**İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY**

# IDENTIFICATION AND CHARACTERIZATION OF CHICKEN NEURONAL P80 KATANIN

**M.Sc. Thesis by**
**Ayşegül YILDIZ, B.Sc.**

**Department: Advanced Technologies in Engineering**

**Programme: Molecular Biology-Genetics and Biotechnology**

**Supervisor : Assoc. Prof. Dr. Arzu KARABAY KORKMAZ**

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**JUNE 2006**

**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# TAVUK NÖRONAL P80 KATANİNİN TANIMLANMASI VE KARAKTERİZASYONU

**YÜKSEK LİSANS TEZİ**

**Ayşegül YILDIZ, B.Sc.**
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**HAZİRAN 2006**

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June, 2006 Ayşegül YILDIZ

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## ABBREVIATIONS

| | |
|---|---|
| AAA | : **A**TPases **A**ssociated with various cellular **A**ctivities |
| ADP | : Adenosine Diphosphate |
| ATP | : Adenosine Triphospahe |
| BLAST | : Basic Local Alignment Search Tool |
| cDNA | : Complementary Deoxyribonucleic Acid |
| CMV | : Cytomegalovirus |
| DEPC | : Diethyl Pyrocarbonate |
| dNTP | : Deoxyribonucleotide Triphosphate |
| EDTA | : Ethylenediaminetetraacetic Acid |
| EtBr | : Ethidium Bromide |
| GC | : Guanine Cytosine |
| gDNA | : Genomic Deoxyribonucleic Acid |
| GFP | . Green Fluorescent Protein |
| GTP | : Guanosine Triphospahte |
| HSV TK | : Herpes Simplex Virus Thymidine Kinase |
| IPTG | : Isopropyl-b D- thiogalactopyranoside |
| LB media | : Luria Bertani Media |
| MAP | : Microtubule Associated Protein |
| MCS | . Multiple Cloning Site |
| mRNA | . Messenger Ribonucleic Acid |
| OD | : Optical Density |
| PCR | : Polymerase Chain Reaction |
| PEG | : Polyethylene Glycol |
| RNase | : Ribonuclease |
| RT-PCR | : Reverse Transcriptase Polymerase Chain Reaction |
| SOC | : Super Optimal Catabolite Repression Broth |
| SV40 | : Simian Virus 40 |
| TAE | : Tris Acetate EDTA |
| TBS | : Tris Buffered Saline |

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## LIST OF SYMBOLS

| | |
|---|---|
| bp | : Base pair |
| E-site | : Exchangeable site |
| kb | : Kilobase |
| kDa | : Kilodalton |
| MOPS | : 3-(N-morpholino)propanesulfonic acid |
| PIPES | : 2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid |
| Tm | : Melting Temperature |
| Tris | : Hydroxymethyl aminomethane |
| UV | : Ultraviolet |
| X-Gal | : 5-brom-4-chloro-3-indolyl-beta-D-galactopyranoside |

**IDENTIFICATION AND CHARACTERIZATION OF CHICKEN NEURONAL P80 KATANIN**

## SUMMARY

Katanin is a heterodimeric protein that severes microtubules by hydrolyzing ATP. Katanin consists of 60 kDa and 80 kDa polypeptides. 60 kDa subunit (p60) has the enzymatic activity to break microtubules, whereas 80 kDa subunit (p80) has a role in localization of the protein complex in the cell.

Katanin has been shown to have roles for microtubule severing in mitotic cells including release of microtubules from centrosome, depolymerization of microtubule minus ends in the mitotic spindle.

In addition to having roles in mitotic cells, katanin is also important for some specialized cell types such as neurons. It is thought that katanin provides a source of non-centrosomal microtubules that are transported into developing axons and dendrites for structural support.

Since katanin reorganizes microtubule arrays in neurons, it provides the cell developing and branching axons and dendrites. This feature of katanin, hence, may be utilized to regenerate injured neurons, if both subunits are better studied and well understood. p60 katanin has been paid more attention than p80, since it is the subunit that has the severing activity itself. Thus, the exact functions of p80 katanin remain to be solved.

In this study, p80 katanin was cloned from *Gallus gallus* (chicken) brain cDNA and then it was transfected into NIH 3T3 cells in order to reveal possible functions of p80.

In the next step of the study, p80 katanin will be transfected into cultured chicken embryo primary neurons so as to observe effects of p80 in neuronal cells. In addition to this, *in situ* hybridization will be performed with cryosections of chicken embryos to be able to visualize distribution of p80 katanin in the cells through different developmental stages of embryos.

**TAVUK NÖRONAL P80 KATANİNİN TANIMLANMASI VE KARAKTERİZASYONU**

## ÖZET

Katanin 60 kDa (p60) ve 80 kDa (p80) büyüklüğünde iki alt üniteden oluşan ve ATP hidrolizleyerek mikrotübülleri parçalayan bir proteindir. p60 enzim aktivitesi ile mikrotübülleri keserken, p80 enzimin hücre içindeki lokalizasyonunda görevlidir.

Mitotik hücrelerde kataninin, mikrotübüllerin sentrozomdan kesilerek serbest bırakılması, mikrotübül eksi uçlarının depolimerize edilmesiyle mitoz esnasında kromozomların kutuplara çekilmesi gibi hücre içi olaylarda görevli olduğu düşünülmektedir.

Mitotik hücrelerin yanısıra katanin nöronlar gibi bazı özelleşmiş hücreler için de oldukça önemlidir. Kataninin sinir hücrelerindeki fonksiyonu, sentrozomda bulunan mikrotübülleri keserek serbest bırakmaktır. Daha sonra bu mikrotübüller gelişmekte ve dallanmakta olan akson ve dendritlere taşınarak onlara yapısal destek sağlamaktadır.

Katanin nöronlardaki mikrotübül ağının yeniden düzenlenmesini sağlayarak hücreye yeni akson ve dendritler kazandırdığından, kataninin bu özelliğinden hasar gören sinir hücrelerinin yenilenmesinde fayda sağlanabileceği düşünülmektedir. Bu da ancak iki alt ünitenin fonksiyonlarının da çok iyi bir şekilde incelenmesi ve anlaşılmasıyla mümkün olabilecektir. Şimdiye kadar p60 katanin, kendi başına mikrotübülleri kesici özelliğe sahip oluşu nedeniyle yapılan çalışmalarda daha fazla yer almış, bu yüzden p80 kataninin fonksiyonları net olarak açıklığa kavuşturulamamıştır.

Bu çalışmada, p80 katanin öncelikle dissekte edilen tavuk beyninden elde edilen cDNA dan klonlanmış ve daha sonra p80'in muhtemel fonksiyonlarını olarak gözlemleyebilmek için ilk olarak kültür halindeki NIH 3T3 hücrelerine transfekte edilmiştir.

Çalışmanın ileri aşamalarında, p80 tavuk embriyosundan elde edilmiş kültür halindeki primer sinir hücrelerine de transfekte edilecek ve bu kez p80 fonksiyonları bu hücrelerde gözlemlenebilecektir. Bu çalışmanın yanısıra, tavuk embriyosundan kriyostatla alınan kesitlerde in situ hibridizasyon uygulanarak p80'in embriyoların farklı gelişim evrelerinde hücre içindeki dağılımları izlenebilecektir.

# 1. INTRODUCTION

## 1.1. Cytoskeleton

Cells organize themselves to be correctly shaped and properly structured internally. They have to be able to rearrange their internal components as they grow, divide and adapt to changing environment. In eucaryotes, these functions are devoted to a very complex system called the “cytoskeleton”.

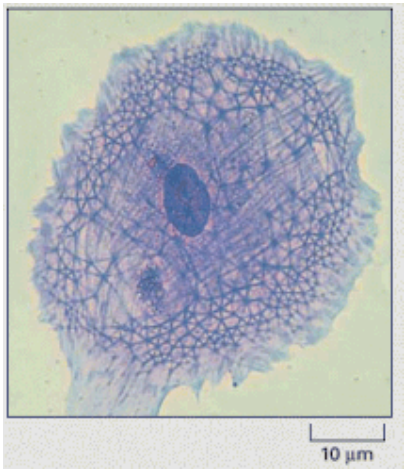


**Figure 1.1:** Cytoskeletal structure in a typical cell (Alberts et al.,2002)

Cytoskeleton pulls the chromosomes apart during mitosis and splits the dividing cell into two. It regulates the movement of entire cells such as sperms and fibroblasts. In addition, it is responsible for the transport of organelles and vesicles from one part of the cell to another. It also enables some specialized cells, such as neurons, to extend an axon and dendrites.

Cytoskeleton consists of three major types of protein filaments. These are microfilaments, intermediate filaments and microtubules. However, there are a lot of accessory proteins that hold these protein filaments together and link them to other cellular components.

## 1.2. Microtubule

### 1.2.1. Structure and Dynamics of Microtubules

Microtubules are made of tubulin subunits. The tubulin subunit is a heterodimer that consists of two closely related globular proteins called α-tubulin and β-tubulin. A microtubule is a hollow cylindirical structure with an outer diameter of 25 nm, formed of 13 parallel protofilaments. Each protofilament is composed of alternating α-tubulin and β-tubulin molecules.

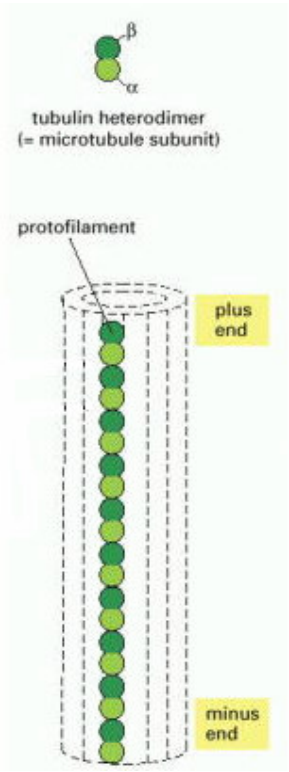


**Figure 1.2:** The structure of a microtubule and its subunit, tubulin (Alberts et al., 2002)

Microtubules are nucleated from γ-tubulin ring complexes found within the pericentiolar region of the centrosomes. Microtubules are nucleated at the centrosome at their minus ends, so the plus ends point outward and grow toward the cell periphery. Microtubules always grow faster from one end than the other end.

Fast-growing end is called “plus end”, while slow-growing end is called “minus end”. Because of the intrinsic structural polarity of microtubules, α-tubulins are exposed at one end (minus end) and β –tubulins are exposed at the other end (plus end).

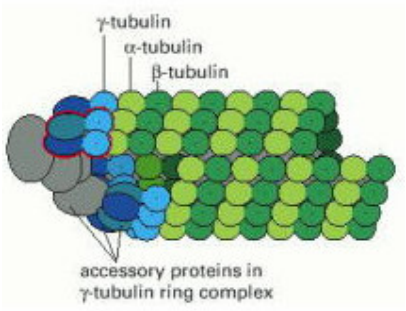


**Figure 1.3:** Polymerization of tubulin nucleated by γ-tubulin ring complexes (Alberts et al., 2002)

Microtubules have a highly dynamic nature that is attributed to regulated growth and shrinkage of the polymer plus ends, a process known as “dynamic instability”. Dynamic instability results from GTP hydrolysis by tubulin; a cap of GTP-containing tubulin subunits at the microtubule end stabilizes the lattice, but exposure of tubulin-GDP at ends destabilizes the polymer (Caplow and Shanks, 1996; Desai and Mitchison, 1997).

Dynamic instability is based on hydrolysis of GTP at nucleotide exchangeable site (E site) of β –tubulin. GTP hydrolysis at E site produces non-exchangeable GDP and makes microtubule end unstable that causes depolymerization of the polymer (Heald and Nogales, 2002; Gadde and Heald, 2004). Another consequence of nucleotide hydrolysis is the unidirectional flux of subunits known as “treadmilling” (Maiato et al., 2004). Treadmillling is the steady state of microtubules at which subunits undergo a net assembly at the plus end and a net disassembly at the minus end at an identical rate.

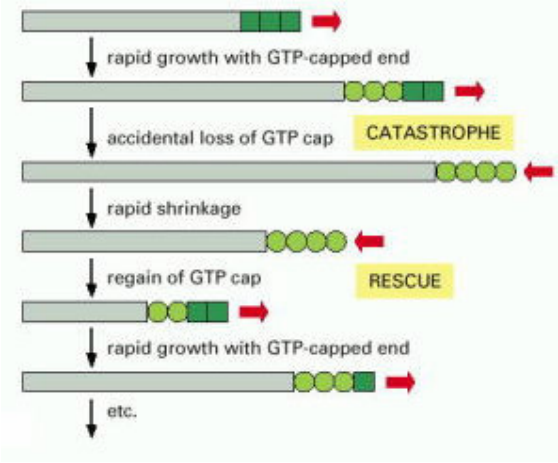


**Figure 1.4:** Dynamic instability as a result of the structural differences between a growing and a shrinking microtubule end (Alberts et al., 2002)

Intrinsic dynamic behaviors of microtubules can be altered by a set of proteins that binds along the sides of the polymer. These microtubule-associated proteins (MAPs) modulate dynamic behavior by binding microtubule wall to promote microtubule polymerization by enhancing the rate of microtubule growth (Drechsel et al., 1992; Kowalski and Williams, 1993). Besides that, some other types of MAPs promote microtubule depolymerization by increasing the frequency of shrinking stages (Hartman et al., 1998).

As mentioned above, MAPs do not only destabilize or stabilize microtubules but some of them can also regulate the interaction of microtubules with other cellular components. MAPs can inhibit interaction of other MAPs or motor proteins with microtubules by binding to microtubules (Baas & Qiang, 2005).

### 1.2.2. Microtubule Functions

Regulation of the dynamic behavior of microtubule filaments allows eucaryotic cells to build a variety of structures from a basic filament system.

Microtubules are required during interphase for organizing intracellular membrane compartments such as Golgi apparatus (Ho et al., 1990) as well as for transporting small membrane carrier vesicles in the secretory pathways (Vale, 1987).

During mitosis, microtubules are primary constituents of the mitotic spindle with hundreds of other accessory proteins functioning togehter and are needed for proper segregation of chromosomes and for specifying the position of the cleavage furrow (Rappaport, 1985).

Microtubules must be arranged into a bipolar aray with their minus ends at the pole and plus ends extending away to capture sister chromatids from opposite spindle poles to allow for directed translocation of chromosomes within the spindle (Rieder and Salmon, 1998). One model for such spindle assembly is the "search-and-capture" model that is based on microtubule dynamic instability. In this proposed model, microtubules emanating from a centrosome undergo cycles of growth and shrinkage, randomly probing cytoplasm until running into a kinetochore to form a stable attachment. When chromosomes segregate, they are carried to distinct poles by shortening microtubules. There are two proposed model to explain microtubule shortening for chromosome trasnport to the poles. One model, termed "pacman" proposes that the kinetochore induces microtubule disassembly at the plus ends, but maintains attachments as the fiber depolymerizes, thus chewing its way to the pole. In another model, termed "traction fiber", poleward microtubule flux is harnessed to move the chromosome. Both mechanisms contribute to depolymerization of kinetochore microtubules (Gadde and Heald, 2004).

