The role and localisation of PAPSS2a and PAPSS2b within zebrafish.

By
Emma Louise McCabe

A thesis submitted to the University of Birmingham for the degree of Master of Research

Centre for Endocrinology, Diabetes, and Metabolism
School of Clinical and Experimental Medicine
College of Medical and Dental Sciences
University of Birmingham
August 2011

ABSTRACT

Sulphation is a crucial modification which is required for normal growth and development. Sulphation reactions are mediated through the universal sulphate donor 3'-phosphoadenosinse 5-phosphosulfate (PAPS). PAPS is synthesised in a two step process by the bi-functional enzyme 3'-phosphoadenosine 5'-phosphosulfate synthase (PAPSS), which contains both APS kinase and ATP sulphurylase. There are two forms of PAPSS, PAPSS1 and PAPSS2. PAPSS2 is important for normal skeletal development and has two different isoforms, PAPSS2a and PAPSS2b. It was hypothesised that knock-downs of the PAPSS2a and PAPSS2b genes would lead to alterations in the phenotype of developing zebrafish embryos.

In Situ hybridisations were undertaken on zebrafish embryos at days 1, 2 and 3 using anti-sense probes for PAPSS2b. This allowed for confirmation of the expression of PAPSS2b within the zebrafish and also to locate the areas where it is expressed. Once expression of PAPSS2b had been confirmed within the zebrafish morpholino knock-downs were also undertaken for both the PAPSS2a and PAPSS2b genes. Four separate concentrations were used for the morpholino knock-downs (10μM, 30μM, 100μM, and 300μM). Splice site and translational blocking morpholinos were used to induce the gene knock-down and a scramble morpholino control was used. Successful knock-down of PAPSS2b was recorded using PCR when 100μM and 300μM concentrations of the morphlolino were used, the development of the embryo were monitored for the first four days of development and those which had the morpholino knock-down of PAPSS2b had a different phenotype to the controls by which their head was slightly smaller. For PAPSS2a the knock-down of the gene was unable to be confirmed due to the PCR not detecting any gene product in any of the injected zebrafish.

ACKNOWLEDGMENTS

I am indebted to my two co-supervisors Dr Vivek Dhir and Dr Ferenc Muller who allowed me to undertake such a diverse project thus allowed me the opportunity to learn about and explore new areas. They provided me with constant support and expert advice spanning all areas of the project.

I would also like to thank Jennifer Roberts, Irene Miguel, and Andreas Zaucker for their help, advice and encouragement throughout the project. Their assistance was invaluable to me throughout, providing expert advice on the area of zebrafish.

ABBREVIATIONS

PAPS = 3'-phosphoadenosine 5-phosphosulfate

PAPSS = 3'phosphoadenosine 5-phosphosulfate synthase

SULT = sulphotransferases

DNA = deoxyribonucleic acid

cDNA = complementary deoxyribonucleic acid

APS kinase = Adenylyl-sulfate kinase

ATP sulphurylase = Adenosine triphosphate sulphurylase

ADP = Adenosine diphosphate

SEMD = spondyloepimetaphyseal dysplasia

LB = Lysogeny Broth

dNTP = Deoxyribonucleotide

PCR = polymerase chain reaction

PBST = phosphate buffered saline with tween 20

bm = brachymorphism

CONTENTS

ABSTRACT 1

ACKNOWLEDGMENTS 2

ABBREVIATIONS 3

1 INTRODUCTION 8

1.1 The role of sulphation in metabolism 8

1.2 3'-phosphoadenosinse 5-phosphosulfate 8

1.3 3'-phosphoadenosine 5'-phosphosulfate synthase 9

1.4 The zebrafish as a model organism 11

1.5 Adrenal gland development in humans and zebrafish 12

1.6 The interaction between endocrine hormones and bone 13

1.7 Hypothesis and aims 14

2 METHODS 15

2.1 Making Agar plates 15

2.2 Transformation 15

2.3 Mini preps 15

2.4 Making an 0.8% Agarose gel 16

2.5 Digestion 17

2.6 PCR 17

2.7 Sequencing 18

2.8 Ligation 18

2.9 Purifying PCR products from gels 18

2.10 Purifying from PCR products from PCR reactions 19
2.11 Obtaining zebrafish embryos 19
2.12 Micro-injection of morpholino solutions into embryos 20
2.13 Preparation of in-situ probes for PAPS localissation 20
2.14 In situ hybridisation 21
2.15 Imaging in situ hybridisation staining 22
2.16 Removing chorions and fixing embryos 23
2.17 RNA extraction and DNase treatment 23
2.18 cDNA synthesis 24
3 RESULTS 25
3.1 Digestion of the PCS^{2+} vector 25
3.2 PCR of PAPSS2b 26
3.3 Colony PCR 29
3.4 In situ hybridisation 32
3.5 Morpholino knock-down of PAPSS2b using splice site and translational blocking morpholinos 37
4 DISCUSSION 43
4.1 In Situ hybridisation of day 1, 2, and 3 zebrafish embryos using PAPSS2b probes 43
4.2 Morpholino knock-down of PAPSS2b using translational blocking and splice site morpholinos 44

4.3 Future work 46

5 REFERENCES 47

TABLE OF FIGURES

Figure 1 The synthesis of PAPS. 9

Figure 2 Digestion of PCS^{2+} vector 25

Figure 3 PAPSS2b mRNA. 27

Figure 4 PAPSS2b and short 28

Figure 5 PAPSS2b short 28

Figure 6 PAPSS2b mRNA insert. 30

Figure 7 Mini-prep 31

Figure 8 In situ day 1 PAPSS2b mRNA probe 33

Figure 9 In situ day 1 PAPSS2b short probe. 33

Figure 10 In situ day 2 PAPSS2b short probe 34

Figure 11 In situ day 2 PAPSS2b mRNA probe 35

Figure 12 In situ day 3 PAPSS2b short probe 36

Figure 13 In situ day 3 PAPSS2b mRNA probe 36

Figure 14 Scramble morpholino and PAPSS2b splice morpholino (300µM) day 3 38

Figure 15 Scramble morpholino and PAPSS2b translational blocking morpholino (300µM) day 3 39

Figure 16 Scramble morpholino and PAPSS2b splice morpholino (300µM) day 4 40

Figure 17 Scramble morpholino and PAPSS2b translational blocking morpholino (300µM) day 4 41

1 INTRODUCTION

1.1 The role of sulphation in metabolism

Sulphation is a crucial modification of many proteins, carbohydrates, and lipids which is required for normal growth and development[7]. Sulphation reactions are mediated through the universal sulphate donor 3'-phosphoadenosinse 5-phosphosulfate (PAPS)[9]. A sulphation reaction consists of the transfer of a sulphate group from PAPS to a wide variety of substrates, and is catalysed by sulphotransferase enzymes[13]. Sulphation is a high-affinity enzymatic process. Notably the entire liver content of PAPS can be used up within 2 minutes[2].

Sulphation has an impact on many physiological processes, including; deactivation and bioactivation of xenobiotics, inactivation of hormones and catecholamines, alteration of structure and function of macromolecules, and elimination of end products of catabolism[13]. Under normal physiological conditions, sulphation is partially regulated by the quantity of PAPS available and the transport mechanisms by which sulphated conjugates enter and leave[2].

1.2 3'-phosphoadenosinse 5-phosphosulfate

PAPS is critical to sulphotransferase reactions, where it acts as a sulphate donor. The synthesis of PAPS in higher animals, including mammals is mediated by the bi-functional enzyme 3'-phosphoadenosine 5'-phosphosulfate synthase (PAPSS)[2], in a two step process. Firstly ATP sulphurylase, catalyses the formation of adenosine 5'-phosphosulfate (APS) from ATP and free sulfate, and secondly APS kinase phosphorylates the previously produced APS to form PAPS[4]. Following this, sulphotransferases (SULT) transfer sulphate, donated by PAPS, to an acceptor substrate. In mammalian cells, PAPS is needed for the sulphonation of proteins, hormones, neurotransmitters, carbohydrates, lipids, drugs and xenobiotics[16].

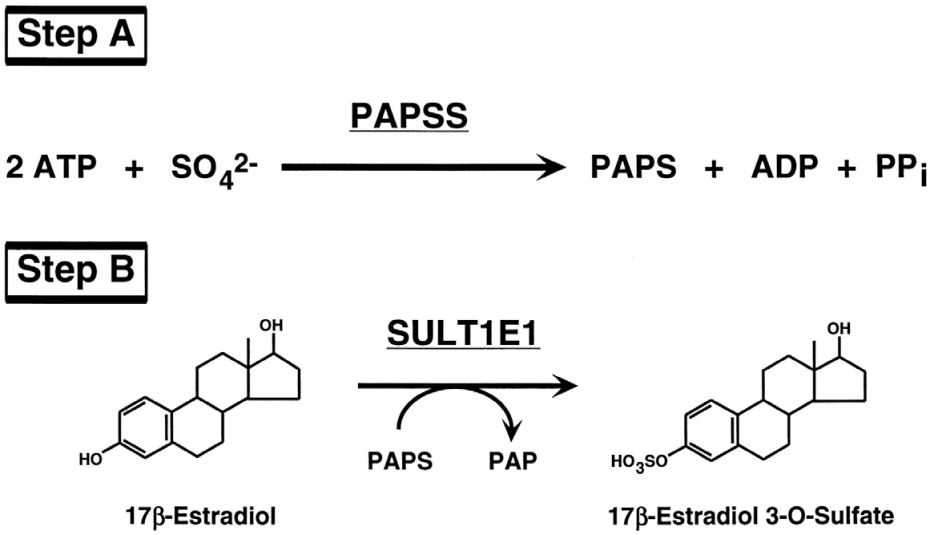

Figure 1 The synthesis of PAPS.
In step A PAPS is produced from ATP and SO_4^{2-} using both the ATP sulphurylase and the APS kinase activities of PAPSS. In step B SULT transfers a sulphate from PAPS (leaving PAP) to an acceptor substrate[5].

1.3 3'-phosphoadenosine 5'-phosphosulfate synthase

As described in 2.2 the PAPSS enzyme produces PAPS by a two stage reaction using both its APS kinase and ATP sulphurylase activities. PAPSS enzymes are crucial for all sulphation reactions. Within bacteria, fungi and yeast the two functionally distinct enzymes (APS kinase and ATP sulphurylase) are located on separate polypeptide chains. Within the marine worm *U.caupo* the mouse and human, APS kinase and ATP sulphurylase consist of a single bi-functional enzyme, PAPSS[4]. The cDNA which encodes the PAPSS gene has also been identified in the nematode *C-elegans*, thus providing further evidence that the fusion of APS kinase and ATP sulphurylase activity onto a single enzyme is conserved within animals[4]. Sequence comparisons have been carried out between sequences with APS kinase and ATP sulphurylase located on a single cDNA and also separate APS kinase and ATP sulphurylase sequences. These comparisons have shown that the bi-functional enzymes have a conserved

organisation, where the APS kinase activity is located to the N-terminus, and the ATP kinase activity is located to the C-terminus of the bi-functional enzymes[4]. The amino acid sequences of APS kinase, and ATP sulphurylase are highly conserved throughout evolution, regardless of whether they have fused to form a single protein, or are encoded by separate genes[4]. These observatiions suggest that vetibrate animal models have a human-like enzyme function and structural relationship. This enables the zebrafish to be a potential model for study of the PAPSS enzyme.

