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MASTER'S RESEARCH DISSERTATION

2015

Regulation of the retinoblastoma binding protein 6 in *Drosophila melanogaster*

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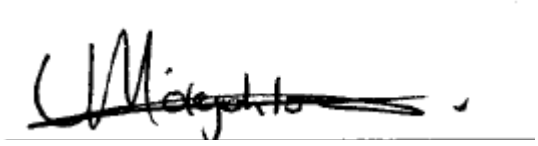
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Declaration

I hereby declare that this dissertation is my own unaided work. It is being submitted to the University of the Witwatersrand, Johannesburg for Master's degree. It has not been previously submitted for any degree or examination at this or any other University.



Lehlogonolo Mokgohloa

13th Day of February 2015

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Dedication

This dissertation is dedicated to my family especially my mother, grandmother, my late grandfather and late uncle, last but not least my first born son Amogelang Mokgohloa.

Research Output

Conference Proceeding

Umar-Faruq Cajee, Brent Oosthuysen, Lehlogonolo Mokgohloa and Monde Ntwasa. (2012). The characterization of *Drosophila melanogaster* RBBP6 protein, SNAMA. Poster presented at:SASBMB/FASBMB Congress 2012, 29 January – 1 February, Champagne Resort, Drakensburg, Kwazulu Natal, South Africa.

Abstract

SNAMA, the protein of interest in this thesis is found in the common model organism *Drosophila melanogaster*, also known as the fruit fly it is also found in all eukaryotic organisms but not in prokaryotes. SNAMA is a 1231 amino acid protein that belongs to the RbBP6 superfamily. Members of this family are characterized by a zinc finger motif, a DWNN domain (domain with no name) and a RING finger motif. The human RbBP6 contains the Rb-binding and p53-binding domains in addition. The mammalian RbBP6 hence interacts with p53 and Rb and it is important for the development and tumorigenesis as a negative regulator of p53. Bioinformatics studies show that transcription of the *Snama* gene is driven by a single TATA-less promoter which give rise to a single 3.9 kb transcript. However, experimental evidence confirming the promoter region has not being published. The main aim of this study was to examine the regulation of *Snama* by identifying the maximal promoter sequence that shows promoter activity in mammalian cell line. This was achieved by using specifically designed primers to amplify the putative *Snama* promoters, ligating promoters in reporter vector (pGL3 basic). The recombinant products used to transfect eukaryotic cells (Cos7, African green monkey cells) and determining the maximal promoter sequence that expresses luciferase activity. The promoter sequences were labeled with biotin attached to the primers and Electrophoretic mobility shift assay (EMSA) was conducted to confirm binding of proteins on the putative promoter fragments. The segment designated promoter 6 has maximal positive activity and many proteins in the cell extract bind to it shown by EMSA. Interestingly the longer fragment designated promoter 7 has less promoter activity. This may suggest that this fragment also contains some repressive elements.

Abbreviations

CO_2 : Carbondioxide

˚C : Degrees Celsius

dH_2O : Distilled water

DMEM : Dulbecco's Modified Eagle Medium

Dmp53 : *Drosophilla melanogaster* p53

DMSO : Dimethyl sulfoxide

DNA : Deoxyribonucleic acid

DREF : DNA replication-related element-binding factor

DRE : DNA replication-related element

DTT : Dithiothreitol

DWNN : Domain with no name

EDTA : Ethylenediaminetetraacetic acid

EMSA : Electrophoretic mobility shift assay

FBS : Fetal bovine serum

HEPES-KOH : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer adjusted with potassium hydroxide

IPTG : Isopropyl-β-D-thiogalactoside, isopropyl-β-D-thiogalactopyranoside

KCl : Potassium chloride

KOAc : Potassium acetate

LA : Luria agar

LAR II : Luciferase assay reagent II

LB : Luria both

MgCl2 : Magneseum chloride

ML	: Milli liters
PBS	: Phosphate buffered saline
PCR	: Polymerase chain reaction
PLB	: Passive lysis buffer
PMSF	: Phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride
penStrep	: pencillin streptomycin
Rb	: Retinoblastoma
RbBP6	: Retinoblastoma binding protein 6
RNA	: Ribonucleic acid
SDS-PAGE	: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TAE	: Tris acetic acid ethylenediaminetetraacetic acid
TBE	: Tris borate ethylenediaminetetraacetic acid
TF’s	: Transcription factors
TSS	: Transcription start site
µg	: Micro grams
µl	: Micro liters
X-GAL	: 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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1. Introduction

Retinoblastoma binding protein 6 (RbBP6) is a protein that is encoded by the RbBP6 gene. The gene is thought to be associated with cell proliferation and such genes are overexpressed in the development of cancer. The regulation of p53 through mdm2 has been reported to be under the control of RbBP6 (Motadi *et al*, 2011). The RbBP6 gene encodes 3 isoforms of the protein and the third isoform (Isoform 3) is down-regulated in human cancers. The overexpression of RbBP6 results in increased G2/M cell cycle arrest whilst the knock-down results in reduced G2/M cell cycle (Mbita *et al*, 2012).

For centuries scientists have been studying the interaction between various organisms, the environment they live in and the intricate details of their systems. Organisms that are used as model organisms are a small fraction of the biodiversity of the earth, although the research that results from the study of these organisms is of great importance and forms the core of the biodiversity knowledge. Independent research communities have a common interest in understanding the evolution, genetics and the development of these organisms. This research also forms the basis of comparisons between closely related and distantly related organisms. These studies have greatly benefited the field of evolutionary biology in which the molecular data has clarified estimates of phylogenetic relationships. Organisms such as the mouse and *Drosophila* are mostly used as model organisms, due to their small size and short generation times (Hedges, 2002; Wong *et al*, 2007).

Insects make ideal model organisms as much is known about their natural history, physiology, genetics and behavior. They are also inexpensive to maintain and reproduce

rapidly (Morgan, 1910). Additionally, insects are of great interest due to their ability to resist pathogenic microorganisms at the cellular and humoral levels (Tweedie *et al*, 2009) hence this study was done to investigate the regulation of RbBP6 in *Drosophila melanogaster*.

1.1. *Drosophila melanogaster*

Drosophila melanogaster which is commonly known as the fruit fly is one of the species which are mostly studied in molecular biology of eukaryotes. Twelve members of the genus *Drosophila* have been completely sequenced (Wong *et al*, 2007) and the sequenced genomes stored in various databases such as flybase (Tweedie *et al*, 2009). *Drosophila melanogaster* belongs to the family Drosophilidae, the order Diptera and it is classified as an insect. The use of *Drosophila* as a model organisms began in the early 1900s. It was used in the generic research on sex linkage and genetic recombination by Thomas Hunt Morgan (Morgan, 1910).

Another useful feature of *Drosophila melanogaster*, is the numerous techniques that can be used to manipulate the organisms genes and this is one of the other reasons *D. melanogaster* is a model organism of choice in genetic studies of eukaryotes. In every organism there are biological processes that need to be maintained to ensure the normal functioning of the organism in events such as death, growth and defense against diseases. Apoptosis and the ubiquitin pathway are some of the processes involved in these actions.

Developments of most of the structures of the adult fly occur during the larval period and take their final form during eclosion. The central nervous system is an exception to this. Discs known as imaginal discs are a group of cells that invaginate from the embryonic ectoderm and form histoblasts and imaginary rings. The familiar external structures of the

adult fly develop from the imaginary discs (figure 1). The life cycle takes about 4-6 weeks. The eggs are about 0.5mm and the larvae take about 5 days to grow. The fruit fly feeds on microorganisms that decompose fruit. The larvae then encapsulate in the puparium and undergo metamorphosis for 5 days. The adults emerge after metamorphosis (Mather *et al*, 2005).

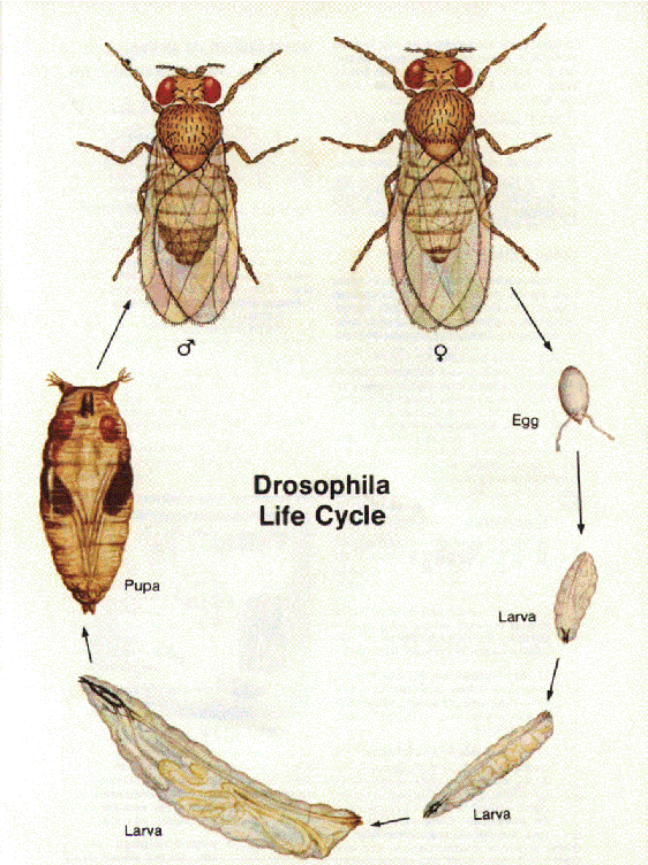


Figure 1: Developmental stages of *Drosophila melanogaster* (Flagg, 1988)**.**

Due to the reasons above *Drosophila melanogaster* was chosen as a model of choice the following study.

1.2. Retinoblastoma binding protein 6 family

This family of proteins is found in eukaryotes and absent in prokaryotes (Pretorius *et al*, 2011) and members of this family are characterized by a zinc finger motif, a DWNN domain (domain with no name) and a RING finger motif. One of the proteins of interest in *D. melanogaster* is SNAMA which is inadequately uncharacterized and believed to belong to the RbBP6 protein family. The human homologue belonging to this family is a 250-kDa human protein known as the human Retinoblastoma binding protein 6 (RbBP6) (Pugh *et al*, 2006). The domain structure of this family of proteins is illustrated in figure 2.

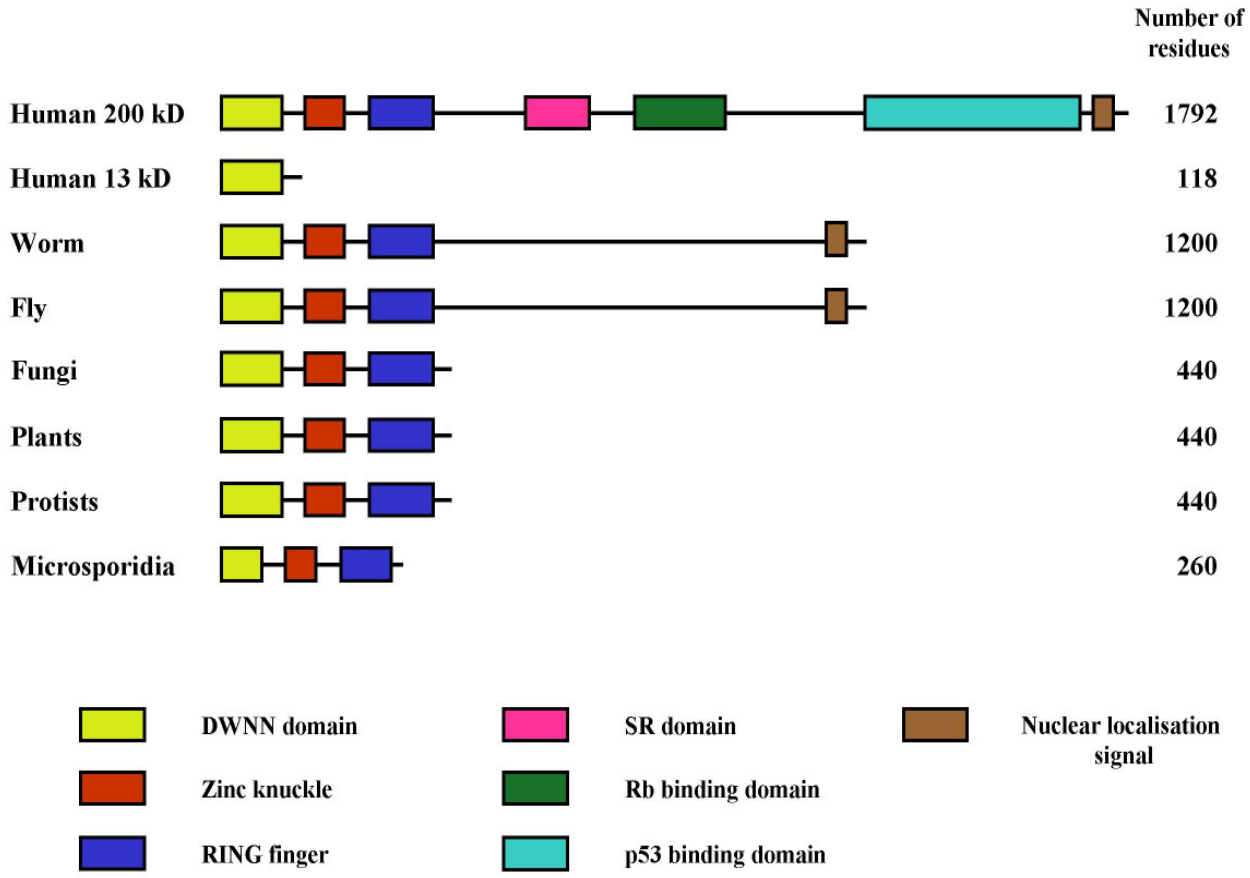


Figure 2: The RbBP6 family of proteins domain structure. Members of this family contain a DWNN domain, a zinc knuckle and a RING figure motif. A long C-terminal extension with p53 is observed in both insects and vertebrates and Rb- interaction domains in humans and mouse (Pugh *et al*, 2006).

The RbBP6 has a ring finger domain and has been identified as a putative E3 ubiquitin ligase. Studies have indicated that it is a multifunctional protein that interacts with p53 and pRb and has been implicated in the processing of pre-mRNA. Although the function of RbBP6, also known as p53 associated cellular protein testes (PACT) derived in mouse, is unclear, it is implicated in tumourigenesis, and is believed to play a role in the cell cycle. The mechanism by which RbBP6's influences the cell cycle is also unclear (Chibi *et al*, 2008). PACT is highly up regulated in esophageal cancer and maybe a promising target for immunotherapy (Li *et al*, 2007).

There are a few proteins known to interact with both tumour suppressor proteins pRb and p53. RbBP6 inhibits the binding of DNA to p53 by binding to wild type p53. RbBP6 also plays a role in facilitating the interaction of p53 and its primary regulator Hdm2. This leads to the ubiquitination and degradation of p53 by the proteasome (Chibi *et al*, 2008).

Chibi *et al* (2008) conducted a study that described the novel interaction between DNA binding protein B (dbpB) and RbBP6. It was found that dbpB falls in the family of cold-shock proteins and is a transcriptional factor that binds to the inverted CCAAT box. It is responsible for cellular functions such as the regulation of transcription and translation in response to stresses that result from extracellular signals. These orthologues of SNAMA have highly similar N-terminal domain configuration, suggesting that SNAMA is a member of this distinct family of proteins and thus plays a role in apoptosis and development in *Drosophila*.

1.3. SNAMA

The name SNAMA is a Xhosa word that means something that sticks like glue. SNAMA was identified during homology searches using a sequence identified from a promoter trap mutagenesis screen for apoptotic genes in Chinese hamster ovary (CHO). The predicted *D. melanogaster* protein consists of 1231 amino acids and a molecular weight of 139 kDa. At the N-terminus SNAMA has the conserved 76 conserved amino acid Domain With No Name (DWNN). This is followed by a C2HC zinc finger motif, a RING finger-like motif and a C-terminal that is highly rich in lysine residues (figure 3) (Mather *et al*, 2005).

