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The Pax-5 Gene is Alternatively Spliced in Trout B Cells

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APPROVAL PAGE

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COMPLIANCE PAGE

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ABSTRACT PAGE

Pax5 is a master regulator of B cell commitment and development, and regulates the expression of a host of genes involved in B cell maturation and activation. Previous studies on mice and human Pax5 have reported on alternatively spliced isoforms that have altered DNA binding (encoded by exons 2 and 3) and trans-activation (TA) potential (encoded by exons 7-10). The study presented here had two goals. Our first goal was to clone and identify trout Pax5 isoforms. cDNA from four trout immune tissues (anterior and posterior kidney, spleen and blood) were screened by nested PCR to search for alternatively spliced Pax5 transcripts. Alternative splicing of trout Pax5 gives rise to at least six alternatively spliced transcripts with deletion of paired domain, trans-activation domain and/or inhibitory domain coding elements. Several splice forms lacking exons 2, 8 or 9 were uncovered. Our second goal was to study the relative expression of Pax5 isoforms during B cell activation. Using semi-quantitative RT-PCR, the change in relative amplification of each isoform transcript was analyzed during LPS activation of splenic and blood derived B cells. Our studies report that spliced Pax5 transcripts lacking exon 2 or 8 are up-regulated during B cell activation with LPS, suggesting these isoforms have roles in terminal B cell differentiation. Conversely, transcripts lacking exon 9 were relatively scarce in LPS activated splenic and blood-derived B cells and probably do not participate in the regulation of terminal differentiation in these tissues. Thus alternatively spliced Pax5 transcripts show unique tissue-specific relative expression patterns in trout B cells.

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This work is dedicated to mappa and everyone that has wished me well.

Acknowledgments

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From unreal to real, from darkness to light, from death to immortality

(Brhadaranyaka Upanishad — I.iii.28)

I would like to take this opportunity to recognize the support of several individuals who helped make this thesis come to fruition. First, I would like to thank my thesis advisor Dr. Patty Zwollo, for providing guidance and the opportunity to pursue my graduate career in a stimulating research environment. I am grateful to Drs Lizabeth Allison and Matthew Wawersik for facilitating my learning experience with constructive input and improving my scientific communication skills throughout my graduate education. Maggie Barr, Alice Harman and Katrina Mott were always willing to enrich my scientific skills and contributed to research productivity. I would like to thank Dr Engstrom for providing training on DNA quantification. Lomax Boyd, Chadd Molloy, Iris Porush and Brian Molloy were always committed towards supporting my education and ideas throughout my graduate education. Finally, I would like to thank my thesis committee Drs Margaret Saha, Lizabeth Allison and Oliver Kerscher whose mentorship facilitated the development of my research project, my professional growth and the quality of this dissertation.

List of Abbreviations used in this work *(in alphabetical order)*

Abbreviation	Expansion
AK (or K1)	Anterior Kidney
cDNA	Complementary DNA
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
DNA	De-oxy ribonucleotide
ELP	Early Lymphoid Progenitor
Full-length* Pax5 or FL*Pax5	Refers to Pax5 transcripts that contains the alternatively spliced exon.
HBSS	Hank's Balanced Salt Solution
HSC	Hematopoietic Stem Cell
LPS	Lipopolysaccharide
PBL	Peripheral Blood Lymphocytes
PCR	Polymerase Chain Reaction
PK (or K5)	Posterior Kidney
RNA	Ribonucleotide
SPL	Spleen
UV	Ultra-Violet
µg	Micro grams
µl	Micro liters

Chapter 1 – Introduction

1.1 Overview of the Pax family of proteins

The **Pa**ired-bo**x** (Pax) family of transcription factors was first identified as a group of proteins that control organogenesis and cell-fate specification in Drosophila. Pax homologs have since been described in various members of the vertebrate lineage; including teleosts, frogs, chicken, mice and humans. Expression of Pax genes is spatially and temporally regulated during embryogenesis and mutation of Pax genes leads to an array of lethal and sub-lethal developmental defects (Wang et al, 2008). Members of the Pax family possess 128 amino acids of conserved DNA binding domain at the amino-terminal, called the paired-box. The mammalian Pax family consists of nine members that have been grouped together based on the presence of three structural domains – Paired, Octopeptide and Homeodomain (figure 1.1) (Mansouri et al, 1999, Noll, 1993). Through these domains and other motifs, Pax proteins interact with DNA and other proteins. Pax5 is a member of the Pax family and is the master regulator of B cell development in the immune system.

Functional Domains: The Pax paired domain was identified as a helix-turn-helix domain in Drosophila paired proteins and consists of two sub-domains – an N-terminal PAI sub-domain and a C-terminal RED sub-domain separated by an unstructured linker region (Czerny et al, 1993, Xu et al, 1999). Both PAI and RED make specific contacts on DNA

independently, and this binding is stabilized by an N-terminal β-turn and the linker region. The paired domain is encoded by exons 2, 3 and 4. The consensus DNA binding site for the paired domain is [(G/T)T(T/C)(C/A)(C/T)(G/C)(G/C)]. Exceptions to this are the DNA binding sites for Pax-6 [TTCACGC] and Pax-8 [GTCAC(G/C)C] (Jun et al, 1996, Czerny et al 1993, Epstein et al 1994). Interestingly, the paired domain is also reported to interact with the HMG domain of Sox proteins (Lang and Epstein, 2003). These interactions induce gene expression more robustly than either transcription factor can alone, as in the case of Pax-3 and Sox-10 at the Mit*f* and Ret promoters (Bondurand et al, 2000) and Pax-6 and Sox-2 at the δ-crystallin promoter (Kamachi et al, 2001).

Like the paired domain, the homeodomain is a sequence-specific helix-turn-helix DNA binding motif that binds the palindromic sequence TAAT$(N)_{2-3}$ATTA called the homeobox (Wilson et al, 1993). Homeodomain containing proteins are divided into two genetic groups. The first group comprises the Hox genes, which are further clustered on the vertebrate genome and their spatial and temporal expression during embryonic patterning. The second group, the 'orphan' group, does not occur in genetic clusters and their expression is restricted to specific tissue types (Simon & Alavian, 2009). The Pax genes belong to the 'orphan' group of Homeodomain-containing proteins along with the transcription factors POU, SIX, OCT, PITX and NKX (for a complete list of homeodomain proteins refer http://research.nhgri.nih.gov/homeodomain). The Pax homeodomain is encoded by exons 6 and 7, except in Group I and II sub-groups.

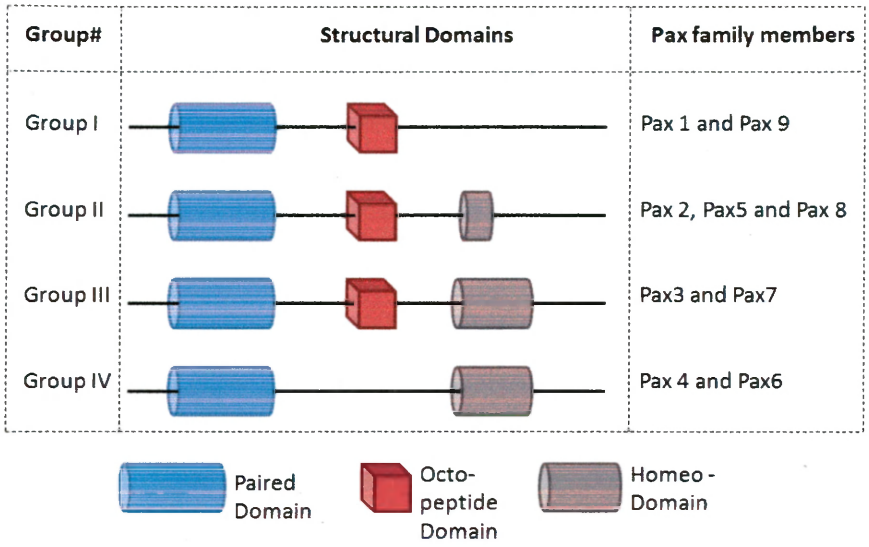


Figure 1.1 Sub-families of Pax proteins classified by presence of structural domains. The nine members of mammalian Pax family are classified into four sub-groups based on presence of paired domain *(PD - blue cylinder)*, octopeptide segment *(OD - red box)* and homeodomain *(HD - grey cylinder)*. Group I members Pax 1 and 9 have PD and OD but lack HD. Group 2 members Pax 2, 5 and 8 have PD and OD but only partial HD. Group 3 members Pax 3 and 7 have all three structural domains. Group 4 members Pax 4 and 6 have PD and HD, but lack OD.

Members of the Pax family also possess an eight amino acid octopeptide motif encoded by exon 5, except Pax-4 and Pax-6 (Noll, 1993). This motif functions as a transcriptional inhibitor, through direct interaction with the Groucho family of transcriptional repressors (Eberhard et al, 2000). Pax 3 and 5 interact with GRG4 (a member of the groucho family) and Lymphoid Enhancer binding factor (LEF-1) to form a repression complex (Jin et al, 2002). Through this complex, Pax proteins are thought to participate in the Wnt signaling pathway, the dysregulation of which is responsible for the development of several types of cancers (Behrens, 2005).

Pax proteins also contain a potent trans-activation domain (TD) at the carboxy-terminus which is rich in proline-serine-threonine (PST) residues. The TD is encoded by exon 8 and partly by exon 9. Most Pax proteins show dose-dependent trans-activation through the

TD, as in Pax-5 and Pax-6 where transcriptional activation is high low concentrations and decreases at higher concentrations (Glasner et al, 1994, Dorfler & Busslinger, 1996). Additionally, Pax-2, Pax-3, Pax-4, Pax-5 and Pax-6 can inhibit transcription through an inhibitory domain (ID) located adjacent to the TD at the carboxy-terminus (Chi & Epstein, 2002, Dorfler & Busslinger, 1996). The inhibitor domain is encoded by part of exon 9 and exon 10. The transcriptional activity of Pax proteins is the result of relative activity between activation and repression functions.

1.2 Role of Pax genes in embryonic development and oncogenesis:

Pax proteins influence cell-fate decisions of multi-potent stem cells. For example, Pax-5 is essential for the commitment of lymphoid progenitors to the B cell lineage (Cobaleda et al, 2007). Pax5 knockout mice show a complete loss of B cell phenotype and an increase in cells of the T cell and natural killer cell lineage, suggesting Pax5 represses the differentiation of uncommitted progenitor cells into non-B lineages (Mikkola et al, 2002, Cobaleda et al, 2007). Similarly, lack of Pax-6 in mice and humans shows complete loss of eye development as the gene is required for the specification of multiple ocular lineages (Marquardt et al, 2001). Interestingly, loss of Pax-7 does not lead to any developmental defects in Pax-$7^{-/-}$ mice, probably because Pax-3 can substitute loss of expression of Pax-7 in skeletal muscle (Seale et al, 2000). However, Pax-7 null mice die shortly after birth, due the inability of skeletal muscles to regenerate. A complete list of Pax proteins and their tissue specific expression is listed in table 1.1.

Pax proteins promote cellular survival and inhibit apoptotic signals during cellular development. Pax 2, 5 and 8 demonstrate anti-apoptotic activity by directly inhibiting p53 expression through a highly conserved enhancer region (Stuart et al, 1995). The p53 family of transcription factors are well known tumor suppressors and are important inhibitors of the cell cycle. Loss of Pax-2 expression by siRNA induces apoptosis in renal carcinoma cells (Gnarra and Dressler, 1995). Similarly, oligonucleotide inhibition of Pax-3 mRNA transcripts leads to increased cell death in rhabomyosarcoma cells (Bernasconi et al, 1996).

Down-regulation of Pax genes is vital for terminal differentiation. Pax5 expression is down-regulated in terminally differentiating B cells. The down-regulation of Pax5 by B lymphocyte inducing factor (Blimp1) allows for the expression of terminal differentiation genes and the re-expression of Pax5-inhibited genes in mature B cells (Shaffer et al, 2002, Delogu et al, 2006). Similarly, Pax3 and Pax7 expression is extinguished prior to terminal differentiation of embryonic myoblasts during myogenesis (Williams et al, 2000, Buckingham et al, 2003).

Table 1.1

Pax gene	Sub-group	Expressed in tissue	Associated Mutation/Disease
Pax -1	I	Skeleton, Sclerotome, Thymus	Kippel-Fell syndrome, Jarcho-Levin syndrome, Salivary gland tumor
Pax-2	II	CNS, Kidney	Papillorenal syndrome, Renal cell carcinoma, Wilms' Tumor, Breast cancer, Kaposi Sarcoma
Pax-3	III	CNS, Neural crest, skeletal muscle	Waardenberg's syndrome, RMS, Eming's sarcoma
Pax-4	IV	Pancreas	Silver-Russell syndrome, Wolcott-Rallison syndrome, Diabetes, Insulinoma

Pax-5	II	CNS, B cells	Large cell lymphoma, Lymphocytic leukemia, Medulloblastoma, Neuroblastoma, Astrocytoma
Pax-6	IV	CNS, Eye, Pancreas	Aniridia, Cataract, Glioblastoma multiform, anaplastic glioblastoma, astrocytic glioma
Pax-7	III	CNS, Cranio-facial, skeletal muscle	RMS, Eming's sarcoma, melanoma, squamous cell lung carcinoma
Pax-8	II	CNS, Kidney, Thyroid	Thyroid dysplasia, Thyroid follicular carcinoma, Wilms' tumor, placental cancer, ovarian serous tumors
Pax-9	I	Skeleton, Cranio-facial, tooth	No thymus, teeth, parathyroid glands, cranio-facial and limb defects

Table 1.1 Tissue-specific expression of Pax genes during embryogenesis and associated defects due to mutations/mis-expression of Pax genes (adapted from Wang et al, 2008).

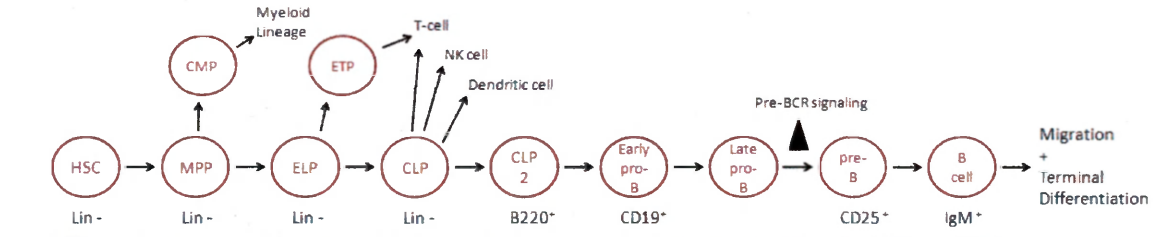
In summary, Pax proteins have important roles in commitment of multi-potent progenitors to specific cell fates, the survival of developing cells and down-regulation of Pax genes is essential for proper terminal differentiation of these cells. Mutations in Pax genes, either due to chromosomal aberration or mis-expression, often leads to cancerous phenotype and/or complete loss of specific cell lineages (see table 1.1 for associated diseases).

1.3 Role of Pax-5 in B cell development

Commitment of hematopoietic progenitors to the mammalian B cell lineage:

Since B cell development has been studied thoroughly in the mammalian system, an overview of mammalian B cell development is provided prior to that in trout. B lymphocytes arise from a pool of multi-potent hematopoietic progenitors that have undergone a strict gene regulatory cascade in order to commit to the B cell lineage. Early stages of hematopoiesis involve the loss of unlimited differentiation potential through the down-regulation of pluri-potency marker c-kit in hematopoietic stem cells

(HSC) and the generation of multi-potent progenitors (MPP). MPPs can give rise to either lymphoid lineage or myeloid lineage in the form of early lymphoid progenitors (ELP) or common myeloid progenitors (CMP) (Kondo et al, 1997, Akashi et al, 2003). Loss of long-term self renewal is marked by the up-regulation of the tyrosine kinase Flt3 in MPP cells (Adolfsson et al, 2001, Christensen et al, 2001). Consequently, MPPs differentiate into ELPs, where the expression of RAG1 and RAG2 coincides with the recombination of D_H-J_H at the immunoglobulin heavy chain (IgH) locus (Igarashi et al, 2002). Recent evidence has shown that ELPs can give rise to both early T-cell progenitors (ETP) in the thymus and /common lymphoid progenitors (CLP) in the bone marrow (Allman et al, 2003).



c-Kit	High	High	High	Low	Low	Low	Low	-	-
Flt3	-	+	+	+	+	-	-	-	-
D_H-J_H	-	-	+	+	+	+	+	+	+
V_H-DJ_H	-	-	-	-	-	+	+	+	+
V_L-J_L	-	-	-	-	-	-	-	+	+
Pax-5	-	-	-	-	+	+	+	+	+
EBF-1	-	-	-	-	+	+	+	+	+
E2a	-	-	-	+	+	+	+	+	-
RAG-1/2	-	-	+	+	+	+	+	+	+

Figure 1.2 Commitment of multi-potent progenitors to the B cell lineage in the bone marrow. The figure highlights the expression of various cell surface markers, transcription factors and Ig gene rearrangement during B cell development in the bone marrow. +/- symbols are not quantitative. Legend: HSC – hematopoietic stem cell, MPP – multi-potent progenitor cell, ELP – early lymphoid progenitor, CLP – common lymphoid progenitor, CMP – common myeloid progenitor, ETP – early T cell progenitor, Pro-B – B cell progenitor cell, Pre-B – B cell precursor cell, Lin – negative for expression of lineage-specific markers. *(Adapted from Busslinger, M., 2004)*

In the presence of appropriate cytokine cues, CLPs can give rise to B cell, T cell, Natural Killer and Dendritic cell lineages (Kondo et al, 1997, Traver et al, 2000). Expression of the B220 receptor in CLPs marks the transition into CLP-2 cells (Martin et al, 2003). Completion of D_H-J_H re-arrangement and the expression of CD19 promote the conversion of CLP-2 cells to early pro-B cells (Li et al, 1996, Tudor et al, 2000, Hardy et al, 1991). Completion of V_H-DJ_H recombination in late pro-B cells induces the expression of Igμ at the cell surface as part of the pre-B cell receptor, which marks the transition into precursor-B cells (pre-B cell) stage. Signaling at the pre-B cell receptor promotes allelic exclusion at the IgH locus, proliferation and cell expansion, and differentiation in to small pre-B cells where light chain recombination occurs (Meffre et al, 2000). Completion of light chain recombination heralds the emergence of immature IgM^+ B cells which migrate from the sterile environment of the bone marrow to peripheral lymphoid organs. (See figure 1.2 for overview of B cell development).

Transcriptional regulation of mammalian B cell development:

The generation of lymphoid progenitors from the HSC depends on at least two main transcription factors: Ikaros and PU.1. The Zn-finger family of transcription factors Ikaros, Helios and Aiolos influence the cell fate decisions of HSC's during hematopoiesis (Georgopoulos, 2002) and is expressed in all hematopoietic lineages, including stem cells and MPPs. Mice that possess a dominant negative allele of Ikaros, generated through the targeted deletion of the N-terminal Zn finger, are unable to give rise to any B, T, NK and DC cells (Georgopoulos et al, 1994, Wu et al, 1997). Thus expression of

Ikaros and its family of transcription factors is essential for the normal development of lymphocytes.

PU.1, a member of the Ets family of transcription factors, is also expressed in many hematopoietic lineages and is thought to work in parallel with Ikaros to regulate lymphocyte development. Retroviral reconstitution experiments have shown that low level of PU.1 induce B cell development in PU.1$^{-/-}$ progenitors, while high level of PU.1 inhibit B cell development and support myeloid cell development (DeKoter et al, 2000). Thus PU.1 regulates the decision of lymphoid versus myeloid fate in multipotent myeloid-lymphoid progenitors.

The regulation of pro-B cell development and survival is regulated by the transcription factors E2a, EBF and Pax-5. The E2a gene is alternatively spliced during B cell development, and gives rise to two basic helix-loop-helix transcription factors E12 and E47 (Murre et al, 1989). E2a$^{-/-}$mice show B cell arrest in the earliest stages of development with lack of D_H-J_H rearrangements and no detectable Pax-5 (Bain et al, 1994, Zhuang et al, 1994, Bain et al, 1997). The EBF gene (Early B cell factor) is expressed in pro-B cell, pre-B cells and mature B cells (Lin et al, 1995). EBF$^{-/-}$ mice also display a phenotype similar to E2a$^{-/-}$ mice, with early developmental arrest in B cells with no detectable levels of Pax5 (O' Riordan et al, 1999). Thus, EBF and E2a are expressed upstream of Pax5 in the B cell program. Interestingly, ectopic expression of EBF and E2a in non-B lymphoid cell lines induces the expression of B cell specific genes (Schlissel et al, 1991, Choi et al, 1996, Kee et al, 1998). Molecular analyses have shown that E2a and

EBF bind the promoters of genes involved in B cell signaling (VpreB, λ5) and surrogate light chain (Igα, Igβ) (Sigvardsson et al, 1997, Sigvardsson, 2000, Gissler and Sigvardsson, 2002).

Commitment of MPPs to the B cell lineage is not complete without Pax5 expression, since Pax-$5^{-/-}$ mice show a developmental arrest in the pro-B cell stage (Urbanek et al, 1994). Culturing Pax-$5^{-/-}$ pro-B cells in the presence of IL-7 showed that these cells retain the ability to develop in to cells of the lympho-myeloid lineage. In the presence of appropriate cytokine signals, these cells can develop into NK cell, macrophages, dendritic cells, osteoclasts and granulocytes (Nutt et al, 1999). Retroviral reconstitution of Pax5 expression in Pax-$5^{-/-}$ pro-B cells restricted the multi-lineage potential and could rescue mature B cell phenotype (Nutt et al, 1999).

Pax5 is first expressed in the hematopoietic system at the pro-B cell stage, and is thought to be downstream of E2a and EBF in the B cell program (Adams et al, 1992). Conditional inactivation of Pax5 leads to loss of identity and function of B cells, and such cells trans-differentiate into macrophage and T cell lineages (Horcher et al, 2001). Cre-mediated gene deletion of Pax5 in committed pro-B cells showed Pax5 is required for both initiation of B cell commitment and maintenance of B cell identity (Mikkola et al, 2002). At the transcriptional level, Pax5 has dual roles as a transcriptional repressor of lineage inappropriate genes and a transcriptional activator of linage appropriate genes. For example, Pax5 based repression of the macrophage lineage factor M-CSFR and the T-cell commitment factor Notch-1, renders committed pro-B cells unresponsive to

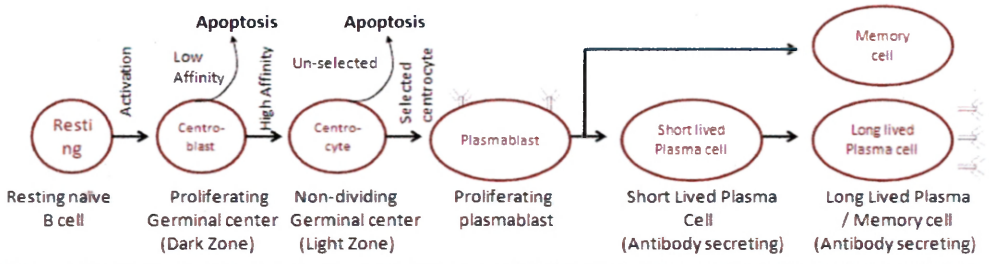
myeloid cytokine M-CSF and Notch ligands (Nutt et al, 1999, Souabni et al, 2002). Alternatively, Pax5 activated a host of B cell specific genes involved in pre-BCR signaling, receptor signaling chain (Igα), the co-stimulatory receptor (CD19) and the central adapter protein BLNK (Nutt et al, 1997, Fitzsimmons et al, 1996, Kozmik et al, 1992, Schebesta et al, 2002). Pax5 also facilitates V_H-DJ_H recombination via long scale contraction of the IgH locus (Nutt et al, 1999, Reynaud et al, 2008).

The transcriptional activity of Pax5 is modulated by its interaction with other transcription factors in a gene context dependent manner. For example, Pax5 is converted into a transcriptional repressor via interaction with Grg4 (a member of the groucho family of transcriptional co-repressors) (Eberhard et al, 2000). The leading hypothesis states the Pax5 trans-activation domain is effectively masked, thus leading to repressor-like function. Conversely, Pax5 functions as a transcriptional activator of mb-1 when it interacts with the Ets family of transcription factors (Garvie et al, 2001).

Activation of mature B cells and terminal differentiation in mammalian sytems:

Immature B cells migrate from an antigen-free environment in the bone marrow to peripheral lymphoid organs for maturation and activation. Naïve resting B cells express membrane IgM and are locked in the G0 phase of the cell cycle. B cell activation takes place in a Thymus independent (TI) or thymus dependent (TD) manner. TD activation occurs through interaction of the B cell receptor with membrane molecules on helper T cells and associated cytokines. On the other hand, TI activation takes place completely independent of T cell stimulation and cytokines. Lipopolysaccharide (LPS) is a TI antigen

that is polyclonal in nature and induces activation of mature B cells. Activated B cells undergo rapid proliferation in the germinal center and are called centroblasts. Densely packed regions of proliferating centroblasts appear darker than the surrounding region of the germinal center (Dark Zone). In the light zone, high affinity non-dividing centrocytes that can bind antigen-antibody complexes on follicular dendritic cells are selected during affinity maturation, while low affinity centrocytes undergo apoptosis.



BLIMP1	-	-	-	+	+	+
MITF	+	-	ND	-	-	-
PAX5	+	+	+	-	-	-
BCL-6	-	-	+	-	-	-
MTA3	-	-	+	-	-	-
BACH2	ND	ND	+	-	+	+
IRF4	-	+	-	+	+	+
XBP1	-	-	-	+	+	+

Figure 1.3 Terminal differentiation of B cell to plasma cells. The figure highlights the expression of various transcription factors involved in B cell development and terminal differentiation during plasma cell development. +/- symbols are not quantitative. ND implies that the expression patterns are not determined.

Positively selected centrocytes undergo immunoglobulin class-switching and mature into plasmablasts or memory cells. Plasmablasts possess membrane bound immunoglobulin and show tell-tale signs of an enlarged endoplasmic reticulum to accommodate the production of massive amounts of immunoglobulin. The terminally differentiated plasma cell lacks membrane bound form of immunoglobulin but produces

secreted form of immunoglobulin and is generally short-lived unless it migrates to an antigen-free niche and receives growth factors the promotes its survival.

Transcriptional regulation of B cell terminal differentiation in mammalian systems:

While Pax5 expression is required for the commitment and identity of B cells, transition of B cells to plasma cells requires the down-regulation of Pax5 (Lin et al, 2002). Activation of activation-induced cytidine deaminase (AID) by Pax5 is an essential part of germinal-center B cells maturation as it initiates somatic hyper-mutation and class-switch recombination processes (Gonda et al, 2003). Pax5 based repression of terminal differentiation genes X-box protein (Xbp-1), J-chain prevents the premature onset of terminal differentiation in maturing B cells (Reimold et al, 1996, Rinkenberger et al, 1996). Interestingly, loss of Pax5 expression alone is not sufficient for the induction of plasma cell fate through Blimp1 (B lymphocyte inducing maturation factor) expression (Horcher et al, 2001). Blimp1 is a Zinc finger transcription factor (Keller and Maniatis, 1992) that interacts with histone de-acetylases (Yu et al, 2000), hGroucho (Ren et al, 1999) and a G9a histone methyltransferase (Gyory et al, 2004) as part of its transcriptional repressive function. Expression of Blimp1 is first detectable in the plasmablast stage of B cell differentiation, where it is thought to be the master regulator of plasma cell differentiation (Martin et al, 2001). Blimp1 expression alone is enough to induce plasmacytic differentiation both in vitro as well as in B cell lines (Shapiro-Shelef, 2003, Shaffer et al, 2002, Turner et al, 1994, Schliephake and Schimpl, 1996). It does this by both inducing the expression of genes involved in plasmacytic differentiation and

suppressing genes involved in B cell identity: Pax5, the proliferative program: c-myc (Lin et al, 1997) and indirectly rescues the expression of Xbp1 through repression of Pax5. Xbp1 acts downstream of Blimp1 in the regulatory cascade and helps expand the secretory apparatus to initiate Ig secretion by plasma cells (Harding et al, 2002). Unlike Blimp1, Xbp1 expression is not enough to initiate differentiation to plasma cell fate.

A host of other transcription factors are involved in the successful production of antibody secreting plasma cells. Notable among these are MITF (Micropthalmia associated transcription factor), BCL-6 (B cell lymphoma protein -6), MTA-3 (metastasis-associated 1 family, member 3), BACH2 and IRF4 (Interferon-regulatory factor 4). MITF is an inhibitor of plasma cell and is crucial for maintaining germinal B cells in resting state. Down regulation of MITF, by IRF4 leads to the expression of Blimp1 in activated B cells (Lin et al, 2004). Like MITF, BCL-6 is also a crucial repressor of Blimp1 and aids in the development of germinal centers of B cell development (Shaffer et al, 2000). BCL-6 based repression of Blimp1 is thought to occur indirectly through the repression of STAT3 which is an inducer of Blimp1 expression (Vasanwala et al, 2002, Reljic et al, 2000). MTA3 interacts directly with BCL-6 to mediate its repressive effects (Fujita et al, 2004). BACH2 is also required for the proper establishment of germinal centers (Muto et al, 2004).

In summary, extinguishing Pax5 expression and up-regulation of Blimp1 and Xbp1 in activated B cells are the major regulatory events surrounding plasmacytic

differentiation. Plasma cells are non-proliferative cells that clonally secrete antibodies, thus completing the B cell humoral immune response.

B cell development and activation in Trout

The stages of B cell development and activation in trout are not well defined but may occur in a manner similar to mammalian B cells. Trout do not possess bone marrow or typical lymph-nodes (Zapata et al, 1995, Zapata et al, 1990). The organs used for B lymphogenesis are different from mammalian B cell genesis. Unlike the bone marrow in mammals, it is suggested that trout use the anterior kidney as a site of primary lymphogenesis (Zapata et al, 1990, Fange et al, 1986). The trout kidney is a ventrally located unpaired organ that extends from cranium at the anterior end of the body to the caudal end. Additionally, the anterior kidney lacks renal function and provides a sterile environment for B cell development. Studies from our lab suggest that trout anterior kidney house proliferating B cell precursors and plasma cells. The presence of antibody secreting cells in the anterior kidney is unexpected and may represent a subset of B1 cells that are characterized by low-levels of Ig secretion even in the absence of activation (Berland & Wortis, 2002).

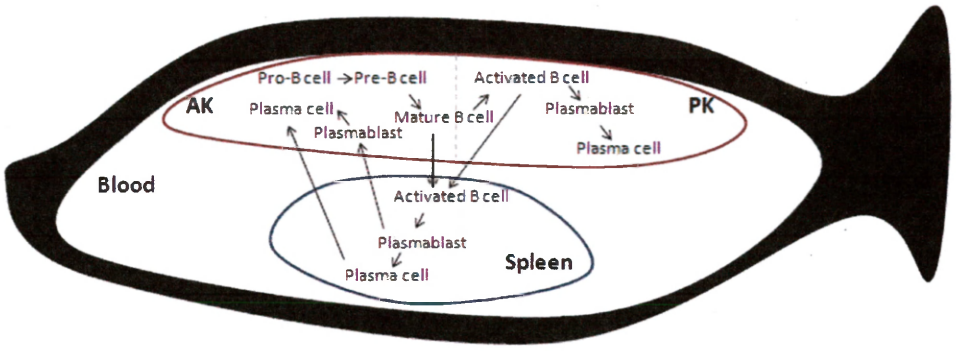


Figure 1.4 Model for the development, migration and activation of B cells in the trout. The anterior kidney houses a repertoire of developing B cells that migrate to the posterior kidney and spleen, where they get activated. Activated B cells terminally differentiate into plasmablasts and antibody secreting plasma cells. It is thought that a small percentage of plasmablasts and plasma cells migrate to the anterior kidney. (adapted from Zwollo et al, 2005)

Developing mature B cells migrate from the anterior kidney to sites of activation at the posterior kidney and spleen via the blood. Unlike the anterior kidney, the posterior kidney possesses renal function and provides an environment for antigen encounter by migrating mature cells (Fange et al, 1986). The posterior kidney was found to contain partially activated B cells and plasmablasts, suggesting it functions as a secondary immune organ. Trout spleen and blood were shown to contain resting B cells with low Ig secretion (Zwollo et al, 2008).

Similar to the posterior kidney, mature B cells get activated and terminally differentiate into plasmablasts and short-lived immunoglobulin secreting plasma cells in the spleen. LPS activation of splenic-B cells showed these cells could produce both membrane and secreted form of IgM, detectable by real-time PCR and flow-cytometric analyses (Zwollo et al, 2008). Conversely, LPS activated blood-derived B cells showed low propensity for generating antibody secreting plasma cells. Taken together, the posterior kidney, spleen and blood serve as secondary immune sites and lack developing B cells. Subsets of

short-lived plasma cells may migrate to the anterior kidney and become long-lived plasma cells. Thus trout kidney serves as a diverse and multifunctional organ for both hematopoiesis and B lymphogenesis.

Although, the predominant immunoglobulin isotypes produced by trout B cells is IgM and IgD, a novel immunoglobulin isotype have been recently reported in a smaller subset of trout B cells – IgT (Hansen et al, 2005). Another novel isotype (IgZ) was recently reported in zebrafish (Flajnik, 2005), suggesting a diverse potential of isotype production in the teleost lineage. Taken together, trout immune tissues contain developmentally diverse and tissue-specific subsets of B cells .

1.4 Alternative splicing of Pax-5 and evolutionary conservation of isoforms

In eukaryotes, nuclear pre-mRNA transcripts contain non-protein coding sequences called introns interspersed between protein coding exons. Formation of functional mature mRNA transcript involves the removal of introns by a mechanism called RNA splicing. Eukaryotic introns are classified as group III introns and their removal is mediated by a large RNA-protein complex called the spliceosome (reviewed by Black, D., 2003). Little is known about the exact composition of the spliceosome, though the main subunits include 5 small uridine rich nuclear ribonucleoprotein particles (U1, U2, U4, U5 and U6) (Nilsen, 2002), seven Sm-like core proteins and over 200 accessory proteins that are added or removed during complex assembly and mRNA splicing. Classically, the intron possesses a GU nucleotide at the 5` splice site, and AG 3` splice site, a conserved

branch point and a polypyrimidine tract. The initial steps of splicing involve the association of U1 snRNP to the GU di-nucleotide and a U2 snRNP associated factor to the polypyrimidine tract. Subsequent recruitment of snRNPs U4, U5, U6 and other core components initiates the assembly of the pre-spliceosome complex. Following assembly, the 2`-OH of an Adenine residue at the branch point attacks the phosphate group at the 5` splice site in a trans-esterification reaction. This leads to the cleavage of the 5` exon and the formation of a lariat structure involving ligation of the 5` end of the intron with the adenine residue at the branch point. The free 3`-OH of the 5` exon then attacks the phosphate group at the 3` end of the intron in a second trans-esterification reaction. This allows for excision of the intron and ligation of the two exons.

Changes in splice site choice arise from changes in the assembly of the spliceosome or functional mutations in the original splice site (Berget, S., 1995, Black, D., 1995). Alternative splicing is a mechanism by which a single gene encodes several unique mRNA products and is a mode by which genes can obtain a high degree of proteomic diversity. Almost 90% of human genes are now known to encode at least two isoforms (Pan et al, 2008, Wang et al, 2008). It is thought that increase in proteomic diversity by alternative splicing serves to augment gene regulatory networks through three main mechanisms: the removal of interaction and localization domains, production of mRNA transcripts with shorter/longer half-lives and production of non-functional mRNA that are targeted for non-sense mediated degradation (Lareau et al, 2004).

Alternative splicing involves the selective inclusion/skipping of nucleotides on an mRNA transcript by way of alternative 5` and 3` splice donor/acceptor sites. There are five main forms of alternative splicing: exon skipping (38% of common alternative splicing events), alternative 3` and 5` splice sites (18% and 8%), intron retention (less than 3%) and other complex events such as mutually exclusive exons, alternative promoter usage and multiple polyadenylation sites (remaining 33%) (Ast, 2004). Regulation of alternative splicing occurs through the interaction of trans-acting splicing regulators present on the spliceosome (>200 subunit ribonucleoprotein) and the cis-acting splice enhancer/silencer sequences on the mRNA transcript (Caceres and Kornblihtt, 2002). Alternative exons often have sub-optimal splice sites and length when compared to constitutive exons (Ast, 2004). Thus regulation of alternative splicing allows for spatial, temporal and tissue specific expression of isoforms.

Alternative spliced Pax5 isoforms were first reported by Zwollo et al in mouse B cells, where four major isoforms are produced: Pax-5a which represents the full-length form, Pax5-b which has deletion of exon 2, Pax-5d which has deletion of exons 6-10 and Pax5-e which contains deletions of both exons 2 and 6-10 (Zwollo et al, 1997). Short and Holland (2008) reported several N-terminal and C-terminal isoforms of the Pax2/5/8 gene, a precursor of vertebrate Pax5, in whole adult amphioxus (a basal chordate) cDNA. Human Pax5 isoforms were first reported by Borson et al (2002) in B cells of multiple myeloma patients, with deletion of exon 2, 8, 9, 7-8 and 7-9. Robichaud et al (2004), confirmed the detection of multiple Pax5 C-terminal isoforms using RT-PCR in normal B cells and lymphoma cells, along with the mutually exclusive use of exon 1b

(Robichaud et al, 2008). Further studies by Arseneau et al (2009), Sekine et al (2007), Oppezzo et al (2005) and Lowen et al (1999) discuss the prevalence, expression and functional studies performed for different isoforms and have been addressed in the discussion section.

Table 1.2

#	Exon	Domain encoded	Humans	Mice	Amphioxus	Identified in Tissue/Cell type
1	1a	Not defined	+	+	+	*All B cells*
2	1b	Not defined	+	+	-	*All B cells*
3	1aΔ2	Partial Paired domain	+	+	+	*B-cell lymphoma, CLL, mouse spleen, MM, Adult amphioxus (whole)*
4	1bΔ2	Partial Paired domain	+	-	-	*Normal B cells, BCL, CLL*
5	Δ2/3	Paired domain	+	-	-	*Normal B cells, BCL, CLL*
6	Δ2/4	Partial PD	+	-	-	*Normal B cells, BCL, CLL*
7	Δ2/8/9	Partial PD, TA and ID	+	-	-	*Normal B cells, BCL, CLL*
8	Δ3	Partial PD	-	-	+	*Adult amphioxus (whole)*
9	Δ3/4a	Partial PD	-	-	+	*Adult amphioxus (whole)*

10	Δ3/8	Partial PD and TA	+	-	-	*Normal B cells, BCL, CLL*
11	Δ4	OD and Partial PD	-	-	+	*Adult amphioxus (whole)*
12	Δ5	Not defined	+	-	-	*Normal B cells, BCL, CLL*
13	Δ5/9	Partial ID	+	-	-	*Normal B cells, BCL, CLL*
14	Δ6-9	TA and ID	+	-	-	*CLL*
15	Δ6-10	TA and ID	-	+	-	*Mouse spleen*
16	Δ6/7/8	HD and TA	+	-	-	*CLL*
17	Δ7	Not defined	+	-	-	*Normal B cells, BCL, CLL, MM*
18	Δ7/8	TA domain	+	-	-	*Normal B cells, BCL, CLL, MM*
19	Δ7-9	TA domain	+	-	-	*Normal B cells, BCL, CLL*
20	Δ7-9	TA and partial ID	+	-	-	*Normal B cells, BCL, CLL*
21	Δ7-10	Partial ID	-	-	+	*Adult amphioxus (whole)*
22	Δ7-p11	TA and ID	-	-	+	*Adult amphioxus (whole)*
23	Δ7/10/p11	Not defined	-	-	+	*Adult amphioxus (whole)*

24	Δ7/10b	Not defined	-	-	+	*Adult amphioxus (whole)*
25	Δ7/10b/p11	Not defined	-	-	+	*Adult amphioxus (whole)*
26	Δ7/p11	Not defined	-	-	+	*Adult amphioxus (whole)*
27	Δ7b	Not defined	-	-	+	*Adult amphioxus (whole)*
28	Δ8	Partial TA domain	+	-	-	*Normal B cells, BCL, CLL, MM*
29	Δ9	Partial TA and ID	+	-	-	*Normal B cells, BCL, CLL, MM*
30	Δ8/9	Partial TA/ID	+	-	-	*Normal B cells, BCL, CLL, MM*

Table 1.2 List of alternatively spliced Pax-5 transcripts reported in different organisms. +/- indicates presence absence, NA – data not available. PD – paired domain, TA – trans-activation domain, ID – Inhibitory domain, HD – homeodomain, OD – Octopeptide segment, Not defined – deleted amino acids encode no known functional domains, Δp-indicates partial deletion, CLL – Chronic Lymphoblastic Leukemia (human), BCL – B-cell Lymphoma (human), MM –Multiple Myeloma B-cells (human).

With the growing number of reports for alternative splicing of Pax5 in different organisms, it is now feasible to focus on cross species conservation of alternative splicing in vertebrates. Evolutionary conservation of isoforms over long periods of time suggests that these isoforms may be positively selected for and implies conserved function (Lareau et al, 2004).

Table 1.2 lists all the alternatively spliced isoforms that have been reported thus far for Pax5. Clearly, human and amphioxus Pax5 yield the most diverse splicing patterns in the vertebrate lineage, where most splicing occurs at the C-terminal end of Pax-5. Human

Pax5 isoforms represent 63% (19 of 30) of total isoforms reported and amphioxus Pax5 isoforms represent 40% (12 of 30) of total isoforms reported. However, between the basal chordate amphioxus and humans, only 2 isoforms are conserved – namely, full-length Pax5 and 1aΔ2. This exon 2 skipping is also conserved in mouse B cells. Conserved Pax5 isoforms represent only 10% (3 of 30) of the reported isoforms for these three organisms. The remaining isoforms are not conserved and represent novel/rare isoforms. The conservation of particular isoforms implies that these sequences have functional roles in vertebrate B cell development. In summary, multiple isoforms of Pax5 have been detected with deletions in the PD, TA and ID. These isoforms possess a wide range of trans-activities and potentially contribute to the intricate regulatory function of Pax5 in B cells.

1.5 Significance of alternative splicing

The regulatory role of alternatively spliced genes in cellular processes like cell cycle/proliferation, differentiation, lineage commitment and apoptosis has previously been under-appreciated. With growing evidence for widespread alternative splicing of genes and evolutionary conservation of alternatively spliced isoforms, alternative splicing has been recognized as an important means of genetic regulation at the post-transcription and translation levels.

Apart from expanding the proteomic diversity of genes, alternative splicing of genes can produce proteins with whole functional domains removed or added. Alternatively spliced isoforms usually function as dominant negative inhibitors and block the function

of the wild-type (Brinkman et al, 2004). For example, alternative splicing of the apoptosis genes Bcl-x, Caspase-9 and Ced-4 yields variants with either pro or anti-apoptotic activity (Wu et al, 2003, Syken et al, 1999). Alternative splicing can also alter protein localization, for example alternative splicing of the fibroblast growth factor receptor gene produces a switch from membrane bound to soluble protein form (Jang, 2002).