Within human tissue two separate PAPSS cDNAs have been identified[1], termed PAPSS1 and PAPSS2. These two isoforms of the PAPSS gene are similar, both consisting of 12 exons, and having virtually identical exon-intron splice junction locations[1]. The lengths of the genes do differ, with PAPSS1 being 108kb, and PAPSS2 145kb[1]. This size difference is also reflected in the size of the transcript, with PAPSS1 having a transcript of 2.7kb and PAPSS2 having a transcript of 4.2kb[1]. The two isoforms also map to chromosomes. PAPPS1 maps to the human chromosome band 4q24, and PAPSS2 maps to 10q22-23[1]. Despite these variations both PAPPS1 and PAPSS2 contain the ATP-sulphurylase domain and an APS-kinase domain critical to its function. Sequence similarity for PAPSS1 and PAPSS2 carried out on the coding regions of the APS kinase and ATP synthase domains have revealed 73% homology within the cDNA, and 76% similarity within the protein sequence[2].

The expression patterns between PAPSS1 and PAPSS2 varies substantially[2]. PAPSS1 is expressed within the testis, pancreas, kidney, thymus, prostate, ovary, small intestine, colon, leukocytes, cartilage and liver[2]. PAPSS2 is predominantly located within in the liver cartilage and adrenal glands[8]. Animal studies have indicated that PAPSS2 is the more

abundant isoenzyme in developing tissues that have a high sulphonation requirement, such as cartilage. PAPSS2 also has two alternatively spliced transcript variants encoding two different isoforms, these are PAPSS2a, and PAPSS2b.

Several studies have indicated that PAPSS2 activity is important for normal skeletal development. It has been found that mutations within PAPSS2 are responsible for abnormal skeletal development in human spondyloepimetaphyseal dysplasia and murine brachymorpism[15]. Pakistani type spondyloepimetaphyseal dysplasia is characterised by short stature, short and bowed lower limbs, mild brachydactyly, kyphoscoliosis, abnormal gait, enlarged knee joints, and precocious osteoarthropathy[2]. Also defects within PAPSS2 have been linked to perturbed foetal bone development[9].

1.4 The zebrafish as a model organism

Over recent years the zebrafish has emerged as a key model organism for the study of developmental processes[17]. Within this project the zebrafish will be used as the model organism to study the localisation of the PAPSS2b gene and also the developmental effects of a PAPSS2b gene knock-down. Research undertaken on zebrafish has led to advances in the fields of developmental biology, oncology, toxicology, reproduction, stem cell and many more.

There are many reasons why zebrafish have been successful as a model organism. Firstly they have a fully sequenced genome, which has been shown to be highly similar to the human genome[7]. This has led to the generation of a dense genetic map allowing for comparative mapping of human genes[11]. Zebrafish and humans also share high similarity in developmental processes, physiology and behaviour[10].

Zebrafish also have rapid embryonic development[10], and transparent embryos which develop outside the mother so they are ideally suited for studying developmental processors as they occur[10]. Due to the permeability and transparency of the zebrafish embryo[18], developing organ primordia in the whole mount specimen can be readily detected in situ hybridizations[19].

Zebrafish also produce many offspring in a short period of time allowing for ease of repetition of experiments, and gene knock-down using morpholino constructs which has made genetic manipulations possible[17].

Zebrafish also contain both PAPSS2a and PAPSS2b isoforms, as seen within humans. Within zebrafish the PAPSS2a and PAPSS2b isoforms originate from a gene duplication event, whereas in humans the two isoforms are formed via alternative splicing.

1.5 Adrenal gland development in humans and zebrafish

The adrenal gland is an area where PAPSS2b has been shown to localise to[21]. For this reason it is hypothesised that this is an area where an effect will be seen with a PAPSS2b gene knock-down in the developing zebrafish.

In humans the adrenal gland formation occurs within the first two months of development, the development within zebrafish is however far quicker. Various groups have shown that interrenal development in the zebrafish shares many conserved molecular and developmental mechanisms with higher vertibrates[19].

Zebrafish belong to the teleost group and therefore do not possess a distinct adrenal gland[11]. They do however contain an intermingled group of cells located in the anterior kidney which produces either catecholamines or steroid hormones[11]. The catecholamine-producing cells are derived from embryonic neural crest cells, while the interenal cells are derived from mesoderm[11] both glucocorticoid and mineralocorticoid are secreted.

In zebrafish there is parallel development of interrenal steroidogenic and catecholamine producing-chromaffin cells which resembles the formation of the adrenal gland in mammals[12]. Also, similar to the mammalian adrenal medulla, the chromaffin component of the interrenal organ is originated from the neural crest through sequential steps of differentiation[12].

1.6 The interaction between endocrine hormones and bone

Bone and cartilage are both areas where PAPSS2b has been shown to localise to during development[21]. For this reason it is hypothesised that the cartilage formation within the zebrafish will be affected by a PAPSS2b gene knock-down.

Bone together with cartilage, makes up the skeletal system which allows for multiple mechanical and metabolic functions, for example providing support for muscle attachment, protecting vital organs, and serving as a reserve of ions[6].

Several studies have indicated that PAPSS2 activity is important for normal skeletal development. A mutation within the PAPSS2 gene causes a skeletal disorder in humans, spondyloepimetaphyseal dysplasia (SEMD), Pakistani type. The clinical signs include short stature which is evident at birth[2].

Other studies have shown that a mis-sense mutation within the PAPSS2 gene leads to brachymorphism (bm) in mice[2]. bm leads to abnormal hepatic detoxification, bleeding times, and postnatal growth. The skeletal phenotype of the bm mice has been linked to reduced sulphation of the proteoglycans of the cartilage extracellular matrix[2].

Deficiency of PAPSS2 results in osteochondrodysplasias. Osteochondrodysplasias are a genetically heterogeneous group of disorders that affect skeletal development, linear growth, and the maintenance of cartilage and bone[8].

1.7 Hypothesis and aims

The project will aim to look into how these clinically important enzymes behave within a zebrafish model, and hence find if the zebrafish is a suitable model organism in which to test the impact of sulphation reactions on bone metabolism. This will include in situ hybridisation of day 1, 2 and 3 embryos using PAPSS2b probes to test the localisation of the gene within the zebrafish. Morpholino knock-downs of both the PAPSS2a and PAPSS2b genes will be used to observe any change in phenotype through the first four days of development.

2 METHODS

2.1 Making Agar plates

10g of LB, 10g of agar and 400ml of water were added to a 500ml bottle. The solution was then autoclaved. The media was left to cool until hand hot, following this ampicillin was added. If the agar had already solidified in the bottle then it was heated up in the microwave until melted. The liquid media was then poured into Petri dishes and left to set at room temperature.

2.2 Transformation

Competent cells (Alpha select, bronze efficiency) were placed on ice to thaw. Once thawed 10ng of plasmid DNA was added to the cells, keeping everything on ice. The cells were then incubated on ice for 30 minutes. Following this the cells underwent heat shock at 37˚C for 30 seconds, using a heat block. They were then placed back on ice for 2 minutes, then 200µl of LB was added to the cells, and then they were incubated at 37˚C using the heat block for 30 minutes and spread on a pre-warmed LB plate with ampicillin. The plate was allowed to dry at room temperature and then incubated at 37˚C overnight.

2.3 Mini preps

The tubes containing the overnight cultures (3.3) were spun down for 5 minutes at maximum speed in the centrifuge at room temperature. The supernant was removed from the tubes leaving the pellet. The pellet was then re-suspended with 250µl of P1 (6.1 g Tris, 3.7 g EDTA-2H2O pH 8.0 w/ HCl/1 liter, 100 µg/ml RNAse A, 10 mg RNase in 100 ml batches, store 4 degrees). Once the pellet had been re-suspended the solution was transferred into an eppendorf. 250µl of P2 (8.0 g NaOH in 900 ml H_2O, 100 ml of 10 % SDS/1 liter, store room temperature) was added to the re-suspension and the eppendorf invert 5/6 times until a blue

colour is consistent. 350µl of N1 was then added and the eppendorf was inverted 5/6 times until the blue colour disappeared. The sample was then spun down for 10 minutes on maximum speed using the micro centrifuge at room temperature. The supernant was added to a spin column and centrifuged for 45 seconds at maximum speed using the micro centrifuge at room temperature and the flow through was discarded. 750µl of buffer PE was then added to the spin column, and centrifuged for 1 minute on maximum speed using the micro centrifuge at room temperature, the flow through was discarded. The sample was centrifuged again for 1 minute at full speed using a micro centrifuge at room temperature and the flow through was discarded. The spin column was placed into a fresh eppendorf, and 50µl of buffer EB (10 mM Tris, pH 8.5) was added. This was then centrifuged for 1 minute at maximum speed using a micro centrifuge at room temperature and then spin column was discarded.

2.4 Making an 0.8% Agarose gel

0.8g of agarose was weighed out using weighing scales, per 100ml of TBE measured using a measuring cylinder. Both of these were added to a conical flask. The mixture was heated in the microwave until the agarose dissolved into the solution, making sure that the solution is checked every 20 seconds and mixed. The solution was left to cool for 10 minutes until hand hot and 5µl of Cybersafe (Invitrogen) per 100ml of the solution was added and mixed. The solution was then poured into the gel mould and left to set for around 30 minutes. The rubber seals and the combs were then removed from the gel mould, and it was placed into the electrophoresis apparatus making sure that it was covered with TBE, adding some if necessary.

5µl of hyperladder 1 was added to the first well using a pipette, and 5µl of the sample/s was added to the following well/s, using a pipette. The gel was electrophoreted at 90V for around 30 minutes.

2.5 Digestion

A typical linearisation digest consisted of 1µl of EcoR1, 6µl of the DNA to be digested, 4µl of buffer 4, and 11µl of water in an eppendorf. This was left at 37˚C using the heat block for 2 hours.

2.6 PCR

10µl of buffer, 5µl of primer 1, 5µl of primer 2, 1µl of dNTP, 2µl of $MgCl_2$, 0.5µl of fusion, 25.5µl of dH_2O, and 1µl of DNA, or 39µl of blue, 1µl of DNA, 5µl of primer 1, 5µl of primer 2 was added to an eppendorf. The primers used were:

Primer	**Primer sequence**
PAPSS2a mRNA	gcagaattcaaactaccttccaactcacctg
PAPSS2a mRNA	gcagaattccgaataagatatacagaaatctcagagac
PAPSS2a cDNA	gcagaattcatgcctggaaacaaaacactg
PAPSS2a cDNA	gcagaattctcactggactttctgtagggaag
PAPSS2a splice (1777)	gcagaattcctccgggactaagatgcgcaag
PAPSS2b mRNA	gcagaattcattaactgtgtcttggtcccttag
PAPSS2b mRNA	gcagaattcgtttattggaatggctgcccgatg
PAPSS2b cDNA	gcagaattcatgtctgggatgaagaagcagag
PAPSS2b cDNA	gcagaattctcactggtctttcttcagagaac
PAPSS2b splice (1685)	gcagaattcaccaggcctcacctctctcg

This was then processed through a PCR cycle according to the following programme:

Stage	**Temperature/C**	**Time/ minutes**
Hold	94	5
Elongation (x35)	94	0.5
	60	0.5
	72	1
Hold (x2)	72	5
End	4	

2.7 Sequencing

Sequencing was carried out by biosciences. The following sequencing primers were used:

Primer	Start (bp)	Primer sequence
PAPSS2a fwd	421	tttgaggtgtttgtgaac
PAPSS2a fwd	841	gacacccttctggaag
PAPSS2a fwd	1260	ggacacacgtcgaag
PAPSS2a fwd	1681	ttttatgacaaagacagac
PAPSS2a rev	280	cgatcctctgaggtg
PAPSS2a rev	770	aacacctgcacccac
PAPSS2a rev	1120	ttctctccaaaacctc
PAPSS2a rev	1540	tcccagcaggatcac
PAPSS2b fwd	431	aagtgtttgtaaatgctc
PAPSS2b fwd	882	tgttccaattgtgcttc
PAPSS2b fwd	1425	catcgtggccattttc
PAPSS2b fwd	1692	cgacaaggaaagacatg
PAPSS2b rev	198	gactagatattcttccag
PAPSS2b rev	647	ggtacaatatcctgttc
PAPSS2b rev	1095	cagccaatccccac
PAPSS2b rev	1490	cgacaatgccattgtac

2.8 Ligation

A typical ligation reaction was carried out by adding 1µl T4 DNA liagase, 1.5µl T4 DNA ligation buffer, 2µl PCS^{2+} (vector), and 10µl DNA insert to an insert followed by incubation overnight at room temperature.