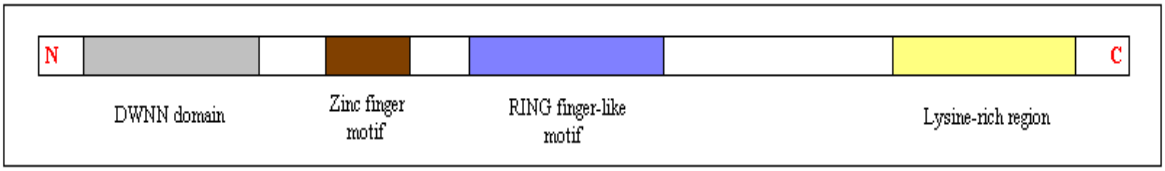


Figure 3: Schematic representation of the internal domain structure of SNAMA in *Drosophila melanogaster* (Mather *et al*, 2005)

The DWNN has an ubiquitin-like fold and is suspected to modify other proteins at least in the human version. This is because this domain exists as an independent module in one of the three isoforms. Although the DWNN domain is highly conserved throughout eukaryotic species, it is absent in prokaryotes (Pugh *et al*, 2006). DWNN has a 22% identity with ubiquitin and has an exposed C-terminal tail that extends beyond the β sheet of ubiquitin but lacks the conserved di-glycine peptide, which is essential for recognition of hydrolases. The di-glycine peptide is replaced by a conserved proline (Mather *et al*, 2005). This may indicate that DWNN is not cleaved off in biological reaction involving SNAMA.

The zinc finger motif tends to be involved in the interaction between protein-DNA and protein-protein (Matthews *et al*, 2001). The RING finger domain of SNAMA is slightly different to other RING finger motifs in that the histidine residue is substituted. This motif is normally found with a serine residue on the fourth ligand (figure 4) (Mather *et al*, 2005). The RING finger ligases have been shown to be RING finger motif dependant and it is suspected that the RING finger-like domain of SNAMA functions as an ubiquitin ligase dispite its uncharacteristic RING figure motif.

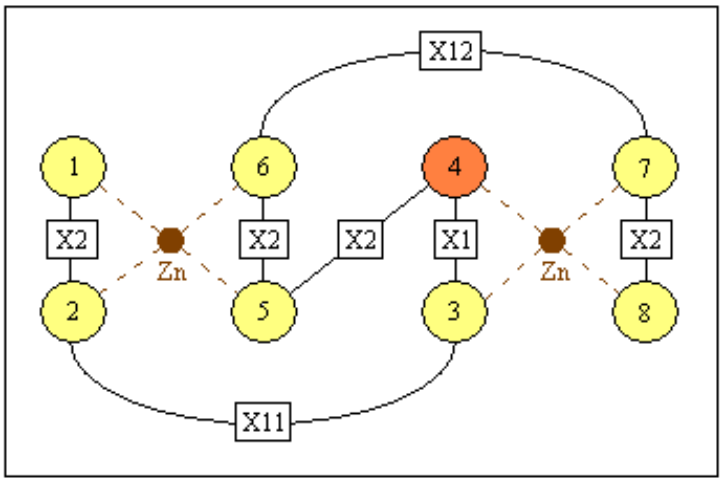


Figure 4: Schematic representation of RING finger motif of SNAMA, Two zinc ions are coordinated by cysteine residues (yellow spheres) and a serine residue (orange sphere) that replace the lysine (Mather *et al*, 2005).

The DWNN domain is found in both SNAMA and the human Retinoblastoma binding protein 6 (RbBP6), indicating homology between SNAMA and the human RbBP6. This protein interacts with p53 and retinoblastoma (Rb) in both mouse and humans and it also functions in the regulation of the cell cycle and the induction of programmed cell death or

apoptosis (Mather *et al*, 2005). A homologue of SNAMA was described in yeast which is involved in the 3' end processing of mRNA (Vo *et al*, 2001).

1.3.1. Work done on SNAMA and its homologues

Although the regulation of SNAMA is not clearly understood, a study done by Hull & Ntwasa (2010) shows that there are similarities between regulation of SNAMA and RbBP6 (Hull & Ntwasa, 2010). SNAMA orthologues are known to bind p53 and its orthologue in mouse, RbBP6 is known to negatively regulate p53 by enhancing Mdm2 which regulated p53 (Hull & Ntwasa, 2010). Hence SNAMA is proposed to play the role of Mdm2 in invertebrates. SNAMA is also believed to suppress apoptosis in early *Drosophila* development. The disruption of SNAMA leads to early apoptosis in *Drosophila* embryos. In the study mentioned above camptothecin and methyl-pyruvate treatment were conducted and transcriptional activation of SNAMA, Dmp53 and Reaper measured during and after recovery from treatment. It was found that the transcription of SNAMA is reduced during treatment but upregulated after recovery. Dmp53 transcriptionally controls Reaper. The camptothecin treatment causes an increase in the transcription of Dmp53 but there was no concomitant increase in Reaper mRNA. The methyl-pyruvate treatment resulted in the accelerated decline of Dmp53 transcription and the activation of SNAMA transcription (Hull & Ntwasa, 2010).

Previously completed work by Ntwasa and Antunes gives insight into transcriptional regulation of *Snama* through the analysis of the core promoter elements surrounding the transcription start site (TSS). The 5' RACE was used to delineate the TSS. Furthermore,

using bioinformatics Mather *et al*., 2005, showed the transcription start site of SNAMA by predicting TSS to be in the region determined by 5' RACE. This transcription start site is believed to be regulated by a TATA-less promoter. The TATA-less promoter region contains core elements Inr and DPE that are needed for binding of the transcription initiation factor TFIID. Antunes work showed that SNAMA is regulated by a single TATA-less promoter that gives rise to an approximately 3.9 kb transcript (Antunes, 2008).

1.4. Apoptosis, programmed cell death

For an organism to function optimally there are a number of processes that need to occur in order to maintain homeostasis. One of these processes, apoptosis, is a mechanism of controlled cell deletion. Damaged, unwanted cells are removed to allow proliferation of normal cells (Wyllie, 1993). Apoptosis appears to play an opposite but complementary role to mitosis in the regulation of animal cell populations. Pathological and physiological environmental stimuli can inhibit or initiate apoptosis. It involves the rapid condensation and budding of the cell by forming apoptotic bodies that are enclosed in a membrane containing well preserved organelles (John Bright & Khar, 1994). Nearby resident cells then digest and engulf the remains of this apoptotic cell. The process involves the cleavage of double stranded nuclear DNA resulting in oligonucleosomal fragments. Inhibitors of mRNA and protein synthesis can suppress the apoptotic process (Kerr *et al*, 1994).

There are two phases that apoptosis can be divided into, the initiation phase and the effector phase. The initiation phase depends on the type of stimulus that induces apoptosis. The effector phase is characterized by DNA fragmentation and morphological changes to the

cells. Both these phases involve the action of specific enzymes called caspases, with caspase 3 playing a key role in the effector phase (Koyama *et al*, 2000).

1.5. Ubiquitination

Damaged proteins are tagged for degradation by a 76 amino acid protein named, ubiquitin. The amino acid sequence of the protein is highly conserved across species and it is found in all eukaryotic cells. Ubiquitin is heat stable due to its three and a half α-helix, a short piece of 3(10)-helix, seven reverse turn and five mixed strands of β sheets (figure 5). The overall structure is tightly bonded by hydrogen atoms.

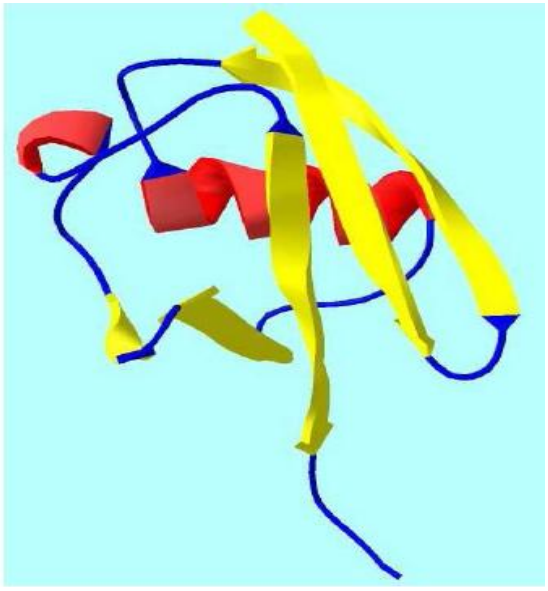


Figure 5: The three dimensional structures of ubiquitin showing its helices (red), 5 β-sheets (yellow) and seven reverse turns (blue).

Ubiquitin tags damaged proteins for degradation by 26S proteasome. The 26S proteasome in the nucleus is covalently attached to histones believed to function in the regulation of transcription. The degradation of proteins by the ubiquitin process involves two consecutive steps which are: (1) tagging of the protein substrate by molecules of ubiquitin and (2) the degradation of the tagged proteins by the 26S proteasome, which recognize ubiquitinated proteins, resulting in the release of reusable ubiquitin and inactive peptides. The tagging involves the attachment of ubiquitin via the glycine on the C-terminal 4-residue tail, to the lysine of the target protein to form an isopeptide bond (Pickart, 2004).

The ubiquitin pathway involves a series of enzymes that aid the binding and the degradation process. The first enzyme in the casade, the E1 activates ubiquitin on its C terminal glycine. The ubiquitin is activated on its C-termial glycine by a thioester bond with cysteine on the active site of E1. The ubiquitin complex is then transferred from E1 to E2, which forms another thioester bond with the complex (Hershko *et al*, 1983). The covalent isopeptide bond between the ε-amino group and α-carboxyl group is catalysed by an E3, ubiquitin ligase. The ε-amino group is found on the side chain of a lysine residue of the protein substrate and the α-carboxyl group is on the C-terminal of ubiquitin. Successive ubiquitin molecules are then attached to one another to form a polyubiquitin chain. The polyubiquitin chain molecules are linked via a Gly^{76}-Lys^{48} isopeptide bond. The 26S proteasome complex recognizes the polyubiquitin chain as a signal for degradation (Hershko & Ciechanover, 1998).

1.5. *Drosophila melanogaster* p53 (Dmp53)

Dmp53 is a 42kDa protein with 385 amino acids. It was identified using its human homologue the human p53 (Ollmann *et al*, 2000). Homology searches in *Drosophila*

identified three isoforms of Dmp53 namely; dp53L, dp53 and dp53n (figure 6). The *dp53L* has two additional exons which make it longer than the other two isoforms and has a sequence on the N-terminal end that is conserved through evolution, the FxxψW. The sequence is required for the activation of gene transcription. Proteins from other vertebrates such as p53, p63 and p73 have the conserved FxxψW sequence, and dp53L share this feature with these proteins unlike dp53 where the FxxψW sequence is absent. The dp53 isoform is produced from an inner promoter and is truncated on the N-terminal. It is the second largest isoform with 385 amino acids. The last isoform, dp53n, is the smallest with only 123 amino acids; it is the splice variant of dp53L with no splicing of intron B (Bourdon *et al*, 2005). The internal promoter is implicated as playing an important role in the control of activities involved with the p53 gene family across species, since the internal promoter sequence is conserved throughout evolution from humans to *Drosophila*.

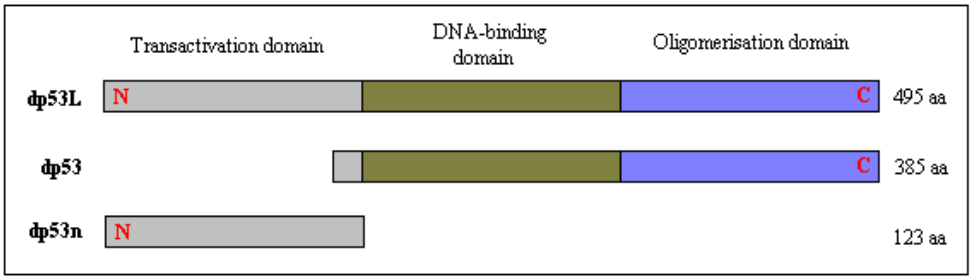


Figure 6: A schematic representation of the three different *Drosophila melanogaster* p53 isoforms: dp53L, dp53, and dp53n (Bourdon *et al*, 2005).

p53 is a tumor suppressor protein that has been characterized primarily as a sequence-specific transcription factor that is mutated in more than half of all human cancers (Levine, 1997). It is usually kept at low concentrations and increased upon cellular stress such as DNA damage and promotes apoptosis. Mouse double minute 2 (Mdm2) protein regulates p53 in mammals and it is believed to be the major physiological antagonist of p53. With the mdm2 gene being

the characterized target of the p53 autoregulatory negative feedback mechanism, where p53 induces the expression of Mdm2, whereas Mdm2 represses p53 activity. This serves as a vital mechanism to inhibit p53 function in normal cells (Lahav *et al*, 2004). Mdm2 not only inhibits p53 but also promotes the degradation of p53 by catalyzing ubiquitination (Haupt *et al*, 1997), acting as an E3 ubiquitin ligase (Honda *et al*, 1997). This is achieved by complex binding with p53 at the N-terminal of Mdm2 and the binding of ubiquitin molecules on the C-terminal end of p53 tagging p53 for degradation (Chen *et al*, 1995).

SNAMA is likely to be a homologue of Mdm2 in *Drosophila* and therefore, a possible candidate for the regulation of Dmp53 and RBF, which is a homologue of Rb. RBF is regulated by ubiquitination and acts downstream of the transcription factor, dE2f, which functions in the regulation of Dmp53. Since the mouse and the human homologues of SNAMA have been shown to bind to p53 and Rb it is suspected that SNAMA interacts with Dmp53 and RBF, possibly playing a critical role in their regulation. Hence, a better understanding of how SNAMA is regulated will give an insight on its role in cancer and apoptosis. Characterisation of the full length promoter sequence of SNAMA would aid in the understanding of the expression and regulation of SNAMA.

1.6. Luciferase Reporter assay for promoter analysis

The luciferase reporter assay technology is useful in investigating questions related to the regulation of genes. Reporters are used to study expression of genes and cellular events that are coupled with expression of genes. In the technology a reporter gene is cloned with a DNA of interest into an expression vector. The vector is then transferred into cells and the presence of the reporter assayed. The presence of the reporter can be assayed by either measuring the

reporter protein or by enzymatic activity. Reporters such as bioluminescence reporters are ideal because they are sensitive and they are quick to measure.

1.7. Luciferase Enzyme

The luciferase enzyme evolved for the nocturnal mating behavior of beetles. This enzyme catalyzes a two-step oxidation reaction in order to produce light between 550-570nm, in the green to yellow region. The two oxidation steps are the activation of luciferyl carboxylate by ATP to yield a mixed reactive anhydride and the creation of transient dioxetane that breaks down into oxyluciferin and carbon dioxide (CO_2). In the second step the transient dioxetane results from the reaction of the intermediate in the first step with oxygen (O_2). The gene that encodes the firefly luciferase is a cDNA, *luc* that contains no introns and is available as a mature enzyme upon translation due to its lack of modification after translation. The luciferase gene, *luc* in the firefly was modified into *luc*+. This was due to the saturation of the peroxisomes, where the luciferase is directed. The saturation resulted in enzyme remaining in the cytoplasm creating a difference in enzyme stability between the peroxisomes and the cytoplasm. This lead to a concentration dependent relationship between protein synthesis and the quantity of luminescence (Sherf & Wood, 1994). The two luciferase reporters of interest are the (i) Firefly and the (ii) Renilla luciferase.

(i) Firefly luciferase, is a 61kDa monomeric enzyme from the firefly (*Photinus pyralis*). It is mostly used as a bioluminescent reporter in molecular biology due to the convenience of the assay, sensitivity and the coupling of protein synthesis with the enzyme activity

(ii) Renilla luciferase is a 36kDa protein which was originally cloned from the marine *Renilla reniformis*. This luciferase does not require post-translation modification for activity. Hence due to renilla luciferase's similarity to the firefly luciferase it can function as a generic reporter immediately after translation.

There are four possible luciferase assays that can be conducted: Bright-Glo, Steady-Glo, One-Glo and Dual-Glo luciferase assay system. The Bright Glo Luciferase assay system quantifies the luciferase expressed by providing 10 fold more light output than other reagents that have extended half-life such as the Steady-Glo single-reporter reagent. The extended half-life of Steady-Glo allows batch processing of assays and does not require luminometers with injectors. The One-Glo luciferase assay also has extended half-life (Wood, 1998).

The fourth assay (Dual-Glo luciferase assay) was the assay of choice since it is engineered to normalize the expression of reporters. The reporters in the assay are experimental (Firefly) and the control (Renilla) reporter which give insight on specific and non-specific cellular responses. The two are widely used as co-reporters for normalization studies due to their sensitivity and quick and easy to use nature. The maximal promoter sequence was identified after using Dual luciferase assay.

Reporter genes can be used for a multitude of processes (figure 7) and they require reporter vectors such as pGL3 and pRL vectors. The pRL vectors contain a cDNA (*Rluc*) Renilla luciferase. There are four vectors belonging to the pGL3 family (see appendix, 5.2) and four belonging to the pRL family (see appendix, 5.2) that contain the luciferase gene.

