic vesicle (tel), the mesencephalic vesicle (mes) and fourth ventricle and along the neuroepithelium of the spinal cord (ne)(x15). D) myogenin was used as a positive control and displayed staining in the somites (S) of E10.5 and E11.5 mouse embryos (x15).
vesicle as well as in the fourth ventricle and dorsal root ganglion of the spinal cord (Figure 3C). Additional staining on the periphery of the mandibular component of the first branchial arch as well as that of the second branchial arch (Figure 3C) was observed. Comparison of the sense labeled embryos (left) with the antisense-labeled embryo (right) reveals otic vesicle staining in both precluding a determination of whether Naip is expressed therein (Figure3C). myogenin was used as another control and expression in the somites was seen as identified by Sassoon et al., (1989) (Figure 3D). The use of myogenin as an antisense control permits a comparison with the staining pattern obtained for Naip and confirms a very different expression pattern for E11.5 embryos (Figure 3C and 3D). No staining was detected in the embryos incubated in the absence of probe or antibody or both (not shown). It is important to note that the level of background obtained varied from embryo to embryo regardless of the probe used even under identical situations (Figure 3C and 3D).

Older embryos (E12.5- E13.5) were hemi-sectioned along the midline to allow a better diffusion of the probe. Hybridization of E12.5 embryos brought to light Naip expression in the spinal cord, CNS, in the nasal epithelium and future digits of the forelimbs and hindlimbs (Figure 4A). A view of the innermost side of the section allows the visualization of specific brain CNS structures expressing Naip. Among these is the wall of the midbrain, the medulla oblongata as well as the wall of the neopallial cortex (Figure 4B). An examination of the dorsal region of E12.5 mouse embryos labeled with the Naip antisense (Figure 4C) and sense probes (Figure 4D) showed Naip transcripts to be present along the spinal cord and in the hindbrain although the exact structures that were labeled are difficult to identify in this manner. In the developing limbs, Naip was
Figure 4: Whole-mount in situ hybridization of E12.5 mouse embryos with a 1038 base pair Naip probe. E12.5 were hemi-sectioned along their midline prior to hybridization with the Naip probe. A) and B) E12.5 embryos showed Naip in the nasal epithelium (n), the future digits of the forelimb (fl) and hindlimb (hl), the spinal cord (sc), the wall of the midbrain (wmb), the medulla oblongata (mo) and the wall of the neopallial cortex (nc) (x10). C and D show a dorsal view of a whole E12.5 embryo labeled with Naip antisense and sense probes respectively (x10). E and F are close ups of the hindlimb and forelimb of E12.5 embryos showing the presence of Naip in the future digits (x40). G) The Naip-DIG-sense labeled embryos showed no specific staining nor did the myogenin DIG-sense labeled embryos (G and H (left))(x10). The myogenin antisense-labeled probe displayed staining in the somites (s), in the intercostals (int) and in other muscle anlagens (m) as previously described by Sassoon et al. (1989).
identified in the future digits of the hindlimbs (Figure 4E) as well as at the dorsal surface of the forelimb (Figure 4F) and in the nasal epithelium. As with whole-mounts, antisense-labeled embryos were always compared to the staining pattern obtained with that of the *Naip* sense labeled embryos (Figure 4G) as well as that of the antisense and sense labeled *myogenin* labeled embryos (Figure 4H).

In the E13.5 mouse embryos *Naip* is still present in the nasal epithelium, the spinal cord and the wall of the midbrain (Figure 5A). Additionally *Naip* was found to be present in the myelencephalon, diencephalon, pons, tongue and the wall of the midbrain (Figure 5B) when compared to the *Naip* sense labeled embryos. The dorsal view of that same section shows strong presence of *Naip* along the entire spinal cord (Figure 5C). Closer examination of the spinal cord of E13.5 mouse embryos labeled with *Naip* antisense probe presented staining in the mantle layer of the spinal cord of the lumbo-sacral region as well as in the dorsal root ganglia (Figure 5D).

3.3) *in situ* hybridization of mouse embryos sections with a $^{33}$P- labeled *Naip* specific riboprobe