2.9 Purifying PCR products from gels

The Cybersafe stained gel was observed under UV light in the dark room and the desired band identified by size and migration was excised using a scalpel removing as little excess gel as possible. The DNA fragment was placed into an eppendorf. The gel was then weighed and 3 volumes of buffer QG to one volume of gel (100mg = 100µl) was added, and incubated at 50˚C for 10 minutes (or until the gel slice has completely dissolved). After the gel slice had

dissolved completely 1 gel volume of isopropanol was added to the sample and mixed. The sample was placed into to the QIAquick column, and centrifuged for 1 minute at full speed at room temperature using a table top centrifuge. The flow through was discarded and 0.75ml of buffer PE was added to the QIAquick column and centrifuged for 1 minute at 13,000rpm at room temperature using a table top centrifuge. The flow through was discarded and the QIAquick column was centrifuged for an additional 1 minute at maximum speed at room temperature using a micro centrifuge. The QIAquick column was then placed into a clean 1.5ml micro centrifuge tube. And 50µl of buffer EB was added to the centre of the QIAquick membrane and then centrifuged for 1 minute at maximum speed at room temperature using a micro centrifuge.

2.10 Purifying from PCR products from PCR reactions

5 volumes of buffer PBI was added to 1 volume of the PCR sample. The QIAquick spin column was placed into a 2ml collection tube. The sample was then placed into the QIAquick column and centrifuged for 1 minute at maximum speed at room temperature in a table top centrifuge. The flow through was discarded and the column was centrifuged for an additional minute at maximum speed, at room temperature using a micro centrifuge. The QIA quick column was then placed into an eppendof and 50µl of buffer EB was added to the centre of the QIAquick membrane and centrifuge the column for 1 minute at 13,000rpm at room temperature using a micro centrifuge.

2.11 Obtaining zebrafish embryos

The day before the egg harvesting the zebrafish were placed into fresh tanks with the males and females separated by dividers with one male and one female per tank. The zebrafish were then left overnight. The following day the dividers were removed from between the zebrafish

and they were left for 15 minutes to lay. Once the eggs had been laid they were transferred into a Petri dish containing fresh water. The eggs were removed from the Petri dish using a pipette and placed in a clean dry Petri dish lid. The excess water was removed from the eggs using a pipette. Using another pipette with a fine tip the eggs were arranged so that none were overlapping.

2.12 Micro-injection of morpholino solutions into embryos

Morpholino solutions were made by adding 2µl of phenol red, 0.5µl of CFP RNA, 1µl of the morpholino of the given concentration (10µM, 30µM, 100uµM, or 300¥M), and 6.5µl of water to an eppendorf and this was kept on ice. Morpholino solution was pippeted into the tip of the needle. The needle was inserted into the embryo and the morpholino solution was then injected into the embryo.

The morpholino sequences used were:

Gene	Target	Morpholino sequence
PAPS2a	Translational blocking	tatccagtgttttgtttccaggcat
PAPS2a	Splice junction	agacctgcagaaacaaacacaaagt
PAPS2b	Translational blocking	ttctctgcttcttcatcccagacat
PAPS2b	Splice junction	cgatcctatcgtcaggacattaaaa
Control	Scramble	ttctatctacgtgtagctgtacttg

2.13 Preparation of in-situ probes for PAPS localissation

10µg of PSC^{2+} plasmid DNA, 5µl of restriction enzyme (Not1 was used to produce the sense probe and BamH1 was used to produce the anti-sense probe), 5µl of 10x buffer and sufficient dH_2O to give a final volume of 50µl was added to an eppendorf. This was incubated for 1.5 hours at 37˚C and then left overnight at 4°C. 2.5µl of 10% SDS and 1ul of Proteinase K

(10ng/µl) was added and incubated for 15 minutes at 55˚C using the heat block. 100µl of 1xTE and 100µl of Phenol/Chloroform was then added and the mixture was mixed by vigorous shaking. This was then centrifuged for 10 minutes at 7000 rpm in a table top centrifuge at 4˚C. The upper phase was placed into a new eppendorf, and 100µl of chloroform was added and the solution was mixed by vigorous shaking. The solution was then centrifuged for 10 minutes in a table top centrifuge at 7000 rpm at 4˚C. The upper phase was transferred into a new eppendorf, and 10µl of Sodium Acetate (pH 5.2) and 250µl of 100% ethanol was added and this was keep for 30 minutes at -80˚C. The solution was then thawed on ice for 15 minutes, and then centrifuged for 10 minutes in a table top centrifuge at 12000 rpm at 4˚C. The supernant was discarded and the pellet was washed with 80% ethanol. This was then centrifuged for 5 minutes in a table top centrifuge with 12000 rpm at 4˚C and the pellet was left to air-dry. The pellet was then re-suspended in 20µl of nuclease free water. 2µl of 10xtranscription buffer, 2µl of 10xDIG RNA labelling mix, 1µl of RNase inhibitor, 1ug of the cut vector template, 2µl of T7 polymerase, and enough nuclease free water to give a final volume of 20µl was added to an eppendorf, and incubated for 2 hours at 37˚C using the heat block. 2µl of RNase free DNase was added and left for 15 minutes at 37˚C using the heat block. 10µl of 7.5M NH_4Ac and 75µl of 100% ethanol was added and this was left at -80˚C for 20 minutes. Following this it was centrifuged for 15 minutes in a table top centrifuge at 12000 rpm at 4˚C. The pellet was washed with 80% ethanol and the pellet was left to air dry. The pellet was then re-suspend in 20µl of hybridisation buffer and stored at -20˚C.

2.14 In situ hybridisation

Dilutions of methanol of 75%, 50%, and 25% using phosphate buffered saline with tween 20 (PBST) were made up. The embryos were washed in 1ml 75% methanol, 50% methanol, and 25% methanol, leaving each for 5 minutes at room temperature. The embryos were then

washed four times in PBST leaving each for 5 minutes at room temperature. 4µl of proteinase K (10µg ml-1) was added and left at room temperature for 10 minutes on the shaker. The embryos were then washed in 1ml of PBST for 5 minutes. 2mls of 4% PFA was added and left for 20 minutes at room temperature. The embryos were then washed four times in PBST, 5 minutes per wash at room temperature. 500µl of hybridisation buffer was added and left for 3 hours in a 70˚C water bath. Then the hybridisation buffer was replaced with fresh hybridisation buffer and 1µl of either the sense or antisense probe. This was then placed in the 70˚C water bath overnight. The embryos were then washed twice for 30 minutes each with 1ml of 50:50 at 70°C in the water bath. The embryos were then washed for 15 minutes each with 1ml of 2xSCC at 70°C in the water bath. The embryos were then washed twice for 30 minutes each with 1ml of 0.2xSCC at 70°C in the water bath. 1ml of blocking buffer was then added and left for 3 hours at room temperature. The blocking buffer was then replaced with 1ml of anti-DIG antibody solution diluted to 1/4000 and left overnight at 4˚C. The embryos were then washed in 1ml of PBST six times for 20 minutes each at room temperature. The embryos were then washed in 1ml of staining buffer twice for 5 minutes each at room temperature, and then transferred to 12 well plates. 1ml of staining solution was then added at room temperature and then placed in the dark. Once the desired amount of staining was been achieved the embryos were washed in 1ml of PBST and then 2mls of 4% PFA was added.

2.15 Imaging in situ hybridisation staining

The PFA was removed and 25% glycerol in PBST was added and left for 1 hour at 4°C. Then the 25% glycerol was removed and 50% glycerol in PBST was added and left for 1 hour at 4°C. Then the 50% glycerol was removed and 75% glycerol in PBST was added and left for 1 hour at 4°C. Then the 75% glycerol was removed and 100% glycerol was added and left overnight at 4C. Slides were set up with 5 pieces of tape layered either side of the slide. A

drop of 100% glycerol was placed in the centre of the slide and on one embryo was placed on to image. The cover slip was placed over the embryo and moved in order to orientate the embryo for a top view and a side view.

2.16 Removing chorions and fixing embryos

1ml of pronase was added to the petri dish containing the embryos in about 1ml of water and left for 10 minutes. The petri dish was shaken to try to remove the embryos from their chorions. This was repeated every 3 minutes until two thirds of the embryos had been removed from their chorions. The petri dish was then placed into a beaker water containing, 1/3 cold water, 1/3 warm water, and 1/3 distilled water. The water was then poured off the embryos leaving them at the bottom of the beaker. The beaker was then tilted to a 45 degree angle and a beakers worth of water was poured down the side. This was repeated until all of the embryos were removed from their chorions. Once all of the embryos were free from their chorions the excess water was removed and the embryos were placed into an eppendorf tube. 1.5ml of 4% PFA was added to the embryos and they were left at 4C overnight. The following day the PFA was removed and 1ml of 25% methanol in PBST was added and left for 5 minutes at room temperature. This was then replaced with 1ml of 50% methanol in PBST and left for 5 minutes at room temperature. This was then replaced with 1ml of 75% methanol in PBST and left for 5 minutes at room temperature. This was then replaced with 1ml of 100% methanol and stored at -20˚C.

2.17 RNA extraction and DNase treatment

15 embryos were added to an eppendorf tube and 100µl TRIZOL was added. The embryos were then homogenised with a pestle. 90µl TRIZOL was then added to each sample and pipetted up and down several times. The sample was then left at room temperature for 5

minutes. 200µl of chloroform was then add and the sample shaken vigorously. It was then left at room temperature for 3 minutes. The sample was then centrifuged at 6000rpm for 10 minutes at room temperature. 400µl of the top phase was then transferred into a fresh eppendorf and 400µl of isopropanol was added. This was then centrifuged at top speed in a microcentrifuge for 30 minutes. The pellet was then washed with 1ml of 75% ethanol and 1ml of ethanol was added. The sample was then centrifuged for 20 minutes. The supernant was removed and the pellet was left to air-dry for 10 minutes. The pellet was then re-suspended in 17µl of nuclease-free water. 2µl of DNase buffer and 1µl DNase was added to the sample, and then incubated at 37˚C for 30 minutes. 2µl of inactivation buffer was then added and the sample was kept at room temperature for 5 minutes. The sample was then centrifuged at 10000rpm for 90 seconds at room temperature in a table top centrifuge, and the supernant was placed into a fresh tube.

2.18 cDNA synthesis

Once the RNA had been extracted it was reverse transcribed so that cDNA for use in the real-time PCR. The reverse transcription generated 50ng of cDNA/µl from 20µl of the initial reaction volume. (2.0µl of 10xRT Buffer, 4.4µl 25mM $MgCl_2$, 4.0µl dNTPs (10mM), 1.0µl random hexamers, 0.4µl RNase inhibitor, 1.25 MultiScribe reverse transcriptase, 7µl RNA, and nuclease free water [the amount of RNA used gave a final amount of 1µg, nuclease-free water was added to bring the combined RNA/water volume to 7µl]). One cycle was used (25˚C for 10 minutes, 37˚C for 60 minutes, and 95˚C for 5 minutes). All reagents were supplied by Applied Biosystems (Warrington, UK).

