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FUNCTION AND REGULATION OF ALTERNATIVE ISOFORMS OF THE TRANSCRIPTION FACTOR PAX-5 DURING B LYMPHOCYTE DIFFERENTIATION

A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

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by

Marina A. Lowen

2000

APPROVAL SHEET

This thesis is submitted in partial fulfillment of

the requirements for the degree of

Master of Arts

Marina A. Lowen

Approved, November 2000

Patty Zwollo

Lizabeth Allison

Diane Shakes

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ABSTRACT

Pax-5, a member of the *Pax* family of transcription factors, plays an important role in B cell development and differentiation. The gene produces four alternatively spliced variants (*Pax-5a*, *Pax-5b*, *Pax-5d*, and *Pax-5e*) including one of the most extensively studied regulators of B-lymphopoiesis, the B-cell-specific activator protein (BSAP). BSAP is encoded by the predominant *Pax-5a* isoform, and while its roles are well defined, the regulatory functions of the remaining Pax-5 isoforms are not clearly understood.

The study presented here pursued two goals. First, the transactivation properties of the alternative isoform Pax-5d were determined *in vivo* using transient transfections. Evidence is provided indicating that Pax-5d has a function opposite to that of Pax-5a and the two isoforms may compete for binding to Pax-5 recognition sequences. The second goal of the project was to investigate the regulatory function of Pax-5 isoforms in resting and LPS-activated B cells using various B cell lines and splenic B lymphocytes. Experimental data demonstrates that, as mature B lymphocytes progress to the plasma cell stage, the ratio of different Pax-5 proteins changes possibly reflecting a shift toward a higher concentration of transcriptionally inert and/or dominant-negative isoforms. Based on these results, the author of this thesis hypothesizes that the alternative splice forms of Pax-5 differentially regulate the activity of Pax-5a/BSAP. Other means of Pax-5a regulation may include post-translational modifications that alter stability and transactivation properties of this isoform.

In addition, this work contains a description of several preliminary studies which include the following: i) development of a novel staggered transfection approach; ii) comparative analyses of the expression patterns of Pax-5 proteins and their DNA-binding activities in activated B cells from young and aged mice; iii) analyses of Pax-5 proteins in LPS-activated immature B cell line WEHI-231; and iv) pilot characterization of the novel Pax-5_x species. The discussion of the results includes a model of Pax-5 regulation and suggestions for future investigations.

LIST OF ABBREVIATIONS

aa: amino acid(s)

BCR: B cell receptor

BSAP: B cell-specific activator protein

CAT: chloramphenicol acetyltransferase

EMSA: electrophoretic mobility shift assay

Ig: immunoglobulin

ivt: *in vitro* translated

LPS: lipopolysaccharide

kb: kilobase

kD: kilodalton

nt(s): nucleotide(s)

O-GlcNAc: *O*-linked N-acetylglucosamine

SDS-PAGE: sodium dodecyl sulfate- polyacrylamide gel electrophoresis

SRB(s): small resting B cell(s)

NOTE TO THE READER

The work presented in this thesis consists of several related projects that explore Pax-5 activity and function. Some of the more interesting findings of these investigations are reported in the form of a manuscript titled *Functional Analyses of Alternative Isoforms of the Transcription Factor Pax-5* (by Marina A. Lowen and Patty Zwollo). This manuscript is currently in preparation for submission and is included here as Chapter II. Due to the format chosen for this thesis, some of the information provided in the manuscript (Chapter II) may also appear in the Literature Review or General Discussion (Chapters I and IV, respectively). In addition, the Discussion section of the manuscript is less detailed than might be expected for a thesis. However, all appropriate considerations concerning the experimental findings of this project are addressed in detail in the General Discussion (Chapter IV), and the reader is encouraged to refer to that section for a more comprehensive examination of the results in light of the relevant research reported in the current literature.

Additional projects that were performed independent of the manuscript are presented in Preliminary Studies (Chapter III). This chapter describes on-going experiments and pilot studies that examine the function of Pax-5 isoforms using alternative approaches. Conclusions based on all the data presented in the thesis are incorporated into the General Discussion (Chapter IV). The collective results of all projects were used to construct the proposed model of Pax-5 function and regulation.

Function and Regulation of Alternative Isoforms of the Transcription Factor Pax-5 During B Lymphocyte Differentiation.

CHAPTER I

Literature Review

B lymphocytes are the major players of the humoral immune response, which is directed to the detection and elimination of extracellular pathogens and foreign antigens. The main distinguishing characteristic of cells of the B lineage is their ability to synthesize and secrete immunoglobulin (Ig) molecules, the antigen-specific antibodies present on the B cell membrane. Interaction of immunoglobulins with antigens triggers activation of B cells followed by their proliferation and differentiation into either antibody-secreting plasma (effector) cells or memory B cells (Rudin and Thompson, 1998; Liberg and Sigvardsson, 1999).

The molecular mechanisms leading to B cell development and activation are complex and involve a number of highly coordinated regulatory signals that direct B lymphocytes through various stages of differentiation. These signals include the external cues provided by extracellular growth factors and cell-cell contact, and intracellular control executed by transcription factors which direct stage-specific gene expression (Reya and Grosschedl, 1998). Transcriptional regulation of B lymphopoiesis has been studied extensively by both molecular and genetic approaches, and a wide variety of the relevant transcription factors have been identified in recent years. The role of many transcriptional regulators has been determined through studies of mice carrying targeted

mutations in genes encoding proteins that direct lineage- and stage-specific gene expression. Mutational analysis has proven invaluable tool in determining genetic targets and elucidating the functional hierarchy and redundancy of specific transcription factors. A number of current investigations focus on identifying intracellular signaling pathways that regulate the activity of transcription factors and their interactions with other proteins. Further understanding of regulatory mechanisms involved in development and differentiation of the B cell lineage will provide increased insight into various aspects of a productive immune response and help to determine a link between B cell anomalies and pathological states including cancer, autoimmune disorders, and immunodeficiencies.