Radio-labeled riboprobe were used to study *Naip* gene expression in mouse embryo sections as sectioning of the whole mounts as well as use of DIG- labeled probes directly on sections failed to produce a visible signal. This method was used to confirm the results of the whole mount *in situ* hybridization experiment as well as to identify novel *Naip* containing tissues.
Figure 5: Whole-mount in situ hybridization E13.5 mouse embryo with a 1038 base pair Naip probe. E13.5 embryos were hemi-sectioned along their midline prior to hybridization with the Naip probe. A1) and A2) Naip was present in the nasal epithelium (ne), the wall of the midbrain (wmb) and the mantle layer of the spinal cord (sc) when the DIG-Naip antisense E13.5 mouse embryos were examined (x10). B1) An inner view revealed additional Naip expression in the myelencephalon (my), the diencephalon (d), the pons (p) and in the tongue when compared to the Naip sense labeled embryos (B2)(x10). C) A dorsal view of E13.5 Naip antisense-labeled mouse embryo reveals staining along the entirety of the spinal cord (x10). D) A close up of the spinal cord area revealed staining in the dorsal root ganglia (drg) (x30), inset (x15).
For E9.5 mouse embryos, *Naip* expression was present in all tissues studied. *Naip* was identified in the neuroepithelial lining of the forebrain (telencephalic vesicle), midbrain (mesencephalic vesicle) and hindbrain (fourth ventricle) (Figure 6A). The presence of *Naip* was determined in comparison to the corresponding *Naip* sense labeled sections where labeling was absent (Figure 6B). Figure 6C and figure 6D show the dark field and light field micrographs of the *Naip* antisense E9.5 labeled neuroepithelium of the neural tube and the mandibular component of the first branchial arch. The neuroepithelium of the neural tube is labeled all the way to the lower extremity as seen in figure 6E and comparison to the *Naip* sense labeled section in figure 6F.

From E9.5 onwards, the *Naip* gene is expressed within certain organs of the developing embryo. E10.5 mouse embryo sections probed with a *Naip* antisense probe showed Naip expression to be similar to that seen in E9.5 mouse sections. Again the neuroepithelium surrounding the telencephalic vesicle, mesencephalic vesicle and fourth ventricle expressed *Naip* (Figure 7A). In addition, *Naip* transcripts were found in cells surrounding the optic stalk. Naip gene expression was observed again in the mandibular component of the branchial arch but was absent in the heart (Figure 7B). Figure 7C and figure 7D show *Naip* expression in the neuroepithelium of the neurotube and in the tail region respectively. None of the *Naip* sense labeled sections showed significant expression (Figure 7, panels on the right).

E11.5 mouse embryo sections labeled with a *Naip* \(^{33}P\) antisense probe also exhibited *Naip* presence at the periphery of the telencephalic vesicle, optic stalk, in the nasal process, in cells surrounding the mesencephalic vesicle, the fourth ventricle and otic vesicle (Figure 8A, B and C). Figure 8D shows *myogenin* expression the somites of
Figure 6: $^{33}$P in situ hybridization of E9.5 sagittal mouse embryo sections with a 1038 base pair Naip probe. A) Naip expression was detected in the tissues of the telencephalic vesicle (tel), mesencephalic vesicle (mes) and fourth ventricle (4thv)(x10). B) Labeling with the corresponding sense probe did not reveal significant background. C) Naip was present in the tissues surrounding the neural lumen (nl) as well as in the maxillary component of the first branchial arch (mba). D and insets of A, B, E and F) Light field micrographs of the counter-stained sections. E) Naip was identified in the neuroepithelium of the neural tube (nte) as seen when compared to the sense labeled section in F.
Figure 7: $^{33}$P in situ hybridization of E10.5 sagittal mouse embryo sections with a 1038 base pair Naip probe. A) Naip expression was high in the neuroepithelial wall of the midbrain (ne) and in the tissues surrounding the telencephalic vesicles (tel), mesencephalic vesicle (mes), the 4th ventricle (4thv), the mandibular component of the first branchial arch (mba) and the optic stalk (os). Panels on the right illustrate sense labeled sections as well as their respective light field micrograph. B) Naip expression was absent in the heart (h) (x10). C and D) Naip was identified in the neuroepithelium of the neural tube (nte) in the tail region (x10). Limb bud (lb). Silver grains present in sense labeled sections are due to background.
Figure 8: *in situ* hybridization of E11.5 sagittal mouse embryo sections with a 1038 base pair *Naip* probe. All panels are laid out such that the antisense labeled section is on the left and the sense labeled sections are on the right. A) *Naip* was detected in the nasal process (np) and at the periphery of the telencephalic vesicle (tel) as well as the optic stalk (os)(x10). B) *Naip* was also present in the mesencephalic vesicle (mes) (x10). C) Strong signal was observed in the tissue surrounding the 4th ventricle (4thV) as well as the otic vesicle (os)(x10). D) E11.5 mouse embryos labeled with \(^{33}\text{P} \) myogenin antisense probes demonstrated its presence in the somites as described by Sassoon *et al.* (1989). E) A sagittal section demonstrating *Naip* presence in the fourth ventricle as well as in the roof of the hindbrain (rhb) (x10). F) *Naip* was also detected in the tissue surrounding the fourth ventricle when using a DIG labeled antisense probe (x20). G) *Naip* was present in the mandibular component of the first branchial arch (mba), in the hepatic primordium (hp) and absent in the heart (h) (x10). H) Again the DIG labeled *Naip* probe displayed the same pattern of expression as that seen with the \(^{33}\text{P} \) labeled probes (x10).
E11.5 embryos. Figure 8E is a sagittal section through the fourth ventricle showing the presence of Naip transcripts at the periphery of the ventricle as well as in the roof of the hindbrain. This pattern was confirmed when a DIG-Naip antisense probe was used (Figure 8F). Naip transcripts were still detectable in the mandibular component of the first branchial arch and in the liver primordia but not in the heart (Figure 8G and H). Parasagittal sections of E11.5 mouse embryo labeled with a DIG-Naip antisense riboprobe revealed Naip expression in the neuroepithelium of the neurotube (Figure 9A), as did the $^{33}$P Naip antisense probe (Figure 9B). Sagittal sections demonstrated the presence of Naip transcripts in the posterior dorsal root ganglia (Figure 9C). Naip sense labeled sections showed little labeling (Figure 9D). Analysis of transverse sections, through the upper portion of the central canal at the level of caudal hindbrain, revealed Naip transcripts to be diffusely present throughout the neural tube (Figure 9E).

