Human Pax-5 C-terminal isoforms possess distinct transactivation properties and are differentially modulated in normal and malignant B cells

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Summary

The transcription factor Pax-5 occupies a central role in B cell differentiation and has been implicated in the development of B cell lymphoma. The transcriptional activation function of Pax-5 requires an intact N-terminal DNA-binding domain and is strongly influenced by the C-terminal transactivation domain. We report the identification and characterization of five human Pax-5 isoforms, which occur through the alternative splicing of exons that encode for the C-terminal transactivation domain. These isoforms arise from the inclusion or exclusion of exon 7, exon 8 and/or exon 9. Three of the Pax-5 isoforms generate novel protein sequences rich in proline, serine and threonine amino acids that are hallmarks of transactivation domains. The Pax-5 isoforms are expressed in peripheral blood mononuclear cells, cancerous and non-cancerous B cell lines, as well as in primary B cell lymphoma tissue. Electrophoresis mobility shift assays demonstrate that the isoforms possess specific DNA binding activity and recognize the PAX-5 consensus binding sites. In reporter assays using the CD19 promoter, the transactivation properties of the various isoforms were significantly influenced by the changes in the C-terminal protein sequence. Finally, we demonstrate, for the first time, that human Pax-5 isoform expression is modulated by specific signaling pathways in B lymphocytes.
**Introduction**

The development of B lymphocytes initiates from a common hematopoietic stem cell progenitor and proceeds according to a specific and coordinated process, which results in the production of mature B cells and antibody secreting plasma cells. This differentiation program is tightly regulated by the precise deployment of stage- and lineage-specific B lymphoid transcription factors that include EBF, E2A, and PAX-5 or the B cell-specific activator protein (BSAP) among others (1,2).

*Pax-5* belongs to the **Paired Box (Pax)** gene family which, in humans, is comprised of nine conserved genes. A hallmark of *Pax* genes is the paired domain, a DNA-binding domain that recognizes and binds to specific DNA sequences (reviewed in Barr, 1997) (3). Previous studies have demonstrated that *Pax* genes are important regulators of embryonic development and organogenesis (reviewed in Underhill 2000 and Dahl, 1997) (4,5). The *Pax* genes influence subordinate gene activity at the transcription level, and thereby modulate cellular processes such as proliferation, differentiation and apoptosis (6-9). Several members of the *Pax* family, such as *Pax-5*, also have crucial functions in the adult organism. Further, *Pax* genes can also act as proto-oncogenes and have the potential to induce neoplastic transformation (10,11).

Tissue screening studies have shown that the *Pax-5* gene is expressed in the embryonic central nervous system (CNS), B lymphocytes and adult testis (12). *Pax-5* expression, in early lymphocyte differentiation, specifies lineage commitment to B
cell development over other hematopoietic cell fates (13). In the absence of Pax-5, B


cell development in mice fails to proceed beyond the proB cell stage (13,14). The


PAX-5 protein or BSAP has been shown to bind to the promoter regions of several B
cell-specific targets including CD19, LEF-1, blk and mb-1, of the Ig genes (reviewed


in Hagman et al. 2000) (15).




Pax-5 belongs to a sub-group of Pax genes that also includes Pax-2 and Pax-8. All

three possess a paired domain, an octapeptide and a partial homeo domain. Previous

studies have shown that all three genes encode for distinct transactivation domain

(TD) rich in proline, serine and threonine (PST) and inhibitory domains in the C-
terminal portion of their respective proteins (16-18). Interestingly, both Pax-2 and

Pax-8 in humans have a number of alternatively spliced isoforms with significantly

altered protein sequence and structure in their respective C-terminal domains (17). In

certain cases, splicing events remove either or both the transactivation and inhibitory

domains whereas in other cases, novel protein domains are generated via shifts in the

reading frame. In most cases, these C-terminal-modified isoforms possess

transactivation (TA) properties that are different from the full-length version of the

protein (17,19). Similarly, alternative splicing events in the N-terminal paired

domain of Pax-6 generate multiple protein isoforms with different DNA-binding

properties (20,21). Murine and human Pax-5 is known to possess a number of

alternatively spliced mRNAs (22-24). In mice, these alternatively spliced mRNAs

produce protein isoforms that modify the amino acid sequence of the full-length

BSAP protein and accordingly, these changes alter the DNA binding affinity and/or

transcriptional activity (25).
In this study, we set out to investigate the possible existence of isoforms in the C-terminal region of the human Pax-5 gene. We report the identification, characterization and expression patterns of these novel Pax-5 isoforms from healthy B lymphocytes as well as B cell lines and primary lymphoma samples. Furthermore, we demonstrate that these isoforms are translated into proteins that possess different TA properties and are generated in response to different cell activation pathways.
Experimental Procedures

Screening of human B-cell cDNA library for Pax-5 isoforms and sequence analysis.
CD19 positive cells were isolated from primary peripheral blood mononuclear cells (PBMCs) from healthy donors with an autoMACS (Miltenyi Biotec, Auburn, CA) apparatus using a CD19 positive selection cocktail. All steps were performed rapidly on ice to minimize any cellular activity and/or RNA degradation. RNA extractions from target cells were done using TRIzol reagent (Invitrogen, Carlsbad, CA) and the standard protocol. RNA extracts were then reverse-transcribed using SuperScript II reverse transcriptase and cloned in the pBluescript plasmid vector. Transformed bacterial colonies were then isolated, characterized and sequenced. DNA sequencing was performed in house using ALFexpress II an automated DNA sequencing (Amersham, Baie d’Urfé, QC).