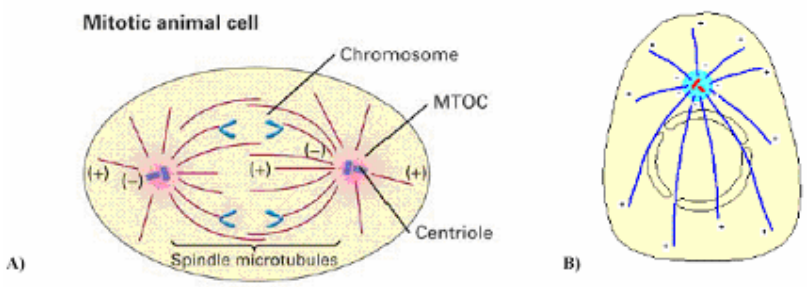


**Figure 1.5:** Orientation of microtubules in a mitotic (A) and an interphase (B) cell (Lodish et al., 2000)

Microtubules have crucial roles in some specialized cell types such as neurons. Neurons use their microtubules not for the formation of a mitotic spindle but rather for the elaboration of an elongated axon (Karabay et al., 2004).

Changes in the configuration and behavior of microtubules within specific regions of the neuron underlie important events in axonal differentiation such as elongation, retraction, navigation, branching and sprouting (Baas and Buster, 2004). Microtubules also provide a support for transporting organelles in both directions within the axon.

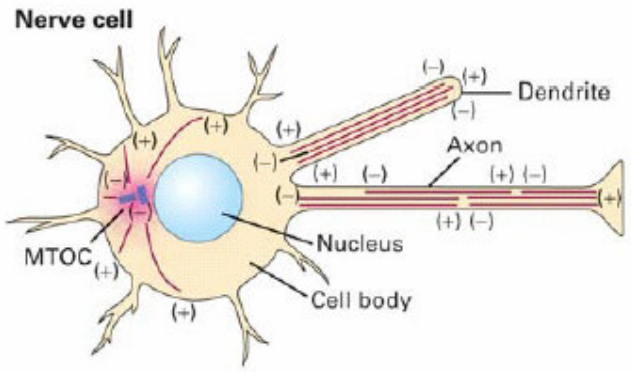


**Figure 1.6:** Microtubule structure and orientation in neurons (Lodish et al., 2000)

Unlike mitotic cells, neurons do not display a radial array of microtubules nucleating from a centrosome (Baas, 1999). Instead, microtubules are abundant throughout the cell body of the neuron (Yu and Baas, 1994). In fact, microtubules destined for the axons and dendrites are nucleated at the centrosome, then released and then actively transported into the processes (Yu et al., 1993). However, the inherent dynamic properties of the microtubules is not sufficient for explaining mechanisms such as release from centrosome and transport in neurons. There are some other important mechanisms that influence the microtubule dynamics. For instance, severing of microtubules and release of their minus ends play an important role in both cases. In addition to that, movement of released microtubules is generated by a minus end directed motor such as cytoplasmic dynein (Keating et al., 1997).

## 1.3. Katanin

### 1.3.1. Structure and Functions of Katanin

Katanin is a member of the AAA adenosine triphosphatase (**A**TPases **A**ssociated with various celular **A**ctivities) superfamily. It was first purified from sea urchin eggs and named "katanin" from katana, the Japanese word for samurai sword (McNally and Vale, 1993). It severes microtubules by using ATP hydrolysis energy.

AAA ATPases disassemble stable protein-protein interactions and play important roles in numerous cellular activities including proteolysis, protein folding, membrane trafficking, cytoskeletal regulation, organelle biogenesis, DNA replication and intracellular motility. The unifying feature of the AAA superfamily is an ATPase domain of ~ 220 amino acids. There is a strong sequence conservation in this domain (~ 30% identity) (Vale, 2000 ; Hartman and Vale, 1999).

AAA proteins function as oligomers, in most cases by forming hexamer rings. In addition, the AAA core structure is highly conserved and that subunit-subunit interactions are likely to be important in the enzymatic mechanism (Vale, 2000).

The AAA enzyme katanin is the only known microtubule-stimulated ATPase (McNally and Vale, 1993). It is a heterodimeric protein that consists of 60 and 80 kDa subunits.

Katanin breaks stable tubulin-tubulin interactions in the wall of a microtubule (Hartman et al.,1998). Severing of a microtubule along its length requires thirteen or so subunits around the circumference of the tubule each to be dissociated from thightly bound neighbours above, below and two sides.

There is a model suggested for microtubule severing by katanin (Hartman and Vale, 1999) : microtubules act as a scaffold upon which katanin oligomerizes as hexameric rings after it has exchanged its ADP for ATP. Once a complete katanin ring is assembled on the microtubule, the ATPase activity of katanin is stimulated.

After ATP hydrolysis and subsequent phophate release, the katanin undergoes a conformational change leading to destabilization of tubulin-tubulin contacts (Quarmby, 2000). Disassembled tubulin subunits are capable of repolymerizing since katanin does not proteolyze or modify tubulin (McNally and Vale, 1993).

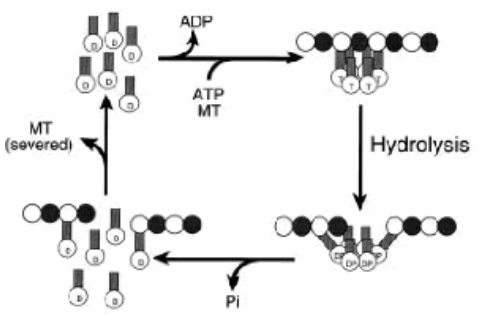


**Figure 1.7:** Model for microtubule severing by katanin (Hartman and Vale, 1999)

Microtubule binding site for katanin hexamer is unknown but there are possible binding sites include the outside of the microtubule, the microtubule lumen or the sides of dimers exposed by holes in the lattice (McNally, 2000). The latter two possibilities suggest that katanin might act on specific defective sites within the microtubule lattice. This hypothesize that katanin exploits local defects and promotes loss of tubulin at the defect site until the two microtubule segments are held together so weakly that mechanically unconstrained microtubules kink at the defect site (Davis et al., 2002).

Oligomerization of katanin monomers is most efficient in the presence of microtubules which act as a scaffold for promoting oligomerizaton. However, ATPase activity of katanin displays a complex stimulation by microtubules. At low microtubule concentrations (< 2μM), ATPase activity increases with increasing microtubule concentration but at higher microtubule concentrations, ATPase activity dicreases until it approaches basal levels because of prevention katanin oligomerization through the sequestration of katanin monomers at discontiguous, low affinity binding sites on microtubules (Hartman et al., 1998; Hartman and Vale, 1999).

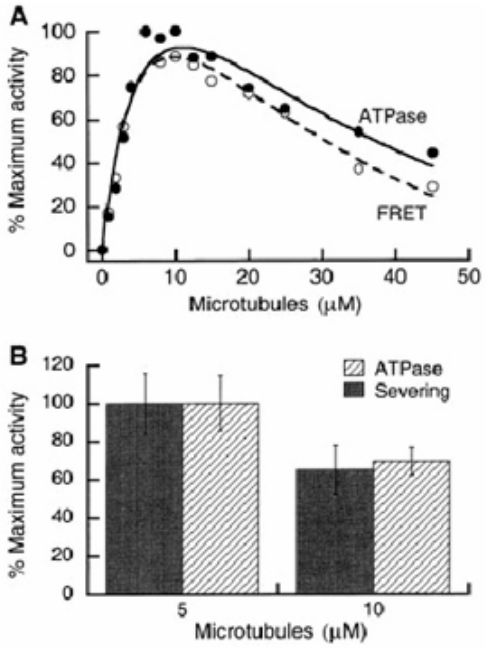


**Figure 1.8:** Correlation between katanin ATPase activity, katanin oligomerization and severing (Hartman and Vale, 1999)

Katanin has potential roles for microtubule severing including release of microtubules from centrosomes (Kitanishi-Yumura and Fukui, 1987; Belmond *et al*., 1990), depolymerization of microtubule minus ends in the mitotic spindle as a component of poleward flux (Mitchison, 1989), acceleration of microtubule turnover at the G2/M transition of the cell cycle and finally providing a source of non-centrosomal microtubules in neurons (Hartman and Vale, 1999 ; Quarmby, 2000).

During cell cycle, katanin is thought to release centrosomal microtubules and facilitate the rapid disassembly of the interphase array as cells enter mitosis. In addition to being localized at centrosomes, katanin is also found in a microtubule-dependent manner at mitotic spindle poles (McNally and Thomas, 1998) and it is thought that katanin plays a crucial role during depolymerization of spindle microtubules at their minus ends, a process important for anaphase chromosome segregation ( Waters et al*.,* 1996 ; Desai et al., 1998).

In some other types of cells that have highly specialized microtubule arrays, such as neurons, katanin is thought to provide an important source of non-centrosomal microtubules that are very critical for the activity of the terminally postmitotic neurons. All microtubules in the neuron are nucleated from centrosome, then released by katanin and conveyed into developing axons and dendrites (Baas, 2005).

The axonal and dendritic processes of neurons require a supply of non-centrosomal microtubules both for structural support and for transport of materials (Quarmby, 2000).

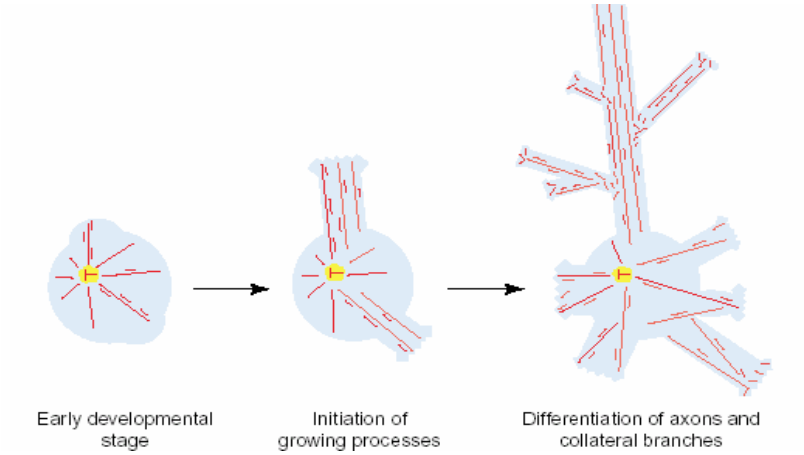


**Figure 1.9:** Microtubule reorganization and redistribution in neurons (Baas, 2005)

Studies on several cell types support a model called “cut and run” for microtubule severing activity of katanin which is very important for microtubule reorganization and regulation in various cell types. This model proposes that long microtubules are relatively immobile, whereas short microtubules are quite mobile. Hence, the long microtubules are severed into short pieces that rapidly move into new configuration in order to transform a cell’s microtubule array from one type of organization to another. After being reorganized, the short microtubules can again elongate and lose their mobility (Baas, 2005).

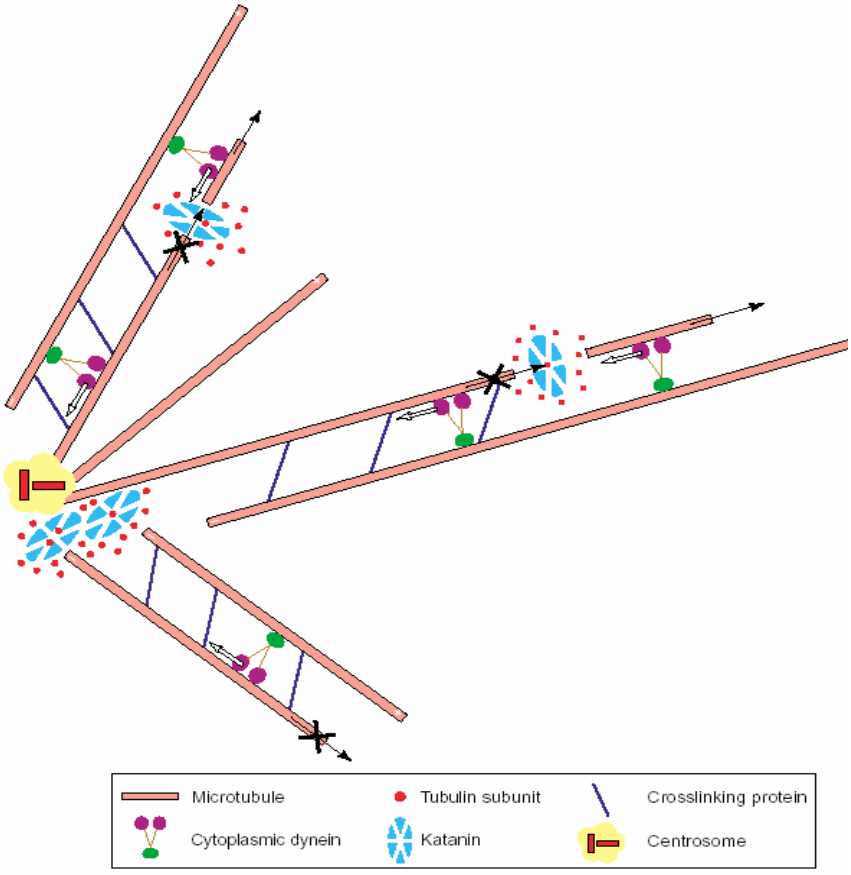


**Figure 1.10:** The 'cut and run' model for microtubule reconfiguration (Baas, 2005)

### 1.3.2. Katanin Subunits

Katanin is a heterodimeric protein that consists of 60 kDa (p60) and 80 kDa (p80) subunits. p60 has the enzymatic activity to severe microtubules by hydrolyzing ATP whereas p80 has a role in localization of the protein complex in the cell.
Due to the presence of a conserved 230-residue C- terminal part, p60 katanin has been shown to be a member of the AAA family (McNally and Vale, 1993).

Rat p60 katanin has been shown to be a 491 amino acid long peptide and it has a conserved C terminal AAA domain (underlined amino acids 249-491; Karabay et al., 2004).

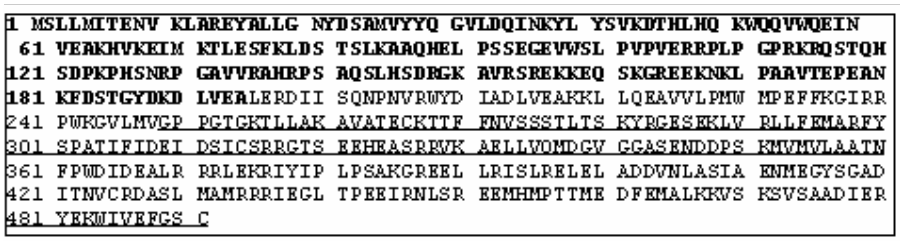


**Figure 1.11:** *Rattus norvegicus* p60-katanin amino acid sequence

p60 katanin functions as an oligomeric ring complex and oligomerizes upon microtubules with its N-terminal domain. Then, ATP hydrolysis occurs and this leads to breakage of microtubule lattice (Hartman and Vale, 1999; Quarmby, 2000).

p80 katanin is composed of an N-terminal WD40 domain, a central proline-rich domain and a C-terminal domain (McNally et al., 2000).