Also, alternative promoter usage can alter mRNA translation rate. In vitro experiments performed by switching promoter sequence on the fibronectin 1 gene showed its expression was almost 10-times higher when paired with its endogenous promoter than with a β-globin promoter (Cramer et al, 1997). Similarly, mRNA transcripts with alternatively spliced 3` un-translated sequence have altered stability and susceptibility to degradation. Additionally, spliced transcripts which generate a premature termination codon are often subject to nonsense-mediated decay. Thus alternatively spliced transcripts may have variant half-lives in an intracellular environment.

Alternatively spliced transcripts are also known to be involved in auto-regulation of gene expression. For example, constitutive splicing of the poly-pyrimidine tract binding protein (PTB) gene produces PTB, which is a regulator of alternative splicing. Increase in PTB protein levels favors the production of un-productive alternatively spliced transcript with a premature-termination codon in exon 11 and is subject to nonsense mediated decay (Wollerton et al, 2004).

Variation in the intracellular level of splicing regulatory factors influence the mode of mRNA splicing and is one explanation for tissue specificity and developmental regulation of alternatively spliced isoforms. A classic example of this was illustrated by Stickeler et al (1999) where stepwise increase in splicing regulator (SR) protein levels correlated with increased alternative splicing of the CD44 gene in mammary tumor cells.

Given its vital role in the regulation of cellular processes, aberrant expression of alternatively spliced isoforms has been implicated in various diseases and cancers. For example, the neurofibromatosis gene (NF1) has one of the highest mutation rates of all human genes and aberrant expression of NF1 leads to the formation of neurofibromas. Almost 32% of neurofibroma cases can be attributed to irregular splicing of NF1 (Ars et al, 2000).

Alternatively spliced isoforms of Pax5 contain selective deletions of one or more functional domains. These isoforms have been tested in functional assays, where they show altered DNA binding and trans-activity when compared to full-length. Isoforms with deletion of DNA binding domain coding exon 2, are unable to bind DNA as shown in EMSA studies for mouse B cells (Zwollo et al, 1997, Lowen et al, 2001). Pax5 isoforms with deletion of trans-activation domain coding exon 8 and 9 have also been shown to have DNA binding ability and trans-activity different from full-length in human normal and multiple myeloma B cells (Robichaud et al, 2004). In one report, human Pax5/Δ8 isoform was shown to bind to the AID promoter and induce AID expression in chronic

lymphoblastic leukemia cell *in vitro* (Oppezzo et al, 2005). Thus Pax-5 isoforms have different and unique trans-activation potentials.

Pax5 trans-activates multiple target genes that are cell surface receptors and involved in pre-BCR signaling. B cell signaling processes are tightly regulated by complex regulatory cascades. Pax5 isoforms can potentially provide a wide range of regulatory control during stages of B cell development and activation, by influencing the expression of Pax5 target genes.

Recently, it has been proposed the alternatively spliced isoforms can be used as biomarkers of cancerous phenotype (Brinkman, 2004). Altered expression of Pax5 isoforms has been reported in B cells from multiple myeloma and lymphoma patients (Borson et al, 2005). Thus Pax5 isoforms may also be used as biomarkers of lymphocytic cancers. Since alternatively spliced transcripts are tissue, stimulus and disease specific, there remains a large scope for using Pax5 isoforms as biomarkers of cancerous phenotype. Detection strategies can include PCR using primers that specifically amplify an alternatively spliced variant or microarrays for detection of a large number of markers in parallel. Since alternatively spliced transcripts are often translated, antibody based strategies may also be used to detect isoform expression (Brinkman et al, 2004). Finally, our study of alternative splicing of Pax5 in trout B cells completes the evolutionary picture of isoform conservation in vertebrates.

1.6 Research aims and hypotheses

The primary aim of this project was to identify and clone alternatively spliced Pax5 transcripts in trout B cells which contain deletions of exons that encode various functional domains, using nested-PCR.

The secondary goal of this project was to study potential changes in the expression of each alternatively spliced transcript versus full-length Pax5 upon activation of splenic and blood-derived B cells. Evaluation of the relative amplification of alternatively spliced Pax5 would implicate the involvement of Pax5 isoforms in the regulatory processes that surround B cell activation.

Based on observed exon skipping patterns of alternatively spliced trout Pax5, we hypothesized that:

1) Alternatively spliced isoforms of Pax5 that skip exon 2, encoding part of the DNA binding domain, would function as dominant negative repressors of Pax5 activity and are expressed in relatively higher quantities during splenic and blood-derived B cell activation.
2) Alternatively spliced isoforms of Pax5 that skip exon 8, encoding part of the trans-activation domain, function as repressors of Pax5 activity and are expressed in relatively higher quantities during splenic and blood-derived B cell activation.

3) Alternatively spliced isoforms of Pax5 that skip inhibitor domain coding exon 9 function as strong trans-activators and their expression is relatively low during splenic and blood-derived B cell activation.

These hypotheses were tested using semi-quantitative PCR analysis of LPS activated cells.

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Chapter 2

Materials & Methods

Chapter 2: Materials and Methods

2.1 Overview of Methods

Harvesting trout immune tissues and activating B cells in culture: The following immune tissues were collected from three to five adult rainbow trout (8-12 inches long; obtained from Casta Line Trout farms) - the anterior kidney (AK or K1), posterior kidney (PK or K5), spleen and peripheral blood (PBL). Tissues were collected in 5mls of Hank's balanced salt solution (HBSS - 137mM NaCl, 5.6mM D-glucose, 5mM KCl, 8.1mM $Na_2HPO_4.2H_2O$ and 20mM Hepes at pH 7.05) and blood was collected in heparinized tubes. Single cell suspensions were obtained by repeated aspiration of tissues with a 10ml syringe followed by passing through a 40nm cell strainer (Falcon/BD biosciences). Erythrocytes were removed from the cell suspension through layering onto histopaque 1077 gradient (Sigma Aldrich) followed by centrifugation at 500g at 4°C for 40 minutes. White blood cells were removed from the interface and washed in 50mls of HBSS prior to being cultured in trout complete medium (TCM) consisting of RPMI 1640 with 10 mM L-glutamine, 10% FCS, 50 g/ml gentamycin, 50 M 2-ME, and the nucleosides adenosine, uracil, cytosine, and guanosine (10 mg/ml; Sigma-Aldrich) at a concentration of 10 million cells per ml of medium, as described in Yui, M.A. & Kaattari S.L. (1987). Cells were activated in culture with the B cell mitogen LPS (055:B5 from E. coli; Sigma) at 100µg/ml. The cells were fed every other day with one-tenth of the culture volume of a 10x tissue culture cocktail containing 500 mg/m gentamycin, 10 mg/ml essential amino acids, 10 mg/ml non-essential amino acids, 70 mM L-glutamine, 70 mg/ml dextrose, 10 mg/ml

nucleosides, and 33% FBS. Cells were harvested on days 0-7, pelleted at 1000rpm in a clinical centrifuge and frozen at -80°C until RNA extraction.

RNA isolation and cDNA synthesis: Total cellular RNA was extracted from frozen pellets of approximately 5 to 10 million human, mouse or trout B cells (either freshly isolated or activated with LPS in culture) using RNeasy mini kit (Qiagen), according to the manufacturer's instructions. Total RNA was quantified on nanodrop ND-1000 spectrophotometer (Thermo Scientific). 0.5µg or 1µg of total RNA was used to generate cDNA using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions.

Nested Polymerase Chain Reaction for screening of O. mykiss alternatively spliced Pax5: Nested PCR was conducted to screen for each alternatively spliced transcript of trout Pax5 on a Perkin-Elmer GeneAmp PCR system 2400. 1.5µl to 3µl of cDNA template was used in the first round of PCR using PCR primers (generated by Integrated DNA Technologies) that externally flank the exons being tested and using Vent® DNA polymerase (New England Biotech) under the following conditions: 1 minute at 94°C, 1 minute at 56-58°C and 1 minute at 72°C for 35 cycles. 1-2µl of amplified product from the first round was then used as template for the second round of PCR using an independent set of internal primers under the following conditions: 1 minute at 94°C, 1 minute at 60-62°C and 1 minute at 72°C for 35 cycles. 10µl of second round PCR product was run on a 2% agarose gel containing Ethidium bromide (0.1µg/ml) and with 0.5µg of

100bp DNA ladder (New England Biotech) as size marker. A list of primers used in the nested PCR screen can be found under Table 2.1.

Nested Polymerase Chain Reaction for screening of human and mouse Pax5/Δ2-8: This method used to isolate alternatively spliced transcripts has been used before by Gorlov and Saunders (2002). Nested PCR was conducted to screen for alternatively spliced Pax5/Δ2-8 in human bone marrow and tonsil cDNA samples, and mouse spleen cDNA on a Perkin-Elmer GeneAmp PCR system 2400. PCR primers were designed based on the published full length cDNA sequence of human Pax5/BSAP (GeneID: 9951919, Ensembl: ENSG00000196092) and Mus musculus Pax5 (GeneID: 118130642, Ensembl: ENSMUSG00000014030). 1.5µl to 3µl of cDNA template was used in the first round of PCR using PCR primers (generated by Integrated DNA Technologies) that externally flank the exon being tested and using Vent® DNA polymerase (New England Biotech) under the following conditions: 1 minute at 94°C, 1 minute at 56-58°C and 1minute at 72°C for 35 cycles. Amplified product from the first round was then used as template for the second round of PCR using an independent set of internal primers under the following conditions: 1 minute at 94°C, 1 minute at 60-62°C and 1 minute at 72°C for 35 cycles. 10µl of second round PCR product was run on a 2% Agarose gel containing ethidium bromide (0.1µg/ml) and with 0.5µg of 100bp DNA ladder (New England Biotech) as size marker. A list of primers used in the nested PCR analysis can be found under Table 2.3.

DNA extraction from low melt agarose: This technique has been adapted from Chory et al (1999). 50-100µl of nested PCR product was electrophoretically separated in a 1% low melt agarose gel and the potential alternatively spliced Pax5 amplicons were excised and stored in TEN buffer (1M Tris pH 8, 0.5M EDTA, 5M NaCl) at 4°C overnight. The mixture was melted at 67°C, added to 1 volume of phenol (pre-warmed to 37°C) and vortex mixed. Samples were spun at 10,000 rpm for 5 minutes at room temperature in a tabletop centrifuge. The upper aqueous layer was harvested and transferred to another aliquot of phenol and the extraction was performed 2 more times. In the last extraction, the upper aqueous layer was added to 1 volume of chloroform (pre-warmed to 37°C) and vortex mixed. Samples were spun at 10,000 rpm for 5 minutes at room temperature in a tabletop centrifuge. The upper aqueous layer was collected and precipitated at -20°C overnight in $1/10^{th}$ volume 3M sodium acetate and two volumes of ice-cold 100% Ethanol (molecular grade). DNA was precipitated by spinning at 10,000 rpm for 30 minutes at 4°C and removing the supernatant. The DNA pellet was washed with one volume of ice-cold 70% ethanol and air dried before re-suspending in TE buffer (10mM Tris, 1mM EDTA – pH 8).

Cloning of alternatively spliced isoforms: 5-50ng of round 2 nested PCR product was ligated into pSC-A-amp/kan cloning vector (Strataclone PCR cloning kit, Stratagene), transformed into Strataclone Solopack™ competent bacterial cells according to the manufacturer's instructions and grown on LB plates (containing 50µg/ml Ampicillin) at 37°C for 16-20 hours. Single colonies were selected and individually re-grown in 5ml of LB broth (containing 50µg/ml Ampicillin) at 37°C for 16-20 hours.

Small scale boiling lysis plasmid preps: Plasmid DNA was extracted from E. coli cultures using small-scale boiling lysis plasmid preps (Ehrt & Schnappinger, 2003) as described below. 5mls of E. coli culture was pelleted at 5000rpm and 4°C for 10 minutes in a clinical centrifuge. The supernatant was discarded and the bacterial cell pellet was resuspended in 70µl of STET buffer (0.1M NaCl, 10mM Tris Cl, 1mM EDTA, 5% Triton X-100) and 5µl of lysozyme (1mg/ml). Cells were lysed by incubating at 95°C for 1 minute followed by centrifugation at 10000rpm for 10 minutes in a microfuge at room temperature. The pellet containing proteins, genomic DNA and cell debris were removed using sterile toothpicks. Plasmid DNA in the supernatant was precipitated by adding one volume of 2- propanol and centrifuging at 10,000 rpm for 15 minutes at 4°C. The supernatant was discarded and DNA pellet was washed with 2 volumes of 70% ethanol. Excess ethanol was removed and the pellet was air dried before resuspending in 40µl of TE buffer (10mM Tris, 1mM EDTA – pH 8).

Restriction digestion with EcoRI: 10µl of plasmid DNA was digested with 200 units of EcoRI restriction enzyme (New England Biotech) at 37°C for 1 hour. 1µl of RNase A (10mg/ml) was added to the reaction mix and the sample was incubated for another 15 minutes at 37°C to remove RNA. 10µl of the digested product was run on a 2% agarose gel with (containing 0.1µg/ml ethidium bromide) to check for requisite DNA insert.

Selection of clones: Clones containing a DNA insert were re-grown overnight in 5mls of LB (containing 50µg/ml Ampicillin) at 37°C and plasmid DNA was extracted using

QIAprep spin miniprep kit (Qiagen), as per the manufacturer's instructions. 1µg of plasmid DNA was digested with EcoRI enzyme (New England Biotech) and run on 2% Agarose gel (containing 0.1µg/ml Ethidium bromide) to re-test for requisite insert, as described above.

Sequencing and analysis: 2-3µg of plasmid DNA, containing the target DNA insert, was shipped for sequencing by the Iowa State University DNA sequence facility (Applied Biosystems 3730xl DNA Analyzer). The DNA insert region was sequenced using T3 universal primers. 3-5 independent clones of each alternatively spliced transcript were sequenced to confirm sequence identity. Alternatively splicing of Pax5 exons was confirmed by aligning the sequenced clone with published full length cDNA sequence of O. mykiss Pax5 (NCBI Gene ID: 185135003) in a Pustell DNA matrix (and ClustalW alignment) using the alignment program MacVector® version 7.2.3.

Semi-quantitative RT-PCR and ratio analysis: RNA was isolated from trout immune tissues as previously described (page 33). A single round of PCR was performed to obtain a relative ratio of alternatively spliced Pax5 isoform to full length Pax5 cDNA using PBL and spleen cDNA. Two sense direction PCR primers were used, that flanked exon boundaries of full length and alternatively spliced Pax5 respectively, along with a common anti-sense PCR primer. PCR was performed using Vent® DNA polymerase (New England Biotech) under the following conditions: 1minute at 94°C, 1minute at 60-62°C and 1minute at 72°C for 32 cycles on a Perkin-Elmer GeneAmp PCR system 2400. 10µl of PCR product was run on a 2% Agarose gel containing Ethidium bromide (0.1µg/ml) and

with 0.5μg of 100bp DNA ladder (New England Biotech) as size marker. PCR amplicons were visualized and recorded on a gel documenting apparatus (Bio-Rad Gel Doc XR CFW1312M) under UV light. Gel images were recorded at 0.5 (± 0.3) seconds UV exposure and band intensities measured using Quantity One® software version 4.6.1 (Discovery Series/Bio-Rad). A list of primers used in the semi-quantitative PCR analysis can be found under Table 2.2.

The ratio of relative intensity of alternatively spliced to full-length* Pax5 amplicon or secreted to membrane IgM and IgT amplicons was calculated and the ratio at each day of LPS activation was then normalized against day 0. All experiments were performed in triplicate, the mean of the resulting values were plotted in a trend line graph with days after LPS activation on the x-axis and relative ratio on the y-axis. Standard deviations from the mean were incorporated for each data point per day.

Table 2.2: Primers used in nested PCR analysis of alternatively spliced Pax5 transcripts.

Primer Name	Direction	Sequence (5` to 3`)	Location	Target isoform	Round of nested PCR
tPax5/5`end.S	Sense	ATG TGC GGC CGC ATG GAA GTA GAG GCC GAG GG	Pax5 exon1a (ATG)	Pax5/1a Δ2 isoform	1
tPax5/528.AS	Antisense	AGC CGA GTC ACT GGA CAC C	Pax5 exon 5	Pax5/1a Δ2 isoform	1
tPax5/e1wt.S	Sense	AGT AGA GGC CGA GGG TCA TG	Pax5 exon 1a	Pax5/1a Δ2 isoform	2
tPax5/E4.AS	Antisense	CTG ACC CGG AGG CTG ATG G	Pax5 exon 4	Pax5/1a Δ2 isoform	2
tPax5b/5`UT.S	Sense	GAT GGA GTC GGC GAA CTA G	Pax5 exon 1b (5`UTR)	Pax5/1b Δ2 isoform	1
tPax5/3`UT.AS	Antisense	CAT CGT TGA TGG GTG GGG TCA	Pax5 3`UTR	Pax5/1b Δ2 isoform	1
tPax5/5`end.S	Sense	ATG GAG ATA GAG AAT ATA GTC GCC	Pax5 exon 1b (ATG)	Pax5/1b Δ2 isoform	2
tPax5/618.S	Sense	ATG TGC GGC CGC GCT ACC CCC CAC ACG TCC CCC	Pax5 exon 6	Pax5/Δ8 isoform	1
tPax5/1104.AS	Antisense	CAT CTG TCC CGT CTG GCT GG	Pax5 exon 10	Pax5/Δ8 isoform	1
tPax5/764.S	Sense	ATG TGC GGC CGC ACC AAT CTG GCC AAT CCA GGG TCA	Pax5 exon 7	Pax5/Δ8 Pax5/Δ9a, 9b and 9c	2

tPax5/964.AS	Antisense	AGG GTG GGA ATA GCA G	Pax5 exon 9	Pax5/Δ8 isoform	2
tPax5/3`end2. AS	Antisense	ATG TGC GGC CGC TCA GTG GCG GTC GTA GGC GG	Pax5 exon 10 (TGA)	Pax5/Δ9a, 9b and 9c	1
tPax5/860.S	Sense	ATG TGC GGC CGC GCT ACC CCC CAC ACG TCC CCC	Pax5 exon 8	Pax5/Δ9a, 9b and 9c	2
tPax5/1104.AS	Antisense	CAT CTG TCC CGT CTG GCT GG	Pax5 exon 10	Pax5/Δ9a, 9b and 9c	2

Table 2.3: Primers used to obtain the relative ratio of alternatively spliced Pax5 transcript to full-length* transcript using semi-quantitative RT-PCR analysis.

Primer Name	Direction	Sequence (5` to 3`)	Location	Target isoform
tPax5/E1-2.S	Sense	GAC GAG CAG GAG GAC ATG G	Pax5 exon 1a/2 boundary	FL*Pax5 (containing exons 1a and 2)
tPax5/E1-3.S	Sense	CGA GCA GGT ACT ATG AGA CAG G	Pax5 exon 1a/3 boundary	Pax5/1a Δ2 isoform
tPax5/e3sp2.AS	Antisense	GAT CGC GGC CGC GTC TCG TAT CTC CCA GGC G	Pax5 exon 3	Pax5/1a Δ2 and FL*Pax5 (containing exons 1a and 2)
tPax5B/E1-2.S	Sense	TGA AGG CGA GAG AAG GAC ATG	Pax5 exon 1b/2 boundary	FL*Pax5 (containing exon 1b and 2)
tPax5/1B2/3.S	Sense	AAG GCG AGA GGT ACT ATG AG	Pax5 exon 1b/3 boundary	Pax5/1b Δ2 isoform
tPax5/7/8.S	Sense	CCA CTG CCA GGT CGA GAC CTC	Pax5 exon 7/8 boundary	FL*Pax5 (containing exon 8)
tPax5/7/9.S	Sense	TCCACTGCCAGGAGGAGAT TTTTC	Pax5 exon 7/9 boundary	Pax5/Δ8 isoform

tPax5/9/10(1018).AS	Antisense	TGT TGG AAC ACT AAC AGG CTG	Pax5 exon 9/10 boundary	Pax5/Δ8 isoform & FL*Pax5 (containing exon 8)
tPax5/8/9.S	Sense	GTA TGG TAC CCG GAG GAG ATT TTT C	Pax5 exon 8/9 boundary	FL*Pax5 (containing exon 9)
tPax5/8(931)/10(1018).S	Sense	TAT GGT ACC CGT GTT CCA ACA G	Pax5 exon 8/10 boundary	Pax5/Δ9a
tPax5/8(920)/10(1018).S	Sense	CTC ACT GAC TGT GTT CCA ACA G	Pax5 exon 8/10 boundary	Pax5/Δ9b
tPax5/8(920)/10(1072).S	Sense	CTC ACT GAC TGG GCT CTT CAC C	Pax5 exon 8/10 boundary	Pax5/Δ9c
tPax5/3`end2.AS	Antisense	ATG TGC GGC CGC TCA GTG GCG GTC GTA GGC GG	Pax5 exon 10 (TGA)	Pax5/Δ9a, Δ9b, Δ9c & FL*Pax5 (containing exon 9)
tHC.S	Sense	CCT TAA CCA GCC GAA AGG G	IgM	secreted & membrane IgM transcript
tHCm.AS	Antisense	CCA ACG CCA TAC AGC AGA G	IgM trans-membrane segment coding region	membrane IgM transcript
tHCs.AS2	Antisense	TGA GGT TCT ATG AAT GGT TCT C	IgM secretory tail coding region	secreted IgM transcript

tIgT.S	Sense	GAG GAC AGT GGC GAG TAC C	IgT	secreted and membrane IgT transcript
tIgtTmem.AS	Antisense	GCT GCC GAA CTC ATC CTC	IgT trans-membrane segment coding region	membrane IgT transcript
tIgTsec.AS	Antisense	GGC AGC AAC ACA AGA CTG AC	IgT secretory tail coding region	secreted IgT transcript

Table 2.4: Primers used to clone human and mouse Pax5/Δ2-8 transcript.

Primer Name	Direction	Sequence (5` to 3`)	Location	Round of nested PCR
hPax5/5`UT.S	Sense	GTG GAA ACT TTT CCC TGT CC	human Pax5 exon 1a (5`UTR)	1
hPax5/3`end.AS	Antisense	GTG CCA TCA GTG TTT GGT GCC	human Pax5 3`end (TGA)	1/2
SD664.S	Sense	ATG GAT TTA GAG AAA AAT TAT CCG	human Pax5 exon 1a (ATG)	2
mPax5/5`UT.S	Sense	CCT CGC TGT CCA TTT CAT CAA GTC C	mouse Pax5 exon 1a (5`UTR)	1
mPax5/3`UT.AS	Antisense	GTA AGT GCT TGG CAC CCG TGG	mouse Pax5 exon 10 (3`UTR)	1
Pax5.ATG1.S	Sense	ATG GAT TTA GAG AA	mouse Pax5 exon 1a (ATG)	2
PP8.S	Sense	AAA TGG ATT TAG AGA AAA ATT AC	mouse Pax5 exon 1a (ATG)	2
mPax5/3`end.AS	Antisense	TCA GTG ACG GTC ATA GGC GGT GGC	mouse Pax5 3`end (TGA)	2

2.5 References:

Chory, J. a. (1999). *Current Protocols in Molecular Biology.* Wiley.

Ehrt S, S. D. (2003). Isolation of plasmids from E. coli by boiling lysis. *Methods Molecular Biology , 235*, 79-82.

Gorlov IP, S. G. (2002). A method for isolating alternatively spliced isoforms: isolation of murine Pax6 isoforms. *Analytical Biochemistry , 308*, 401–404.

Kaattari SL, Y. M. (1987). Polyclonal activation of salmonid B lymphocytes. *Developmental and comparitive immunology , 11* (1), 155-165.

Chapter 3

Results

Chapter 3: Results

3.1 Screening for alternatively spliced isoforms of Pax5 with deletion of the paired domain:

The N-terminal DNA-binding paired domain is a strongly conserved feature among members of the Pax family and is encoded by the second and third exons of the Pax5 gene (figure 3.1) (Czerny, Schaffner and Busslinger, 1993). Alternatively spliced transcripts of Pax5 that lack a paired domain are hypothesized to have low DNA binding ability and may function as dominant transcriptional co-repressors of Pax5 downstream targets. To screen for alternatively spliced isoforms of Pax5 that lack the paired domain coding region (either exon 2 or exon 3), two independent sets of primers were designed based on the published complete cDNA sequence of *Oncorhynchus mykiss* Pax5 (Genbank accession number NM_001124682) that flank the 5` end of the transcript.

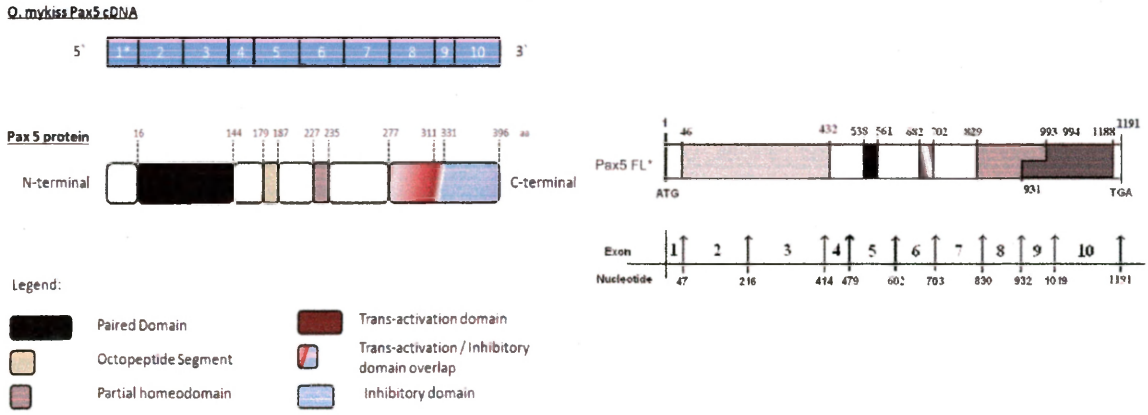


Figure 3.1: Schematic of Pax5 cDNA exon structure and conserved functional domains on the Pax5 protein. O. mykiss Pax5 cDNA is 1191 nucleotides long and consists of 10 exons that encode 5 conserved functional domains. The exon boundaries of O. mykiss Pax5 cDNA was determined by aligning the

published full-length Pax5 sequences of mouse, human and O. mykiss. Nucleotides 46-432 (Exons 2, 3 and partial 4) code for the DNA binding paired domain, 538-561 (partial Exon 5) code for the octopeptide segment, 682-703 (partial Exon 6) code for the partial homeodomain, 829-993 (Exon 8 and partial 9) code for the trans-activation domain and 994-1188 (partial Exon 9 and 10) code for the inhibitory domain.

Using a nested PCR approach (figure 3.2), the first set of primers which externally flank exons 2 and 3 was used to screen for alternatively spliced Pax5 in cDNA from freshly isolated anterior kidney cells (as described in methods). Both rounds of PCR product were electrophoretically separated and DNA bands obtained corresponded to a longer paired domain containing Pax5 amplicon of size 450 nucleotides and a smaller 281 nucleotide long amplicon (see figure 3.2). A 190 nucleotide long amplicon (indicated by an asterisk in figure 3.2) was also obtained, which upon sequence analysis was found to be not related to Pax5.

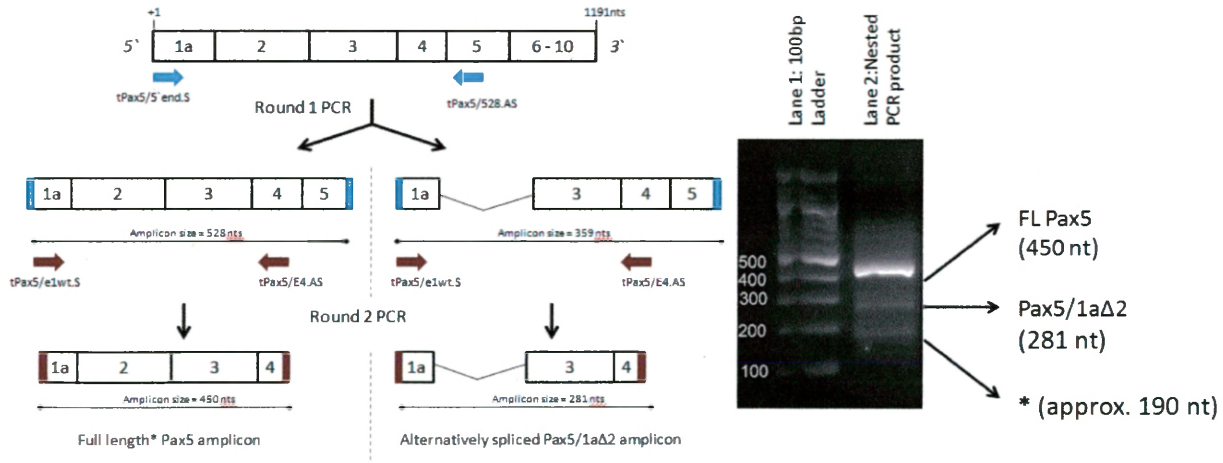


Figure 3.2: Nested PCR strategy for Pax5/1aΔ2 transcript. The first round of nested PCR on AK day 0 cDNA used primers that flank exons 2 to 4. The product from this round was then used in a second round of PCR with an independent set of internal primers that flank exons 2 and 3. The product was electrophoretically separated on a 2% agarose gel with (0.1µg/ml Ethidium bromide). The first lane contains a 0.5µg of 100bp ladder (New England Biotech) and the second lane contains 10µl of second round PCR product. Two amplicons of sizes 450 nucleotides and 281 nucleotides was obtained, corresponding to exon 2 containing and exon 2 deleted Pax5 transcripts. Also, a 190 nucleotide amplicon

was obtained (indicated by * in gel picture). Sequence analysis showed that this amplicon was not Pax5 related. BLAST searches performed for this amplicon showed no sequence homology with genes in the NCBI database.

Subsequent cloning and sequencing of both amplicons revealed that the longer amplicon (clone # 350E2-U461207) was paired domain containing Pax5 (with exons 2 and 3) and the shorter amplicon (clone # E23A0a_T3_492426) was an alternatively spliced transcript of Pax5. A pustel DNA matrix alignment of the paired domain containing Pax5 clone with the published full-length Pax5 cDNA sequence showed complete homology, corresponding to full length Pax5 containing exons 2 and 3. A pustel DNA matrix alignment of the alternatively spliced Pax5 clone with the published full length Pax5 cDNA sequence showed homology in regions corresponding to exons 1a, 3 and 4 with a 169 nucleotide gap between nucleotides 47 to 216 (See Appendix I alignment 2), corresponding to a deletion of exon 2.

Three more independent clones (clone # C02_E56D5_R-2_497304, A02_E2A0a3_R-2_497311 and C02_E2A0a6_R-2_497313) were sequenced to confirm the identity of alternative spliced Pax5 with deleted exon 2 [Pax5/1aΔ2]. A total of seven clones sequenced displayed a splicing pattern distinctive of Pax5/1aΔ2, suggesting that this alternatively spliced transcript may be relatively abundant to full-length Pax5 in B cells. Clones containing tPax5 sequence are listed in table 3.1.

In a previous study by Zwollo et al (un-published data), a cDNA library screen for full-length O. mykiss Pax5 lead to the discovery of putative alternate exon 1b. We designed a nested PCR strategy to test if Pax5 transcripts with exon 1b also deleted exon 2. The

first set of primers flank the entire Pax5 (with exon 1b) transcript and was used to screen for all alternatively spliced transcripts of Pax5 in AK day 0 cDNA from freshly isolated anterior kidney cells. Amplicons of size 300-500 and less than 300 nucleotides were extracted separately from low melt agarose. This DNA was then used as template in the nested round using an independent set of internal primers that flank exons 2 (figure 3.3). Both rounds of PCR product were electrophoretically separated and DNA bands were obtained corresponding to a longer paired domain containing Pax5 amplicon of size 358 nucleotides and a smaller 189 nucleotide amplicon (see figure 3.3). A 240 nucleotide amplicon was obtained that was found to be not-Pax5 related (indicated with an * in figure 3.3).

Cloning and sequence analysis of the potential Pax5/1bΔ2 amplicon is yet to be performed. The difference in size between the longer 358 nucleotide amplicon and the shorter 189 nucleotide amplicon correspond to the size of deleted exon 2 (179 nucleotides and supports the conclusion that the shorter amplicon is indeed Pax5/1bΔ2.

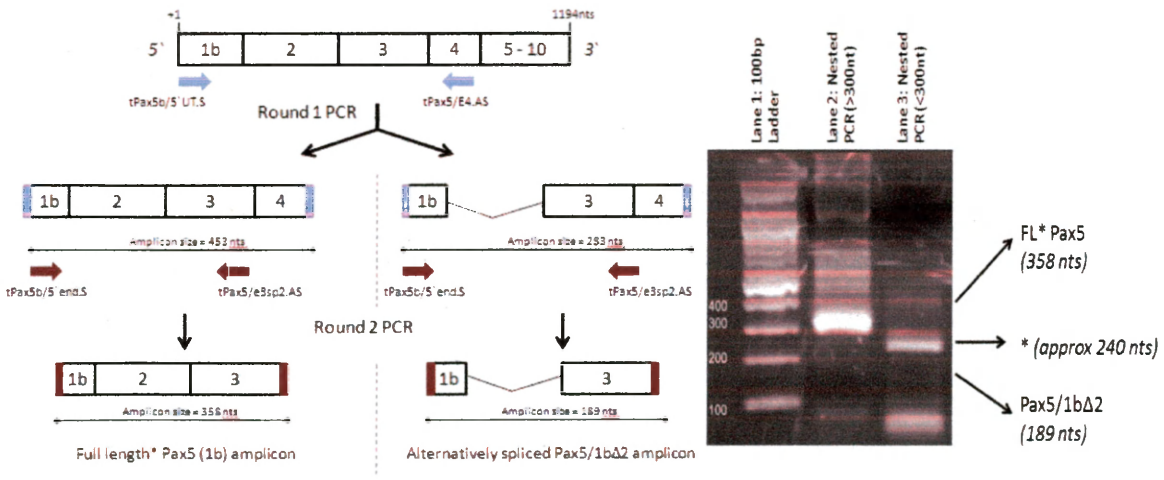


Figure 3.3: Nested PCR strategy for Pax5/1bΔ2 transcript. The first round of nested PCR on AK day 0 cDNA used primers that flank the entire Pax5/1b transcript. The product from this round was then separated electrophoretically on a 1% low melt agarose gel (0.1μg/ml Ethidium bromide). Amplicons of size 500-300 nucleotides and less than 300 nucleotides were extracted and used as template in a second round of PCR with an independent set of internal primers that flank exons 2. The products were electrophoretically separated on a 2% agarose gel with (0.1μg/ml Ethidium bromide). The first lane contains a 0.5μg of 100bp ladder (New England Biotech); the second lane contains 10μl of nested PCR product using round 1 amplicons of size 500-300 as template; the third lane contains 10μl of nested PCR product using round 1 amplicons less than 300 nucleotides in size as template. Two amplicons of sizes 358 nucleotides and 189 nucleotides were obtained, corresponding to exon 2 containing and exon 2 deleted Pax5/1b transcripts. Also, a 240nt amplicon was obtained that was not Pax5 related. BLAST searches performed for this amplicon showed no sequence homology with genes in the NCBI database.

3.2 Screening for alternatively spliced isoforms of Pax5 with deletion of the trans-activation domain:

The trans-activation domain of Pax5 has been described as a region rich in proline, serine and threonine residues (PST rich) (Dorfler and Busslinger, 1996) and its position on the *Oncorhynchus mykiss* (Rainbow trout) Pax5 cDNA sequence was determined based on ClustalW alignments with published mouse (Genbank ID: 118130642) and human Pax5 cDNA (Genbank ID: 9951919) (Alignment 2.1). Exons 7, 8 and exon 9 encode the trans-activation domain of O. mykiss Pax5 (See figure 3.1). An alternatively spliced isoform of Pax5 that lacks the trans-activation domain will have an intact inhibitory domain at its carboxy-terminal and may function as a dominant transcriptional repressor of Pax5 downstream targets. To screen for alternatively spliced isoforms of Pax5 containing a deletion of the trans-activation domain coding region, two independent sets of primers were designed that flank exons 7, 8 and 9 externally (see Figure 3.4). This work was done together with an undergraduate student Alice Harman.

Using a nested PCR approach, the first set of primers which flank exons 7, 8 and 9 broadly was used to screen for alternatively spliced Pax5 transcripts in cDNA obtained from posterior kidney cells that had been stimulated with LPS for 7 days. Product from this round of PCR was then used as template in the second round using an independent set of internal primers that flanks exon 8. Nested PCR product was electrophoretically separated and the DNA bands obtained corresponded to a longer exon 8 containing Pax5 amplicon of size 200 nucleotides and another amplicon that was roughly 102 nucleotides smaller (figure 3.4).

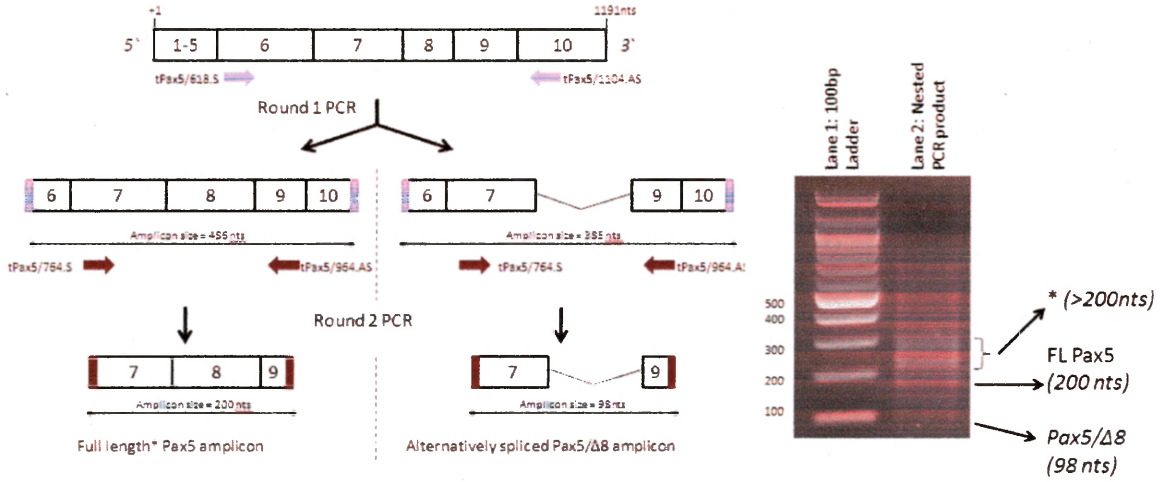


Figure 3.4: Nested PCR strategy for Pax5/Δ8 transcript. The first round of nested PCR on AK day 7 cDNA used primers that flank exons 7 to 9. The product from this round was then used in a second round of PCR with an independent set of internal primers that flank exons 8. The product was electrophoretically separated on a 2% agarose gel with (0.1µg/ml Ethidium bromide). Lane 0 contains 0.5µg of 100bp ladder (New England Biotech) and lane 1 contains 10µl of second round PCR product. Two amplicons of sizes 200 nucleotides and 98 nucleotides (faintly visible) were obtained, corresponding to exon 8 containing and exon 8 deleted Pax5 transcripts. Two additional amplicons greater than 200 nucleotides were also obtained, consequent sequencing of which revealed that they were not related to Pax5 (indicated with an asterisk symbol).

The shorter amplicon of size 98 nucleotides was barely discernable suggesting that it is present at lower relative levels than full-length Pax5 transcript.

Subsequent cloning and sequencing of both amplicons revealed that the longer amplicon was exon 8 containing Pax5 and the shorter amplicon (clone # A6_T3_504023) was an alternatively spliced transcript of Pax5 with deleted exon 8. A Pustel DNA matrix alignment of the Pax5/Δ8 clone with the published Pax5 cDNA sequence showed homology of exons 7 and 9, with a 102 nucleotide long gap between nucleotides 830 to 931 on FL* Pax5 (See Appendix I : Alignment 4), corresponding to a deletion of exon 8.

Another independent clone (clone # A5_T3_504022) was sequenced to confirm the identity of alternative spliced Pax5 with deleted exon 8 [Pax5/Δ8]. *(For a full list of sequenced clones see table 3.1)*

3.3 Screening for alternatively spliced isoforms of Pax5 with deletion of the inhibitory domain:

The inhibitory domain of Pax5 is located at the C-terminal end of the protein and is encoded by exons 9 and 10 (minimal inhibitory domain by nucleotides 994-1188, complete inhibitory domain by nucleotides 931-1188; see figure 3.1) (Dorfler and Busslinger, 1996). Alternatively spliced isoforms of Pax5 that lack the inhibitory domain will have an intact trans-activation domain at the carboxy-terminal and may function as more potent transcriptional activators of Pax5 downstream targets. To screen for alternatively spliced transcripts of O. mykiss Pax5 that contain a deletion of the inhibitory domain coding region, two independent sets of primers were designed that

flank the 3` end of the transcript, specifically exons 9 and 10 (see Figure 3.5). This work was done together with an undergraduate student Alice Harman.

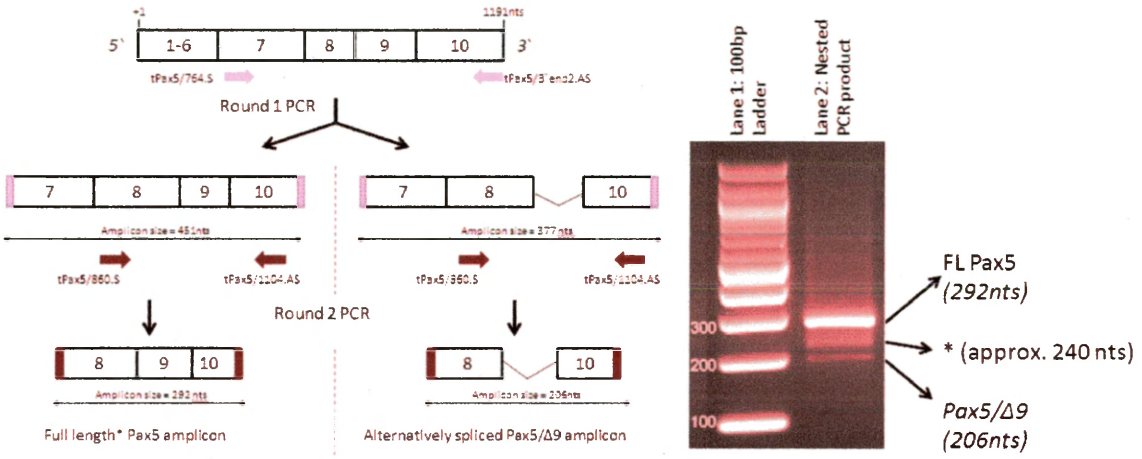


Figure 3.5: Nested PCR strategy for Pax5/Δ9 transcript. The first round of nested PCR on AK day 7 cDNA uses primers that flank exons 8 and 9. The product from this round was then used in a second round of PCR with an independent set of internal primers that flank exon 9. The product was electrophoretically separated on a 2% Agarose gel with (0.1µg/ml Ethidium bromide). Lane 1 contains a 0.5µg of 100bp ladder (New England biotech) and lane 2 contains 10µl of second round PCR product. Two amplicons of sizes 268 nucleotides and 182 nucleotides were obtained, corresponding to exon 9 containing and exon 9 deleted Pax5 transcripts.