Cell lines and stimulation procedures.
The lymphoid cell lines Raji, 293T and Jurkat E6.1 were obtained from the American Type Culture Collection (Rockville, MD). REH and Nalm-6 were kindly provided by Dr. Edward A. Clark, University of Washington Medical Center, Seattle and the TA cell line, which is derived from EBV-immortalized healthy mature B cells, was kindly provided by Dr. André Darveau, University Laval, Québec. Cell lines were maintained in complete culture medium made of RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), L-glutamine (2 mM), penicillin G (100 U/ml), and streptomycin (100 µg/ml) (Invitrogen, Carlsbad, CA). PBMCs from healthy donors were isolated by Ficoll-Hypaque (Pharmacia,
Piscataway, NJ) density gradient centrifugation. CD19 positive cells were isolated from PBMCs with an autoMACS (Miltenyi Biotec, Auburn, CA) apparatus using a CD19 positive selection cocktail. Specific B cell sub-populations were isolated by flow cytometry (FACSCalibur, BD Biosciences, Mississauga, ON) cell sorting using CD10, CD19, CD20 and CD38-labeled antibodies. Patient lymph nodes biopsies were obtained with informed consent and subsequently selected from a library of B cell lymphoma diagnoses from the Pathology Department of the Dr. Georges L. Dumont Hospital. Murine cells were obtained from the excision of spleen and thymus of Balb/C mice (Charles River, Montréal, QC). Cells were either left untreated or treated (as described in figure legends) with either phorbol 12-myristate 13-acetate (PMA, at 20 ng/ml; Sigma, Oakville, ON)/Ionomycin (I, at 1 µM; Calbiochem, LaJolla, CA) or with phytohemagglutinin (PHA, at 3 µg/ml; Sigma). For time course experiments, Raji cells were synchronized through serum deprivation using culture medium containing 1% FBS for 16 hours.

**Nested PCR analyses.**

The PCR analyses were conducted through nested reactions using two different sets of Pax-5-specific primers (Table 1). The reaction mixtures were composed of 1.5 μg of DNA, 200 μM of each four deoxynucleotide triphosphates along with MgCl₂ (2 mM), AmpliTaq buffer (1X) and 1.25 U of AmpliTaq Gold DNA polymerase (Roche, Branchburg, NJ). This reaction mixture was then denatured (10 min at 94°C) followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C and finally 10 min at 72°C for complete polymerization. The primer pairs used for these
experiments included BSAP 5’ and BSAP 3’ (binding regions exon 1 and 10 respectively) which amplify full-length human Pax-5 (1200 base pair fragment) and a nested pair of primers Exon 5 and Exon 10 AS (binding regions exon 5 and 10 respectively). The primers amplifying full-length murine Pax-5 correspond to the oligos identified mBSAP 5’ and mBSAP 3’ where the nested oligos are mExon 5 and mExon 10 AS. Each RT-PCR reaction was performed in at least two separate experiments. As controls, primer sets were used to amplify DNA fragments from either the G6PDH gene or the β-tubulin gene (Table 1).

**Plasmids and cloning.**

The plasmids CD19-luciferase (26) was kindly provided by M. Busslinger (Research Institute of Molecular Pathology, Vienna, Austria). All Pax-5 isoform variants were cloned into pBlueScript (Stratagene) and subsequently subcloned into the pcDNA3.1 expression vector (Invitrogen) using a directional cloning technique involving the XhoI, XbaI and NotI restriction sites depending on the insert’s original orientation.

**Transfections and reporter gene assays.**

Transient transfections of the 293T cell line using the Polyfect™ transfection kit (Qiagen, Mississauga, ON) were performed according to the manufacturers’ instructions. Briefly, cells were seeded into 6 well plates (2 x 10^5/well) 48 h pre-transfection where they received a total of 2µg of plasmid DNA that was complexed with the provided transfection reagent. Cell extracts were then performed accordingly to the analysis intended. Reporter gene assays were conducted using the Dual-
Luciferase System™ (Promega, Madison, WI). Transfections included 200 ng of Renilla luciferase (pRL) (Promega) as a control. After 24 hours post-transfection, cells were lysed in a lysis buffer (Promega) by shaking at room temperature for 30 minutes. Lysates were then analyzed with a luminometer (BMG Fluostar Optima) to measure luciferase activity. Relative reporter activity was calculated and normalized based on Renilla-luciferase activity which reflects transfection efficiency. At least three separate experiments were performed each in triplicates.

**Western blot analysis.**

Cells (2 x 10⁶) were lysed using 0.5 ml of RIPA lysis buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS along with freshly added 10 µl/ml PMSF [10 mg/ml], 30 µl/ml aprotinin and 10 µl/ml sodium orthovanadate [100 mM]). Cell extracts were then incubated on ice for 20 min and pre-cleared of cellular debris by centrifugation at 10 000 x g. Thereafter, 80 µg of protein extract was mixed with 20 µl of 2X electrophoresis sample SDS-PAGE buffer, heated at 95°C for 5 min and separated on a 12% SDS-polyacrylamide gel. Proteins were then transferred to a PVDF membrane (Millipore, Bedford MA). The membrane was then blocked with 3% gelatin and incubated with either anti-C- or anti-N-terminal Pax-5 antibodies (Santa Cruz Biotech., Santa Cruz, CA) followed by HRP-conjugated secondary antibodies (Sigma). Signals were revealed with an enhanced chemiluminescent kit according to the manufacturer’s protocol (Amersham).