3 RESULTS

3.1 Digestion of the PCS^{2+} vector

Probes were required for the PAPSS2a and PAPSS2b genes in order to carry out in situ hybridisations. The genes of interest needed to be ligated into the vector PCS^{2+} to form a construct. The EcoR1 site was selected as the point of ligation.

The successful digestion of the vector by EcoR1 allows for the gene of interest to be ligated into it. The PCS^{2+} cloning vector was digested using EcoR1 as described in section 3.6. The product was then run on a gel to confirm that the digestion had been successful (figure 2).

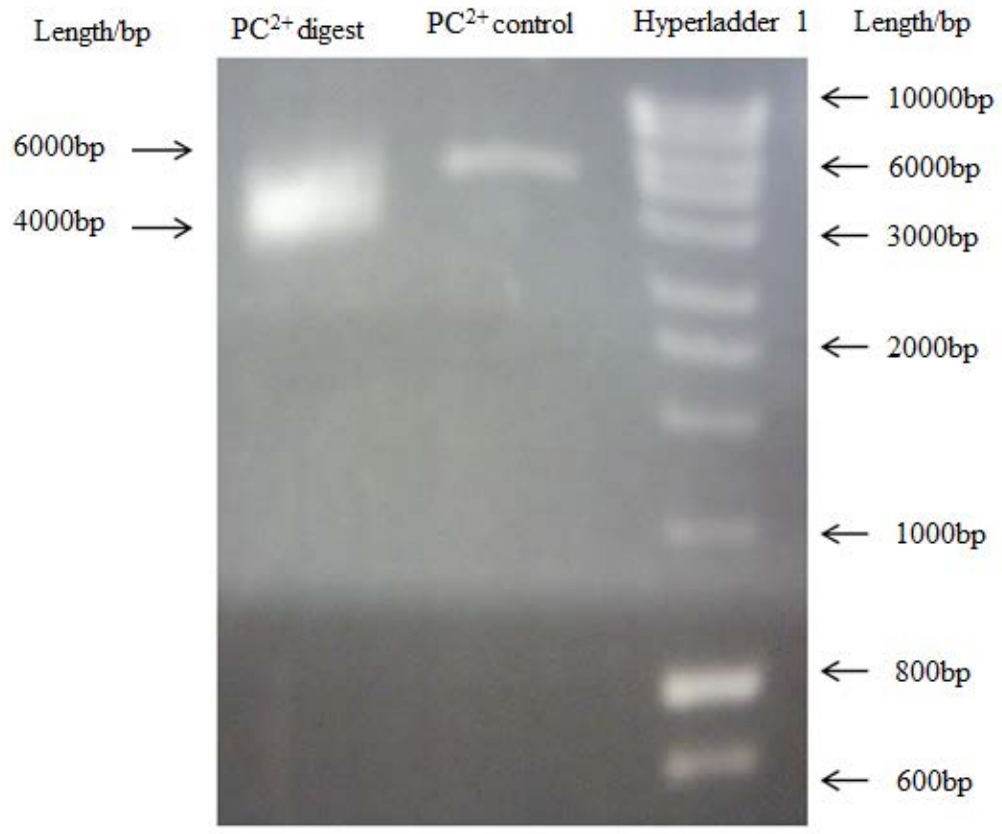

Figure 2 Digestion of PCS^{2+} vector
The image of the 0.8% gel confirms that the PCS^{2+} vector was successfully digested. An undigested control was also used. The digest is shown in the first lane, and the undigested control is shown in the second lane. As can be seen the control has a size of around 600bp and the digest has a size between 4000bp and 6000bp

3.2 PCR of PAPSS2b

PCR was carried out using self-designed primers shown in 3.7. The PCR was performed in order to obtain gene products to be ligated into the PCS^{2+} vector so that they could be used for the in situ hybridisation experiments. We aimed to produce gene products of three different sizes for both PAPSS2a and PAPSS2b. The first was a full length mRNA including the UTR, the second was only the coding section of the gene, and the third was a short segment from the 3' end of the gene (short).

PCR was performed using both fusion and blue methods (3.7). The temperatures used included 41˚C, 50˚C, 52˚C, 54˚C, 55˚C, 60˚C, 63˚C, and, 65˚C. The templates used were either 2 and 3 day embryo cDNA, PAPSS2b mRNA which I had produced as outlined in 3.7 or adult zebrafish ovary cDNA. The volume of $MgCl_2$ was also varied with 2µl, 4µl, and 6µl used. The elongation times were also varied between 30 seconds and 1.5minutes. Different primers for PAPSS2b were also used.

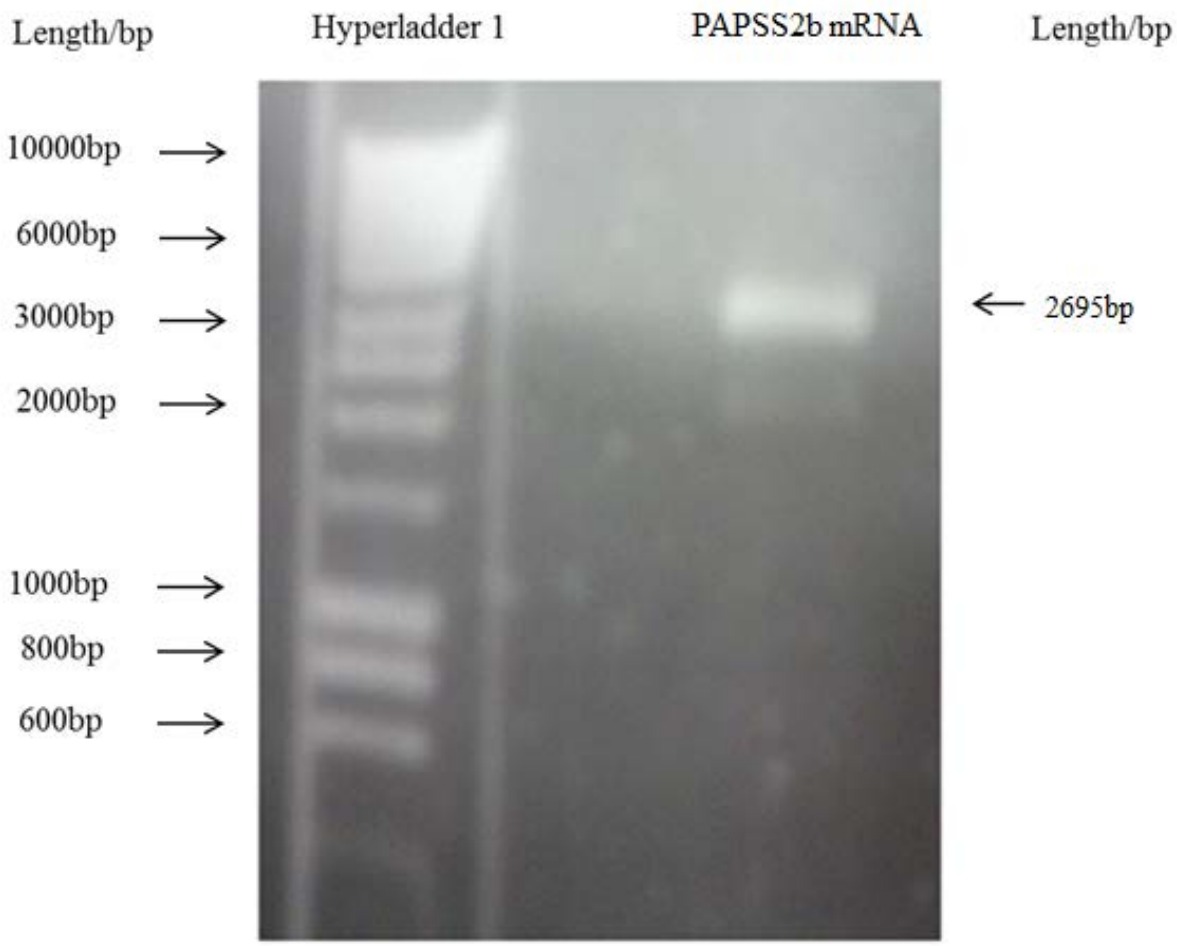

Figure 3 PAPSS2b mRNA.
The image shows the 0.8% gel image of the bands which had been produced by the PCR while using fusion mix, with a temperature of 55˚C and using 2/3 day cDNA. The primers which were used were for PAPPS2a; full length mRNA, coding region, and short, and PAPSS2b; mRNA, cDNA, and short. The band showing that PAPSS2b mRNA has successfully been amplified can clearly be seen. The PAPSS2b mRNA band is located at 2695bp in size.

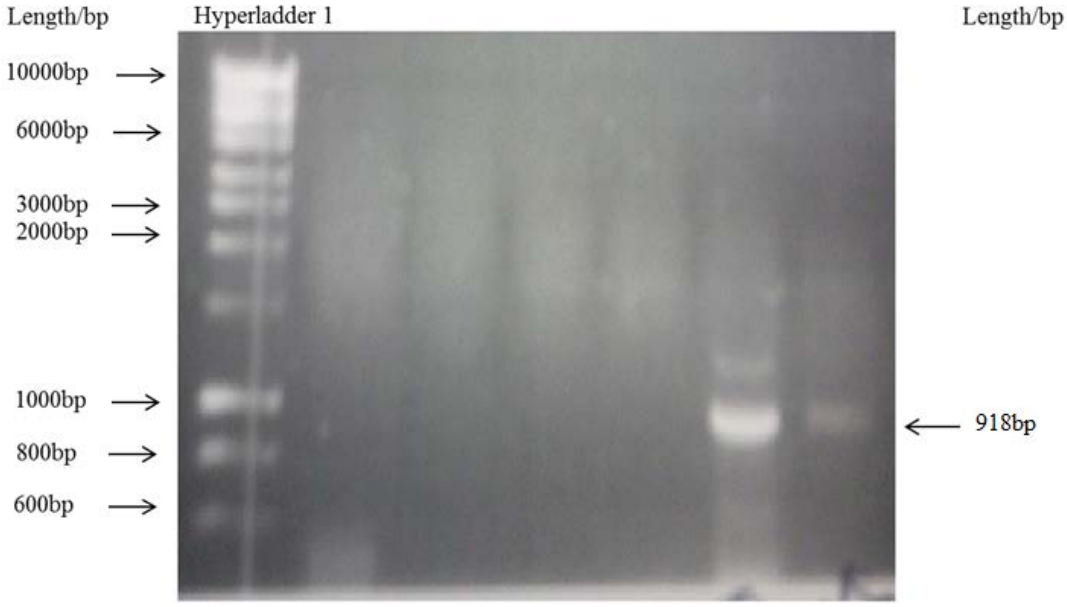

Figure 4 PAPSS2b and short

The image shows the 0.8% gel image of the PCR reactions which had been produced by the PCR while using fusion mix, with a temperature of 60˚C and using 2/3 day cDNA. The primers which were used were for PAPPS2a; mRNA, cDNA and short, and PAPSS2b; mRNA, cDNA, and short. The band showing that PAPSS2b short has successfully been amplified can clearly be seen in the fifth row from the hyperladder 1. The PAPSS2b short band is located around 918bp in size.