Figure 10 A and B demonstrate the presence of Naip transcripts in the lateral ventricle of E12.5 mouse embryo sections as seen in a parasagittal and sagittal section respectively. At this stage of development, Naip RNA was still present in the nasal epithelium (Figure 10C) and in the wall of the midbrain (Figure 10D). Naip was also still identified around the third ventricle and fourth ventricle in both sagittal (Figure 10 E and F) and parasagittal sections (Figure 10G). Naip expression continued in the liver (Figure 11A) and in the posterior dorsal root ganglion (Figure 11C). The heart still did not display the presence of Naip transcripts (Figure 11A) however the mantle layer of the spinal cord (Figure 11B) and the future digits of the hindlimb (Figure 11D) did.

Analysis of six-micrometer thick sections of E13.5 mouse embryos showed very faint labeling with the $^{33}$P Naip antisense probe (Figure 12 A and B, center panels). In an
Figure 9: $^{33}P$ in situ hybridization of E11.5 mouse embryo sections with a 1038 base pair Naip probe with focus on the staining in the neuroepithelium. A), B) and C) Naip was detected in the neuroepithelium of the neural tube (ne) as well as in the posterior dorsal root ganglions (drg). A) Results from DIG-Naip antisense probe as compared to the sense labeled section on the right (x10). B), C) The panels on the left are the dark field of the Naip antisense labeled sections and the panels on the right are the respective light field exposures. D) Dark field micrograph of Naip sense labeled sections did not show specific staining of the neuroepithelium (ne) (x10). E) Transverse sections through the central canal (cc) revealed Naip to be localized in the neural tube (nt) when compared to the sense labeled sections (right panel). Inset of right panel is the light field exposure of the sense labeled section.
Figure 10: $[^{33}P] \text{in situ hybridization of E12.5 mouse embryo head sections with a}$ 1038 base pair Naip probe. Left panels are antisense-labeled sections and right panels are sense labeled sections. A) and B) are parasagittal and sagittal sections respectively of the lateral (lat)ventricle. Naip expression was found at the periphery of the lateral ventricle as well as in the roof of the neopallial cortex (nc)(x10). C) Naip transcripts were also detected in the nasal epithelium of sagittal sections (n). D) The walls of the midbrain (wmb) as well as the caudal mesencephalic vesicle (mes) were found to be Naip positive (x10). E) and F) are sagittal sections showing the presence of Naip in the third ventricle (3v) as well as in the fourth ventricle (4thv)(x10). G) The labeling of the fourth ventricle continues through to parasagittal sections (x10). H) Myogenin antisense labeled sections demonstrated its presence in the somites (x10).
Figure 11: $^{33}$P in situ hybridization of E12.5 mouse embryo sagittal sections with a 1038 base pair Naip probe. Left panels are antisense-labeled sections and right panels are the sense labeled counterparts. A) Naip was distributed in the liver (l) and not the heart (h) of E12.5 mouse embryo sections (x10). B) and C) The mantle layer of the spinal cord (msc) as well as the dorsal root ganglia (drg) were positive for the presence of Naip (x10). D) The future digits of the hindlimb also showed the presence of Naip.
Figure 12: *in situ* hybridization of E13.5 mouse embryo sagittal sections with a 1038 base pair *Naip* probe. Panels on the left are dark field micrographs of $^{33}$P *Naip* antisense-labeled 16 μm sections with their respective light field exposure inset. Panels on the far right are dark field exposures of 6 μm thick sections labeled with a $^{33}$P *Naip* sense probe. Panels in the center are either 6 μm thick sections labeled with a $^{33}$P *Naip* antisense probe (A and B) or 6 μm thick sections labeled with a DIG-*Naip* antisense probe (C, D, and E). All insets are the respective light field exposures of the sections. A) *Naip* was found in the surrounding tissue of the fourth ventricle (4thv) as well as in the nasal epithelium (n) (B), in the wall of the midbrain (wmb), in the neighboring of the mesencephalic vesicle (mes) (C) and in the striatum (st) (D) (x10). E) *Naip* was present in the mantle layer of the spinal cord (msc)(x10).
effort to confirm the results seen in the whole mount in situ hybridization experiments, thicker sections were hybridized with the Naip probes under the same conditions as their six-micron counterparts. In E13.5 mouse embryo sections, Naip transcripts were detected in the tissues surrounding the fourth ventricle (Figure 12A), in the nasal epithelium (Figure 12B), in the wall of the midbrain (Figure 12C), in the striatum and the cells neighboring the lateral ventricle (Figure 12D). DIG-Naip antisense labeled probes mirrored the results obtained with the $^{33}$P labeled probes (Figure 12 C, D and E, center panels). Naip RNA was also identified in the mantle layer of the spinal cord (Figure 12 E) as seen in the whole mount hybridization experiments (Figure 5B). Naip antisense labeling of transverse sections through the medulla oblongata, in the rostral spinal cord, established the presence of Naip transcripts in the mantle layer (Figure 13A). This pattern was also observed in more rostral transverse sections where Naip RNA was again identified in the mantle layer of the spinal cord but also in the dorsal root ganglia and the notochord (Figure 13B). Figure 13C reveals the presence of Naip transcripts in cells surrounding the residual lumen of the anterior lobe of the pituitary (previously the Rathke’s pouch) while Figure 13D demonstrated Naip RNA in olfactory epithelium of the nasal cavity of antisense labeled transverse sections through the head. Sagittal sections (Figure 13E) and transverse sections (Figure 13F) through the liver of E13.5 mouse embryo labeled with antisense Naip riboprobe show the presence of the genes transcripts in this tissue.