**Preparation of nuclear extracts and Electrophoretic Mobility Shift Assays (EMSA).**
Nuclear extracts were prepared according to the described microscale preparation protocol (27). Briefly, 10⁶ cells were washed with ice-cold PBS and resuspended in 400 µl of cold buffer A (10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM dithiothreitol [DTT], and 0.5 mM phenylmethylsulfonyl fluoride [PMSF]). The cells were allowed to swell on ice for 15 min, after which 25 µl of a 10% solution of Nonidet NP-40 was added, and the tube was vigorously vortexed for 10 sec. The homogenate was centrifuged for 10 sec at 12 000 x g. The supernatant fraction was discarded and the pellet was resuspended in 50 µl of cold buffer C (20 mM HEPES-KOH [pH 7.9], 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF) and incubated at 4°C for 15 min on a shaking platform. Cellular debris were removed by centrifugation at 12 000 x g for 20 min at 4°C and the supernatant fractions were stored at -70°C until used. EMSA experiments were performed as described in Ruscher et al. 2000 (28) with modifications. Briefly, 10 µg of nuclear protein extracts were incubated for 30 min at room temperature in 15 µl of the binding buffer (100 mM HEPES [pH 7.9], 40% glycerol, 10% Ficoll, 250 mM KCl, 10 mM dithiothreitol, 5 mM EDTA, 250 mM NaCl, 2 µg poly [dl-dC], 10 µg nuclease-free bovine serum albumin fraction V) containing 1 ng of Cy5-labeled double stranded DNA (dsDNA) oligonucleotide. The following consensus binding site dsDNA oligonucleotides were synthesized and used as probes and/or competitors: Pax-5 (5’-[Cy-5]-GAATGGGGCACTGAGGCGTG-3’), AP-1 (5’-CGCTTGATGACTCAGGCCGTG-3’). DNA-protein complexes were resolved from polyacrylamide gels fragment analysis software combined with a ALFexpress II
apparatus. Cold competition assays were carried out by adding a 100-fold molar excess of unlabeled dsDNA oligonucleotide simultaneously with the labeled probe.

*Sequence accession numbers.*

The newly identified sequences were thereafter submitted to GenBank and filed as Homo sapien BSAP alternatively spliced mRNAs: full-length *Pax-5* (Accession # AY463952), *Pax-5Δ7* variant (AY463953), *Pax-5Δ8* variant (Accession # AY463954), *Pax-5Δ9* variant (AY463955), *Pax-5Δ7/8* variant (AY463956), and *Pax-5Δ7/8/9* variant (Accession # AY463957).
Results

Identification of novel Pax-5 isoforms in normal human B-cells.

Our studies of Pax-5 gene expression in normal and malignant B cells lead us to investigate possible alterations in the 3’ coding region of the Pax-5 mRNA. A cDNA library was constructed from a pool of CD19+ cells isolated from the peripheral blood of 13 healthy donors. The library was screened by RT-PCR using Pax-5-specific primers that span exons 5 through 10. On the basis of cDNA length, six distinct variants of Pax-5 cDNA clones were identified (Fig. 1A). Sequence analysis confirmed that one group of Pax-5 cDNA clones was identical in length to the published coding sequence for the full-length (FL) human Pax-5 mRNA (herein referred to as Pax-5 FL). Interestingly, the remaining five groups correspond to novel Pax-5 cDNAs with discrete gaps in the 3’ coding region of the gene sequence. These gaps correspond precisely to deletions of exon 7, exon 8, exon 9, exons 7 and 8 or exons 7, 8 and 9 (herein referred to as Pax-5Δ7, Pax-5Δ8, Pax-5Δ9, Pax-5Δ7/8 and Pax-5Δ7/8/9, respectively).

Translation of these novel alternatively-spliced mRNAs into their putative protein sequences revealed several noteworthy characteristics. Figures 1B and C illustrate the exon boundaries and corresponding protein domains of the full-length Pax-5, whereas figure 1D shows the exon and protein domain alignment of the five alternatively-spliced isoforms. The Pax-5Δ8 and Pax-5Δ9 isoforms both use in-frame fusions of flanking exons and conserve the same reading frame and stop codon as the Pax-5 FL.
transcript. Pax-5Δ8 produces a protein lacking 34 amino acids (aa) entirely within the transactivating domain whereas Pax-5Δ9 produces a protein lacking 29 aa that spans the end of the transactivating domain and the beginning of the inhibitory domain. Strikingly, all Pax-5 isoforms with a deletion of exon 7 (i.e. Pax-5Δ7, Pax-5Δ7/8 and Pax-5Δ7/8/9) undergo a shift in the reading frame that generates novel protein sequences (solid box). The deletion of exon 7 in Pax-5Δ7 causes a reading frame shift immediately after exon 6 which, in turn, terminates translation prematurely in exon 8. This results in the production of a novel protein sequence of 28 aa. Reading frame shifts are also observed in Pax-5Δ7/8 and Pax-5Δ7/8/9 which both begin at exon 6 splice donor junction and extend past the full-length Pax-5 termination codon in exon 10 to generate novel protein sequences of 64 and 35 aa respectively. It is noteworthy that in isoforms Pax-5Δ7, Pax-5Δ7/8 and Pax-5Δ7/8/9, the new sequences contain a higher ratio of PST in comparison to the TD of Pax-5 FL (Table 2).

Expression of alternatively spliced Pax-5 isoforms in human B cells.