1. Stages of B lymphocyte development

Generation of an effector B cell is preceded by several stages of maturation as determined by distinctive growth factor requirements, sequential Ig gene rearrangements, and expression of specific sets of cell-surface markers (reviewed in Kruisbeek and Storb, 1994; Rolink and Melchers, 1996; Reya and Grosschedl, 1998; Liberg and Sigvardsson, 1999; Hagman *et al.*, 2000). Progression through these stages occurs in the bone marrow and does not require the presence of antigen. The earliest B cell progenitors (**pro-B cells**) arise from lymphoid stem cells which originate from pluripotent hematopoietic stem cells committed to the lymphoid lineage (Figure I.1). The commitment to lymphoid development is regulated by several transcription factors which include basic helix-loop-helix proteins E2A and early B cell factor (EBF), Ets family transcription factor PU.1, and zinc-finger containing products of the *Ikaros* gene (Reya and Grosschedl, 1998;

Rudin and Thompson, 1998). The main characteristic of pro-B cells is the expression of a transmembrane protein tyrosine phosphatase B220 and surface antigens AA4.1 and CD43 (Reya and Grosschedl, 1998). The Ig genes of these progenitors remain in germline configuration and produce sterile transcripts from the heavy-chain (IgH) locus (Liberg and Sigvardsson, 1999).

Proliferation and differentiation of pro-B cells into precursor B cells (**pre-B cell** stage) is accompanied by activation of the Ig gene recombination machinery and rearrangements at the IgH loci, which is facilitated by the expression of recombinase enzymes Rag-1 and Rag-2. B cell precursors are defined by the presence of the pre-B cell receptor (pre-BCR) on the plasma membrane. The pre-BCR of early pre-B cells consists of a rearranged IgH chain associated with surrogate light chains, λ5 and VpreB, and accessory polypeptides Igα and Igβ. In addition to pre-BCR components, pre-B cells express genes which code for proteins involved in pre-BCR signaling such as the Src family tyrosine kinases (Blk, Lyn, Syk, and Fyn), CD19, CD20, CD22, and CD72 (Li *et al.*, 1993; Liberg and Sigvardsson, 1999). Functional pre-BCR and its signaling components play an important role in positive selection of pre-B cells with productive IgH gene rearrangement (Melchers *et al.*, 1995).

Positively selected pre-B cells (late pre-B cells) undergo Ig light (IgL) chain recombination and enter the next stage of differentiation, the **immature B cell** stage. Cells that failed to generate a functionally rearranged IgH gene will undergo developmental arrest followed by programmed cell death, or apoptosis (Rudin and Thompson, 1998; Scaffidi *et al.*, 1999). Immature B lymphocytes possess a fully formed and functional B-cell receptor (BCR) represented by a membrane-bound IgM with a

particular antigenic specificity. At this stage, the cells are negatively selected against self-specificity and may undergo receptor editing which rescues some of the autoreactive B lymphocytes through an additional round of IgL gene rearrangement (Melchers *et al.*, 1995; Hertz and Nemazee, 1998). After receptor editing, all the cells with an autoreactive IgM are eliminated by apoptosis within the bone marrow (Rudin and Thompson, 1998). The surviving lymphocytes differentiate into **mature B cells** characterized by the presence of antigen-specific IgM and IgD coexpressed on the membrane, and are capable of responding to cognate antigenic stimulation.