Naip transcripts were still present in the roof of the midbrain, the cells surrounding the mesencephalic vesicle (Figure 14A), the roof of the neopallial cortex (Figure 14B) in antisense probed sagittal section of E14.5 mouse embryos. The lip
Figure 13: \(^{33}\text{P} \text{ in situ} \) hybridization of transverse and sagittal sections of E13.5 mouse embryos hybridized with a 1038 base pair Naip probe. Panels on the left are the dark field exposures of the Naip antisense labeled probes while those on the right are the sense labeled sections. Insets are the respective light field exposure when available. A) Transverse section through upper head region of E13.5 mouse embryo showed Naip to be present in the mantle layer of the spinal cord (sc) (x10). B) Transverse sections through the medulla oblongata revealed Naip transcripts in the mantle layer of the spinal cord (msc), the dorsal root ganglia (drg) and the notochord (ntc) (x10). C) And D) In transverse sections through the head, Naip was found in a vestigial structure of the Rathke’s pouch (R) as well as in the developing nasal epithelium (x10). E) And F) Sagittal and transverse sections through the liver (li) expressed Naip (x10). Optic chiasma (oc), olfactory epithelium (oe), abdominal wall (aw).
Figure 14: *in situ* hybridization of transverse and sagittal sections of E14.5 mouse embryos hybridized with a 1038 base pair *Naip* probe. Panels on the left are the dark field exposures of the *Naip* antisense labeled probes while those on the right are the sense labeled sections. Insets are the respective light field exposure when available. Sagittal sections through the head region of E14.5 mouse embryo showed *Naip* to be present in the roof of the midbrain (rmb)(A), the neopallial cortex (nc) of the lateral ventricle (B) and in the developing lip area (C)(x10). Sagittal sections through the body probed with a $^{33}$P *Naip* antisense probe, revealed transcripts in the mantle layer of the spinal cord (msc)(D) while no signal was detected in the heart (h). (E) (x10). Transverse sections through the head of the E14.5 mouse embryos labeled with $^{33}$P *Naip* antisense probe, again demonstrated *Naip* to be present in the mantle layer of the spinal cord (F) as well as in the neural layer of the retina (nr)(G) (x10). H) *Naip* was detected in the developing intestine with an antisense DIG-labeled probe (x10).
(Figure 14C) as well as the mantle layer of the spinal cord (Figure 14D) was also shown to express Naip. The heart of E14.5 mouse embryos did not display the presence of Naip RNA (Figure 14E). Transverse sections at the level of the eye of E14.5 mouse embryos labeled with Naip antisense riboprobe revealed the presence of the Naip message in the spinal cord (Figure 14F), the neural layer of the retina (Figure 14G). DIG-Naip antisense labeled probes revealed the presence of transcripts in the developing intestinal tissue of E14.5 embryos (Figure 14H).