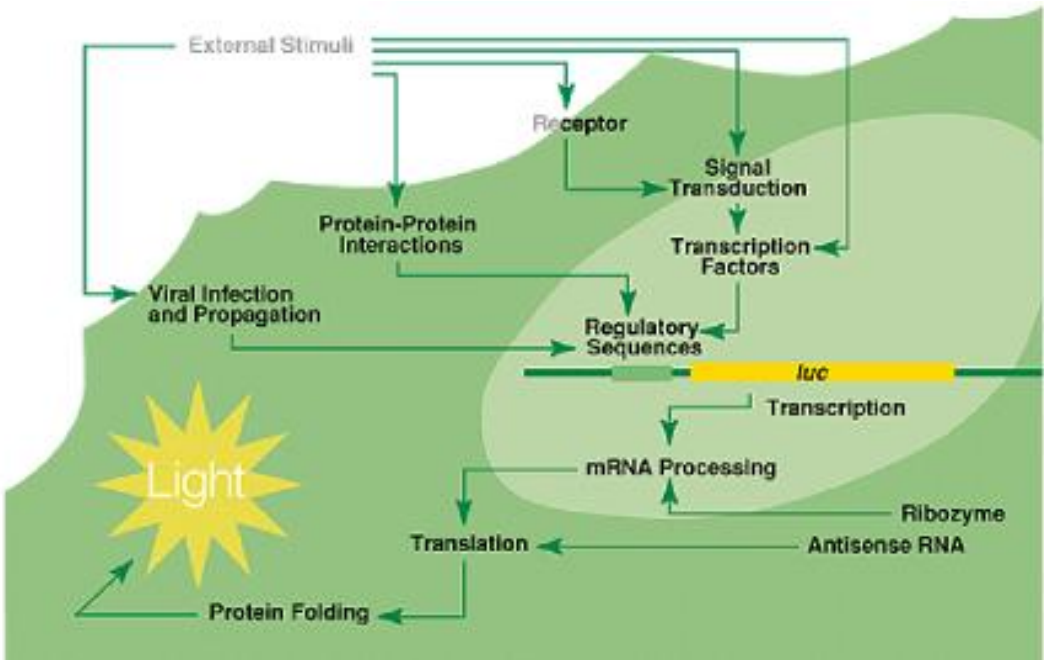


Figure 7: Schematic representation of a range of applications for reporter genes in cellular biology (Sherf & Wood, 1994).

1.8. Aims

This study aimed to examine the regulation of the *Snama* gene in *Drosophila melanogaster* by identifying the maximal promoter sequence using designed *Snama* primers. Sequentially truncated regions of the putative promoter sequence of *Snama* were individually amplified and cloned into both pGem-T-easy and pGL3 basic reporter vector. Eukaryotic cells (Cos7 cell lines) were transfected with the pGL3 clones and assayed for luciferase expression. The longest promoter sequence which induced luciferase expression was considered to contain the core promoter elements of *Snama*. Promoter sequences were also used for EMSA to identify whether there are proteins that bind to the biotin labeled probes. These proteins would then represent the transcription factors that bind to these promoters. The proteins that bind were to be identified by mass spectroscopy and would give insight of their interaction with SNAMA.

2. Materials and Methods

2.1. Growth media

Table 1: Growth media

Media	**Composition**
Apple juice agar	1.25% sucrose; 2% agar; 0.025% streptomycin; 0.15% methylparaben (3g dissolved in 30 ml ethanol and made up to a litre); 25% apple juice (v/v).
Luria-Bertani broth (LB)	1% tryptone powder; 0.5% yeast extract powder; 1% NaCl; mix and autoclave.
LB agar (LA)	LB + 1.5% agar; autoclave.
Cos7 cells (mammalian cells)	3:1 ratio of DMEM : Ham's F12, 10%FBS, 1% penStrep

2.2. Buffers and solutions

Table 2: Molecular biology buffers and solutions. All reagents were stored at room temperature and made up with deionized water unless indicated.

Buffer	**Composition**
Coomassie blue stain	0.1% Coomassie blue R-250; 40% ethanol; 10% glacial acetic acid.
DNA loading dye (10X)	0.21% Bromophenol blue; 0.21% xylene cyanol FF; 50% glycerol; 0.2 M EDTA, pH

	8.0; store at 4ºC.
Electrode (running) buffer (5X)	125 mM Tris base; 1 M glycine; 17 mM SDS; pH to 8.3 with HCl.
Embryo wash (10X)	0.7% NaCl, 0.04% triton-100
Grinding buffer	5% sucrose, 80mM NaCl, 100mM Tris pH 8.5,0.5% SDS, 50mM EDTA
Miniprep solution I	5 mM sucrose; 10 mM $Na_2EDTA\text{-}2H_20$; 25 mM tris; pH to 8.0 with NaOH.
Miniprep solution II	0.2 M NaOH; 1% SDS; prepare fresh.
Miniprep solution III	3 M sodium acetate, pH 4.8 made up with acetic acid.
Phosphate buffered saline (PBS), 50X	1.4 M NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4, pH to 7.3 with HCl.
Protein extraction (Nuclear) Buffer I plus inhibitors	1mM DTT, 0.1mM EDTA, 15mM HEPES-KOH pH 7.6, 10mM KCl, 5mM $MgCl_2$, 100ug/ml PMSF, 2ug/ml Aprotinin, 50ug/ml Leupeptin, 0.35M Sucrose
Protein extraction (Nuclear) Buffer II plus inhibitors	0.1mM EDTA, 15Mm HEPES-KOH pH 7.6, 10mM KCl, 5mM $MgCl_2$. 0.8M Sucrose, 1mM DTT, 50ugml Leupeptin, 2ug/ml Aprotinin, 100ug/ml PMSF
SDS sample buffer (5X)	1 M Tris-HCl, pH 6.8; 10% glycerol; 10% SDS; β-mercaptoethanol; 1% bromophenol blue; store at 4ºC. For Native gel (SDS

	sample buffer + 0.024g/ml Urea)

2.3. Chemicals and kits

Table 3: Chemicals, enzymes and their suppliers

Chemical/Enzymes/Kit	**Supplier catalogue numbers**
Agarose	SBS Genetic co. GA4-100
Dual-Luciferase® Reporter Assay System	Promega E1910
GeneRuler™ 100bp DNA ladder plus	Fermentas SM0321
LightShift Chemiluminescent	Thermo scientific 89880
PageRuler™ prestained protein ladder	Fermentas SM0671
pGEM®-T Easy vector system	Promega A1380
pGL3 basic reporter vector	Promega E1751
pRL Renilla Luciferase Reporter Vectors	Promega E2241
Streptravidin® agarose	Sigma alderich S1638-1ML
Trypsin/Versene	Highveld biological 205A
Zymoclean Gel DNA Recovery kit	Zymo research corporation D4001S

2.4. Cell culture

Table 4: Cell culture

Strain	**Supplier**
Cos7 cells (mammalian)	Elsabe

2.5. Bacterial strains

Table 5: Bacterial strains

Strain	**Genotype**
Escherichia coli XL-1 Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)]

2.6. Bioinformatics analysis of *Snama* gene

The *Snama* gene was analysed using DNAman and following analysis the primers for putative *Snama* promoter designed. The designed primers were purchased from Inqaba Biotec and are indicated in table 6 below.

Table 6: Oligonucleotides used for PCR and sequencing. All the primers were used unlabeled and labeled with biotin except for the reverse primer.

Oligonucleotide	**Sequence**
Snama primer 3	5'-CAGAAATGCAGATCTGCTGCC-3'
Snama primer 4	5'-CGTATTTACTGCAGATCTAGAG-3'

Snama primer 5	5'-GACTAAATTAGATCTGCATCGA-3'
Snama primer 6	5'-GCAAAGATCTAAAAGGCGGTG-3'
Snama primer 7	5'-GCCTCCTTAAGATCTATTCG-3'
Snama reverse primer	5'-GAACGTGAAGCTTAAGCCC-3'

2.7. Isolation of *Drosophila melanogaster* genomic DNA

Fifty adult fruit flies reared at 25˚C on standard commercial apple juice agar with yeast were homogenized in 500µl grinding buffer. The homogenate was incubated at 70˚C for 30 minutes. Following incubation, 87µl of 8M KOAc was added and the mixture was placed on ice for 30 minutes and centrifuged at 13000g for 10 minutes. The supernatant was collected and the DNA was precipitated with isopropanol. The tube was left to stand at room temperature for 10 minutes, spun at room temperature for 10 minutes at 13000g and the supernatant discarded. The DNA pellet was washed with ethanol and left to dry 37˚C for 2-3 minutes. Subsequently the pellet was resuspended in 100ul nuclease free water (Bender *et al*, 1983). The genomic DNA was stored in the -20˚C freezer until needed.

2.8. Amplification of putative *Snama* promoters

Snama promoters were amplified by Polymerase chain reaction (PCR) for 40 cycles. PCR is a technique used to amplify a DNA fragment of interest, in order to isolate and produce more of this target DNA for further experimentation. PCR is a three step process which consists of denaturation, annealing and elongation. During denaturation, DNA is heated until it separates into two strands to allow the primer to anneal. Taq polymerase catalyses the elongation of the two strands. DNA isolated from the fruit fly was used in the PCR run and primers which were

specific for putative promoters of *Snama*. The PCR mixture and the PCR conditions are indicated in the following tables.

Table 7: The reaction mixture used for PCR

Reagent	**Concerntration of stock solution**	**Volume added (µl)**	**Concerntration in final mixture (50µl)**
dH_2O	-	38	-
Taq buffer	10X	5	1X
dNTP's	10mM	1	0.2mM
$MgCl_2$	25mM	2	1mM
Forward primer	100µg/µl	1	2 µg/µl
Reverse primer	100µg/µl	1	2 µg/µl
Template DNA	100ng/µl	1	2ng/µl
Taq polymerase	5 units/µl	1	5 units

Table 8: The temperature profiles used for PCR

Cycle	**Temperature (◦C)**	**Duration (sec)**
Taq polymerase activation	94	600
Denaturation	94	30
Annealing	60	30
Extention	72	60
Final extension	72	420
Holding step	4	∞

2.9. Gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA bands according to their molecular sizes. The products from the PCR were separated on a 1% agarose gel, dissolved in 1x Tris-Acetic acid Ethylenediaminetetraacetic acid (TAE). The separated PCR fragments were recovered and DNA bands were extracted from agarose gels with the aid of gel extraction kits. The promoter sequences were gel purified using the Zymoclean gel recovery kit following manufacturer's instructions. The purified DNA was used to perform for ligations.

2.10. Ligation of promoter sequences

Ligations were conducted to insert the purified DNA into a vector for expression purposes. The extracted promoter sequences were ligated into the pGEM-T easy cloning vector. These promoter sequences were then digested out of pGEM-T Easy vector with *Eco*RI (a sticky end cutter, Fermentas) and blunt ended with Klenow enzyme (Roche). The pGL3 basic vector was linearized using SmaI (a blunt end cutter, Roche) and dephosphorylated. The blunt ended promoter sequences were ligated into the dephosphorylated vector and the ligation reactions were used for transformation.

2.11. Transformation of products

The ligated promoter sequences in pGEM-T Easy were transformed into competent *Escherichia coli* bacterial cells. Transformed cells were platted on luria bertani agar (LA) amended with 50ug/ml ampicillin, 0.2 mM IPTG and 40ug/ml X-gal for selection of blue and white colonies.

The transformation was conducted according to the procedure of (Sambrook & Russell, 2001). Controls were used to confirm success of the transformation. Clones containing recombinant plasmid DNA were indicated by white colonies.

Fresh bacterial cells (XL-1 Blue cells) were made competent as per (Sambrook & Russell, 2001) method, prior to the transformation in order to allow efficient transformation. Plasmid DNA was isolated using the alkaline lysis method (Birnboim, 1983). Double digestions were performed with HindIII and Kpn1 (Fermentas) in order to confirm the presence of the promoter sequences in the pGL3 basic vector.

2.12. Small scale plasmid preparation

Plasmid DNA was isolated using the alkaline lysis method (Birnboim, 1983) and the DNA concentration was determined based on the absorbance of light at 260 nm. The prepared mini-prep was used for digestions. The digestion reactions were conducted in order to verify the availability of the promoter sequences (insert). After successful digestion (verified from agarose gel electrophoresis, 1% agarose) the miniprep was sent for automated sequencing in order to verify that the promoter sequences were correct and no mutations had occurred. Large scale plasmid preparations (maxipreps) were performed in order to have promoter sequences in large amounts for further experimentation. The recombinant DNA was used for transfection of eukaryotic cells, Cos7 cell line.

2.13. Transfection of eukaryotic cells with recombinant pGL3 basic reporter vector

Cos7 cells ($5x10^5$ cells/ml) were seeded in 60mm tissue culture dishes at 37˚C, 5% CO_2 prior to transfection. A total of 5ug of DNA (pGL3 basic vector recombinant and pRL-TK 1:20) was diluted in cell growth media with neither antibiotic (penStrep) nor serum (FBS) to a volume of 150ul. To this 30ul of superfect transfection reagent (Qiagen) was added and the mixture was incubated for 10 minutes at 25˚C. While complexes were forming media was aspirated from cells and cells were washed with 1X PBS. Following the formation of complexes 1ml of media with serum (FBS) and antibiotics (penStrep) was added and mixed. The total volume was added to the cells in 60mm dishes with cells and incubated for 3hours at 37˚C 5% CO_2. After incubation the media containing the remaining complexes was aspirated and cells washed with 1X PBS. Fresh growth media was added to the cells and cells were incubated under normal growth conditions (37˚C and 5% CO_2) for 48hours. After 48hours the luciferase assay was performed.

2.14. Expression of luciferase

The pGL3 luciferase reporter vector has been used to quantify the level of gene expression in eukaryotic cells. The vector contains a modified coding region for *Photinus pyralis* luciferase that has been optimized for monitoring the activity of transcription of eukaryotic cells that are transfected as its backbone is designed for increased expression (Groskreutz *et al*, 1995). It also lacks a eukaryotic promoter and the resulting luminescence is rapid, sensitive and quantitative in nature.

The Dual-Glo Luciferase Assay Syatem was used to quantify the level of expression of luciferase in the pGL3 luciferase reporter vector system.The Dual-Glo Luciferase Assay System was used to measure the two assays, the Firefly (Experimental, pGL3 basic) and Renilla (Positive Control, pRL-TK). Luminescence in the luciferase assay was measured with a luminometer. For the assay growth media was aspirated from the cells and the cells were washed with 1X PBS prior to the addition of 400µl of passive lysis buffer (PLB). Cells were scraped off and added to an eppendorf tube. 100µl of Luciferase Assay Reagent II (LARII) was added to each well of the 96 well luminometer plate. The PLB lysates were mixed and 20µl of each reaction was added to the LARII in the 96 well plate and the luminescence of the Firefly luciferase was measured. The plate was removed and 100µl of Stop and Glo reagent added and the luminescence of the Renilla luciferase was measured.

2.15. Preparation of protein extracts

Prior to protein extraction, *Drosophila melanogaster* embryos aged from 0-6 hours were collected from an apple juice agar and yeast. The embryos were washed off the dish with 1X embryo wash, dechorionated using 5% bleach and rinsed with 1X embryo wash. The embryos were rinsed with dH_2O and transferred to a microcentrifuge tube and left to settle. The rinse solution was aspirated and buffer I with inhibitors was added and the mixture was kept on ice. The embryos were homogenized using 7-10 strokes of a tight fitting pestle on ice at 4˚C until the suspension became milky. The homogenate was poured into a microcentrifuge tube and spun at 7.700x g for 15 minutes at 4˚C to pellet the yolk and the nuclei. The supernatant was removed (cytoplasmic extraction) and stored at -70˚C. The top white layer of the yolk which contained the nuclei was removed without disrupting the yolk. Buffer I with inhibitors was added to the nuclei and homogenized with 4 strokes of the pestle.

The homogenate was layered on top of buffer II plus inhibitors in a new microcentrifuge tube and centrifuged at 1.310x g for 30 minutes at 4˚C. The supernatant was aspirated and the pellet resuspended in buffer I and stored at -70 ˚C until needed.

2.16. EMSA

Electrophoresis mobility shift assay (EMSA) is the use of electropherisis to detect complexes between nucleic acid and protein. The electrophoresis is carried out on either a polyacrylamide or an agarose gel. Generally complexes of labeled nucleic acids and proteins migrate slower than unbound nucleic acids. The assay is a rapid and sensitive method used to detect nucleic acids and protein complexes (Hellman & Fried, 2007). An EMSA kit (Thermo scientific) was used with biotin labeled putative SNAMA promoters. The assay generally involved the preparation of binding reactions, electrophoresing of these reactions on a native polyacrylamide gel in 0.5X TBE and the transfer of the DNA to a nylon membrane. This is followed by the crosslinking of DNA to the membrane and the detection of biotin-labeled DNA by chemiluminescence. The protocol was carried out as per manufacturer's instructions.