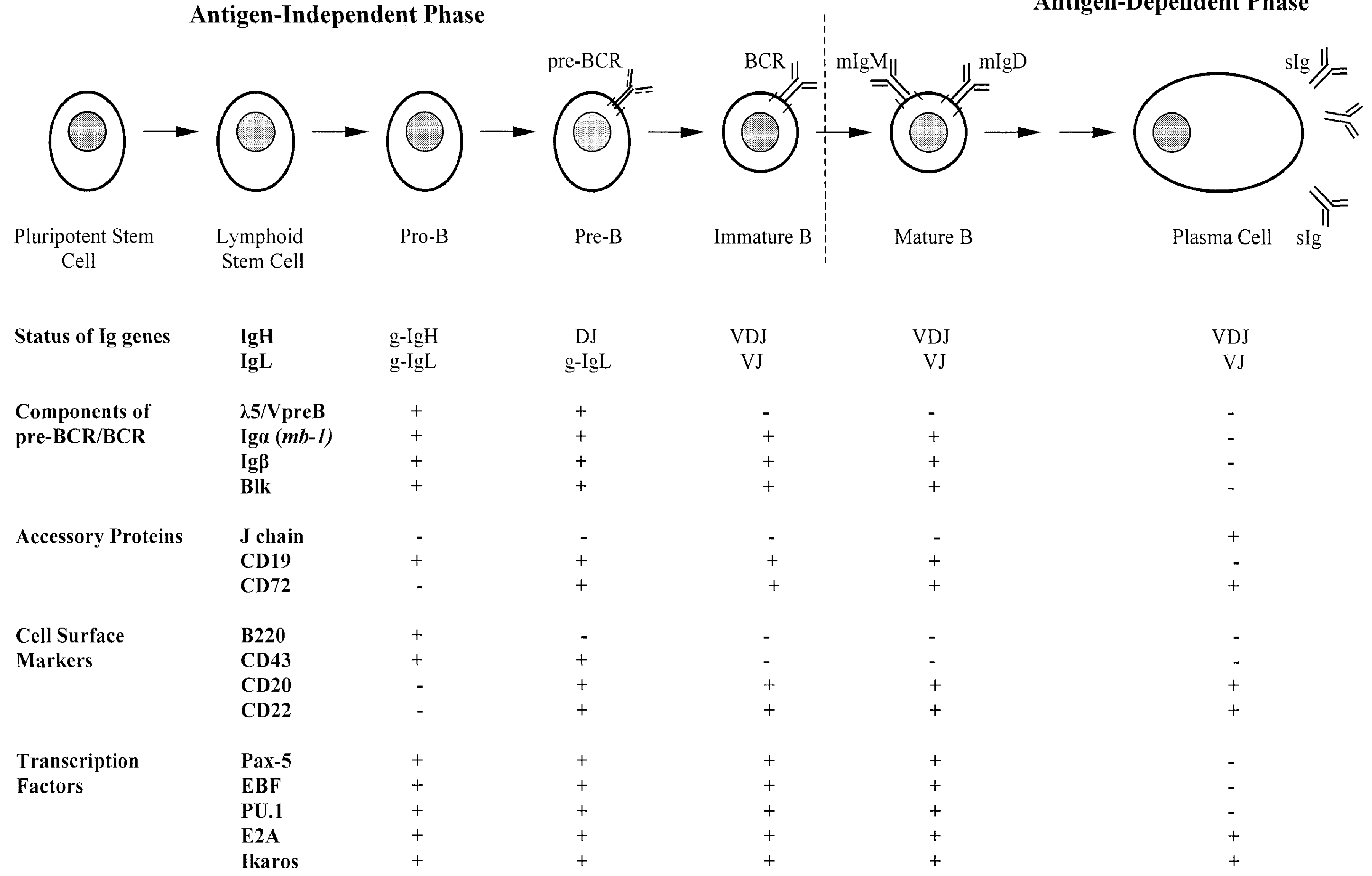
Mature, naive B cells emerge from the bone marrow into the circulating blood and lymph and enter the secondary lymphoid organs where they can either undergo antigen-induced activation or, in the absence of antigen, be eliminated by apoptosis (Kruisbeek and Storb, 1994). The mature B cell stage, as well as all subsequent stages of B cell development, is antigen-dependent. During the antigen-dependent phase, differentiation of mature B lymphocytes may either be initiated by cross-linking of the B cell antigen receptor complex (thymus-dependent antigens), or through direct stimulation by certain cytokines in the presence of B cell mitogens such as bacterial lipopolysaccharide (LPS) (thymus-independent antigens). The signal-transduction pathways are quite different for each route of activation and, in many cases, are not fully understood. Activation of B cells by the thymus-dependent mechanism is antigen-specific and requires interaction with helper T cells within germinal centers that form in the spleen and lymph nodes in response to antigenic stimulation (Rudin and Thompson, 1998). The activation process triggers a number of important changes including Ig isotype class switching, antibody affinity maturation, plasma cell differentiation, and

generation of memory B cells (Liberg and Sigvardsson, 1999; Hagman *et al.*, 2000). In contrast, mitogenic stimulation is independent of antigenic specificity of B cells and does not require contact with T lymphocytes. The immune response to thymus-independent antigens is generally weaker due to a lack of class switching and absence of long-lived memory cells (Hodgkin and Basten, 1995). Regardless of the type of antigen, activation signals always trigger a cascade of events that result in proliferation and further differentiation of mature B lymphocytes into **plasma cells**, highly differentiated effector cells capable of antibody secretion (Kruisbeek and Storb, 1994; Liberg and Sigvardsson, 1999).

While plasma cells have been studied extensively, the characterization of **memory B cells** remains elusive due to a limited number of well-defined membrane markers specific for this population and the consequent difficulties in isolation and purification of these cells. The differentiation pathways leading to formation of memory cells are poorly understood. Some evidence suggests that generation of either plasma or memory B cells is influenced by extracellular signals provided by certain cytokines (e.g., IL-6) or receptor ligands (e.g., CD40L, OX40L) (Arpin *et al.*, 1995; Dutton *et al.*, 1999). Other findings indicate that memory B cells may arise from a different lineage than plasma cells (Klinman, 1997). In general, memory B lymphocytes possess a characteristic CD27 cell-surface marker in addition to diverse isotypes of Ig, various adhesion molecules, and high levels of complement receptors (Dutton *et al.*, 1999; Agematsu *et al.*, 2000). Memory B lymphocytes represent a population of long-lived high-affinity resting cells which, upon their re-exposure to target antigen, can

differentiate into antibody-secreting plasma cells constituting the basis for a secondary immune response (Rudin and Thompson, 1998).

Figure I.1 (see next page): Stages of normal murine B cell development. Distinct stages of B lymphocyte development are determined by the status of immunoglobulin (Ig) genes and expression of the specific sets of genes (Liberg and Sigvardsson, 1999; Hagman *et al.*, 2000). Antigen-independent differentiation of B cells from hematopoietic stem cells into immature B cells takes place in the bone marrow. During these stages, formation of a functional pre-BCR and BCR on the cell surface facilitates positive and negative selection of lymphocytes with productive Ig rearrangements. Activation and differentiation of mature B lymphocytes into plasma cells is an antigen-dependent process which occurs in the peripheral lymphoid organs and culminates in secretion of large quantities of antigen-specific antibodies (sIg). Expression patterns of selected cell surface markers and accessory proteins, as well as several important transcription factors, are indicated. *IgH*, immunoglobulin heavy-chain loci; *IgL*, immunoglobulin light-chain loci; *g-IgH(L)*, immunoglobulin in germline configuration; *mIg*, membrane-bound immunoglobulin molecule; *sIg*, secreted immunoglobulin molecule.