E16.5 mouse embryos labeled with antisense Naip riboprobe had a very different pattern of gene expression. The neopallial cortex composed of the cortical plate and the marginal zone of the lateral ventricle expressed Naip RNA (Figure 15A). Sagittal sections of E16.5 embryos labeled with $^{33}$P antisense Naip probe (Figure 15-1) or with a DIG-Naip antisense probe (Figure 15-3) revealed its presence in the lip and primordia of follicles of vibrissae associated with the lip. The epithelial cells of the intestinal villi expressed Naip as seen with a $^{32}$P antisense Naip probe (Figure 15C-1) and with a DIG-Naip antisense probe (Figure 15C-4). The neural layer of the retina as well as the developing lenses were strongly labeled by Naip antisense probe (Figure 15D). In the spinal cord of E16.5 mouse embryos no labeling was seen with the Naip antisense riboprobe (Figure 15E).
Figure 15: in situ hybridization of E16.5 sagittal mouse embryo sections with a Naip probe. A) 1-$^{33}$P silver grain dark field antisense expression of Naip in the marginal zone (mz) of the lateral ventricle of the brain (x10). Next panels are respectively, the light field, toluidine blue counter-stained $^{33}$P antisense-labeled section (2), the $^{33}$P dark field sense labeled micrograph (3) and its light field counterpart (4) (x10). B) 1- in the upper lip area, $^{33}$P antisense Naip silver grain dark field micrographs showed the transcripts to be present in the primordia follicles of the vibrissae (v) when compared to the sense labeled dark field micrographs (2). Inset to Figure B-1 is the light field exposure of the $^{33}$P antisense labeled section. B-3 results from the labeling with a DIG-antisense labeled probe also showing vibrissae staining (x10). C) 1- Dark field micrograph of the $^{33}$P antisense Naip labeled section-showing expression in the intestinal epithelium (i). 2- light field micrograph of the $^{33}$P antisense Naip labeled section showed in C1. 3- Dark field micrograph of the $^{33}$P sense Naip labeled section-demonstrating absence of signal. 4- section from the labeling with a DIG-antisense labeled probe also showing intestinal epithelium staining. Inset, DIG-sense labeled section (x10). D) Dark field micrograph of $^{33}$P antisense Naip labeled eye section showing distribution in the neural layer of the retina (nr) as well as in the lens (ls). The Naip sense probe did not show any staining. Insets are the respective light field micrographs (x10). E) Dark field micrograph of $^{33}$P Naip antisense-labeled spinal cord did not detect the presence of Naip when compared to the sense labeled section (left panel)(x10).
### Table 5: Summary of the distribution of Naip transcripts during murine embryogenesis.

Presence of signal is indicated as (+) and absence of signal is indicated as (-).
4. DISCUSSION

Naip is expressed at low levels throughout the developing mouse

In keeping with previous reports, the data presented here shows that even in the embryo, the overall expression of Naip is low, as 60 cycles of PCR were needed to visualize any amplification product. The presence of Naip2 during murine embryogenesis was confirmed by the use of primers specific for this locus. This is the only Naip copy whose expression was verified in this study as all other primer pairs used amplified Naip1, Naip2 and Naip3 and possibly the remaining Naip loci. Naip1 specific primers were used to try and determine if the neuronal copy of Naip is expressed during murine embryogenesis however, this experiment failed. This may have occurred because of very low levels of expression of Naip1 at the stages examined. The low abundance of Naip1 is probably due to the fact that the copy of this gene is not critical during development as proven by Naip1 (-/-) knock out mice, which are viable with no developmental CNS abnormalities (Dr. M. Holcik, personal communications). However, there is an increase in the cell death of the CA3 neurons of the hippocampus of the Naip1 (-/-) mice after administration of kainic acid (Dr. M. Holcik, personal communications). No significant difference was seen in the RT-PCR products from various developmental periods suggesting that the level of Naip expression is low in all stages examined.

Detection of Naip transcripts using whole-mount in situ hybridization

In this study, we present data on the expression of Naip during murine embryogenesis. The whole mount in situ hybridization analysis revealed an expression pattern for Naip during development. Unfortunately this method did not allow us to
delineate which copies of *Naip* were being expressed during the embryo’s development as attempts to hybridize with *Naip1* or *Naip2* specific probes failed. This could have been a result of the absence of the *Naip1* copy but this is not true of the *Naip2* copy as its presence was documented by RT-PCR (figure 2C). The absence of signal was more likely due to an inability for the probe to properly bind to its target or to a very low expression, which could not be visualized by the DIG whole mount *in situ* hybridization method.