Rat p80 (Yu *et al.*, 2005) contains six WD40 repeats. WD40 repeats are conserved domains that consist of 40-60 amino acid which are initiated by a glycine-histidine (GH) dipeptide and end with a tryptophan-aspartic acid (WD) dipeptide at C terminus. 40 amino acid conserved core sequence is between the GH and the WD dipeptides (Li and Roberts, 2001).

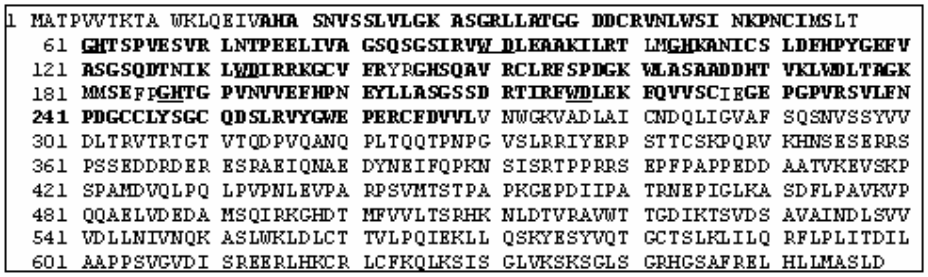


**Figure 1.12:** *Rattus norvegicus* p80-katanin amino acid sequence

The N-terminal WD40 domain of p80 targets the enzyme to the centrosome (Hartman et al., 1998) and it is also required for spindle pole localization. On the other hand, this N-terminal WD40 domain acts as a negative regulator of microtubule disassembly activity (McNally et al., 2000).

The C-terminal region of p80 did not exhibit significant amino acid identity to any previously described protein. This C-terminal 130 amino acid part of p80 is suggested to be involved in the dimerization with p60 (Hartman et al., 1998). Although p60 can severe microtubules even in the absence of p80, further investigations indicated that severing is

more efficient in the presence of p80 (McNally et al., 2000; Yu et al., 2005). It may be due to enhancement of p60's microtubule affinity by increasing the number of microtubule binding sites with p80 ( McNally et al., 2000).

### 1.4. Aim of the Study

Katanin is thought to have important roles in variety of cell types. In mitotic cells, katanin reorganizes microtubules, whereas it is responsible for elongation and elaboration of axons and possibly branching of dendrites in neurons.

Since neurons are terminally differentiated, they can not divide. Hence their injuries cause irreversible paralysis of body parts.

Nerve regenerations may become possible only if there are newly branching axons and dendrites that require reorganization of microtubule structure within a cell. This mechanism requires severing proteins, like katanin.

Katanin is a heterodimeric protein that consists of 60 kDa (p60) and 80 kDa (p80) subunits. p60 has the enzymatic activity to break the microtubule lattice. p80 has no severing activity of its own but it targets p60 to the centrosome. However, recent studies revelaed that p80 subunit is widely distributed throughout all compartments of the neuron, suggesting that it may have possible additional functions besides targeting, such as increasing or supressing severing activity of p60 by binding to this subunit with its different domains.

Since regulation mechanism of severing activity is an important and unressolved issue, p80 katanin was chosen for this study to better understand its possible role in this mechanism.

On the other hand, chicken (*Gallus gallus*) was chosen as a model organism to study p80 function. After the analysis of chicken genome, it was understood that about 60% of the chicken protein-coding genes have human equivalents. Hence, chicken genome gives surprising insights into human genome and it can be used as an invaluable tool to better identify and characterize similar sequences in humans.

In addition to this similarity, chicken embryo dorsal root ganglion (DRG) is the most suitable model system to determine the katanin function, since it does not have intrinsic branching feature.

For these purposes, it was first aimed to clone p80 katanin from chicken brain. After that, it was aimed to transfect cloned p80 into cultured fibroblasts and chicken embryo primary neurons such as DRGs to elucidate the possible functions of p80 katanin in regeneration of neurons.

Furthermore, chicken embryo is a unique model to study vertebrate development. Thus, it was also aimed to take cryosections from chicken embryos and label these sections with riboprobes specifically designed for p80 katanin mRNA. It would enable us to visualize distribution of p80 katanin in cells with *in situ* hybridization.

# 2. MATERIALS AND METHODS

## 2.1. MATERIALS

### 2.1.1. Equipments

The equipments used in the study are demonstrated in the table below:

**Table 2.1.** Equipments

| | |
|---|---|
| Electrophoresis Gel System | E-C Apparatus Corporation, EC250-90 Minicell Primo |
| Thermal Cycler | Applied Biosystems, GeneAmp PCR System 2700 |
| Microcentrifuge | Beckman Coultier |
| Water Bath | Memmert |
| UV Transilluminator | BioRad UV Transilluminator 2000 |
| UVIPhotoMW Version 99.05 for Windows 95 & 98 | UVItec Ltd. |
| Shaker | Forma |
| pH Meter | Mettler Toledo MP220 |
| Precision weigher | Precisa 620C SCS |
| Vortex | Heidolph, Reaxtop |
| DNA Sequencer | Applied Biosciences 3100-Avant |

### 2.1.2. Chemicals

The chemicals used in the study are shown in the table below:

**Table 2.2.** Chemicals

| | |
|---|---|
| 3-(N-morpholino)propanesulfonic acid(MOPS) | Acros Organics |
| DEPC<br>IPTG | AppliChem |
| PIPES<br>MgSO4 | BDH Laboratory Supplies |
| GC Melt | Clontech |
| MgCl2<br>PEG4000<br>dNTP mix<br>DNA Ladder<br>DNA Loading Dye | Fermentas |
| Isopropanol<br>CaCl2<br>Glycerol<br>NaCl<br>Glacial Acid | Fluka |
| P60-p80 specific primers | Integrated DNA Technologies |
| M13F/R universal primers | Invitrogen |
| Tryptone | Lab M TM |
| Glucose<br>EDTA<br>EtBr<br>Tris base<br>Yeast extract<br>Agar<br>KCl<br>MgCl2 | Merck |
| DNA Ladder | Promega |
| Agarose<br>RNA Loading Dye | Sigma |

### 2.1.3. Enzymes and Buffers

Enzymes and their buffers which were used in the study are shown in the table below:

**Table 2.3.** Enzymes and buffers

| | |
|---|---|
| Taq Polymerase<br>Kpn I restriction enzyme<br>Sal I restriction enzyme<br>Sma I restriction enzyme<br>Hind III restriction enzyme<br>BamHI Buffer<br>10 X Buffer Tango<br>Taq Polymerase Buffer | Fermentas |
| T4 Ligase<br>T4 Ligase Buffer | New England Biolabs |

### 2.1.4. Kits

Special kits used in this study are demonsrated in the table below:

**Table 2.4.** Kits

| | |
|---|---|
| High Pure RNA Tissue Kit | Roche |
| cDNA Synthesis Kit | Fermentas |
| Qiaquick Gel Extraction Kit | Qiagen |
| High Pure Plasmid Purification Kit | Roche |
| Big Dye Terminator v 3.1 Cycle Sequencing Kit | Applied Biosystems |
| InsT/A Cloning Kit | Fermentas |

### 2.1.5. Buffers and Solutions

Buffers and solutions used in the study are shown in the table below:

**Table 2.5.** Buffers and solutions

| | Contents |
|---|---|
| TAE Buffer (50X) | 40 mM Tris base |
| | 20 mM glacial acetic acid |
| | 1 mM EDTA (pH 8.0) |
| | $H_2O$ |
| 5X Formaldehyde Gel Running Buffer | 0.1 M 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.0 |
| | 50 mM Sodium acetate |
| | 5 mM EDTA pH 8.0 |
| | $H_2O$ |
| $CaCl_2$ Solution | 60 mM $CaCl_2$ |
| | 10 mM PIPES |
| | 15% glycerol |

### 2.1.6. Bacterial strains

*Escherichia coli* (*E.coli*) strain XL1 Blue [recA1 endA1 gyrA96 thi-1 hsdR17 supE44relA1 lac [F' proAB $lacI^qZ\Delta M15$ Tn10 ($Tet^r$)]], Novagen

### 2.1.7. Bacterial culture media

LB medium was prepared by dissolving 10 gram (gr) tryptone, 5 gram yeast extract, and 10 gram NaCl in distilled water. Distilled water was added to a final volume of 1 liter

(l). The LB medium was sterilized by autoclaving for 15 minutes. In order to make selection media, antibiotic was added to the LB medium according to the concentration described in Table 2.1, and the antibiotic containing LB was stored at 4ºC.

LB-agar plate was prepared by adding 15 gram/l of agar to LB medium and sterilized by autoclaving as described above.

SOC medium was used to cultivate *E.coli* for 1 hour after temperature shock during transformation. It was prepared by dissolving 2 gr of tryptone, 5 gr of yeast extract, 0.058 gr of NaCl, 0.0186 gr of KCl, 0.095 gr of $MgCl_2$, 0.24 gr of MgSO4, 0.36 gr of glucose in distilled water. Distilled water was added to a final volume of 100 mililiter (ml). The SOC medium was sterilized by autoclaving at 120°C for 15 minutes. When required, antibiotic was added to the medium in order to make selection (Table 2.6.).

**Table 2.6.** Stock and working solution of antibiotics

| Antibiotic | Stock solution concentration | Working concentration |
|---|---|---|
| Kanamycin | 10miligram(mg)/ml in water | 50 microgram (µg)/ml |
| Amphicillin | 10 mg/ml in water | 50 µg/ml |

### 2.1.8. T/A cloning vector

pTZ57R/T is a specifically designed cloning vector. The vector has been pre-cleaved with Eco32I (an isoschizomer of EcoRV) and treated with terminal deoxynucleotidyl transferase to create 3'-ddT overhangs at both ends. When a PCR fragment with 3'-dA overhangs is ligated into the vector, a circular molecule with two nicks is produced.The circular product can be used directly to transform *E.coli* cells with high efficiency. An additional advantage of this approach is that the T-overhangs prevent recircularization of the vector during the ligation procedure. As a result, the yields of the recombinants are typically as high as 90%. The DNA insert can be readily excised from the versatile polylinker of pTZ57R/T and subcloned into other vectors, as well as sequenced using standard M13/pUC primers.

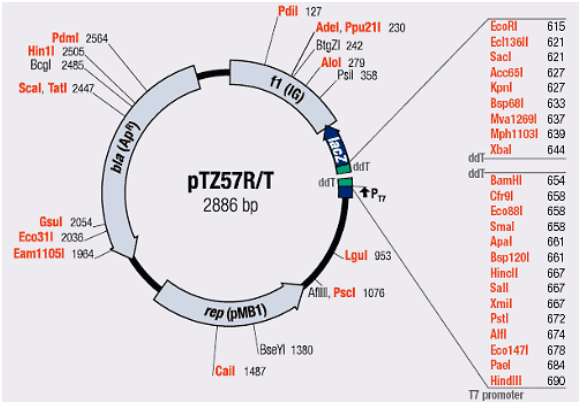


**Figure.2.1:** pTZ57R/T cloning vector map.

### 2.1.9. pEGFP-N1 Expression Vector

pEGFP-N1 encodes a red-shifted variant of wild-type GFP (1–3) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nanometer (nm); emission maximum = 507 nm.) pEGFP-N1 encodes the GFPmut1 variant which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences. Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site to further increase the translation efficiency in eukaryotic cells. The MCS in pEGFP-N1 is between the immediate early promoter of CMV ( PCMV IE) and the EGFP coding sequences. Genes cloned into the MCS will be expressed as fusions to the N-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T antigen. A neomycin-resistance cassette (Neor), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418.

A bacterial promoter upstream of this cassette expresses kanamycin resistance in E. coli. The pEGFP-N1 backbone also provides a pUC origin of replication for propagation in E. coli and an f1 origin for single-stranded DNA production.

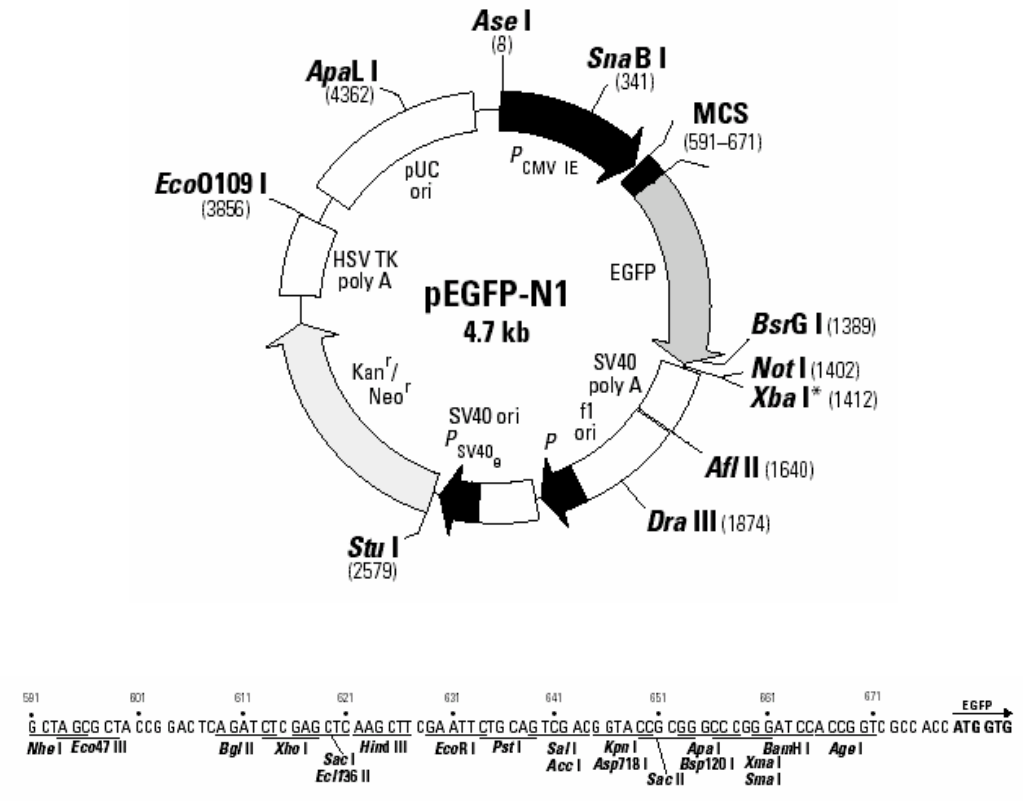


**Figure.2.2**: pEGFP-N1 vector map and multiple cloning site.

## 2.2. METHODS

### 2.2.1. Brain dissection

Adult chicken (*Gallus gallus*) was sacrificed. After providing a sterile environment, the skin and underlying tissue were carefully removed from cranium and the skull was exposed. Then the skull was cut into small pieces with bone cutting and dissecting scissors and brain was removed by using fine forceps and a probe.