Using a nested PCR approach, the first set of primers which flank exons 9 and 10 broadly was used to screen for alternatively spliced Pax5 in cDNA obtained from day 7 anterior kidney cDNA. Product from this round of PCR was then used as template in the second round using an independent set of internal primers that flank exons 9. Round II PCR product was electrophoretically separated and the DNA bands obtained corresponded to a longer inhibitory domain containing Pax5 amplicon of size 268 nucleotides and another amplicon of size 182 nucleotides (see figure 3.5).

Subsequent cloning and sequencing of both amplicons revealed that the longer amplicon (clone# E9500a2_T3_407321) contains complete exon 9 and the shorter amplicon (clone# E9180a3_R-2_497320) was an alternatively spliced transcript of Pax5 that lacked exon 9 (Pax5/Δ9a). A Pustel DNA matrix alignment of the Pax5/Δ9a clone with the published Pax5 cDNA sequence showed homology of exons 8 and 10, with an 86 nucleotide gap between nucleotides 932 to 1018 (See alignment 5), corresponding to a deletion of exon 9.

An independent clone (clone # A9_T3_504026) was sequenced from AK day 0 and Spleen day 0 cDNA to confirm the identity of alternative spliced Pax5 with deleted exon 9 [Pax5 Δ9a] as listed in table 3.1.

Two other clones were sequenced that used alternative splice donor and acceptor sites, and were different from those at the exon 8/9 and exon 9/10 junctions. The first cryptically spliced clone (A10_T3_504027) uses a 5` splice donor site at nucleotide 920 inside exon 8 and 3` splice acceptor site at the classical exon 9/10 boundary (see alignment 6). This clone was called Pax5 Δ9b and has a truncation off an additional 11 nucleotides from the TD coding region.

The other cryptically spliced clone (clone # D02_E56D56_T3_497305) uses a 5` splice donor site at nucleotide 920 in exon 8 and a 3` splice acceptor site at nucleotide 1072 inside exon 10 (see alignment 6). This clone was called Pax5 Δ9c and has a deletion of an additional 11 nucleotides from the TD coding region and 54 nucleotides from the ID coding region.

2 more independent clones of Pax5 Δ9b and 9 more independent clones of Pax5 Δ9c (clone IDs: G01_E56D56_R-2_497305, E02_E56D5x_T3_497306, D02_E9100a5_R-2_497314, E02_E9100a7_R-2_497315 and G02_E9160a1_R-2_497317) were sequenced from AK day 0, day 7 and Spleen day 0 cDNA to confirm the identity of cryptically spliced transcripts Pax5 Δ9b and Pax5 Δ9c. All clones are listed in table 3.1.

In summary, six alternatively spliced transcripts of O. mykiss Pax5 were isolated from several immune tissues with deletions of regions that encode the paired domain, trans-activation domain and inhibitory domain. A schematic of all the alternatively spliced transcripts and the position of their deletions can be found in figure 3.18.

3.4 Summary of cloned alternatively spliced Pax5 transcripts:

In total, six novel alternatively spliced transcripts of trout Pax5 were cloned and sequenced in this study (figure 3.6).

Figure 3.6

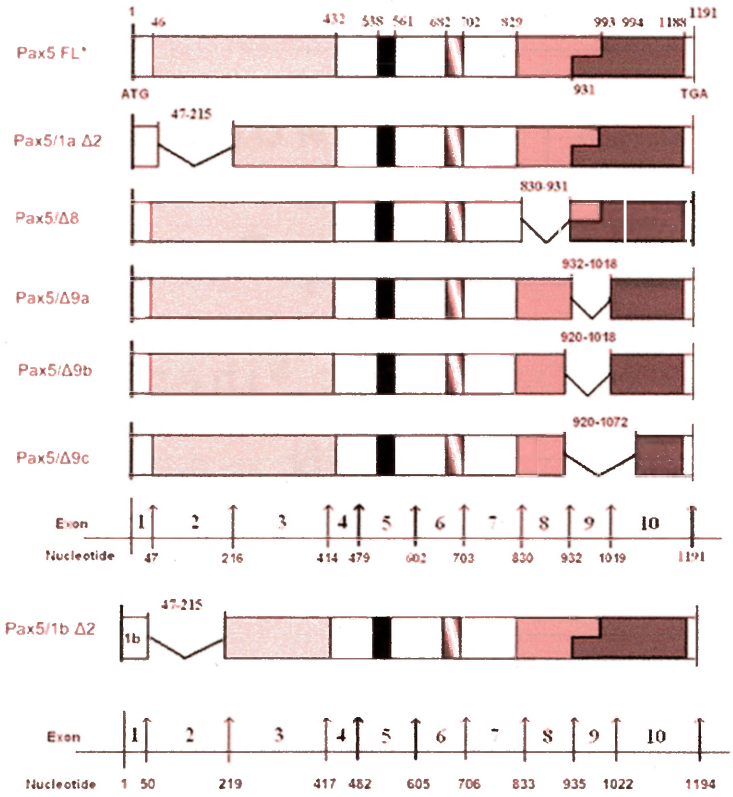


Figure 3.6 Summary of alternatively spliced transcripts of trout Pax5 cloned in this study. The panel schematically shows cDNA transcripts of the six common alternatively splice variants of O. mykiss Pax5. The full-length form of Pax5 contains 10 exons and spans over 1191 nucleotides (with exon 1a). Nucleotides 46-432 encode a DNA binding paired domain (light grey), nucleotides 538-561 encode the octopeptide segment (black), nucleotides 682-702 encode the partial homeodomain (striated box), nucleotides 829-930 and 829-993 encode the minimal and maximal trans-activation domains (medium grey), nucleotides 931- 1188 and 994 to 1188 encode the maximal and minimal inhibitory domains (dark grey). Deletion of an exon on the alternatively spliced transcripts results in the truncation of a respective functional domain encoding region. The lower panel shows an alternatively spliced transcript of Pax5 with mutually exclusive use of exon 1b and deletion of exon 2.

3.5 Relative ratio of alternatively spliced Pax5 isoforms to full length* Pax5 in the Spleen

Previous studies (Zwollo et al, 2008) suggest that O. mykiss spleen lacks developing B cells and houses mostly resting, mature B cells. The same study showed that LPS activation of splenic cells shows a high capacity of producing plasma cells. This suggests that the spleen might serve as a secondary immune organ and during infections produces a diverse population of terminally differentiating B cells. In our study, the relative abundance of each Pax5 isoform was estimated in splenic B cells using a semi-quantitative RT-PCR approach. Freshly collected splenic B cells were cultured in the presence of the B cell mitogen lipopolysaccharide (LPS) and collected from days 0 to 7 for total RNA extraction and cDNA synthesis. LPS polyclonal activated B cells by cross-linking the IgM receptor on B cells and inducing activation of B cells.

Sense primers were designed that flank the exon-exon boundary of full length* or alternatively spliced Pax5, allowing for the individual amplification of each transcript. A standard RT-PCR reaction was performed using both sets of sense primers and a common antisense primer. The relative ratio of the alternatively spliced Pax5 isoform (with a deleted exon) to full length* Pax5 isoform (in which the alternatively spliced exon is retained in the transcript) was obtained semi-quantitatively. A similar approach was also used to estimate the relative ratio of secreted to membrane IgM and IgT across 7 days of activation with LPS. The comparison of both data sets were used to study the

change in relative amounts of alternative spliced to full length isoform during B cell activation.

3.5.1 Relative ratio of secreted to membrane IgM in the spleen:

As described above, secreted and membrane IgM transcripts were amplified in triplicate RT-PCR reactions using spleen cDNA from LPS activated cells between Days 0 and 7. Two amplicons were obtained: 598 nucleotide amplicon corresponding to secreted IgM and 373 nucleotide amplicon corresponding to membrane IgM transcripts (figure 3.7a).

Figure 3.7a

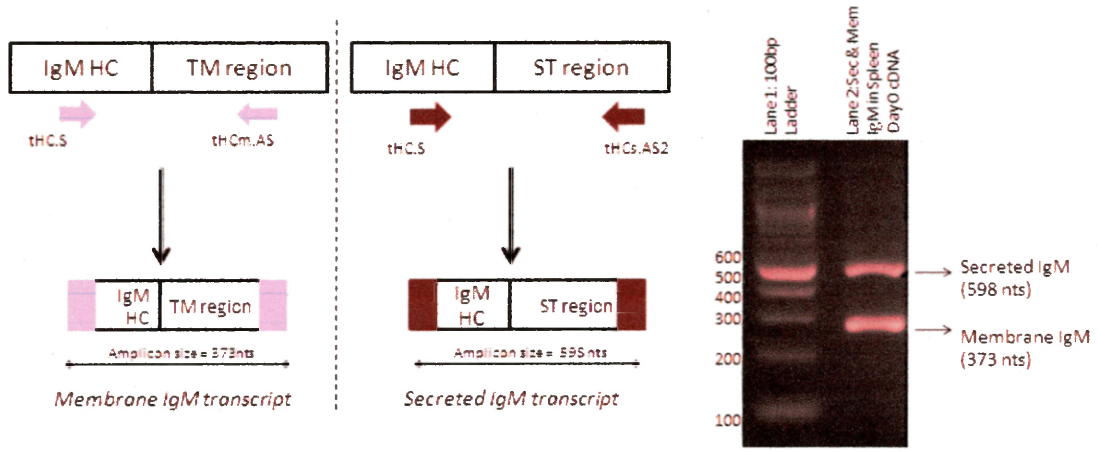


Figure 3.7b

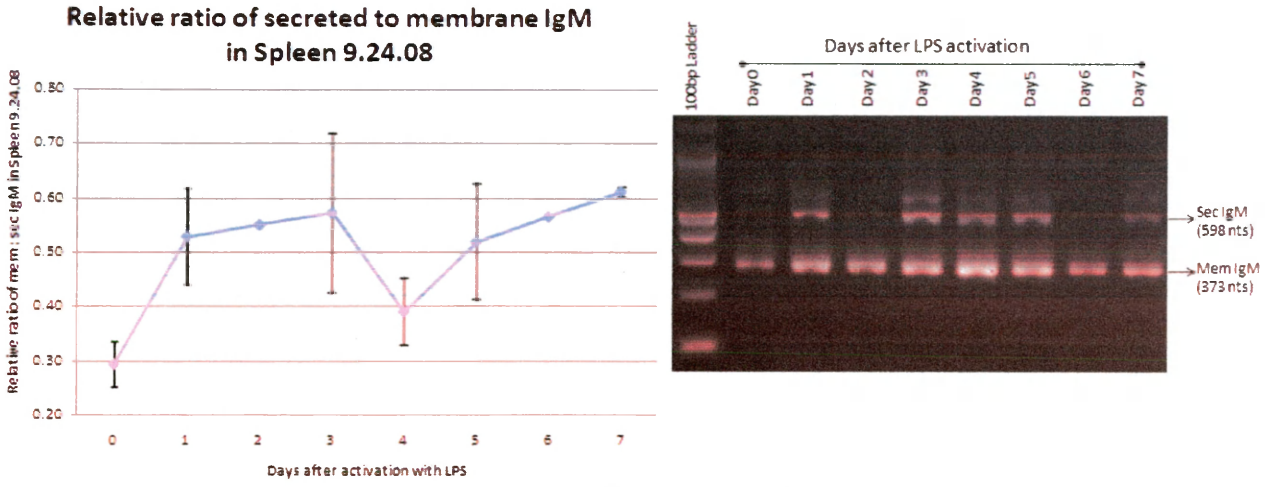


Figure 3.7 Relative ratio of secreted to membrane IgM in spleen. (a) Semi-quantitative PCR strategy to amplify secreted and membrane IgM transcripts using a common sense primer and antisense primers that recognize either the trans-membrane (TM) segment coding region or the secretory tail (ST) coding region. The product is electrophoretically separated on a 2% agarose gel (0.1µg/ml Ethidium bromide). Two amplicons of sizes 598 nucleotides and 373 nucleotides are obtained corresponding to secreted IgM and membrane IgM transcripts respectively. (b) The left panel shows the relative ratio of secreted to membrane IgM transcript in cDNA from splenic cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows electrophoretically separated secreted and membrane IgM transcripts on a 2% agarose gel; lane 1 = 0.5µg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from splenic cells that have been LPS activated from 0-7 days respectively.

At day 0 the ratio of secreted to membrane IgM is less than 1, indicating that there is greater membrane IgM transcript amplification than secreted IgM transcript amplification in freshly isolated splenic B cells (figure 3.7b). Upon activation with LPS, the relative ratio of secreted to membrane IgM increases between Day 0 and Day 1; with a small overall increase from days 1 through 7. This is consistent with known effects of LPS activation in trout B cells, which shifts IgM transcript levels from membrane to secreted IgM in terminally differentiating B cells. However, it is interesting

to note that the relative ratio of secreted to membrane IgM does not exceed 1, indicative of a fairly high level of IgM in the cells.

3.5.2 Relative ratio of secreted to membrane IgT in the spleen:

The immunoglobulin molecule IgT has been discovered quite recently and is reported to be unique to the teleost B cell lineage (Hansen et al, 2005). We sought to use IgT expression as a comparative method of studying the expression of alternatively spliced isoforms of Pax5 during B cell activation. As previously described for IgM expression study, secreted and membrane IgT transcripts were amplified in triplicate RT-PCR reactions using spleen cDNA from LPS activated cells between day 0 (freshly isolated) and day 7. Two amplicons were obtained: 451 nucleotide amplicon corresponding to secreted IgT and 271 nucleotide sized amplicon corresponding to membrane IgT (see figure 3.8a).

At day 0 (no LPS activation), the relative ratio of secreted to membrane IgT is marginally greater than 1, indicating that both transcripts are almost equally amplified in freshly isolated splenic B cells (see figure 3.8b). Upon LPS activation, the relative ratio of secreted to membrane IgT increases and is highest between days 6 and 7. It is interesting to note that the relative ratio of secreted to membrane IgT drops to around 1 at day 5 of LPS activation. Although the reason for this is unknown, possible explanations have been offered in the discussion section.

Figure 3.8 (a)

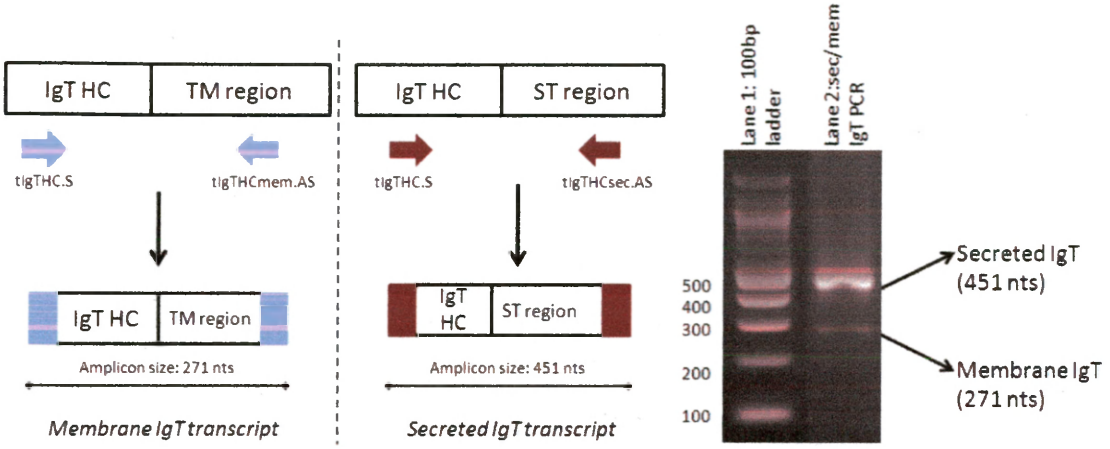


Figure 3.8(b)

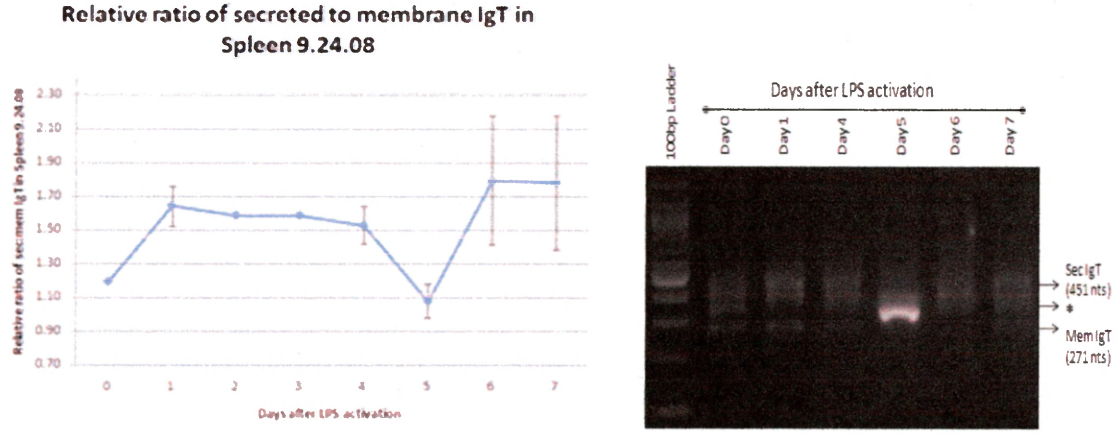


Figure 3.8 Relative ratio of secreted to membrane IgT in spleen. (a) Semi-quantitative PCR strategy to amplify secreted and membrane IgT transcripts using a common sense primer and antisense primers that recognize either the trans-membrane (TM) segment coding region or the secretory tail (ST) coding region. The product is electrophoretically separated on a 2% agarose gel (0.1µg/ml Ethidium bromide). Lane 1 contains 0.5µg of 100bp ladder (New England Biotech), Lane 2 contains 10µl of IgT in blood day 4 cDNA. Two amplicons of sizes approx 451 nucleotides and 271 nucleotides are obtained corresponding to secreted IgT and membrane IgT respectively. (b) The left panel shows the relative ratio of secreted to membrane IgT transcript in cDNA from splenic cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows electrophoretically separated secreted and membrane IgT transcripts on a 2% agarose gel; lane 1 = 0.5µg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from splenic cells that have been LPS activated for days 0, 1, 4, 5, 6 and 7 respectively. An asterisk symbol denotes

unidentified amplicons, approx 350 nts in size which are not specific to IgT and have been excluded from the analysis. Further sequence analysis is required to determine the identity of these amplicons.

3.5.3 Relative ratio of Pax5/1aΔ2 to full-length* Pax5 in the spleen:

Pax5/1aΔ2 is an alternatively spliced isoform of Pax5 that lacks the paired domain coding region exon 2 and contains the alternative exon 1a. In order to study the expression of this alternatively spliced transcript relative to full-length* Pax5 in the spleen, triplicate RT-PCR reactions were performed using cDNA from splenic B cells that had been activated with LPS in culture between 0 and 7 days. Two amplicons were obtained of sizes 399 nucleotides and 230 nucleotides, corresponding to full-length*Pax5 and Pax5/1aΔ2 respectively (See figure 3.9a).

Figure 3.9 (a)

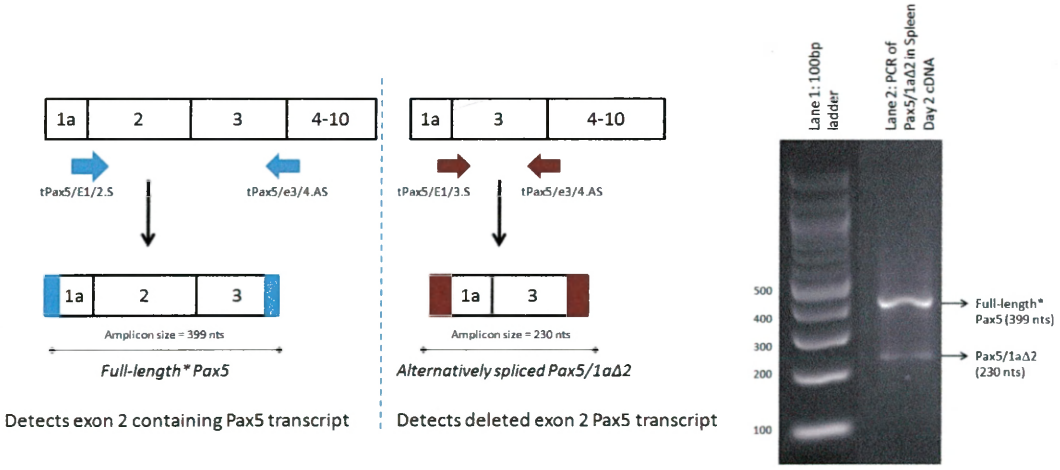


Figure 3.9 (b)

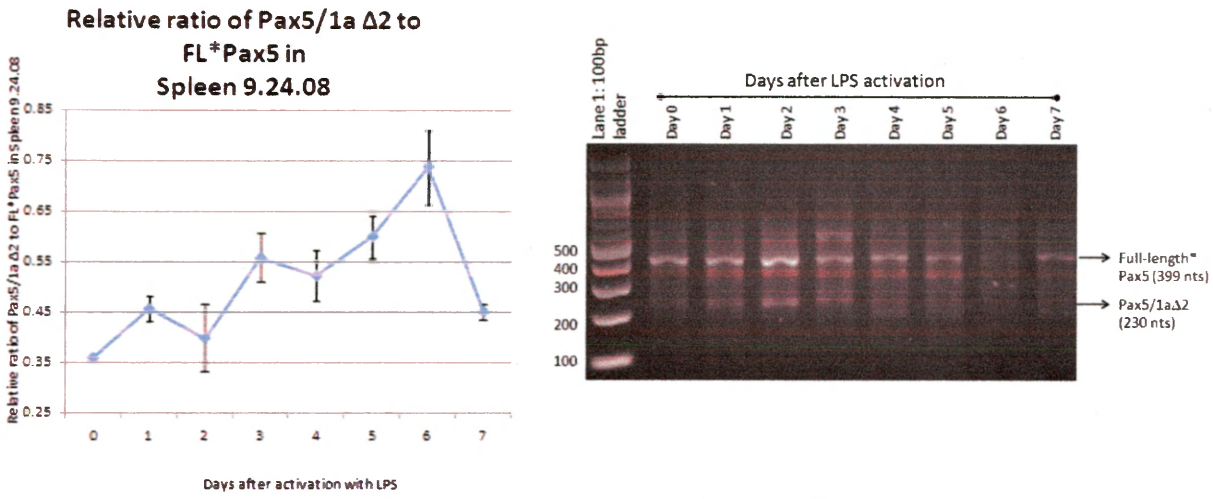


Figure 3.9 Relative ratio of Pax5/1aΔ2 to FL*Pax5 in the spleen. (a) Semi-quantitative PCR strategy to amplify Pax5/1aΔ2 and FL*Pax5 transcripts using a common antisense primer and sense primers that recognize either the exon 1a-2 junction of FL*Pax5 or the exon 1a-3 junction of Pax5/1aΔ2. The product is electrophoretically separated on a 2% agarose gel (0.1µg/ml Ethidium bromide). Lane 1 contains 0.5µg of 100bp ladder (New England Biotech), Lane 2 contains 10µl of PCR product in spleen day 2 cDNA. Two amplicons of sizes approx 399 nucleotides and 230 nucleotides are obtained corresponding to FL*Pax5 and Pax5/1aΔ2 respectively. (b) The left panel shows the relative ratio of Pax5/1aΔ2 to FL*Pax5 transcripts in cDNA from splenic cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows the PCR product that has been electrophoretically separated on a 2% agarose gel; lane 1 = 0.5µg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from splenic cells that have been LPS activated for days 0-7 respectively.

At day 0 (no LPS activation), the relative ratio of Pax5/1aΔ2 to full-length* Pax5 is approximately 0.36 (less than 1), indicating that the alternatively spliced transcript is present at much lower relative levels than full-length* Pax5 (see figure 3.9b). Upon activation with LPS, there is a steady increase in the relative ratio of Pax5/1aΔ2 to full-length* Pax5 until day 6 where it is almost double that at day 0. This suggests that there is a shift towards using more of the alternative isoform Pax5/1aΔ2 during later stages of B cell activation.

3.5.4 Relative ratio of Pax5/1bΔ2 to full-length* Pax5 in the spleen:

Pax5/1bΔ2 is an alternatively spliced isoform of Pax5 that lacks the paired domain coding region exon 2 and contains the alternative exon 1b. In order to study the expression of this alternatively spliced transcript relative to full-length* Pax5 in the spleen, triplicate RT-PCR reactions were performed using cDNA from splenic B cells that had been activated with LPS in culture between 0 and 7 days. Two amplicons were obtained of sizes 330 nucleotides and 161 nucleotides, corresponding to full-length*Pax5 and Pax5/1bΔ2 respectively (See figure 3.10a).

At day 0 (no LPS activation), the relative ratio of Pax5/1bΔ2 to full-length* Pax5 is approximately 0.36 (less than 1), indicating that the alternatively spliced transcript is present at much lower amounts relative to full-length* Pax5 (figure 3.10b). Upon activation with LPS, there is an increase in the relative ratio of Pax5/1bΔ2 to full-length* Pax5 until day 3; where it almost doubles. This suggests that there is a shift towards using the alternative isoform Pax5/1bΔ2 in the initial stages of LPS activation.

Figure 3.10 (a)

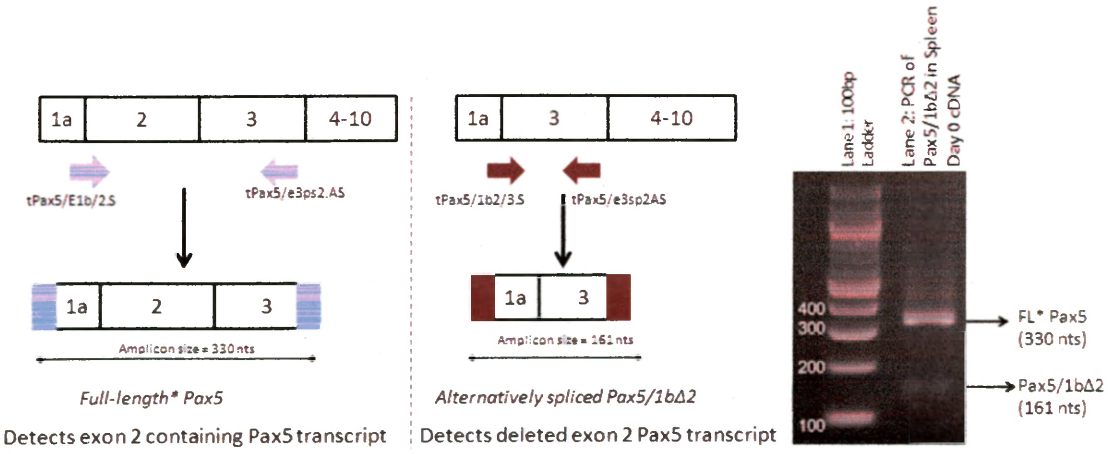


Figure 3.10 (b)

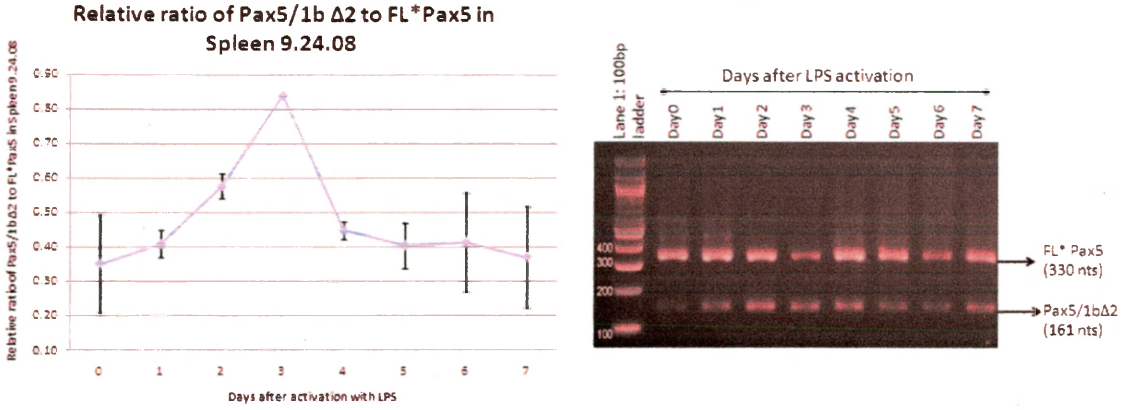


Figure 3.10 Relative ratio of Pax5/1bΔ2 to FL*Pax5 in the spleen. (a) Semi-quantitative PCR strategy to amplify Pax5/1bΔ2 and FL*Pax5 transcripts using a common antisense primer and sense primers that recognize either the exon 1b-2 junction of FL*Pax5 or the exon 1-3 junction of Pax5/1bΔ2. The product is electrophoretically separated on a 2% agarose gel (0.1µg/ml Ethidium bromide). Lane 1 contains 0.5µg of 100bp ladder (New England Biotech), Lane 2 contains 10µl of PCR product in spleen day 2 cDNA. Two amplicons of sizes approx 330 nucleotides and 161 nucleotides are obtained corresponding to FL*Pax5 and Pax5/1bΔ2 respectively. (b) The left panel shows the relative ratio of Pax5/1bΔ2 to FL*Pax5 transcripts in cDNA from splenic cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows the PCR product that has been electrophoretically separated on a 2% agarose gel; lane 1 = 0.5µg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from splenic cells that have been LPS activated for days 0-7 respectively.

At day 0 (no LPS activation), the relative ratio of Pax5/1bΔ2 to full-length* Pax5 is approximately 0.36 (less than 1), indicating that the alternatively spliced transcript is present at much lower amounts relative to full-length* Pax5 (figure 3.4b). Upon activation with LPS, there is an increase in the relative ratio of Pax5/1bΔ2 to full-length* Pax5 until day 3; where it almost doubles. This suggests that there is a shift towards using the alternative isoform Pax5/1bΔ2 in the initial stages of LPS activation.

3.5.5 Relative ratio of Pax5/Δ8 to full-length* Pax5 in the spleen:

Pax5/Δ8 is an alternatively spliced isoform of Pax5 that lacks a part of the trans-activation domain encoded by exon 8. In order to study the expression of this isoform changes relative to full-length* Pax5 in the spleen, triplicate RT-PCR reactions were performed using cDNA from splenic B cells that had been activated with LPS in culture between 0 and 7 days. Two amplicons were obtained of sizes 228 nucleotides and 126 nucleotides, corresponding to full-length*Pax5 and Pax5/Δ8 respectively (See figure 3.11a).

At Day 0 (no LPS activation) the relative ratio of Pax5/Δ8 to full-length* Pax5 is approximately 0.37 (less than 1), indicating that the alternatively spliced transcript is present at much lower amounts relative to full-length* Pax5 (figure 3.11b). Upon activation with LPS, the relative ratio of Pax5/Δ8 to full-length* Pax5 increases gradually and is highest around day 6. This may suggest a role for Pax5/Δ8 during later stages of B cell activation.

Figure 3.11a

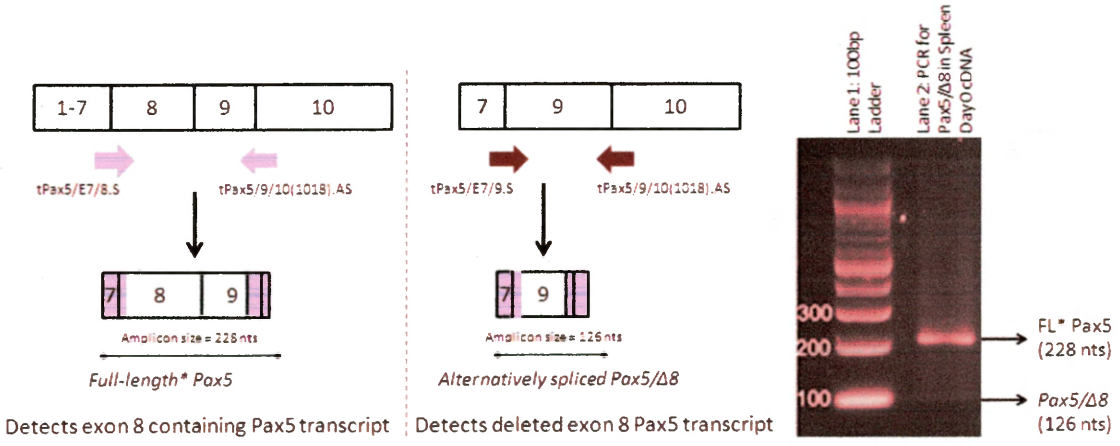


Figure 3.11b

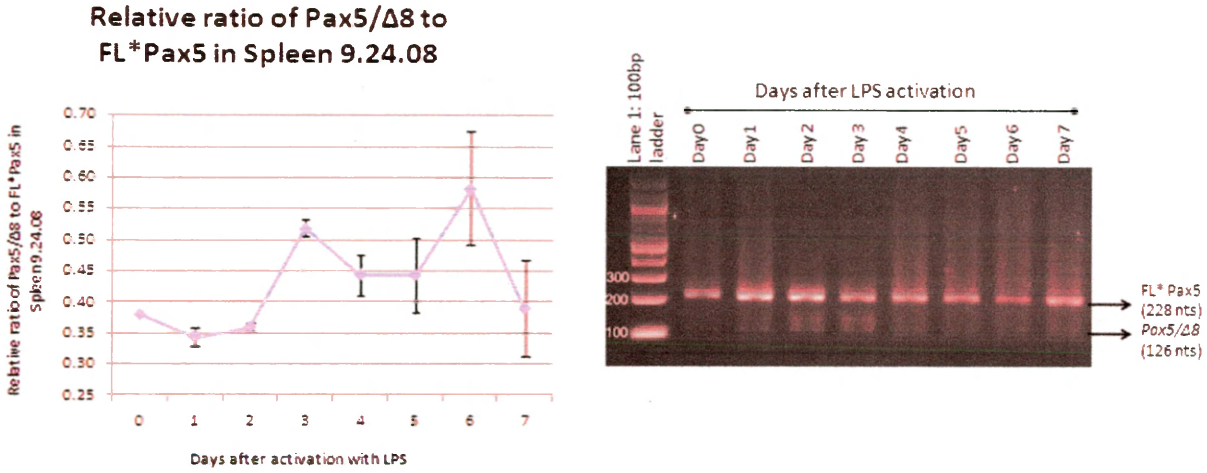


Figure 3.11 Relative ratio of Pax5/Δ8 to FL*Pax5 in the spleen. (a) Semi-quantitative PCR strategy to amplify Pax5/Δ8 and FL*Pax5 transcripts using a common antisense primer and sense primers that recognize either the exon 7-8 junction of FL*Pax5 or the exon 7-9 junction of Pax5/1bΔ2. The product is electrophoretically separated on a 2% agarose gel (0.1μg/ml Ethidium bromide). Lane 1 contains 0.5μg of 100bp ladder (New England Biotech), Lane 2 contains 10μl of PCR product in spleen day 0 cDNA. Two amplicons of sizes approx 228 nucleotides and 126 nucleotides are obtained corresponding to FL*Pax5 and Pax5/1bΔ2 respectively. (b) The left panel shows the relative ratio of Pax5/Δ8 to FL*Pax5 transcripts in cDNA from splenic cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows the PCR product that has been electrophoretically separated on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from splenic cells that have been LPS activated for days 0-7 respectively.

3.5.6 Relative ratio of Pax5/Δ9a, Pax5/Δ9b and Pax5/Δ9c to full length* Pax5 in the spleen:

The alternatively spliced transcripts of Pax5 that lack complete or part of exon 9 encode Pax5 protein isoforms with truncated C-terminal inhibitory domains. In order to study the expression of these isoform changes relative to full-length* Pax5 in the spleen, triplicate RT-PCR reactions were performed using cDNA from splenic B cells that have

been activated with LPS in culture between 0 and 7. A PCR for the relative ratio of Pax5/Δ9a to full-length* Pax5 should yield two amplicons of sizes 269 nucleotides and 183 nucleotides, corresponding to full-length*Pax5 and Pax5/Δ9a respectively (See figure 3.12a).

Only 1 amplicon of size 269 nucleotides was detected corresponding to full-length*Pax5, where the amplicon corresponding to Pax5/Δ9a was undetectable for most days of LPS activation (See figure 3.12b). This suggests that the alternatively spliced transcripts of Pax5 with deletion of complete exon 9 occur in minute relative quantities when compared to the full-length* Pax5 transcript.

Figure 3.12a

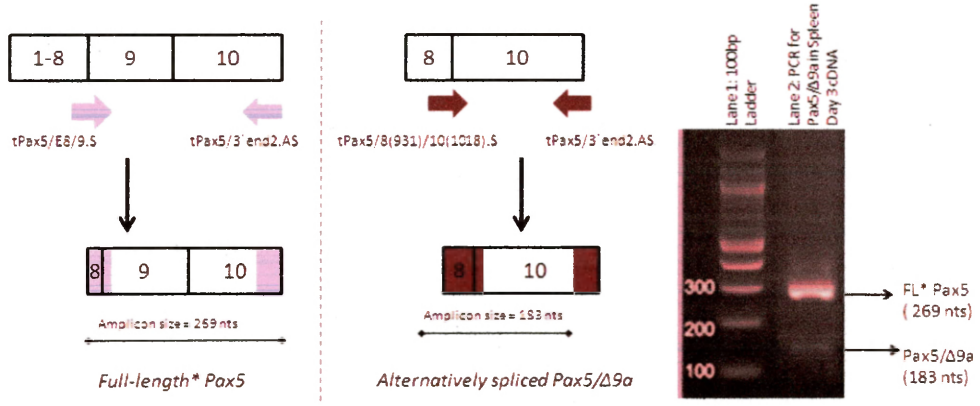


Figure 3.12b

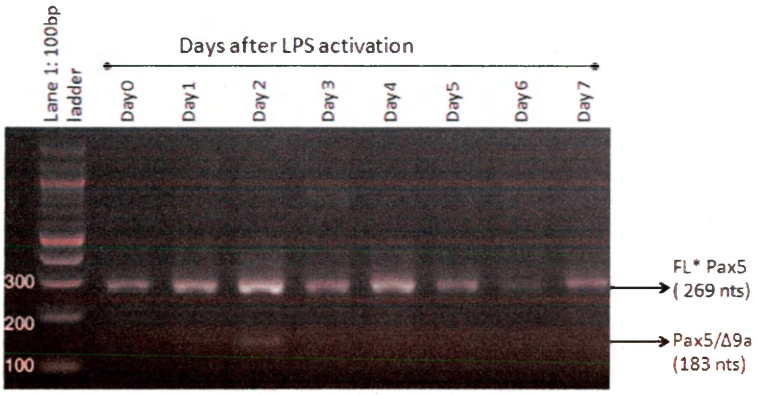


Figure 3.12 Relative ratio of Pax5/Δ9a to FL*Pax5 in the spleen. (a) Semi-quantitative PCR strategy to amplify Pax5/Δ9a and FL*Pax5 transcripts using a common antisense primer in exon 10 and sense primers that recognize either the exon 8-9 junction for FL*Pax5 or the exon 8-10 junction for Pax5/Δ9a transcripts. The product was electrophoretically separated on a 2% agarose gel (0.1μg/ml Ethidium bromide). Lane 1 contains 0.5μg of 100bp ladder (New England Biotech), Lane 2 contains 10μl of PCR product in spleen day 3 cDNA. Two amplicons of sizes approx 269 nucleotides and 183 nucleotides are obtained corresponding to FL*Pax5 and Pax5/Δ9a respectively. (b) The left panel shows the relative ratio of Pax5/Δ9a to FL*Pax5 transcripts in cDNA from splenic cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows the PCR product that has been electrophoretically separated on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from splenic cells that have been LPS activated for days 0-7 respectively.

The same approach was used to measure the relative ratio of Pax5/Δ9b or Pax5/Δ9c vs. full-length* Pax5, using sense primers that recognize the exon 8-10 junctions unique to each alternatively spliced transcript. In both cases, amplification of alternatively spliced transcript was not detected (data not shown).

3.5.7 Summary of the relative ratio of alternatively spliced Pax5 transcripts to full-length Pax5 in the Spleen:

Figure 3.13 outlines the different relative amplification of each alternatively spliced Pax5 transcript with respect to their full-length counterparts during LPS activation of splenic B cells. During activation, both IgM and IgT shift toward an increase in relative secreted transcript, suggesting that splenic B cells are responsive to LPS treatment which is in agreement with previous studies (Zwollo et al, 2008). This increase in relative secreted Ig levels can be correlated with an increase in alternative isoform expression during LPS activation as can be observed in the case of Pax5/1aΔ2, Pax5/1bΔ2 and Pax5/Δ8 for the spleen. This correlation suggests a link between the expression of alternatively spliced isoforms during splenic B cell activation, and will discussed in later sections.

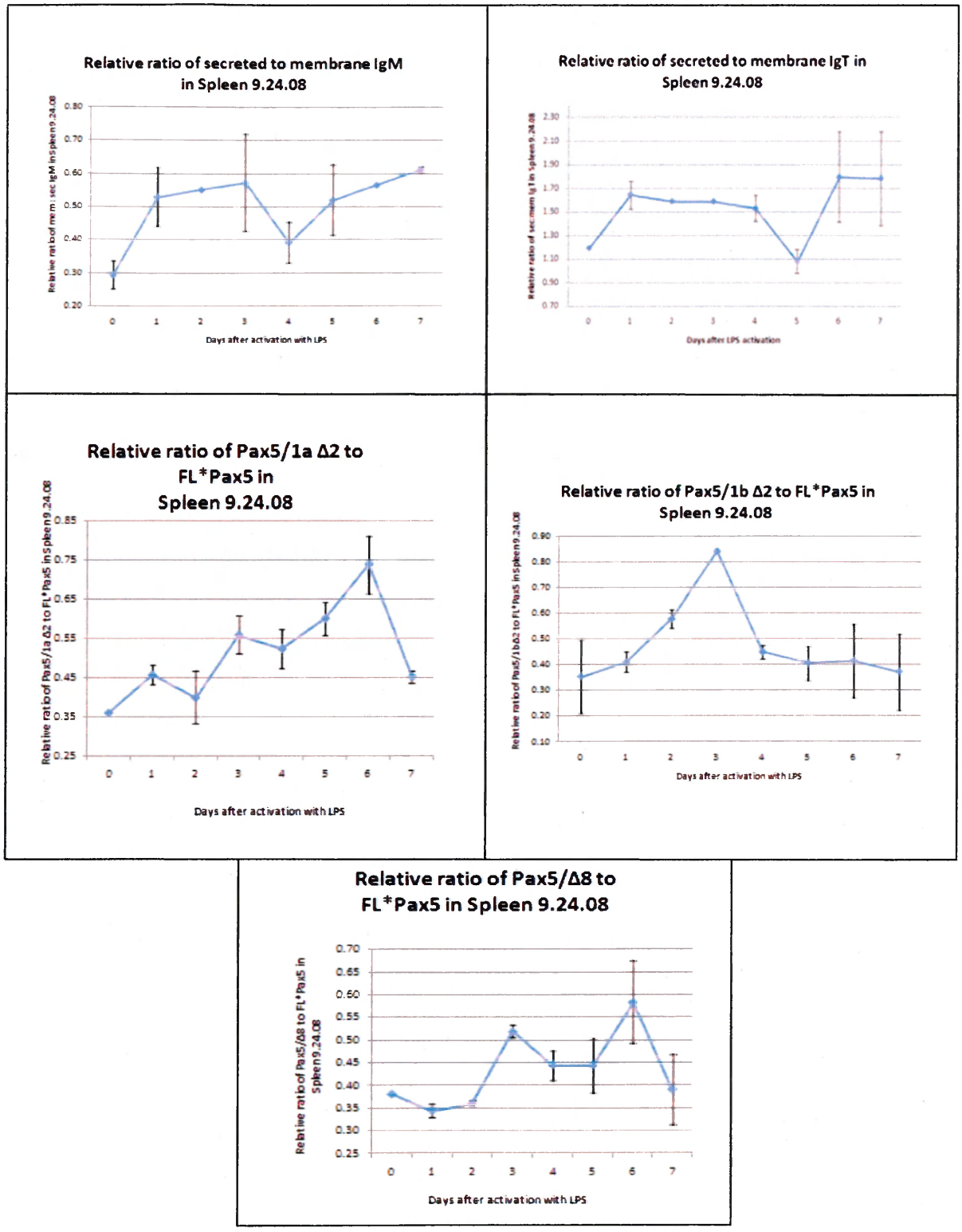


Figure 3.13 Summary of the relative ratio of alternatively spliced Pax5 transcripts to full-length Pax5 in the spleen.