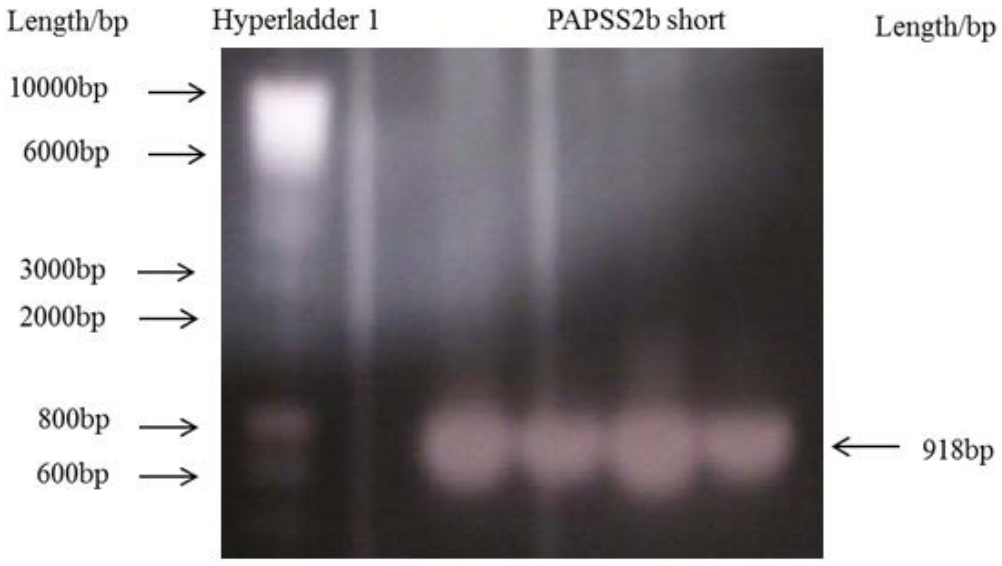

Figure 5 PAPSS2b short

The image shows the 0.8% gel image of the PCR reactions which had been produced by the PCR while using fusion mix, with a temperature of 41˚C and using the mRNA that I had produced as the template. The primers which were used were for PAPSS2b; cDNA, and short. The bands showing that PAPSS2b short has successfully been amplified can clearly be seen in the second, third, forth and fifth lanes from the hyperladder 1. The PAPSS2b short band is 918bp in size.

I was able to successfully produce a cDNA band representing the full length mRNA and the shortened version of the mRNA for the PAPSS2b gene using differing conditions for the PCR. The samples which had been run on the gel containing the mRNA could then be excised from the gel and purified. These mRNA samples were then ligated into the PCS^{2+} vector.

3.3 Colony PCR

Following the ligation of the vector and the PCR amplified insert the resulting vector was transformed into *E.Coli*, and then plated on agar plates. From here 25 colonies were selected for colony PCR. Following this the PCR was run on 8% gel and four colonies were selected which were identical to the positive control. A negative control of water was also used.

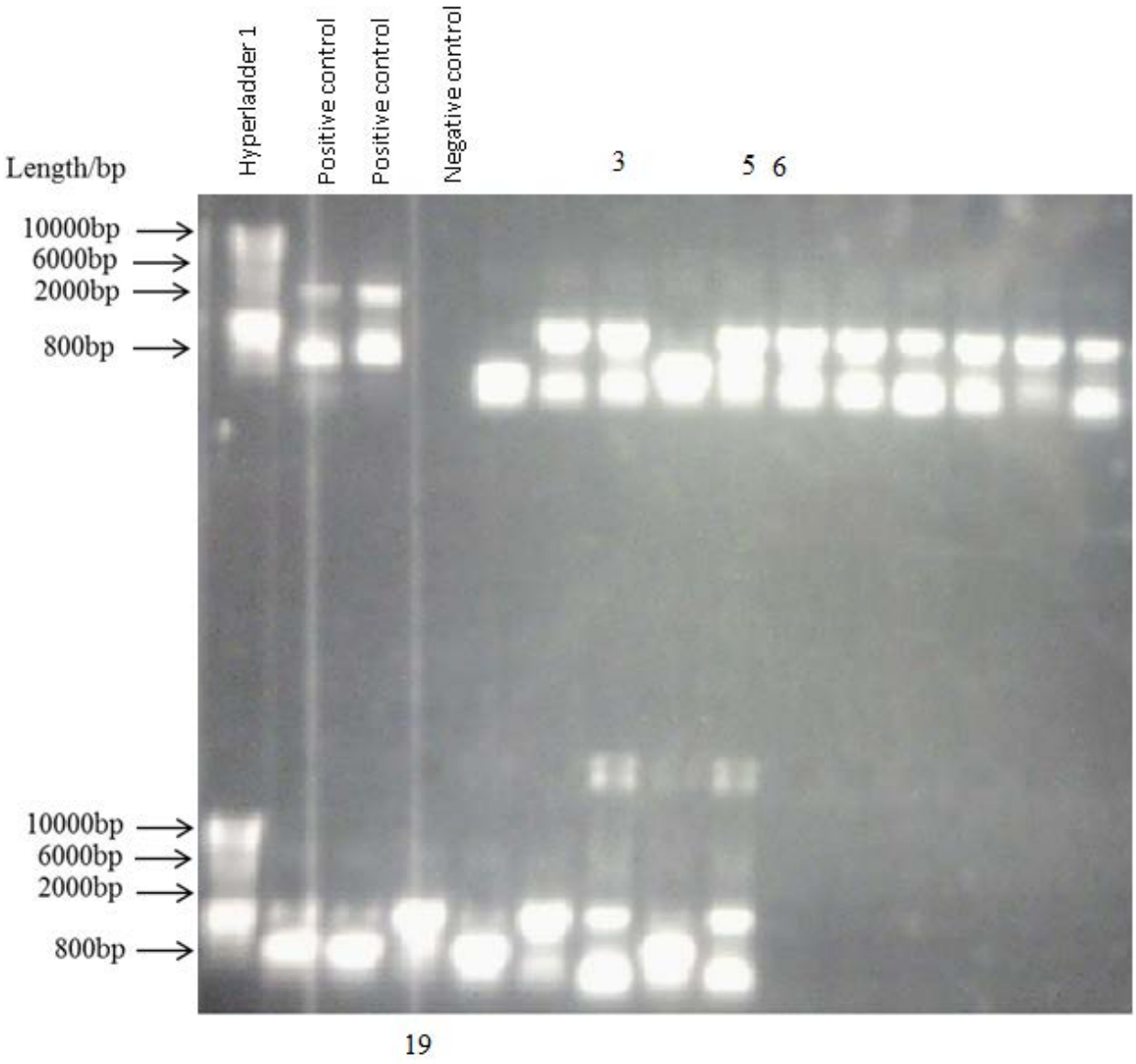

Figure 6 PAPSS2b mRNA insert.
The image shows the 8% gel print out of the solutions which had been produced by the colony PCR for the transformations which included the PAPSS2b mRNA insert. The third, fifth, sixth and nineteenth colony was selected.

Figure 4 shows which of the colonies contained the vector. The four which were selected were chosen due to firstly containing the insert and secondly because they most closely resembled the positive control.

Plasmid DNA was extracted by mini-prep as described in section 3.4 and the presence of the insert was confirmed by restriction digest with EcoR1 as described in section 3.6.

Figure 7 Mini-prep
The image shows the 0.8% gel image of the PCR reaction which had been produced by the mini-prep. These are the mini-preps of the colonies selected from figure 4. As can been seen three of the colonies still contain the insert.

As can be seen the insert is still present within three of the colonies and these can be used for further experiments. The sample was then excised from the gel, purified, and then sent for sequencing to confirm that the sequence is correct. The following primers were used for the sequencing:

PAPSS2b fwd	431	aagtgtttgtaaatgctc
PAPSS2b fwd	882	tgttccaattgtgcttc
PAPSS2b fwd	1425	catcgtggccattttc
PAPSS2b fwd	1692	cgacaaggaaagacatg
PAPSS2b rev	198	gactagatattcttccag
PAPSS2b rev	647	ggtacaatatcctgttc
PAPSS2b rev	1095	cagccaatccccac
PAPSS2b rev	1490	cgacaatgccattgtac

This allowed for a comparison between the genomic sequence and the cloned sequence which had been produced to confirm that the sequence was correct. Sequencing analysis confirmed that the insert was correct and could therefore be used successfully in future work.

3.4 In situ hybridisation

Embryos were selected and fixed at day 1, day 2, and day 3 following fertilisation. In situ hybridisation was then carried out on these embryos and images obtained. Sense and anti-sense probes which had previously been produced for PAPSS2b mRNA and PAPSS2b short transcript were used to identify the location of the PAPSS2b gene within the zebrafish at these time points. The sense probes provided a control, whereas the anti-sense probes allowed for the location of the PAPSS2b gene expression at the given time points to be revealed. This also allowed for confirmation that the PAPSS2b was being expressed within the zebrafish at these developmental stages.

Figure 8 In situ day 1 PAPSS2b mRNA probe
The images show the distribution of the PAPSS2b gene within the zebrafish 24 hours post-fertilisation with the use of the PAPSS2b mRNA probe. The blue colour indicates the staining with the anti-sense probe. At this time there is staining of the brain, immature eye, and somite.

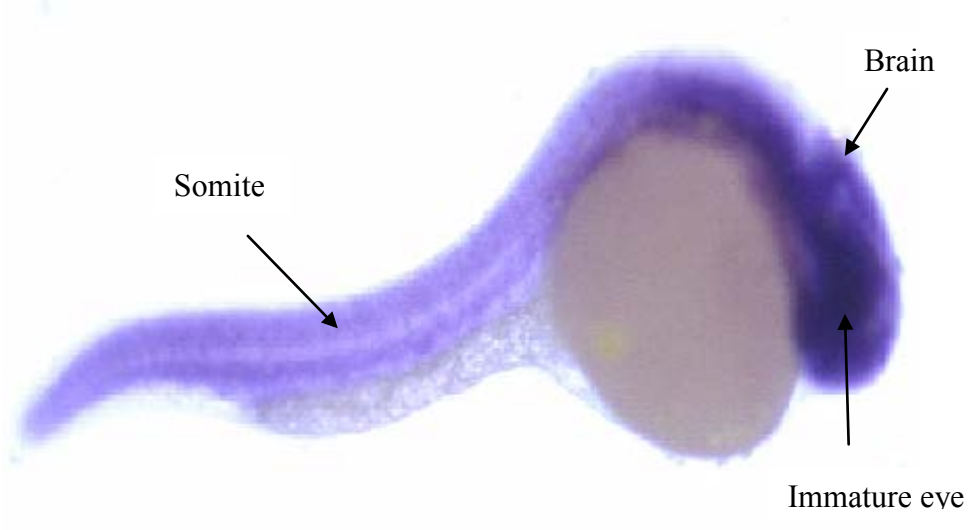

Figure 9 In situ day 1 PAPSS2b short probe.
The images show the distribution of the PAPSS2b gene within the zebrafish 24 hours post-fertilisation with the use of the PAPSS2b short probe. The blue colour indicates the staining with the anti-sense probe. At this time there is staining of the brain, immature eye, and somite.