2.17. Treatment of Cos7 cells with compounds

The Cos7 cells were treated with different compounds and concentrations see table below:

Table 9: Concentrations of additives used to treat Cos7 cells.

	Cos7 (1)	Cos7 (2)	Cos7 (3)
Camptothecin	1uM	5 uM	10 uM
Methyl pyruvate	1mM	1mM	1mM
DMSO	1%	1%	1%

The table above shows the concentrations of the various additives used to treat Cos7 cells. The differences in the responses of the cells to these additives may be a consequence of these differences in concentrations.

3. Results

In the first instance these results describe the analysis of the *Snama* promoter and establishes the segment with maximal promoter activity in Cos cells. Previous bioinformatics analysis of the putative regulatory region of *Snama* using the BDGP Neural Network Promoter Prediction programme, located on the web at http://www.fruitfly.org/seq_tools/promoter.html (Reese, 2001), indicated that the promoter region was located upstream of the start site predicted to be at position 1608 bp upstream from the transcriptional start site and had a score of 0.97 (Mather, 2005). The software relies on common eukaryotic 5' elements such as TATA boxes. Furthermore R. Antunes (Antunes, 2008) confirmed experimentally the TSS of *Snama* to be upstream the translational start site, using 5' RACE. Based on this information I created truncated versions of the putative promoters regions beginning at the TSS to -672. Secondly EMSA shows that the promoter fragment with maximal activity binds the most proteins in cell extract compared to other fragments. Thirdly using exCELLegence technology this work showed that camptothecin (DNA damaging agent) causes death of cells and slow recovery of surviving cells. This work also shows that in a SNAMA promoter-dependent manner methyl pyruvate accelerates, somewhat the recovery process.

3.1. Cloning of promoter fragments

Primers were used to obtain truncated versions of the predicted *Snama* promoter to access the longest possible sequence upstream of the *Snama* transcription start site that could function as a promoter. The primers were designed to give rise to a series of sequences that were roughly 100 bp truncations of the promoter region and each other (Table 8).

Table 10: Positions and sequences of *Snama* primers

Sequence Name	Position	Sequence
Primer Reverse	-19	5' GGGCTTTGGCGTCACGTTC 3'
Primer 1 (P1)	-50	5' CCCGCGTGTGCGTTCGCAT 3'
Primer 2 (P2)	-129	5'CTAATAAAGTTCAATACTTTCACTT 3'
Primer 3 (P3)	-231	5' CAGAAATGCAGAATTGCTGCC 3'
Primer 4 (P4)	-332	5' CGTATTTACTGCAGCTTAAGAG 3''
Primer 5 (P5)	-454	5' GACTAAATTAGGATTGCATCGA 3'
Primer 6 (P6)	-552	5' GCAAATATCGAAAAGGCGGTG 3'
Primer 7 (P7)	-672	5' GCCTCCTTACAATCCATTCG 3'

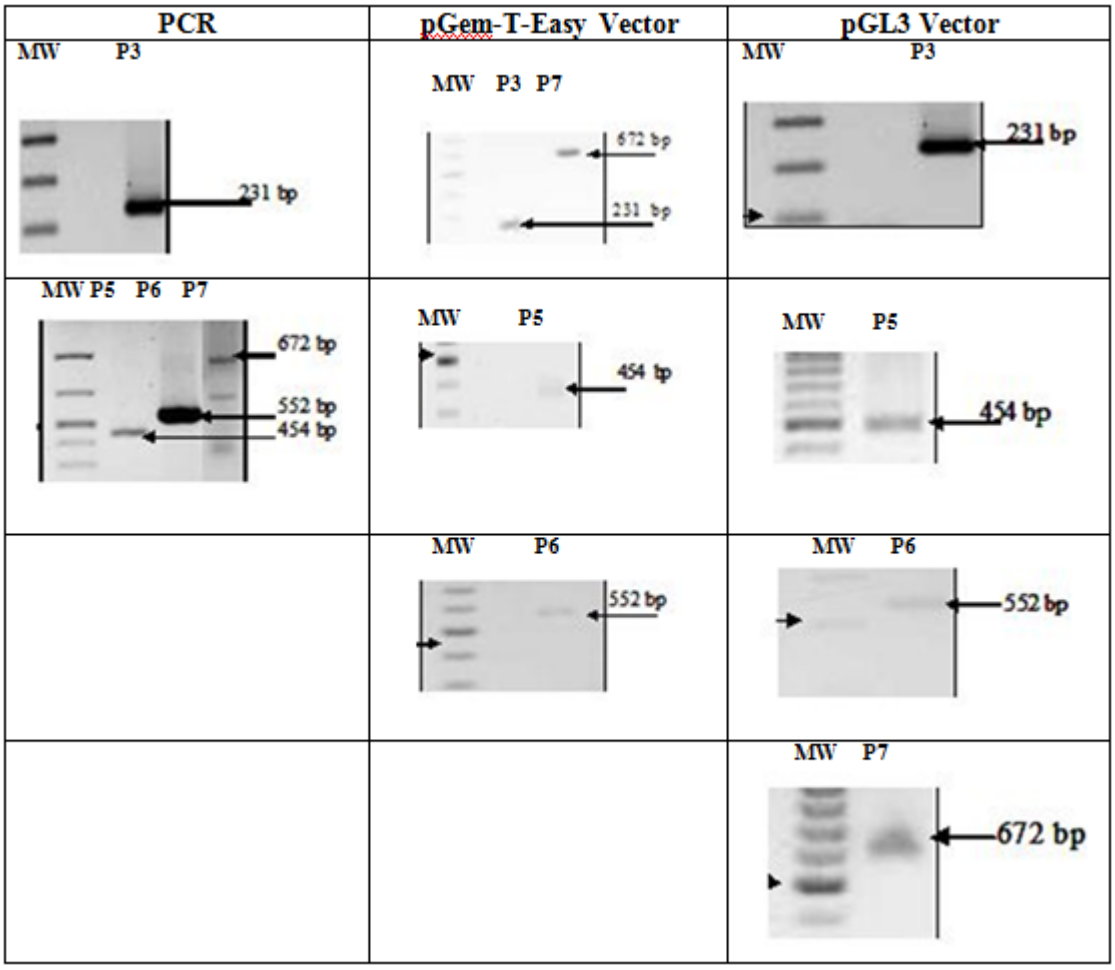
PCR was conducted to amplify the putative *Snama* promoter sequences. Promoters 3, 5, 6 and 7 of *Snama* were amplified and the products of PCR run on a 1% agarose gel (Table 9, Column 1). The sequences were purified using a gel extraction kit from Qiagen and sent for sequencing and then cloned into pGem-T-easy. Amplification of promoter 4 was however unsuccessful hence no further experiments were conducted with this primer sequence. The bands for promoters 3 and 6, show bright bands of good quality DNA, while only faint bands were obtained for promoter 5 and 7. As expected the product obtained for primer 7 and the reverse primer was just smaller than 700 bp (672bp) with the product obtained using primer 6 being approximately 100 bp shorter (552bp). The product obtained using primer 5 is a further 100 bp shorter (454 bp) while that obtained using primer 3 is a further 200 bp shorter (231 bp).

3.2. Luciferase assays to determine transcription activity

To verify that the sequences were successfully cloned into the pGem-T-Easy, the vector was digested with restriction enzyme (*Eco*RI) from Roche (Table 9, Column 2) and visualized on a 1% agarose gel. The sizes of the restricted plasmid and insert were verified by comparing them with the molecular weight marker. The liberated inserts were purified and the cloned into the reporter vector pGL3 basic. The pGL3 basic vector contains the luciferase reporter gene. Cloning of the putative promoter sequences placed the luciferase gene under the control of these promoters. The sequences were digested out of the reporter vector by double digestion, using the restriction enzymes *Hind*III and *Kpn*I. This was done in order to verify that the sequences had been ligated into the reporter vector (Table 11, Column 3).

The Luciferase assay was to determine the maximal region to drive expression. Furthermore, EMSA was used to test whether this region can interact with proteins. Currently we are in the process of using affinity Chromatography and mass spectroscopy to determine and identify the proteins shown by EMSA to interact with the promoters.

Table 11: Gel electrophoresis results of *Snama* putative promoters to verify the clones. PCR products were cloned in pGem-T-Easy and pGL3 vectors.



3.3. Determination of promoter activity by Luciferase reporter assay

To determine the fragment with maximal promoter activity, the dual luciferase assay was carried out. The results are shown in figure 8. Cells were cultured until they reached a density of $1x10^5$ cells/ml (in 5 ml) and then they were transfected with 5µg of DNA. The ratio of the experimental vector to the control vector was 1:20. The dual luciferase assay measured firefly

and renilla luciferase sequentially using the Dual luciferase system from Promega (cat# E1910).

The assay was carried out in triplicates following the instructions of the manufacturer. The data was pooled together to plot the graph using relative ratios (see below). The promoters that were investigated were promoters 5, 6 and 7. The graph shows that despite being the longest promoter sequence, promoter 7 was found not to express much luciferase activity in relation to the control (pGL3 basic). The second longest truncated promoter, promoter 6 was found to promote significant luciferase activity. The third truncation promoter 5 was found to behave like promoter 7 and did not show significant difference compared to the control. Therefore, promoter 6 was found to be the maximal promoter sequence expressing luciferase. The relative ratios used to plot the graph were calculated as follows:

$$X = \frac{\text{Control firefly luminescence}}{\text{Control renilla luminescence}}$$

$$Y = \frac{\text{Experimental firefly luciferase}}{\text{Experimental renilla luciferase}}$$

$$\text{Relative Ratio} = \frac{Y}{X}$$

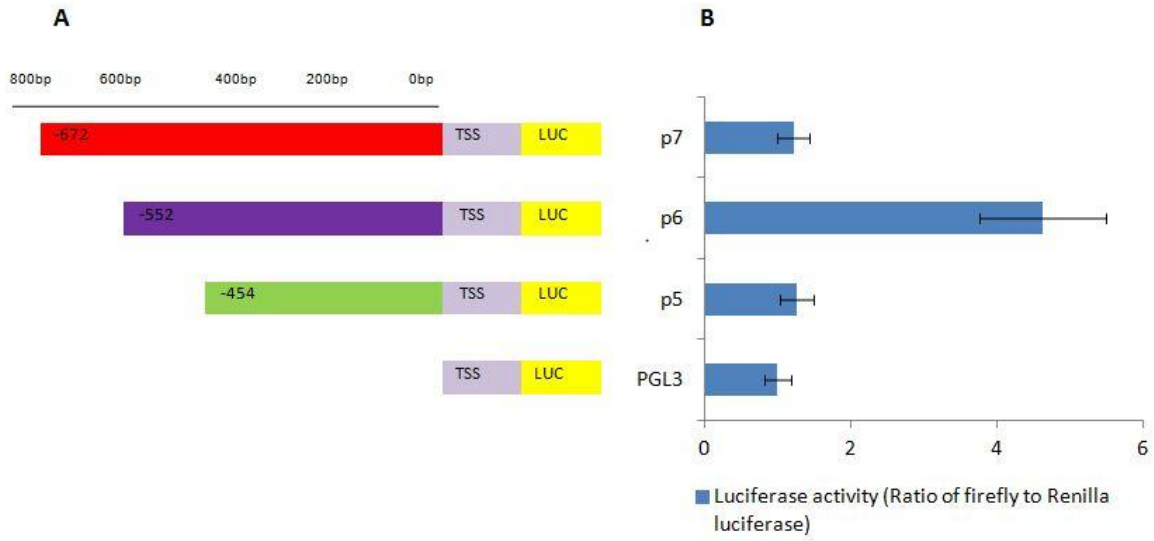


Figure 8: Schematic representation of the primer positions in the upstream untranslated region of *Snama* and putative promoter activity. **(A)** Schematic representation of putative *Snama* Promoters (p5, p6 and p7), the positions are relative to the transcription start site (TSS)+1**. (B)** Dual luciferase assay to determine the maximal promoter sequence. Measurement of Luminescence activity of Firefly and *Renilla* luciferase activity indicated that the maximal promoter sequence required to initiate *Snama* transcription and expression, was promoter 6. Despite promoter 7 being the larger sequence that contained promoter 6 the luciferase activity detected, was no more than the activity detected in the control pGL3 in the figure.

3.4. Possible Transcription Factors determined by Electrophoretic Mobility Shift Assay (EMSA).

Having assessed the putative transcription region of *Snama*, potential transcription factors were identified using EMSA. PCR was performed using biotin labeled primers, in order to label promoter sequences with a probe which could be detected via chemiluminescence. The amplification of biotin labeled DNA sequences was confirmed via electrophoresis. These

PCR products of biotin labeled primers 3, 6 and 7 are shown in figure 9, the amplification with primer 5 was however unsuccessful. Promoter 6 produced a bright band of good quality DNA whilst the other two promoters (promoter 3 and 7) produced faint but visible bands.

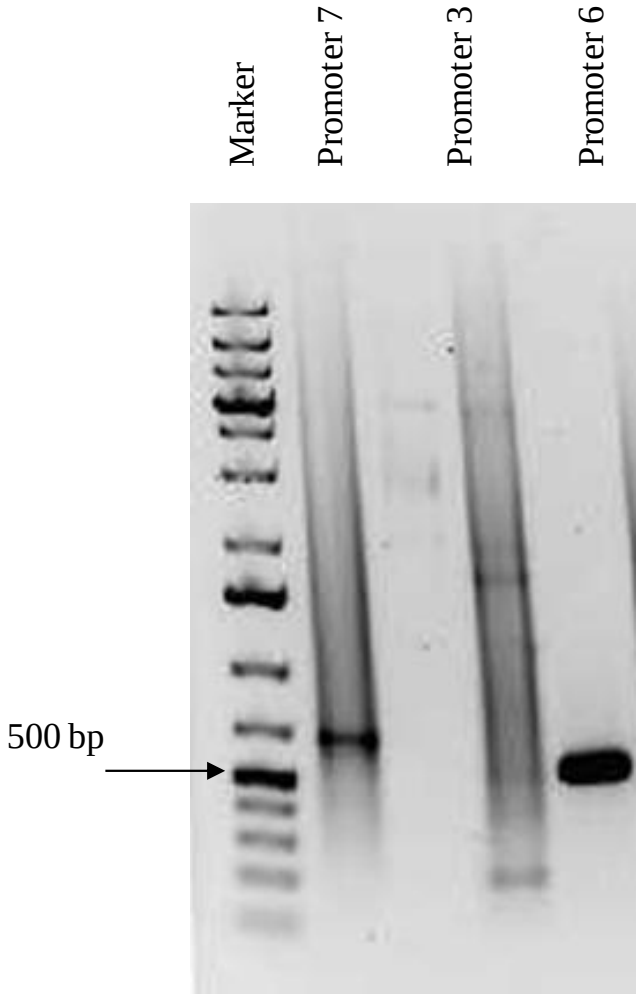


Figure 9: Agarose gel of PCR products of *Snama* putative promoters with biotin labeled primers. The gel shows amplified promoters 3, 6 and 7. The products are the expected size.

Nuclear protein extracts from *Drosophila* embryos were reacted with the biotin labeled putative promoter sequences. To establish whether any proteins bound to the promoters, an EMSA was carried out by running the reactions on a 6% native polyacrylamide gel and transferring the DNA to a nylon membrane. The biotin labeled DNA was detected by chemiluminescence and the results are shown in figure 10. The shift on the film indicates that there are proteins that bound to the biotin labeled DNA sequences.

The results that were obtained for the control system were as expected as per manufacturer's manual. Control 1 consisting biotin-EBNA control DNA in lane 1 showed no shift. Control 2 in lane 2 consists of biotin-EBNA control DNA and EBNA extract, contains sufficient target protein to effect binding and shift of the biotin-EBNA DNA as compared to the shift detected in control 1. Finally control 3 consisting of biotin-EBNA control DNA, EBNA extract and excess unlabeled EBNA DNA is found in lane 3, this result demonstrates that the signal shift observed in control 2 can be prevented by competition from excess non-labeled DNA. For all the promoter reactions shifted bands were observed for the promoter sequences. This observation shows a shift of DNA in all three was due to binding of proteins. As expected the unlabeled promoters, with no biotin label, gave no signal.