3.6 Relative ratio of alternatively spliced Pax5 isoforms to full-length* Pax5 in the blood

Previous studies in O. mykiss have demonstrated that the blood contains populations of resting/non-Ig secreting B cells and lacks terminally differentiated B cells or plasma cells (Zwollo et al, 2008). Even with LPS activation the blood had a lower propensity of generating activated B cells, plasmablasts or plasma cells. In our study, the relative abundance of each Pax5 isoform was estimated in the blood using a semi-quantitative RT-PCR approach. Freshly collected blood-derived B cells were cultured in the presence of the B cell mitogen lipopolysaccharide (LPS) and collected from days 0 to 7 for total RNA extraction and cDNA synthesis. LPS polyclonal activated B cells by cross-linking the IgM receptor on B cells and inducing activation of B cells.

Sense primers were designed that span the exon-exon boundary of full length* or alternatively spliced Pax5, allowing for the individual amplification of each transcript. A standard RT-PCR reaction was performed using both sets of sense primers and a common antisense primer. The relative ratio of the alternatively spliced Pax5 isoform (with a deleted exon) to full length* Pax5 isoform (in which the alternatively spliced exon is retained in the transcript) was obtained semi-quantitatively. A similar approach was also used to estimate the relative ratio of secreted to membrane IgM and IgT across 7 days of activation with LPS. The comparison of both data sets were used to study the change in relative amounts of alternative spliced to full length isoform during B cell activation.

3.6.1 Relative ratio of secreted to membrane IgM in the blood:

As described above, secreted and membrane IgM transcripts were amplified in triplicate RT-PCR reactions using cDNA from blood-derived B cell that have been activated with LPS for 7 days. Two amplicons were obtained: 598 nucleotide amplicon corresponding to secreted IgM and 373 nucleotide amplicon corresponding to membrane IgM (see figure 3.14a).

Figure 3.14 (a)

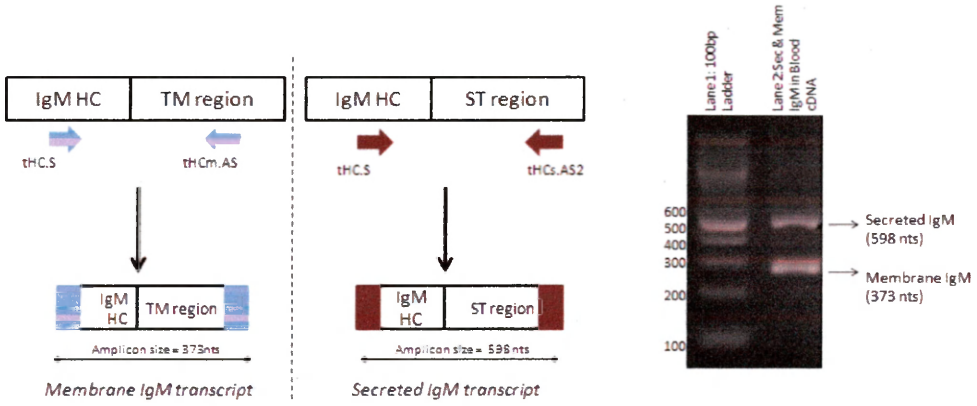


Figure 3.14 (b)

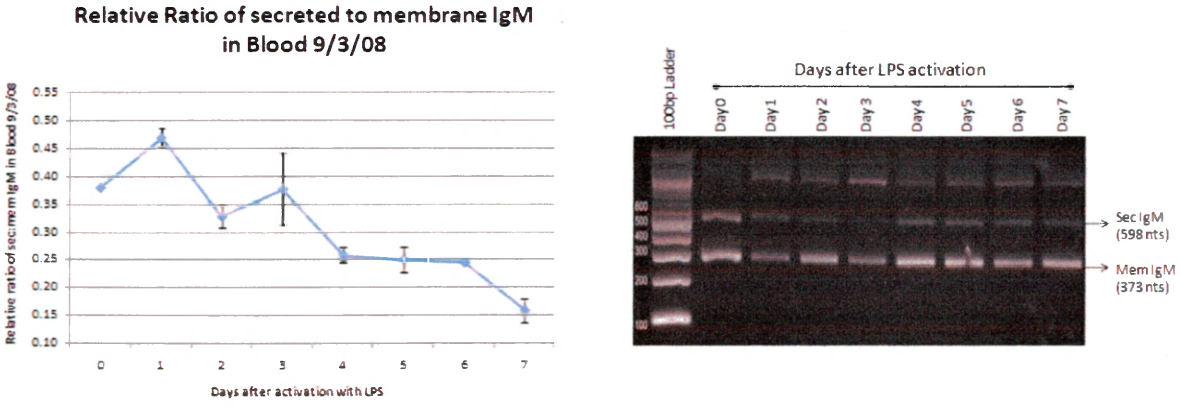


Figure 3.14 (c)

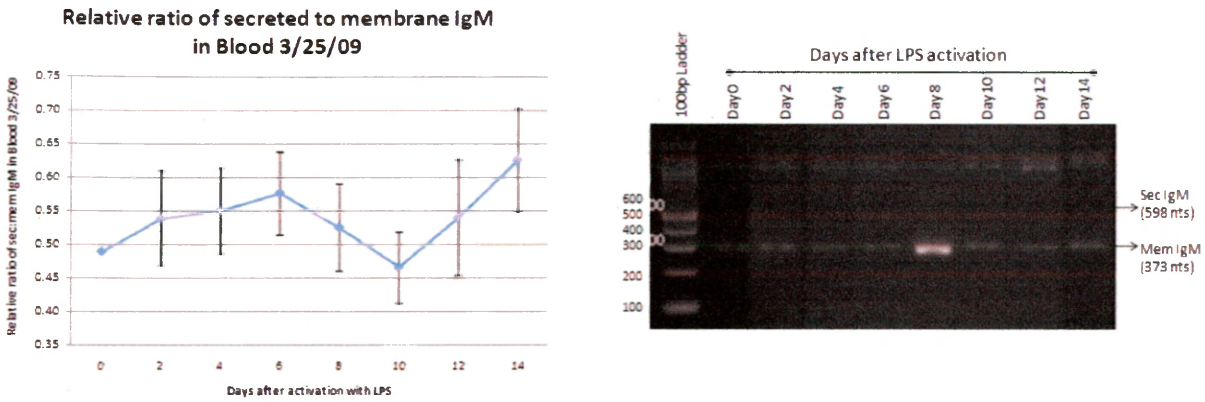


Figure 3.14 Relative ratio of secreted to membrane IgM in blood. (a) Semi-quantitative PCR strategy to amplify secreted and membrane IgM transcripts using a common sense primer and antisense primers that recognize either the trans-membrane (TM) segment coding region or the secretory tail (ST) coding region. The product is electrophoretically separated on a 2% agarose gel (0.1µg/ml Ethidium bromide). Two amplicons of sizes approx 600 nucleotides and 280 nucleotides are obtained corresponding to secreted IgM and membrane IgM respectively. (b) The left panel shows the relative ratio of secreted to membrane IgM transcript in cDNA from PBL cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows electrophoretically separated secreted and membrane IgM transcripts on a 2% agarose gel; lane 1 = 0.5µg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated from 0-7 days respectively. (c) The left panel shows the relative ratio of secreted to membrane IgM transcript in cDNA from PBL cells that have been stimulated with LPS between 0-14 days in culture. The right panel shows electrophoretically separated secreted and membrane IgM transcripts on a 2% agarose gel; lane 1 = 0.5µg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated on even days from 0,2,4,6,8,10,12,14 days respectively.

At day 0 (no LPS activation), the relative ratio of secreted to membrane IgM is approximately 0.38 (less than 1), indicating that there is greater membrane IgM transcript than secreted IgM transcript in un-activated blood B cells. Immediately after activation, the relative ratio of secreted to membrane IgM increases slightly at day 1. After day 1, the relative ratio of membrane to secreted IgM progressively reduces towards later days of LPS activation. This is consistent with the Zwollo et al (2008) study

where blood derived B cells showed low potential for generating plasma cells upon activation with LPS.

To further investigate if blood derived B cells get activated by LPS beyond the resolution of 7 days, an independent sample of blood derived B cells were activated with LPS in culture for 14 days. Using the same experimental setup, semi-quantitative RT-PCR analysis of the independent blood sample (figure 3.14c) revealed:

1) The relative ratio of secreted to membrane IgM is always less than 1 for all days of LPS activation observed.
2) The difference in the relative ratio of secreted to membrane IgM between the lowest at day 0 and the highest at day 14 is almost negligible.

This supports the hypothesis that blood derived B cells have low potential for terminal differentiation during LPS activation.

3.6.2 Relative ratio of secreted to membrane IgT in the blood

As in the splenic B cell study, we sought to use IgT expression as a comparative method of studying the expression of alternatively spliced isoforms of Pax5 during B cell activation. As previously described for IgM expression study, secreted and membrane IgT transcripts were amplified in triplicate RT-PCR reactions using spleen cDNA from LPS activated cells between days 0 and 7. Two amplicons were obtained: 451 nucleotide amplicon corresponding to secreted IgT and 271 nucleotide amplicon corresponding to membrane IgT (see figure 3.15a).

Figure 3.15 (a)

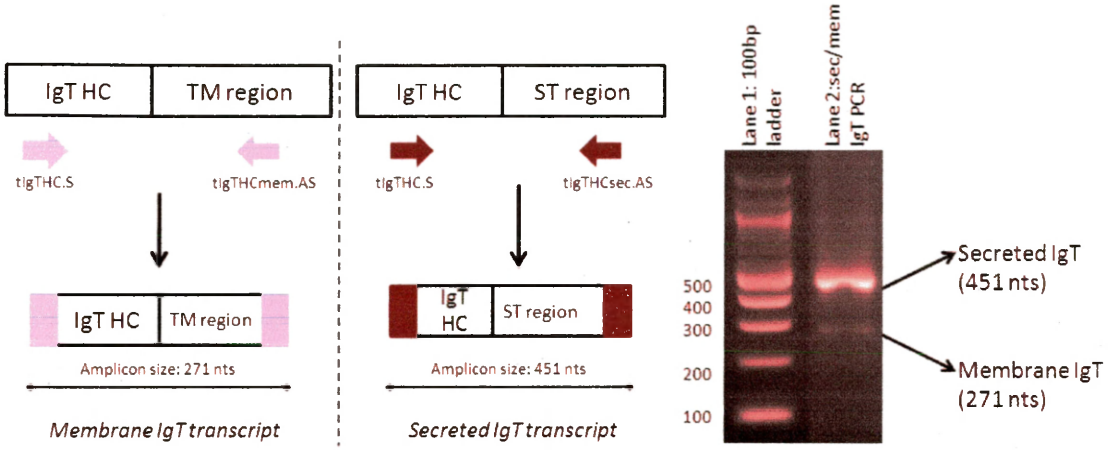


Figure 3.15 (b)

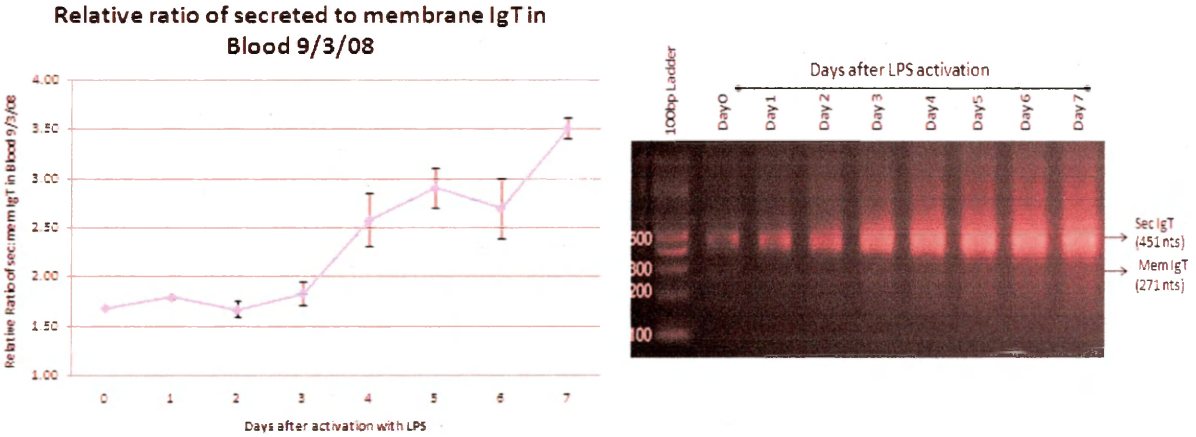


Figure 3.15 (c)

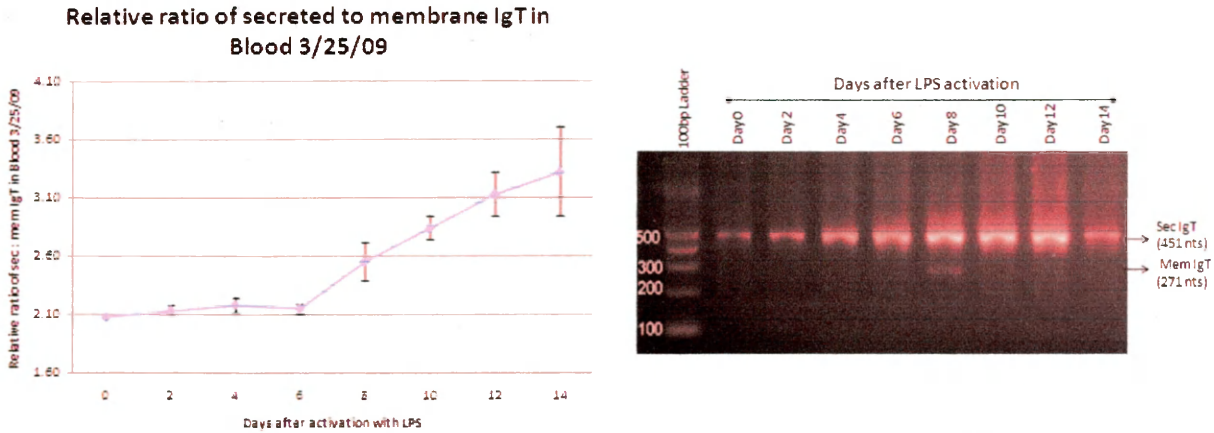


Figure 3.15 Relative ratio of secreted to membrane IgT in blood. (a) Semi-quantitative PCR strategy to amplify secreted and membrane IgT transcripts using a common sense primer and antisense primers that recognize either the trans-membrane (TM) segment coding region or the secretory tail (ST) coding region. The product is electrophoretically separated on a 2% agarose gel (0.1μg/ml Ethidium bromide). Lane 1 contains 0.5μg of 100bp ladder (New England Biotech), Lane 2,3 and 4 contains 10μl each of membrane, secreted and both membrane and secreted IgT from blood day 0 cDNA. Two amplicons of sizes approx 451 nucleotides and 271 nucleotides are obtained corresponding to secreted IgT and membrane IgT respectively. (b) The left panel shows the relative ratio of secreted to membrane IgT transcript in cDNA from blood cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows electrophoretically separated secreted and membrane IgT transcripts on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from blood cells that have been LPS activated for days 0, 1, 4, 5, 6 and 7 respectively. (c) The left panel shows the relative ratio of secreted to membrane IgT transcript in cDNA from PBL cells that have been stimulated with LPS between 0-14 days in culture. The right panel shows electrophoretically separated secreted and membrane IgT transcripts on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated on even days from 0,2,4,6,8,10,12,14 days respectively.

At day 0, the relative ratio of secreted to membrane IgT is approximately 1.69 (greater than one) indicating that the secreted transcript is present at greater levels than the membrane transcript in un-activated blood-derived B cells. Amplification of membrane IgT was detectable in very low relative levels. After activation with LPS, the relative ratio remains unchanged until day 3, but then increases rapidly to greater than 2 fold higher at day 7 (figure 3.15b).

The same trend was observed when the relative ratio of secreted to membrane IgT was obtained from an independent sample of blood derived B cells that were activated with LPS for 14 days (See figure 3.15c). The relative ratio of secreted to membrane IgT remains unchanged in the initial days of activation with LPS, but almost doubles after day 6. Once again the secreted IgT transcript seems to be present at greater levels than

the membrane IgT transcript throughout activation – the lowest relative ratio being 2.08 at day 0.

3.6.3 Relative ratio of Pax5/1aΔ2 to full-length* Pax5 in the blood:

Pax5/1aΔ2 is an alternatively spliced isoform of Pax5 that lacks the paired domain coding region exon 2 and contains the alternative exon 1a. In order to study the expression of this isoform relative to full-length* Pax5 in the blood, triplicate RT-PCR reactions were performed using cDNA from blood-derived B cells that had been activated with LPS in culture between 0 and 7 days. Two amplicons were obtained of sizes 399 nucleotides and 230 nucleotides, corresponding to full-length*Pax5 and Pax5/1aΔ2 respectively (See figure 3.16a).

Figure 3.16 (a)

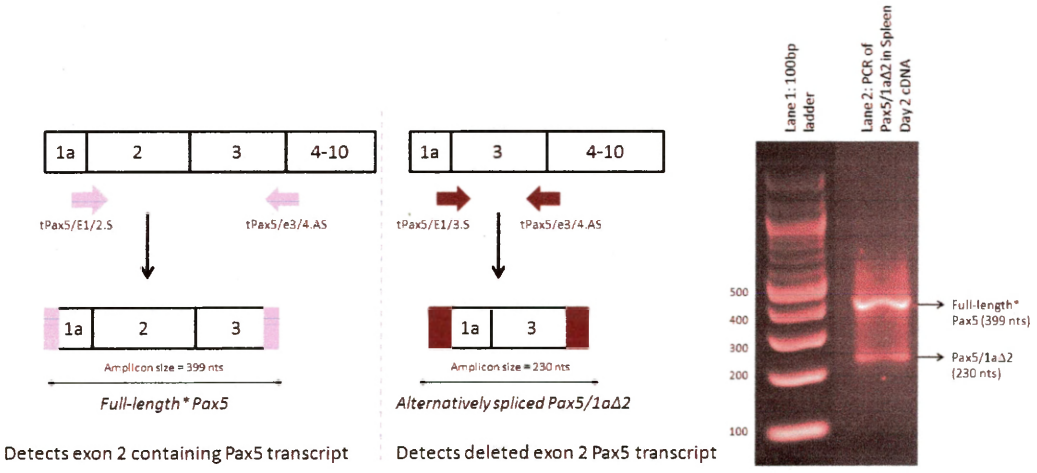


Figure 3.16 (b)

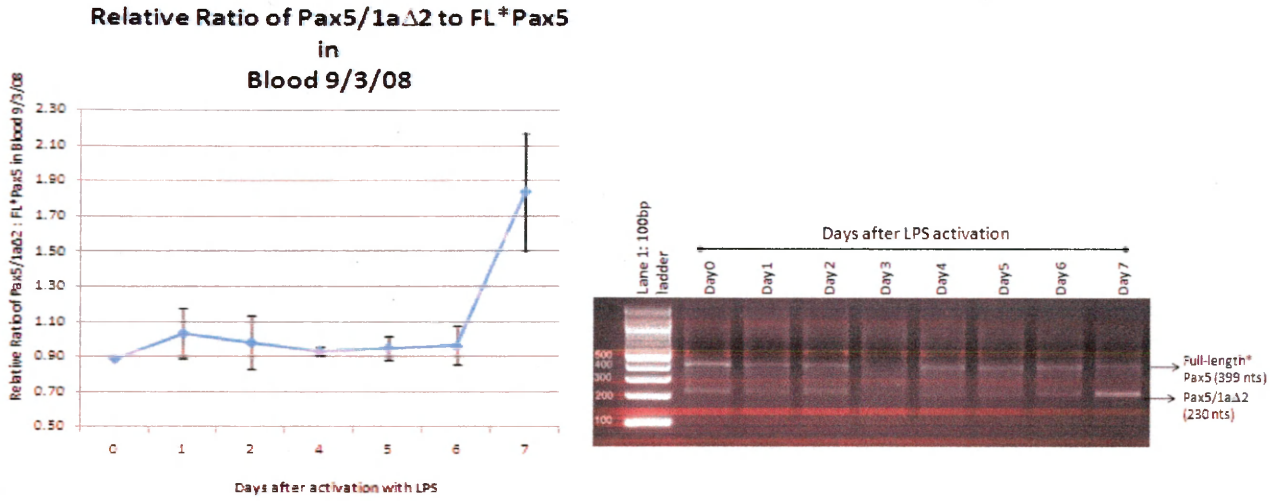


Figure 3.16 (c)

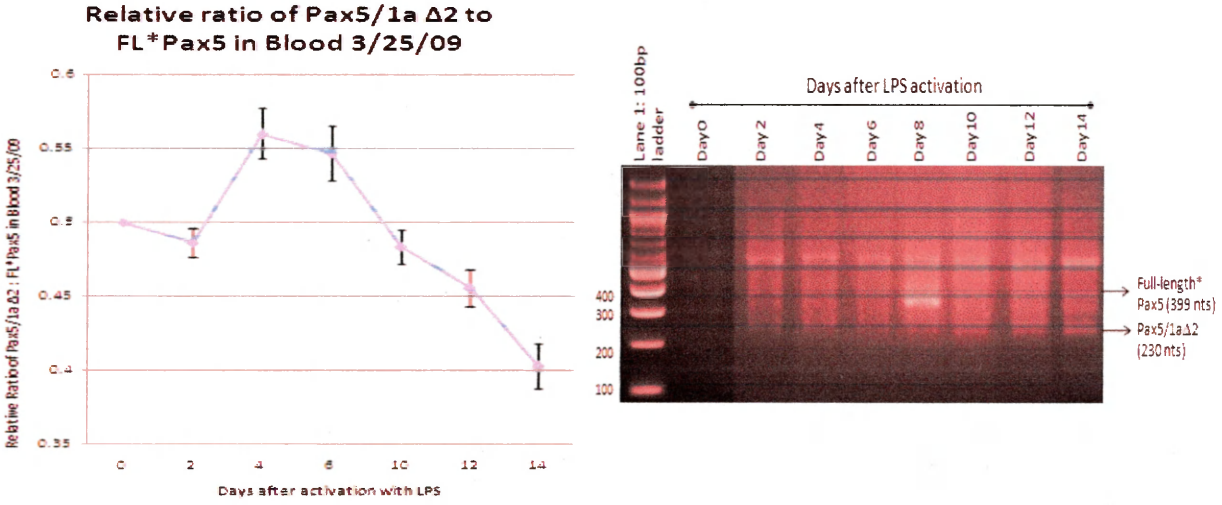


Figure 3.16 Relative ratio of Pax5/1aΔ2 to FL*Pax5 in the blood. (a) Semi-quantitative PCR strategy to amplify Pax5/1aΔ2 and FL*Pax5 transcripts using a common antisense primer and sense primers that recognize either the exon 1a-2 junction of FL*Pax5 or the exon 1a-3 junction of Pax5/1aΔ2. The product is electrophoretically separated on a 2% agarose gel (0.1µg/ml Ethidium bromide). Lane 1 contains 0.5µg of 100bp ladder (New England Biotech), Lane 2 contains 10µl of PCR product in blood day 2 cDNA. Two amplicons of sizes approx 399 nucleotides and 230 nucleotides are obtained corresponding to FL*Pax5 and Pax5/1aΔ2 respectively. (b) The left panel shows the relative ratio of Pax5/1aΔ2 to FL*Pax5 transcripts in cDNA from PBL cells that have been stimulated with LPS for 0-7 days in culture. The right

panel shows the PCR product that has been electrophoretically separated on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated for days 0-7 respectively. (c) The left panel shows the relative ratio of Pax5/1aΔ2 to FL*Pax5 transcript in cDNA from PBL cells that have been stimulated with LPS between 0-14 days in culture. The right panel shows electrophoretically separated Pax5/1aΔ2 and FL*Pax5 transcripts on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated on even days from 0,2,4,6,8,10,12,14 days respectively.

At day 0 (no LPS added), the relative ratio of Pax5/1aΔ2 to full-length* Pax5 is approximately 0.89 indicating that the full length transcript is only slightly more than the alternatively spliced isoform. Following activation with LPS, the ratio remains unchanged until after day 6 (figure 3.16b). This suggests that Pax5/1aΔ2 may have a contributing role in later stages of blood-derived B cell activation.

To test this hypothesis, we replicated this experiment in an independent sample of blood derived B cells that were activated with LPS for 14 days (See figure 3.16c). Analysis of the data from this experiment revealed a trend where although the relative ratio of Pax5/1aΔ2 to full-length* Pax5 remained unchanged during initial days of LPS activation, a downward trend forms towards later stages of activation. Also, this experiment showed almost twice as much full length* Pax5 than Pax5/1aΔ2 isoform on most days of LPS activation.

3.6.4 Relative ratio of Pax5/1bΔ2 to full-length* Pax5 in the blood:

Pax5/1bΔ2 is an alternatively spliced isoform of Pax5 that lacks the paired domain coding region exon 2 and contains the alternative exon 1b. In order to study the expression of this isoform relative to full-length* Pax5 in the blood, triplicate RT-PCR

reactions were performed using cDNA from blood-derived B cells that have been activated with LPS in culture between 0 and 7 days. Two amplicons were obtained of sizes 330 nucleotides and 161 nucleotides, corresponding to full-length*Pax5 and Pax5/1bΔ2 respectively (See figure 3.17a).

Figure 3.17 (a)

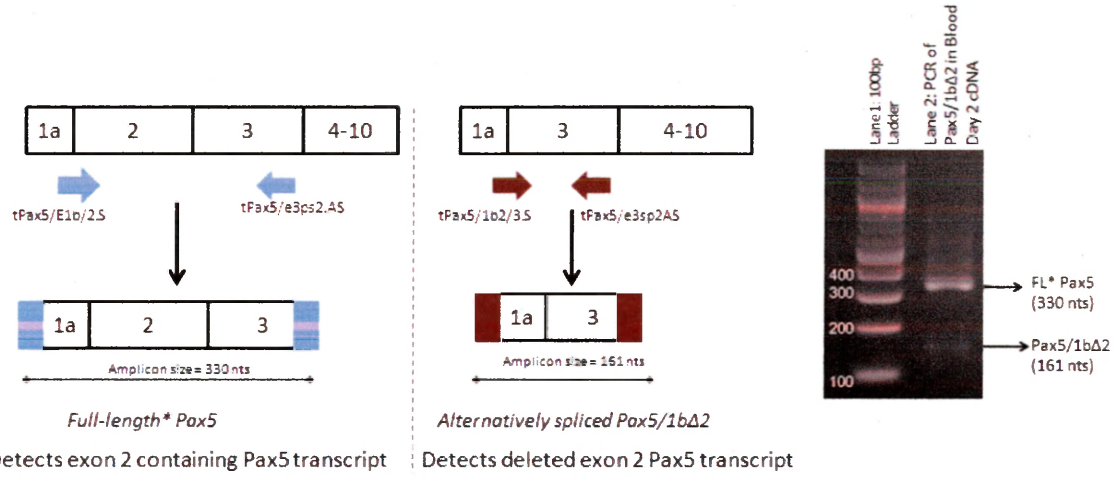


Figure 3.17 (b)

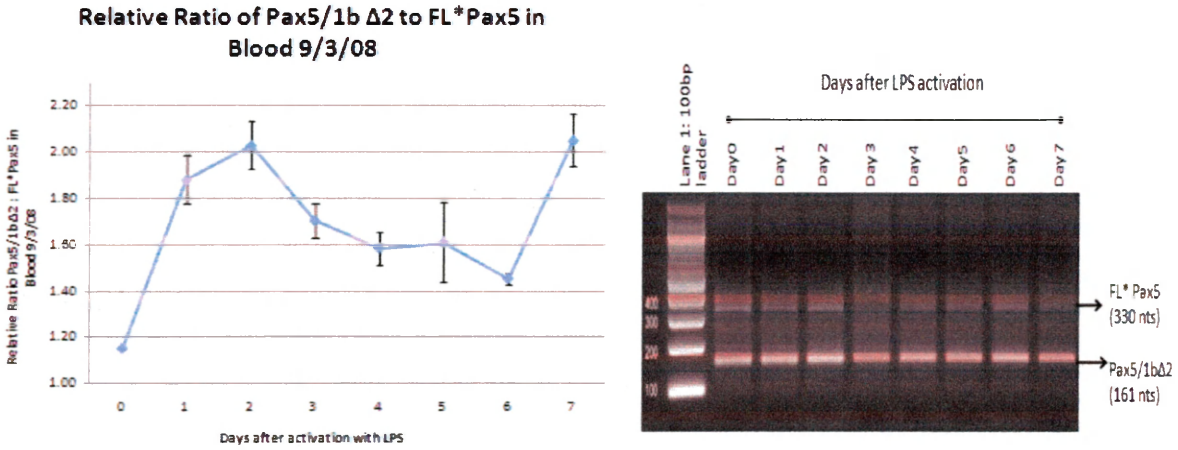


Figure 3.17 (c)

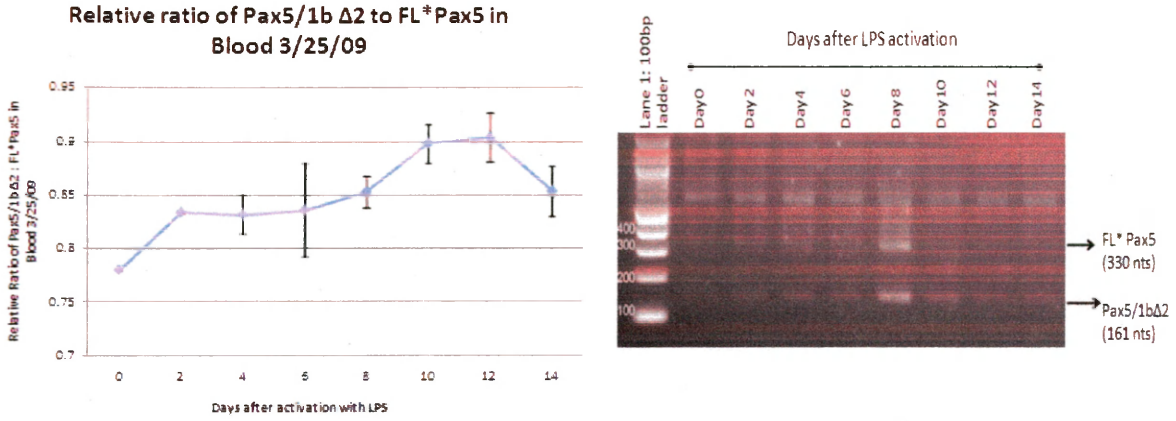


Figure 3.17 Relative ratio of Pax5/1bΔ2 to FL*Pax5 in blood (a) Semi-quantitative PCR strategy to amplify Pax5/1bΔ2 and FL*Pax5 transcripts using a common antisense primer and sense primers that recognize either the exon 1b-2 junction of FL*Pax5 or the exon 1-3 junction of Pax5/1bΔ2. The product is electrophoretically separated on a 2% agarose gel (0.1μg/ml Ethidium bromide). Lane 1 contains 0.5μg of 100bp ladder (New England Biotech), Lane 2 contains 10μl of PCR product in blood day 0 cDNA. Two amplicons of sizes approx 330 nucleotides and 161 nucleotides are obtained corresponding to FL*Pax5 and Pax5/1bΔ2 respectively. (b) The left panel shows the relative ratio of Pax5/1bΔ2 to FL*Pax5 transcripts in cDNA from PBL cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows the PCR product that has been electrophoretically separated on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated for days 0-7 respectively. (c) The left panel shows the relative ratio of Pax5/1bΔ2 to FL*Pax5 transcript in cDNA from PBL cells that have been stimulated with LPS between 0-14 days in culture. The right panel shows electrophoretically separated Pax5/1bΔ2 and FL*Pax5 transcripts on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated on even days from 0,2,4,6,8,10,12,14 days respectively.

At day 0 (no LPS added), the relative ratio of Pax5/1bΔ2 to full-length* Pax5 is approximately 1.15, indicating that the alternatively spliced isoform is present in slightly greater relative quantities than the full-length* transcript in blood-derived B cells (figure 3.17b). Following activation with LPS, there is an increase in the relative ratio of Pax5/1bΔ2 to full-length* Pax5 with highest levels at days 2 and 7.

This trend was observed again when the experiment was repeated in an independent sample of blood-derived B cells that had been activated with LPS for 14 days in culture (see figure 3.17c). The relative ratio of Pax5/1bΔ2 to full-length* Pax5 is highest around days 10 and 12 of PBL stimulation with LPS, suggesting that Pax5/1bΔ2 may participate in late stages of B cell activation.

3.6.5 Relative ratio of Pax5/Δ8 to full-length Pax5 in the blood:

Pax5/Δ8 is an alternatively spliced isoform of Pax5 that lacks a part of the trans-activation domain coding region exon 8. In order to study the expression of this isoform changes relative to full-length* Pax5 in the blood, triplicate RT-PCR reactions were performed using cDNA from blood-derived B cells that have been activated with LPS in culture between 0 and 7 days. Two amplicon were obtained of size 198 nucleotides and 98 nucleotides, corresponding to full-length* Pax5 and Pax5/Δ8. (See figure 3.18a)

Figure 3.18 (a)

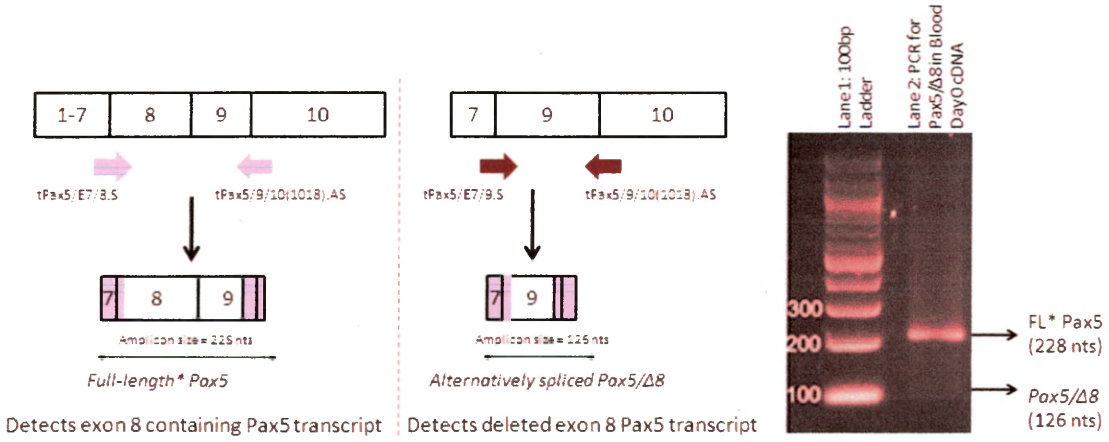


Figure 3.18 (b)

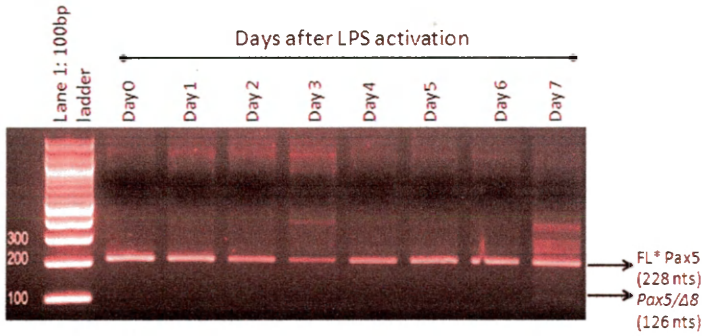


Figure 3.18 (c)

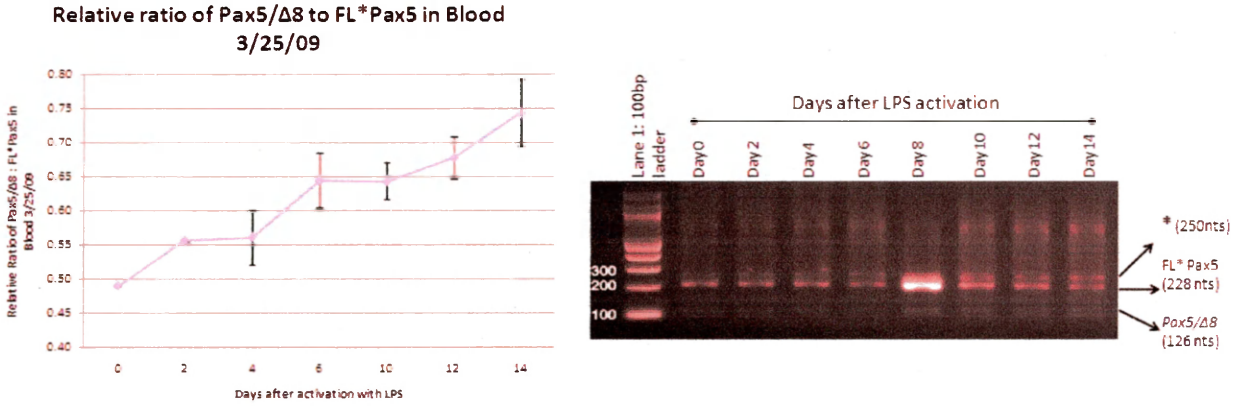


Figure 3.18 Relative ratio of Pax5/Δ8 to FL*Pax5 in the blood. (a) Semi-quantitative PCR strategy to amplify Pax5/Δ8 and FL*Pax5 transcripts using a common antisense primer and sense primers that recognize either the exon 7-8 junction of FL*Pax5 or the exon 7-9 junction of Pax5/1bΔ2. The product is electrophoretically separated on a 2% agarose gel (0.1μg/ml Ethidium bromide). Lane 1 contains 0.5μg of 100bp ladder (New England Biotech), Lane 2 contains 10μl of PCR product from PBL day 0 cDNA. Two amplicons of sizes approx 198 nucleotides and 98 nucleotides are obtained corresponding to FL*Pax5 and Pax5/1bΔ2 respectively. (b) shows a semi-quantitative PCR for Pax5/Δ8 and FL*Pax5 transcripts in cDNA from PBL; the PCR products have been electrophoretically separated on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated for days 0-7 respectively. Pax5/Δ8 transcripts were undetectable. (c) The left panel shows the relative ratio of Pax5/Δ8 to FL*Pax5 transcript in cDNA from PBL cells that have been stimulated with LPS between 0-14 days in culture. The right panel shows electrophoretically separated Pax5/Δ8 and FL*Pax5 transcripts on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL

cells that have been LPS activated on even days from 0,2,4,6,8,10,12,14 days respectively. An asterisk denotes amplified product greater than 250 nts that are not Pax5 products.

Pax5/Δ8 was undetectable in cDNA from a set of PBL that had been stimulated with LPS for 7 days in culture 9 (Figure 3.18b), but the FL*Pax5 transcript was abundant. This suggests that Pax5/Δ8 transcript is present in very low relative quantities in the blood.

In an independent set of PBL cells that have been stimulated in culture with LPS for 14 days, the Pax5/Δ8 transcript was detectable (figure 3.18c), though once again FL*Pax5 transcript were better amplified. At day 0, the relative ratio of Pax5/Δ8 to full-length* Pax5 is approximately 0.49 (less than 1), indicating that there is twice as much full length than alternatively spliced Pax5/Δ8 transcript in blood B cells. Upon activation with LPS the relative ratio gradually increases and is highest at day 14.

3.6.6 Relative ratio of Pax5/Δ9a to full-length* Pax5 in the blood:

The alternatively spliced transcripts of Pax5 that lack complete or part of exon 9 encode Pax5 protein isoforms with truncated C-terminal inhibitory domains. In order to study the expression of these isoform changes relative to full-length* Pax5 in the blood, triplicate RT-PCR reactions were performed using cDNA from blood-derived B cells that have been activated with LPS in culture between 0 and 7 days. Two amplicon of sizes 269 nucleotides and 183 nucleotides was detected corresponding to full-length*Pax5 and Pax5/Δ9a (See figure 3.19a).