		Pro-B	Pre-B	Immature B	Mature B	Plasma Cell
Status of Ig genes	**IgH**	g-IgH	DJ	VDJ	VDJ	VDJ
	IgL	g-IgL	g-IgL	VJ	VJ	VJ
Components of pre-BCR/BCR	**λ5/VpreB**	+	+	-	-	-
	Igα (*mb-1*)	+	+	+	+	-
	Igβ	+	+	+	+	-
	Blk	+	+	+	+	-
Accessory Proteins	**J chain**	-	-	-	-	+
	CD19	+	+	+	+	-
	CD72	-	+	+	+	+
Cell Surface Markers	**B220**	+	-	-	-	-
	CD43	+	+	-	-	-
	CD20	-	+	+	+	+
	CD22	-	+	+	+	+
Transcription Factors	**Pax-5**	+	+	+	+	-
	EBF	+	+	+	+	-
	PU.1	+	+	+	+	-
	E2A	+	+	+	+	+
	Ikaros	+	+	+	+	+

Figure I.1

2. Pax-5 (BSAP) as the "master regulator" of B cell development

2.1 Pax family of genes.

A number of transcription factors regulate lineage- and differentation-stage-specific expression of the genes involved in B lymphopoiesis (Reya and Grosschedl, 1998; Liberg and Sigvardsson, 1999). Among these factors are the products of the *Pax-5* gene which encodes one of the most critical transcriptional regulators, the B-cell specific activator protein (BSAP). *Pax-5* is a member of a family of genes which encode nuclear transcription factors involved in development, organogenesis, morphogenesis, and pattern formation (Strachan and Read, 1994). The *Pax* family consists of nine members (*Pax-1* through *Pax*-9) all of which share an evolutionarily conserved N-terminal DNA-binding region of 128 amino acids comprising the paired domain (Walther *et al.*, 1991). The *Pax* genes have been classified into four paralogous groups (Figure I.2) based on structural similarities within the paired domain and on the presence or absence of the centrally-located octamer and homeodomain motifs (Dahl *et al.*, 1997). Genes within each subclass show similar patterns of expression during embryogenesis and are highly homologous in their paired domain sequence (Walther *et al.*, 1991).

The *Pax-2*, *Pax-5*, and *Pax-8* genes comprise a group of closely related *Pax* genes which contain a complete octapeptide motif and a truncated homeodomain (Figure I.2; Adams *et al.*, 1992; Strachnan and Read, 1994). The paired domains within the Pax-2/5/8 subclass show 90-95% homology in the amino acid sequence, and, as a result, the three proteins recognize almost identical DNA sequences (Walther *et al.*, 1991; Kozmik *et al.*, 1993). Since the spatial and temporal expression patterns of the *Pax-2*, *Pax-5*, and *Pax-8* genes overlap partially during early mouse development (Nornes *et al.*, 1990;