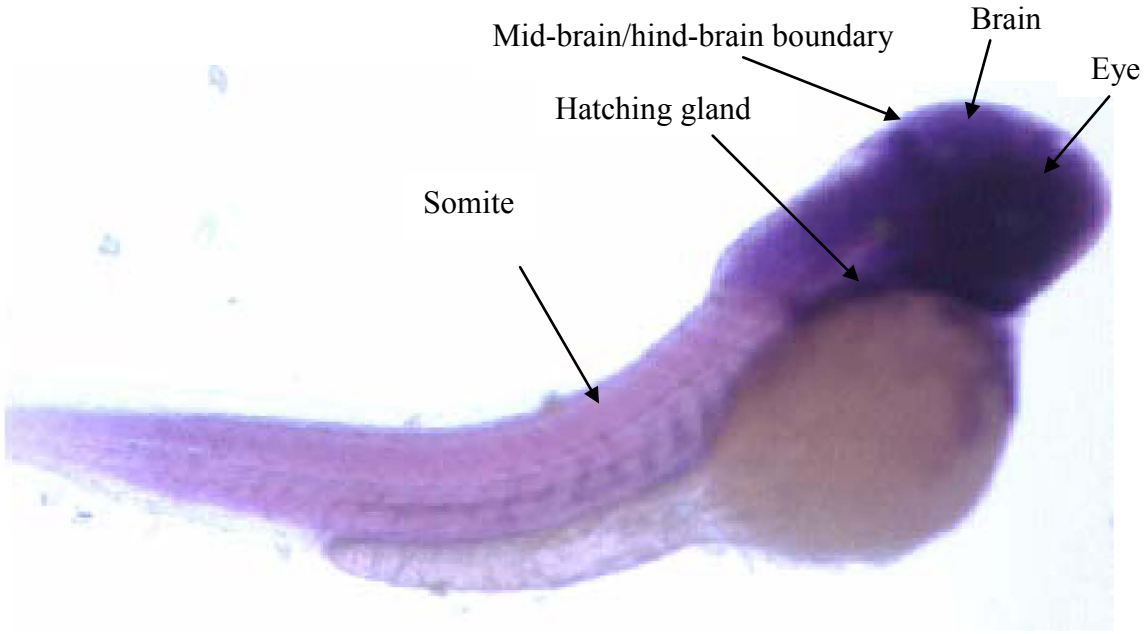

Figure 10 In situ day 2 PAPSS2b short probe
The images show the distribution of the PAPSS2b gene within the zebrafish 48 hours post-fertilisation with the use of the PAPSS2b short probe. The blue colour indicates the staining with the anti-sense probe. At this time there is staining of the brain, mid-brain/hind-brain boundary, eye, hatching gland, and somite.

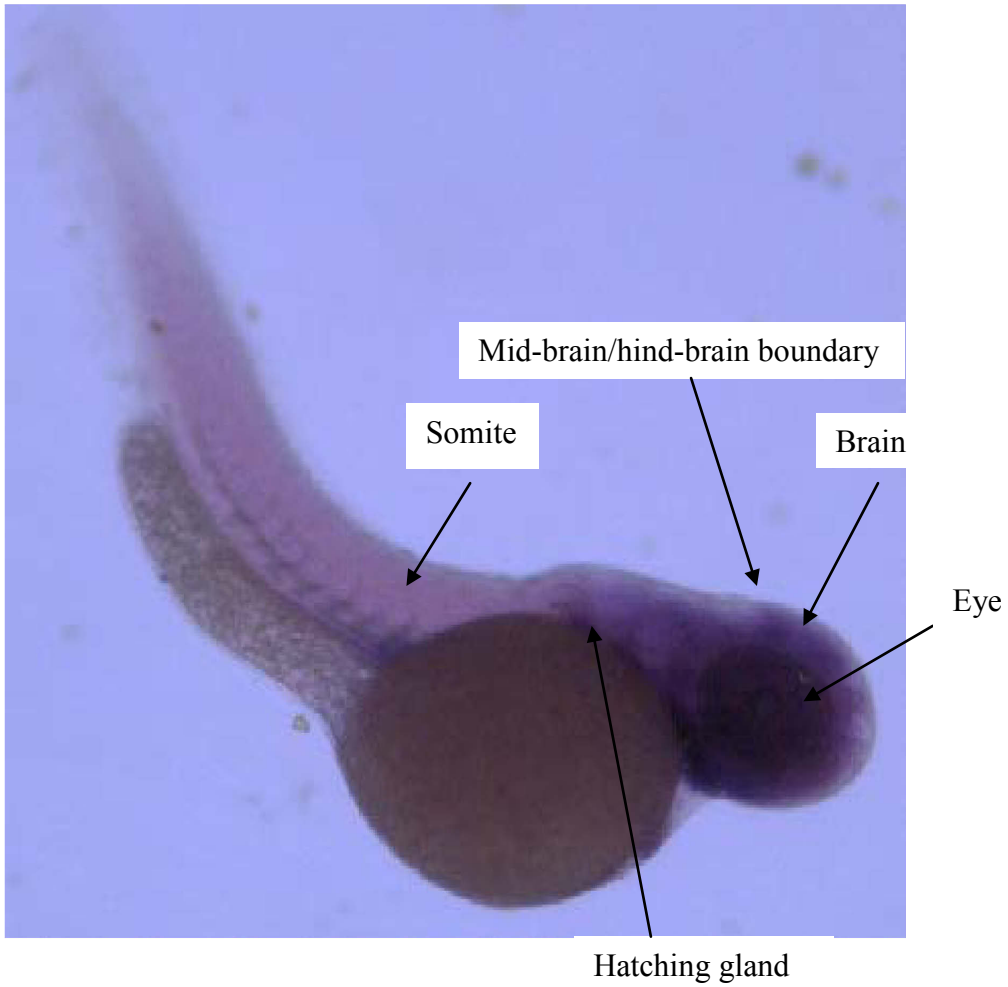

Figure 11 In situ day 2 PAPSS2b mRNA probe
The images show the distribution of the PAPSS2b gene within the zebrafish 48 hours post-fertilisation with the use of the PAPSS2b mRNA probe. The blue colour indicates the staining with the anti-sense probe. At this time there is staining of the brain, mid-brain/hind-brain boundary, eye, hatching gland, and somite.

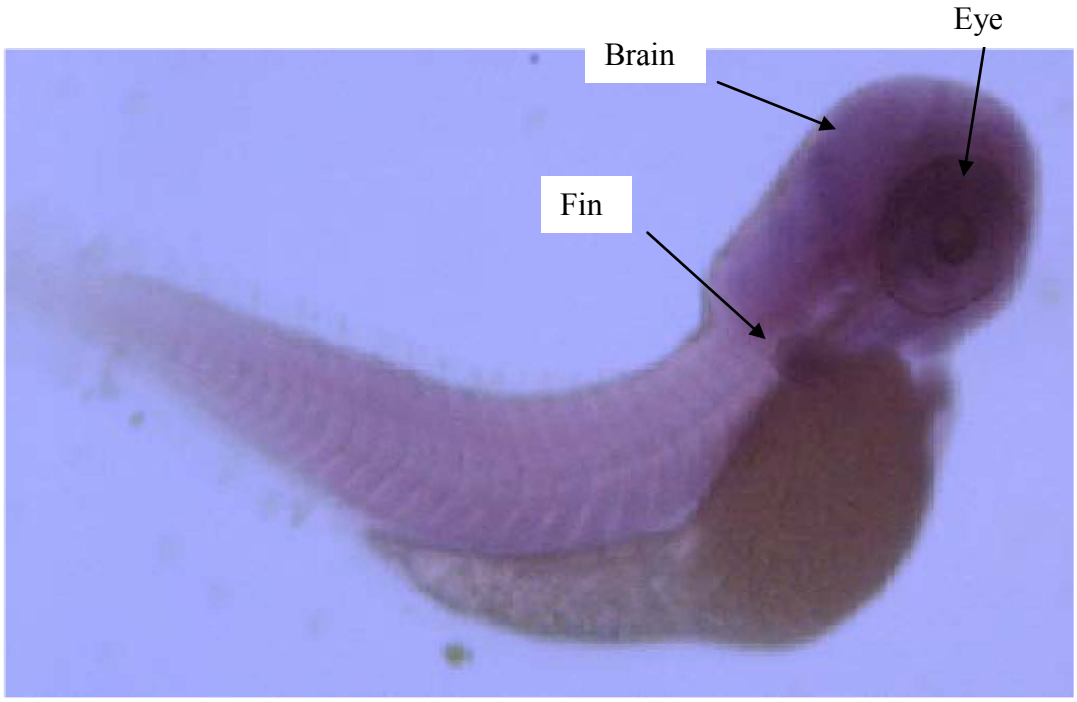

Figure 12 In situ day 3 PAPSS2b short probe
The images show the distribution of the PAPSS2b gene within the zebrafish 72 hours post-fertilisation with the use of the PAPSS2b short probe. The blue colour indicates the staining with the anti-sense probe. At this time there is staining of the brain, eye, and fin.

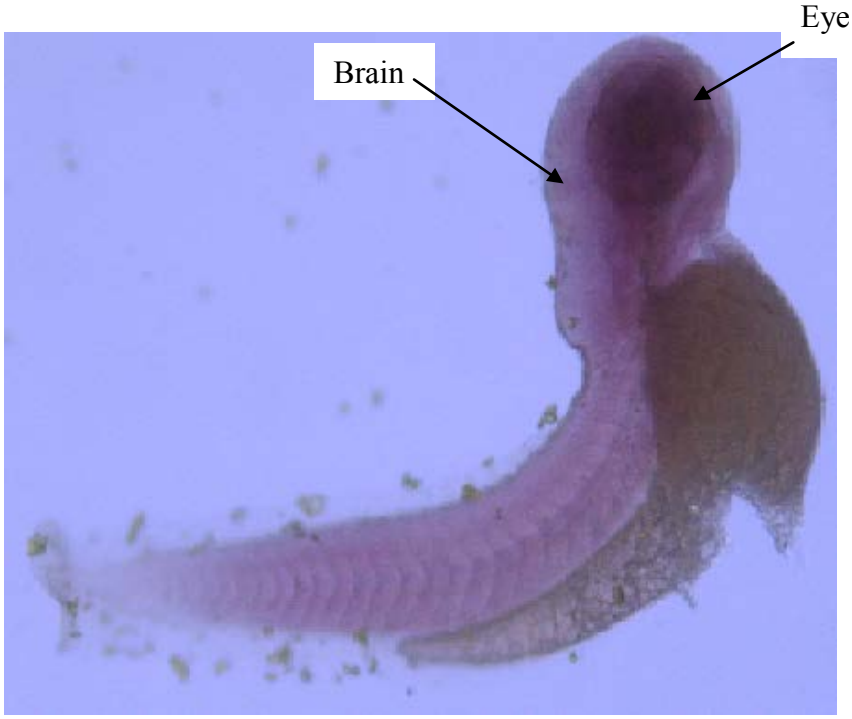

Figure 13 In situ day 3 PAPSS2b mRNA probe
The images show the distribution of the PAPSS2b gene within the zebrafish 72 hours post-fertilisation with the use of the PAPSS2b mRNA probe. The blue colour indicates the staining with the anti-sense probe. At this time there is staining of the brain, and eye.

The in situ hybridisation confirmed the presence of the PAPSS2b gene to be present within the zebrafish during days 1, 2, and 3. Both short and mRNA probes were used in order to see if it was possible to get more specific staining with different length probes. At day 1 the PAPSS2b gene is present in the brain, the immature eye, and the somite as shown in figures 8 and 9. Figure 8 shows PAPSS2b presence confirmed by use of the mRNA probe and figure 9 shows PAPSS2b presence confirmed by the short probe. At day 2 the PAPSS2b gene is present in the brain, mid-brain/hind-brain boundary, the eye, the hatching gland, and the somite as shown in figures 10 and 11. Figure 10 shows PAPSS2b presence confirmed by use of the short probe and figure 11 shows PAPSS2b presence confirmed by the mRNA probe. At day 3 the PAPSS2b gene is present in the brain, the eye, the fin as shown in figure 12 using the short probe. Also at day 3 the PAPSS2b gene is shown to be present in the brain and the eye as shown in figure 13

3.5 Morpholino knock-down of PAPSS2b using splice site and translational blocking morpholinos

The morpholino knock-down of the PAPSS2b gene was performed using splice site and translational blocking morpholinos to see if the knock-down resulted in a phenotypic change in the developing embryo. The splice site morpholino was positioned across an intron exon boundary and resulted in exon skipping leading to a functional mutation. The translational blocking morpholino was positioned over the ATG start site and resulted in a decrease in gene product being produced.