Figure 3.19 (a)

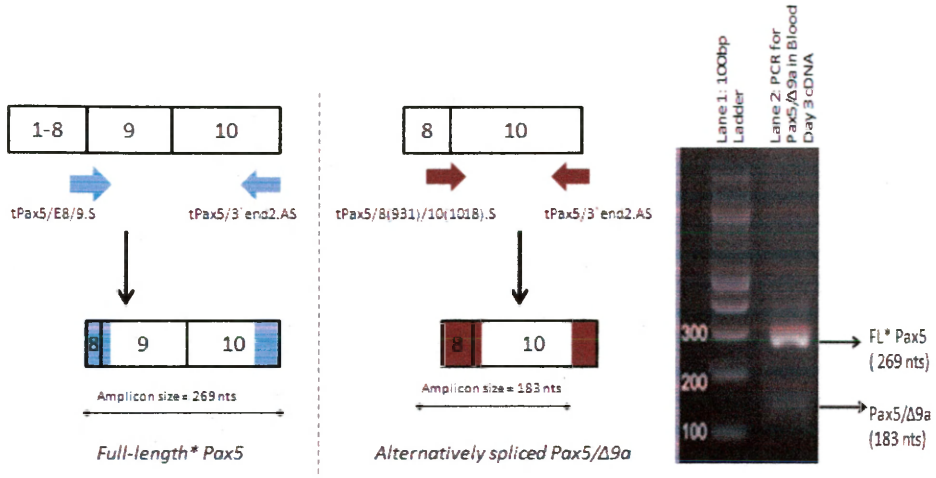


Figure 3.19 (b)

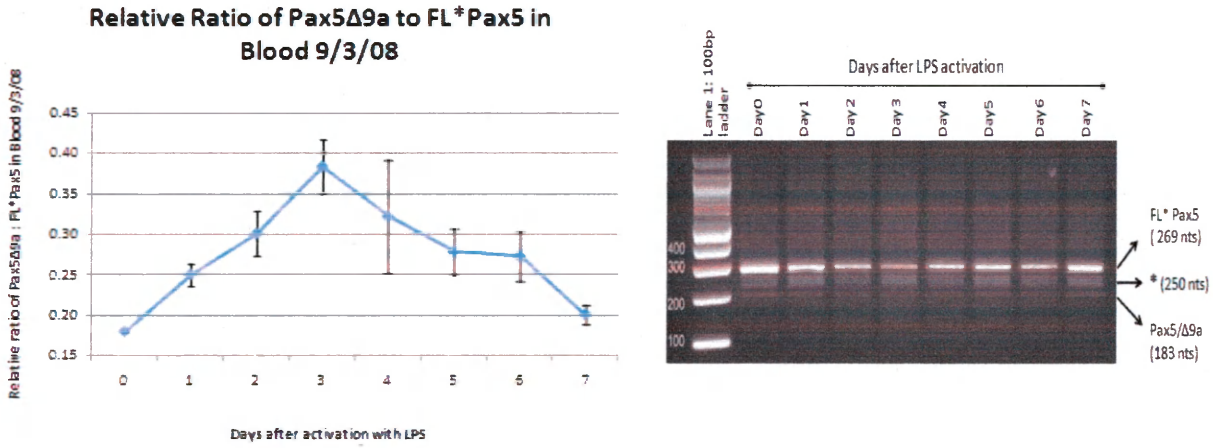


Figure 3.19 (c)

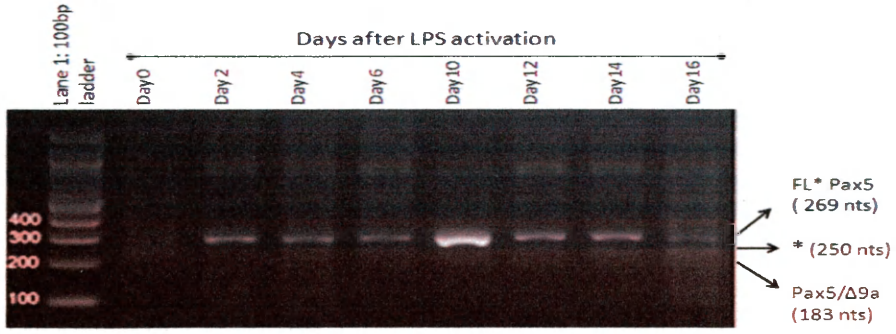


Figure 3.19 Relative ratio of Pax5/Δ9a,b or c to FL*Pax5 in the blood. (a) Semi-quantitative PCR strategy to amplify Pax5/Δ9a, 9b or 9c and FL*Pax5 transcripts using a common antisense primer and sense primers that recognize either the exon 8-9 junction of FL*Pax5 or the exon 8-10 junction of Pax5/Δ9 transcripts. The product is electrophoretically separated on a 2% agarose gel (0.1μg/ml Ethidium bromide). Lane 1 contains 0.5μg of 100bp ladder (New England Biotech), Lane 2 contains 10μl of PCR product in pbl day 3 cDNA. Two amplicons of sizes approx 269 nucleotides and 183 nucleotides are obtained corresponding to FL*Pax5 and Pax5/Δ9a respectively. (b) The left panel shows the relative ratio of Pax5/Δ9a to FL*Pax5 transcripts in cDNA from splenic cells that have been stimulated with LPS for 0-7 days in culture. The right panel shows the PCR product that has been electrophoretically separated on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from splenic cells that have been LPS activated for days 0-7 respectively. An asterisk denotes PCR product or size 240 nts that is not-Pax5. (c) The left panel shows the relative ratio of Pax5/Δ9a to FL*Pax5 transcript in cDNA from PBL cells that have been stimulated with LPS between 0-14 days in culture. The right panel shows electrophoretically separated Pax5/Δ9a and FL*Pax5 transcripts on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated on even days from 0,2,4,6,8,10,12,14 days respectively. (c) shows the electrophoretic separation of Pax5/Δ9a and FL*Pax5 transcripts on a 2% agarose gel; lane 1 = 0.5μg 100bp DNA ladder (New England Biotech), Lanes 2-9 = cDNA from PBL cells that have been LPS activated on even days from 0,2,4,6,8,10,12,14 days respectively. Pax5/Δ9a transcripts were undetectable.

At day 0 (not LPS activated), the relative ratio of Pax5/Δ9a to full-length* Pax5 is approximately 0.18 (<1) (Figure 3.19b). This suggests that the alternatively spliced transcripts with deleted exon 9 occur in very small relative quantities when compared to the full-length* Pax5 transcript in PBL cells. Upon activation, the relative ratio increases and peaks at Day 3.

In a repeat of the experiment in an independent PBL sample that had been activated with LPS for 14 days in culture, Pax5/Δ9a transcripts were undetectable while the FL*Pax5 transcripts were measurable (figure 3.19c). This suggests that Pax5/Δ9a transcripts are present in relatively lower amounts in PBL cells.

3.6.7 Relative ratio of cryptically spliced Pax5 isoforms Pax5/Δ9b and Pax5/Δ9c to full-length* Pax5 in the blood:

Semi-quantitative RT-PCR experiments performed to measure the relative ratio of cryptically spliced Pax5 isoforms (Pax5/Δ9b and Pax5/Δ9c) to full-length* Pax5 were unable to detect alternatively spliced product in both un-activated and LPS activated blood-derived B cells (data not shown). This suggests that these isoforms are expressed in minute relative quantities in the blood when compared to the full-length* Pax5 transcript.

In both independent sets of experiments on blood B cells, the relative amount of secreted IgM is lower than membrane IgM and decreases or remains unchanged during LPS activation. This is in agreement with previous reports (Zwollo et al, 2008) where blood derived B cells do not elicit a strong activation response when treated with LPS. It is interesting to note that there is a relative increase of secreted IgT with respect to membrane IgT. Reasons for different response for these Ig isotype have been discussed in later sections. Even though a strong secreted Ig amplification was absent during LPS activation of blood B cells, the relative amplification of alternatively spliced transcript to full-length Pax5 seem to increase following activation, as was observed in splenic B cells. This supports the hypothesis that alternatively spliced isoforms of Pax5 are involved in B cell activation and progression of activated mature B cells towards terminally differentiated plasma cells.

3.6.8 Summary of the relative ratio of alternatively spliced Pax5 transcripts in the blood

Figure 3.20 (a)

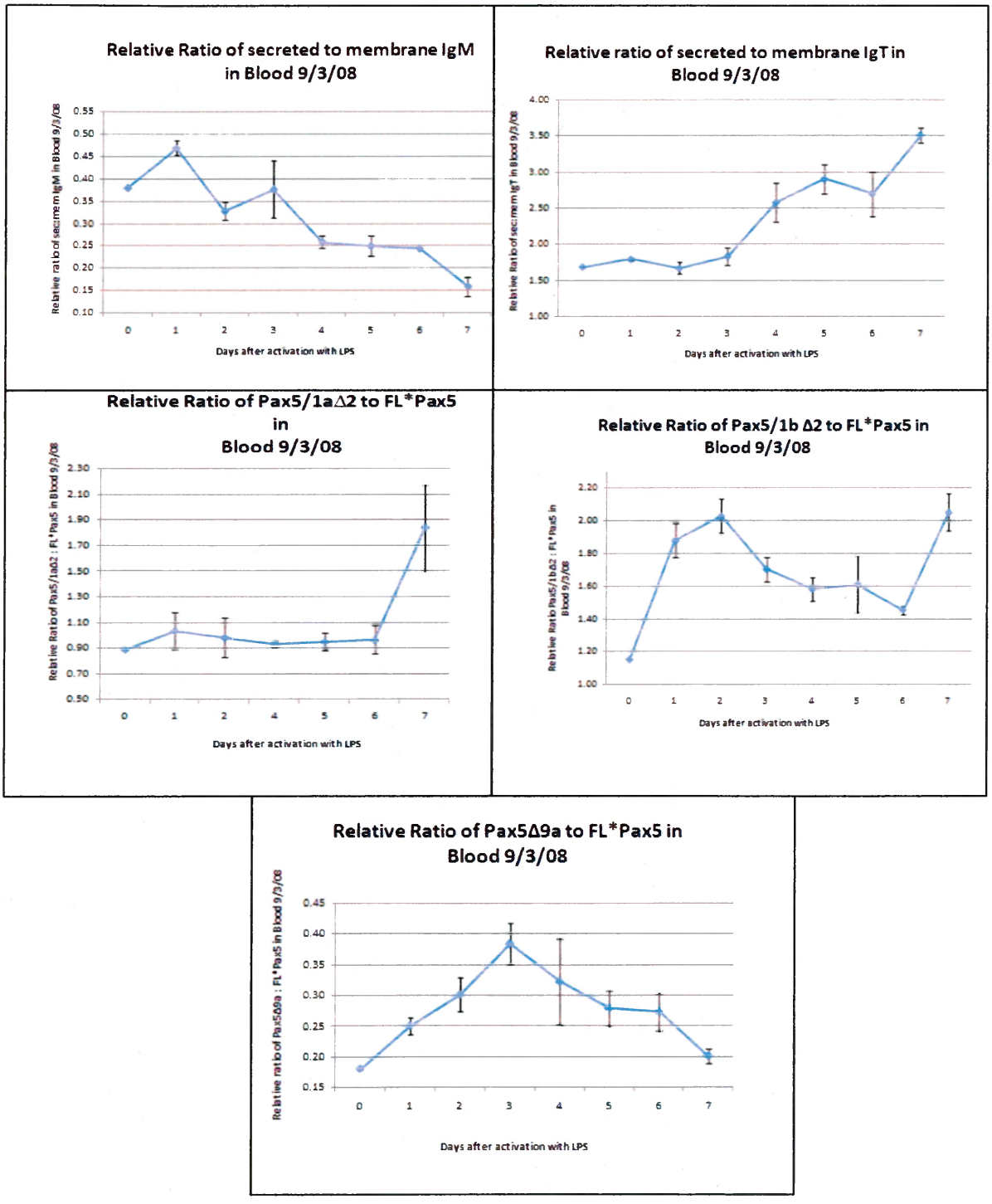


Figure 3.20 (b)

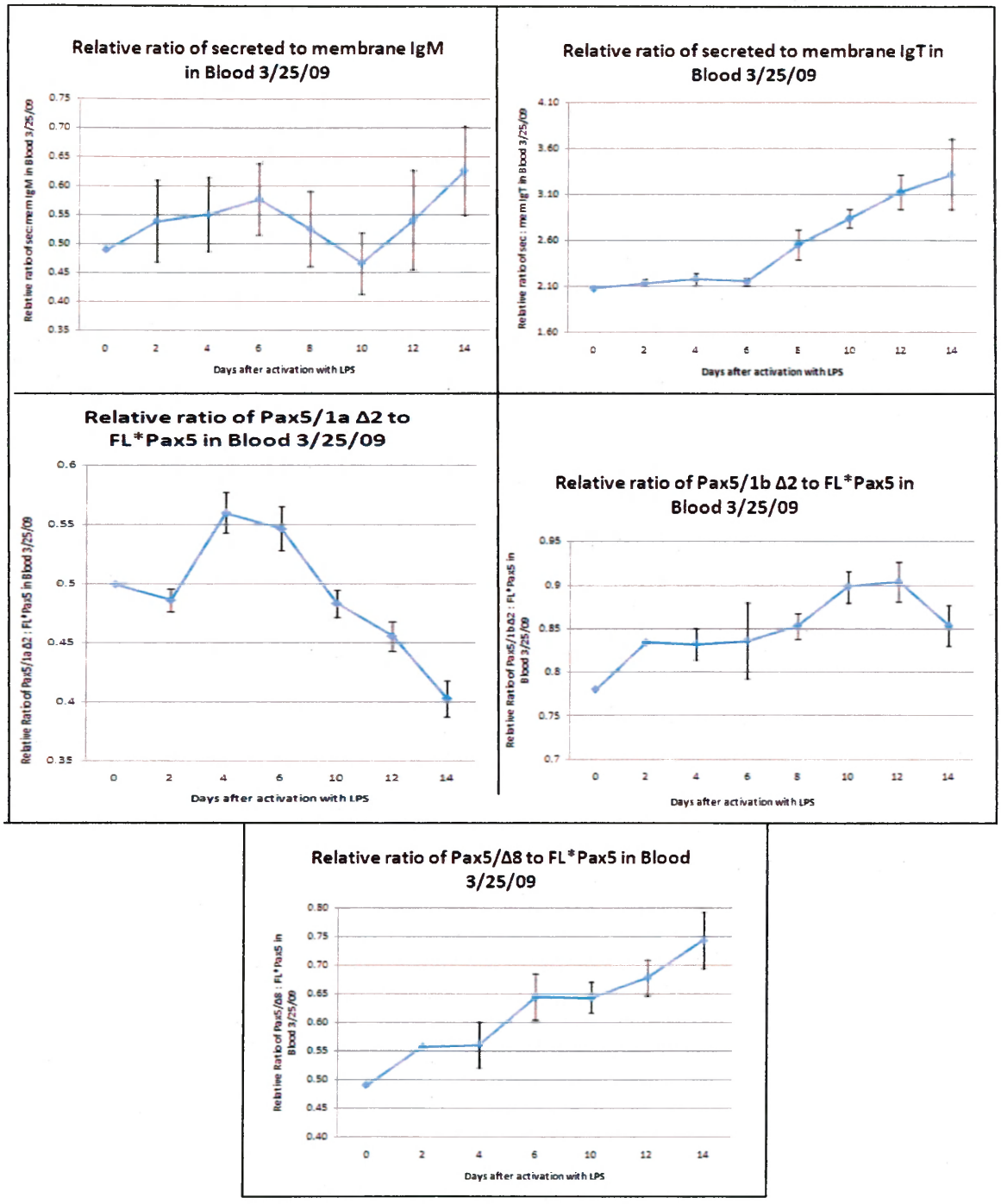


Figure 3.20 Relative ratios of alternatively spliced transcripts to full length Pax5 in the Blood (a) The relative ratio of secreted to membrane IgM and IgT, alternatively spliced transcript to full length Pax5 for 1aΔ2, 1bΔ2 and Δ8 between Days 0 to 7 of LPS activation. (b) The relative ratio of secreted to membrane IgM and IgT, alternatively spliced transcript to full length Pax5 for 1aΔ2, 1bΔ2 and Δ8 between Days 0 to 14 of LPS activation.

3.7 Using nested PCR to detect Pax5/Δ2-8 in human and mouse immune tissues

A novel alternatively spliced transcript of O. mykiss Pax5 was cloned (Alice Harman, unpublished data) that contained only exons 1a, 9 and 10, and contained an in-frame deletion of 885 nucleotides (exons 2 through 8). The deleted exons encode the paired domain, the octopeptide segment, the partial homeodomain and the trans-activation domain (figures 3.1 and 3.4). The resulting transcript solely encodes the maximal inhibitory domain. Based on this analysis, we predict that Pax5/Δ2-8 may function as a co-repressor and contribute to Pax5's inhibitory role in the B cell program. Since predominant alternatively spliced variants of genes with in-frame deletions are often conserved across the vertebrate lineage (Lareau et al, 2004) we sought to identify human and mouse analogues of Pax5/Δ2-8.

Figure 3.21

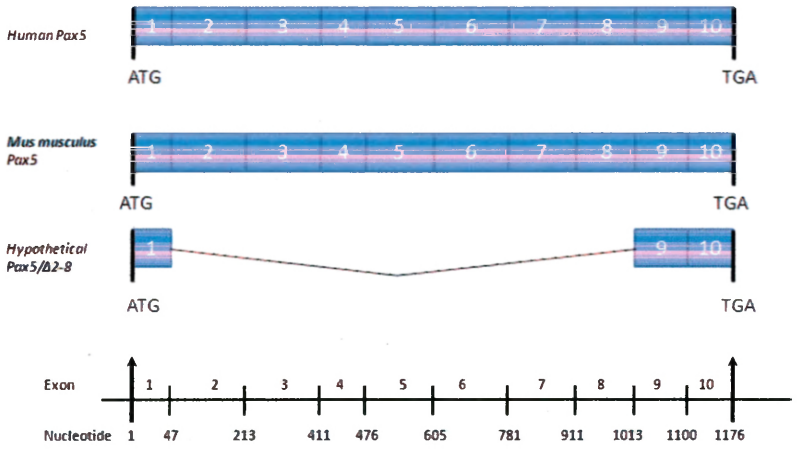


Figure 3.21 Hypothetical human and mouse Pax5/Δ2-8 transcript. Mouse and human Pax5 show a high degree of homology and consist of 10 exons spread over 1176 nucleotides. Human and mouse Pax5/Δ2-8 analogues would encode a complete inhibitory domain but would lack all other conserved functional domains of full-length Pax5.

Human and mouse Pax5/Δ2-8 sequence variants were artificially generated in silico from the published cDNA sequenced of human Pax5 (Genbank ID: 9951919) and mus musculus Pax5 (Genbank ID: 118130642). PCR primers that span the exon 1-9 junction of human and mouse Pax5 were generated and two sets of independent PCR Primers were designed for each organism that flank exons 1-10. Primer sequences are provided in table 3.

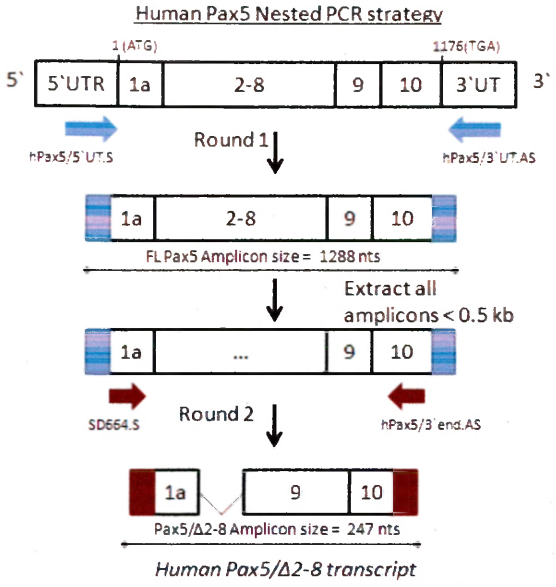
A nested RT-PCR strategy was employed to screen human bone marrow and tonsil cDNA, and mouse spleen cDNA for alternatively spliced transcript Pax5/Δ2-8 (see figure 3.22a). Amplified product from the first round was electrophoretically separated on a 1% low melt agarose gel and amplicons less than 0.5kb were extracted using standard phenol: chloroform extraction *(see Methods).* This product was then used as template for the nested round of PCR with an independent set of internal primers. Two strategies were used to detect alternatively spliced Pax5/Δ2-8: The first strategy used internal primers that flank exon 1 through 10 of human and mouse Pax5, while the second strategy used a sense primer that spans the exon 1-9 junction of mouse Pax5/Δ2-8 and an antisense at the 3` end of exon 10. Figure 3.21b shows the electrophoretically separated nested products of Pax5 in human bone marrow and tonsil cDNA; figure 3.20c shows nested products of Pax5 in mouse spleen. DNA bands were visible with sizes corresponding to human Pax5/Δ2-8 (247 nts) and mouse Pax5/Δ2-8 (210 nts).

Nested PCR fragments that corresponded with human and mouse analogues of alternatively spliced Pax5/Δ2-8 transcript were cloned into pSC vectors (Strataclone). 13

clones of the hypothetical human Pax5/Δ2-8 amplicon and 7 clones of the hypothetical mouse Pax5/Δ2-8 amplicon were sequenced (For a complete list of clones refer table 3.2). Unfortunately none of these clones contained expected Pax5/Δ2-8 sequence. A BLAST analysis of these sequences revealed no homology with known genes in the NCBI database.

One mouse clone (ID: B10_T3_567252) was of particular interest as it possessed parts of exon 1 and complete exon9, specifically: 10 nucleotides of the 3` end of exon 1 was ligated to the 5` end of exon 9. This was determined by aligning the clone with both artificially generated mouse Pax5/Δ2-8 sequence and the published mouse Pax5 cDNA sequence (See appendix I : Alignment 8). Although this initially seemed like evidence for the usage of exon 1 to 8 splicing in mouse Pax5, it was noted that the clone lacked exon 10. Also only one version of this clone was obtained, despite repeated attempts to sequence independent clones. This clone was generated using a nested strategy with exon 1-9 spanning primer; raising the question if the clone was the result of the exon 1-9 junction primer annealing to the 5` end of exon 9. Hence this was not sufficient evidence for Pax5/Δ2-8 in mouse cells.

Figure 3.22a



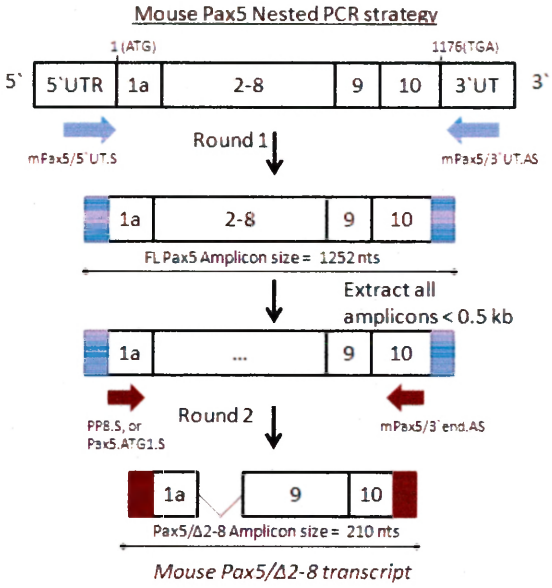


Figure 3.22b

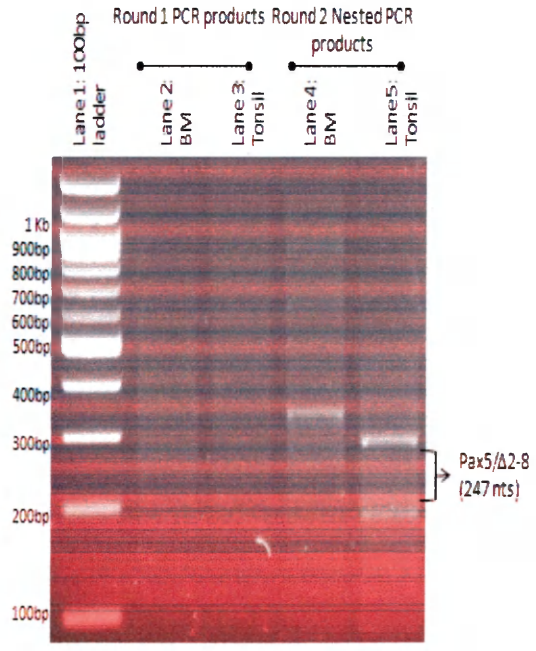


Figure 3.20c

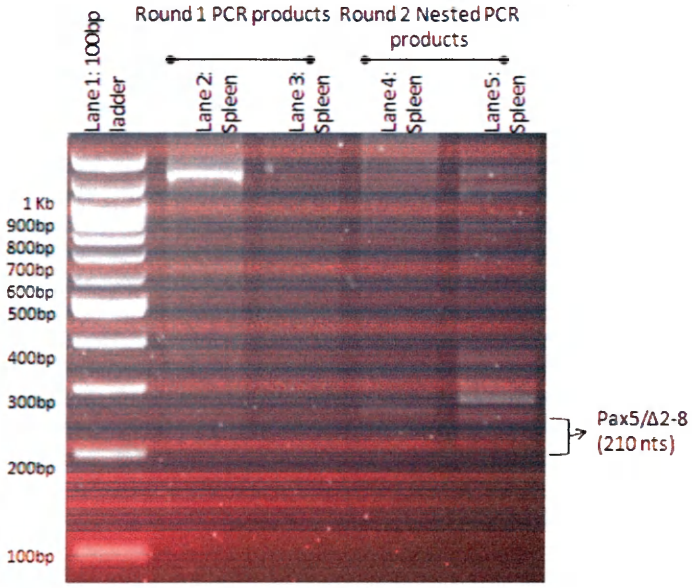


Figure 3.22 Nested PCR for human and mouse Pax5/Δ2-8. (a) Nested PCR strategy for detecting Pax5/Δ2-8 analogues in human and mouse cDNA. (b) Nested PCR products were electrophoretically separated on a 2% agarose gel (0.1µg/ml Ethidium bromide) Lane 1 contains 0.5µg of 100bp ladder (NEB), Lanes 2 and 3

contain round 1 nested PCR product from human BM and tonsil cDNA respectively, Lane 4 and 5 contain round 2 nested PCR product (using product <0.5kb from lanes 2 and 3 as template respectively). Several amplicons of size around 247 nucleotides were visible in lane 5, corresponding to predicted human Pax5/Δ2-8 amplification. (c) Nested PCR products were electrophoretically separated on a 2% agarose gel (0.1μg/ml Ethidium bromide) Lane 1 contains 0.5μg of 100bp ladder (NEB), lanes 2 and 3 contain round 1 nested PCR product from 2 independent samples of mouse splenic cDNA, lanes 3 and 4 contains round 2 nested PCR product (using product <0.5kb from lanes 6 and 7 as template respectively). Several amplicons of size around 210 nucleotides were visible in lane 5, corresponding to predicted mouse Pax5/Δ2-8 amplification.

Other mouse and human clones (see table 3.2 for a complete list of clones) contained only small regions of homology (usually exon 9) with human Pax5 when aligned with the published human Pax5 cDNA and artificially generated human Pax5/Δ2-8 sequences. These regions correspond to PCR primers and are often fused with sequences of DNA that were non-Pax5. A BLAST of these unknown sequences against the database of human, mouse and other nucleotides was inconclusive.Further efforts are required to clone these novel and seemingly rare transcripts of alternatively spliced Pax5/Δ2-8 in mouse and human cDNA.

Table 3.1: List of clones sequenced to screen for trout Pax-5 isoforms.

#	Exon	Clone ID	S primer	AS primer	Isoform result	Tissue	Sequence Date
1	2	350E2-U461207	tPax5/e1wt.S	tpax5/e3sp2.AS	FL* Pax5 with Exon 1a and 2	PK Day 5	6/2/08
2	Δ2	E23A0a_T3_492426	tPax5/e1wt.S	tPax5/E4.AS	Pax5/1a Δ2	AK Day 0	6/2/08
3	Δ2	E2A0b_T3_497310	tPax5/e1wt.S	tPax5/E4.AS	Pax5/1a Δ2	AK Day 0	6/23/08
4	Δ2	E2A0a3_T3_497311	tPax5/e1wt.S	tPax5/E4.AS	Pax5/1a Δ2	AK Day 0	6/23/08
5	Δ2	E2A0a5_T3_497312	tPax5/e1wt.S	tPax5/E4.AS	Pax5/1a Δ2	AK Day 0	6/23/08
6	Δ2	E2A0a6_T3_497313	tPax5/e1wt.S	tPax5/E4.AS	Pax5/1a Δ2	AK Day 0	6/23/08
7	Δ2	6_T3_531756	tPax5/562.S	tPax5/909.AS	Pax5/1a Δ2	AK Day 7	12/8/08
8	Δ2	7_T3_531757	tPax5/562.S	tPax5/909.AS	Pax5/1a Δ2	AK Day 7	12/8/08
9	8	A7_T3_504024	tPax5/764.S	tPax5/1104.AS	FL* Pax5 with exon 8	Spl Day 0	7/23/08
10	Δ8	A5_T3_504022	tPax5/764.S	tPax5/1104.AS	Pax5/Δ8	Spl Day 0	7/23/08
11	Δ8	A6_T3_504023	tPax5/764.S	tPax5/1104.AS	Pax5/Δ8	Spl Day 0	7/23/08
12	9	E9500a2_T3_497321	tPax5/764.S	tPax5/1104.AS	FL*Pax5 with Exon 9	Spl Day 0	6/23/2008
13	9	E9500a7_T3_497322	tPax5/764.S	tPax5/1104.AS	FL*Pax5 with Exon 9	Spl Day 0	6/23/2008
14	9	E9500a9_T3_497323	tPax5/764.S	tPax5/1104.AS	FL*Pax5 with Exon 9	Spl Day 0	6/23/2008
15	Δ9	E9180a3_T3_497320	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9a	Spl Day 0	6/23/2008
16	Δ9	A9_T3_504026	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9a	Spl Day 0	7/23/08
17	Δ9	A10_T3_504027	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9b	Spl Day 0	7/23/08

18	Δ9	E9160a1_ T3_497317	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9c	Spl Day 0	6/23/08
19	Δ9	E9100a5_ T3_497314	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9c	Spl Day 0	6/23/08
20	Δ9	E9100a7_ T3_497315	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9c	Spl Day 0	6/23/08
21	Δ9	E56D56_ T3_497305	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9c	Spl Day 0	6/23/08
22	Δ9	E56D5X_ T3_497306	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9c	Spl Day 0	6/23/08
23	Δ9	E9S6_ T3_492437	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9c	AK Day 7	6/2/2008
24	Δ9	E9S7_ T3_492438	tPax5/764.S	tPax5/1104.AS	Pax5/Δ9c	AK Day 7	6/2/2008

Table 3.2: List of clones sequenced to screen for mouse and human Pax5/Δ2-8.

#	Clone ID	Size nts	Cloning Sense Primer	Cloning Antisense Primer	Tissue	Sequence date
1	H6_a2_T3_582604	248	SD664.S	hPax5/3`end.AS	human Tonsil	7/28/2009
2	H6a16_T3_582605	248	SD664.S	hPax5/3`end.AS	human Tonsil	7/28/2009
3	H6a5_T3_582606	150	SD664.S	hPax5/3`end.AS	human Tonsil	7/28/2009
4	H6a13_T3_582607	150	SD664.S	hPax5/3`end.AS	human Tonsil	7/28/2009
5	H6a14_T3_582608	150	SD664.S	hPax5/3`end.AS	human Tonsil	7/28/2009
6	H1a12_T3_582609	292	hPax5/5`UT.S	hPax5/3`end.AS	human Bone Marrow	7/28/2009
7	H1a13_T3_582610	292	hPax5/5`UT.S	hPax5/3`end.AS	human Bone Marrow	7/28/2009
8	H1a15_T3_582611	292	hPax5/5`UT.S	hPax5/3`end.AS	human Bone Marrow	7/28/2009
9	H1a9_T3_582612	190	hPax5/5`UT.S	hPax5/3`end.AS	human Bone Marrow	7/28/2009
10	H1a11_T3_582613	190	hPax5/5`UT.S	hPax5/3`end.AS	human Bone Marrow	7/28/2009
11	R1_T3_577845	350	hPax5/5`UT.S	hPax5/3`end.AS	human Bone Marrow	7/7/2009
12	R2_T3_577846	300	hPax5/5`UT.S	hPax5/3`end.AS	Human Bone Marrow	7/7/2009
13	A1_T3_577847	270	PP8.S	mPax5/3`end.AS	Mouse spleen (-1|-3)	7/7/2009
14	A2_T3_577848	270	PP8.S	mPax5/3`end.AS	Mouse spleen (-1|-3)	7/7/2009
15	A3_T3_577849	270	PP8.S	mPax5/3`end.AS	Mouse spleen (-1|-3)	7/7/2009
16	H1_T3_577860	270	PP8.S	mPax5/3`end.AS	Mouse spleen	7/7/2009

					(-1|-3)	
17	B7_T3_567249	100	mPax5/E1/E9.S	mPax5/1110.AS	Mouse spleen (-1|-3)	5/28/2009
18	B8_T3_567250	100	mPax5/E1/E9.S	mPax5/1110.AS	Mouse spleen (-1|-3)	5/28/2009
19	B9_T3_567251	600	mPax5/E1/E9.S	mPax5/1110.AS	Mouse spleen (-1|-3)	5/28/2009
20	B10_T3_567252	160	mPax5/E1/E9.S	mPax5/1110.AS	Mouse spleen (-1|-3)	5/28/2009

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Chapter 4

Discussion

Chapter 4: Discussion

The transcription factor Pax-5 is known as the master regulator of B cell development, as it is responsible for the commitment of lymphoid progenitors to the B cell lineage (Delogu et al, 2006) and its sustained expression is essential for B cell identity (Cobaleda et al, 2007). This is evidenced in the expression pattern of *Pax-5* during B cell development, where it is first turned on in the progenitor B cell stage and remains expressed until terminal differentiation of B cells into plasma cells (Busslinger et al, 2004). As a transcription factor, Pax5 serves a primary purpose of inducing the expression of a number of secondary transcription factors that further reinforce the B cell program, such as Irf8 and Ikxf3 (Aiolos) (Nera et al, 2006), as well as those involved in adhesion and pre-BCR signaling like CD19, CD79a and BLNK (Kozmik et al, 1992, Fitzsimmons et al, 1996, Schebesta et al, 2002). Pax-5 also serves a secondary function of inhibiting the expression of lineage inappropriate genes, such as the T-lineage receptor Notch-1 (Souabni et al, 2002), the macrophage lineage commitment factor csf1r (Tagoh et al, 2006), genes associated with multi-potency like flt3 (Holmes et al, 2006) and plasmacytic differentiation gene Xbp-1 (Dent et al, 1997, Reimold et al, 2001).

The basis for Pax5's dual function as a transcription activator and a repressor has been attributed to the presence of two distinct modular domains at its C-terminus: a potent trans-activation domain (TA) that is rich in proline, serine and threonine (PST) residues and an adjacent inhibitory domain (ID) that exerts a negative influence on the trans-activation domain (Dorfler and Busslinger, 1996). It is thought that gene context-

dependent trans-activity of Pax5 is the result of masking of inhibitory domain elements through interactions with other co-factors present at the gene promoter or enhancer locus. The same may then be true for Pax-5's inhibitory activity, where masking of TA domain elements abrogates trans-activation ability.

Here, we present an alternative scenario in which alternatively spliced protein products of Pax5 in trout may selectively impose trans-activating or inhibitory effects on the transcription of downstream target genes. Alternative splicing of Pax5 can yield a host of isoforms with varying degrees of TA potentials. Combined expression of these isoforms permits Pax5 to exert subtle changes in gene expression that are necessary to ensure B-lineage commitment, identity and terminal differentiation.

In this study, we cloned and sequenced several alternatively spliced transcripts of trout Pax5 with deletion of exons that code for the DNA-binding paired domain, the TA domain and the inhibitory domain. Using semi-quantitative PCR analysis, we then studied the relative amplification of each isoform relative to full length Pax5 in LPS activated splenic and blood-derived B cells from trout. Based on the relative expression pattern of each spliced transcript during B cell activation, we propose a model wherein Pax5 isoforms are selective transcriptional activators or inhibitors.

IgM and IgT production in trout splenic and blood-derived B cells

In order to correlate isoform usage with activation state of B cells in splenic and blood-derived B cells, we first measured the relative ratio of secreted to membrane IgM and IgT across 7 days of LPS activation. LPS activation of B cells is known to cause a shift

towards increased secreted Ig mRNA and protein production. Our studies of the relative ratio of secreted to membrane IgM in splenic B cells show an increase in the secreted IgM transcript immediately after and throughout activation. This confirms that splenic B cells react predictably towards LPS treatment in trout. Conversely, the relative ratio of secreted to membrane IgM in blood-derived B cells decreases after LPS activation and there is shift towards increase in membrane IgM. This too is in agreement with previous studies, where blood-derived B cells were found to be less responsive to LPS treatment and have low potential for generating Ig secreting plasma cells (Zwollo et al, 2008).

In order to confirm that the trend observed in Ig transcript amplification was a true reflection of LPS activation, total secreted IgM was measured for LPS activation splenic and blood-derived B cells using ELISA. These studies were performed by a graduate student, Maggie Barr. ELISA studies showed that in both splenic and blood-derived B cells a gradual increase of secreted IgM could be detected after 2 days of LPS activation, consistent with known effects of LPS activation. It was noted using flow-cytometric analysis of blood-derived B cells that while the number of secreted Ig^+ cells increased gradually post-activation; there was also a corresponding increase in membrane IgM^+ cells (data not shown). This addresses why the relative amplification of secreted to membrane IgM in our semi-quantitative studies for blood-derived B cells was always less than 1.

We also performed semi-quantitative studies on the relative ratio of secreted and membrane transcripts of a recently identified IgT immunoglobulin isotype. Relative

amplification of secreted IgT in splenic B cells increased immediately after LPS activation and remains elevated throughout. In the blood, the relative amplification of secreted IgT remains unchanged during the initial days of LPS activation but increases suddenly after day 3. This is in agreement with delayed Ig response observed in trout blood-derived B cells (Zwollo et al, 2008).

The average amplification of total membrane and secreted IgM was greater than that of IgT, consistent with reports that the subset of IgT producing B cells are lower than those that produce IgM (Hansen et al, 2008). Based on these studies, we concluded that LPS stimulation of splenic and blood-derived B cells does indeed induce B cell activation.

4.1 *Alternatively spliced Pax5 isoforms which lack complete Paired domain (PD)*

In our study using trout B cells, two types of alternatively spliced Pax5 transcripts were cloned that lack exon 2. These Δ exon 2 transcripts contained either exon 1a or exon 1b, named Pax5/1aΔ2 and Pax5/1bΔ2 respectively. Trout Δ2 isoforms have an incomplete PD and are hypothesized to have impaired DNA binding ability.

Alternatively spliced Pax5/Δ2 with exon 1a

From all clones containing Pax5 sequence, 1aΔ2 was by far the most abundant alternatively spliced Pax5 species, where almost 30% of clones sequenced were 1aΔ2 (see Chapter 3 Results - Table 3.1 for a complete list of clones). This is in agreement with our semi-quantitative studies in splenic and blood-derived B cells where, barring the full-length isoform, 1aΔ2 has the strongest relative amplification of all the alternatively spliced variants. Our studies on splenic B cells showed an increase in the relative amount of 1aΔ2 after 6 days of LPS activation. Interestingly, blood-derived B cells also show an increase in the relative amount of Pax5/1aΔ2 after 6 days of LPS activation. This suggests that the 1aΔ2 isoform may play a role in the genetic events associated with terminal B cell differentiation.

Alternatively spliced isoforms of Pax5 lacking part of the paired domain have been reported in mouse studies (mouse Pax5b and Pax5e), where skipping of exon 2 leads to a shift in the reading frame and a premature stop in exon 3 (Zwollo et al, 1997).

Messenger RNAs with a premature stop codon close to exon junctions are normally targeted for degradation by non-sense mediated decay (Hillman et al, 2004). It is likely that mouse 1aΔ2 transcripts use a second downstream ATG start codon located inside exon 3, and yield isoforms with a truncated paired domain. This implies that these isoforms may behave as dominant negative co-repressors of Pax5 activity by competing for co-factors necessary for normal Pax5 function. Western blot analysis could detect mouse 1aΔ2 isoforms (Pax 5b and 5e) in mouse B cell lines showing that these alternatively spliced Pax5 transcripts are translated (Zwollo et al, 1997, Lowen et al, 2001).

The 1aΔ2 isoform has also been reported in human B cells, where differences were observed between the relative expression of human 1aΔ2 transcript in healthy donors and patients with multiple myeloma (Borson, 2002). However, due to a high degree of variability between the relative expressions of 1aΔ2 between individuals, no consistent pattern could be established in both normal and multiple myeloma patients. Recently, another group established a relative expression pattern of 1aΔ2 in normal human B cells by using micro-fluidics technology to enhance the sensitivity of detecting amplified transcripts by RT-PCR (Arseneau et al, 2009). Arseneau et al noted that in both lymphoma and normal B cells the full length isoform was more abundant than 1aΔ2, and is in agreement with our previous studies in mice. Arseneau et al also showed polysomal association of human 1aΔ2 transcripts with ribosomes in EHEB cells, suggesting that the human 1aΔ2 transcript is translated. In a related study, Santoro et al (2009) noted a greater incidence of the 1aΔ2 isoform and single point mutations in B cell pre-cursor

acute lymphoblastic leukemia (BCP-ALL) cells, but were unsuccessful in elucidating a consistent relative expression pattern of 1aΔ2 that was characteristic for ALL. Santoro et al reported that 29 of 100 ALL patients had detectable amounts of 1aΔ2 by quantitative RT-PCR. In these patients the median ratio of full-length Pax5 to 1aΔ2 was 25:96, implying a higher relative amount of the alternatively spliced isoform in ALL patients. This directly contradicts the Arseneau study and thus yet, no conclusions can be made about aberrant 1aΔ2 isoform expression and disease incidence.

Recently, a similar exon 2 skipping pattern was reported in amphioxus Pax2/5/8; the evolutionary ancestor of vertebrate Pax5 (Short & Holland, 2008); demonstrating conservation of the 1aΔ2 isoform along the vertebrate lineage. However, no comments were made about the relative abundance of 1aΔ2 in amphioxus tissues.

Mouse, human and amphioxus 1aΔ2 are analogous to trout 1aΔ2 in the pattern of exon 2 skipping. As in mouse 1aΔ2, exon 2 skipping in trout Pax5 leads to a shift in the reading frame and a premature stop in exon 3 (nucleotides 248-250). This likely results in the use of a second ATG start codon (nucleotides 221-223), located in exon 3. The expression pattern of trout 1aΔ2 isoform during LPS activation suggests that it may also function as a dominant negative co-repressor of target downstream genes, like CD19 and blk. The down-regulation of these target genes may contribute to the de-repression of genes involved in the B cell terminal differentiation program like Blimp1 and Xbp-1, by Pax5 (See introduction, figure 1.1). Thus the 1aΔ2 isoform may be associated with processes that trigger the terminal differentiation program.

Zwollo et al, 1997 performed functional studies using EMSA assays of mouse Pax5b and Pax5e using high-affinity Pax5-binding sites to demonstrate that these isoforms lacked DNA binding ability in-vitro. In a follow up study on the function of mouse Pax5 Δ2 isoforms (Lowen et al, 2001), co-transfection of the Pax5e isoform along with full length isoform synergistically enhanced the transcriptional activity of the full length isoform in a dose dependent manner. This suggests certain Pax5 isoforms may auto-regulate Pax5 activity in B cells.

Alternatively spliced Pax5/Δ2 with exon 1b

Trout Pax5 with exon 1b sequence was serendipitously cloned in a previous library screen (Zwollo et al, unpublished data). Preliminary studies performed to amplify exon 1b using RT-PCR revealed a second amplicon whose size differs from the full-length by approx 180 nucleotides. This difference is approximately the size of exon 2, suggesting that this second amplicon arose from a Pax5/1bΔ2 transcript *(cloning studies and sequence analysis of 1bΔ2 are yet to be performed).* Our studies on the relative amplification of 1bΔ2 in trout B cells also showed an increase immediately after LPS stimulation. This elevation peaks at day 3 for spleen. In contrast the relative amount of 1bΔ2 in the blood almost doubles following LPS stimulation and remains elevated, unlike in the spleen. The increased expression of the 1bΔ2 isoform during activation of B cells suggests that it may also be involved in the genetic regulation of terminal B cell differentiation, similar to the 1aΔ2 isoform.

Studies on alternatively spliced Pax5 with exon 1b are extremely scarce. Recently, Arseneau et al (2009) used micro-fluidics technology to obtain high-resolution separation of PCR amplified Pax5 transcripts. Arseneau et al reported a human 1bΔ2 spliced variant that could be detected in B cells from chronic lymphoblastic leukemia, lymphoma and healthy patients. In each case, the full-length was amplified in greater relative amounts than 1bΔ2, which is agreement with our studies in trout. Their data suggested that 1bΔ2 amplification differed in CLL, lymphoma and healthy patients, however, the researchers did not comment on performing relative amplification studies for this isoform.