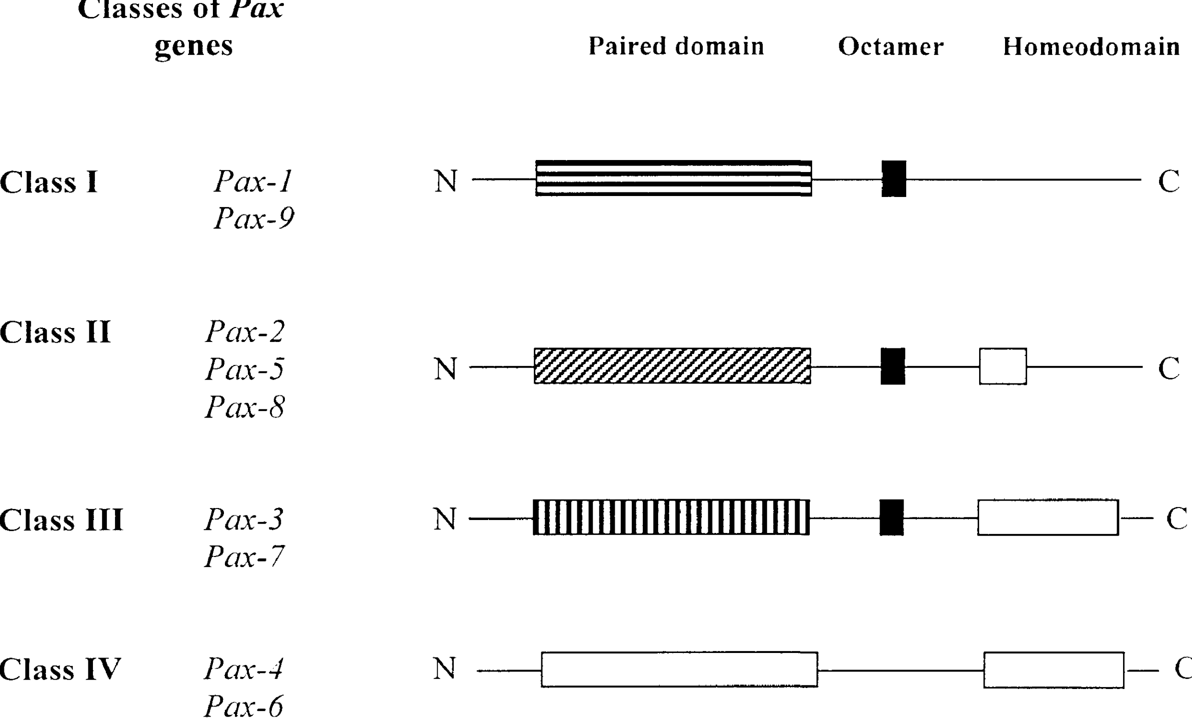


Figure I.2: Classes of *Pax* family of genes. Vertebrate *Pax* genes have been classified into four classes (I through IV) based on the presence or absence of the octamer sequence and homeodomain homology region. *Pax-1* and *Pax-9* genes have no homeodomain and thus belong to the same class, Class I. Genes of Class II, *Pax-2*, *Pax-5*, and *Pax-8,* contain a partial homeodomain with only one α-helix present. *Pax-3* and *Pax-7* form Class III and possess the octamer sequence and the complete homeodomain. Class IV is represented by *Pax-4* and *Pax-6* which have the complete homeodomain but lack the octamer. Genes within each class contain a highly homologous DNA-binding motif, the paired domain. The binding specificity of the paired domain varies between the classes, as is depicted by different patterns.

Plachov *et al.*, 1990; Asano and Gruss, 1992), their products may have either cooperative or redundant function in regulation of target genes. For example, cooperation of *Pax-2* and *Pax-5* has been shown to be essential for normal midbrain and cerebellum development (Urbanek *et al.*, 1997), while *Pax-2* and *Pax-8* have been postulated to regulate the same genes in kidney cells (Kozmik *et al.*, 1993).

2.2 BSAP: discovery, expression patterns, and functions

The transcription factor Pax-5 was originally identified as a mammalian homolog of the sea urchin tissue-specific activator protein (TSAP) which is involved in regulation of late histone gene (H2A-2.2) expression in sea urchins (Barberis *et al.*, 1990). The expression of the newly discovered factor was observed in cells of the B lymphoid lineage with the exception of terminally differentiated plasma cells. Furthermore, this TSAP homolog was shown to bind DNA in nuclear extracts derived from pro-B, pre-B, and mature B lymphocytes. As no expression or DNA-binding activity was detected in cells of either erythroid or T lymphoid lineages, the novel protein was named B-cell specific activator protein (BSAP) (Barberis *et al.*, 1990). Further biochemical purification and characterization of BSAP revealed that it is encoded by the *Pax-5* gene (Adams *et al.*, 1992).

The earliest expression of *Pax*-5 is detected in the developing central nervous system where it is temporally and spatially regulated (Asano and Gruss, 1991; Adams *et al.*, 1992). *Pax*-5 transcripts are also found in fetal liver and, later, in all B-lymphoid tissues and testis of adult mice (Adams *et al.*, 1992). Within the B cell lineage, *Pax-5* is expressed during early stages of B cell development, from the pro-B-cell up to the mature

B-cell stage, but is greatly downregulated or absent in plasma cells (Barberis *et al.*, 1990).

Some important regulatory functions of Pax-5 were determined through the analysis of *Pax-5-/-* knockout mice (Urbanek *et al.*, 1994). Consistent with its expression pattern, *Pax-5* has been shown to play an essential role in B cell lymphopoiesis and midbrain development. Inactivation of the Pax-5 gene in mouse resulted in abnormalities of the posterior midbrain region and in a complete block of B cell development. Significantly, *Pax-5-/-* mutant mice failed to produce small pre-B, mature B, and plasma cells, although they could still generate B220+ cells indicating developmental arrest at the pro-B stage. The lack of Pax-5/BSAP was also correlated with alterations in the expression of some of its target genes, most notably the *CD19* gene which was not expressed in the knockout mice (Urbaneck *et al.*, 1994; Nutt *et al.*, 1997). The *Pax-5* mutation also inhibited V_H-to-D_HJ_H recombination and antibody production, indicating the involvement of this transcription factor in the regulation of expression and rearrangement of Ig-gene (Urbanek *et al.*, 1994; Nutt *et al.*, 1997). Thus, the study of *Pax-5-/-* mutant mice revealed that Pax-5 is a critical factor for progression of B cell differentiation beyond the pro-B stage (Figure I.3).

In addition to its role in B-lymphopoiesis, Pax-5 has been implicated in activation and proliferation of B lymphocytes. Introduction of Pax-5 anti-sense oligonucleotides into mature B cells causes decreased BSAP expression and subsequently leads to a significant reduction in LPS-induced cell proliferation (Wakatsuki *et al.*, 1994). This observation is in agreement with other studies of the *Pax* gene family which indicate that one of the important functions of *Pax* genes is initiation of cell proliferation (Dahl *et al.*,

1997). Deregulation of these genes often results in cell transformation and development of tumors. Thus, altered expression of *Pax-5* has been detected in medulloblastomas, astrocytomas, and some lymphomas (Kozmik *et al.*, 1995; Stuart *et al.*, 1995a; Busslinger *et al.*, 1996; Mahmoud *et al.*, 1996; Krenacs *et al.*, 1998). Furthermore, Pax-5, as well as Pax-2 and Pax-8, have been shown to suppress *p53*, a tumor suppressor gene that is essential for controlled cell proliferation, apoptosis, and protection against oncogenic transformation (Stuart *et al.*, 1995b; Lambert *et al.*, 1998). Based on this finding, it has been proposed that high levels of Pax-5 expression are required for prevention of p53-mediated apoptosis during early stages of B cell development when rapid cell growth is necessary for attainment of critical cell mass (Stuart *et al.*, 1995b). Conversely, the reduced expression of Pax-5 alleviates p53 inhibition allowing for lymphocyte selection and further differentiation. During the final stages of B cell development, downregulation of Pax-5 activity is required for terminal differentiation of mature activated B cells into plasma cells that are capable of Ig isotype class switching and high antibody production (Usui *et al.*, 1997; Cogne *et al.*, 1994; Stuber *et al.*, 1995).