To investigate the Pax-5 isoform expression patterns from individual donor samples, specific primer pairs were designed to enable the amplification of 1) the entire coding region of the Pax-5 cDNA and 2) a nested product spanning exon 5 through exon 10. For this experiment, primary CD19+ B cells from 13 healthy donors were analyzed individually (Fig. 2A). As a control, cloned FL Pax-5 cDNA was amplified in parallel and yielded an anticipated 602 bp product containing the entire coding sequences of exons 6 through 10. The nested RT-PCR of donor samples revealed distinct amplification products that were shorter in length compared to the control Pax-5 FL.
In addition, the isoform expression profiles were different in nearly each individual, and no correlation could be established between the observed Pax-5 isoform expression patterns and either donor age, gender or race (data not shown).

We then set out to investigate the Pax-5 isoform expression profiles in various B cell lines. RT-PCR analysis was used to examine the isoform expression profiles from immature (Nalm6 and REH /lanes 1 and 2 respectively) as well as mature (TA cell line and Raji /lanes 3 and 4 respectively) cells from the B cell lineage (Fig. 2B). Multiple Pax-5 amplicons that were shorter than the amplified Pax-5 FL control were observed. Interestingly, the Pax-5 FL variant did not appear to be the predominant isoform in some of the cell lines tested.

Since several studies have shown that aberrant expression of Pax-5 is implicated in the development of a certain type of B cell lymphomas, namely lymphoplasmocytoid lymphoma, we investigated Pax-5 isoform expression in RNA extracts from lymph nodes of patients diagnosed with B cell lymphoma using nested RT-PCR. As depicted in Figure 2C, most of the B cell lymphoma assessed expressed a single predominant isoform. In a majority of these samples, except for lane 2, Pax-5 FL was the sole isoform present whereas the splice variant Pax5Δ8 was the sole isoform in the remaining cases. The observed expression profiles of Pax-5 isoforms have yet to reveal any consistent correlation with the patients’ medical diagnosis.

In the past decade, numerous studies have correlated the expression of the orthologous Pax-5 gene in the development of murine B cells. To investigate the
presence of alternatively spliced transcripts in the C-terminal region of Pax-5 in mouse, we performed an identical nested RT-PCR experiment using murine-specific primers targeting the corresponding region of the Pax-5 transcript. RNA extracts from murine thymus and spleen were examined in parallel with human PBMCs and Raji cells as controls. The nested amplification of the 3’ end of the murine Pax-5 transcript revealed the presence of a single 600 bp amplified fragment in murine thymus (lane 1) and spleen (lane 2) samples (Fig. 2D). In contrast, no amplification of human Pax-5 fragments was detected using murine-specific primers in neither human PBMCs (lane 3) nor Raji (lane 4) cell samples; although amplification of a positive control, β-tubulin (280 bp) was achieved in all samples (data not shown). These results suggest that differences in Pax-5 isoform expression exist between human and mouse hosts.

Pax-5 isoforms are translated in human B cells.

To confirm the anticipated putative protein sequences from the human Pax-5 isoforms, we performed Western blot analysis on total protein extracts from 293T cells transiently transfected with the cloned human Pax-5 isoforms (Fig. 3A). Using an anti-human PAX-5 antibody specifically recognizing the N-terminal region of the protein (Fig. 3A, top panel), all isoforms appeared at their expected molecular weight (lanes 1 through 6). Subsequently, the membrane was striped and reprobed with an anti-PAX-5 antibody specific for the C-terminal region of the protein (Fig. 3A, middle panel). As expected, only isoforms Pax-5 FL, Pax-5Δ8, and Pax-5Δ9, which possess an in-frame exon 10, were revealed (lanes 1, 3 and 4, respectively).
Conversely, isoforms $Pax5\Delta 7$, $Pax5\Delta 7/8$ and $Pax5\Delta 7/8/9$ were not recognized by the C-terminal antibody. As a control the blot membrane was probed with an anti-tubulin antibody (Fig. 3A, bottom panel).

Evidence for the expression of lower molecular weight PAX-5 protein variants in B-cell samples was also obtained by Western blot analysis. Total cell lysates from B cell lines (Raji, TA and REH), a T-cell line (Jurkat), a lymph node, as well as from 293T cells transiently transfected with various $Pax-5$ isoforms were immunoblotted with an anti-PAX-5 antibody directed against the N-terminal region (Fig. 3B). In contrast to the Jurkat T cell line (lane 5), all B cell extracts, as well as transfected control cells (lanes 6 through 9), expressed the PAX-5 protein. In addition, the anti-PAX-5 antibody revealed protein bands at lower molecular weights in extracts from lymph node (lane 1) and REH (lane 4) cells. These smaller proteins have molecular weights of approximately 27 kDa and 35 kDa, which correspond to the calculated MW of the isoforms $Pax-5\Delta 7$ and $Pax-5\Delta 7/8$. Moreover, these lower MW proteins co-migrate with transfected $Pax-5\Delta 7$ and $Pax-5\Delta 7/8$, respectively, in the control samples (lanes 7 and 9). These results demonstrate that lower MW variants of the PAX-5 protein are expressed in the various samples tested and do not represent products generated from partial proteolysis.