The Pax5 1b exon has been reported in human and mouse studies, but there is a dearth in the literature on its functional significance. The suppression of exon 1b containing human Pax5 in REH cell lines showed an increase in CD19 mRNA and cell surface expression (Robichaud et al, 2008). CD19 is a component of the B-cell receptor (BCR) complex and plays key roles in cell signaling events surrounding B-cell proliferation and differentiation. Suppression of exon 1b containing Pax5 also lead to an arrest in proliferation and increased susceptibility of B cells to apoptosis. Based on these studies, we hypothesize that trout 1bΔ2 isoform functions differently from 1aΔ2 during terminal differentiation. The sustained expression of 1bΔ2 even during later days of activation in trout suggests that this isoform may be involved in the survival of B cells as they terminally differentiate into plasma cells. Transfection assays will have to be performed to test the function of 1bΔ2 on Pax5 activity.

Structural analysis of Pax-5 Δ2 isoforms

NMR and X-ray crystallography studies of the highly conserved paired domain have provided interesting insight about Pax paired domain structure and its mechanism of DNA binding. Studies by Xu et al (1999) in Pax6 showed that the highly conserved Pax paired domain is bi-partite in structure and is composed of two helix-turn-helix (HTH) motifs separated by a short linker region. The N-terminal HTH motif, referred to as PAI in the literature, contains two conserved cysteine residues that control the PAI sub-domain's interaction with DNA in a redox-regulated manner. Oxidation of the conserved cysteine residues by gluthionylation completely abrogates DNA binding ability (Xu et al, 1999). The C-terminal HTH motif, referred to as RED in the literature, forms contacts with DNA independently and regardless of the redox state (Cao et al, 2005). Additionally, a β-hairpin at the N-terminal and the linker region provide specific contacts at the minor groove level and stabilize DNA binding by PAI and RED (Xu et al, 1999). Recently, Codutti et al (2008) reported that the Pax-8 PAI and RED domains retain a fair degree of tertiary structure even when unbounded by DNA. Interestingly, Pax-5 Δ2 isoforms are predicted to lack both conserved cysteine residues in the PAI sub-domain and the N-terminal β-hairpin. This drastically reduces the available DNA binding interface for the truncated paired domain, suggesting that Δ2 isoforms may be unable to adopt sufficient contacts on DNA that enable docking into the minor groove to ensure stable DNA binding.

Other alternatively spliced transcription factors with altered DNA-binding

Deletion of paired domain encoding exons is relatively new to the Pax group of genes and has been extensively studied in only one other member – *Pax6*, which is expressed in the developing olfactory bulb and eye. Use of alternative promoters control the expression of paired domain containing Pax6 and the paired domain lacking 'Paired-less' Pax6 (Kim & Lauderdale, 2006). Unlike Pax5, Pax 6 isoforms contain an intact DNA binding homeodomain. Thus, 'Paired-less' Pax6 retains its DNA binding ability even without a paired domain. However, over-expression of paired-less Pax6 caused a micropthalmic phenotype in Pax-6(+/+) and Pax-6(+/-) mice, suggesting that a delicate balance between both Pax-6 isoforms is necessary for normal eye development.

Alternatively spliced isoforms with altered DNA binding ability have also been reported in the zebrafish Oct-3 like transcription factor pou2 (Takeda et al, 1994). POU domain of transcription factors are important genetic regulators during early development. Alternative splicing of the *pou2* gene yields isoforms that either contain the DNA binding POU domain or contain incomplete POU domain structure (t-pou2). As a result of this splicing pattern, t-pou2 lacks DNA binding ability and over-expression of this isoform lead to complete or partial developmental arrest due to incomplete gastrulation. This provides further evidence for neofunctionalization of alternatively spliced isoforms and warrants further investigation of Pax5 isoform function on overall Pax5 activity in B cells.

4.2 *Alternatively spliced Pax5 isoforms that lack complete Trans-activation Domain (TD)*

Alternatively spliced Pax5/Δ8

Our studies in trout B cells are the first to report the Δ8 isoform in vertebrates other than humans, since analogues of Δ8 have not yet been discovered in murine and amphioxus systems (Zwollo et al, 1997, Short and Holland, 2008). Although deletion of exon 8 in Pax5 does not shift the reading frame or stop codon usage, the resulting isoform resembles full-length but lacks most of the minimal trans-activation domain (34 amino acid deletion) (Dorfler and Busslinger, 1996) *(see Chapter 3 – Results: Figure 3.1 for location of minimal TD)*. Based on this structural analysis, we predict that the Δ8 isoform functions as a transcriptional repressor of Pax5 activity. Trout Δ8 clones were obtained from splenic B cells and represent only 8% of clones with Pax5 sequence, suggesting that this isoform may be relative rare in freshly isolated splenic B cells (see table 3.1 for a complete list of clones). This is in agreement with our semi-quantitative studies, where full-length Pax5 is always amplified in greater relative amounts than Δ8 transcript in both freshly isolated and LPS activated splenic and blood-derived B cells. In the spleen, the relative amplification of Δ8 increased gradually throughout LPS activation and peaked at day 6. Interestingly, blood-derived B cells also showed a gradual increase in the relative amplification of Δ8 during LPS activation, but unlike the spleen the relative ratio remained elevated even after 14 days of LPS activation. This

suggests that, like the Δ2 isoform, Δ8 isoforms may also be involved in the transcriptional regulation of terminal B cell differentiation.

The Δ8 isoform was first described by Borson et al (2002) in patients with multiple myeloma (MM), where (like trout Δ8) skipping of exon 8 conserves both the reading frame and stop codon usage. Borson et al noted that in a total of 6 MM patients and 3 normal patients the full-length isoform was predominant and only trace amounts of Δ8 isoform was detectable through RT-PCR analysis. This is in agreement with our own studies in trout splenic and blood-derived B cells. Borson et al also reported that Pax5 isoform expression profiles differed in normal and MM patients but sample limitations and variations between individual patient's isoform patterns prevented the establishment of a distinct expression profile for each. Consequently, Robichaud et al (2004) attempted to use RT-PCR analysis to establish an isoform expression profile for B cell lymphoma patients and noted that in 8 of 11 patients tested the full-length isoform was predominantly expressed, whereas in the remaining three Δ8 was the most predominant isoform. Although a consistent expression profile for Pax5 isoforms in lymphoma patients could not be established, Robichaud et al used western blot analysis to show that the Δ8 isoform was indeed translated.

Following with the theme of studying Pax5 isoform expression in B cell lymphomas, Oppezzo et al (2005) found that Δ8 isoforms may regulate AID (activation-induced cytidine deaminase) expression in chronic lymphoblastic leukemia (CLL) B cells. In normal B cells, AID initiates somatic hyper-mutation and class-switch recombination

during early B lymphogenesis. Oppezzo et al reported that the full-length isoform was predominantly amplified in CLL B cells with constitutive AID expression. However, full-length amplification was reduced in CLL B cells without constitutive AID expression and the emergence of Δ8 isoform was detectable in 33 of 54 patients. This suggests that Δ8 isoform functions as a transcriptional repressor by competing with full-length Pax5 for binding sites on the AID promoter.

Sadakane et al (2006) also reported the presence of full-length and Δ8 isoform in 14 children with acute lymphoblastic leukemia (ALL) and healthy donors using RT-PCR and western blot analysis. More recently, Santoro et al (2009) noted that full-length Pax5 was often the only detectable isoform in cord-blood and bone marrow-derived progenitor cells from healthy patients, and that Δ8 isoform was detectable only in mature B cells from peripheral-blood.

Robichaud et al (2004) performed functional DNA binding studies to show that the Δ8 isoform could efficiently bind DNA. Interestingly, the trans-activation ability of Δ8 isoform was comparable to full-length human Pax5. This is unexpected since more than 50% of the TD is missing from the Δ8 isoform, suggesting that important trans-activation elements may reside in regions outside those encoded by exon 8. Alternatively, deletion of 34 amino acids from the TA domain might alter protein folding and overall 3-dimensional protein conformation at the C-terminal end, thus mask important elements in the inhibitory domain.

Taken together, these results suggest that although the Δ8 isoform is present in lower transcript levels relative to full-length, Δ8 isoform probably functions as a transcription factor that represses Pax5 activity in normal and malignant B cells.

Other alternatively spliced transcription factors with altered trans-activation

Skipping of trans-activation domain coding exons has been reported in one other member of the Pax family – Pax-7. The transcription factor Pax-7 helps convert multi-potent stem cells to the myogenic lineage and is vital for skeletal muscle myogenesis and regeneration (Buckingham and Relaix, 2007). Mao et al (2008) report a Δ8 spliced variant of Pax-7 in chick cells, that results in a 22 amino acid deletion in the isoforms TD. QT-PCR studies for the full-length and Δ8 variants in developing chick embryos showed that they had similar expression profiles until day 12, where Δ8 amplification was highest. Co-transfection and luciferase reporter studies performed by Mao et al showed that this Δ8 isoform had lower trans-activation potential than both full-length Pax7 and an empty vector used as control. This suggests that Pax-7 Δ8 isoform probably also functions as a transcriptional repressor during chick muscle myogenesis.

Alternatively spliced isoforms with truncated trans-activation domains frequently show reduced or complete loss of trans-activation ability, as is the case for p63 isoforms (Petitjean et al, 2008). p63 proteins are responsible for the development of stratified epithelia during bone morphogenesis and are structurally similar to the p53 tumor suppressor family of proteins. It has been demonstrated, *in vitro* and *ex vivo,* that Pax5 directly represses p53 expression through a Pax5 binding site in its un-translated first

exon (Stuart et al, 1995), though no such evidence has been presented for p63. Alternative splicing of p63 produces isoforms that lack an N-terminal trans-activation domain and these isoforms act as dominant negative inhibitors of isoforms that possess the intact trans-activation domain (Yang et al, 1998). This inhibition is thought to be the result of accumulation of inactive hetero-oligomers or competition for specific response elements on DNA binding sites (Westfall et al, 2003). We suggest that this mechanism of inhibition is precisely what Δ8 isoforms employ to negatively regulate Pax5 activity in B cells.

4.3 *Alternatively spliced Pax5 isoforms that lack complete Inhibitory Domain (ID)*

Alternatively spliced Pax5/Δ9a

In our search for alternatively spliced variants of trout Pax5 with deletions at the 3` end, 8% of clones containing Pax5 sequence had a deletion of complete exon 9 (Pax5/Δ9a) (for a complete list of clones refer table 3.1). Exon 9 encodes 29 amino-acids at the C-terminal end of Pax5, which compose of a 21 amino-acid region where C-terminal elements from of the TA domain overlap with N-terminal elements of the ID domain and an additional 8 amino-acids from the N-terminal part of the ID domain (Dorfler and Busslinger, 1996) *(See Chapter 2 – Results: Figure 3.1)*. Skipping of exon 9 conserves both the reading frame and stop codon usage, and the resulting isoform incurs truncations in both the TA and ID domains. Since removal of inhibitory elements from the ID domain may be counteracted by removal of activating elements from the TA domain (or vice versa), it is not feasible to predict the trans-activation/inhibitory activity of the Δ9a isoform without functional data from reporter gene expression/transfection assays.

Our relative amplification studies in trout splenic and blood-derived B cells indicate that Δ9a is produced in minute quantities relative to full-length in these tissues. In blood-derived B cells, the relative ratio of Δ9a increases marginally during LPS activation and peaks around day 3. Δ9a amplification was undetectable in most days of LPS activated splenic B cells, except day 2. At this point it is unclear if the Δ9a isoform is involved in

regulatory processes that govern terminal differentiation of B cells; however, previous reports about the human Δ9a isoform may help shed some light on this matter.

Pax5 Δ9a isoforms were first reported by Borson et al (2002) in normal and malignant human B cells, where different isoform profiles were observed in each case. Consequently, Robichaud et al (2004) reported that human Δ9a isoform could be detected by Western blot, indicating that Δ9a is indeed translated. In all human cell lines tested by Robichaud et al, the full-length isoforms predominated over Δ9a, as is evidenced in our studies in trout B cells. Robichaud et al observed that activation of several B cell and primary lymphoma cell lines with the mitogens PHA and PMA/ionomycin always lead to reduced amplification of full length and different patterns of isoform expression, though no distinct pattern could be elucidated for each. Sekine et al (2007) studies on the amplification of human Pax5 isoforms in human cord blood-derived B cell progenitors also concluded that the full length form was most predominant, and that Δ9a amplification was detectable only after one week of co-culture.

Finally, Arseneau et al (2009) reported that multiple C-terminal isoforms, including Δ9a, could be detected in 10 chronic lymphoblastic leukemia patients using RT-PCR. Arseneau et al also reported anti-N-terminal Pax5 antibodies could detect a 40kDa protein in western blot studies that could either be Δ8 or Δ9a (since the predicted molecular weight for both isoforms is 40kDa). Furthermore, polyribosomal association

analysis suggests that Δ9a mRNA is probably recruited to the translation apparatus, where it is translated into protein form.

In vitro mutagenesis and transient transfection experiments by Dorfler and Busslinger (1996) showed that removal of inhibitory domain elements enhanced the trans-activity of Pax5 by 8 fold. This suggests that Pax5 isoforms with truncated inhibitory domains would have greater trans-activity than the full-length form. In striking contrast to this experiment, Robichaud et al (2004) found that human Δ9a isoforms could bind DNA and induce expression of a luciferase reporter construct at levels a little lower but somewhat comparable to that of full-length Pax5. This implies that truncation of the inhibitory domain does not relieve its negative regulation on trans-activation domain function. Recently, Sekine et al (2007) also tested the trans-activation potential of human Pax5 C-terminal isoforms using a luciferase reported fused with high affinity Pax5 binding sites. Δ9 isoforms showed a 7 fold induction of luciferase expression over controls, while full-length isoform showed a 9 fold induction. These data support the view that Δ9 isoform is a slightly less potent trans-activator when compared to full-length.

Cryptically spliced isoforms Pax5/Δ9b and Pax5/Δ9c

Our studies report for the first time two cryptically spliced variants of Pax5/Δ9, unique to trout, that use alternative 5` donor and 3` acceptor sites. The first variant (Δ9b) uses a 5` splice acceptor that is 12 nucleotides upstream of the exon 8/9 junction and a 3` splice donor site at the normal exon 9/10 junction. This unique splice pattern does not alter the reading frame and the resulting isoform would lack 33 amino-acids at the C-

terminal end of Pax5 composed of: 4 amino-acids from the TA domain, 21 amino-acids containing elements of both the TA and ID domains and 8 amino-acids from the N-terminal part of the ID domain. Comparison of the primary protein structure of Δ9a and Δ9b isoforms reveals that an additional 4 amino acids of the TA domain are lacking in Δ9b. Only 8% of clones containing Pax5 sequence were Δ9b, suggesting that it is rare in abundance. This conclusion is supported by our semi-quantitative studies where Δ9b amplification is undetectable in splenic and blood-derived B cells, where full-length Pax5 is predominantly amplified.

The second cryptically spliced variant (Δ9c) uses a 5` splice acceptor that is 12 nucleotides upstream of the exon 8/9 junction and a 3` splice donor site that is 54 nucleotides downstream of the exon 9/10 junction. Like in Δ9b, the splice pattern for Δ9c conserves the reading frame and the resulting isoform would lack 51 amino-acids at the C-terminal end of Pax5. These residues are composed of a 4 amino-acids from the TA domain, 21 amino-acids containing elements of both the TA and ID domains and 26 amino-acids from the N-terminal part of the ID. Almost 30% of clones containing Pax5 sequence were Δ9c, suggesting that it is more abundant than Δ9a and Δ9b. However, our semi-quantitative studies were once again unable detect Δ9b amplification in LPS activated splenic and blood-derived B cells and full-length Pax5 was predominant.

The lack of Δ9b and Δ9c amplification in LPS activated splenic and blood-derived B cells suggests that expression of these spliced variants may be limited to specific B cell developmental stages in other immune tissues, like the anterior and posterior kidney.

We predict that the Δ9b might have a marginally lower trans-activity than Δ9a due to a deletion of 4 additional amino acids from the TA domain. Alternatively, we predict that Δ9c might have greater trans-activity than Δ9a, since almost a third of the ID is removed in Δ9c. It remains to be seen if the cryptically spliced Δ9b and Δ9c isoforms do exhibit altered trans-activity.

4.4 Model of Pax5 isoform mediated regulation of the B cell program

Based on our PCR studies in trout B cells and the resulting predictions on isoform function, alternatively spliced isoforms of trout Pax5 can be broadly classified under two categories: transcriptional activators (Isoforms: Δ9c; and possibly Δ9a and Δ9b) or transcriptional inhibitors (Isoforms: 1aΔ2, 1bΔ2 and Δ8).

Figure 4.1 (a)

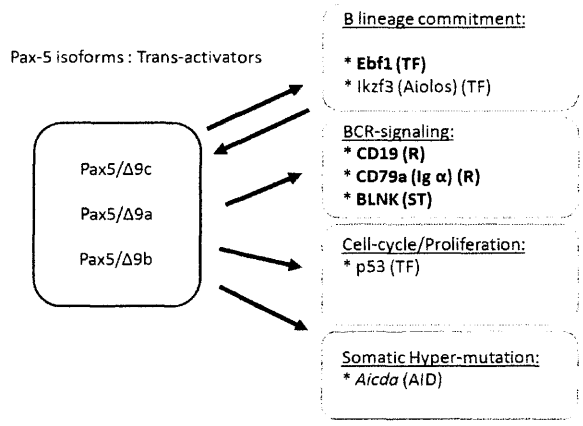

Figure 4.1 (b)

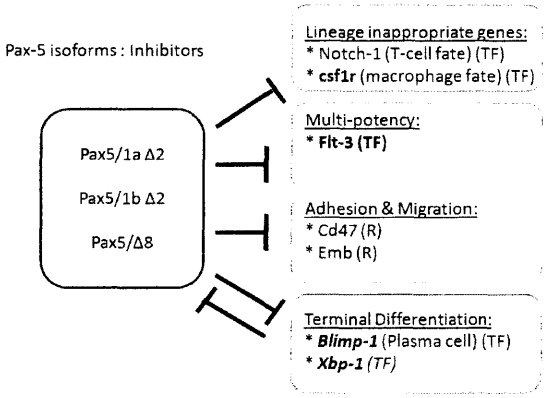

Figure 4.1 Model of Pax5 isoform mediated regulation of the B cell program. (a) Pax-5 isoforms Δ9a, Δ9b and Δ9c function as trans-activators that directly or indirectly induce the expression of genes involved in B-lineage commitment, BCR-signaling, cell cycle and proliferation and somatic hyper-mutation. (b) Pax-5 isoforms 1a Δ2, 1b Δ2 and Δ8 function as transcriptional repressors that directly or indirectly inhibit the expression of genes involved in other lineage commitment programs, multi-potency, adhesion / migration and terminal differentiation. In parenthesis (TF) = transcription factor, (R) = receptor, (ST) = secretory tail. **Boldface** = Genes that are direct targets of Pax5, *Italics* = Genes that are under Pax5 control in mature B cells.

Pax5 isoforms with putative trans-activator function:

Based on semi-quantitative analysis and data from the literature, isoforms Δ9a, Δ9b and Δ9c are most likely to induce transcriptional activation in target genes. Genes involved in commitment of lymphoid progenitors to the B lymphocyte lineage like Early B cell factor (Ebf1) E2a and Aiolos are reported to be both regulated by Pax5 and also exert regulatory influences on Pax5 expression. The early B cell factor promotes the commitment of lymphoid progenitors to the pro-B cell stage (Roessler et al, 2007). Roessler et al found Pax5 binding sites on the Ebf1 promoter implying a direct mode of regulation by Pax5 and its isoforms. On the other hand, Ikzf3 (Aiolos) plays an important role in the pre-B cell and mature B cell stages of differentiation and is controlled by Pax5 (Nera et al, 2006). Δ9 isoforms of Pax5 may help induce and sustain the expression of these transcription factors during early B cell development. Pax5 binding sites have also been found on genes for integral components of the BCR-signaling complex – namely, CD19 and CD79a and BLNK (Kozmik et al, 1992, Nutt et al, 1997, Schebesta et al, 2002). BCR signaling is a tightly regulated process that provides the necessary cues for B cell differentiation towards mature B cell state. Pax-5 has also been suggested to be involved in the direct regulation of cell-cycle through tumor suppressor p53 (Stuart et al, 1995). Finally, Pax5 isoforms may also regulate the expression of activation-induced cytidine deaminase (AID) which is responsible for somatic hyper-mutation and class switch recombination in developing B cells. Taken together, isoforms Δ9a, Δ9b and Δ9c may contribute to Pax5 control of important regulators of the B cell program.

Pax5 isoforms with putative Inhibitory function:

Pax5 repressive activity has been best documented in the suppression of non B lineage genes such as Notch-1, an important T-cell fate inducer (Souabni et al, 2002), and csf1r, which induced macrophage fate (Tagoh et al, 2006). Recently, Pax5 was also reported to repress the multi-potency inducing transcription factor flt3, thus restricting the lymphoid progenitors to the B cell lineage (Holmes et al, 2006). Pax5 also represses migration and adhesion receptor genes CD47 and Emb (Carotta et al, 2008), implying that Pax5 activity is pivotal for the migration of progenitor B cells and commitment towards mature B cells stage. Finally, Pax5 also represses genes involved in terminal differentiation of B cells in to plasma cells through B lymphocyte inducing maturation protein (Blimp1) and X-box protein (Xbp1) (Mora-Lopez et al, 2007). Pax5 isoforms 1aΔ2 and 1bΔ2 may contribute to repressive activity as dominant negative co-repressors since both isoforms are predicted to have impaired DNA binding ability. Pax5 isoform Δ8, however, may engage in a more direct regulation of the above genes.

4.5 Evolutionary Conservation of Pax5 isoforms

An interesting observation can be made about the conservation of certain isoforms across evolutionary time. Comparison of alternatively spliced transcripts in various organisms has revealed that predominant splice variants are well conserved and rarer isoforms are less conserved (reference Lareau et al, 2004). This suggests that conserved isoforms are functionally important. Alternatively spliced isoforms of Pax5 have been reported in mammalian systems (in mice and humans), in amphioxus (a basal chordate)

and now, in teleosts (O. mykiss). The mutually exclusive use of exon 1a or 1b has been reported in all 4 systems (Busslinger et al, 1996, Short & Holland, 2008).

Alternatively spliced variants of Pax5 with exon 1a and deletion of the paired domain coding exon 2 are conserved from basal chordates to mammals (Zwollo et al, 1997, Borson et al, 2002, Short & Holland, 2008). Interestingly, alternatively spliced variants of Pax5 with exon 1b and deletion of exon 2 have been reported in humans (Arseneau et al, 2009) and now in trout, but are absent in mice and amphioxus. Similarly, alternatively spliced variants that lack exon 8 or exon 9 have been reported in humans and now in teleosts but not in mice or amphioxus – although, two splice variants of mouse Pax5 (mouse Pax5d and Pax5e) were reported to lack exons 6-10 that encode the TD and ID (Zwollo et al, 1997) and 7 different C-terminal isoforms of amphioxus Pax5 encode truncations of the TD and ID (Short & Holland, 2008).

A common effect of alternative splicing is altered localization of the resulting isoform (Chi & Epstein, 2002). Human Pax5 possesses a nuclear localization sequence (NLS) in exon 5 (encoded by nucleotides 585 to 603) that interacts with the nuclear transport protein importin α1 (Kovac et al, 2000). Alternative splicing of exon 5 can produce an isoform lacking NLS and would not be transported into the nucleus after translation. Unfortunately, our search for trout Pax5 isoforms with deleted NLS coding exon 5 returned no results. Also, reports of Pax5 isoforms in other organisms support the view that exon 5 is always constitutively spliced. Many novel and rare isoforms of Pax5 have been reported that are unique to the organism it was reported in but are largely absent

in other species, either because they have not been discovered yet or are no longer produced. For example, mouse Pax5d and Pax5e have a deletion of exon 6-10 and instead possess a shorter novel sequence at their 3` end (Zwollo et al, 1997). Similarly, multiple C-terminal isoforms have been reported in humans that have a deletion of consecutive exons, Δ7/8, Δ8/9, Δ7/8/9 (Robichaud et al, 2004, Arseneau et al, 2009). These isoforms have not been found in other systems, suggesting that they are not conserved. These isoforms are translated, as shown in western blot and polysomal analyses, but their function is yet to be determined. It is likely that these isoforms may be tissue specific or limited to finite stages of B cell development and activation.

4.6 Future Directions

In this study, we cloned six alternatively spliced variants of trout Pax5 and studied their relative expression in splenic and blood-derived B cells. A preliminary study of the relative amplification of these isoforms in B cells obtained from the anterior kidney revealed a complex pattern of isoform expression. We attribute this complexity to the observation that the anterior kidney houses multiple populations of developing B cell (Zwollo et al, 2008). To overcome this barrier, the study will be repeated on tissues that have been separated by mass/density on Percoll gradients, as has been previously described (Zwollo et al, 2005). This will improve the resolution with which we can measure the relative amounts of spliced variants in B cells from the anterior kidney. Since B cells in like stages of development are separated together using Percoll gradients, relative isoform amplification would also reveal potential associations

between isoform expression and B cell developmental stages. These isoforms can then be used as markers of B cell development.

Another area of interest lies in establishing relative isoform transcript levels in normal and malignant B cell lines using a quantitative real-time PCR or a semi-quantitative RT-PCR approach. Establishment of an isoform expression pattern in B cell lines would provide important clues about potential association of isoforms with B cell lymphomas and other lymphocyte diseases.

There is some concern that alternatively spliced Pax5 mRNA transcripts detected far exceed those reported as translated protein. Not all human isoforms are readily detected by Western blot analysis. Arseneau et al (2009) recently described a polysomal association analysis that identifies translated mRNA. This technique relies on the fact that translated mRNAs can be separated from un-translated messenger transcripts by virtue of their weight added on by ribosomes. Ribosomes attached to the translated RNA make them denser and allow for separation of the polysomal fraction of translated mRNA. Detection of Pax5 spliced transcripts within this pool of translated mRNA would indicate if it is translated.

Due to design limitations, we were unable to screen for deletions/truncations of exon 10 and the 3` UTR of trout Pax5 using a nested PCR strategy. Alternative exon 10 usage has been reported in the basal chordate amphioxus (Short and Holland, 2008). Alternative splicing of the un-translated region has been known to alter the stability, localization and efficiency of translation of pre-mRNA transcripts (Kim et al, 2008).

Interaction of Pax5 isoforms with alternatively spliced 3` ends with the gene expression of downstream targets can potentially influence the kinetics of B cell proliferation and differentiation.

Functional 'in-vitro' Studies

To further investigate the functional role of each alternatively spliced isoform, EMSA studies of each isoform with target Pax5 binding sites from CD19 promoter can be performed to test DNA binding ability. Pax5 that possess an intact paired domain are known to possess DNA binding ability, and are hypothesized to function as dominant negative repressors of Pax5 activity. In our studies, 4 Pax5 isoforms possess an intact DNA binding domain; each must be tested for efficient DNA binding so that their role in B cell activation can be elucidated.

The trans-activation potential of each isoform must be evaluated using transient transfection or co-transfection assays and a reporter gene system, such as the CAT or luciferase assays. Based on the deletion of certain exons we have made predictions about the trans-activity of each Pax5 isoform and classified them as activators and repressors (or co-activators and co-repressors). Evaluating the trans-activation ability of an isoforms is the first step towards understanding the role of Pax5 isoforms in the genetic regulation of the B cell.

Using RNAi, micro-RNA or ribozyme suppression system to selectively knockout isoform production, as recently described by Robichaud et al, 2008, may be a useful tool to study the role of Pax5 isoforms in B cell activation and terminal differentiation events.

Analysis of functional role of Pax5 isoforms would help tease apart Pax-5's complex regulatory control over the 140 or more down-stream target genes involved in B cell development.

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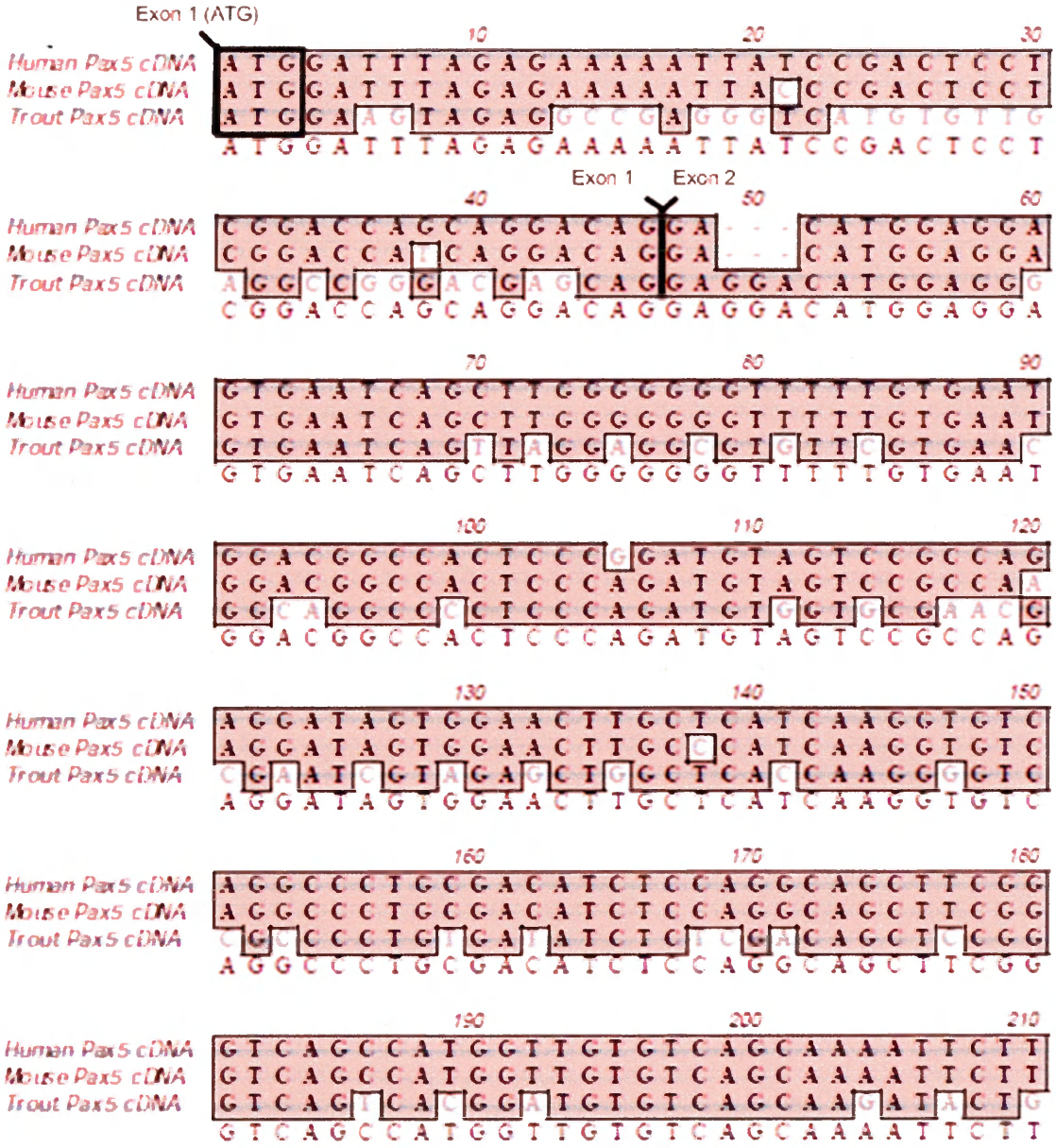
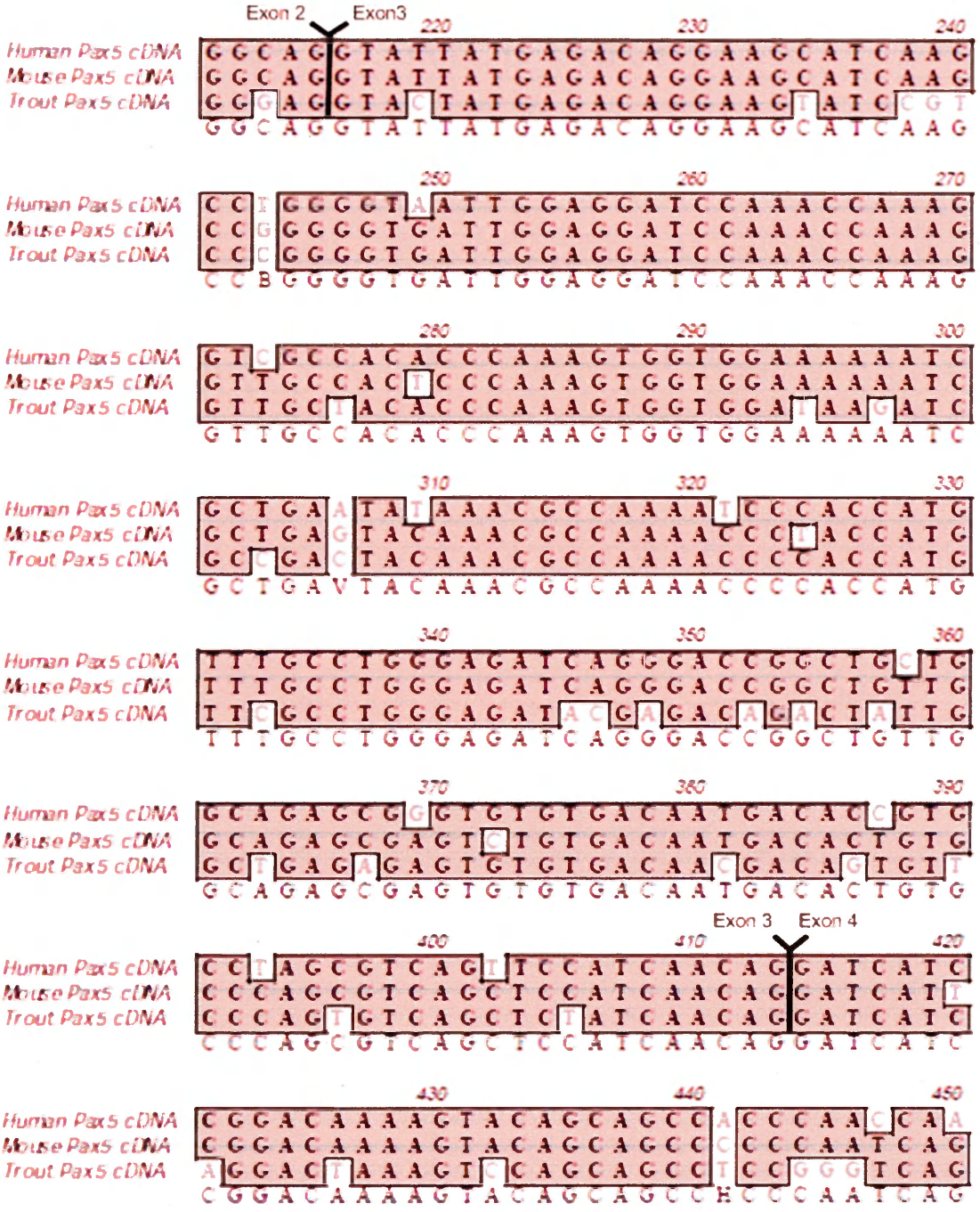
Appendix

1. Multiple Alignments of Human, mouse and trout full-length Pax5. cDNA sequence of mouse and human Pax5 exon junctions were used to predict trout Pax5 exon junctions.




Exon 2
Exon3
Exon 3
Exon 4
Human Pax5 cDNA
Mouse Pax5 cDNA
Trout Pax5 cDNA
220
230
240
250
260
270
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290
300
310
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330
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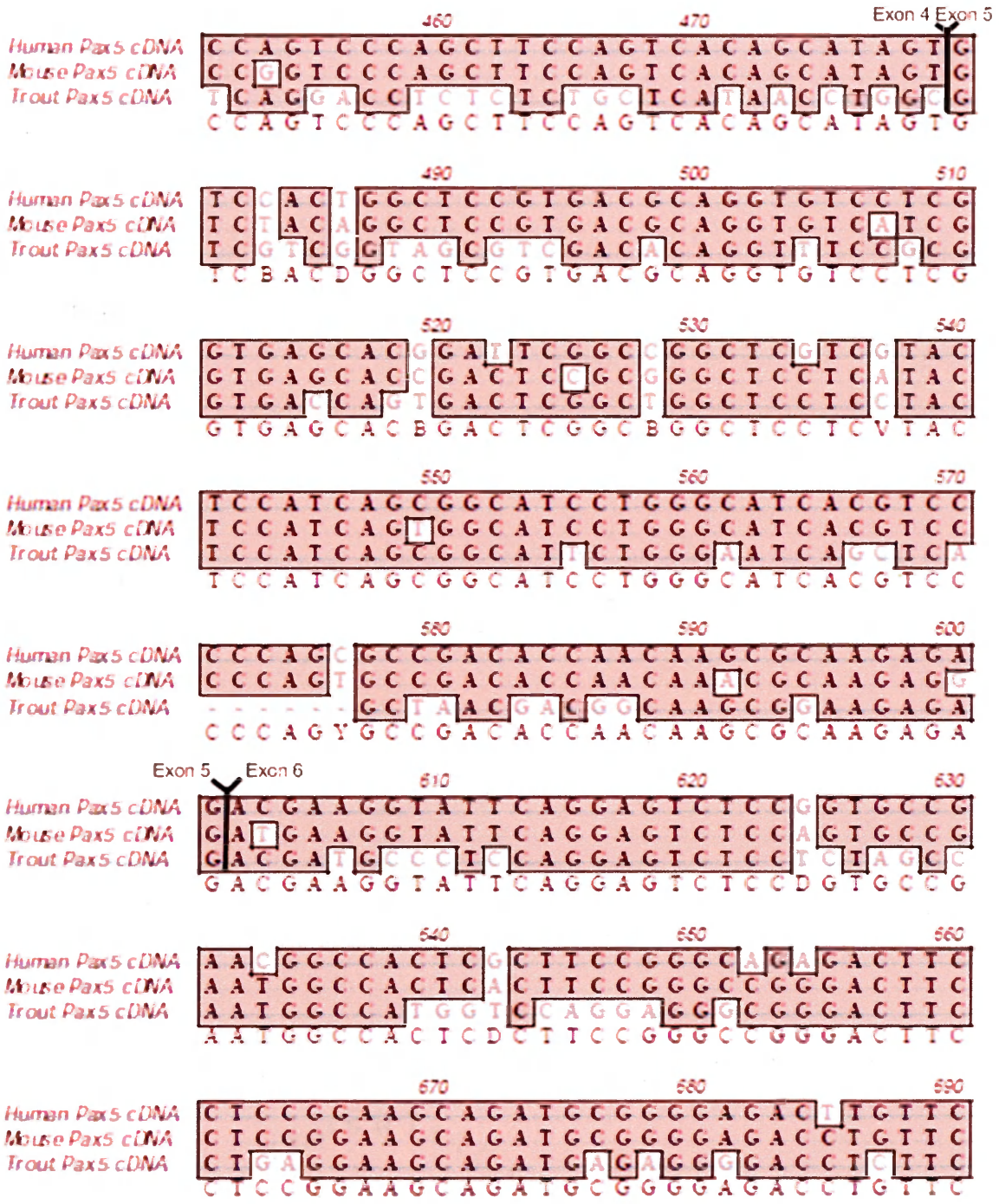
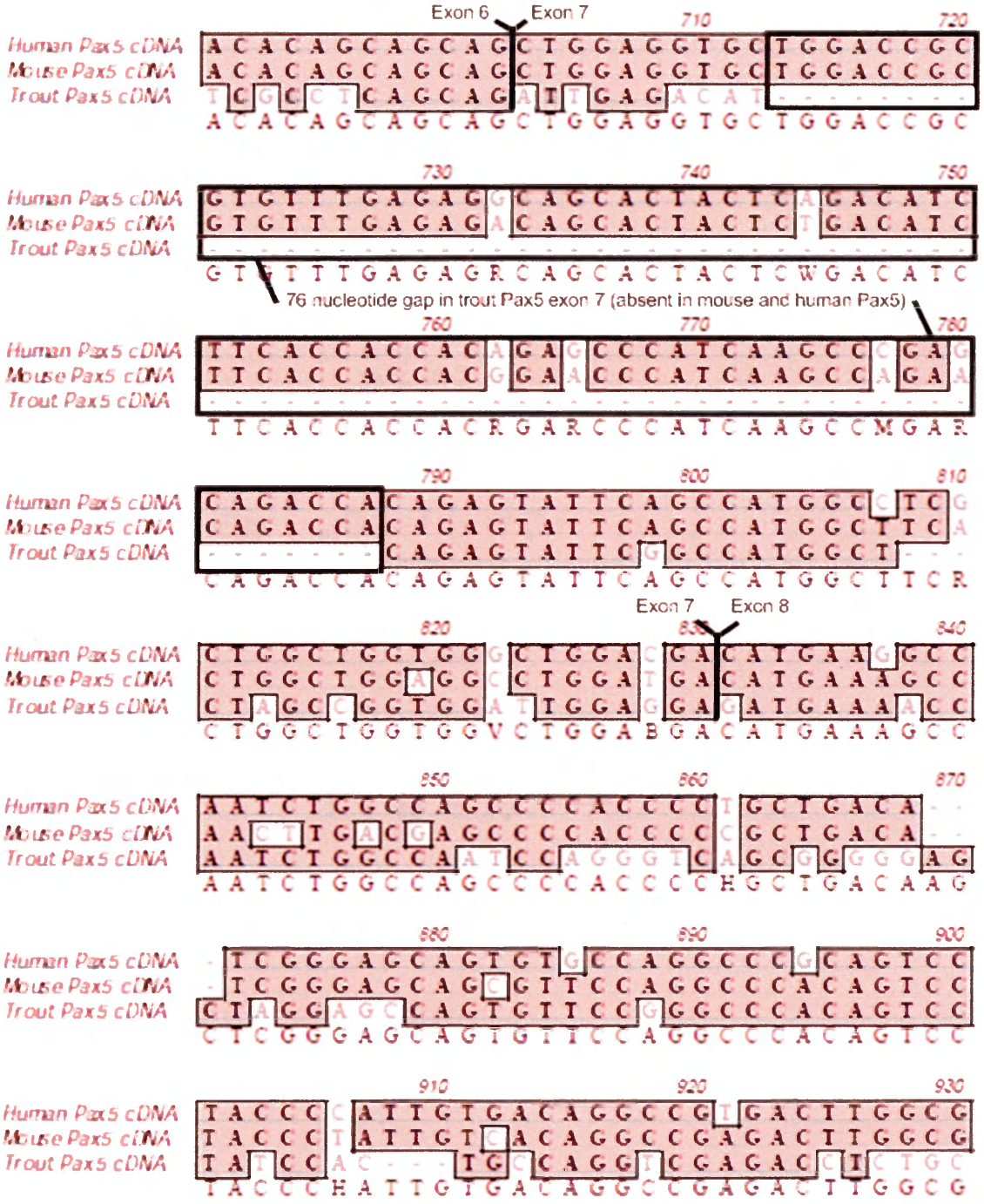
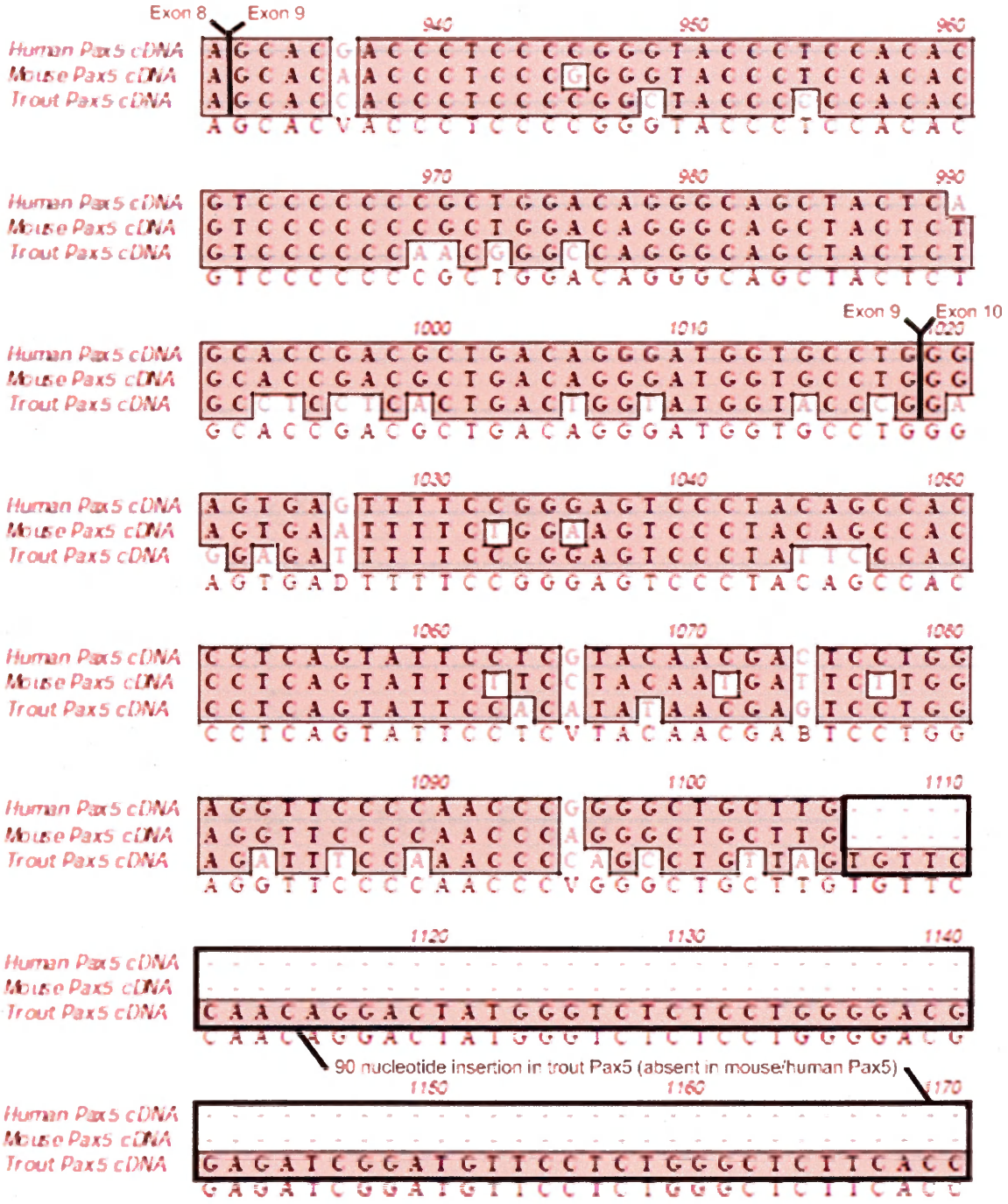
350
360
370
380
390
400
410
420
430
440
450