A recent study by Nutt *et al.* (1999) has revealed an essential role of Pax-5 in B-lineage commitment during the earliest stages of B lymphopoiesis. *Pax-5-/-* pro-B cells (derived from *Pax-5* knockout mice) were shown to have characteristics of a hematopoietic progenitor with broad lymphomyeloid developmental potential, including the expression of genes from different lineage-affiliated programs. Under specific *in vitro* conditions with provided growth requirements, the uncommitted progenitors can be induced to differentiate into distinct cell lineages such as monocytes, granulocytes and natural killer cells. However, reconstitution of Pax-5 activity in *Pax-5-/-* pro-B cells

leads to repression of lineage-promiscuous gene expression and subsequent restriction of developmental plasticity in favor of the B-lymphoid lineage. Thus, during early differentiation events, Pax-5 simultaneously functions as an activator of B-cell specific genes and a repressor of inappropriate expression of other hematopoietic genes. Functions of Pax-5 important for B lymphocyte development and differentiation are summarized in Figure I.3.

2.3 Pax-5 target genes

Pax-5 binding sites have been identified on the promoters of several B-cell-specific putative target genes including *CD19*, *mb-1*, *VpreB*, *λ5*, *Ig J* chain, and *blk* (reviewed in Hagman *et al.*, 2000). In addition, Pax-5 plays an important role in regulation of the Ig genes: binding sites have been identified on *Ig* heavy chain *3' Cα* and *Ig* light chain *κ3'* enhancers, *Ig α* and *ε* germline promoters, and in multiple *Ig* switch regions. Other Pax-5 targets include the *p53* tumor suppressor gene (Stuart *et al.*, 1995b), genes encoding transcription factors hXBP-1 (Reimold *et al.*, 1996), LEF-1 and N-myc (Nutt *et al.*, 1998), as well as the gene encoding the cell surface protein PD-1 (Nutt *et al.*, 1998).

Depending on the target gene and/or developmental stage of the B cell, Pax-5 may function as an activator, repressor or docking protein as determined by the presence and activity of other transcription factors. Among the positively regulated Pax-5 targets are genes encoding pre-BCR/BCR components and molecules associated with BCR signaling such as the CD19 co-stimulatory receptor (Kozmik *et al.*, 1992), components of the surrogate light chain VpreB and λ5 (Okabe *et al.*, 1992; Tian *et al.*, 1997), and protein

Figure I.3: Functions of Pax-5 (BSAP) in B cell differentiation (see next page). *Pax-5* is expressed during early stages of B cell development (from pro-B to mature B), but greatly downregulated in plasma cells. At the earliest stages of differentiation, Pax-5 is thought to direct the commitment of hematopoietic lymphoid progenitors toward the B-lymphoid lineage by repressing lineage-promiscuous genes such as *M-CSF-R*. During antigen-independent stages of B cell development, Pax-5 regulates a number of B-cell specific genes that encode pre-BCR/BCR components and proteins involved in BCR signaling. B cell development is blocked at the pro-B cell stage in *Pax-5-/-* knockout mice. Pax-5 has also been speculated to have a function in pro-/pre-B cell proliferation; however, the mechanism of this regulation is unclear. During antigen-dependent phase, Pax-5 is involved in activation and proliferation of mature B lymphocytes and Ig heavy chain class switch recombination. Downregulation of Pax-5 expression during the plasma stage cell results in relief of its repressor function, which is especially important for production and secretion of antibodies. (+), positively-regulated Pax-5 target genes; (-), negatively-regulated Pax-5 target genes.