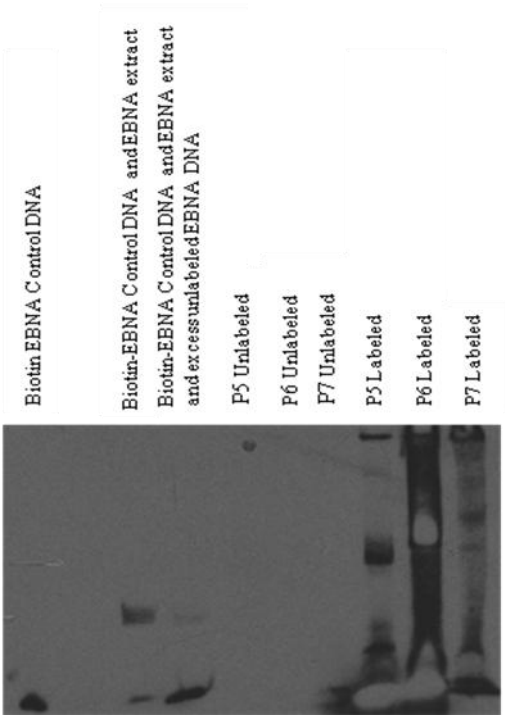


Figure 10: X-ray film showing detection of mobility shift of labeled promoter sequences reacted with nuclear protein extracts. Mobility shift assay of Biotin labeled and unlabeled DNA sequences of *Snama* promoters are indicated in the different lanes. Lane 1-3 indicates the EBNA control system, Biotin-EBNA Control DNA, Biotin- EBNA Control DNA and EBNA extract and Biotin-EBNA Control DNA and EBNA extract with excess unlabeled EBNA DNA respectively. The biotin-EBNA control DNA shows no shift, biotin-EBNA control DNA and EBNA extract shows a shift and the biotin-EBNA control DNA and EBNA extract and excess unlabeled EBNA DNA shows minimal shift due to competition. Lane 4-6 shows the unlabeled DNA fragments P5, P6 and P7. These unlabelled promoters do not give any signal. Finally, lanes 7-9 containing the labeled promoters P5, P6 and P7, indicate a mobility shift due to the binding of *Drosophila* nuclear proteins to the promoters.

3.5. Bioinformatics analysis of Transcription binding sites

Using the Model Inspector software from Genomatix, the possible TF's matches that could bind to *Snama* were predicted and the predictions are indicated in the figure below (figure 11) whereby positions of the transcription factors and the strands they are located is indicated.

Model Name	Position	Strand
HAND_GATA_01	26 - 54	(+)
PBXC_MYOD_01	50 - 29	(-)
BRNF_RXRF_01	60 - 12	(-)
CAAT_SP1F_01	76 - 112	(+)
ETSF_SP1F_05	78 - 112	(+)
EBOX_SREB_01	216 - 489	(+)
GREF_OCT1_01	231 - 184	(-)
CEBP_EBOX_01	239 - 216	(-)
SORY_PAX6_01	592 - 549	(-)
GATA_NKXH_01	651 - 623	(-)
MYOD_VTBP_01	871 - 809	(-)

Figure 11: Possible Transcription factors (TF's) of *Snama* as predicted by model inspector software from Genomatix**.**

The sequence that was used for the Model Inspector is shown in the figure below with all the sequences of the possible transcription factors indicated. The transcription start site (TSS) is also indicated in the figure (figure 12).

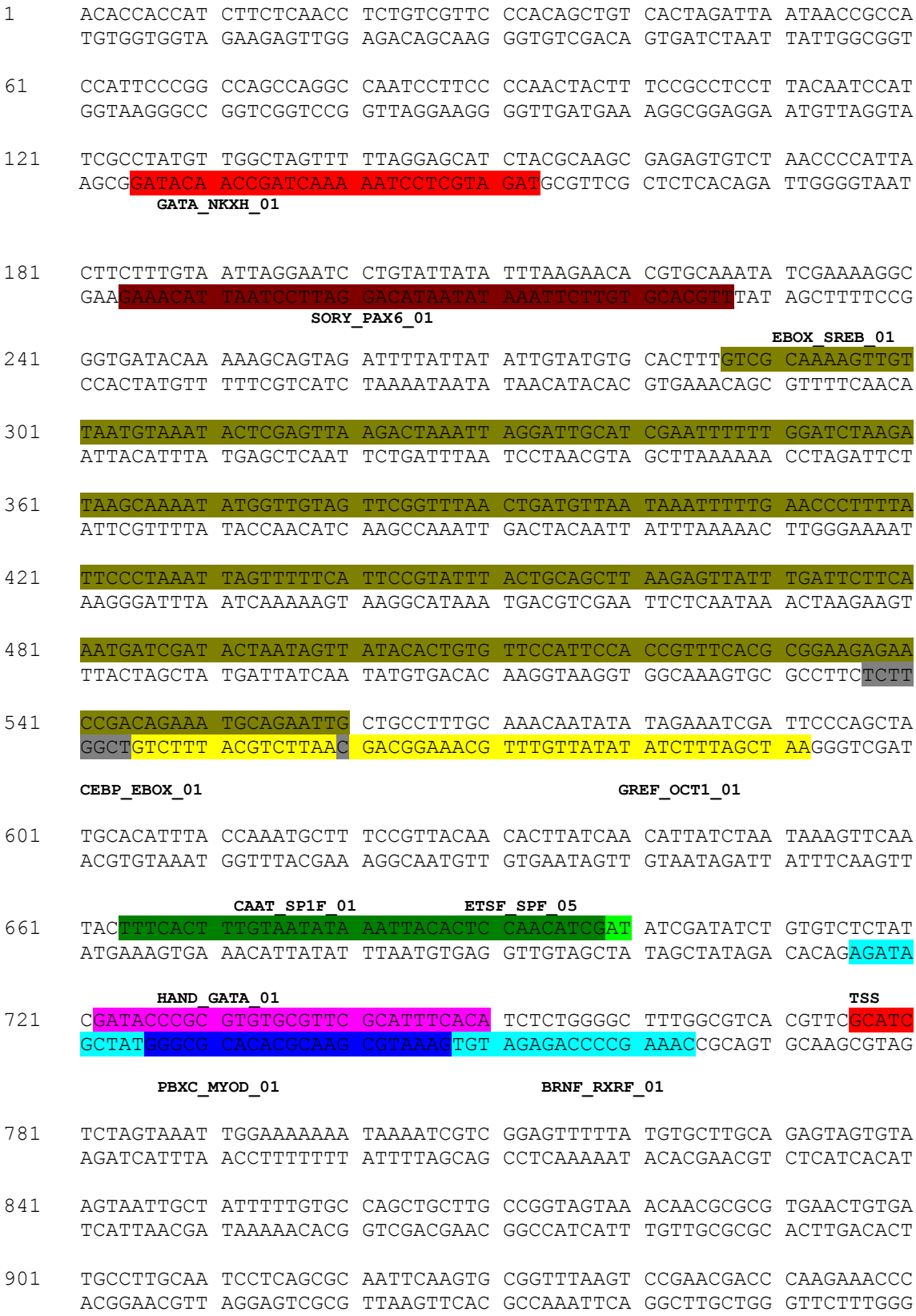


Figure 12: *Snama* sequences indicating the possible transcription factors. The TSS is indicated in red and labelled TSS. The possible transcription factors are labelled and indicated in various colours.

3.6. The effect of cells treated with DNA damaging agents.

Growth of Cos7 cells, derived from the kidney of the African green monkey, was monitored using the Xcelligence system (Acea). This system monitors growth of cells in real time. The normal growth pattern of these cells is observed in figure 13. The experiment was carried out in normal growth media under normal growth conditions and was performed in duplicates. The graph shows the expected shape for healthy growing cells. It depicts the growth trends for cell growth; the lag phase, log phase (exponential phase), stationary phase and eventually the death phase. The results show that the cells used were healthy and that the doubling rate for the cells is approximately 8 hours in the log phase.

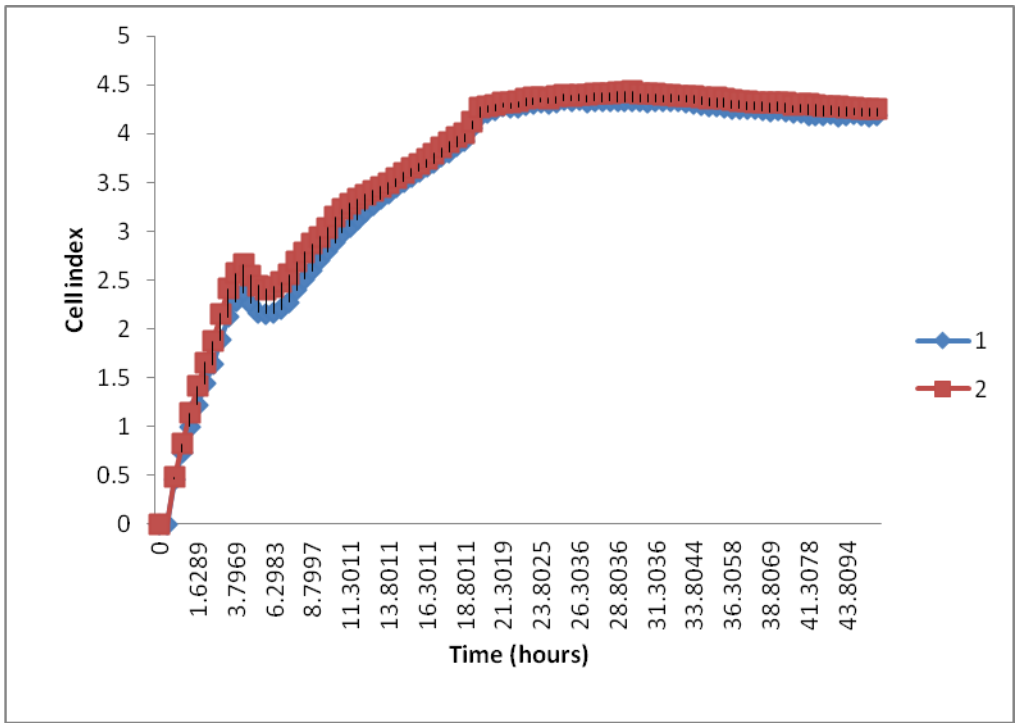


Figure 13: The growth curve of primary Cos7 cells. The cells were reared at 37˚C in normal growth media. From the curve it could be determined that the cells display an average doubling time of approximately 8 hours**.**

Previous work in our lab showed that methyl pyruvate improved the survival of cells that recover from treatment with agents such as camptothecin. This phenomenon was accompanied by changes in the expression patterns of Dmp53 and SNAMA (Hull & Ntwasa, 2010).

As the xCELLigence system (Acea) is a system that allows real time monitoring of cells whilst conducting experiments. The system continuously and non-invasively detects cell responses throughout the experiment without the use of exogenous labels that can potentially disrupt the natural cell environment. The system also allows for better target for treatments with agents as the growth of cells is monitored in real time when the cells are in the incubator. The technology uses gold plated plates with two separable sections, the upper and lower chamber. Cells are seeded in the upper chamber and move to the lower chamber through the microporous membrane. Cells adhere to the microelectrode sensors and lead to an increased impedance that is measured in real time. Applications for the system include;

- Cell invasion and migration assays
- Compound and cell-mediated cytotoxicity
- Cell adhesion and spreading
- Cell viability, proliferation, and differentiation
- Receptor-mediated signaling
- Virus-mediated cytopathogenicity
- Cardiotoxic compound effects

- High-throughput screening

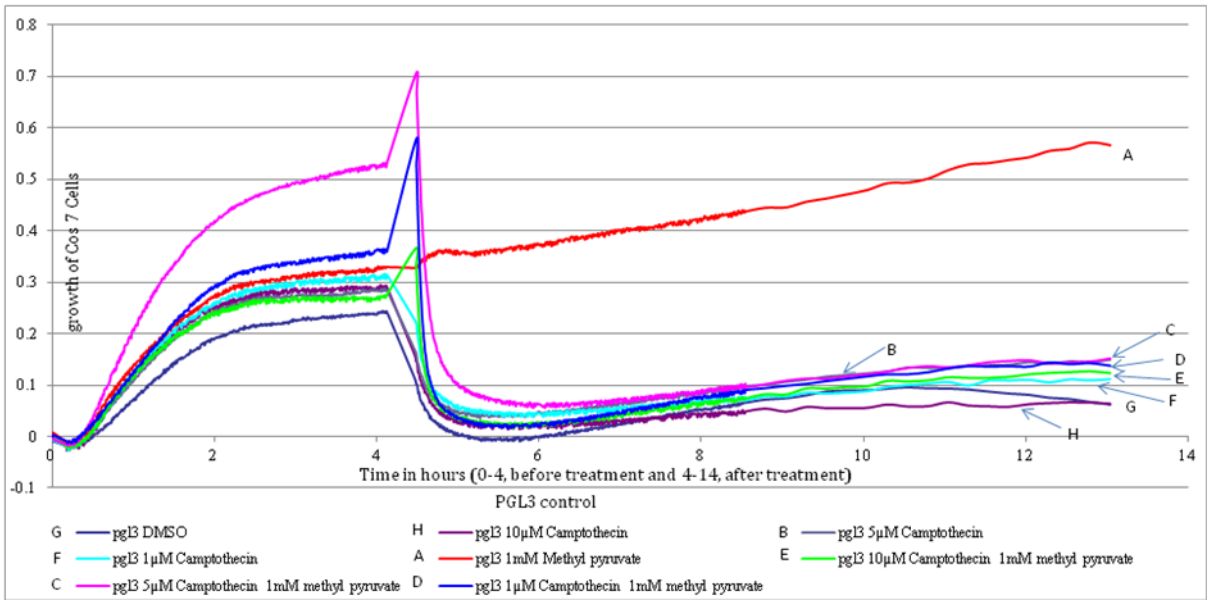
- Continuous quality control of cells in culture (www.aceabio.com).

Figure 14 (A-D) shows the effect of different concentrations of camptothecin on the growth of transfected Cos7 cells. It appears that regardless of the concentration used the cells mortality levels increase following camptothecin exposure. The higher the concentration the faster the cells die. However, the DMSO control showed the presence of the solvent killed the cells at rates similar to the lower concentration (1µM) of camptothecin. Therefore, we cannot conclusively state that the increase in mortality is due to the presence of camptothecin. So in this experiment we observe that:

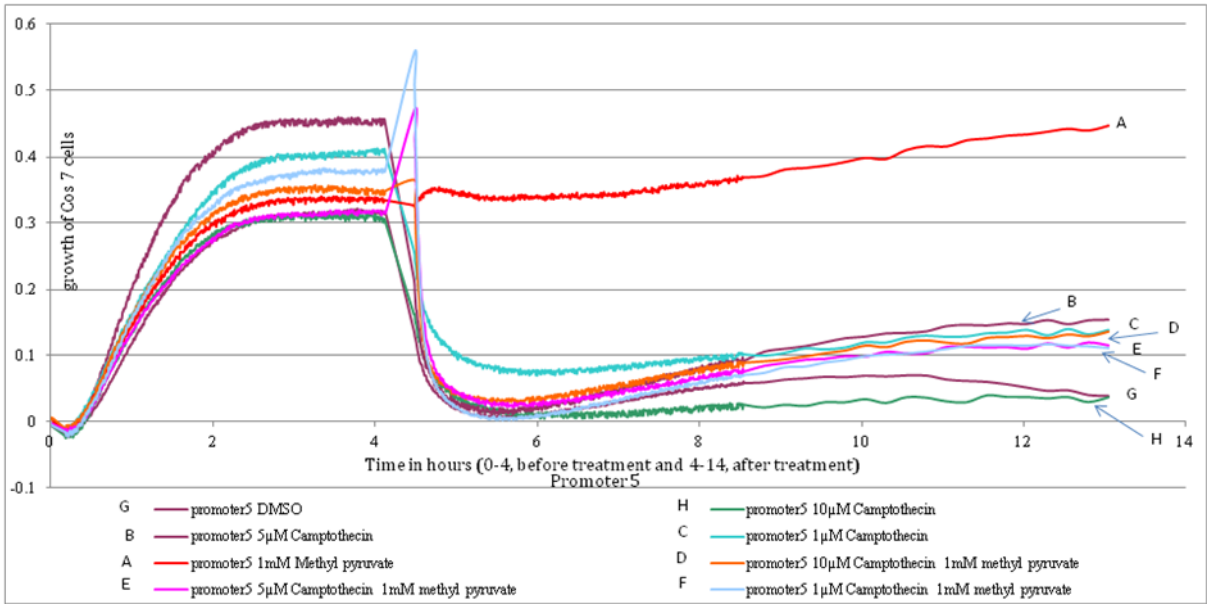
(i) DMSO, the solvent used for camptothecin, inhibited cell multiplication (figure 14)

(ii) It is evident methyl pyruvate has the same effect as normal untreated cells. The cells were not affected and grew normally