In Situ hybridisation was also carried out on the embryos injected with the morpholios at days 1, 2, and 3, using both the mRNA probe and the short probe. The staining from the in situs produced from the morpholinos was the same as was seen for the control embryos at the

respective time points (data not shown). The embryos were injected with morpholinos at differing concentrations in order to see the effect of a morpholio knock down of the PAPSS2b gene within the developing zebrafish. Both splice acceptor and translational blocking targeted morpholinos were used at four differing concentrations (10µM, 30µM, 100µM, and 300µM) in order to find the optimum concentration. The changes in the patterns of development were compared to embryos which had been injected with a scramble morpholino at the corresponding concentration which acted as the control. The embryos were imaged at day 3, and day 4 as these showed developmental changes in the zebrafish.

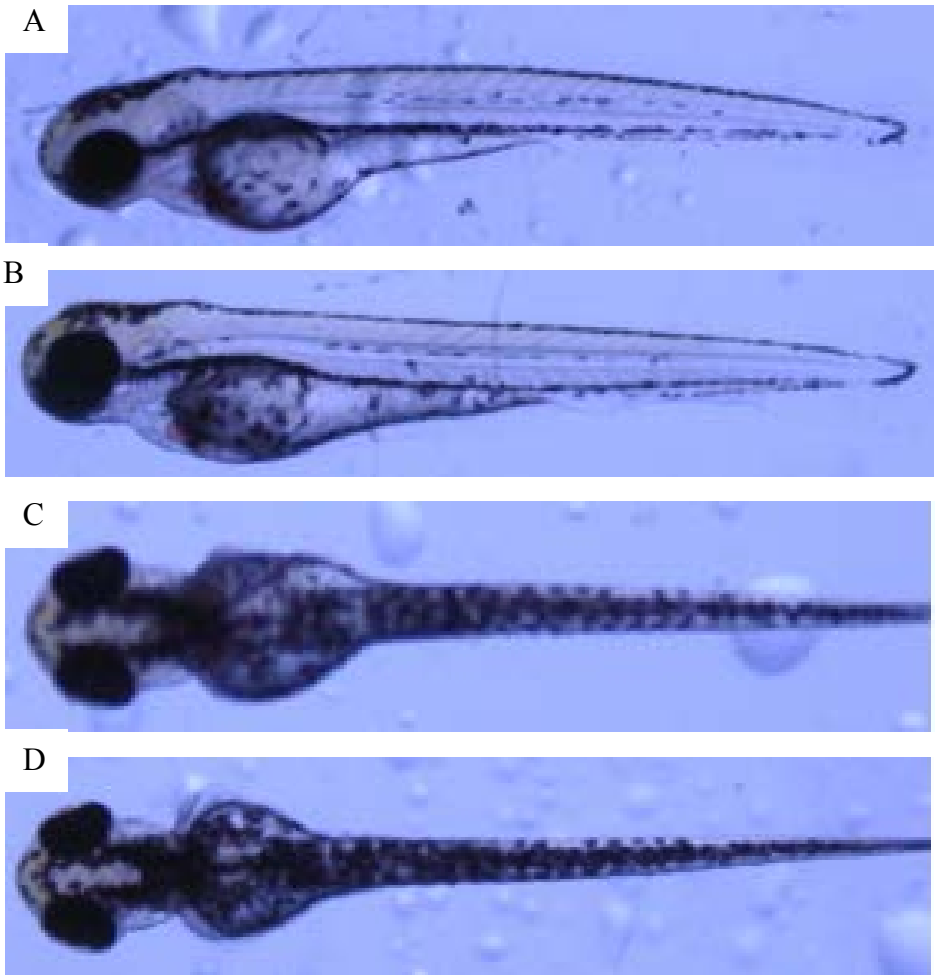

Figure 14 Scramble morpholino and PAPSS2b splice morpholino (300µM) day 3
The images show the scramble morpholino (A, C) and the PAPSS2b splice morpholino (B, D) phenotypes at day 3 following injection. In B the PAPSS2b splice morpholino appears to have a squashed head in camparison to A and also the eye appears closer to the top of the head.

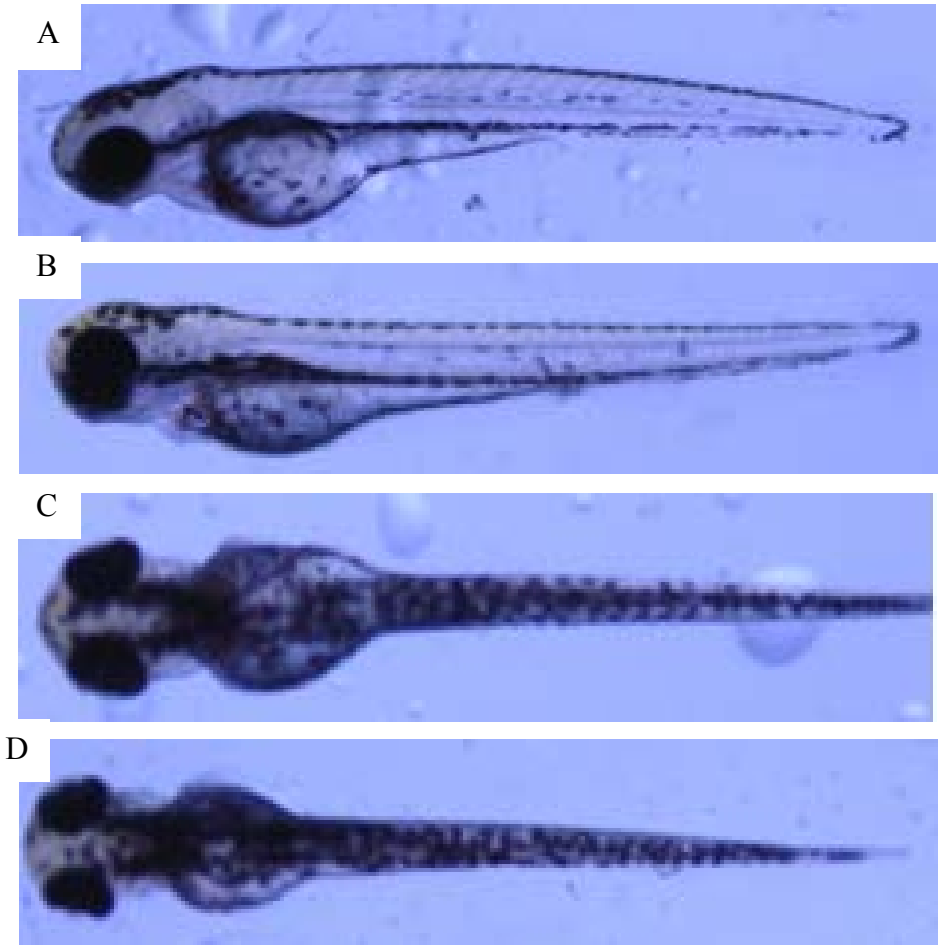

Figure 155 Scramble morpholino and PAPSS2b translational blocking morpholino (300µM) day 3

The images show the scramble morpholino (A, C) and the PAPSS2b translation blocking morpholino (B, D) phenotypes at day 3 following injection. In B the PAPSS2b translational blocking morpholino appears to have a squashed head in camparison to A and also the eye appears closer to the top of the head.

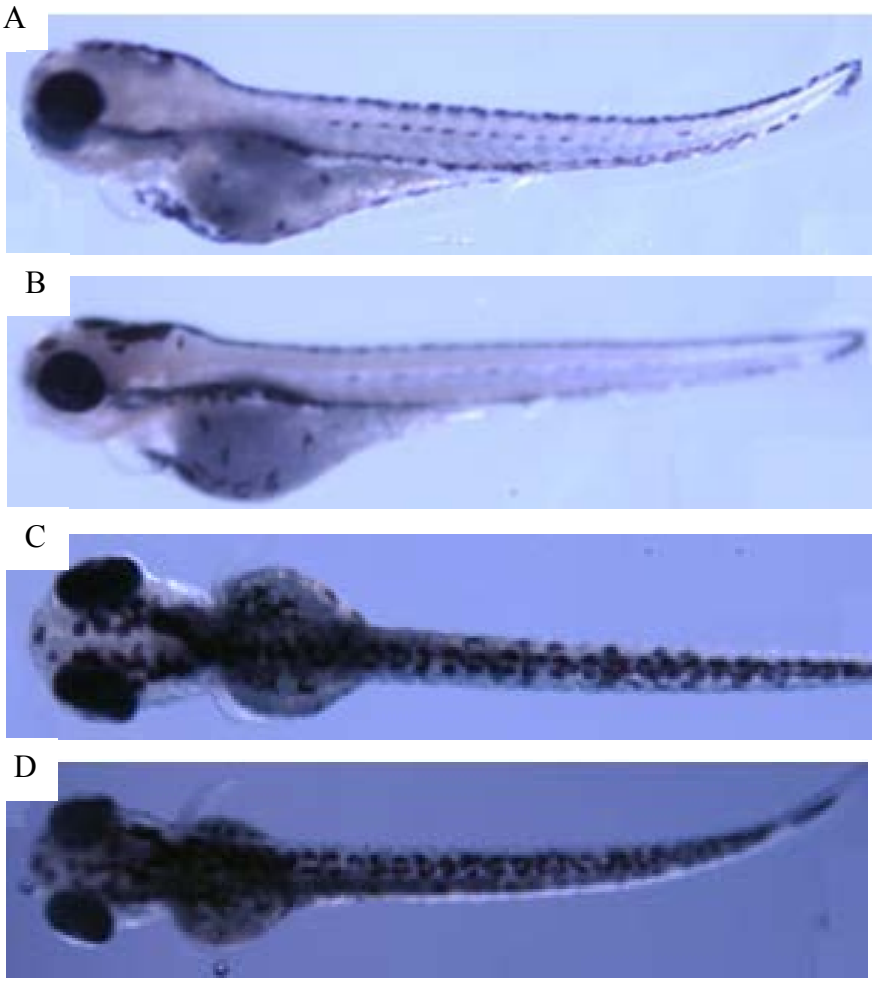

Figure 166 Scramble morpholino and PAPSS2b splice morpholino (300μM) day 4
The images show the scramble morpholino (A, C) and the PAPSS2b splice morpholino (B, D) phenotypes at day 4 following injection. The head of the splice morpholino (B) appears to be smaller than that of the control scramble morpholino, A.