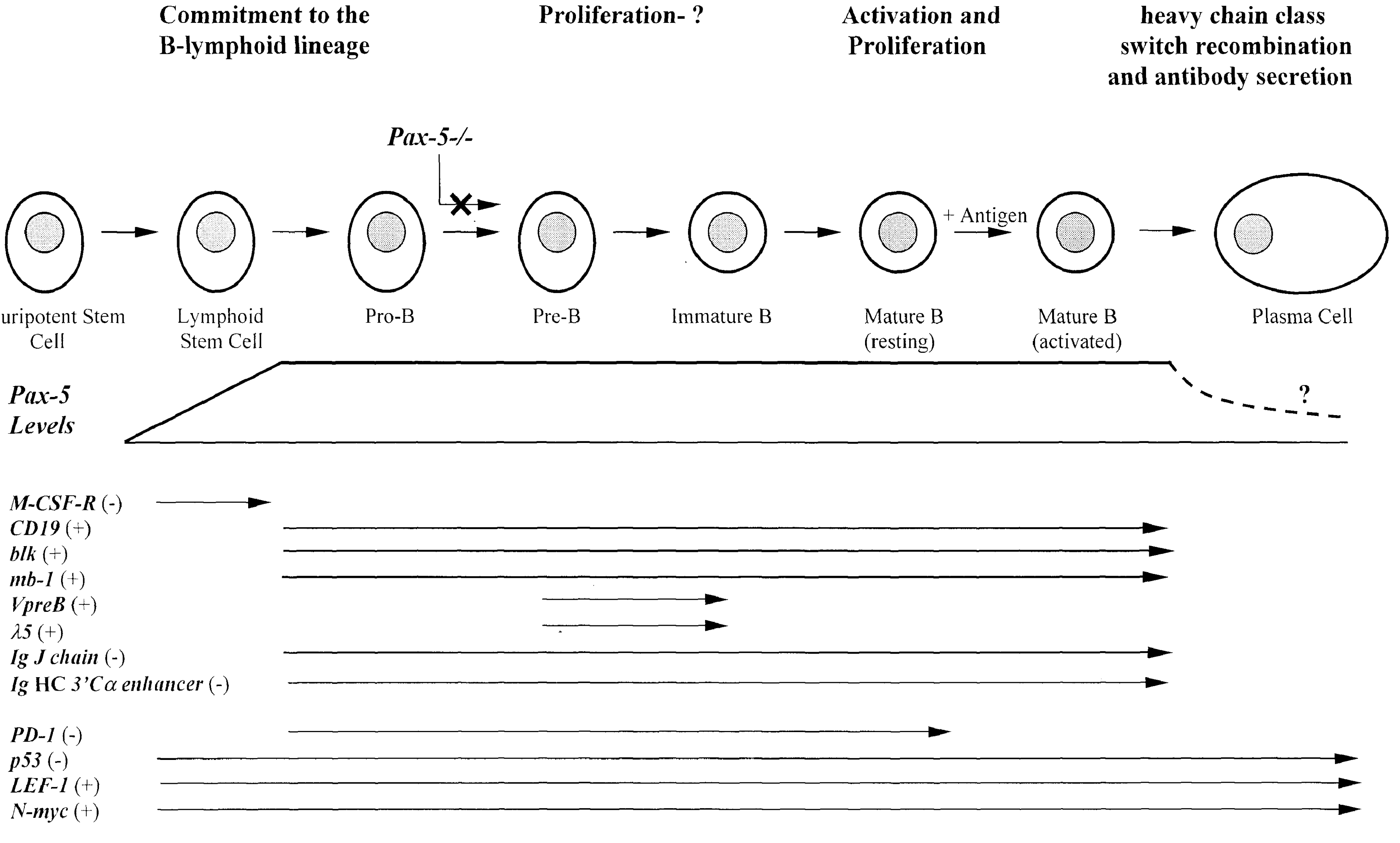


Figure I.3

tyrosine kinase Blk (Zwollo and Desiderio, 1994). In contrast, overexpression of Pax-5 inhibits the expression of the *Ig J-chain* gene (Rinkenberger *et al.*, 1996), while its downregulation activates the IgH 3'α enhancer (Singh and Birshtein, 1993; Neurath *et al.*, 1994), illustrating in both cases the repressor function of Pax-5. Pax-5 also functions as a repressor of *PD-1* and *hXBP-1* genes (Reimold *et al.*, 1996; Nutt *et al.*, 1998) and may be a key factor in inhibition of alternative lineage choices during commitment of hematopoietic progenitors to B lymphopoiesis (see Section ***2.2***; Nutt *et al.*, 1999).

As a docking protein, Pax-5 forms a complex with Ets-1 and functions as a recruiter in positive regulation of the *mb-1* gene which encodes the Igα subunit of the BCR (Fitzsimmons *et al.*, 1996). Similarly, it can interact with co-repressors of the Groucho family and may recruit them for inhibition of the negatively regulated Pax-5 target genes, including the *M-CSF-R* gene that encodes the myeloid cytokine M-CSF receptor (Eberhard *et al.*, 2000). Curiously, the transcription of some genes (*mb-1*, *PD-1*, *LEF-1*) can be regulated with equal efficiency by either full-length Pax-5 protein or the truncated paired domain polypeptide PRD (Nutt *et al.*, 1998). This finding is an indication of the recruiter function of Pax-5 and yet another piece of evidence for the intricate complexity of pathways and interactions involved in B-cell-specific gene regulation.

2.4 Pax-5 protein functional domains

The main distinguishing characteristic of the *Pax* family of transcription factors is the paired domain. Mutational analysis of Pax-5 and its DNA recognition sequences revealed that this 128-amino acid DNA-binding motif has a bipartite structure with

distinct amino- and carboxy-terminal domains that bind to half-sites in adjacent major grooves of the DNA helix (Czerny *et al.*, 1993). This structure has been confirmed by X-ray crystallographic analysis of the paired domain-DNA complex, which has also demonstrated that each subdomain is composed of a helix-turn-helix motif resembling the structure of the homeodomain (Xu *et al.*, 1995). For different members of the *Pax* family, the specificity of DNA-binding is achieved through recognition of differential target sequences as well as through cooperation between the paired domain and the homeodomain (Jun and Desplan, 1996). Those *Pax* proteins that contain a full homeodomain (i.e., Pax-3, Pax-4, Pax-6, and Pax-7) have the ability to utilize that region in different combinations with either one or both paired subdomains for differential binding of DNA sequences (Jun and Desplan, 1996). In contrast, DNA-binding of the proteins without a complete homeodomain, such as the members of Pax-2/5/8 subfamily, has to rely entirely on both subdomains of the paired motif (Czerny *et al.*, 1993; Epstein *et* al., 1994; Xu *et al.*, 1995).