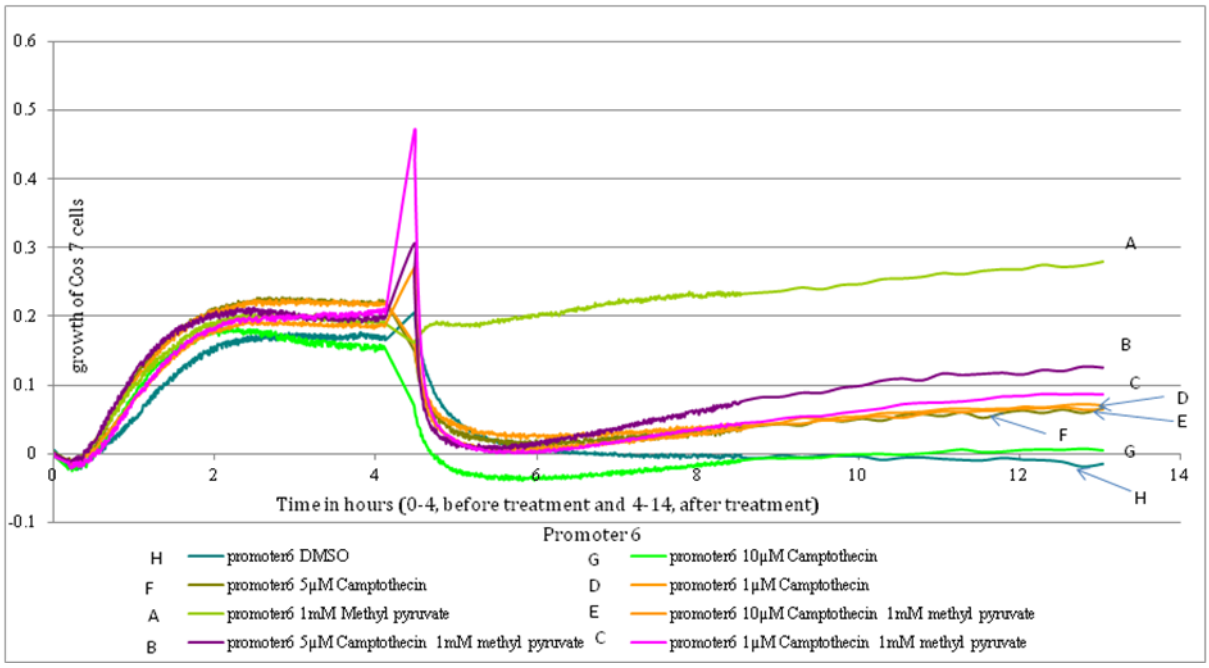
(iii) Furthermore, methyl pyruvate tends to improve the recovery process of DNA damaged cells. This is evident in all the experiments using methyl pyruvate treatments


growth of Cos 7 Cells
Time in hours (0-4, before treatment and 4-14, after treatment)
PGL3 control
G pgl3 DMSO
H pgl3 10µM Camptothecin
B pgl3 5µM Camptothecin
F pgl3 1µM Camptothecin
A pgl3 1mM Methyl pyruvate
E pgl3 10µM Camptothecin 1mM methyl pyruvate
C pgl3 5µM Camptothecin 1mM methyl pyruvate
D pgl3 1µM Camptothecin 1mM methyl pyruvate

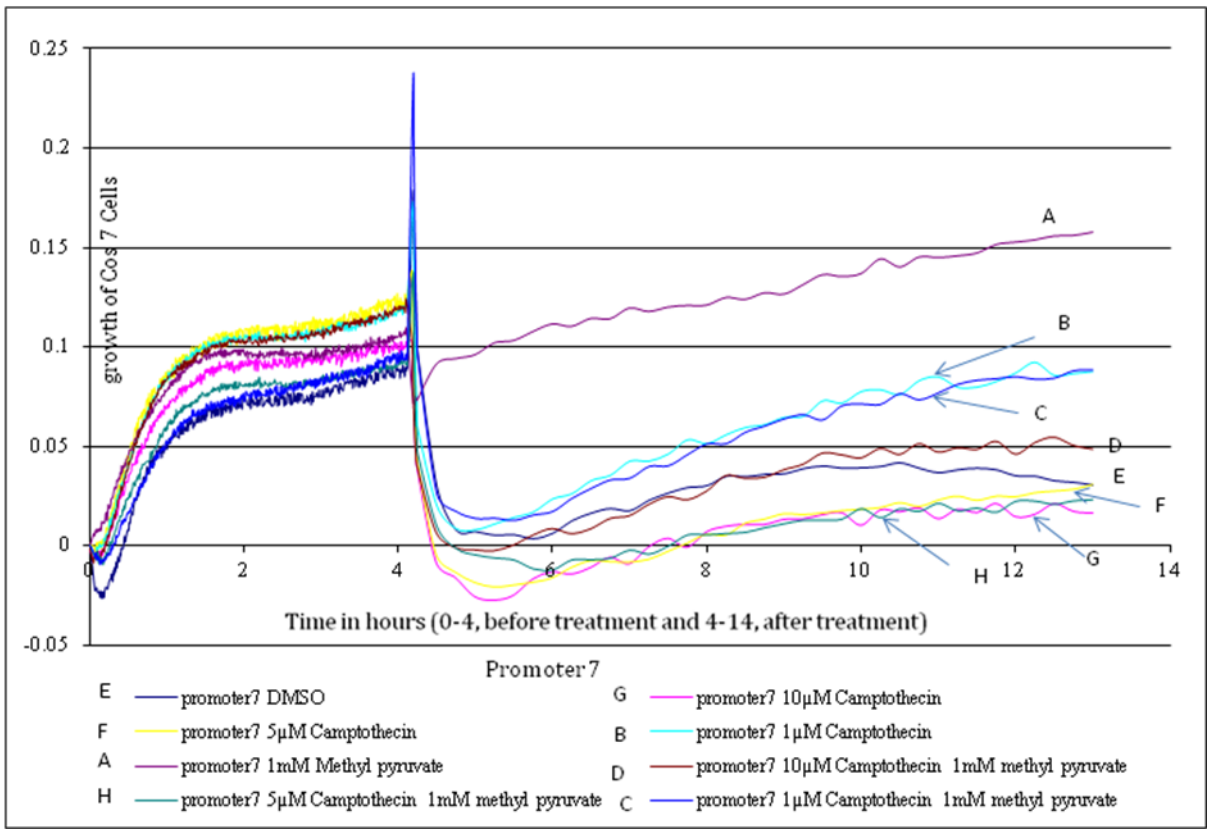
A


growth of Cos 7 cells
Time in hours (0-4, before treatment and 4-14, after treatment)
Promoter 5
G promoter5 DMSO
H promoter5 10µM Camptothecin
B promoter5 5µM Camptothecin
C promoter5 1µM Camptothecin
A promoter5 1mM Methyl pyruvate
D promoter5 10µM Camptothecin 1mM methyl pyruvate
E promoter5 5µM Camptothecin 1mM methyl pyruvate
F promoter5 1µM Camptothecin 1mM methyl pyruvate

B


0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
growth of Cos 7 cells
0
2
4
6
8
10
12
14
A
B
C
D
E
F
G
H
Time in hours (0-4, before treatment and 4-14, after treatment)
Promoter 6
H promoter6 DMSO
G promoter6 10µM Camptothecin
F promoter6 5µM Camptothecin
D promoter6 1µM Camptothecin
A promoter6 1mM Methyl pyruvate
E promoter6 10µM Camptothecin 1mM methyl pyruvate
B promoter6 5µM Camptothecin 1mM methyl pyruvate
C promoter6 1µM Camptothecin 1mM methyl pyruvate

C


0.25
0.2
0.15
0.1
0.05
0
-0.05
growth of Cos 7 Cells
0
2
4
6
8
10
12
14
A
B
C
D
E
F
G
H
Time in hours (0-4, before treatment and 4-14, after treatment)
Promoter 7
E promoter7 DMSO
G promoter7 10µM Camptothecin
F promoter7 5µM Camptothecin
B promoter7 1µM Camptothecin
A promoter7 1mM Methyl pyruvate
D promoter7 10µM Camptothecin 1mM methyl pyruvate
H promoter7 5µM Camptothecin 1mM methyl pyruvate
C promoter7 1µM Camptothecin 1mM methyl pyruvate

D

Figure 14: Treatment of Cos7 cells, transfected with plasmids containing different potential *Snama* promoters, with camptothecin, methyl pyruvate or a combination of both. In all cases the cells continue to grow at an exponential rate even after the addition of 1mM methyl pyruvate.. The DMSO control shows that DMSO at 1% final concentration is having a lethal effect on all cells. The cells exposed to higher camptothecin concentrations show a slight increase in mortality compared to DMSO alone. Methyl pyruvate seems to increase the survival rate of cells exposed to higher camptothecin concentrations.

4. Discussion

In this work the putative promoter region of *Snama* has been shown experimentally to drive transcription of a fused luciferase gene. Furthermore, electrophoretic mobility shift assays show that a subset of cellular proteins binds to this region suggesting the presence of transcription factor binding sites in this region. This is the first time that *Snama* promoter activity has been reported. Therefore, a window has been opened for further studies on the regulation of *Snama*. The bioinformatics assessment of potential transcription factor binding sites is limited by the fact that a mammalian database was searched. Further studies should focus on *Drosophila* Transfac databases perhaps using the Patch programme provided by Biobase.

4.1. The region immediately upstream the SNAMA TSS has promoter activity.

The main aim of this study was to determine the maximum promoter sequence needed to drive expression of *Snama*. The truncated promoter segments of *Snama* were tested for promoter activity using the luciferase assays. Clear promoter activity was indicated by the fragment named ‘promoter 6’ (P6).

The dual luciferase assay measures the change in luminescence when gene expression has occurred. For improved accuracy this assay also incorporates renilla luminescence measurement which can be used as an internal experimental control. The smaller truncations of the putative promoter region (Promoters 1, 2, 3 and 4) showed little or no ability to drive

transcription of either luciferase or renilla. This could be due to the fact that they lack the sites for core promoter elements required for minimal promoter activity. Promoter 5, located at position 454 upstream of the TSS (see figure 8) showed the least significant activity. This promoter sequence appears to have a TATA box at around position 195 upstream of the TSS (see figure 12). The TATA box is the binding site for DNA polymerase during initiation of transcription but requires the presence of core general transcription factors to initiate basal transcription activity. These may be lacking in this fragment.

4.2. Factors binding to promoter fragment represented by promoter (P6) seem to have positive impact on gene transcription.

The promoter sequence in *Snama* designated promoter 6 (P6) extends to position 552 upstream of the TSS and gave rise to the greatest Luciferase activity indicating the ability to induce the highest levels of transcription. These transcription levels were significantly higher than those observed in the control. We can therefore, confidently state that the positive regulatory region of *Snama* is located 552 bases upstream from the transcriptional start site.

When EMSA was conducted, P6 appeared to bind more nuclear proteins than P5 and P7 with P7 binding more proteins than P5. Notably, despite P7 being the longest promoter sequence and containing the full length sequence of P6 and an additional sequence it gave lower activity than P6, with EMSA showing less proteins binding as compared to P6. This leads us to conclude that this region probably contains a negative regulatory element which inhibits the transcription of the *luciferase* gene.

4.3. Promoter analysis

A bioinformatics search reveals potential sites for binding of transcriptional factors that regulate cell proliferations, patterning along the anteroposterior axis, mesoderm development and sex determination. An analysis of the transcription factors identified in this process shows that they have common functions in multiple associated biological processes. Many of these transcription factor models interact with each other through the JAK-STAT pathway to control developmental processes (Johnson *et al*, 2011; Liu *et al*, 2009). RNA PolII promoters usually consist of multiple binding sites for transcription factors which mediate the promoter function. Frameworks are sets of sequences that share a common orientation and distance organization between a set of related sequences.

Model Name	Position	Strand	*Drosophila* homolog	Function	Reference
HAND GATA	26-54	+			
HAND			Hand	1) Negative regulator of apoptosis. 2) Transcription regulation. 3) Embryonic\ larval development 4) Hemopoisis	(Han & Olson, 2005)
			GATA factor Pannier	1) Development 2) Cellular biosynthesis regulation 3) Pigment metabolism	(Fromental-Ramain *et al*, 2008)
			GATA factor Serpent	1) Development including 2) Hemapoisis 3) Immune system 4) Cellular process	(Muratoglu *et al*, 2007)
PBXC MYOD	50-29	-			
MYOD			nautilus CG10250	1) Transcription regulation 2) Muscle development	(Berkes *et al*, 2004), (Enriquez *et al*, 2012)
			PBX Ultrabithorax	1) Development 2) RNA metabolism	(Assa-Munt *et al*, 1993; Duboule, 2007)
BRNF RXRF	60-12	-			
			Multiple POU domain containing proteins exist in *Drosophila*. For example drifter	1) Development 2) Antimicrobial peptide synthesis 3) Positive regulation of transcript elongation from RNA polymerase II promoter	(Andersen & Rosenfeld, 2001; Assa-Munt *et al*, 1993)
			Ultraspiracle shares 47.9 % identity with the RXRF homolog from the locust *L migratora*	1) Development 2) Metabolism of organic compounds 3) Reproduction	(Ahuja *et al*, 2003)
CAAT SP1F	76-112	+			

SP1F			D-Sp1 Armadillo like helical Zinc finger containing Transcription Factor	1) Imaginal disc-derived leg morphogenesis 2) Regulation of transcription	(Narayan *et al*, 1997), (Schaeper *et al*, 2010), (Tseng *et al*, 2013)
ETSE SP1F	76-112	+			
			There are at least 9 members of the ETS transcription factor family present in *Drosophila* an example is the protein Pointed	1) Development 2) RNA metabolism 3) Reproduction	(Boisclair Lachance *et al*, 2014)
EBOX SREB	216-489	+		SREB Transcription factors are involved in 1) cholesterol biosynthesis 2) Fatty acid uptake and biosynthesis 3) membrane biosynthesis	
			srebp	1) Phagocytosis 2) Fatty acid biosynthesis 3) Positive regulation of transcription 4) Cell morphogenesis 5) Signaling pathways	(Parraga *et al*, 1998)
			An example of a Transcription factor that binds to E Boxes Bmal1/ Cycle	1) Biological regulation 2) rhythmic process; response to stimulus; positive regulation of cellular biosynthetic process; regulation of RNA metabolic process; positive regulation of nucleobase-containing compound metabolic process; response to organic substance; response to nutrient levels; locomotory behavior;	(Abruzzi *et al*, 2011)

				response to starvation; negative regulation of cellular biosynthetic process; adult behavior; cellular process; single-organism developmental process.	
			Another example of a protein that binds to E boxes cMyc diminutive, MyoD	1) Development 2) Cell cycle regulation 3) Reproduction	(Parisi *et al*, 2013)
GREF OCT1 POU TF family	231-184	-	Nubbin	1) Development 2) Regulation of transcription from RNA polymerase II promoter	(Li & Popadić, 2004)
CEBP EBOX	239-216	-			(Rørth *et al*, 2000)
CCAAT-enhancer-binding proteins (or C/EBPs)			slbo-PB C/EBP	1) Border follicle cell migration 2) JAK STAT kinase regulation 3) Cell fate determination 4) Transcription regulation 5) regulation of transcription from RNA polymerase II promoter	
SORY PAXB	592-549	-	eyeless	Development	(Tanaka-Matakatsu *et al*, 2015)
GATA NKXH	651-623	-		NKX-homeodomain transcription factors regulate organ development	

NKXH			Tinman	1)	Development commitment	(Ryu *et al*, 2011)
MYOD VTVB	871-819	-	nautilus CG10250			(Berkes *et al*, 2004; Enriquez *et al*, 2012)
(vertebrate TATA binding Protein)			TBP-related factor	1)	Transcription regulation	(Kedmi *et al*, 2014)

Figure 15: Trascription fators (TF's) and their functions.