### 2.2.2. RNA Isolation from Brain Tissue

Isolating intact RNA is a prerequisite for the analysis of gene expression. Frequently applied techniques like Reverse Transcriptase-PCR (RT-PCR), Northern blotting, and RNase protection require the use of intact undegraded RNA. Tissue samples are disrupted and homogenized in the presence of a strong denaturing buffer containing guanidine hydrochloride to instantaneously inactivate RNases, and to ensure isolation of intact RNA. After adding ethanol, RNA binds selectively to a glass fiber fleece in the presence of a chaotropic salt (guanidine HCI). Residual contaminating DNA is digested by DNase I, applied directly on the glass fiber fleece. During a series of rapid "wash-and-spin" steps to remove contaminating cellular components the RNA remains bound to the glass fiber fleece. Finally, low salt elution removes the nucleic acids from the glass fiber. "Roche High Pure RNA Tissue Kit" was used for this purpose and protocol is as described below:

1-400 µl Lysis/Binding Buffer and the appropriate amount of frozen tissue (maximum 20–25 mg)was added to a nuclease-free 1.5 ml microcentrifuge tube; the tissue was disrupted and homogenized using a homogenizer.
2-Lysate was centrifuged for 2 minutes (min) at maximum speed in a microcentrifuge and only the collected supernatant was used for subsequent steps .
3-200 µl absolute ethanol was added to the lysate supernatant and mixed well.
4-The High Pure Filter Tube and the Collection Tube were combined and the entire sample was pipetted in the upper reservoir.
5-It was centrifuged for 30 seconds (s) at maximal speed (13,000 x *g*) in a standard table top microcentrifuge.
6-The flowthrough was discarded and again the Filter Tube and the used Collection Tube were reassembled.
12-300 µl Wash Buffer I I was added to the upper reservoir of the Filter Tube, and centrifuged for 2 min at maximum speed (approx. 13,000 x *g*) to remove residual Wash Buffer.

13-The column was carefully removed from the Collection Tube so that the column did not contact the flowthrough as this would result in carryover of ethanol. Residual ethanol may interfere with subsequent reactions.
14-The Collection Tube was discarded. The Filter Tube was inserted in a nuclease-free 1.5 ml reaction tube.
15-100 µl Elution Buffer was added to the upper reservoir of the Filter Tube.
16-It was centrifuged 1 min a t 8000 x *g*. The microcentrifuge tube contained the eluted RNA was stored at -80°C for later analysis.

### 2.2.3. Denaturing RNA Formaldehyde Gel

The overall quality of an RNA preparation may be assessed by electrophoresis on a denaturing agarose gel; this will also give some information about RNA yield. A denaturing gel system is suggested because most RNA forms extensive secondary structure via intramolecular base pairing, and this prevents it from migrating strictly according to its size.

In order to check the integrity and purity of isolated RNA, denaturing RNA formaldehyde gel was prepared as follows:

- 500 ml 5X Formaldehyde gel running buffer was prepared.

5X formaldehyde gel running buffer :
0.1 Molar (M) 3-(N-morpholino)propanesulfonic acid (MOPS) (pH 7.0)
50 miliMolar (mM) Sodium acetate
5 mM EDTA (pH 8.0)

10.3 gr MOPS was dissolved in 400 ml DEPC-treated 50 mM sodium acetate. pH was adjusted to 7.0 with 2N NaOH. Then 10 ml DEPC-treated 0.5 M EDTA (Ph 8.0) was added. The volume of solution was adjusted to 500 ml with DEPC-treated water. Finally, the solution was filter-sterilized by using 0.2 micron Milipore filter and stored at room temperature protected from light.

- 2.2 M %37 Formaldeyhe was prepared form 12,3 M formaldehyde stock solution.
- Then, 50 ml formaldehyde agarose gel was prepared as follows:

0.5 gr. Agarose was heated in 31 ml DEPC-treated water until dissolved, then it was cooled to 60 °C. 10 ml 5X formaldehyde gel running buffer and 9 ml formaldehyde solution were added to the mixture.

- The prepared gel was poured using a comb that will form wells. Then gel was assembled in the tank, an appropriate amount of 1X MOPS running buffer was added to cover the gel by a few millimeters.
- RNA sample was prepared by using Sigma RNA sample loading buffer (5X) :

1 volume of RNA sample and 2 –5 volume of sample loading buffer were mixed. Then mixture was heated to 65°C for 10 minutes.After that, it was chilled on ice immediately.

- Before loading the sample, the gel was prerun for 5 minutes at 5V/cm. After that sample was immediately loaded into the lane of the gel. The gel was run for 40 minutes at 70 V/cm. Then, it was visualised on a UV transilluminator.

### 2.2.4. Determination of the purity and integrity of RNA

To check the isolated RNA for any genomic DNA contamination, control Polymerase Chain Reaction(PCR) was performed by using RNA as template. The reaction was prepared in a sterile PCR tube by adding the compounds shown in Table 2.7. below:

**Table 2.7.** Compounds of control PCR reaction

| Contents | Amount | Volume, µl |
|---|---|---|
| 10XTaq reaction buffer | 1X | 2,5 |
| 10 mM dNTP mixture | 0,2 mM each | 0,5 |
| Template (RNA) | 10 pg-1 µg | 1 |
| Primer (forward) | 1 µM | 0.25 |
| Primer (reverse) | 1 µM | 0.25 |
| Taq polymerase | 1,25u/50 µl | 0.5 |
| Water | X | 20 |
| Total reaction volume | | 25 |

The PCR reaction was performed using a thermal cycler with the program in Table 2.8.:

**Table 2.8.** Control PCR conditions

| Cycles | Temperature | Time |
|---|---|---|
| 1 | 94 ºC | 2 minutes |
| 35 | 94 ºC | 30 seconds |
| | 61 ºC | 30 seconds |
| | 68 ºC | 2 minutes |
| Final extention | 68 ºC | 7 minutes |
| Final hold | 4 ºC | ---------- |

### 2.2.5. cDNA Synthesis

"Fermentas The RevertAid™ First Strand cDNA Synthesis Kit" was used to synthesize cDNA from isolated RNA. Kit is designed for preparation of full-length first strand cDNA from RNA templates.

The RevertAid™ first strand cDNA synthesis kit relies on a genetically engineered version of the Moloney Murine Leukemia Virus reverse transcriptase (RevertAid™ M-MuLV RT) with low RNase H activity. This allows the synthesis of full-length cDNA from long templates (up to 13kb). RevertAid™ M-MuLV RT synthesizes first strand cDNA at sites determined by the type of primer used.

In this study, oligo(dT)18 primers were used. Thus, only mRNA's with 3'- poly(A) tails were used as templates for cDNA synthesis. cDNA Synthesis procedure was performed by following instructions of the manufacturer:

1-The reaction mixture in Table 2.9. was prepared in a tube on ice :

**Table 2.9.** cDNA synthesis reaction mixture contents

| Contents | Amount | Volume, µl |
|---|---|---|
| Total RNA | 10ng-5 µg | 10 µl |
| Oligo(dt)18 primer | 0.5 µg/ µl | 1 µl |
| DEPC-treated water | | 1 µl |
| Total reaction volume | | 12 µl |

2-The mixture was mixed gently and spinned down for 3-5 seconds in a microcentrifuge.

3-The mixture was incubated at 70°C for 5 minutes, Then it was chilled on ice.

4-Reaction tube was placed on ice and the compounds shown in Table 2.10. was added :

**Table 2.10.** cDNA synthesis reaction mixture contents (continued)

| Contents | Amount | Volume , µl |
|---|---|---|
| 5x Reaction buffer | 1X | 4 µl |
| Ribonuclease inhibitor (20 U/ µl) | 20 U | 1 µl |
| 10 mM dNTP mix | 1 mM | 2 µl |
| Total reaction volume | | 19 µl |

5-This reaction mixture was incubated at 37°C for 5 minutes.

6-1 µl reverse transcriptase enzyme (200u/ µl) was added. 20 µl mixture was incubated at 45 °C for 60 minutes.

7-The reaction was stopped by heating the mixture to 70°C for 10 minutes to inactivate the enzyme.

8-It was stored at -20°C.

### 2.2.6. Determination of the integrity of cDNA

PCR reaction was performed to see whether or not the synthesized cDNA worked well. For this purpose 587 bp part of beta-actin gene which is a house-keeping one, was cloned from cDNA. The result was seen with agarose gel electrophoresis.

### 2.2.7. Agarose Gel Electrophoresis

Electrophoresis is used to separate molecules by some property. DNA molecules are seperated based on their size. DNA has a negative charge in solution, so it will migrate to the positive pole in an electric field. In agarose gel electrophoresis the DNA is forced to move through a sieve of molecular proportions that is made of agarose. The result is that large pieces of DNA move slower than small pieces of DNA. The place in the gel that the DNA migrated to is observable under ultraviolet light when the current is turned off and the gel is stained with ethidium bromide.

To prepare 1% agarose gel, 0.4 g of low melting point:

- Agarose was dissolved in 40 ml (small gel) 1x TAE (Tris-acetate-EDTA) buffer.
- The agarose was solubilized in a microwave oven until the agarose was completely dissolved.
- Gel was cooled to $\leq$ 45ºC and ethidium bromide was added to a final concentration of 0.5 μg/ml and mixed through gentle swirling.
- The agarose gel was then poured into a horizontal gel tray, and a comb for forming the sample slots was placed into the gel.
- The gel was solidified for about 30 minutes and then placed into an electrophoresis tank, where the gel was covered by 1x TAE buffer used to make the gel.

The DNA was mixed with loading dye and the sample was placed into a well on the agarose gel. As fragment size control, a MassRuler™ DNA Ladder, Mix (80bp-10kb) and Promega Benchtop 1 kb DNA Ladder were used. Electrophoretic separation was achieved by constant current at 90 mV for 40 minutes.

DNA within agarose gels is only visible when stained with ethidium bromide and can then be visualized under UV light. The gel was placed onto an UV ilumuminator that emits UV light at 302 nm and photographed with a camera connected to a computer. Image files were saved with UVIPhotoMW Version 99.05 for Windows 95 & 98, UVItec Ltd. and subsequently analyzed. The size of the DNA was determined by comparing their mobility with the fragments of the MassRuler and Promega.

### 2.2.8. Primer Design

The gene of interest has to be amplified from genomic or vector DNA by PCR. The first step is the design of the necessary primers. There are some important features while designing the primers :

- Primer Sequence : The 3'-end of the primer molecule has a crucial role for the specificity and sensitivity of PCR It is recommended not to have 3 of more G or C bases at this position. This may stabilize nonspecific annealing of the primer and also it should not have a 3' thymidine, since it is more prone to mispriming than the other nucleotides. Primer pairs should be checked for complementarity at the 3'-end. This often leads to primer-dimer formation.

- Primer length : A primer length of 18-30 bases is usually optimal for most PCR applications. Shorter primers could lead to increased amplification of nonspecific PCR products.

- Melting temperature ($T_m$): The specificity of PCR depends on the melting temperature ($T_m$) of the primers.

- GC content : The GC content of a primer should be between 40 and 60%.

The 3 primer pairs that were designed in the light of these informations are shown in Table 2.11. below:

**Table 2.11.** PCR Primer Pairs

| Primer name | Primer sequence | Tm (ºC) | GC% |
|---|---|---|---|
| Forward primer 1 (Chp80F1) | 5'CGGGAGATGGCAGCGGC3' | 62,5 | 76 |
| Forward primer 2 (Chp80F2) | 5'GCAGCCTTGATTTCCATCCTTACG3' | 58,3 | 50 |
| Forward primer 3 (Chp80F3) | 5'CAAAGGAAGCAGTGAAGCCTAACC3' | 58,2 | 50 |
| Rewerse primer 1 (Chp80R1) | 5'GCAGATGTTTGCCTTGTGACC3' | 57 | 52,4 |
| Rewerse primer 2 (Chp80R2) | 5'AGGTTGGTTAGGCTTCACTGCTTCC3' | 61 | 52,2 |
| Rewerse primer 3 (Chp80R3) | 5'TCACTCCAGCACAGCCATGAG3' | 59,5 | 57,1 |

### 2.2.9. PCR Cloning

Several PCR reactions were performed in order to clone katanin p80 subunit from chicken brain cDNA. Cloning of p80 subunit was achieved by touchdown, hotstart PCR.

Touchdown PCR is a method which increases specificity of PCR reactions. Touchdown PCR cycles begin with higher annealing temperatures than the calculated annealing temperatures of primers. The annealing temperature is decreased by 1 degree Celsius for every subsequent 2 cycles. The primer will anneal at the highest and therefore, least-permissive of nonspecific binding temperature at which it is able. Thus, the first sequence amplified is the one between the regions of greatest primer specificity; it is most likely that this is the sequence of interest. These fragments will be further amplified during subsequent cycles at lower temperatures, and will swamp out the nonspecific sequences to which the primers will bind at those lower temperatures. If the primer initially binds to the sequence of interest at a low temperature, subsequent rounds of PCR can be performed upon the product to further amplify those fragments.

The HotStart PCR involves withholding Taq DNA Polymerase until it has been heated to 95° C. This reduces the occurrence of mis-primes, primer dimers, and premature annealing. A hotstart and touchdown PCR was performed in a sterile PCR tube by adding the compounds shown in the table below:

**Table 2.12.** Compounds of PCR reaction for p80 cloning

| Contents | Amount | Volume, µl |
|---|---|---|
| 10x PCR buffer | 1x | 2,5 |
| MgCl2 (25mM) | 3 mM | 1,5 |
| 10x GC Melt | 1x | 2,5 |
| dNTP(10mM each) | 0,2 mM each | 0,5 |
| Chp80F1 primer | 1 mM | 0,5 |
| Chp80R1 primer | 1 mM | 0,5 |
| Template DNA | 10pg-1µg | 1 |
| Taq DNA Polymerase | 1,25u/50 µl | 0,5 |
| ddH2O | X | 15,5 |
| Total reaction volume | | 25 |

The PCR reaction was performed using a thermal cycler with the program in the table below and the result of PCR reaction was observed by agarose gel electrophoresis:

**Table 2.13.** PCR reaction conditions for p80 cloning

| Cycles | Temperature | Time |
|---|---|---|
| 1 | 94 ºC | 2 minutes |
| 3 | 94 ºC | 30 seconds |
| | 67 ºC | 30 seconds |
| | 68 ºC | 3 minutes |
| 3 | 94 ºC | 30 seconds |
| | 66 ºC | 30 seconds |
| | 68 ºC | 3 minutes |
| 3 | 94 ºC | 30 seconds |
| | 65 ºC | 30 seconds |
| | 68 ºC | 3,5 minutes |
| 3 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 3,5 minutes |
| 6 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 4 minutes |
| 6 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 4,5 minutes |
| 5 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 5 minutes |
| 5 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 5,5 minutes |
| 6 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 6 minutes |
| Final extention | 68 ºC | 10 minutes |
| Final hold | 4 ºC | ---------- |

### 2.2.10. Determination of nucleic acid concentration

Concentrations of nucleic acids were measured by gel electrophoresis. After running several amounts of nucleic acids on agarose gel along with a known amount of DNA, the intensity of the sample band was compared to the band with the known amount of DNA. DNA ladders provided such a standard for a more accurate estimation of DNA concentration.