In view of the low levels detected for *Naip* by RT-PCR, a number of parameters were tested to optimize the DIG whole mount *in situ* hybridization method. Among these were the incubation time in proteinase K, the nature of the blocking agent used prior to hybridization with the DIG antibody and the ratio of the substrates. These parameters were analyzed in order to obtain staining with the least amount of background while preserving the integrity of the embryos structures. For example the ratio of NBT/BCIP was varied. Most published reports employ a 1:1.9 ratio of BCIP/ NBT. In accordance with Arcellana-Panlilio and Schultz (1994) we found that omission of NBT greatly reduced the level of background although increasing the time required for the colorimetric reaction to take place. To circumvent this problem we used a 1:0.9 ratio of BCIP/NBT allowing a better control over the rate at which the colour developed. We also found that background levels varied from sample to sample as well as with the probes utilized.

Once the DIG-*Naip* labeled whole mounts were obtained they were sectioned. Unfortunately these attempts failed, as the signal was always lost. Moreover, sectioning of the embryos prior to hybridization with a DIG-*Naip* labeled probe revealed the presence of *Naip* only in a subset of those structures that were shown to be *Naip* positive.
by whole mount *in situ* hybridization. Discrepancies between the analysis of the sectioned slides and the whole mounts may have been the result of the physical limitations of the techniques themselves. For example, signals may have been detected in the whole mounts because of the presence of several layers of DIG-*Naip* positive cells. However, upon sectioning the layering is reduced such that the amount of DIG-*Naip* positive cells present on a section may not be sufficient to be visualized by the DIG technique and would thus be perceived as an absence of signal.

*Naip* is expressed throughout the developing mouse CNS: primarily in non-apoptotic regions.

Radiolabeled riboprobes were utilized on sectioned embryos in an attempt to delineate the exact *Naip* expressing structures. $^{33}$P-UTP was used to label our riboprobes because it allows the detection of low abundance mRNAs with less background than does $^{35}$S (Faulkner-Jones, 1993). This method corroborated the pattern observed in the whole mount *in situ* hybridization in that CNS and spinal cord expression was detected in E9.5 to E13.5 sections. Given NAIP protein’s previously described expression in the rat central nervous system as well as in the spinal cord, the detection of the mouse *Naip* transcripts in these developing tissues was expected (Xu et al., 1997).

Signals were found at varying levels in the developing CNS in all stages examined suggesting a role for *Naip* in normal brain function and development. E9.5 to E14.5 embryos were found to express *Naip* transcripts in the neuroepithelium of the fourth ventricle, telencephalon and mesencephalon. The expression in the brain of E16.5 embryo sections decreased notably relative to E14.5 expression and was restricted to the cortical and marginal layers of the lateral ventricle. The cortical plate is composed of
post-mitotic cells and plays a decisive role in the organization of the definitive cortex (Naruse and Keino, 1995). In the murine embryonic cortex, dying cells are rare at E10.5 but by E14.5, comprise 70%. This number decreases to 50% by E18.5. The majority of the dying cells are found in proliferative zones rather than in regions of post-mitotic neurons (Blaschke et al., 1996). The expression of anti-apoptotic genes in the developing cortical plate is not uncommon as both Bcl-2 (Novack and Korsmeyer, 1994) and Al (Carrio et al.; 1996) have been localized in these regions at E16.5 of mouse development. The absence of the Al and Bcl-2 transcripts in the highly apoptotic region of the intermediate zone and high expression of the proteins in the proliferative region of the cortex is consistent with a role for these genes in brain development (Carrio et al., 1996). Similarly, Naip may be acting as an anti-apoptotic mediator in these important areas of the developing cortex. It may be significant that Naip functions in a distinct pathway from that of the Bcl-2 family members, as it is interacting with and inhibiting Caspase-3 (J. Maier, pers. communications). Studies on the distribution of Caspase-3 mRNA and activated Caspase-3 protein, as well as the appearance of apoptotic cells in the developing cerebral cortex of E18.5 mice have revealed that Caspase-3 mRNA was most abundant in the cortical plate while apoptotic cells and activated Caspase-3 protein were both mostly located in the proliferating ventricular zone (Urase et al., 1998). While activation of Caspase-3 is not observed for the death of post-mitotic cells of the cortical plate at E18.5, it is essential in the apoptosis of the neuroepithelium of E10.5-11.5 and undifferentiated proliferative neurons of E18.5 mice (Urase et al., 1998). Given Naip’s inhibition of Caspase-3, it is possible that it protects against the activation of this pro-apoptotic caspase in the cells of the cortical plate.
Naip is expressed in the progenitors of the spinal cord.

A central and as yet unanswered question in the molecular pathogenesis of SMA is whether NAIP loss exacerbates the clinical phenotype (Burghes, 1998; MacKenzie, 1998). Naip CNS expression was observed from E9.5 to E14.5 in the neuroepithelium of the neural tube and spinal cord. E12.5 and E13.5 hemi-sectioned embryos, which underwent the whole mount in situ protocol, revealed the presence of Naip in the mantle layer of the spinal cord. This pattern of expression was confirmed with transverse sections through the spinal cord of E13.5 revealing a very disperse expression pattern of the transcript (Figure 13b). The mantle layer of the embryonic spinal cord contains postmigratory and migrating young neurons as well as post-mitotic young neurons and glioblasts eventually becoming the gray matter of the mature spinal cord. Although this study did not permit the precise identification of Naip positive cells, it is interesting to consider the implication of the presence of Naip in the future anterior horn of the spinal cord with respect to the pathogenesis of SMA.