$Pax-5$ isoforms have DNA-binding ability and transcriptional activation properties. To further assess the role and function of the lower MW PAX-5 protein variants, we first investigated the DNA-binding capacity of these proteins through electrophoretic
mobility shift assays (EMSA). Nuclear extracts from transiently transfected 293T cells with the various human PAX-5 isoforms were incubated with a Cy-5-labeled DNA probe corresponding to the PAX-5 consensus binding sequence. The formation of multiple shifted PAX-5/DNA complexes was observed (lanes 3 to 8) (Fig. 4A). The specificity of these complexes was confirmed using a 100-fold molar excess of unlabelled PAX-5 consensus DNA probe (lanes 9 to 14). Conversely, the PAX-5/DNA complexes were preserved in the presence a 100-fold excess of a non-specific DNA probe corresponding to the AP-1 consensus motif (lanes 15 to 20). These results strongly suggest that each of these human PAX-5 isoforms possess DNA-binding properties similar to PAX-5 FL.

Given the fact that all tested PAX-5 isoforms seem to recognize and bind a specific DNA consensus motif, we analyzed their TA potential using a luciferase-based reporter assay. 293T cells were co-transfected with each isoform construct individually, along with DNA from pRL (Renilla luciferase construct) and CD19-Luc (a PAX-5 inducible reporter construct). The cells were then lysed, and the transcriptional activation activity was assessed (Fig. 4B). Interestingly, all PAX-5 isoforms induced reporter gene activity. Further comparison revealed that PAX-5 FL had the highest activity with a nearly 16-fold induction of activity compared to non-induced reporter controls. Both PAX-5Δ8 and PAX-5Δ9 proteins produced a 14-fold increase of transcriptional activity whereas PAX-5Δ7/8/9, PAX-5Δ7/8 and PAX-5Δ7 produced progressively lower levels of induction of 7.5-, 5- and 2.5-fold, respectively.
Cell activation modulates Pax-5 isoform expression in human B cells.

PAX-5 is an important regulator of B cell development and its expression is developmentally regulated accordingly. As previous studies were not designed to examine the expression patterns of the C-terminus isoforms, we sought to investigate the regulating parameters governing the expression of the different Pax-5 isoforms.

First, we set out to determine if, individually, these isoforms are differentially regulated during specific stages in B cell development. Accordingly, stage-specific B cell subpopulations were purified from different healthy donors by FACS using two different cell surface antigen expression profiles. The first cell population (CD19+, CD20+, CD10- and CD38-) selected mature B cell or plasmocytes, whereas the second subset (CD19+, CD20-, CD10- and CD38+) isolated cells at an earlier stage of B cell differentiation known as ProB1 cells. Nested RT-PCR was then performed on each cell subset from different donors to determine whether the expression of Pax-5 isoforms is regulated in a developmental stage-dependent manner. In mature B cell isolates from each donor, no detectable expression of Pax-5 was observed (lanes 1 and 2) (Fig. 5A). As a control, a 600bp G6PDH amplicon was detected in each donor (lanes 3 and 4 respectively). This result supports several studies (29,30) on the absence of Pax-5 expression in mature B cells or plasmocytes. On the other hand, Pax-5 isoform profiling of the ProB1 cell subset from several donors demonstrated a different isoform expression pattern in each sample (Fig. 5B). Further, RT-PCR results revealed different patterns of Pax-5 isoform expression in a given B cell subset collected from single donors at the different time points at 2 week intervals (data not shown).
It has previously been shown that murine N-terminal isoforms of Pax-5 can be modulated by changes in cell activation state by the use of LPS (25). In view of these findings, we analyzed the Pax-5 isoform expression patterns from human B cells undergoing various activation processes. In our analysis, B cells from a primary lymphoma and several B cell lines were stimulated using mitogenic agents known to activate specific intracellular signaling pathways. PHA was used to non-specifically activate cell surface receptors whereas PMA/ionomycin was used to induce the intracellular mediators PKC and calcium mobilization respectively. Isoform expression profiles from untreated and treated cells were examined using nested RT-PCR (Fig. 5C). In all cases, except for the TA cell line, cellular activation modified the expression patterns of Pax-5 isoforms. Furthermore, the use of PHA versus PMA/ionomycin led to distinctively different expression profiles, thus providing evidence that Pax-5 isoform expression is differentially influenced by distinct signaling and cell activation pathways. A time course stimulation experiment was also done using PHA (3μg/ml) on synchronized Raji cells. The results show reversion back to resting state expression pattern of Pax-5 isoforms within a few days post-treatment (data not shown).
Discussion

The *Pax-5* gene is crucial for appropriate B cell development where it interacts with a number of nuclear factors to regulate gene expression. In this report, five alternatively-spliced *Pax-5* transcripts were identified and characterized. These *Pax-5* isoforms were found in B cells isolated from healthy peripheral blood samples, malignant B cells and B cell lines.

The *Pax-5* isoforms are generated through alternative splicing in the 3’ region of the *Pax-5* mRNA and result in the exclusion of different combinations of exons 7, 8 and 9. These changes alter the C-terminus of full-length PAX-5 protein, and in each case, produce shorter protein variants. Furthermore, in 3 cases, *Pax-5Δ7*, *Pax-5Δ7/8* and *Pax-5Δ7/8/9*, the alternative splicing events cause a shift in the reading frame that result in novel protein sequences. These novel sequences contain a higher ratio of PST residues which are often found in the transactivation domains of various PAX proteins (17,31-33). Accordingly, these modifications could have important consequences on protein activity and subsequently upon target genes. Studies conducted by Kozmik et al, (1993) show that despite the identical DNA-binding properties of various *Pax-8* isoforms studied, modifications in the C-terminal region resulted in different TA potentials (17). These latter results also suggested that the isoforms could differentially regulate *Pax-8* targeted genes. Similarly, the composition and structural modifications brought to *Pax-5* isoforms by deleted peptide regions as well as by shifted reading frames could also bring changes in the
interaction with co-acting proteins such as TLE4, importin α1 and Ets, which have all been shown to be essential collaborators with BSAP (34-36).