### 2.2.11. DNA Fragment Isolation from Agarose

Nucleic acids bind specifically to the surface of glass or silica materials in the presence of a chaotropic salt. The binding reaction occurs due to the disruption of the organized structure of water molecules and the interaction with the nucleic acids. Since the binding process is specific for nucleic acids, impurities are efficiently washed away, and the pure DNA is simply eluted with Tris buffer or water.

DNA fragments from agarose gels were isolated by using "QIAquick Gel Extraction Kit". The QIAquick system combines the convenience of spin-column technology with the selective binding properties of a silica-gel membrane. Isolation procedure was performed by following instructions of the manufacturer.

1. The DNA fragment from the agarose gel was excised with a clean, sharp scalpel.
2. The gel slice was weighed in a colorless tube. Three volumes of agarose volume solubilization buffer was added to one volume of gel (100 mg ~ 100 µl).
3. It was incubated at 50°C for 10 min (or until the gel slice has completely dissolved).
4. After the gel slice was dissolved completely, it was checked that the color of the mixture was yellow.
5. One gel volume of isopropanol was added to the sample and mix.
6. A QIAquick spin column was placed in a provided 2 ml collection tube.
7. The sample was applied to the QIAquick column to bind DNA, and centrifuged for 1 min.
8. Flow-through was discarded and QIAquick column was placed back in the same collection tube.

9. 0.5 ml of agarose volume solubilization buffer was added to QIAquick column and centrifuged for 1 min.
10. 0.75 ml of washing buffer was added to QIAquick column to wash and centrifuged for 1 min.
11 The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min at ≥10,000 x *g* (~13,000 rpm).
12. QIAquick column was placed into a clean 1.5 ml microcentrifuge tube.
13. 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O was added to the center of the QIAquick membrane to elute DNA and the column was centrifuged for 1 min at maximum speed.

### 2.2.12. Preparation of Chemically Competent Cells (calcium chloride method)

Since DNA is a very hydrophilic molecule, it will not normally pass through a bacterial cell's membrane. In order to make bacteria take in the plasmid, they must first be made "competent" to take up DNA. This is done by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium.We homemade competent cells according to the following protocol, provided by Sambrook *et al* (Sambrook *et al.*, 1989).

- Working aseptically, XL1 Blue cells (taken from a glycerol stock culture) were streaked out on an LB plate and incubated overnight at 37°C.
- The next day, one bacterial colony was picked and inoculated into 10 ml of LB medium containing tetracyclin in a Falcon tube, and the overnight culture was grown.
- The next day 100 ml LB medium was inoculated with 4 ml of overnight culture solution and was incubated at 37°C in a rotatory shaker. Cell density was measured by a spectrophotometer at $OD_{600}$. When an $OD_{600}$ of 0.6 was reached, the bacteria were transferred to 50 ml prechilled sterile ultracentrifuge tubes and incubated on ice for 10 min.
- The cells were spun down at 1600 *x g* for 10 minutes at 4ºC, the supernatant was discarded

- Bacterial pellet was resuspended in 10 ml ice-cold $CaCl_2$ (pH 7, the solution was filter sterilized through a filter of 0.45 μm pore size) and incubated on ice for 30 minutes.
- Centrifugation was performed again for 5 minutes at the same speed as previously, and the cells were resuspended in 2 ml of $CaCl_2$.
- The cells were used immediately for transformation and/or distributed into prechilled sterile microfuge tubes. The competent cells were stored at – 80ºC in 40 μl aliquots.

**Table 2.14.** $CaCl_2$ solution

| Contents | Concentration | Amount |
|---|---|---|
| $CaCl_2$ | 60 mM | 0.33 g |
| PIPES | 10mM | 0.15 g |
| Glycerol | 15% | 7.5 ml |
| water | X | up to 50 ml |

### 2.2.13. T/A Cloning

T/A Cloning takes advantage of the terminal transferase activity of some DNA polymerases such as Taq polymerase. This enzyme adds a single, 3'-A overhang to each end of the PCR product. This makes it possible to clone this PCR product directly into a linearized cloning vector with single, 3'-T overhangs. The PCR products with dA overhang, are mixed with this vector in high proportion. The complementary overhangs of "T" vector and PCR product will be ligated with T4 DNA ligase.

In order to clone p80 into a cloning vector, "Fermentas InsT/Aclone™ PCR Product Cloning Kit" was used. The Kit is based on a specially designed (linearized and ddT-tailed) cloning vector pTZ57R/T. The 3'-ddT overhangs prevent recircularization of the vector during ligation, resulting in high cloning yields. Ligated PCR fragment is directly transformed into competent XL1Blue cells. Besides that recombinant clones can be identified by blue/white selection, since the vector is *lacZ* genetically marked.

### 2.2.13.1. Ligation for T/A Cloning

Ligation is the process in which linear DNA fragments join together with covalent bonds. More specifically, DNA ligation involves creating a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another.

T4 DNA ligase is the enzyme that is used to ligate DNA fragments, which originates from the T4 bacteriophage. This enzyme will ligate DNA fragments having overhanging, cohesive ends that are annealed together.

Ligation mixture was made by adding compounds depicted in the table below and ligation mixture was incubated overnight at room temperature:

**Table 2.15.** Compounds of ligation reaction for T/A cloning

| Contents | Amount | Volume, µl |
|---|---|---|
| pTZ57R/T Plasmid DNA (0,055 µg/ µl) | 0,165 µg | 1,5 |
| Insert DNA | 0,755 µg | 5 |
| 10 x buffer | 1x | 1,5 |
| Ligase | 20000u/ µl | 0,5 |
| Total reaction volume | | 15 |

### 2.2.13.2. Transformation for T/A Cloning

The term, transformation, is used to describe the introduction of plasmid DNA into bacterial cell which can be performed either by an electrical or by a chemical method. In this study, the chemical method which consists of a heat shock to introduce the DNA into the host was used. A short protocol is as follows:

1-40 µl of cells per transformation were thawed on ice.

2-1/5 volume of a DNA Ligation reaction was added to each tube. The mixture was incubated on ice for 30 minutes.

3-Then tubes were heat shocked in a 42°C water bath for 45 seconds.

4-Tubes were returned to ice for 2 minutes.

5-80 µl SOC was added to each tube and the eppendorf tube was shaked at 250 rpm for 1 hour at 37°C.

6-IPTG/X-Gal solution was added to the mixture so as to select recombinant proteins via blue/white selection method.

7-100 µl of the reaction mixture was plated onto LB agar containing selective antibiotics and incubated at 37ºC overnight (16 hours).

### 2.2.13.3. Colony PCR for T/A Cloning

Colony PCR is used to determine whether or not a specific colony on a plate has desired sequence. Either universal primers or sequence specific primers can be used for this purpose. In this study, both M13 F/R Universal primers (Invitrogen) and p80 sequence specific primers were used. A brief procedure of colony PCR is shown below:

- After overnight incubation of transformed cells at 37 °C, ten white colonies which were supposed to have the sequence of interest were selected.
- Each of ten colonies was picked and resuspended in 50 µl sterile water in sterile eppendorf tubes.
- Then resuspended cells were heated for 5 minutes at 100°C to disrupt the bacterial cells.
- A PCR master mix was prepared for all ten colonies by adding the compounds in the table below:

**Table 2.16.** Contents of colony PCR reaction for T/A cloning

| Contents | Amount | Volume, µl |
|---|---|---|
| 10x PCR buffer | 1x | 25 |
| MgCl2 (25mM) | 3 mM | 15 |
| 10x GC Melt | 1x | 25 |
| dNTP(10mM each) | 0,2 mM each | 5 |
| M13 forward primer | 1 mM | 5 |
| M13 reverse primer | 1 mM | 5 |
| Taq DNA Polymerase | 1,25u/50 µl | 2 |
| ddH2O | X | 158 |
| Total reaction volume | | 240 |

- Prepared master mix was distributed equally into each tube and added to PCR tubes.
- After that, 1 µl of resuspended cells of each colony was added to PCR mixture.
- Colony PCR reactions were performed using a thermal cycler with the program in Table 2.17. and the results of PCR reactions were observed on %1 agarose gel:

**Table 2.17.** Colony PCR conditions for T/A cloning

| Cycles | Temperature, °C | Time |
|---|---|---|
| 1 | 94 | 2 minutes |
| 6 | 94 | 30 seconds |
| | 55 | 30 seconds |
| | 68 | 3 minutes |
| 6 | 94 | 30 seconds |
| | 55 | 30 seconds |
| | 68 | 3,5 minutes |
| 6 | 94 | 30 seconds |
| | 55 | 30 seconds |
| | 68 | 4 minutes |
| 6 | 94 | 30 seconds |
| | 55 | 30 seconds |
| | 68 | 4,5 minutes |
| 5 | 94 | 30 seconds |
| | 55 | 30 seconds |
| | 68 | 5 minutes |
| 5 | 94 | 30 seconds |
| | 55 | 30 seconds |
| | 68 | 5,5 minutes |
| 6 | 94 | 30 seconds |
| | 55 | 30 seconds |
| | 68 | 6 minutes |
| Final extention | 68 | 10 minutes |
| Final hold | 4 | ---------- |

### 2.2.14. Plasmid DNA Preperation ( small scale)

Plasmid mini preparation was performed using “Roche High Pure Plasmid Isolation Kit” for small-scale (mini) preparations, following instructions of the manufacturer. The principle of this purification is as follows: alkaline lysis releases plasmid DNA from bacteria and RNase removes all the RNA in the lysate. Then, in the presence of a chaotropic salt (guanidine HCl), plasmid DNA binds selectively to glass fiber fleece in a centrifuge tube.

The DNA remains bound while a series of rapid "wash-and-spin" steps remove contaminating bacterial components. Finally, low salt elution removes the DNA from the glass fiber fleece.

The protocol is as follows:

- A single bacterial colony was picked and inoculated into 6 ml LB media (with amphicilline) containing Falcon tube, and grown overnight with shaking (250 rpm) at 37ºC.
- The following day, 4 ml of the culture was distributed into 2 eppendorf tubes (2 ml each tube), and the bacteria were recovered by centrifugation for 5 minutes at 14.000 x *g*. The supernatants were discarded.
- The bacterial pellet was resuspended in 50 µl of suspension buffer in each eppendorf tube separately and then collected to one eppendorf tube (250 µl suspension buffer in total). Suspension buffer contains RNase which removes bacterial RNA.
- To lyse the cells, 250 µl lysis buffer was added (contains NaOH), mixed by inverting the tube 6 times and incubated at room temperature for up to 5 minutes.
- Lysis was stopped by addition of 350 µl ice-cold binding buffer. Tube was again inverted 6 times and incubated on ice for up to 5 minutes.
- The mixture was centrifuged for 10 minutes at 14.000 x *g* and the supernatant was transferred to a filter tube. Chromosomal DNA was precipitated with cellular debris during centrifugation and this supernatant contains the plasmid DNA.
- Again centrifugation for 1 minute at maximum speed was performed. Plasmid DNA is bound to the glass fibers pre-packed in the filter tube. Supernatant was discarded from the collection tube.
- To wash the cells, 700 µl of wash buffer was added to the filter tube and centrifuged at maximum speed for 1 minute. Supernatant from collection tube was discarded.
- To elute the DNA 100 µl elution buffer was added, and the DNA solution was obtained by centrifugation for 1 minute at full speed.

### 2.2.15. DNA Sequencing

DNA sequencing enables to perform a thorough analysis of DNA because it provides the most basic information about the sequence of nucleotides.

DNA sequencing method is based on the use of dideoxynucleotides(ddNTP's) in addition to the normal nucleotides (NTP's) found in DNA. Dideoxynucleotides are essentially the same as nucleotides except they contain a hydrogen group on the 3' carbon instead of a hydroxyl group (OH). These modified nucleotides, when integrated into a sequence, prevent the addition of further nucleotides. (Speed, 1992).This occurs because a phosphodiester bond cannot form between the dideoxynucleotide and the next incoming nucleotide, and thus the DNA chain is terminated. In this method, all the reactions start from the same nucleotide and end with a specific base. Thus in a solution where the same chain of DNA is being synthesized over and over again, the new chain will terminate at all positions where the nucleotide has the potential to be added because of the integration of the dideoxynucleotides (Russell, 2002). In this way, bands of all different lengths are produced and DNA fragments are marked and the sequence can be detected by excitation with a laser and detection with photodiodes in a sequencing machine.

The constructed plasmid was verified by DNA sequencing using Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems). The sequence reaction was prepared in a sterile PCR tube by adding the compounds shown in the Table 2.18 and PCR was carried out using the program in Table 2.19:

**Table 2.18.** Contents of DNA sequencing PCR reaction

| Contents | Amount | Volume, µl |
|---|---|---|
| Big dye reaction mix | X | 2 |
| 5X sequence mixture | X | 2 |
| Template DNA | 360 ng | 1 |
| M13forward or reverse | 25 mM | 0.5 |
| Water | X | 4.5 |
| Total reaction volume | X | 10 |

**Table 2.19.** Sequencing PCR conditions

| Cycles | Temperature | Time |
|---|---|---|
| 1 | 95 ºC | 5 minutes |
| 40 | 95 ºC | 45 seconds |
| | 55 ºC | 45 seconds |
| | 60 ºC | 4 minutes |
| Final extention | 4 ºC | |

### 2.2.16. Sequence PCR Purification

After sequence PCR was completed, PCR product was purified by using following protocol:

1- 2 µl 3 M NaAc and 50 µl 95% cold ethanol and PCR product were mixed in an eppendorf tube.
2- The mixture was incubated for 30 minutes on ice.
3- Then it was centrifuged for 30 minutes at 14.000 rpm.
4- Supernatant was discarded and pellet was resuspended in 250 µl 70% cold ethanol.
5- It was centrifuged for 30 minutes at 14.000 rpm.
6- Supernatant was discarded and pellet was dried at 95 ºC for 5 minutes.
7- Then pellet was resuspended in 20 µl formamide and denatured at 95 ºC for 2-3 minutes.

### 2.2.17. Sequence Alignments

Nucleotide alignments were made with ClustalW tool, available at http://www.ebi.ac.uk/clustalw/index.html and also with BLAST tool, available at http://www.ncbi.nlm.nih.gov/BLAST/ (nucleotide-nucleotide BLAST (blastn)).

### 2.2.18. Subcloning of p80-katanin into DsRed2-N1 expression vector

#### 2.2.18.1. DNA cleavage with restriction endonucleases for pDSRed2-N1 vector

Restriction endonucleases were first discovered in *E. coli* strains that appeared to be restricting the infection by certain bacteriophages. Restriction enzymes therefore are believed to be a mechanism evolved by bacteria to resist viral attack and to help in the removal of viral sequences. A restriction enzyme cuts only double-helical segments that contain a particular nucleotide sequence, and it makes its incisions only within that sequence known as a "recognition sequence". Once it encounters its particular specific recognition sequence, it will bond to the DNA molecule and makes one cut in each of the two sugar-phosphate backbones of the double helix. The positions of these two cuts, both in relation to each other, and to the recognition sequence itself, are determined by the identity of the restriction endonuclease used to cleave the molecule in the first place. Some enzymes make strand incisions immediately opposite one another, producing "blunt end" DNA fragments. Most enzymes make slightly staggered incisions, resulting in "sticky ends", out of which one strand protrudes.