Given SMN’s central role in SMA pathogenesis and the role we propose for NAIP, a comparison of the expression of these two genes is instructive. SMA is characterized by the loss of motor neurons in the anterior horn of the spinal cord and lower brain stem (Towfighi et al., 1985). Battaglia et al., (1997) and Tizzano et al., (1998) have shown that SMN, the SMA causative gene, is expressed in the very cells affected in SMA. Tizzano’s et al (1998) demonstration of the presence of SMN in the spinal cord of both the human fetus and adult as well as in the adult cortex is consistent with a role for SMN in normal neuron ontogenesis and maintenance. Battaglia’s et al., (1997) study of SMN distribution during rat and monkey development revealed different
levels of SMN expression among different motor neurons. They suggest that the differences in expression levels might explain the survival of the motor neurons in the milder forms of SMA. As SMN2 can generate full-length SMN, it rescues the neuropathic effect of SMN1 deletion thus modulating the severity of SMA (Lefebvre et al, 1997). Correlation has been made between the copy number of SMN2 and SMA severity but we cannot exclude the possibility of the existence of other modifying genes as patients with similar number of SMN2 exhibit variations in their phenotype (Lorson et al, 1998; Scharf et al., 1998). The presence of Naip transcripts in the developing spinal cord aids in implicating this gene in the pathogenesis of SMA.

Furthermore the course of SMA suggests early losses of functioning in motor neurons followed by increases in the stability of surviving motor neurons (Crawford and Pardo, 1996). This has led to the idea that SMA is a disease resulting from defects in the apoptosis seen during development (Sarnat 1984, Oppenheim, 1991). During normal human development, motoneuron loss occurs between 11 weeks and 25 weeks of gestation with the greatest decline occurring between 12 and 16 weeks (Forger and Breedlove, 1987). We have witnessed Naip’s expression during murine embryo spinal cord formation to be at its peak at the time where motor neuron loss should be at its greatest, that is with the zenith of motor neuron cell death occurring at E14 (Lance-Jones, 1982). Naip mRNA expression in the spinal cord was very strong until E14.5 and became undetectable at E16.5. Whether or not Naip has an antiapoptotic function during the second trimester of gestation remains to be studied but it is apparent that it does have a role in spinal cord development. The drop in the level of Naip transcript expression in the
spinal cord, early in the third trimester of gestation, was not surprising as previous studies had documented *NAIP* levels to be low in the adult human spinal cord (Roy *et al*; 1995).

*Naip* is expressed in other tissues involved in SMA such as the dorsal root ganglia

In addition to motoneurons, attrition of dorsal root ganglia is also seen in SMA. Neuropathological studies from various groups have documented ballooned neurons and chromatolytic neurons in the dorsal root ganglia of some type I SMA patients despite the absence of detectable clinical sensory abnormalities in SMA (Murayama *et al*., 1991; Towfighi *et al*., 1985). *SMN* has been documented in the dorsal root ganglia (Tizzano *et al*; 1998). Likewise, *Naip* transcripts were also found in the dorsal root ganglia, indicating that *Naip* is also expressed in the peripheral nervous system. The implication of *Naip* and SMN presence in the dorsal root ganglia with regard to SMA is still unclear, however it suggests that they have a role in the development of the sensory neurons.

*Naip* transcripts showed a uniform expression in the dorsal root ganglia of E11.5 mouse embryos that continued in E13.5 embryos. Similarly, *Caspase-3* mRNA showed strong and uniform expression in the dorsal root ganglia of E11.5 mouse embryos whereas activated Caspase-3 positive cells showed a more restricted pattern of expression which coincided with the spatio-temporal appearance of apoptotic cells (Urabe *et al*, 1998). Activation of Caspase-3 proteases causes apoptosis of DRG neurons (Mukasa *et al*; 1997) and *Naip*’s involvement in promoting the survival of neurons in the dorsal root ganglia, with respect to caspase mediated cell death, will only be clarified with a study on the distribution of Naip protein within this tissue.
Naip message is expressed in other tissues not affected in SMA

The nasal epithelium and the developing eye are two other sensory areas where Naip transcripts were found. In the nasal area Naip expression was always diffuse until E16.5 at which stage it is found in the primordia of follicles of vibrissae associated with the lip. Naip transcripts were found in the neural layer of the retina as well as in the lens of the embryos from E11.5 to E16.5. Both the olfactory epithelium as well as the developing retina are organs that undergo cell death during their development (Capello et al., 1999; Young, 1984). Cell death in the retina of the mouse occurs during the first two weeks after birth, while programmed cell death has been reported in the nasal placode epithelium from as early as E10.5 (Grindley et al.; 1995, Young, 1984). The function of Naip in these tissues remains unclear.