Our Western blot and EMSA analyses demonstrate that alternatively-spliced transcripts are indeed translated, and possess specific DNA binding activity towards the consensus PAX-5 binding sequence. Therefore, all PAX-5 isoforms have the potential to recognize and bind to identical DNA targets. Significant differences in the C-terminal transactivation domains, however, lead us to believe that the TA potential would vary considerably among isoforms. Indeed, this was demonstrated in transient co-transfection reporter assays where the isoforms PAX-5Δ7/8, PAX-5Δ7/8/9 and, in particular, PAX-5Δ7 have markedly reduced TA potentials compared to PAX-5 FL. These results are of particular interest since the alternative reading frames employed generate protein sequences with no obvious sequence similarity to known proteins. On the other hand, its C-terminal position and high PST ratio would suggest strong TA activity. Although the TA properties of these novel isoforms were low compared to PAX-5 FL, these results relate only to the CD19 reporter construct. We believe that the relative TA properties of PAX-5 isoforms are influenced by the promoter/enhancer environment. In support of this, our preliminary data reveal that in different promoter/enhancer environments, the TA potential of PAX-5Δ7 surpasses that of PAX-5 FL (R. Morin and R.J. Ouellette, unpublished data). It will therefore be of particular interest to study the TA potentials of PAX-5 isoforms on a wider spectrum of known targets.
Reporter assays also demonstrate that PAX-5Δ8 and PAX-5Δ9 have TA properties nearly equal to those of PAX-5 FL. In light of the important contribution of exon 8 to the TD (16), we had anticipated a markedly reduced activity in the case of PAX-5Δ8. The exclusion of exon 8 in PAX-5Δ8 removes the first 34 aa of the TD, which leaves less than a third of the domain to provide the TA function. Therefore, important and independently functional transactivation elements must be present in exon 9 and/or in exons preceding exon 8. For PAX-5Δ9, similar TA potentials are also observed despite the exclusion of exon 9 which removes the remaining third of the TA domain as well as a small portion of the inhibitory domain. In this case, the loss of transactivation elements could be counteracted by the loss of inhibitory elements both present in exon 9. Once again, TA elements encoded by one exon, in this case exon 8, are capable of autonomous activity in the absence of neighbouring exon-encoded elements. Thus, it appears from these results that the TD of PAX-5 is composed of at least two separate sub-domains delimited by exons 8 and 9 rather than a single domain as previously described (16). The modular organization of the TD is reminiscent of the transactivation domains of rel, (37) and AhR (38,39) where the deployment of various modules allows for increased functionality in cellular processes. Therefore, it appears that the generation of a large number of isoforms provides a spectrum of PAX-5 proteins that intrinsically possess different TA potentials thus increasing the scope of possibilities for protein-enhancer as well as protein-protein interactions.

The aberrant expression of Pax-5 has been correlated with the development of various B cell lymphomas, and thus it was of interest to investigate Pax-5 isoform
expression in such tissues. Interestingly, we routinely observed one predominant isoform, usually \(Pax-5\) \(FL\) or \(Pax-5\Delta8\), in individual lymphoma samples. Thus, it appears that a restricted number of \(Pax-5\) isoforms are deployed in cases of B cell lymphoma whereas multiple isoforms are routinely observed in healthy samples. It is noteworthy that Borson et al. (2002) (24), reported the expression of a greater variety of isoforms in multiple myeloma (MM) cells relative to healthy cells. Hence, it will be of importance to investigate the mechanisms by which the clonal propagation of MM cells triggers the expression of multiple \(Pax-5\) isoforms, whereas the clonal propagation of lymphoma cells leads the restriction of expression to a particular \(Pax-5\) isoform. We are currently investigating questions raised by these findings.

Alternatively-spliced \(Pax-5\) isoforms have also been described in murine tissue (22). The murine \(Pax-5\) isoforms identified to date, however, are quite different from the human isoforms described in this study and that of Borson et al. (2002) (24). Alignments of human and murine \(Pax-5\) nucleotide and amino acid sequences demonstrated a high level of conservation between the two species, especially at exon-intron junctions, and suggested that if the alternatively spliced transcripts were present in mice, they could produce \(Pax-5\) variants similar to their human counterparts (data not shown). Despite many attempts however, we have been unable to detect any \(Pax-5\) transcripts with 3′ modifications in murine tissues. There is now a growing body of evidence that suggests differences in isoform expression patterns exist between humans and mice. For example, Hardy and O’Neil (2004) demonstrated that alternatively spliced murine \(Irak2\) transcripts are present in mice, but not in humans (40). Further, Baranova et al, (2003) identified human-specific
transcripts of the \textit{RFP2} gene (41). Finally, genome-wide studies suggest a low
conservation level of alternative splicing among humans, mice and rats (42,43).