In this study, p80 was cloned into pTZ57R/T cloning vector, but, in reverse orientation. Hence, it had to be subcloned into pEGFP-N1 fluorescent expression vector changing its orientation. Selection of appropriate restriction enzymes for this purpose should have been done according to the following requirements:

- they have to cut both T/A and expression vectors;
- their sequence in the vectors should be different, i.e. 5' restriction site on T/A vector should be in 3' compared to another restriction site on expression vector;
- restriction endonucleases should not cut inside the p80 subunit, preferably.

Since there were not any suitable enzymes that provided these requirements, p80 had to be first subcloned into another suitable fluorescent expression vector to change its orientation. DsRed2-N1 vector and Sal I and Kpn I enzymes met the requirements for restriction reaction.

Normally DNA was cleaved at 37ºC for 4 hours with 1 X buffer (supplied by the manufacturer).

**Table 2.20.** Restriction reaction mixture

| Contents | Amount | Volume, µl |
|---|---|---|
| Plasmid DNA (DsRed2-N1) | 3 µg | 20 |
| Sal I | 3 units | 2 |
| Kpn I | 8 units | 2 |
| BamHI buffer (10x) | 1x | 3 |
| Water | | 3 |
| Total reaction volume | | 30 |
| Plasmid DNA(pTZ57R/T-p80) | 4 µg | 20 |
| Sal I | 3 units | 2 |
| Kpn I | 8 units | 2 |
| BamHI buffer (10x) | 1x | 3 |
| Water | | 3 |
| Total reaction volume | | 30 |

### 2.2.18.2. Ligation

After restriction reaction was completed, digested DNA fragments were run on agarose gel and desired DNA bands were isolated from agarose as decribed before.

Ligation reaction was performed by adding compounds in the table below:

**Table 2.21.** Ligation reaction mixture

| Contents | Amount | Volume, µl |
|---|---|---|
| Plasmid DNA (DsRed2-N1) | 100 ng | 1 |
| Insert DNA(p80) | 300ng | 5 |
| 10 x buffer | 1x | 1 |
| Ligase | 20000u/µl | 1 |
| Water | | 2 |
| Total reaction volume | | 10 |

### 2.2.18.3. Transformation

Ligation mixture was transformed into competent XL1 Blue cells as described in T/A Cloning section. The only difference is that IPTG/X-Gal solution was not added while plating transformation mixture, since plasmid DNA did not have blue/white selection system.

### 2.2.18.4. Colony PCR

10 colonies were selected from transformation plate and resuspended in water. Then a PCR master mix was prepared by adding the compounds in the table below:

**Table 2.22.** Colony PCR reaction mixture

| Contents | Amount | Volume, µl |
|---|---|---|
| 10x PCR buffer | 1x | 25 |
| MgCl2 (25mM) | 3 mM | 15 |
| 10x GC Melt | 1x | 25 |
| dNTP(10mM each) | 0,2 mM each | 5 |
| Chp80F1 primer | 1 mM | 5 |
| Chp80R1 primer | 1 mM | 5 |
| Taq DNA Polymerase | 1,25u/50 µl | 2 |
| ddH2O | X | 158 |
| Total reaction volume | | 240 |

Prepared master mix was distributed equally into each tube and added to PCR tubes. After that, 1 µl of resuspended cells of each colony was added to PCR mixture. Colony PCR reactions were performed using a thermal cycler with the program in the Table 2.23. and result of PCR reaction was observed on agarose gel :

**Table 2.23.** Colony PCR conditions

| Cycles | Temperature | Time |
|---|---|---|
| 1 | 94 ºC | 2 minutes |
| 3 | 94 ºC | 30 seconds |
| | 67 ºC | 30 seconds |
| | 68 ºC | 3 minutes |
| 3 | 94 ºC | 30 seconds |
| | 66 ºC | 30 seconds |
| | 68 ºC | 3 minutes |
| 6 | 94 ºC | 30 seconds |
| | 65 ºC | 30 seconds |
| | 68 ºC | 3,5 minutes |
| 6 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 4 minutes |
| 6 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 4,5 minutes |
| 5 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 5 minutes |
| 5 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 5,5 minutes |
| 6 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 6 minutes |
| Final extention | 68 ºC | 10 minutes |
| Final hold | 4 ºC | ---------- |

### 2.2.19. Subcloning of p80-katanin into pEGFP-N1 expression vector

#### 2.2.19.1. Restriction reaction for pEGFP-N1 vector

Restriction enzymes that were suitable for cleaving pEGFP-N1 vector and DsRed2N1-p80 at the same time were Sma I and Hind III. A sequencial restriction was performed since Sma I works at 30ºC while Hind III works at 37ºC.

Firstly, restriction reaction was performed for Sma I for 2 hours at 30ºC then Hind III and some other compounds were added to mixture and incubated for 2 hours at 37ºC. Restriction reactions for Sma I and Hind III are shown in the tables below:

**Table 2.24.** Restriction reaction mixture for Sma I

| Contents | Amount | Volume, µl |
|---|---|---|
| Plasmid DNA(pEGFP-N1) | 4 µg | 20 |
| Sma I | 1,2 units | 1 |
| 10xBuffer Tango | 1x | 2,5 |
| Water | | 1,5 |
| Total volume | | 25 |
| DsRed2N1-p80 | 3 µg | 20 |
| Sma I | 1,2 units | 1 |
| 10xBuffer Tango | 1x | 2,5 |
| Water | | 1,5 |
| Total volume | | 25 |

**Table 2.25.** Restriction reaction mixture for Hind III

| Contents | Amount | Volume, µl |
|---|---|---|
| PlasmidDNA(pEGFP-N1)reaction mixture | 4 µg | 25 |
| Hind III | 10 units | 2 |
| 10xBuffer Tango | 1x | 0,5 |
| Water | | 2,5 |
| Total volume | | 30 |
| DsRed2N1-p80 reaction mixture | 3 µg | 25 |
| Hind III | 10 units | 2 |
| 10xBuffer Tango | | 0,5 |
| Water | | 2,5 |
| Total volume | | 30 |

### 2.2.19.2. Ligation

Digested DNA fragments were run on agarose gel and desired DNA bands were isolated from agarose as decribed before.

Since Sma I creates blunt ends, PEG solution that is known to enhance blunt-end ligation was added to the ligation reaction. Ligation reaction was performed by adding compounds in the table below:

**Table 2.26.** Ligation reaction mixture contents

| Contents | Amount | Volume, µl |
|---|---|---|
| Plasmid DNA (pEGFP-N1) | 50 ng | 1 |
| Insert DNA(p80) | 200 ng | 7 |
| 10 x buffer | 1x | 1,5 |
| Peg 4000 | %10 | 1,5 |
| Ligase | 20000u/ µl | 2 |
| Water | | 2 |
| Total reaction volume | | 15 |

### 2.2.19.3. Transformation

Ligation mixture was transformed into competent XL1 Blue cells as described previous sections.

### 2.2.19.4. Colony PCR

10 colonies were selected from transformation plate and resuspended in water. Then a PCR master mix was prepared by adding the compounds in Table 2.27:

**Table 2.27.** Colony PCR reaction mixture

| Contents | Amount | Volume, µl |
|---|---|---|
| 10x PCR buffer | 1x | 25 |
| MgCl2 (25mM) | 3 mM | 15 |
| 10x GC Melt | 1x | 25 |
| dNTP(10mM each) | 0,2 mM each | 5 |
| Chp80F1 primer (20 micromolar) | 1 mM | 5 |
| Chp80R1 primer (20 micromolar) | 1 mM | 5 |
| Taq DNA Polymerase | 1,25u/50 µl | 2 |
| ddH2O | X | 158 |
| Total reaction volume | | 240 |

Prepared master mix was distributed equally into each tube and added to PCR tubes. After that, 1 µl of resuspended cells of each colony was added to PCR mixture. Colony PCR reactions were performed using a thermal cycler with the program in the table below and the result of PCR reaction was observed on agarose gel:

**Table 2.28.** Colony PCR conditions

| Cycles | Temperature | Time |
|---|---|---|
| 1 | 94 ºC | 2 minutes |
| 3 | 94 ºC | 30 seconds |
| | 67 ºC | 30 seconds |
| | 68 ºC | 3 minutes |
| 3 | 94 ºC | 30 seconds |
| | 66 ºC | 30 seconds |
| | 68 ºC | 3 minutes |
| 6 | 94 ºC | 30 seconds |
| | 65 ºC | 30 seconds |
| | 68 ºC | 3,5 minutes |
| 6 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 4 minutes |
| 6 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 4,5 minutes |
| 5 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 5 minutes |
| 5 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 5,5 minutes |
| 6 | 94 ºC | 30 seconds |
| | 64 ºC | 30 seconds |
| | 68 ºC | 6 minutes |
| Final extention | 68 ºC | 10 minutes |
| Final hold | 4 ºC | ---------- |

## 3. RESULTS

### 3.1. RNA Isolation From Chicken Brain

Adult chicken brain was dissected. 20 mg brain tissue was first weighed then it was disrupted and homogenized by a homogenizer in the lysis buffer. RNA isolation was performed from lysate by using "Roche High Pure RNA Tissue Kit".

Integrity and purity of the isolated RNA were checked by agarose denaturing gel electrophoresis technique. 18S and 28S Ribozomal RNAs appeared as two sharp bands on denaturing gel (Figure 3.1).


**Figure 3.1**: Isolated 18S and 28S rRNA bands on denaturing agarose gel.

In addition to this, PCR was also performed by using isolated RNA as a template to see whether there was genomic DNA (gDNA) contamination. PCR result was observed on agarose gel and no amplification product was seen on the gel, except primer dimers due to annealing of primer pairs to each other as a result of complementary sequences (Figure 3.2). It confirmed that there was not genomic DNA contamination in the RNA preparation.

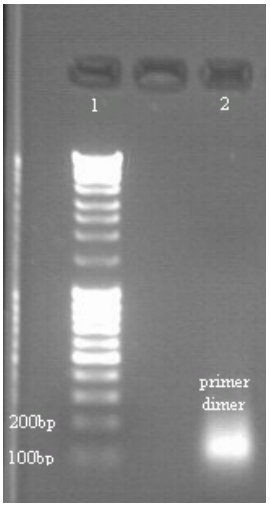


**Figure 3.2:** PCR was performed to check genomic DNA contamination using RNA as a template. (Lane 1: Fermentas Mass Ruler Mix, DNA Marker, Lane 2: PCR result).

### 3.2. cDNA Synthesis From Isolated RNA

cDNA was synthesized from chicken brain RNA by using "Fermentas RevertAid™ H Minus First Strand cDNA Synthesis Kit" which is designed for reverse transcriptase PCR. Oligo(dT) primers were used for this purpose.

After cDNA synthesis reaction, a control PCR was performed to see if synthesized cDNA would work well. For this reason, a 587 bp fragment of beta-actin gene which is a housekeeping one, was cloned with beta-actin specific primers. After agarose gel electrophoresis, 587 bp fragment was observed on the gel ( Figure 3.3), as expected.

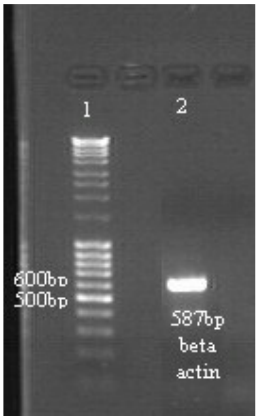


**Figure 3.3:** Control PCR for sytnhesized cDNA. (Lane 1: Fermentas Mass Ruler Mix, DNA Marker, Lane 2: 587 bp beta-actin band).

### 3.3. PCR Cloning of Katanin p80 Subunit

In order to clone 1974 bp neuronal katanin p80 subunit, specific primers were designed at first. Predicted mitotic katanin p80 sequence data from http://www.ncbi.nih.gov/ (NCBI- XM_413997.) was used to design the primers.

After numerous PCR reactions were performed for optimization, 1974 bp katanin p80 subunit was cloned by using specifically designed primers. Reaction mixture was run on agarose gel and 1974 bp band was observed ( Figure 3.4).

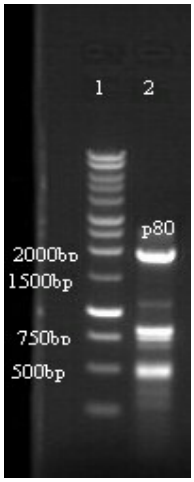


**Figure 3.4:** Cloned p80 katanin subunit was run on agarose gel. (Lane 1: Promega BenchTop 1 kb DNA Ladder, Lane 2: top band indicates p80 and other lower bands indicates unspecific amplification products).

Cloned p80 was then ligated into pTZ57R/T cloning vector included in "Fermentas Ins T/A Cloning Kit". Ligation was performed mixing DNA at a 1:5, vector:insert molar ratio into 15 µl reaction volume. This construct, is then, transfected into competent *E.Coli* XL1Blue Strain and 10 of the transformation colonies were selected from selective ampicillin plates by using blue/white screening method. Colony PCR was performed with these colonies using M13 Forward/Reverse Universal primers (Invitrogen). After that, PCR reactions were run on agarose gel and it was shown that all of ten colonies had the 1974 bp p80 insert (Figure 3.5). Plasmid-p80 construct was then purified from one of these colonies with "Roche High Pure Plasmid Isolation Kit".

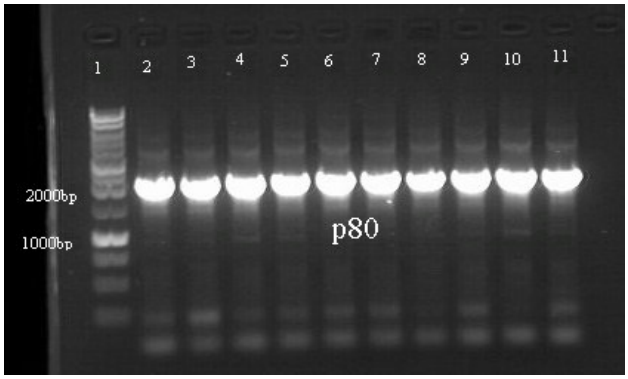


**Figure 3.5:** Colony PCR results were observed by agarose gel electrophoresis.( Lane 1: Promega BenchTop 1 kb DNA Ladder, Lane 2-11: PCR results of different colonies).

### 3.4. DNA Sequencing

Sequence PCR was performed in order to confirm the sequence of p80 katanin by using both M13F/R Universal Primers and sequence specific primers. After PCR purification of the product, sequencing was carried out with Applied Biosciences 3100-Avant DNA Sequencer.