Naip was also localized in the first and second mandibular component of the branchial arch of E11.5 embryos. During development, cells migrate and proliferate around the pharynx, and meet the opposite arch (Craigmyle and Presley, 1975). The ectoderm covering the branchial arches is one of the cell types involved in the initial fusion between the arches however, this cell population is absent from the fusion zone and one of the proposed mechanism for removal of the epithelia cells is programmed cell death (Shuler, 1995). Our data does not allow the identification of which cell type of the developing mandibular arch is Naip positive but when considering the tendency for apoptosis in that area it is possible to postulate a role for Naip.

Results from both in situ hybridization techniques were also concordant in the developing future digits of E12.5 mouse embryo hindlimbs (Figure 4E and Figure 11D). The cell death of the interdigital zones of the developing limbs is a well-characterized
finding. *Naip*'s presence in areas in the future digits, an area that is not prone to undergo programmed cell death, is consistent with a role of *Naip* as an anti-apoptotic agent. As reported by Novack and Korsmeyer (1994), the anti-apoptotic protein Bcl-2 is also located in the digital zones of E12.5 embryos. They suggest that cells lacking Bcl-2 are susceptible to the cell death signal thus down regulation of Bcl-2 in the interdigital space may be important for the programmed cell death to occur. However Bcl-2 knockout mice have normal limb development thus suggesting that other factors are required for the development of digits. *Naip* may be one such factor.

*Naip* expression was very high in the developing intestine of E16.5 embryos. At E14.5, the epithelium is undifferentiated but by E16.5, the tissue convolutes with developing villi. *Naip* transcript expression is restricted to the villi unlike Bcl-2, the expression of which is restricted to the progenitor cells at the base of the villi (Novack and Korsmeyer, 1994). *Naip* is once again present in cells that have ceased to divide (Traber, 1994) but which are migrating to the villus tip. Absorptive enterocytes and goblet cells are extruded into the intestinal lumen of the adult mouse (Traber, 1994), an event shown to occur at the tip of the rat villus where apoptotic cuffs are formed in contrast to the mid-villus and crypt cells which are non-apoptotic (Westcarr et al, 1999). It is unclear which of the six murine *Naip* loci are expressed in the intestine. Delineation of the protein's role in the intestinal tract will likely be clarified with the identification of the responsible loci and analysis of mouse models null for these gene copies.
Conclusion

*Naip* has a distinct expression pattern in the developing mouse embryo when compared to other modulators of apoptosis, such as Bcl-2, and to other members of the IAP family such as Survivin (Adida *et al.*, 1998). When contemplating *Naip*’s phylogenetic relationship to the other BIR containing genes, it is evident that it is in a class by itself (Deveraux and Reed, 1999). While other IAPs members such as XIAP have had a strong inhibition of apoptosis documented or a role in regulating cell proliferation (e.g. Survivin), *Naip* is comparatively distinct at the sequence level and in addition to inhibiting apoptosis it is clearly developmentally regulated. Our data has showed a *Naip* expression pattern of developmental stage-specific expression in various organs of the mouse embryo. Interestingly, some of the tissues where *NAIP* expression has been revealed in the adult such as the lungs and the heart are void of *Naip* during mouse embryogenesis. These data suggest a role for *Naip* in the specialized function of these organs in adults rather than in their development. Other tissues, such as the brain, show low expression throughout adulthood. This involvement of a gene in many tissues other than the one(s) linked to the disease to which the gene is associated is not uncommon in genes involved in neurodegenerative disorders. One such example is Huntington disease gene in the rat where, despite the regional specificity of the degeneration in Huntington’s disease, the gene is expressed in organs not implicated in the progression of the disease (Strong *et al*; 1993).

*Naip* has a widespread expression in various tissues of the body. Some of the *Naip* positive tissues are the tissues that are affected by the very specific pathology of SMA. One model of SMA pathogenesis involves motor neuron attrition in the second and
possibly third trimester of gestation. Our observation of *Naip* transcripts in the spinal cord between E9.5 and E14.5 (equivalent to the second trimester) is consistent with a role for *Naip* in modifying SMA severity. In some tissues, *Naip*’s temporal expression coincides with the onset of programmed cell death. The role of *Naip* in all of the tissues where it was found remains to be elucidated. Our results suggest a developmental regulation of *Naip* expression. While *Naip*’s implication in the pathogenesis of SMA may be due to its developmentally regulated expression, its exact involvement in the pathogenesis of the disease remains to be clarified; comparison of SMN deficient mice with SMN deficient/NAIP null mice will help clarify this issue.
REFERENCES