These transcription factors include the *Drosophila* homologs of Hand. Hand is involved in the negative regulation of apoptosis, embryonic and larval development and the regulation of transcription. It is activated by the GATA factors, Pannier and Serpent both of which are also involved in development (Han & Olson, 2005). A binding site for Brn POU domain transcription factors (*BRNF)* was also identified. Pou domain containing proteins are DNA binding transcription factors that function in the neuroendocrine system as well as in organ development (Muratoglu *et al*, 2007). There are multiple POU domain- containing proteins found in *Drosophila*. The POU domain interacts with other proteins and in the case of the SNAMA promoter, it is predicted to allow the binding of a RXRF transcription factors. These transcription factors are involved in a variety of signalling pathways which are primarily related to metabolism, cell differentiation and cell death

Another transcription factor predicted to bind to the regulatory region of *Snama* is Nautilus. This is the *Drosophila* homolog of the MyoD. MyoD binds to myogenin promoter through domains which interact specifically with an adjacent protein complex containing the homeodomain protein Pbx. This transcription factor is involved in muscle development as well as the regulation of transcription (Fromental-Ramain *et al*, 2008). Here Nautilus is predicted to be under the control of either Pbx homeodomain proteins such as ultrabithorax (Enriquez *et al*, 2010) or POU domain proteins such as Nubbin in *Drosophila.* As mentioned previously Pou domain containing proteins have a wide variety of functions including development (Li & Popadić, 2004; Muratoglu *et al*, 2007). Other proteins that were predicted to associate with MyoD to potentially initiate SNAMA transcription are the vertebrate TATA

binding Protein VTVB (Tapscott *et al*, 1992). The *Drosophila* homolog TBP related factor 1 is also predicted to be involved in transcriptional regulation (Kedmi *et al*, 2014)

The SP1 transcription factor in humans is involved in cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling (Chae *et al*, 2014). The Sp1 homolog could interact with a member of the ETS transcription factor family or directly through a CAAT box, whose presence was identified in the promoter region (Gegonne *et al*, 1993). The ETS family is known to be involved in cellular differentiation, cell cycle control, cell migration, cell proliferation and apoptosis (Boisclair Lachance *et al*, 2014).

The Sterol Regulatory Element-Binding Proteins (SREBPs). SREBPs have a structure similar to E-box-binding helix-loop-helix (HLH) proteins (Parraga *et al*, 1998). A *Drosophila* homolog known as Srebp or Helix loop helix protein 106 is involved in the innate immune system, metabolism and cellular morphogenesis (Parraga *et al,* 1998). Further E box binding proteins present in *Drosophila* that are associated with development include the Cycle protein which controls cell cycle progression and development (Abruzzi *et al*, 2011) and the diminutive protein (Parisi *et al*, 2013). Another transcription factor family that is predicted to potentially associate with the E box contained within the SNAMA promoter region is the CCAAT-enhancer-binding proteins (or C/EBPs). A *Drosophila* homolog is responsible for Border follicle cell migration, cell fate determination and transcription regulation (Parisi *et al*, 2013) The PAXb transcription factor is also predicted to have a potential binding site within

the *Snama* promoter region. The *Drosophila* homolog of this protein Eyeless is involved in the development of the eye and general organ development (Tanaka-Matakatsu *et al*, 2015).

Binding sites were also identified for Tinman, a NKX homeodomain factor. These transcription factors operate in a positive feedback loop with GATA transcription factors and are responsible for heart development in vertebrates and in *Drosophila* it is involved in the development of the cardiac and digestive system (Ryu *et al*, 2011).

Previous studies have highlighted roles for SNAMA in nucleic acid metabolism, transcription during development and cell cycle control. Many of the transcription factor frameworks identified above play roles in these processes. Although, these transcription factors binding sites are predicted by a non *Drosophila* database, they have credible *Drosophila* homologues. This could be confirmed by a more rigorous analysis using invertebrate transcription factor databases.

4.4. Role in apoptosis

The real time cell assays performed using the xCELLigence system laid the groundwork for future studies on the role played by SNAMA during apoptosis. These initial trials showed that transfection with the reporter plasmids fused to the promoter did not alter the effects that camptothecin, methyl pyruvate or a combination of both on the Cos7 cells. These compounds had the same effect on the cells with or without the transfection with the reporter plasmids. They were performed as a pilot study based on earlier reports that SNAMA undergoes altered

regulation upon camptothecin and methylpyuravte treatment of adult *Drosophila*. Future work can be carried out with the promoter fused to *Snama* and followed by treatment of the cells with these compounds. Alteration in the expression of *Snama* would then be correlated with changes in cell growth and/or survival. As the xCELLigence assays show, camptothecin alone and in combination with methyl pyruvate, resulted in cells presenting a typical apoptosis response growth curve this treatment on cells that overexpress *Snama* would help to confirm the role played by SNAMA during apoptosis.

The hypothesis is that SNAMA plays a role in apoptosis through its association with p53 and its functional similarities with Mdm2. In *Drosophila* no Mdm2 homolog has been detected but the mouse homolog of RbBP6, PACT/P2P-R, can negatively regulate p53 by enhancing the activity of Mmd2. Both *mdm2* null mutants and *pact*$^{-/-}$ mice die during embryogenesis and display widespread apoptosis (Li *et al*, 2007). Deletion of p53 is able to rescue these mutants (de Oca Luna *et al*, 1995).

While these phenotypes are similar, the phenotype of the *pact-/-* mice is more severe indicating that PACT has additional functions to promoting the activity of Mdm2. For example, the *pact-/-* embryos can only be partially rescued by p53 deletion resulting in embryos with a distinct anterior-posterior pattern suggesting an important role for PACT in mouse embryonic patterning. Taken together these facts indicate the crucial importance of refining the biological role of SNAMA.

4.5. Conclusion

The putative promoter region of SNAMA drives gene expression of the luciferase gene. In addition, the possible transcription binding sites were found in this region. However further investigation is still required to elucidate the finer aspects of the regulation of *Snama*. The findings of the present study should contribute to understanding the regulation of *Snama* and potentially lead to the development of drugs that can be used to treat life threatening conditions such as cancer since SNAMA seems to be involved in apoptosis.

4.6. Future Work

Replacing the luciferase gene with the *Snama* in the pGL3 vector prior to transfection might give insight to the direct effect of *Snama* on cell growth and survival and to the mechanism by which SNAMA responds to DNA damage. Understanding the interaction between *Snama* and the promoters can possibly lead to the identification of the transcription factors involved which will give better insight of the positive or negative role the proteins play in gene regulation.

Since DMSO was found to induce apoptosis in the genotoxic DNA damage experiment, across all the promoters investigated, it masks the effect that the promoter may have in increasing the levels of apoptosis. It would be better to use an alternative solvent to observe the effect of the promoters on *Snama* in a more insightful manner. Such an alternative would be irinotecan which is a water soluble derivative of camptothecin.