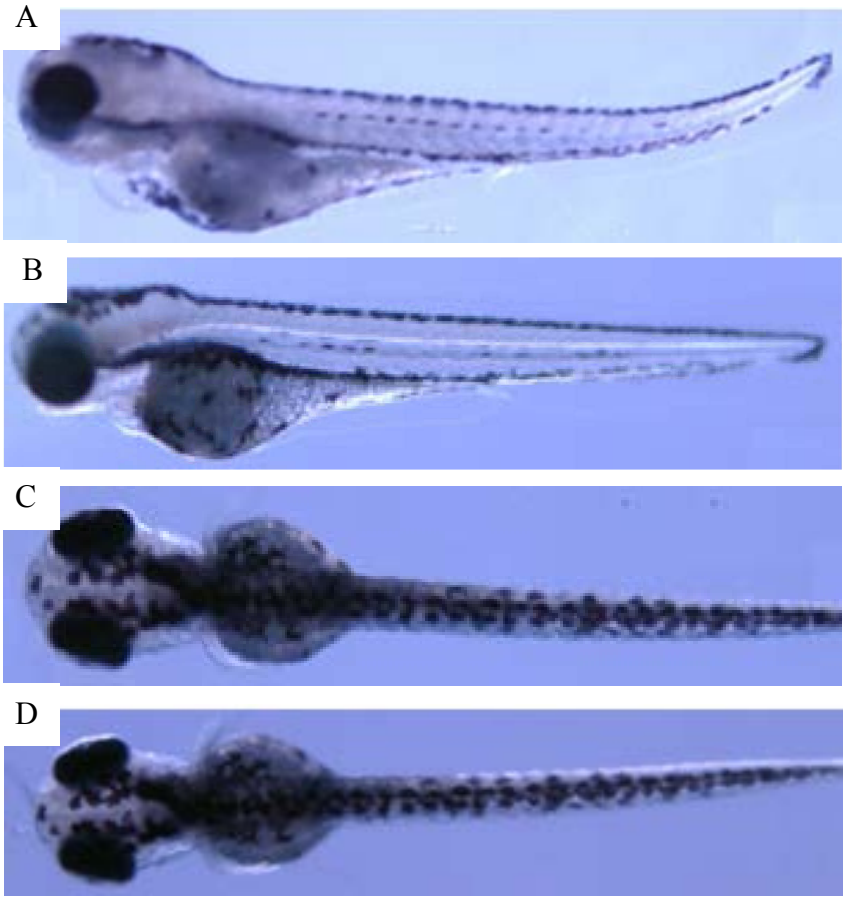

Figure 1717 Scramble morpholino and PAPSS2b translational blocking morpholino (300µM) day 4
The images show the scramble morpholino (A, C) and the PAPSS2b translational blocking morpholino (B, D) phenotypes at day 4 following injection. The translational blocking morpholino, B appears to have a smaller and more compact head than the control, scramble morpholino, B.

When the translational blocking morpholino (figure 15 and 17) and the scramble morpholino (figure 14 and 16) were used for PAPSS2b there was a change in phenotype when a concentration of 300µM was used at days 3 and 4. The morpholino heads appear smaller and also more compacted. The eyes are also closer to the top of the head at day 3. There were a total of 52 embryos injected with the 300µM scramble morpholino which were analysed and at days 3 and 4, of these two showed the smaller head phenotype. There were a total of 54 embryos injected with the 300µM translational blocking morpholino of which 32 showed the

smaller head phenotype at day 3, and 37 showed the phenotype at day 4. There were a total of 51 embryos injected with the 300μM splice site morpholino of which 25 showed the smaller head phenotype at day 3, and 29 showed the phenotype at day 4.

4 DISCUSSION

4.1 In Situ hybridisation of day 1, 2, and 3 zebrafish embryos using PAPSS2b probes

Day 1, 2, and 3 zebrafish embryos were successfully stained using both full length mRNA PAPSS2b probes and also short length PAPSS2b probes, which had previously been produced (3.7). This allowed for both confirmation of the presence of PAPSS2b at these developmental stages and also for the localisation of PAPSS2b to be established within the developing zebrafish embryo.

The in situ hybridisations were carried out at days 1, 2 and 3 post-fertilisation. Two separate probes were used, a probe for full-length mRNA and a short probe for PAPSS2b which had been cloned into the PCS^{2+} vector. Both sense and anti-sense probes were used for each of the constructs, for each the staining was stopped at differing time points in order to observe the full staining profile. Also once there was staining observed with the sense probe all of the staining samples were stopped as this is an indication that non-specific staining was starting to take place. The in situ hybridisations revealed at what point during development the PAPSS2b gene is expressed and its localisation.

At day 1 staining was localised to the muscle, the eye and the somite of which muscle is the major derivative. At day 2 the staining was primarily located within the brain, the mid-brain/hind brain barrier, the eye and the hatching gland. On day 3 the staining was seen within the brain, the eye, the fin and the blood vessels. Also the eye showed layering in its staining. On day three effective and accurate staining was harder due to the later stage embryos being harder to permiablise. This may lead to areas which have PAPSS2b expression not being stained and therefore overlooked. Ideally the in situs would have been repeated three times in

order to draw accurate comprehensive conclusions, although due to time restrictions this was not possible. The location of the staining is different to where the expression of PAPSS2b has been found in previous studies. Previous studies have shown it to be expressed in liver, cartilage and adrenal glands[8] which is not where it was found in these in situs. Also animal studies have indicated that PAPSS2 is the more abundant isoenzyme in developing tissues that have a high sulphonation requirement, such as cartilage. The difference could be due to a species difference or due to the staining of these areas being hard to distinguish. Therefore future work could involve using staining markers for the liver and interrenal organ in order for a more precise location of the staining to be found.

4.2 Morpholino knock-down of PAPSS2b using translational blocking and splice site morpholinos

The morpholinos used were a translational blocking morpholino, a splice morpholino and a scramble morpholino which acted as the control. The morpholinos were injected at four concentrations (10μM, 30μM, 100μM, and 300μM) for the PAPSS2b gene. The morpholinos at 10μM and 30μM did not give a successful knock-down of the PAPSS2b gene. This could be due to several factors, firstly it could be due to an injection error where the morpholino solution was not injected into the correct place. Alternatively the morpholino concentrations used may not have been high enough to lead to a successful knock-down of the gene as the gene may have been produced in too high a quantity for the morpholino to be able to decrease the overall product enough. When the higher concentrations were used (100μM, and 300μM) there was successful knock-down of the PAPSS2b gene by both the splice morpholino and the translational blocking morpholino, however the knock-down was only slight for 100μM and there was considerable knock-down for 300μM. The development of the treated embryos was

observed throughout the following four days following injection and images were taken at days 3 and 4 to document the appearance.

There was no clear change in the phenotype of the morpholino treated embryos with respect to the scramble morpholino treated control embryos at 10μM, 30μM and 100μM concentrations. When 300μM concentration was used there was a noticeable change in the phenotype although subtle (figures 14, 15, 16, and 17) for PAPSS2b knock-down. This was that the heads of the embryos injected with the translational blocking morpholino and the splice morpholino were smaller and more compact that those injected with the scramble morpholino control. Also at day three the eyes appeared closer to the top of the head when compared to the control. The number of morpholino injected embryos exhibiting this phenotype was quantified and shown to be substantial. Due to the size of the head being smaller in the PAPSS2b knock-down embryos this could be a result of a defect in the cartilage development within this area as it has already been shown that cartilage development is affected by a PAPSS2b gene mutation in a previous study[2]. This fits in well with previous studies in mice which have shown that when there are defects in the PAPSS2 gene there are consequential bone defects.

This project has started to look into the functions of the PAPSS isoforms in zebrafish, however since these are pilot studies further studies will be required to confirm the exact nature of their role.

4.3 Future work

Future work to continue this project could include a double knock-down of the morpholino where both PAPSS2a and PAPSS2b are both knocked-down within the same embryo. This is due to the hypothesis that PAPSS2a could be compensating for the knock-down of PAPSS2b and that PAPSS2b could be compensating for the knock-down of PAPSS2a. So by knocking down both of the genes simultaneously it would eliminate this possible compensation. Also due to the phenotype change seen with the morpholino knock-down at 300μm being slight it could be that the phenotype is subtle and this could be investigated further. For example with bone, liver, or interrenal staining. Also latter stages of development could be investigated in order to see if there is more effect of a gene knock-down latter in development.

5 REFERENCES

1. Xu Z-H, Otterness D.M, Freimuth R.R, Carlini E.J, Wood T.C, Mitchell S, Moon E, Kim U-J, Xu J-P, Siciliano M.J, Weinshilboum R.M. 2000. Human 3 -Phosphoadenosine 5 -Phosphosulfate Synthetase 1 (PAPSS1) and PAPSS2: Gene Cloning, Characterization and Chromosomal Localization. Biochemical and Biophysical Research Communications. 268 (2) 437-444(8)

2. Stelzer. C, Brimmer. A, Hermanns. P, Zabel. B, Dietz. U. H. 2007. Expression profile of PAPSS2 (3′-phosphoadenosine 5′-phosphosulfate synthase 2) during cartilage formation and skeletal development in the mouse embryo. Developmental Dynamics. 236 (5), 1313–1318.

3. Kimmel. C. B, Ballard. T. F, Kimmel. S. E, Ullmann. B, Schilling. T. F. 1995. Stages of Embryonic Development of the Zebrafish. Developmental dynamics.

4. Julliena. D, Crozatierb. M, Kasa. E. 1997. cDNA sequence and expression pattern of the Drosophila melanogaster PAPS synthetase gene: a new salivary gland marker. Mechanisms of Development 68 179–186.

5. Xu. Z, Wood. T. C, Adjei. A. A, Weinshilboum. R. M. 2001. Human 3′-Phosphoadenosine 5′-Phosphosulfate Synthetase: Radiochemical Enzymatic Assay, Biochemical Properties, and Hepatic Variation. DMD. 29 (2) 172-178

6. Du. S. J, Frenkel. V, Kindschi. G, Zohar. Y. 2001. Visualizing Normal and Defective Bone Development in Zebrafish Embryos Using the Fluorescent Chromophore Calcein. Developmental Biology 238, 239–246

7. Bakkers. J. 2011. Zebrafish as a model to study cardiac development and human cardiac disease. Cardiovascular research. 19(2), 279–288.

8. Venkatachalam. K. V. 2003. Human 3'-phosphoadenosine 5'-phosphosulfate (PAPS) Synthase: Biochemistry, Molecular Biology and Genetic Deficiency. IUBMB Life. 55, 1-11,

9. Dawson. P. A. 2011. Sulfate in fetal development, Seminars in cell and developmental biology.

10. To. T, Hahner. S, Nica. G, Rohr. K, Hammerschmidt. M, Winkler. C, Allolio. B. Pituitary-Interrenal Interaction in Zebrafish Interrenal Organ Development Molecular Endocrinology 21(2):472–485

11. McGonnell. I. M, Fowkes. R. C. 2006. Fishing for gene function – endocrine modelling in the zebrafish. Journal of endocrinology. 189 425-439

12. Liu. Y, 2007. Interrenal Organogenesis in the Zebrafish Model. Organogenesis. 3(1): 44–48.

13. Klaassen. C. D, Boles. J. W. 1997. Sulfation and sulfotransferases 5: the importance of 3'- phosphoadenosine 5'-phosphosulfate (PAPS) in the regulation of sulfation. The FASEB Journal. 11, 404-418,

14. Tao. T, Peng. J. 2009. Liver development in zebrafish (Danio rerio). Genomics. 36, 325−334.

15. Besset. S, Vincourt. J, Amalric. F, Girard. J. 2000. Nuclear localization of PAPS synthetase 1: a sulfate activation pathway in the nucleus of eukaryotic cells. The FASEB Journal.14:345-354

16. Sekulic. N, Dietrich. K, Paarmann. I, Ort. S, Konrad. M, Lavie. A. 2007. Elucidation of the active conformation of the APS kinase domain of human PAPS synthetase 1. Journal of molecular biology. 367(2), 488-500.

17. Elks. P. M, Loynes. C. A, Renshaw. S. A. 2011. Measuring Inflammatory Cell Migration in the Zebrafish. Methods in molecular biology. 2011, Volume 769, 261-275,

18. Jing. L, Zon. L. I. 2011. Zebrafish as a model for normal and malignant hematopoiesis. Dis Model Mech. 4(4): 433–438.

19. Liu. Y. 2007. Interrenal Organogenesis in the Zebrafish Model. Organogenesis. 3(1), 44–48.

20. PAPSS1 – A New Treatment Target for HIV Infection. Innovation. 2008. Martin Lehr.

21. H Fuda. H, Shimizu. C, Lee. Y. C, Akita. H. 2002. Characterization and expression of human bifunctional 3'-phosphoadenosine 5'-phosphosulphate synthase isoforms. Biochemical Journal. 365(2): 497–504