We initially wanted to verify whether isoform profiles might reflect individual
differences in ratios of B cell sub-populations at various stages of differentiation and
therefore surveyed \textit{Pax-5} isoform expression in a sample of healthy donors. As we
were unable to associate isoform expression patterns with a specific B cell sub-
population, we then hypothesized that isoform modulation could be influenced by cell
activation states. Accordingly, stimulation of calcium-dependant and independent
signaling pathways altered the resting state pattern of isoform profiles in various B
cell backgrounds. Similar observations have been reported, for example, a differential
modulation of the murine \textit{Pax-5} isoforms, \textit{Pax-5d} and \textit{Pax5e} was observed in
response to LPS activation (25). Further, Lynch and Weiss (2000) demonstrated that
CD45 splicing in T-cells responds to changes in activation through the actions of
protein kinase C and Ras (44), and the alternative splicing of CD44 is influenced by
activation via the ERK MAP-kinase pathway (45) in both B cells and T-cells.
Similarly, Sgambato et al. (2003) observed that the neuronal expression of the ania-6
isoforms was modulated by signaling pathways that converge on the cAMP response
element binding protein (CREB) (46). Finally, we note that Borson et al. (200) (24)
reported an increase in \textit{Pax-5} isoform expression in multiple myeloma patients
following treatment with anti-cancer agents. Clearly, these treatments produced
changes in cell activation states, not unlike PHA or P/I, which influenced the isoform
expression pattern. Given its central role of in B cell activation, it seems likely that
the Pax-5 gene, itself, could be influenced by intracellular transduction events. Taken together, these data strongly suggest that Pax-5 isoform deployment is a stoichiometrically dynamic process that represents real-time responses to physiological stimuli.

The discovery of alternatively-spliced Pax-5 isoforms in the wide variety of normal and malignant B cell samples tested emphasizes their important contribution to the overall function of the gene. Whether the function of these isoforms is accessory or complementary to the full length version is not known at this time, however, the data obtained suggest that these lower MW versions of PAX-5 are an integral part of the spectrum of B cell activity. These findings are: 1) the presence of multiple isoforms in most B cell samples from healthy donors; 2) in a number of healthy donors, the full length version of Pax-5 is absent leaving only shorter isoforms to perform Pax-5-mediated functions; 3) the apparent clonal expression of a single isoform in cases of B cell lymphoma; 4) the apparent modulation of isoform patterns in activated B cells when compared to their naive counterparts and 5) the activation of distinct cellular pathways with mitogenic agents (PHA or P/I) triggers different isoform expression profiles. Taken together, these observations suggest a carefully controlled mechanism for the production of these Pax-5 isoforms, rather than a random or aberrant process. Interestingly, we note that upon the comparison of both transcription and translation of Pax-5 isoforms in a given cell type, we observed that the expressed products of one did not always reflect the pattern in the other. This could be explained by the fact that not all mRNA are tunnelled into protein synthesis. In some cases, mRNA transcripts are pooled and influence self-regulating mechanisms as aptamers and
riboswitches (47-49). These results could also be a consequence of a bias amplification by PCR of shorter fragments. Further investigation of these observations will bring more insight into these issues. We believe that this further argues in favor of a tightly regulated mechanism for the deployment of Pax-5 isoforms.

Alternative splicing is being increasingly accepted as an important means for the expansion of the variability of gene products and associated functions. The deployment of protein isoforms is likely an evolutionary adaptation as a mean to increase the functionality of a given gene, which provides a rapid response to cellular requirements during development and other processes such as cellular proliferation, differentiation and apoptosis. In light of our findings, we can thus anticipate that the finding of multiple human Pax-5 isoforms will help elucidate BSAP’s role and complexity in transcriptional regulation as well as in B cell processes and development. The further characterization of the various human Pax-5 isoforms will bring new insight to BSAP’s function in transcriptional regulation, in cellular processes and development, and also in Pax-5-related oncogenesis.
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### Table 1: Oligonucleotide primer sequences used in this study

| Primer name          | Sequence (5’ to 3’)                                      |
|----------------------|----------------------------------------------------------|
| BSAP 5’              | GTCCATTCCATCAAGTCCTG                                     |
| BSAP 3’              | CTCCAAGGGTCAGTGACGG                                      |
| Exon 5               | GACACCAACAAGCGCAAGA                                      |
| Exon 10 AS           | GGTCAGTGACGGTCATAGGC                                     |
| mBSAP 5’             | GACCATCAGGACAGGACA                                       |
| mBSAP 3’             | TGTAGGAAGAATACTGAGGGTG                                    |
| mExon 5              | GACACCAACAAACGCAAGA                                      |
| mExon 10 AS          | AGGGCTGTAATAGTATGGG                                      |
| G6PDH                | GTCAACGGATTTGGGTCTGTATT                                  |
| G6PDH AS             | AGTCTTCTGGGTGGCAGTGAT                                   |
| β-Tubulin            | ATGAGGGAAATCGTGCACTTGCA                                  |
| β-Tubulin AS         | CATGGTGCCGGGGCTCCAGATC                                  |
| β-Tubulin (nested)   | TGTTTGTCTACTTCTCTCTG                                    |
| β-Tubulin (nested)AS | TACCCATCCACGACCC                                      |
Table 2. Relative amounts of proline, serine and threonine in PAX-5 isoforms

| Isoform | Total size (aa) | Total % P-S-T | % P-S-T in Trans domain | Trans domain size (aa) |
|---------|----------------|---------------|-------------------------|-----------------------|
| FL      | 391            | 28.1          | 141                     | 36.2                  |
| Δ7      | 288            | 25.7          | 38                      | 39.5                  |
| Δ8      | 357            | 27.0          | 107                     | 34.6                  |
| Δ9      | 362            | 27.4          | 112                     | 35.7                  |
| Δ78     | 324            | 28.7          | 74                      | 46.0                  |
| Δ789    | 295            | 26.8          | 45                      | 44.5                  |
Figure legends