5. Appendix

5.1. SNAMA sequence

```
1          GCATTTCACATCTCTGGGGCTTTGGCGTCACGTTCGCATCTCTAGTAAATTGGAAAAAAA
1                                          TSS
61         TAAAATCGTCGGAGTTTTTATGTGCTTGCAGAGTAGTATTTCTTTCATATGCAACTATGT
21                                                                M  S
121        CGGTACACTATAAATTTAAGAGTACACTCAACTTTGATACAATTACTTTTGATGGACTTC
41            V  H  Y  K  F  K  S  T  L  N  F  D  T  I  T  F  D  G  L  H
181        ACATTTCTGTCGGGGACTTAAAAAGGGAGATTGTGCAGCAGAAGCGACTGGGCAAAATCA
61            I  S  V  G  D  L  K  R  E  I  V  Q  Q  K  R  L  G  K  I  I

241        TCGACTTTGATCTCCAAATAACAAATGCGCAGAGTAAAGAAGAATACAAGGACGATGGGT
81            D  F  D  L  Q  I  T  N  A  Q  S  K  E  E  Y  K  D  D  G  F

301        TCCTTATTCCCAAAAACACAACGCTGATCATATCGCGCATCCCCATCGCCCATCCCACAA
101           L  I  P  K  N  T  T  L  I  I  S  R  I  P  I  A  H  P  T  K

361        AAAAGGGCTGGGAGCCACCAGCAGCAGAAAATGCCTTTTCGGCGGCGCCTGCCAAGCAGG
121           K  G  W  E  P  P  A  A  E  N  A  F  S  A  A  P  A  K  Q  D

421        ACAACTTCAACATGGACCTGTCCAAAATGCAAGGCACGGAGGAGGACAAAATCCAGGCCA
141           N  F  N  M  D  L  S  K  M  Q  G  T  E  E  D  K  I  Q  A  M

481        TGATGATGCAGAGCACAGTCGACTATGATCCTAAGACGTACCATCGTATTAAAGGACAAT
161           M  M  Q  S  T  V  D  Y  D  P  K  T  Y  H  R  I  K  G  Q  S

541        CGCAAGTGGGAGAAGTTCCCGCATCCTACCGATGCAACAAATGCAAGAAAAGCGGACACT
181           Q  V  G  E  V  P  A  S  Y  R  C  N  K  C  K  K  S  G  H  W

601        GGATCAAAAACTGTCCCTTTGTGGGGGGAAAGGACCAGCAAGAGGTCAAACGGAATACTG
201           I  K  N  C  P  F  V  G  G  K  D  Q  Q  E  V  K  R  N  T  G

661        GTATTCCGCGGTCTTTCCGCGACAAGCCAGATGCGGCTGAGAACGAATCAGCCGATTTTG
221           I  P  R  S  F  R  D  K  P  D  A  A  E  N  E  S  A  D  F  V

721        TGCTGCCTGCTGTACAAAACCAAGAGATACCGGAGGATCTGATATGCGGCATATGCCGAG
241           L  P  A  V  Q  N  Q  E  I  P  E  D  L  I  C  G  I  C  R  D

781        ATATATTCGTCGATGCTGTCATGATACCCTGCTGCGGAAGTTCCTTTTGTGACGACTGTG
261           I  F  V  D  A  V  M  I  P  C  C  G  S  S  F  C  D  D  C  V

841        TGCGAACCTCCTTATTGGAGTCAGAGGATAGTGAGTGCCCCGATTGCAAGGAGAAGAACT
281           R  T  S  L  L  E  S  E  D  S  E  C  P  D  C  K  E  K  N  C

901        GTTCGCCTGGCTCCCTGATACCTAATCGGTTCTTGAGGAATTCGGTGAACGCCTTTAAAA
301           S  P  G  S  L  I  P  N  R  F  L  R  N  S  V  N  A  F  K  N

961        ATGAGACTGGGTATAACAAAAGCGCGGCTAAGCCAGCTGCAGTAAAAAATGAGGAAAAAC
321           E  T  G  Y  N  K  S  A  A  K  P  A  A  V  K  N  E  E  K  P

1021       CTCCTGTTGAAAAAGAAGTGGAGAAAAAGCCAGTCGCGGAGGTGGAACCCGAAGAGACTG
341           P  V  E  K  E  V  E  K  K  P  V  A  E  V  E  P  E  E  T  E

1081       AGGTGAAACCTGAAAAGCAAAAAGAATCCGAAACCAATGGCAGTAATCCGCCAAAATCGG
361           V  K  P  E  K  Q  K  E  S  E  T  N  G  S  N  P  P  K  S  E
```

```
1141      AATCTCCAGAGCCTCCCGCAACCACAGAACCATCACAGAAGGAGAAAGATAAATATGATT
381          S  P  E  P  P  A  T  T  E  P  S  Q  K  E  K  D  K  Y  D  S

1201      CAGACTACGAGGATAACATTACCATAAAAATGCCCCAGCCTGCAGCTGATTCTACAACAG
401          D  Y  E  D  N  I  T  I  K  M  P  Q  P  A  A  D  S  T  T  V

1261      TGCCCAGCAAAAGATCCCCCAGTTATTCCCACAGAAGTGAATCCTCTCATCGACGGGACA
421          P  S  K  R  S  P  S  Y  S  H  R  S  E  S  S  H  R  R  D  R

1321      GGTCGGATTATGTTTCCGATCACGATCACAAGCACCAACGTCCATCAAAATCGGAGTCTG
441          S  D  Y  V  S  D  H  D  H  K  H  Q  R  P  S  K  S  E  S  V

1381      TTAACAAGGATCGCAGTCTCCTGCCCTTGCCCATTGGCACCCTGCCTAGCTACCAGGGCC
461          N  K  D  R  S  L  L  P  L  P  I  G  T  L  P  S  Y  Q  G  H

1441      ACATGATGGCCGAATCAGAAGAAGCTCGTCGATCGAGTGCCTATAAGCCCCCTTATATGC
481          M  M  A  E  S  E  E  A  R  R  S  S  A  Y  K  P  P  Y  M  Q

1501      AAATGCAGCGGGGCCCACCTCCTATGCACATGATGAGTCACCACATGCCAGCCTACAACA
501          M  Q  R  G  P  P  P  M  H  M  M  S  H  H  M  P  A  Y  N  N

1561      ACGGGTTTAACAACATGGGACAGAGGCCTCCCCTCAGCTATGTGCCGTATCAAAACCAAT
521          G  F  N  N  M  G  Q  R  P  P  L  S  Y  V  P  Y  Q  N  Q  S

1621      CCGTACACCCAATGCGTGCGCCGTACGGATCTGCAGGCGGAGGTATGAATATGAATATGT
541          V  H  P  M  R  A  P  Y  G  S  A  G  G  G  M  N  M  N  M  S

1681      CACAACCATTTCAGTCCCCAAATTTAGCCTCGATATACCAAGGGGTGGCAGCGAAGGTCG
561          Q  P  F  Q  S  P  N  L  A  S  I  Y  Q  G  V  A  A  K  V  G

1741      GTTCCGGTCCCATTGACGATCCGTTGGAGGCCTTCAATCGCATCATGAAGGAGAAGGAGC
581          S  G  P  I  D  D  P  L  E  A  F  N  R  I  M  K  E  K  E  R

1801      GGAAGAAGGTGGACCGCTTTCGAAGCTCTGACCGCCACAGGTCAAGGTCCCCGGATAGAC
601          K  K  V  D  R  F  R  S  S  D  R  H  R  S  R  S  P  D  R  Q

1861      AAAGGCACCGCTTTAAGTCTCCCATGTACGAAAAGGACAACTCCAGGGATAATCTCAAGG
621          R  H  R  F  K  S  P  M  Y  E  K  D  N  S  R  D  N  L  K  D

1921      ACAAAAGACCGCGATCCCGGGAAAGGAAGCGAGAACATAGCTACGAACGGCATATACGCC
641          K  R  P  R  S  R  E  R  K  R  E  H  S  Y  E  R  H  I  R  H

1981      ACCCTCGTTCTAGTCGCCAGCCGAATGATGGCTCTAAGTCCCCAGGTGGCAGAATCAAAA
661          P  R  S  S  R  Q  P  N  D  G  S  K  S  P  G  G  R  I  K  R

2041      GATCTGGACATCGTCGCTCTGCATCTCCAAAGCCGGGCTACAAGAGTGATTACAGAGACA
681          S  G  H  R  R  S  A  S  P  K  P  G  Y  K  S  D  Y  R  D  K

2101      AGCCGTACAACAAGCCTAGTGCTCCCAAAACGGAGGCAGTTGAGCCTCCTCCCCCCGGAT
701          P  Y  N  K  P  S  A  P  K  T  E  A  V  E  P  P  P  P  G  F

2161      TCGAGCCGTTGCAGCTGACGGATGAAGACGGCTACAGGAACAAGCACCCGACCAGTTCGG
721          E  P  L  Q  L  T  D  E  D  G  Y  R  N  K  H  P  T  S  S  E

2221      AAGCATCACAAAGCAGCAAGGGTGATAGCAGCAAGAAGAGAGGGGAAAACAGGCACGAAG
741          A  S  Q  S  S  K  G  D  S  S  K  K  R  G  E  N  R  H  E  E

2281      AGGCGCCACGAAAGAGGCACAGGTCTCGCAGCATTAGCAAGGAACCGAAGCCGAATGACA
761          A  P  R  K  R  H  R  S  R  S  I  S  K  E  P  K  P  N  D  S

2341      GCAACTACAGGAGCCTGACTCCACCAGCAAAGATCACCACACCGAAAATGACTGCTGCCC
781          N  Y  R  S  L  T  P  P  A  K  I  T  T  P  K  M  T  A  A  Q

2401      AGTTGAGGCAACGCGAAAGTTCACCGAAGACGCCGGAAAAGAGTCACGACGATTATCTGA
801          L  R  Q  R  E  S  S  P  K  T  P  E  K  S  H  D  D  Y  L  T
```

```
2461      CCGCGAAGGCCAGAATTATGGCCTCCCAGCCCGTCATCAACGACACGGAAATGGAGACCA
821          A  K  A  R  I  M  A  S  Q  P  V  I  N  D  T  E  M  E  T  N

2521      ATGTGGGCAAGGAGAACAAGGCCAAGAGTCCGTTGTCAAAAGATCGCAAGAAGAAGAAGA
841          V  G  K  E  N  K  A  K  S  P  L  S  K  D  R  K  K  K  K  K

2581      AGGACAAGGACAAGGCTGAGCGCAAGAAAAACAAGAAGGACAAGCGCGCTAAGAAGGAGA
861          D  K  D  K  A  E  R  K  K  N  K  K  D  K  R  A  K  K  E  K

2641      AAGGGGATCGCCAGAAGAAGAGCTCCTCAGTTAATCGATCTGACTCGGATATTAACAACA
881          G  D  R  Q  K  K  S  S  S  V  N  R  S  D  S  D  I  N  N  S

2701      GCTCACTAATGAACGAGTCAAATTATAAAGTATTGTCTCCCAGGGCTCAAAGTCCCAGCA
901          S  L  M  N  E  S  N  Y  K  V  L  S  P  R  A  Q  S  P  S  I

2761      TTGAGATCAATGCGGCTCAACTTTCCCCTACTCACAACGCTACTGAAAACGTTAATCCGA
921          E  I  N  A  A  Q  L  S  P  T  H  N  A  T  E  N  V  N  P  K

2821      AGAGTCATTCCATCCTTACTGTGGGTGCTGCTAGCGACGATAATCTTGGCCCAAGAAGCA
941          S  H  S  I  L  T  V  G  A  A  S  D  D  N  L  G  P  R  S  K

2881      AACTCAGCGAGGCTAATTCTGTCAATCTATCCAAATGGGAAATCGACGAGAATATCTTAG
961          L  S  E  A  N  S  V  N  L  S  K  W  E  I  D  E  N  I  L  G

2941      GTTTGGAGGATTCCTCCAAAAAAGCTGCCGGGGCCTCCGACGATCCGTCGGAAATAACTT
981          L  E  D  S  S  K  K  A  A  G  A  S  D  D  P  S  E  I  T  S

3001      CAGACGTCCTGCGAAAGGCTGAGAACGCAATATTTGCAAAGGCTATTAATGCCATCAGGC
1001         D  V  L  R  K  A  E  N  A  I  F  A  K  A  I  N  A  I  R  P

3061      CTATGGAGTTTCAAGTTATTATCAATTCCAAGGACAACAGCAAGGACCGCTCCGTAGTTC
1021         M  E  F  Q  V  I  I  N  S  K  D  N  S  K  D  R  S  V  V  R

3121      GAAGTGACAAGGATCGCTCCTCCTCACCCAGGCGTAACAACAGCAGCAGGTCGGTAAAGG
1041         S  D  K  D  R  S  S  S  P  R  R  N  N  S  S  R  S  V  K  D

3181      ATAGGCTGGGCACCAAGATTTCCAATGATAGAAGCCGTTCGCGAGACAAGTCGAAGGGCA
1061         R  L  G  T  K  I  S  N  D  R  S  R  S  R  D  K  S  K  G  R

3241      GGCGCCGGGCCGCAAGGAGCTCCGACGACGATGCGAACCGCGGCAGGTCGGATCGTCATG
1081         R  R  A  A  R  S  S  D  D  D  A  N  R  G  R  S  D  R  H  G

3301      GCAGCCGGAAGAGGGACAACAGATCCCGCGACAGGGCGGCGCCTTCAGAGAAGAGGCAGG
1101         S  R  K  R  D  N  R  S  R  D  R  A  A  P  S  E  K  R  Q  E

3361      AGCGTTCGTACAAGCGAAGCTCGCCGGAGGACGACAAGCTGAGGCGCCAGAACAAGGAAC
1121         R  S  Y  K  R  S  S  P  E  D  D  K  L  R  R  Q  N  K  E  Q

3421      AGTCCGAATCCAAGCACGGAAAGCATGATCAAAACAATAGCGACGACTCGGATCGGAGGG
1141         S  E  S  K  H  G  K  H  D  Q  N  N  S  D  D  S  D  R  R  A

3481      CGGCCAAAAACACCAAGTCCAGCGACAGCCGAGTGGTCTCCTCTGTAACAGCCGTGGTTG
1161         A  K  N  T  K  S  S  D  S  R  V  V  S  S  V  T  A  V  V  A

3541      CTCCTCCCAAACCCTGTCGTCCAGACAACCCGTTCCGCAAGTTCGTCGACACCAGTTCGT
1181         P  P  K  P  C  R  P  D  N  P  F  R  K  F  V  D  T  S  S  S

3601      CGAGCAGCTTAGTTGTAAAATATGATAACACGATACAGAAGGAGGGCGCGTCCTCGGACA
1201         S  S  L  V  V  K  Y  D  N  T  I  Q  K  E  G  A  S  S  D  N

3661      ACGGCATGGAGCACAGGAAGCAGAGGGATAAGAAGCTGAAGAAACATTCAAAATATTCGT
1221         G  M  E  H  R  K  Q  R  D  K  K  L  K  K  H  S  K  Y  S  S

3721      CAACCGATTCGTTGAAGAGCGAGAAGCGCAAGGATCCGAAGAGCAAAAAGAAGAGCAAGA
1241         T  D  S  L  K  S  E  K  R  K  D  P  K  S  K  K  K  S  K  I
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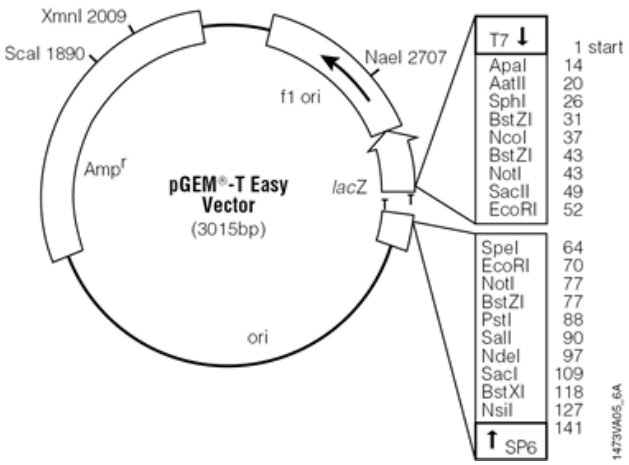
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3781      TTTTGAAGAAGAAGAAAAAATCAAAGAAGTAGGTTACGGTAGGCTACGAGATAAGAATGA
1261         L  K  K  K  K  K  S  K  K  *  V  T  V  G  Y  E  I  R  M  I

3841      TATAAATATTGAAGATTAATGTGTACAAATCAAAGATTTTTAATGTATGTATTTATCATG
1281         *  I  L  K  I  N  V  Y  K  S  K  I  F  N  V  C  I  Y  H  A

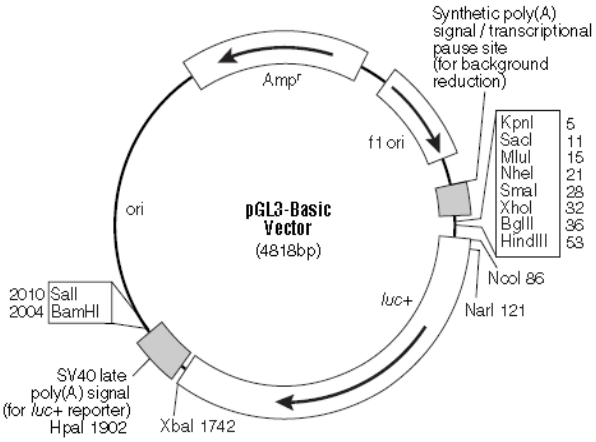
3901      CAACTATAAGTATACAAATAAAACAGAACTACTC
1301         T  I  S  I  Q  I  K  Q  N  Y
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The nucleic acid and amino acid sequence of SNAMA with DWNN, zinc and RING finger motif in green, blue and red respectively. The transcription start site is highlighted in a purple block.

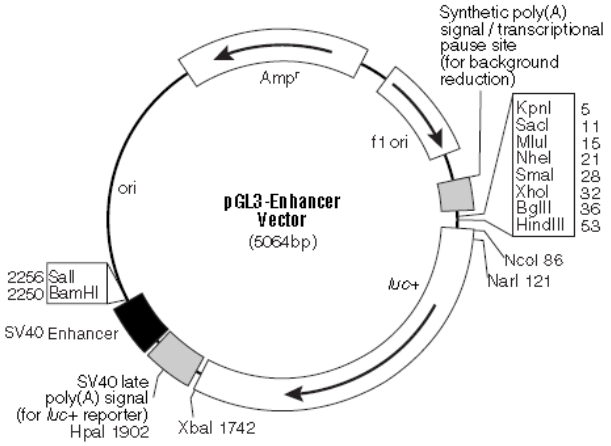
5.2. Vectors



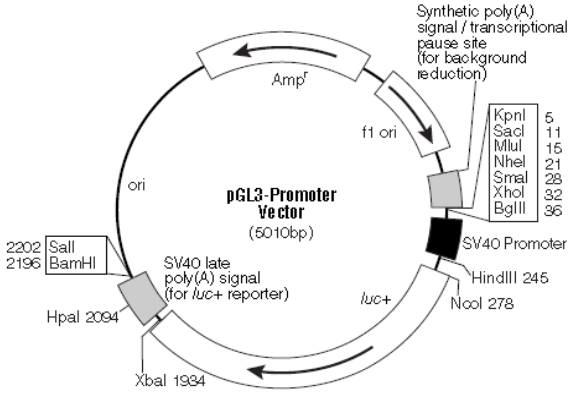
pGem-T-Easy circle map (Promega)



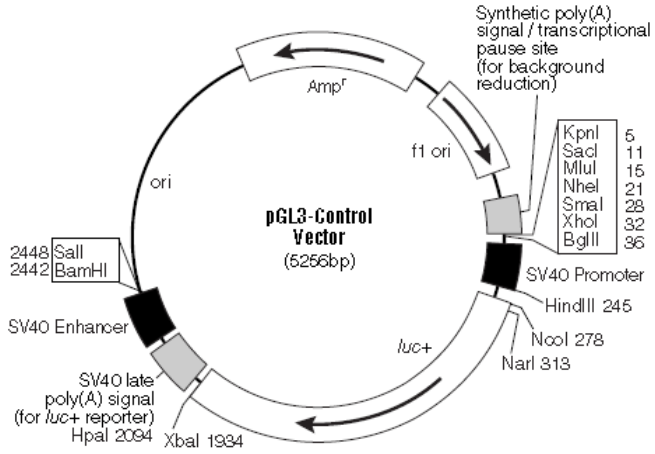
pGL3-Basic vector circle map (Promega)



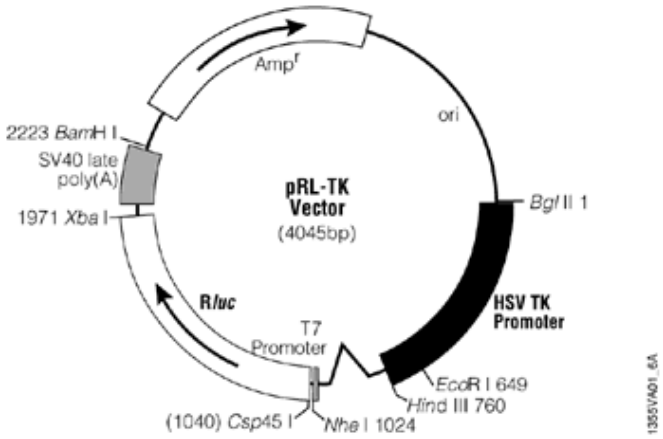
pGL3-Enhancer vector circle map (Promega)



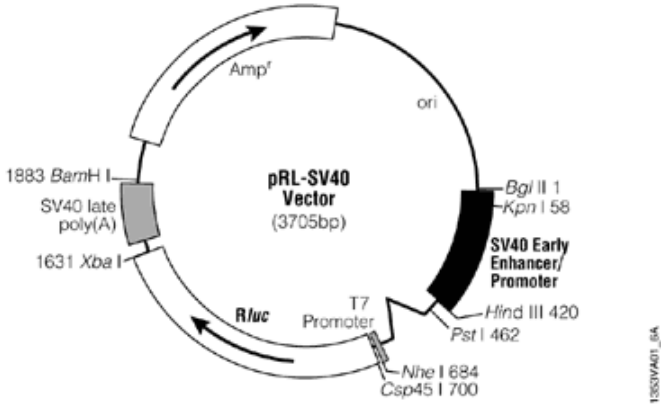
pGL3-Promoter vector circle map(Promega)



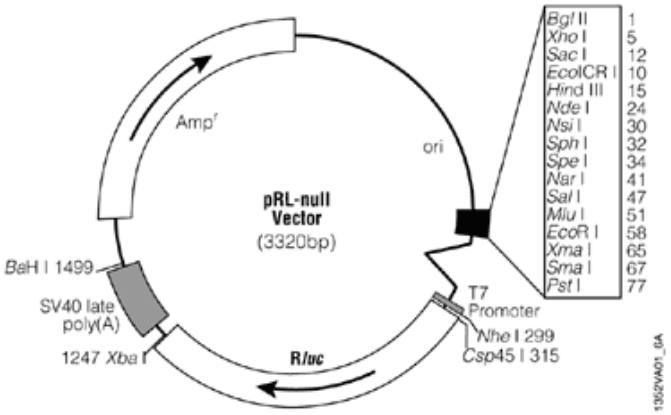
pGL3-Control vector circle map (Promega)



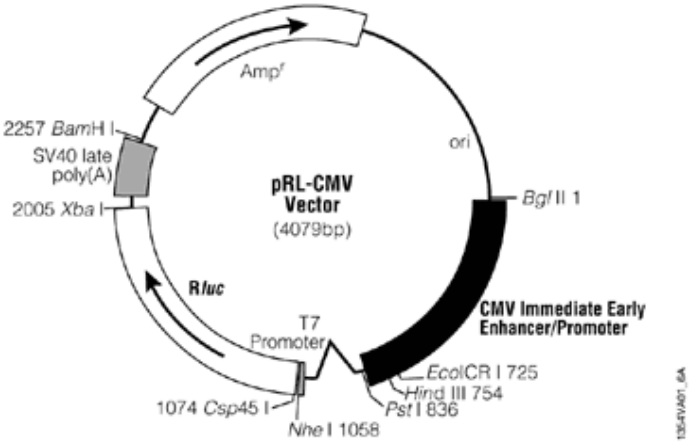
pRL-TK vector circle map (Promega)



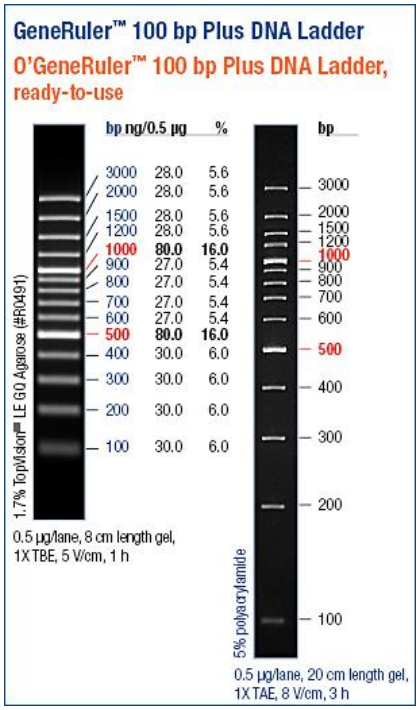
pRL-SV40 vector circle map (promega)



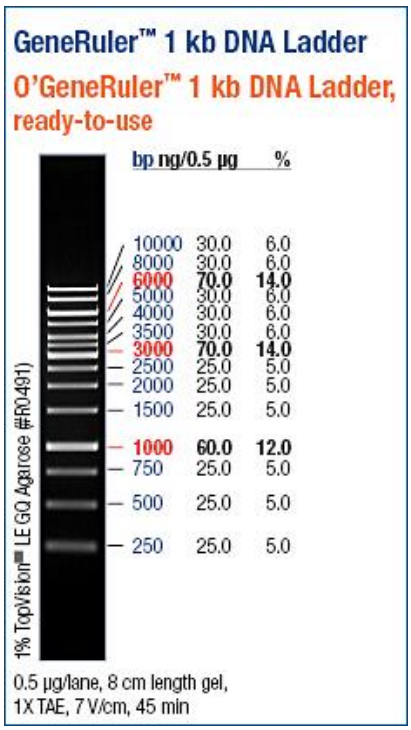
pRL-null vector circle map (promega)



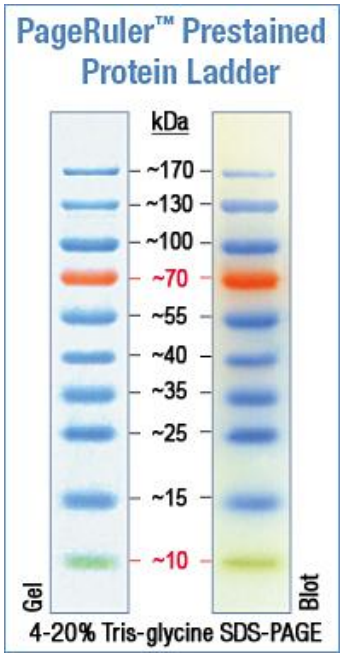
pRL-CVM vector circle map (promega)



GeneRuler 100bp (Fermentas)



GeneRuler 1kb (Fermentas)



PageRuler prestained protein ladder (Fermentas)

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