The obtained nucleotide sequence for chicken neuronal p80 katanin was submitted to GenBank with the accession number DQ410670. Sequence result was aligned with a sequence of p80 cloned from mitotic chicken bursal lymphocyte cells (Source: http://www.ncbi.nih.gov/ Reference number: NCBI- NM_001030559) by using ClustalW tool, available at http://www.ebi.ac.uk/clustalw/index.html and BLAST tool, available at http://www.ncbi.nlm.nih.gov/BLAST/ (nucleotide-nucleotide BLAST (blastn)).Alignment results showed high similarity of 99% between two p80 sequences.

Sequence of cloned chicken katanin p80 is shown in the Table 3.1. below:

**Table 3.1.** Chicken neuronal p80 katanin sequence

| | |
|---|---|
| ATGGCAGCGGCCGTCGTCACCAAGACGGCCTGGAAGCTGCAGGAGATCGTAGCTCACAGC | 60 |
| AGCAATGTGTCCTCATTAGTCCTGGGGAAGAGCACGGGCCGGCTGCTGGCAACTGGAGGA | 120 |
| GATGACTGTCGGGTCAACGTGTGGTCAGTTAACAAGCCCAACTGCGTCATGAGCTTGACA | 180 |
| GGCCACACGACACCCATTGAGAGCCTACAGATCAGTGCAAAGGAAGAACTCATTGTTGCA | 240 |
| GGGTCCCAGTCAGGGTCCATTCGAGTCTGGGACCTGGAAGCTGCCAAAATTCTCCGTACG | 300 |
| TTACTTGGTCACAAGGCAAACATCTGCAGCCTTGATTTCCATCCTTACGGAAGTTTCGTG | 360 |
| GCATCTGGCTCTTTAGATACAGACATTAAGCTCTGGGACGTACGAAGAAAAGGCTGCATC | 420 |
| TTCAAGTATAAGAGCCACACACAAGCAGTGAGATGTCTTCGGTTTAGTCCTGATGGCAAG | 480 |
| TGGTTGGCCTCTGCTGCCGATGATCACACTGTGAAGCTGTGGGATCTGACTGCTGGGAAG | 540 |
| GTAATGTTTGAGTTTACAGGACATTCTGGCCCAGTCAACGTCGTTGAATTCCATCCCAGT | 600 |
| GAATACCTTTTGGCTTCTGGCAGCTCTGACAGGACCATTCGTTTCTGGGACTTGGAGAAG | 660 |
| TTTCACGTTGTGAGCTGTATTGAAGAGGAGGCTACTCCTGTCAGGTGTATTCTTTTCAAC | 720 |
| CCAGATGGCTGCTGCTTGTATGGTGGCTTCCAGGATTCTCTGCGTGTGTACGGCTGGGAG | 780 |
| CCAGAGCGCTGTTTCGATGTGGTCGTAGTGAACTGGGGAAAAGTAGCTGACTTATCTGTC | 840 |
| TGCCACAACCAGCTGATAGGAGTTTCCTTTGCACAAAGCACAGTCTCTTCCTTTGTTGTG | 900 |
| GATCTCAGCAGAGTCACCAAGTCAGGTTCAGTTCCTCATGGGCTGCTCAGGAACAACGAG | 960 |
| CTTCTGGCTCAGCCCACTCCCACAGGGTCCTCCCTTCGTCGCAGCTATGACAGACCCTCA | 1020 |
| ACTAGCTGCAGCAAGCCTCAGAGAGTGAAGCACAGTTCAGAGAGCGAGAGGCGCAATCCC | 1080 |
| AGCAGTGAAGAGGACCGGGATGAGAAGGAATCCAAGGCTGAGATCCAGAACCCAGAGGAT | 1140 |
| TACAAAGAGATCTTCCAGCCCAGGAATGCCATCTCTCGAACTCCTCCTCATATCAATGAG | 1200 |
| CCCTTTCCAGCCCCCCCAGAGGATGAGCCCATAACTGCAAAGGAAGCAGTGAAGCCTAAC | 1260 |
| CAACCTGTGGAAGTCCAGACCCCGCTGCCAACGCAAGAGCTTCCTGAGACATTTCAGAGG | 1320 |
| CCACCAATTGCTTCCTCAACTCCTATGCCCAGAGCAGAGCCATCAGTCATTCCTGCAGCC | 1380 |
| AGGAACGAGCCCATTGGCCTGAAAGCCTCTGACTTCCTACCAGCTCTGAAAAACCAAAGC | 1440 |
| CAGGCTGAACTCACGGATGAAGAAATCATGTCCCAGATCAGGAAAGGCCACAAGACTGTG | 1500 |
| TGCATGGTGCTCACCAGCCGCCACAAGAATCTGGACACTGTGAGGGCTGTATGGAGCACC | 1560 |
| AGTGACATGAAGAACTCTGTGGACGCTGCGGTAGCAACCAACGACCTGTCTGTTGTTGTG | 1620 |
| GACCTCTTGAACATTGTCAACCAAACTGCATCTCTCTGGAAGTTGGATTTATGCACCGTA | 1680 |
| GTTCTGCCTCAAATAGAAAAACTTCTCCAAAGTAAATATGAAAGTTATGTGCAAACGGGC | 1740 |
| TGCACCTCCTTGAAACTCATCCTTCAGAGATTCCTGCCACTGATCACCGACATACTTGCT | 1800 |
| GCACCACCTTCTGTTGGAGTGGACATCACCAGAGAGGAGAGGCTCCATAAATGCAGGCTG | 1860 |
| TGCTACAAGCAGCTGAAAAACATCAGCAACATTGTCAAGAACAAATCCGGGCTCAGCGGC | 1920 |
| CGCCACGGTAGTGCCTTCAGAGAACTGCATCTCCTCATGGCTGTGCTGGAGTGA | 1974 |

DNA sequence of p80 was converted into amino acid sequence by using TRANSLATOR tool, available at http://www.justbio.com/tools.php. P80 amino acid sequence is shown in Table 3.2. below:

**Table 3.2.** Amino acid sequence of chicken neuronal p80 katanin

"MAAAVVTKTAWKLQEIVAHSSNVSSLVLGKSTGRLLATGGDDCRVNVWSVNKPN
CVMSLTGHTTPIESLQISAKEELIVAGSQSGSIRVWDLEAAKILRTLLGHKANICSLDF
HPYGSFVASGSLDTDIKLWDVRRKGCIFKYKSHTQAVRCLRFSPDGKWLASAADDH
TVKLWDLTAGKVMFEFTGHSGPVNVVEFHPSEYLLASGSSDRTIRFWDLEKFHVVSC
IEEEATPVRCILFNPDGCCLYGGFQDSLRVYGWEPERCFDVVVVNWGKVADLSVCH
NQLIGVSFAQSTVSSFVVDLSRVTKSGSVPHGLLRNNELLAQPTPTGSSLRRSYDRPST
SCSKPQRVKHSSESERRNPSSEEDRDEKESKAEIQNPEDYKEIFQPRNAISRTPPHINEP
FPAPPEDEPITAKEAVKPNQPVEVQTPLPTQELPETFQRPPIASSTPMPRAEPSVIPAAR
NEPIGLKASDFLPALKNQSQAELTDEEIMSQIRKGHKTVCMVLTSRHKNLDTVRAVW
STSDMKNSVDAAVATNDLSVVVDLLNIVNQTASLWKLDLCTVVLPQIEKLLQSKYES
YVQTGCTSLKLILQRFLPLITDILAAPPSVGVDITREERLHKCRLCYKQLKNISNIVKNK
SGLSGRHGSAFRELHLLMAVLE"

### 3.5. Subcloning of p80 into pEGFP-N1 Fluorescent Expression Vector

P80 was cut out from pTZ57R/T cloning vector with appropriate restriction enzymes in order to be subcloned into pEGFP-N1 expression vector. The orientation of p80 had to be changed while subcloning it into pEGFP-N1 since it was ligated in reverse orientation into pTZ57R/T. Appropriate restriction enzymes for this purpose were not easily available to us. Hence p80, first, cut out and ligated into a suitable vector, DsRed2-N1 to change solely its orientation.

Kpn I and Sal I restriction endonucleases were chosen to be the most appropriate ones for this purpose. Kpn I and Sal I cut out p80 from pTZ57R/T by cutting the vector from positions 627. and 667. , respectively. DsRed2-N1 was also cut with Sal I and Kpn I at positions 639 and 649., respectively.

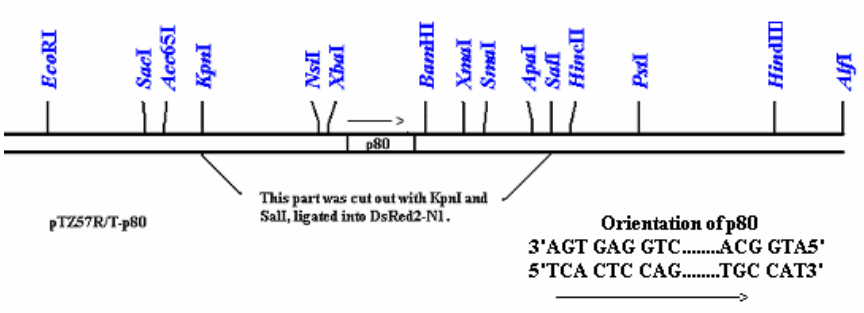


**Figure 3.6**: Restriction map for pTZ57R/T-p80 construct.

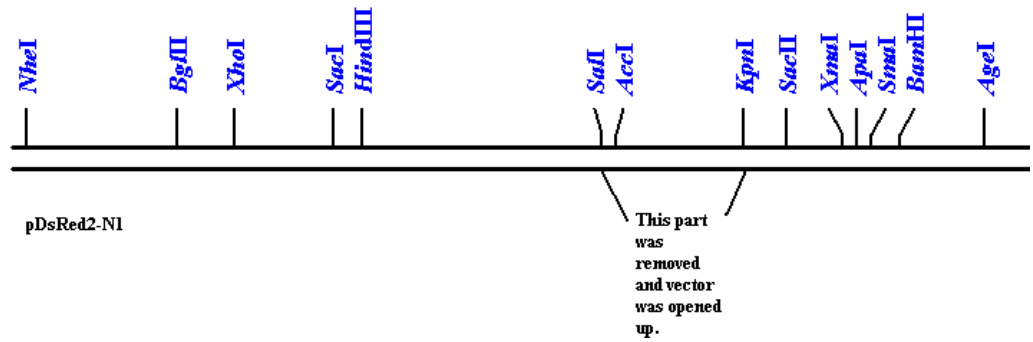


**Figure 3.7:** Restriction map for pDsRed2-N1 vector.

Restriction reactions were performed with these enzymes for both pTZ57R/T- p80 and DsRed2-N1. After restrictions were completed, they were run on agarose gel and fragments close by size to pTZ57R/T vector part (2,9 kb) and p80 insert part (1,98 kb) and opened up DsRed2-N1 part (4,7 kb) appeared (Figure 3.8). Then p80 and opened up DsRed2-N1 were extracted from agarose gel for ligation procedure.

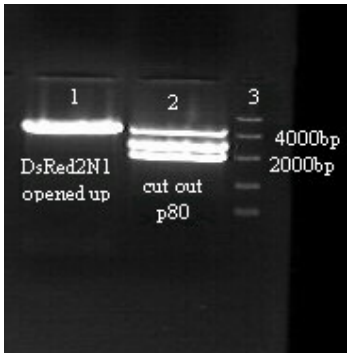


**Figure 3.8:** Restriction-digestion of DsRed2N1 vector and pTZ57RT-p80 construct. (Lane 1: 4,7 kb. opened up DsRed2N1, Lane 2:bottom band belongs to 1980 bp. p80, Lane 3: Fermentas, Fast Ruler, High Range Dna Ladder).

p80-DsRed2-N1 construct was obtained by ligation reaction and this construct was transformed into *E.Coli* XL1 Blue Strain.10 of the transformation colonies, then, were selected in order to perform colony PCR. PCR results were observed on agarose gel (Figure 3.9). It was shown that all ten colonies had the insert. p80-DsRed2-N1 was purified from transformation colonies with "Roche High Pure Plasmid Purification Kit".

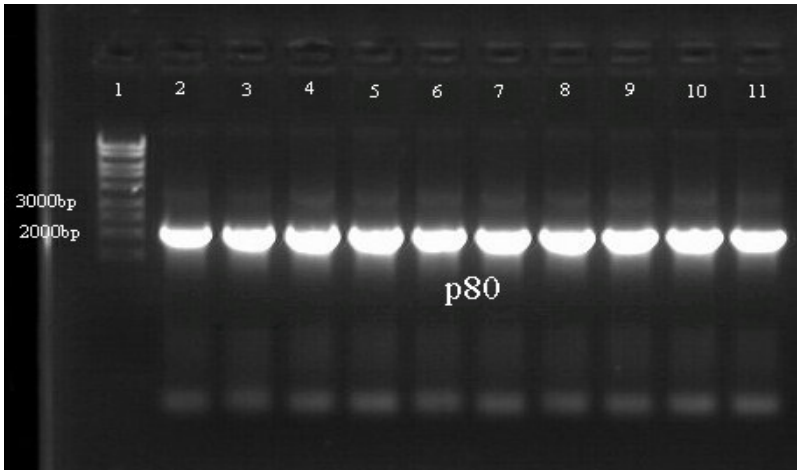


**Figure 3.9:** Transfomation colonies were checked by colony PCR for the presence of p80-DsRED2-N1 construct. (Lane 1: Fermentas, Mass Ruler, High Range DNA Ladder, Lane 2-11: PCR results of different colonies,).

After that, p80 was cut out from DsRed2-N1 with Hind III and Sma I restriction endonucleases, while pEGFP-N1 was opened up with the same enzymes. Hind III cut DsRed2-N1 at position 622, whereas Sma I cut at position 658.

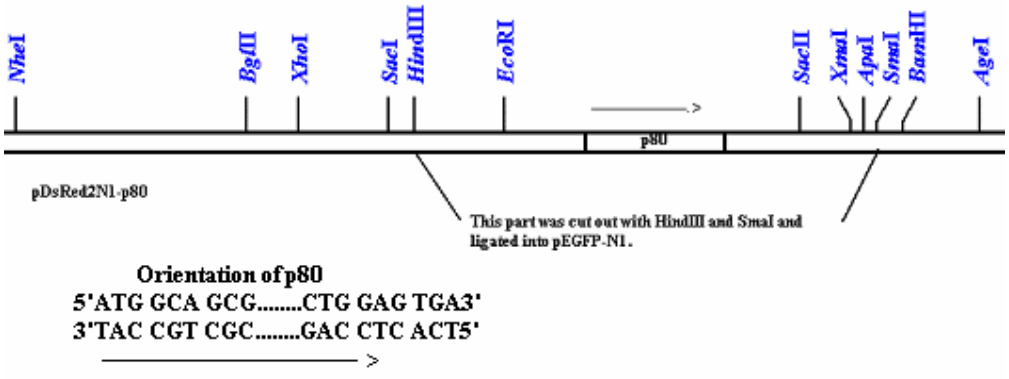


**Figure 3.10:** Restriction map for pDsRed2-N1-p80 construct.

Similar to that, Hind III and Sma I opened up pEGFP-N1 by cutting at positions 623 and 659 bp., respectively.