**Figure 1. Identification and analysis of human *Pax-5* isoforms.** (A) Human *Pax-5* isoforms were identified through the use of RT-PCR analysis in cDNA libraries from CD19+ PBMCs derived from healthy adult donors. *Pax-5* variants were subsequently sequenced and identified as: *Pax-5* full-length (FL) (lane 1), *Pax-5Δ7* (lane 2), *Pax-5Δ8* (lane 3), *Pax-5Δ9* (lane 4), *Pax-5Δ7/8* (lane 5), and *Pax-5Δ7/8/9* (lane 6). (B) The cloned isoforms were mapped according to exon/intron junctions in reference to the human *Pax-5* locus. Ten exons are labeled E1 to E10, and exon sizes are given below exon number. The precise size of exon 10 is unknown, but is likely >8 kb. (C) Functional domains of the PAX-5 protein (PD/Paired Domain, O/Octapeptide, HD/Homeo Domain, TD/Transactivation Domain and ID/Inhibition Domain.) were compared and aligned to the putative protein sequences (16), (D) of the novel *Pax-5* isoforms. Numbers delimiting protein domains correspond to the position in nucleotides whereas the solid lines indicate deleted exons. Black boxes correspond to novel protein sequences.

**Figure 2. Multiple *Pax-5* isoforms are expressed in human B cell lineages.**

Through the use of RT-PCR amplifying the region spanning exons 5 through 10, multiple *Pax-5* isoforms expression profiles were detected and identified in: (A) human primary CD19+ B cells (healthy adult donors lanes 1 to 13); (B) various immature (Nalm-6 and REH/lanes 1 and 2 respectively) as well as mature (TA and Raji/lanes 3 and 4, respectively) B cell lines; and (C) a range of malignant B cell
tissues (tissues 1 to 11). An attempt to identify various Pax-5 isoforms in mouse (D) was also conducted using RT-PCR with mouse-specific primers targeting similar regions of Pax-5 in murine thymus (lane 1) and spleen (lane 2) in comparison to human CD19+ B (lane 3) and Raji cells (lane 4). Lane “M” designates a 100 bp DNA ladder whereas “FL” is an amplified control product resulting from a RT-PCR on the cloned Pax-5 full-length isoform.

**Figure 3. Translation of human PAX-5 isoforms.** (A) Western blot analysis using an anti-PAX-5 antibody specific to either the N- (upper panel) or the C-terminal regions (middle panel) of the protein was conducted in 293T cells transiently transfected with the Pax-5 isoform clones. The membrane was additionally probed for tubulin as a control (lower panel). (B) Western blot analysis was also performed on total cellular extracts from human lymph node (lane 1), Raji (lane 2), TA (lane 3), REH (lane 4), and Jurkat cells (lane 5) using an anti-PAX-5 antibody directed against the N-terminal region of the protein. The detected complexes were compared to the migration patterns from control extracts being 293T cells transfected with Pax-5 FL (lane 6) or isoforms Pax-5Δ7 (lane 7), Pax-5Δ8 (lane 8), and Pax-5Δ7/8 (lane 9).

**Figure 4. Functional analysis of the novel human Pax-5 isoforms.** (A) Electrophoretic Mobility Shift Assays were conducted on nuclear extracts from 293T cells transfected with the cloned Pax-5 isoforms. Extracts were incubated with the PAX-5-specific dsDNA oligonucleotides labeled with Cy5 and the DNA-protein complexes were resolved and analyzed on a 4% polyacrylamide gel encompassed in
an ALFexpress II apparatus. Cold competition assays were carried out by adding a 100-fold molar excess of unlabeled dsDNA oligonucleotide corresponding to the PAX-5 (lanes 9 to 14) or the AP-1 (lanes 15 to 20) consensus binding motif. Lanes 1 and 2 were loaded with free probe (FP) and extracts from 293T cells (C-) transfected with the expression vector alone respectively. (B) The transactivation potential from each PAX-5 isoform was assessed using a dual-luciferase reporter gene system. Reporter genes from firefly as well as renilla luciferase were co-transfected in 293T cells, along with each Pax-5 isoform construct, individually, and analyzed for transcriptional activity using a BMG luminometer. The results were calculated in fold induction over the activity of control cells (transfected with the vector alone) and normalized according to non-incucible renilla luciferase activity. The calculated data and errors are plotted and represent four different sets of experiments.

Figure 5. Developmental and activation state dependency of Pax-5 isoform expression. The Pax-5 isoform expression profiles were assessed using RT-PCR analysis in distinct human B cell subpopulations. (A) RNA extracts from the plasmocyte subset (CD19+, CD20+, CD10- and CD38-) from two different adult donors were analyzed for Pax-5 (lanes 1 and 2) as well as G6PDH (lanes 3 and 4) expression. (B) RNA extracts from ProBl cells (CD19+, CD20-, CD10- and CD38Dim) were also tested in 6 different infant donors for Pax-5 isoform expression. (C) Pax-5 isoform expression patterns were then examined in various B cell lines either untreated or stimulated for 8 hours with 3μg of phytohemagglutinin (PHA, at 3 μg/ml) or with phorbol 12-myristate 13-acetate/ionomycin (P/I, at 20 ng/ml and 1 μM
respectively). "M" and "FL" designate a 100 bp DNA ladder and amplified PCR control product from the cloned Pax-5 full-length isoform respectively.
A

anti-N-terminal

anti-C-terminal

anti-tubulin

B

B
Human pax-5 C-terminal isoforms possess distinct transactivation properties and are differentially modulated in normal and malignant B cells
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