Exon 4 Exon 5
Exon 5 Exon 6
Human Pax5 cDNA
Mouse Pax5 cDNA
Trout Pax5 cDNA


Exon 6
Exon 7
Exon 7
Exon 8
Human Pax5 cDNA
Mouse Pax5 cDNA
Trout Pax5 cDNA
76 nucleotide gap in trout Pax5 exon 7 (absent in mouse and human Pax5)


Exon 8
Exon 9
Exon 9
Exon 10
Human Pax5 cDNA
Mouse Pax5 cDNA
Trout Pax5 cDNA
940
950
960
970
980
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1000
1010
1020
1030
1040
1050
1060
1070
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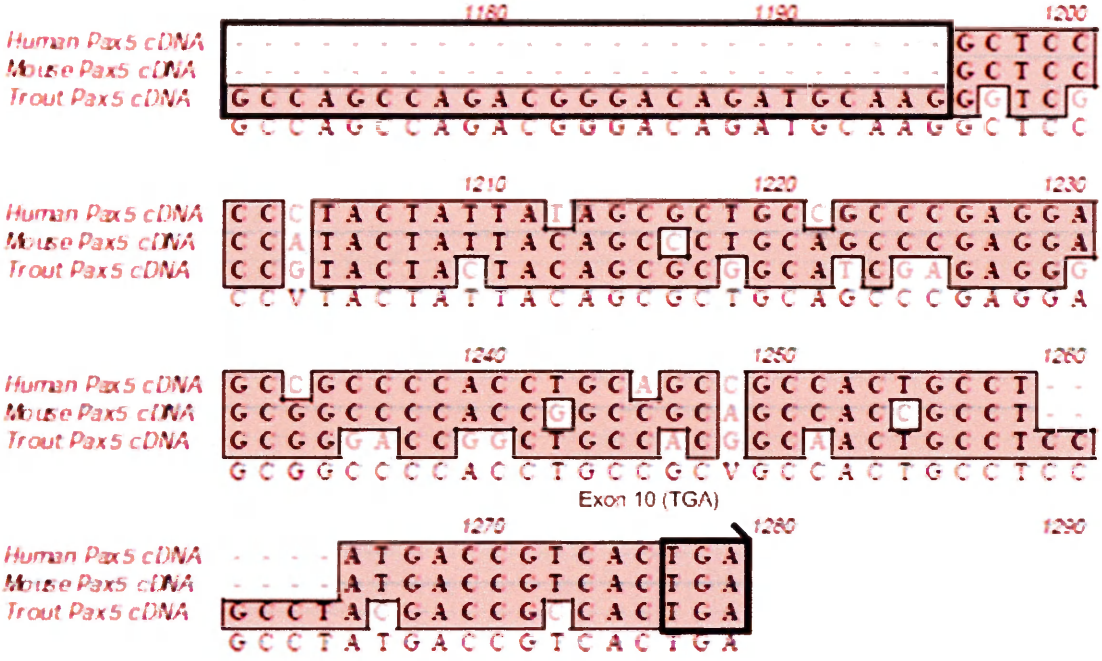
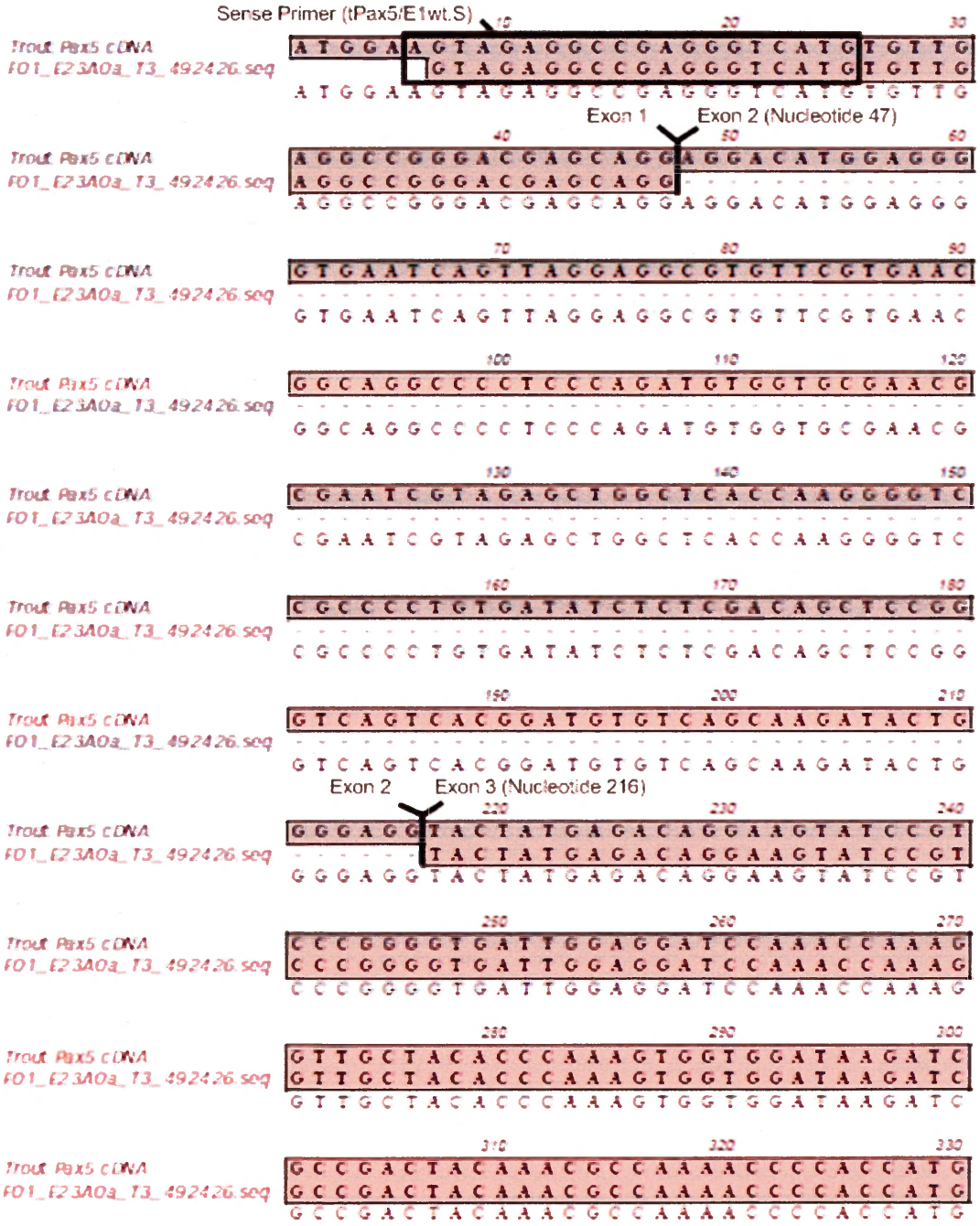
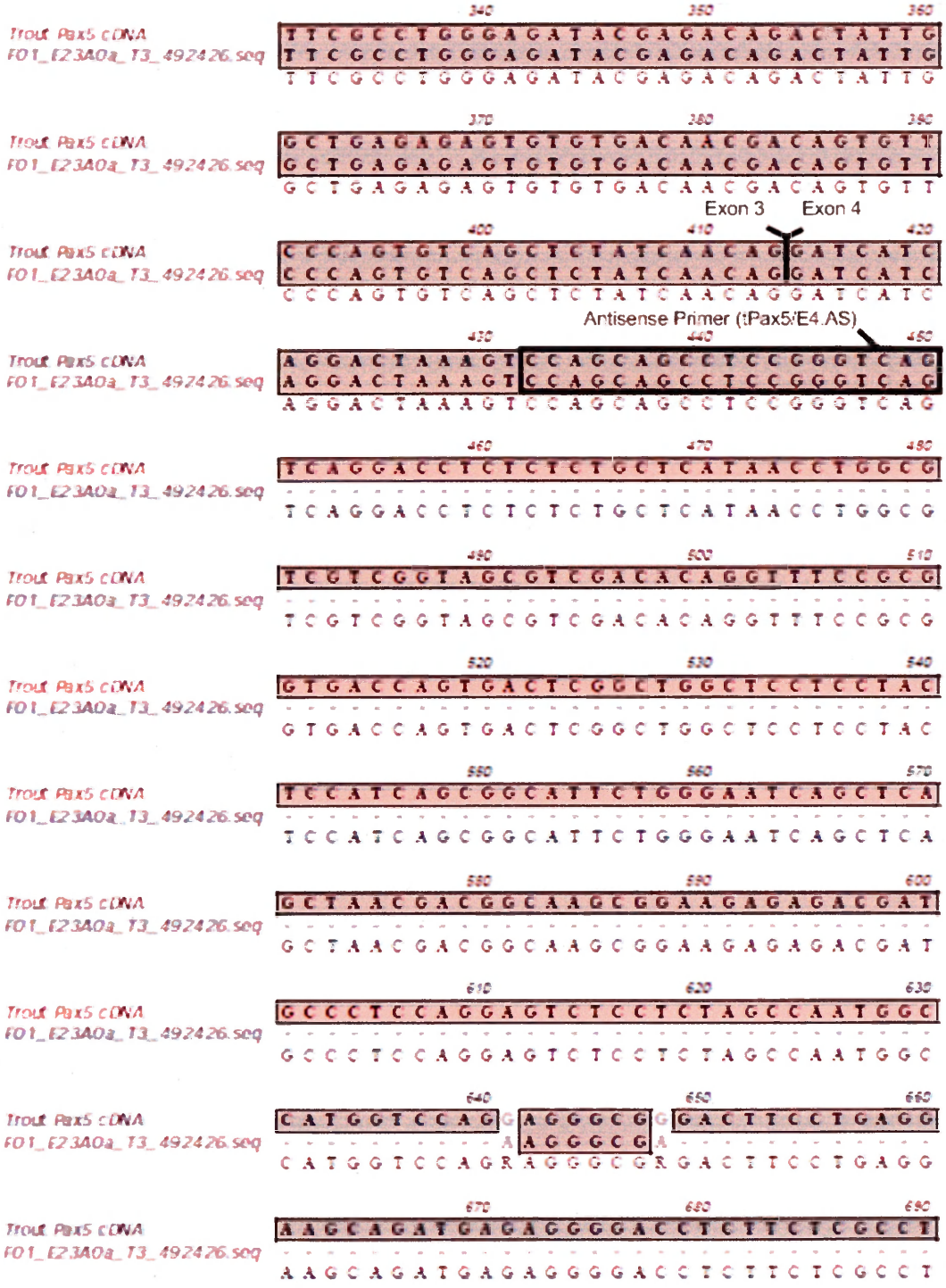
90 nucleotide insertion in trout Pax5 (absent in mouse/human Pax5)


1180
1190
1200
Human Pax5 cDNA
Mouse Pax5 cDNA
Trout Pax5 cDNA
1210
1220
1230
1240
1250
1260
Exon 10 (TGA)
1270
1280
1290

2. Formatted multiple alignment of full-length trout Pax5 and Pax5/1aΔ2 clone (contains exon 1a with deleted exon 2 (Nucleotides 47-216)- E23A0a_T3_492426).

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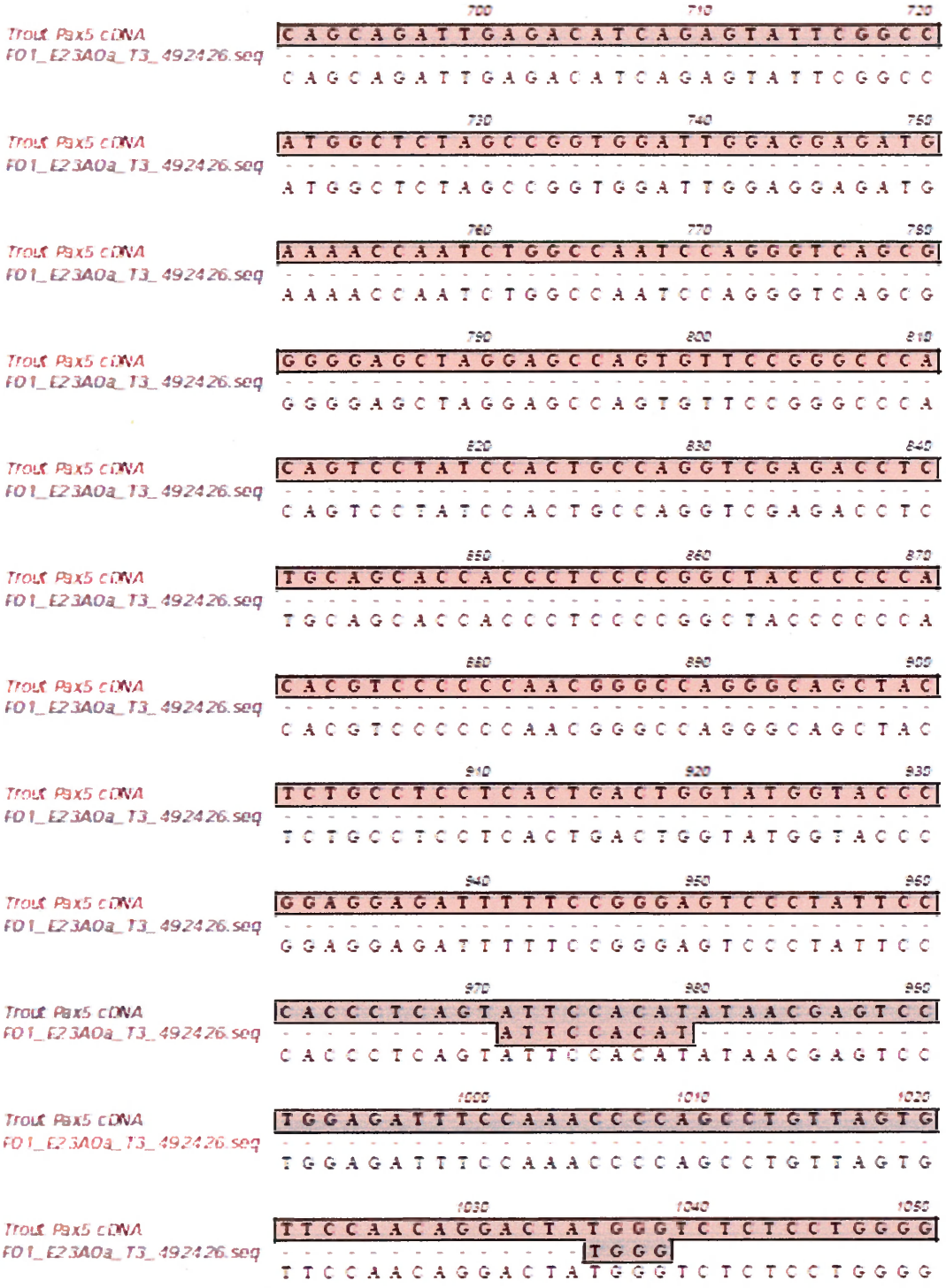
Exon 3
Exon 4
Antisense Primer (tPax5rE4.AS)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730 740 750
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760 770 780
AAAACCAATCTGGCCAATCCAGGGTCAGCG
790 800 810
GGGGAGCTAGGAGCCAGTGTTCCGGGCCCA
820 830 840
CAGTCCTATCCACTGCCAGGTCGAGACCTC
850 860 870
TGCAGCACCACCCTCCCCGGCTACCCCCCA
880 890 900
CACGTCCCCCCCAACGGGCCAGGGCAGCTAC
910 920 930
TCTGCCTCCTCACTGACTGGTATGGTACCC
940 950 960
GGAGGAGATTTTTCCGGGAGTCCCTATTCC
970 980 990
CACCCTCAGTATTCCACATAATAACGAGTCC
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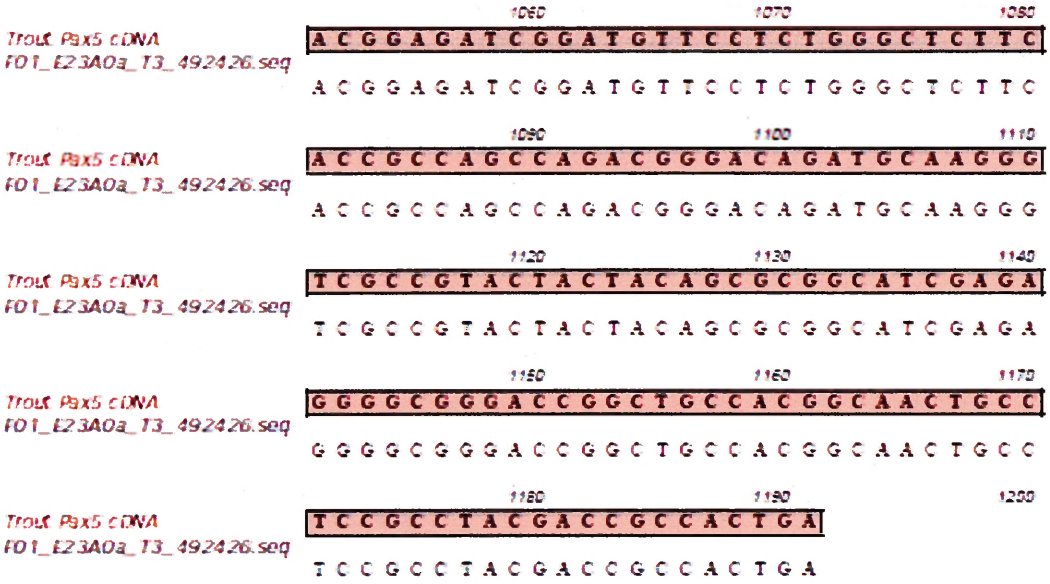
TGGG


1060
1070
1080
Trout Pax5 cDNA
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ACGGAGATCGGATGTTCCTCTGGGCTCTTC
1090
1100
1110
Trout Pax5 cDNA
FD1_E23A0a_T3_492426.seq
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ACCGCCAGCCAGACGGGACAGATGCAAGGG
1120
1130
1140
Trout Pax5 cDNA
FD1_E23A0a_T3_492426.seq
TCGCCGTACTACTACAGCGCGGCATCGAGA
TCGCCGTACTACTACAGCGCGGCATCGAGA
1150
1160
1170
Trout Pax5 cDNA
FD1_E23A0a_T3_492426.seq
GGGGCGGGACCGGCTGCCACGGCAACTGCC
GGGGCGGGACCGGCTGCCACGGCAACTGCC
1180
1190
1200
Trout Pax5 cDNA
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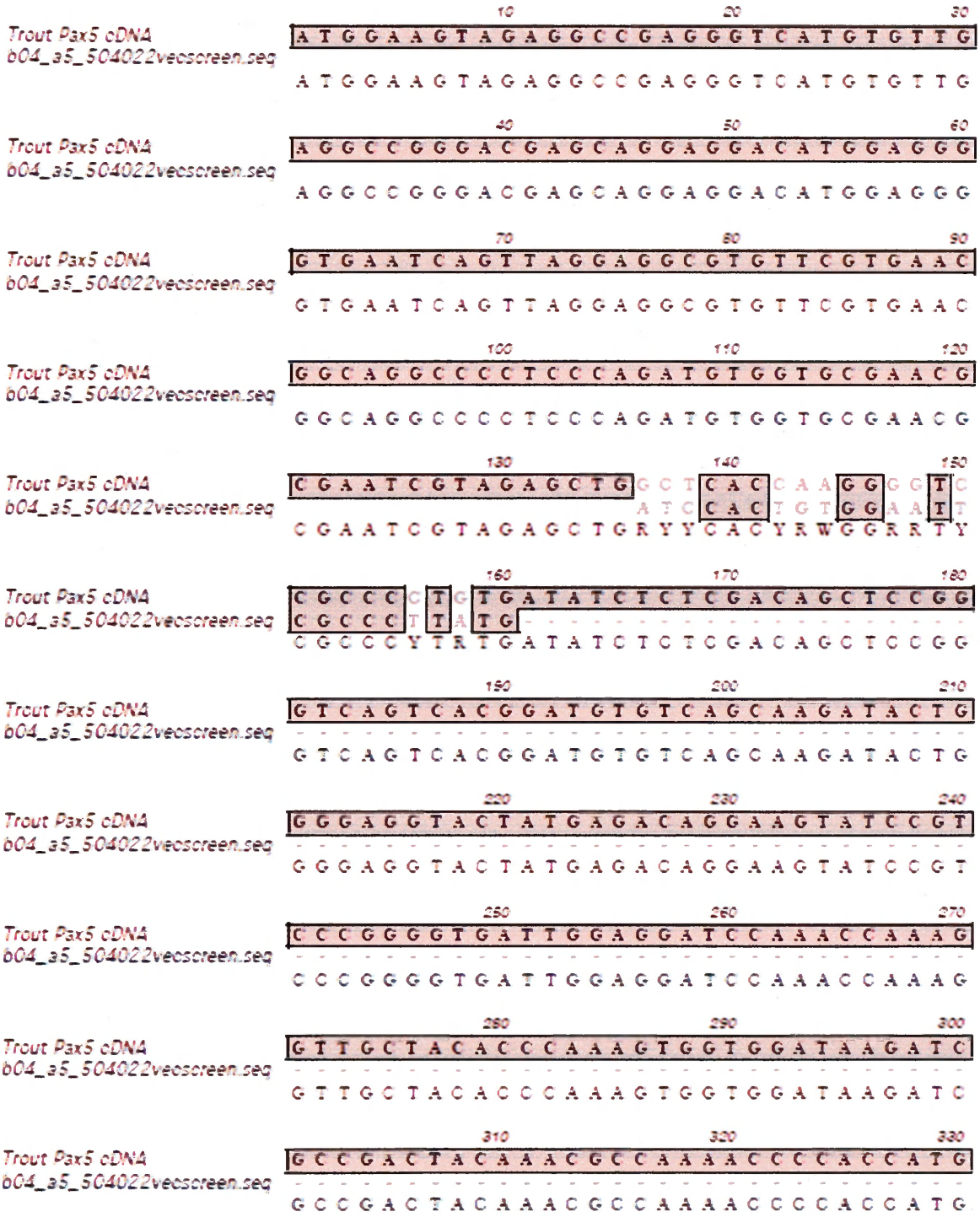
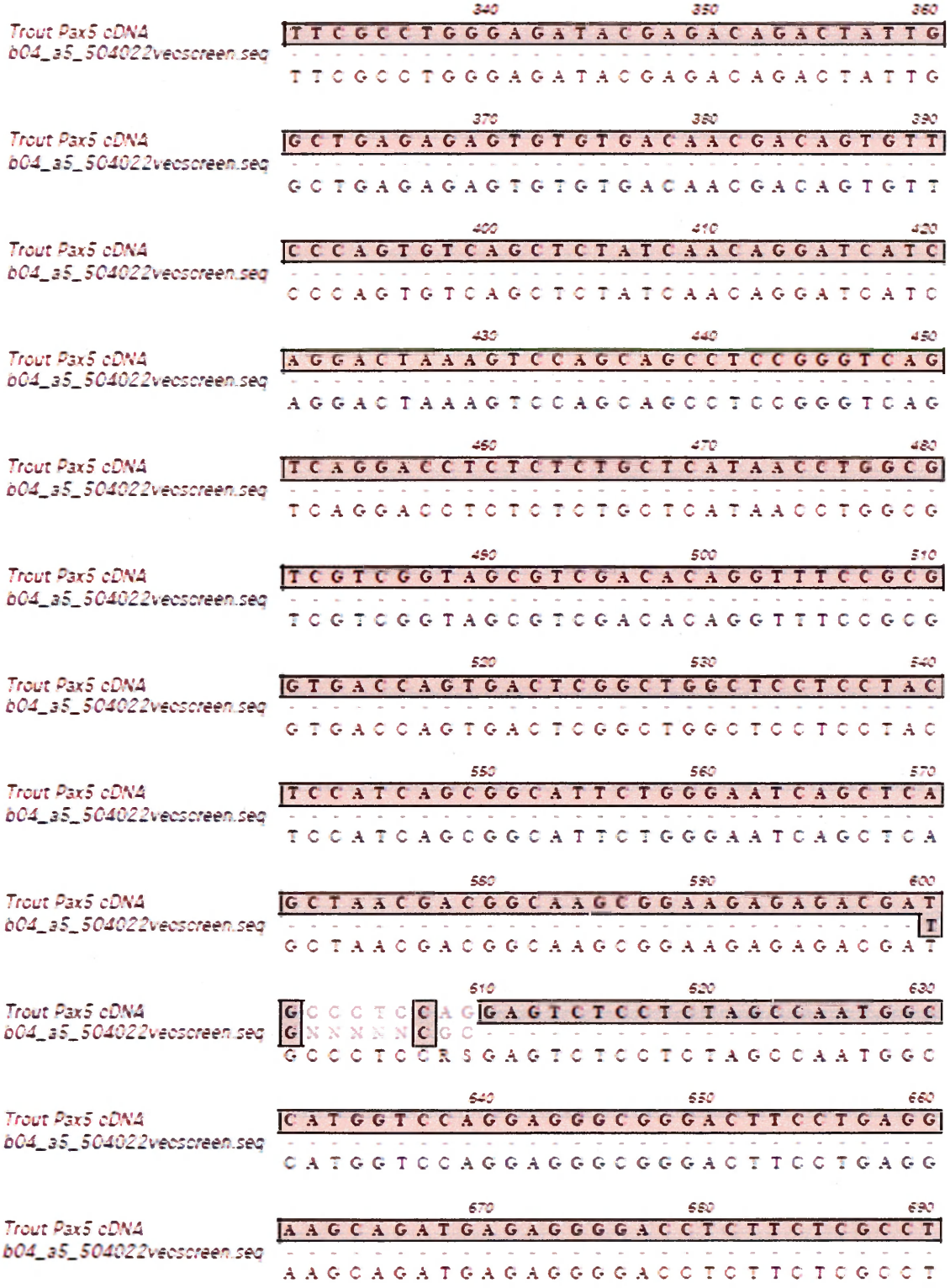
TCCGCCTACGACCGCCACTGA
TCCGCCTACGACCGCCACTGA

3. Formatted multiple alignment of full-length trout Pax5 and Pax5/Δ8 clone (with deleted exon 8 (Nucleotides 832-931) - b04_a5_ 504022).

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GCTGAGAGAGTGTGTGACAACGACAGTGTT
400 410 420
CCCAGTGTCAGCTCTATCAACAGGATCATC
CCCAGTGTCAGCTCTATCAACAGGATCATC
430 440 450
AGGACTAAAGTCCAGCAGCCTCCGGGTCAG
AGGACTAAAGTCCAGCAGCCTCCGGGGTCAG
460 470 480
TCAGGACCTCTCTCTGCTCATAACCTGGCG
TCAGGACCTCTCTCTGCTCATAACCTGGCG
490 500 510
TCGTCGGTAGCGTCGACACAGGTTTCCGCG
TCGTCGGGTAGCGTCGACACAGGTTTCCGCG
520 530 540
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GTGACCAGTGACTCGGCTGGCTCCTCCTAC
550 560 570
TCCATCAGCGGCATTCTGGGAATCAGCTCA
TCCATCAGCGGCATTCTGGGAATCAGCTCA
580 590 600
GCTAACGACGGCAAGCGGAAGAGAGACGAT
T
GCTAACGACGGCAAGCGGAAGAGAGACGAT
610 620 630
GCCCTCCAGGAGTCTCCTCTAGCCAATGGC
GNNNNCGC
GCCCTCCRSGAGTCTCCTCTAGCCAATGGC
640 650 660
CATGGTCCAGGAGGGCGGGACTTCCTGAGG
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670 680 690
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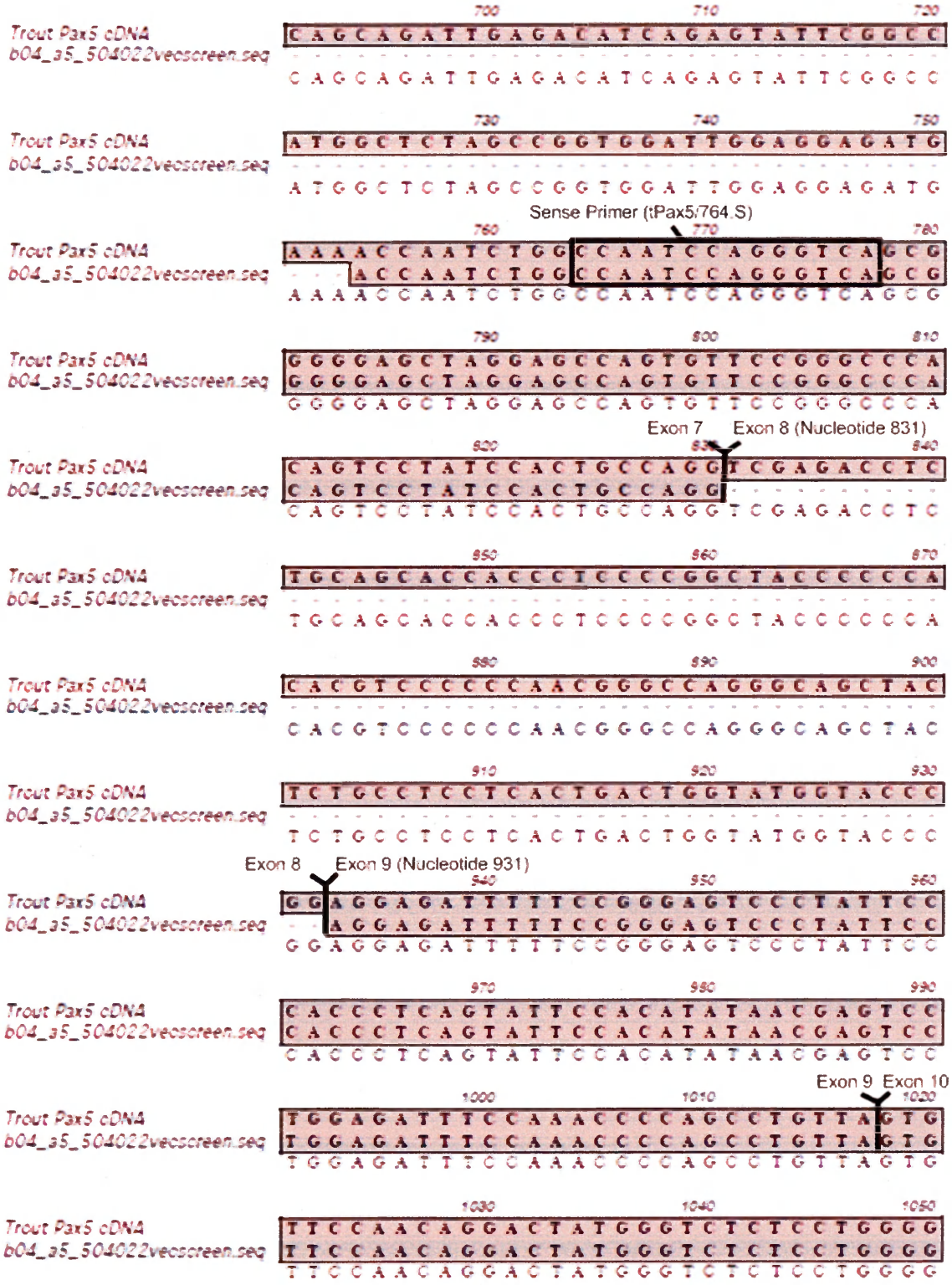
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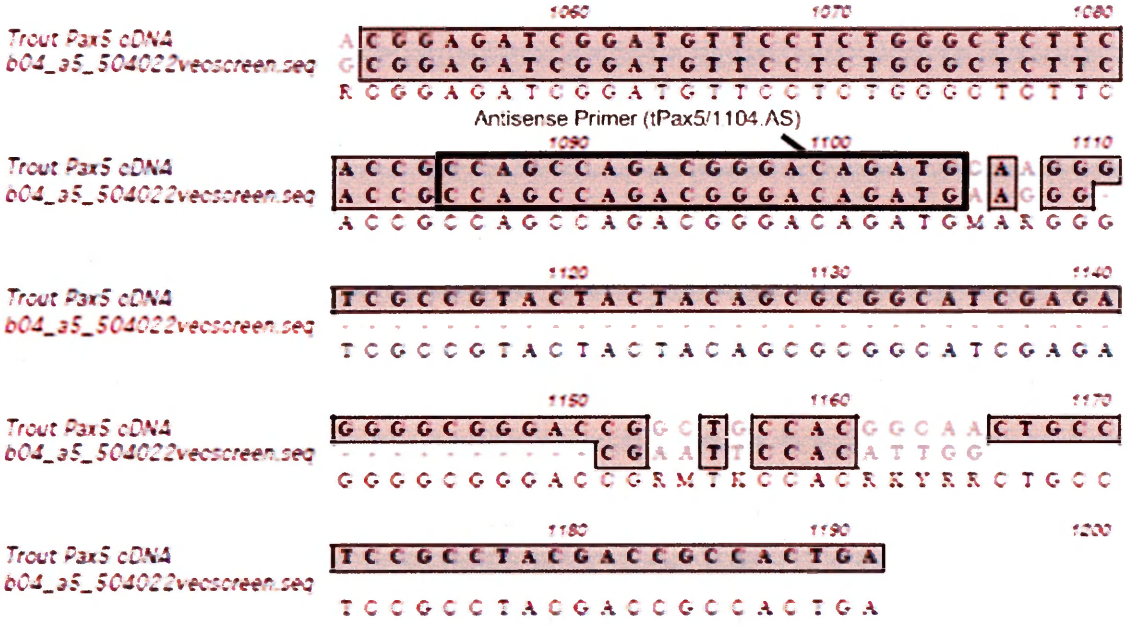
Sense Primer (tPax5i764.S)
Exon 7
Exon 8 (Nucleotide 831)
Exon 8
Exon 9 (Nucleotide 931)
Exon 9
Exon 10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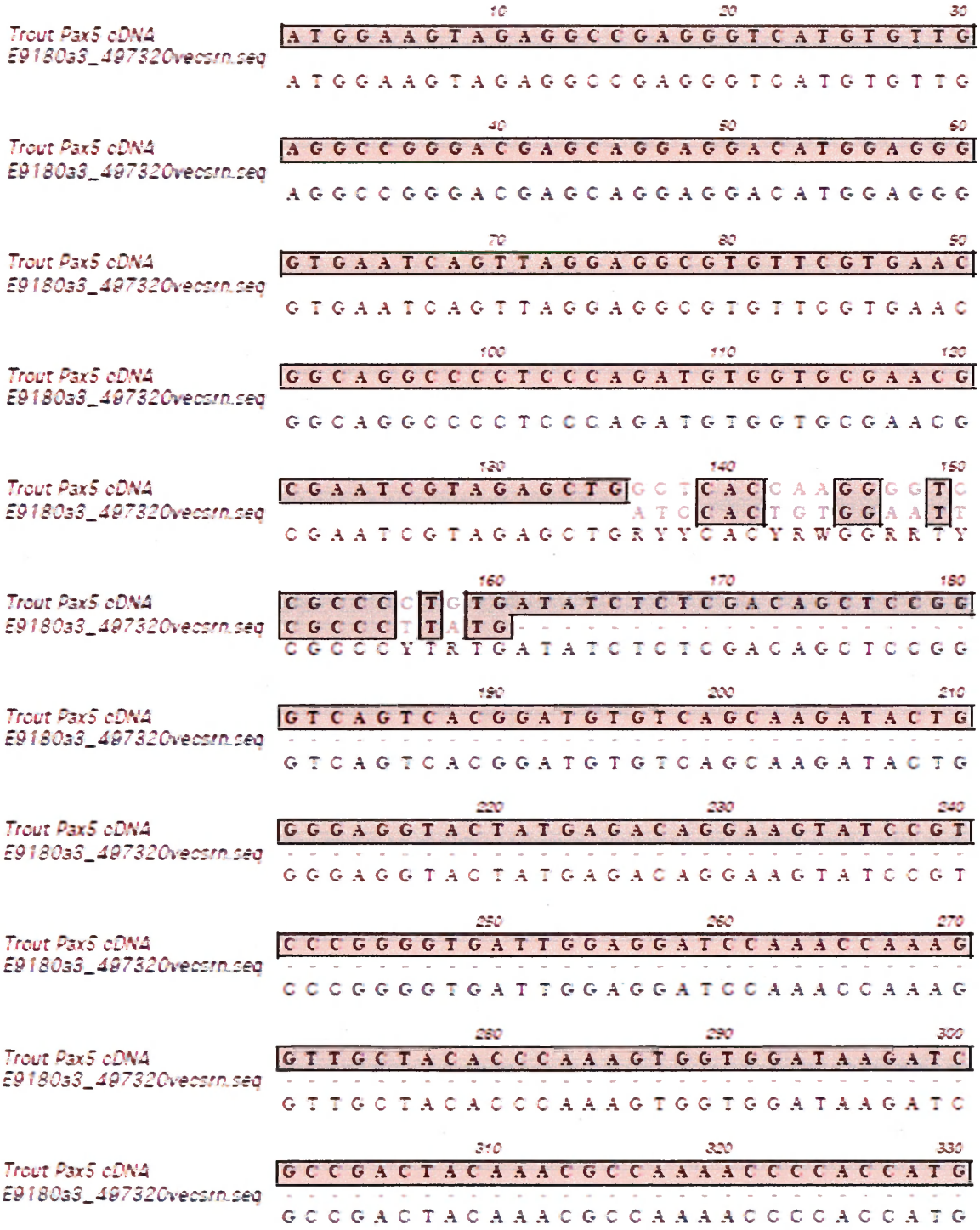
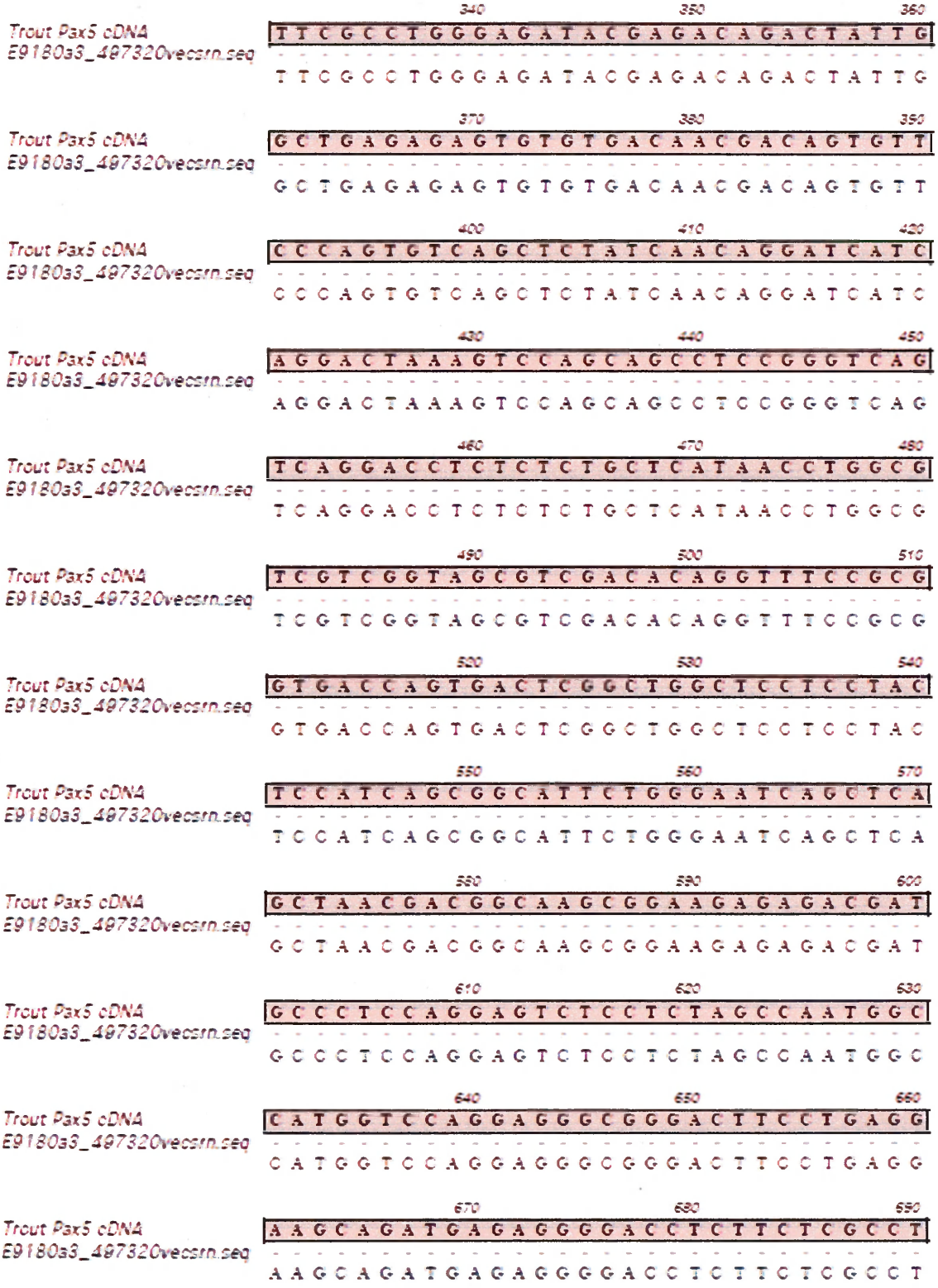
Antisense Primer (tPax5/1104.AS)

4. Formatted multiple alignment of full-length trout Pax5 and Pax5/Δ9a clone (with deleted exon 9 (nucleotides 932-1018) – E9180a3_497320).