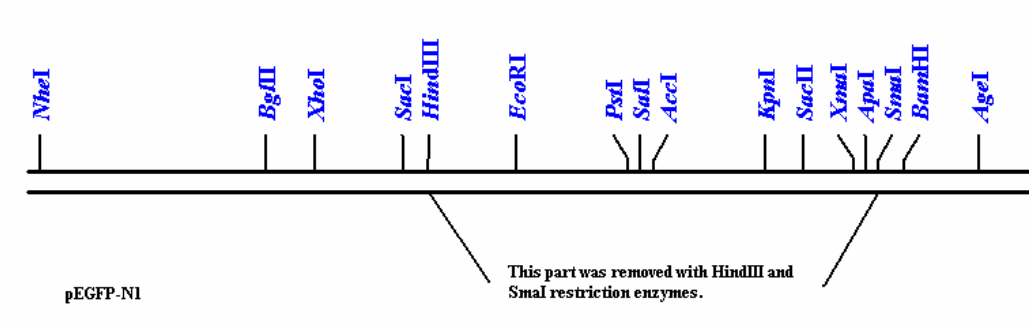


**Figure 3.11:** Restriction map for pEGFP-N1 vector.

Restriction mixtures were run on agarose gel and bands indicating p80 part and opened up pEGFP-N1 part were extracted from agarose gel (Figure 3.12).

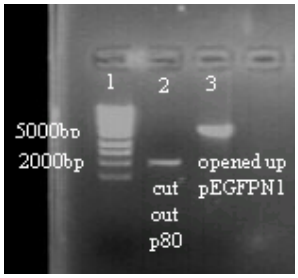


**Figure 3.12:** Restriction mixtures that were run on agarose gel. (Lane 1: Fermentas, Mass Ruler, High Range Dna Ladder, Lane 2: cut out p80 band, Lane 3: opened up pEGFP-N1 vector band).

p80 was ligated into pEGFP-N1 and then transformed into *E.Coli* XL1 Blue Strain.

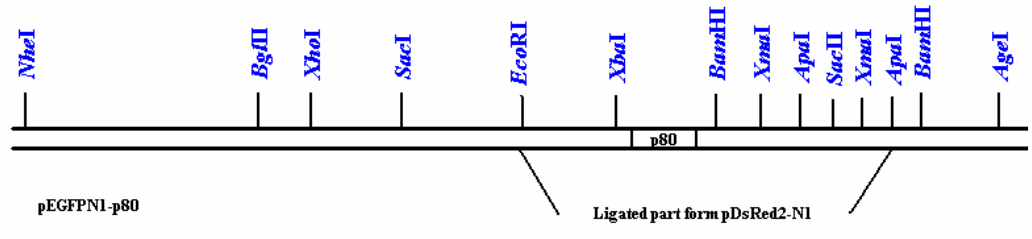


**Figure 3.13:** pEGFP-N1-p80 construct map.

ten of the transformation colonies, then, were selected in order to perform colony PCR. PCR results were observed on agarose gel (Figure 3.14).

It was shown that all ten colonies had the desired insert but only one colony had a bright band, the other p80 bands were very weak due to taking approximate amounts of cells from colonies while performing colony PCR. p80-pEGFP-N1 was then purified from transformants by using "Roche High Pure Plasmid Purification Kit".

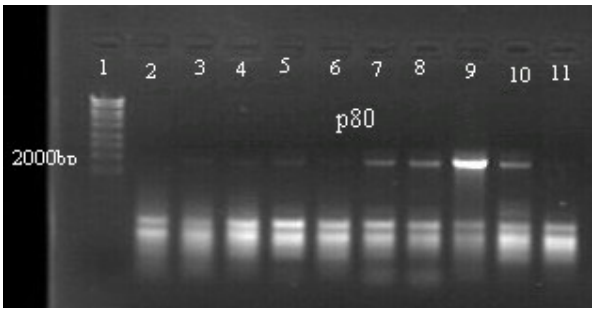


**Figure 3.14:** Colony PCR results after transformation of pEGFP-N1-p80 construct. (Lane 1: Fermentas, Mass Ruler, High Range Dna Ladder, Lane 2-9: Colony PCR results).

# 4. DISCUSSION

## 4.1. RNA Isolation

After cDNA was synthesized from isolated RNA, several PCR optimizations were performed with this cDNA in order to clone p80 subunit but every time a smear occured from beginning to the end of the lane that were loaded with PCR product on the gel (Figure.4.1).

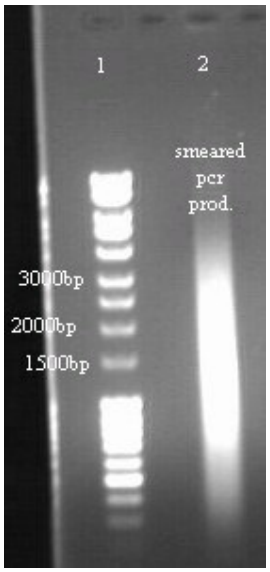


**Figure 4.1:** PCR product was run on agarose gel. (Lane 1: Fermentas Mass Ruler Mix, DNA Marker, Lane 2 : Smeared PCR product).

Since this smear could be resulted from gDNA which might have been isolated together with RNA from brain tissue, a control PCR was performed by using RNA as template to understand if there was any DNA contamination. It was expected to see any amplification as a band or a smear on gel in case of gDNA contamination. This control PCR resulted in smeared PCR product caused by gDNA (Figure.4.2).

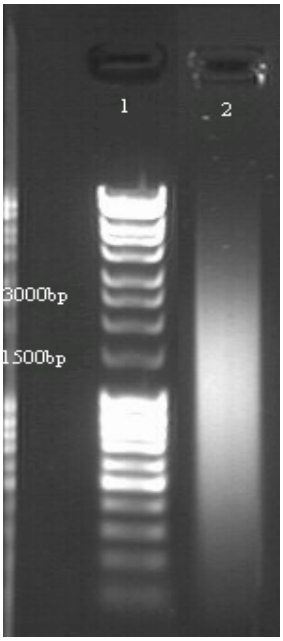


**Figure 4.2:** Control PCR for gDNA contamination. (Lane 1 : Fermentas Mass Ruler Mix, DNA Marker, Lane 2 : Smear indicated gDNA contamination during RNA isolation).

When RNA is being isolated from tissues, genomic DNA (gDNA) in the cells is isolated together with RNA. Since gDNA interferes with further steps like cDNA synthesis and PCR cloning, it should be eliminated during RNA isolation. Therefore, there is a DNase I treatment step in the RNA isolation procedures to digest residual contaminating DNA. According to the followed "Roche High Pure RNA Tissue Kit" manufacturer's instructions, DNase I treatment should be done for 15 minutes at room temperature after lysis of the cells but, in this experiment, it was seen that this time was not enough to get rid of all gDNA. Even though purified RNA was checked on denaturing agarose gel to see its purity and integrity, gDNA contamination could not be seen on the gel. Since gDNA was too large, gel running time was not enough for gDNA to migrate through the gel. Hence, it could not be visualized under UV Transilluminator.

Thus, a new RNA isolation was performed and DNase I incubation time was extended from 15 minutes to 45 minutes for higher efficiency. The achieved RNA was again used as template for control PCR and any PCR product was not seen on agarose gel, except primer dimers (Figure.4.3).

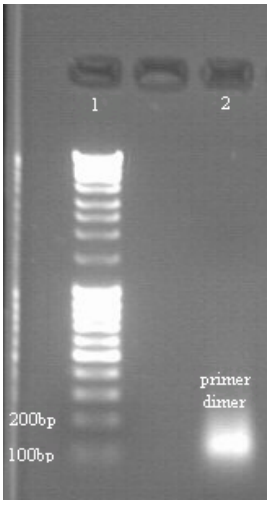


**Figure 4.3:** Control PCR result by using RNA as a template after new RNA isolation. (Lane 1 : Fermentas Mass Ruler Mix Range DNA Marker, Lane 2 : No PCR product was observed).

### 4.2. PCR Optimization

Firstly, a 740 bp -C terminal part and a 943 bp middle part of p80 that contained the four conserved WD 40 repeats were chosen to be cloned. Since it is easier to clone smaller fragments with PCR than bigger ones, choosing these two fragments of p80 was thought to be advantageous for the beginning of the study. For this purpose, specific internal primers were used. Since the exact chicken neuronal p80 sequence was not known, a predicted p80 sequence that was submitted on http://www.ncbi.nlm.nih.gov/entrez/query.fcgi, was used in order to design the specific primers. Melting temperatures of primer pair for 740 bp part were as follows:

Chp80F3: 58,2 °C

Chp80R3: 59,5 °C

Melting temperatures of primer pair for 943 bp part were as follows:

Chp80F2: 58,2 °C

Chp80R2: 59,5 °C

Hence, 54 °C was used as annealing temperature of the reactions and an extention time ranging from 2 minutes to 4,5 minutes was used in PCR.

As a result, 740 bp part appeared as a sharp and bright band on the gel whereas 947 bp part band was very weak with a smear on the lane. In order to optimize this, annealing temperature was increased from 54 °C to 57 °C and an extention time ranging from 3 minutes to 6 minutes was used to increase the specificity of the annealing and to dicrease the smearing. PCR result was observed on agarose gel and 947 bp bright band was appeared. After sequence analysis of these two regions of p80, four conserved WD 40 repeats were observed, as expected.

These preliminary results ensured p80 existence in cDNA library which made further steps easier.

Melting temperatures of the primers that were used to clone complete coding sequence of p80:

Chp80F1:62,5 °C

Chp80R3:59,5°C

59 °C annealing temperature was first chosen in order to clone p80. It was expected to see 1974 bp band that would indicate p80 on agarose gel after PCR, but there were unspecific 500 bp and 750 bp bands and a very weak 1974 bp band (Figure.4.4).

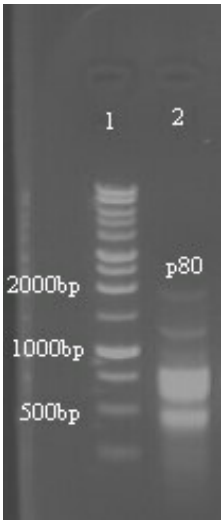


**Figure 4.4:** PCR result for complete coding sequnce of p80. (Lane 1: Promega BenchTop 1 kb DNA Ladder, Lane 2: Top weak band idicates p80 and lower brighter bands indicate unspecific products).

Since p80 DNA sequence is 46% GC base-rich, it was possible for two strands of DNA not to dissociate completely during denaturation step of PCR. Because there is three hydrogen bonds between G and C bases and these bonds are stronger then the bonds between A and T bases thus it is difficult to dissociate. As a result of incompleted dissociation of two strands, there may have been partial cloning of p80 and it may have been seen as smaller unspecific products on agarose gel. A PCR component known as "GC Melt" was used so as to make dissociation of two strands easier. Its efficiency was controlled by performing the same reactions with and without GC Melt at the same time. It was seen that GC Melt made dissociation of two strands easier.

Another adjustment was done to increase the primer annealing specificity. A "touchdown PCR" was performed with an annealing temperature ranging from 62 °C to 60 °C to determine the most suitable and critical annealing temperature for the primers. This time, expected 1974 bp. band was observed on the gel but it was weak and there were 500 bp. and 750 bp. bands again (Figure.4.5).

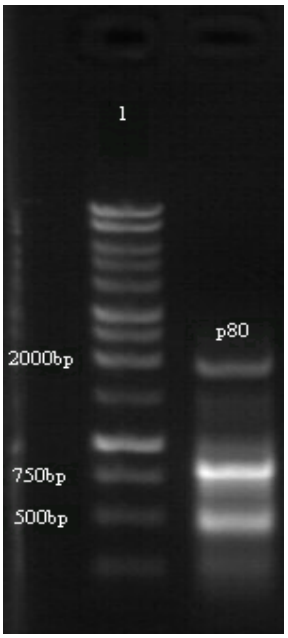


**Figure 4.5:** Touchdown PCR (62°C-60°C) results. (Lane 1: Promega BenchTop 1 kb DNA Ladder, Lane 2 : top weak band idicates p80 and lower brighter bands indicate unspecific products).

Different touchdown PCR reactions were performed to get rid of unspecific smaller bands and to have a brighter desired band. Finally, a touchdown PCR with an annealing temperature range between 67 °C and 64 °C provided the best result. Although unspecific bands did not disappeared completely, they got very weak and a sharp 1974 bp. band was observed on the gel. It was sufficient for sequence analysis. After sequencing, it was shown that obtained sequence had a 99% similarity with the submitted mitotic p80 sequence. Consequently, chicken neuronal p80 sequence was cloned for the first time.

Unspecific smaller fragments' sequences were, too, analyzed and it was shown that these fragments were not a region or a part of p80 but they belonged to two different proteins that have roles in nerve system, too.

### 4.3. Restriction-Digestion Reactions

Since p80 was in reverse orientation in pTZ57R/T cloning vector, restriction enzymes should have been chosen in a way that the orientation of p80 would change while ligating into pEGFP-N1. The suitable enzymes for this purpose were not avaliable. Hence, it was thought to subclone p80 into another suitable vector in order to change the orientation at first. It would make subcloning p80 into pEGFP-N1 expression vector possible. The most appropriate vector was DsRed2-N1 for this purpose and p80 was first ligated into DsRed2N1 in its normal orientation then it was cut out with suitable restriction endonucleases and ligated into pEGFP-N1.

## 5. CONCLUSIONS

Since katanin is a microtubule severing protein, it may be utilized to regenerate injured neurons. p80 subunit of katanin is thought to have an important role in regulation of the severing activity of katanin. However, exact functions of p80 subunit are not well understood. In order to better understand its possible role in regulation mechanism, in this study, chicken neuronal p80 katanin was successfully cloned from brain tissue for the first time. Sequence of p80 was analyzed and submitted to GenBank with the accession number DQ410670 ( Yildiz A., Baas P.W., Karabay A.). After that, p80 katanin was subcloned into pEGFP-N1 fluorescent expression vector in the right orientation to be able to visualize its functions in the living environment, and pEGFP-N1-p80 construct was obtained.

Cell culture studies are currently underway. Obtained construct is being transfected into HeLa cells for preliminary studies. Then, it will also be transfected into chicken embryo primary neurons to determine the possible roles of katanin in neuron regeneration. Furthermore, *in situ* hybridization will be carried out with chicken embryo cryosections to visualize p80 katanin distribution in cells.

These studies will enable us to better understand the vital roles of katanin in neurons. In the light of these studies, katanin may become a key to the riddle of repair and regeneration of injured neurons in the future.

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**RESUME**

Ayşegül Yıldız was born in Karabük in 1981. After getting her high school diploma from Pendik High School in 1999, she has continued her undergraduate degree at Marmara University, Faculty of Science and Letters, Department of Biology in 1999. She had her Bachelor degree in 2003. She has continued to her graduate studies at Advanced Technologies in Molecular Biology –Genetics and Biotechnology program. She is still pursuing her studies in the same department.