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TTCGCCTGGGAGATACGAGACAGACTATTG
370 380 390
Trout Pax5 cDNA
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GCTGAGAGAGTGTGTGACAACGACAGTGTT
GCTGAGAGAGTGTGTGACAACGACAGTGTT
400 410 420
Trout Pax5 cDNA
E9180a3_497320vecsrn.seq
CCCAGTGTCAGCTCTATCAACAGGATCATC
CCCAGTGTCAGCTCTATCAACAGGATCATC
430 440 450
Trout Pax5 cDNA
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AGGACTAAAGTCCAGCAGCCTCCGGGTCAG
460 470 480
Trout Pax5 cDNA
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TCAGGACCTCTCTCTCTGCTCATAACCTGGCG
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490 500 510
Trout Pax5 cDNA
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TCGTCGGTAGCGTCGACACAGGTTTCCGCG
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520 530 540
Trout Pax5 cDNA
E9180a3_497320vecsrn.seq
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GTGACCAGTGACTCGGCTGGCTCCTCCTAC
550 560 570
Trout Pax5 cDNA
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TCCATCAGCGGCATTCTGGGAATCAGCTCA
580 590 600
Trout Pax5 cDNA
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GCTAACGACGGCAAGCGGAAGAGAGACGAT
610 620 630
Trout Pax5 cDNA
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GCCCTCCAGGAGTCTCCTCTAGCCAATGGC
640 650 660
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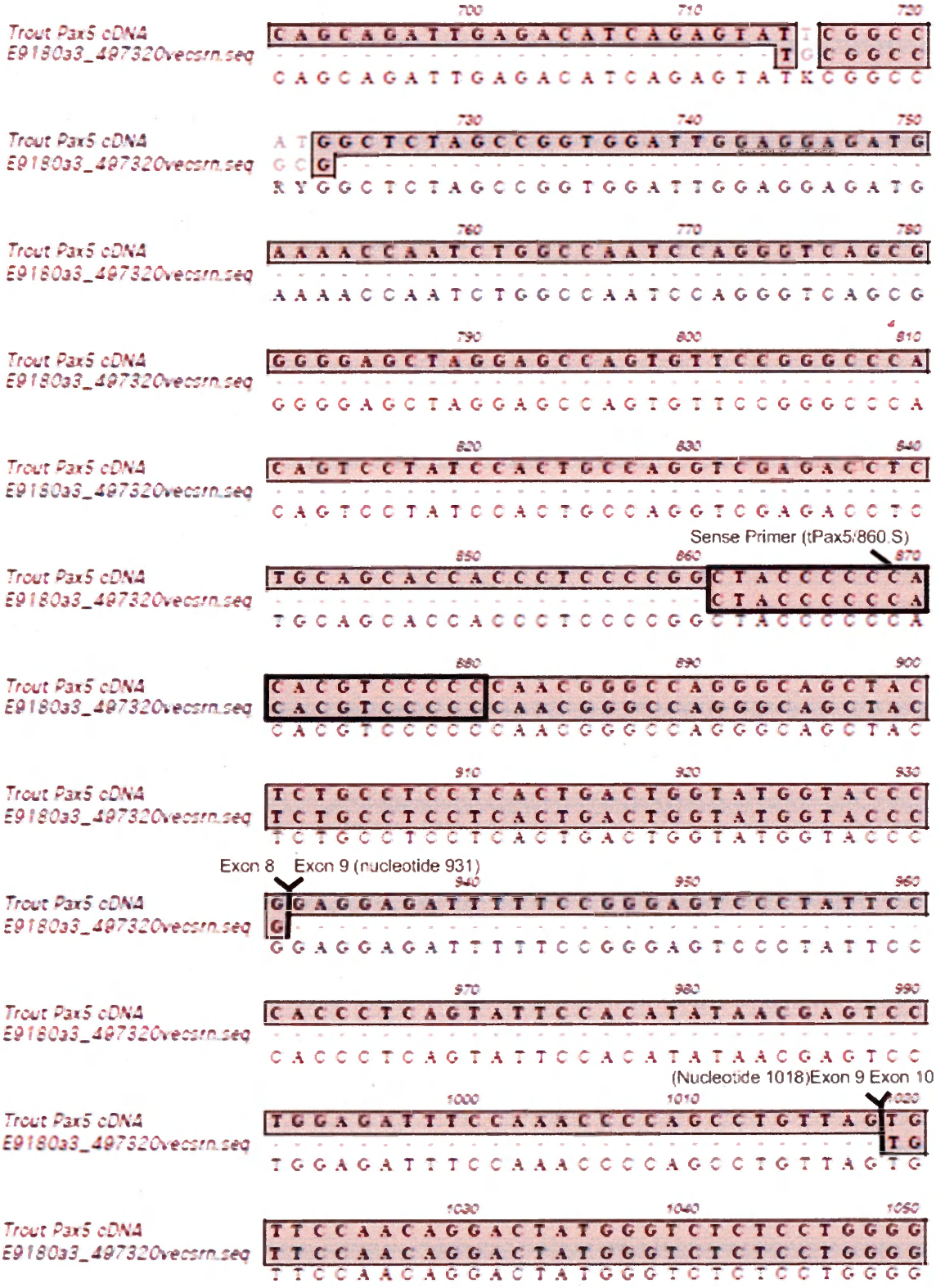
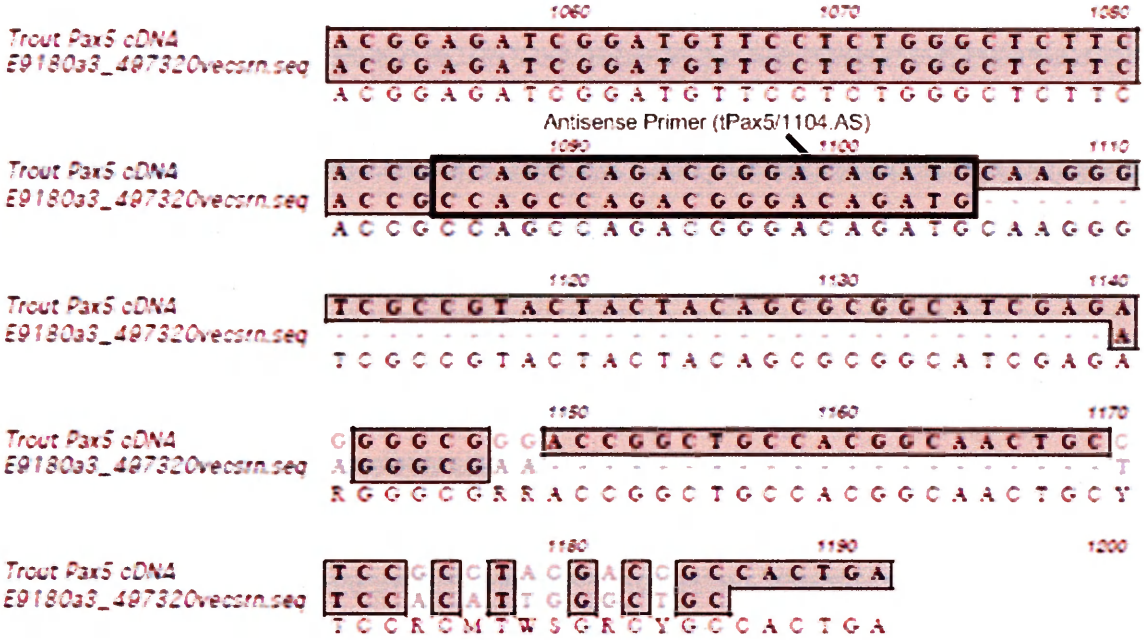
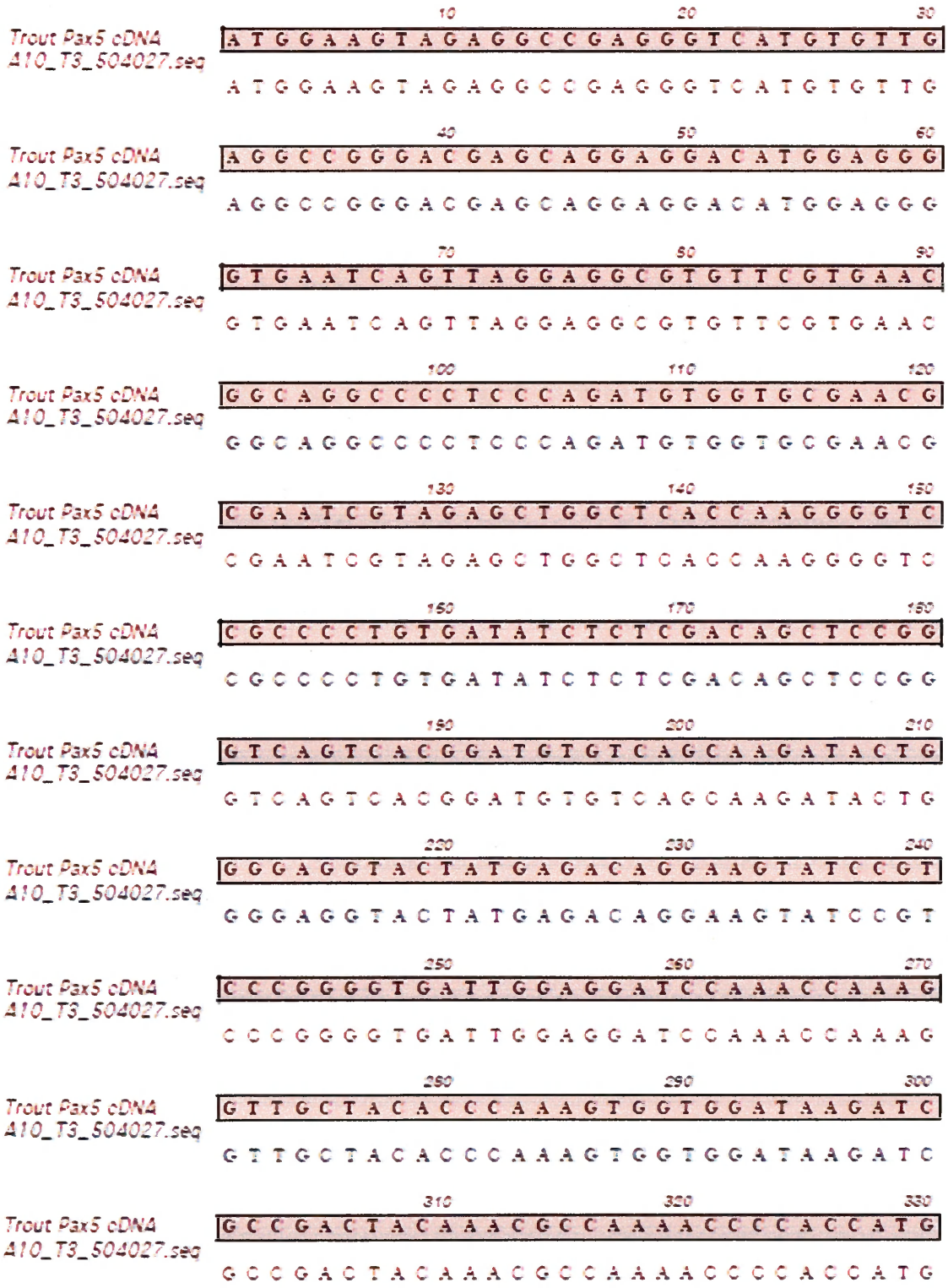
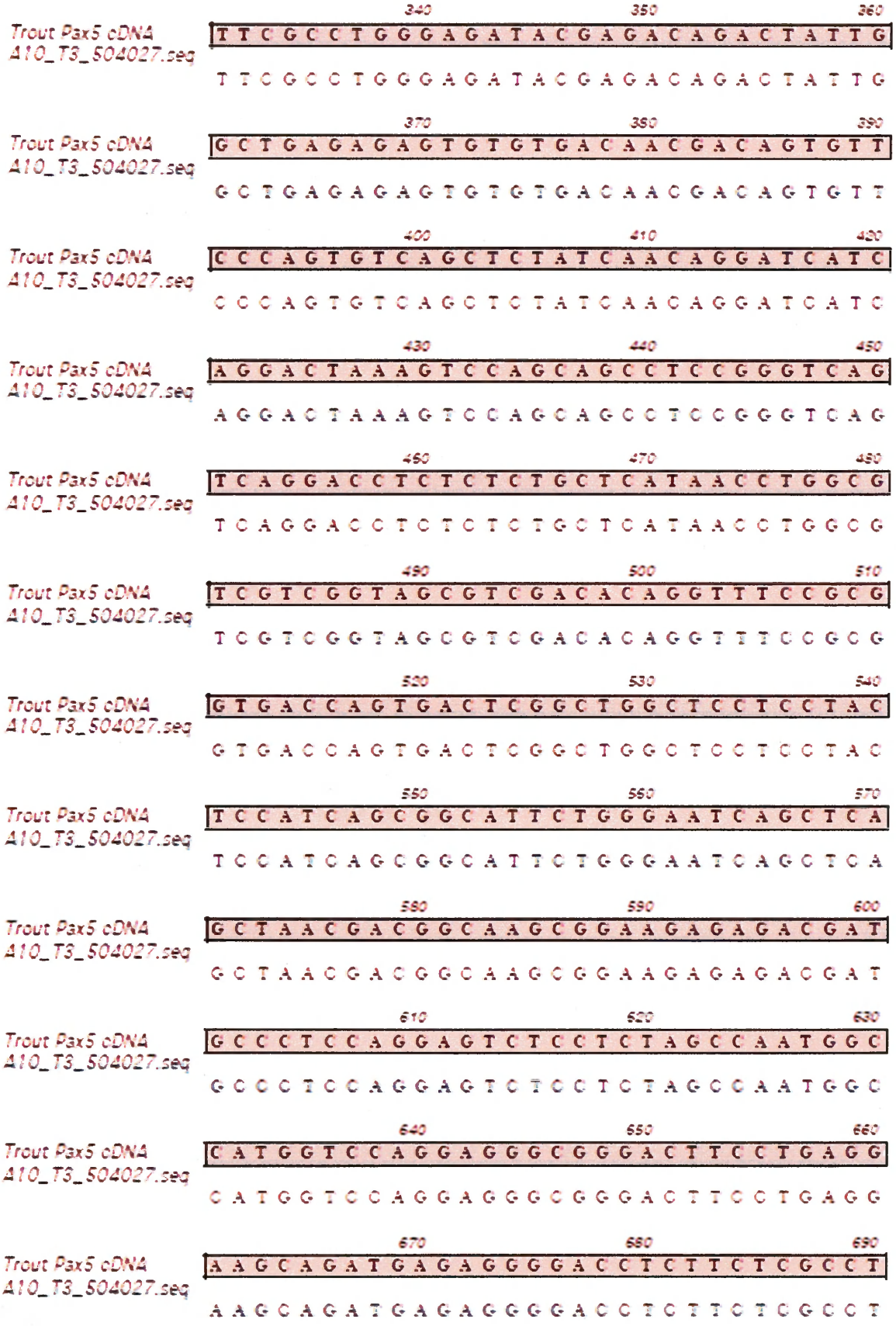
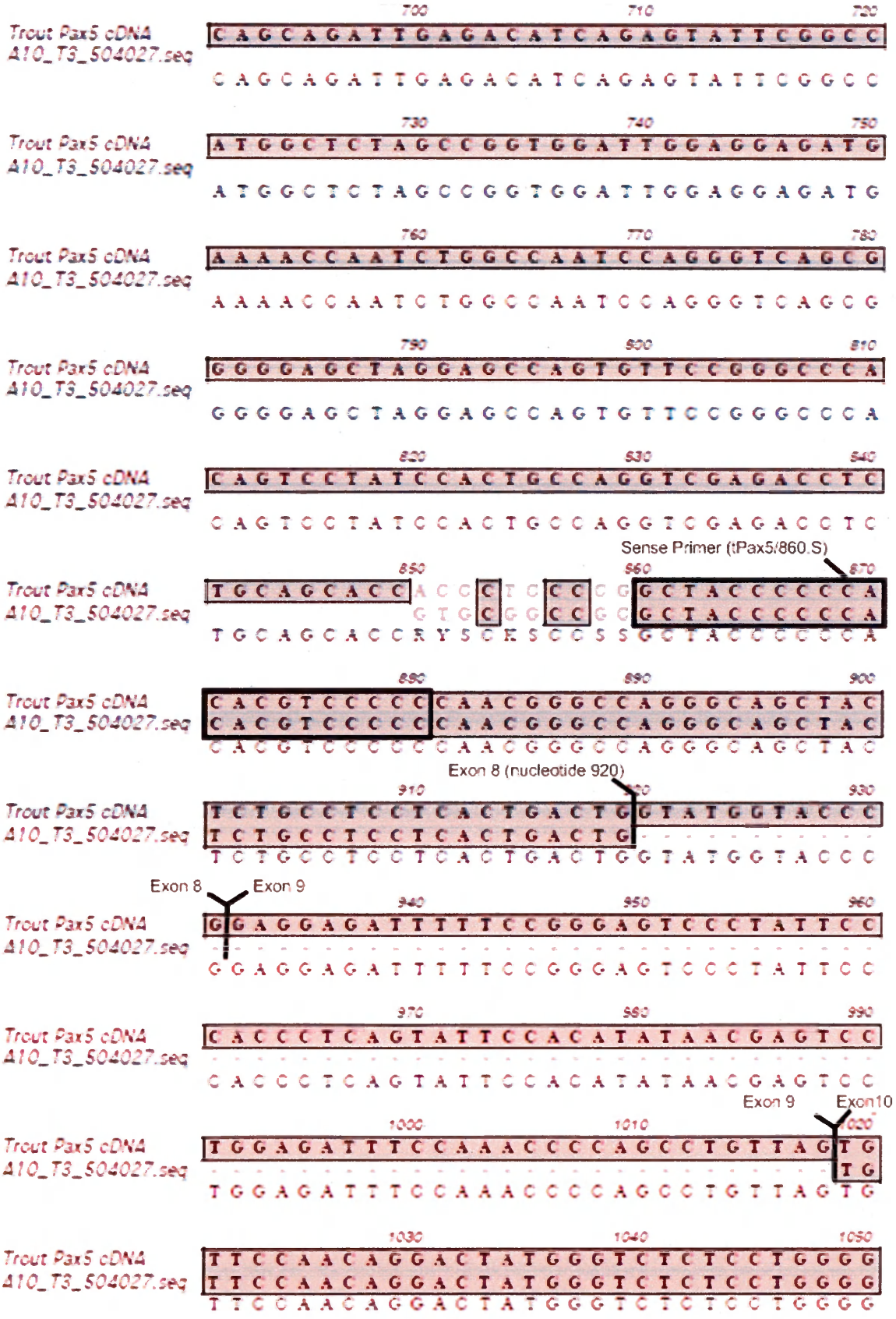

Trout Pax5 cDNA
E9180a3_497320vecsrn.seq
Sense Primer (tPax5/860.S)
Exon 8
Exon 9 (nucleotide 931)
(Nucleotide 1018)Exon 9 Exon 10

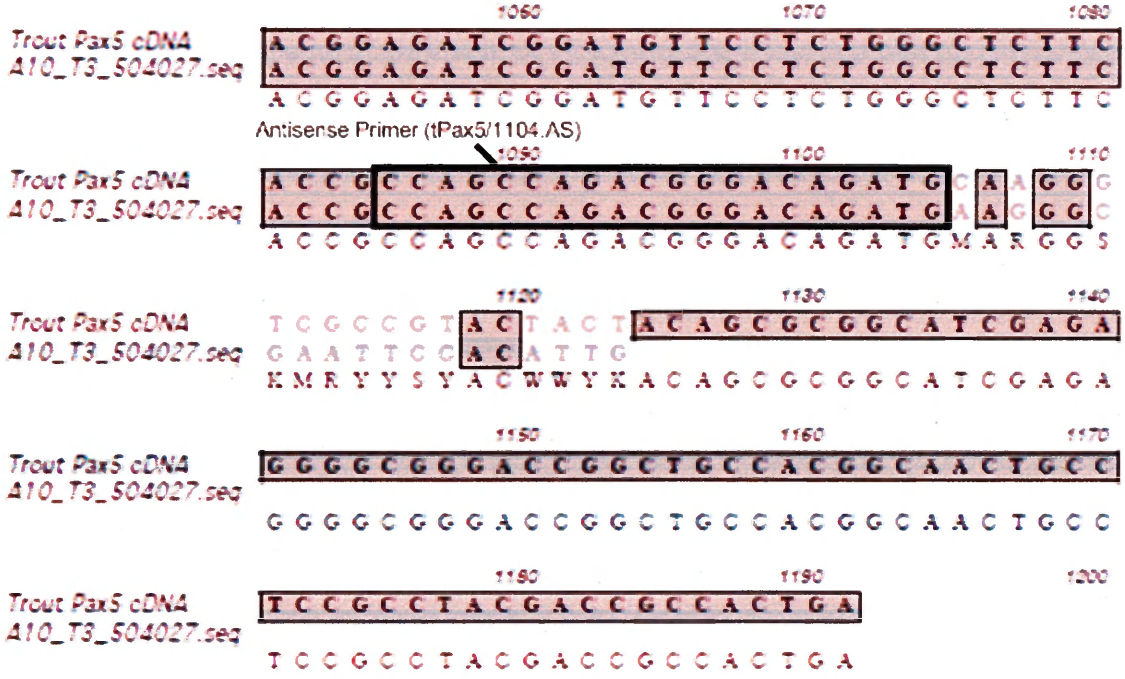

Trout Pax5 cDNA
E9180a3_497320vecsrn.seq
1060
1070
1080
Antisense Primer (tPax5/1104.AS)
1090
1100
1110
1120
1130
1140
1150
1160
1170
1180
1190
1200

5. Formatted multiple alignment of full-length trout Pax5 and Pax5/Δ9b clone (with deleted exon 9 (nucleotides 920 to 1018) – A10_T3_504027).

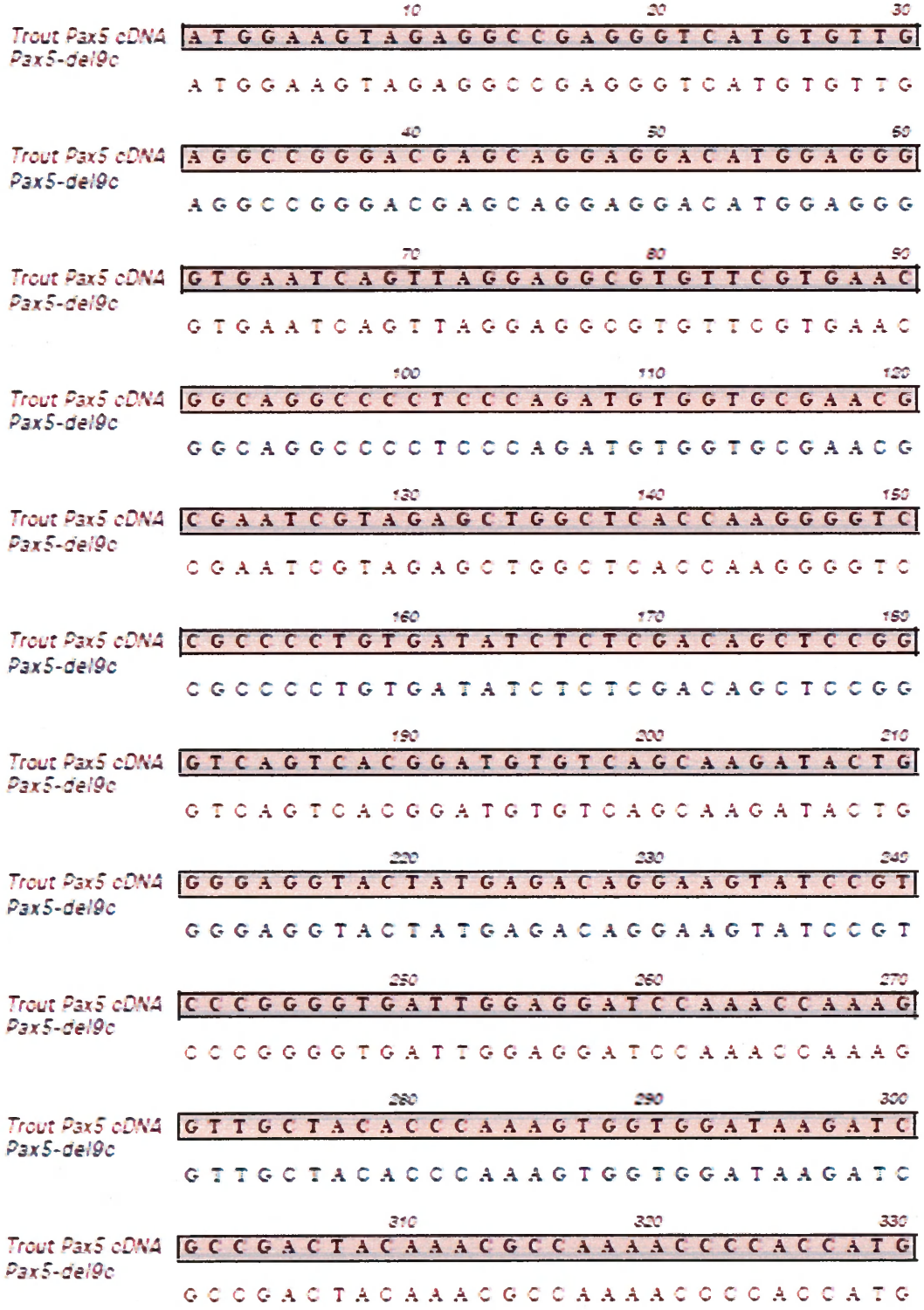


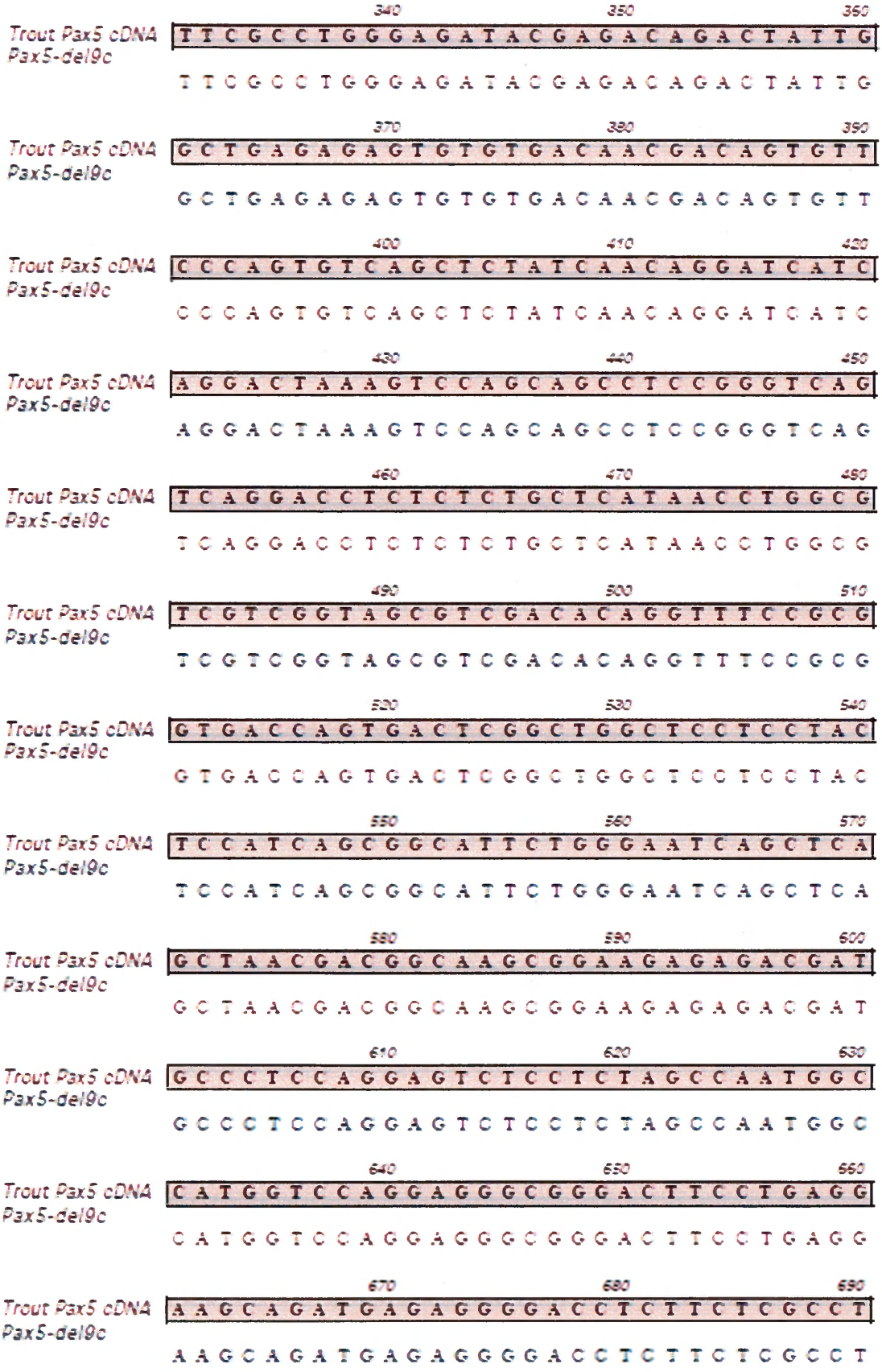

340
350
360
Trout Pax5 cDNA A10_T3_504027.seq
TTCGCCTGGGAGATACGAGACAGACTATTG
TTCGCCTGGGAGATACGAGACAGACTATTG
370
380
390
Trout Pax5 cDNA A10_T3_504027.seq
GCTGAGAGAGTGTGTGACAACGACAGTGTT
GCTGAGAGAGTGTGTGACAACGACAGTGTT
400
410
420
Trout Pax5 cDNA A10_T3_504027.seq
CCCAGTGTCAGCTCTATCAACAGGATCATC
CCCAGTGTCAGCTCTATCAACAGGATCATC
430
440
450
Trout Pax5 cDNA A10_T3_504027.seq
AGGACTAAAGTCCAGCAGCCTCCGGGTCAG
AGGACTAAAGTCCAGCAGCCTCCGGGTCAG
460
470
480
Trout Pax5 cDNA A10_T3_504027.seq
TCAGGACCTCTCTCTGCTCATAACCTGGCG
TCAGGACCTCTCTCTGCTCATAACCTGGCG
490
500
510
Trout Pax5 cDNA A10_T3_504027.seq
TCGTCGGTAGCGTCGACACAGGTTTCCGCG
TCGTCGGTAGCGTCGACACAGGTTTCCGCG
520
530
540
Trout Pax5 cDNA A10_T3_504027.seq
GTGACCAGTGACTCGGCTGGCTCCTCCTAC
GTGACCAGTGACTCGGCTGGCTCCTCCTAC
550
560
570
Trout Pax5 cDNA A10_T3_504027.seq
TCCATCAGCGGCATTCTGGGAATCAGCTCA
TCCATCAGCGGCATTCTGGGAATCAGCTCA
580
590
600
Trout Pax5 cDNA A10_T3_504027.seq
GCTAACGACGGCAAGCGGAAGAGAGACGAT
GCTAACGACGGCAAGCGGAAGAGAGACGAT
610
620
630
Trout Pax5 cDNA A10_T3_504027.seq
GCCCTCCAGGAGTCTCCTCTAGCCAATGGC
GCCCTCCAGGAGTCTCCTCTAGCCAATGGC
640
650
660
Trout Pax5 cDNA A10_T3_504027.seq
CATGGTCCAGGAGGGCGGGACTTCCTGAGG
CATGGTCCAGGAGGGCGGGACTTCCTGAGG
670
680
690
Trout Pax5 cDNA A10_T3_504027.seq
AAGCAGATGAGAGGGGACCTCTTCTCGCCT
AAGCAGATGAGAGGGGACCTCTTCTCGCCT

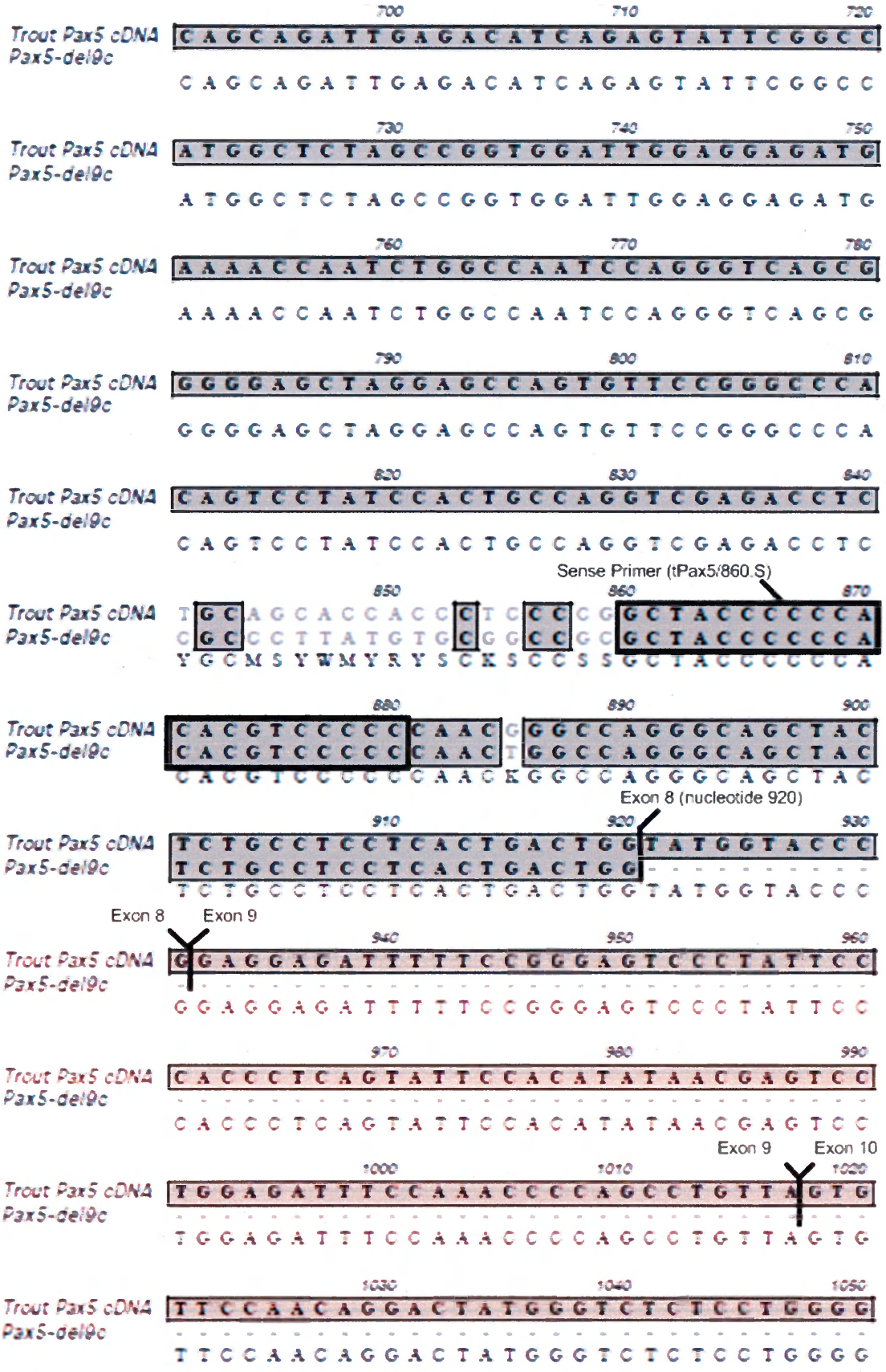

Trout Pax5 cDNA A10_T3_504027.seq
Sense Primer (tPax5/860.S)
Exon 8 (nucleotide 920)
Exon 8
Exon 9
Exon 9
Exon10

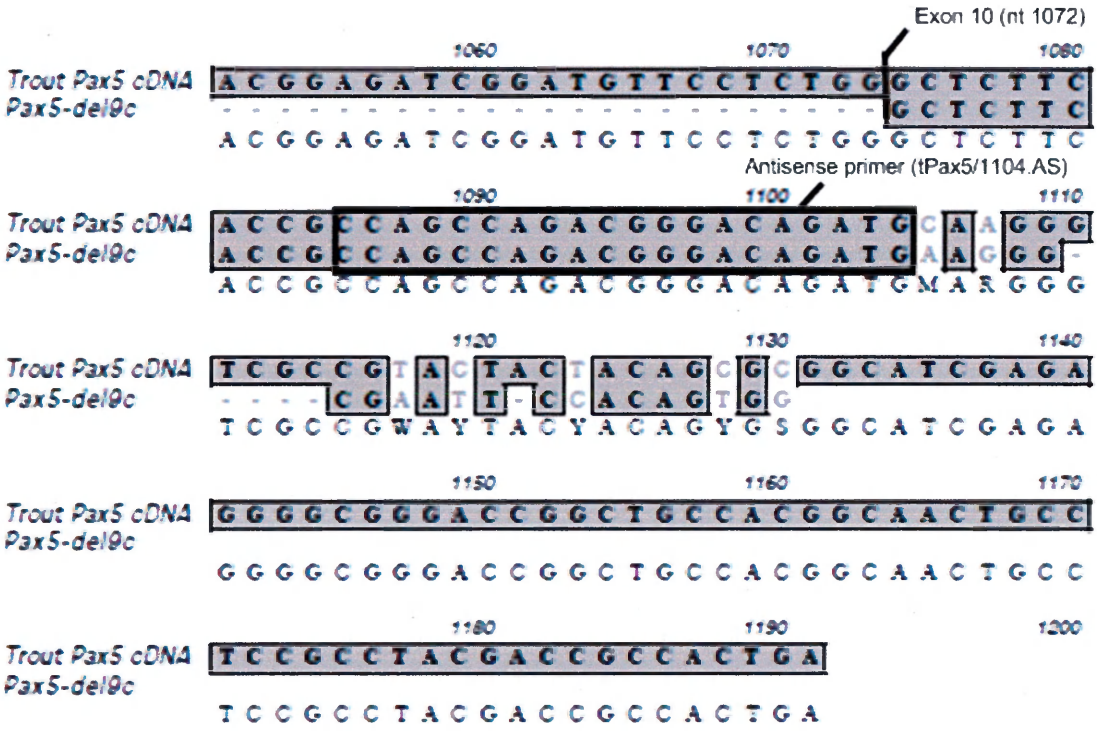

1060
1070
1080
Trout Pax5 cDNA
A10_T3_504027.seq
Antisense Primer (tPax5/1104.AS)
1090
1100
1110
1120
1130
1140
1150
1160
1170
1180
1190
1200

6. Formatted multiple alignment of full-length trout Pax5 and Pax5/Δ9c clone (with deleted exon 9 (nucleotides 920 to 1072) – E9160a1_T3_497317).




340 350 360
Trout Pax5 cDNA
TTCGCCTGGGAGATACGAGACAGACTATTG
Pax5-del9c
TTCGCCTGGGAGATACGAGACAGACTATTG
370 380 390
Trout Pax5 cDNA
GCTGAGAGAGTGTGTGACAACGACAGTGTT
Pax5-del9c
GCTGAGAGAGTGTGTGACAACGACAGTGTT
400 410 420
Trout Pax5 cDNA
CCCAGTGTCAGCTCTATCAACAGGATCATC
Pax5-del9c
CCCAGTGTCAGCTCTATCAACAGGATCATC
430 440 450
Trout Pax5 cDNA
AGGACTAAAGTCCAGCAGCCTCCGGGTCAG
Pax5-del9c
AGGACTAAAGTCCAGCAGCCTCCGGGTCAG
460 470 480
Trout Pax5 cDNA
TCAGGACCTCTCTCTGCTCATAACCTGGCG
Pax5-del9c
TCAGGACCTCTCTCTGCTCATAACCTGGCG
490 500 510
Trout Pax5 cDNA
TCGTCGGTAGCGTCGACACAGGTTTCCGCG
Pax5-del9c
TCGTCGGTAGCGTCGACACAGGTTTCCGCG
520 530 540
Trout Pax5 cDNA
GTGACCAGTGACTCGGCTGGCTCCTCCTAC
Pax5-del9c
GTGACCAGTGACTCGGCTGGCTCCTCCTAC
550 560 570
Trout Pax5 cDNA
TCCATCAGCGGCATTCTGGGAATCAGCTCA
Pax5-del9c
TCCATCAGCGGCATTCTGGGAATCAGCTCA
580 590 600
Trout Pax5 cDNA
GCTAACGACGGCAAGCGGAAGAGAGACGAT
Pax5-del9c
GCTAACGACGGCAAGCGGAAGAGAGACGAT
610 620 630
Trout Pax5 cDNA
GCCCTCCAGGAGTCTCCTCTAGCCAATGGC
Pax5-del9c
GCCCTCCAGGAGTCTCCTCTAGCCAATGGC
640 650 660
Trout Pax5 cDNA
CATGGTCCAGGAGGGCGGGACTTCCTGAGG
Pax5-del9c
CATGGTCCAGGAGGGCGGGACTTCCTGAGG
670 680 690
Trout Pax5 cDNA
AAGCAGATGAGAGGGGACCTCTTCTCGCCT
Pax5-del9c
AAGCAGATGAGAGGGGACCTCTTCTCGCCT


Trout Pax5 cDNA
Pax5-del9c
Sense Primer (tPax5/860.S)
Exon 8 (nucleotide 920)
Exon 8
Exon 9
Exon 9
Exon 10


Exon 10 (nt 1072)
1060
1070
1080
Trout Pax5 cDNA
Pax5-del9c
Antisense primer (tPax5/1104.AS)
1090
1100
1110
1120
1130
1140
1150
1160
1170
1180
1190
1200