Despite the differences in binding mechanisms and subclass-specific preferences for binding certain sets of nucleotides, consensus sequences for the Pax proteins show an unusual amount of degeneracy. For example, medium- or low-affinity binding sites for Pax-5/BSAP occur every 1-2 kb in the mouse genome; however, the only sites that have functional relevance are those found in the vicinity of B cell-specific genes (Busslinger and Urbanek, 1995). Furthermore, none of the naturally occurring Pax-5 binding sites conform completely to the consensus recognition sequence (Czerny *et al.*, 1993; Busslinger and Urbanek, 1995). Therefore, to ensure regulation of specific genes and to

enhance DNA-sequence specificity, the *Pax* transcription factors must engage in additional interactions with various partner proteins (Hagman *et al.*, 2000).

The transactivation function of Pax-5 is regulated by the C-terminal regulatory module comprised of Ser/Thr/Pro-rich transactivation domain (aa 304-358) and the adjacent repressor sequence (aa 358-391) (Dörfler and Busslinger, 1996). The inhibitory domain has been proposed to function as a DNA context-specific "switch" that determines whether Pax-5 acts as an activator or a repressor of transcription of a particular gene (Dörfler and Busslinger, 1996). Interestingly, similar negative regulatory domains appear to be common in inducible transcription factors that are activated by intracellular signaling events. Although a connection between transcriptional activation and signal transduction has not been found for Pax-5, it is conceivable that such a link could have an important function in regulation of gene transcription during B cell activation.

To date, the exact mechanism of differential regulation of Pax-5 target genes remains unclear. It has been established that regulation of many Pax-5 target genes requires recruitment and cooperative interactions with partner-proteins (Fitzsimmons *et al.*, 1996; Eberhard *et al.*, 2000). Alternatively, transcription of some positively regulated genes might be initiated through the direct contact between the basal transcription machinery and Pax-5 functional domains other than the C-terminal regulatory module (Dörfler and Busslinger, 1996). The homeodomain homology motif (aa 229-251) and the octamer sequence (aa 179-186) are the likely candidates for such interactions, as both have been shown to be involved in complex formation with other proteins. For example, the partial homeodomain can bind the TATA-binding (TBP) and the retinoblastoma (Rb)

proteins (Eberhard and Busslinger, 1999). Incidentally, the Rb protein is known to repress activity of several transcription factors by either inhibiting their interaction with the basal transcription machinery or by promoting the formation of inactive chromatin (Weintraub *et al.*, 1995; Luo *et al.*, 1998). The octamer sequence is involved in interaction of Pax-5 with Groucho proteins which repress Pax-5 transcriptional activity (Eberhard *et al.*, 2000). In addition, the presence of the octapeptide motif has been shown to attenuate transactivation function of Pax-2 and several other Pax proteins (Lechner and Dressler, 1996).

2.5 Pax-5 isoforms

The *Pax-5* gene produces four alternatively spliced variants: *Pax-5a*, *Pax-5b*, *Pax-5d*, and *Pax-5e* (Figure I.4; Zwollo *et al.*, 1997). The full length Pax-5 is known as the B cell-specific transcription factor BSAP, and corresponds to the Pax-5a isoform. Alternative isoforms of the *Pax-5* gene include transcripts with incomplete DNA-binding domains (*Pax-5b* and *Pax-5e*), and the variants in which a region containing the homeodomain and the C-terminal regulatory module (aa 203-391) has been replaced with the novel sequence (*Pax-5d* and *Pax-5e*). It has been shown that only the Pax-5a and Pax-5d isoforms can interact with the Pax-5 binding site (Zwollo *et al.*, 1997). Due to the deletions in the paired domain, Pax-5b and Pax-5e are unable to bind to DNA and must regulate their target genes by indirect mechanisms or via interactions with other factors. At the present, the function of the novel sequence (aa 203-244) is unknown, but has been hypothesized to have an inhibitory role in regulation of transcription (Anspach *et al.*, submitted).

The four Pax-5 isoforms exhibit different levels and patterns of expression throughout B-cell development and may vary in their function and transactivation properties. In agreement with previous findings, the levels of Pax-5a appear to be high during all stages of B cell development except the plasma cell stage. Pax-5d levels are low in B cell lines, but easily detectable in resting splenic B cells (Anspach *et al.*, submitted). Normal resting B cells, as well as early B cell lines, contain very low levels of Pax-5e and Pax-5b protein. Curiously, Pax-5b levels increase during the late stages of B cell development and the protein remains detectable even in the plasma cell line (Zwollo *et al.*, 1997); however, the significance of this is unclear. The relative levels of alternative *Pax-5* transcripts have been estimated by screening λ phage libraries containing spleen or B cell cDNA from the mature B cell line A20/2J (Zwollo *et al.*, 1997). In 45 Pax-5-containing clones isolated from the spleen library, 35 represented isoform *Pax-5a* (78%), seven clones were *Pax-5d* (15.6%), two were *Pax-5b* (4.4%), and one was identified as *Pax-5e* (2%) (Zwollo *et al.*, 1997). It is not unlikely that relative levels of Pax-5 isoforms vary depending on the developmental stage or activation status of B cells.

In addition to alternative splicing, each *Pax-5* transcript can produce proteins from one of two available translational start sites: a proximal ATG codon (at nucleotide 1) and a distal ATG codon (at nucleotide 325) (Figure I.4; Zwollo *et al.*, 1997). Use of the proximal ATG codon generates isoforms Pax-5a and Pax-5d from the corresponding transcripts. Translation initiation from the distal codon on either *Pax-5a* or *Pax-5b* transcripts produces a 41-kD protein with a partial DNA-binding domain. Likewise, the Pax-5e isoform can arise from either *Pax-5d* or *Pax-5e* transcript with translation starting

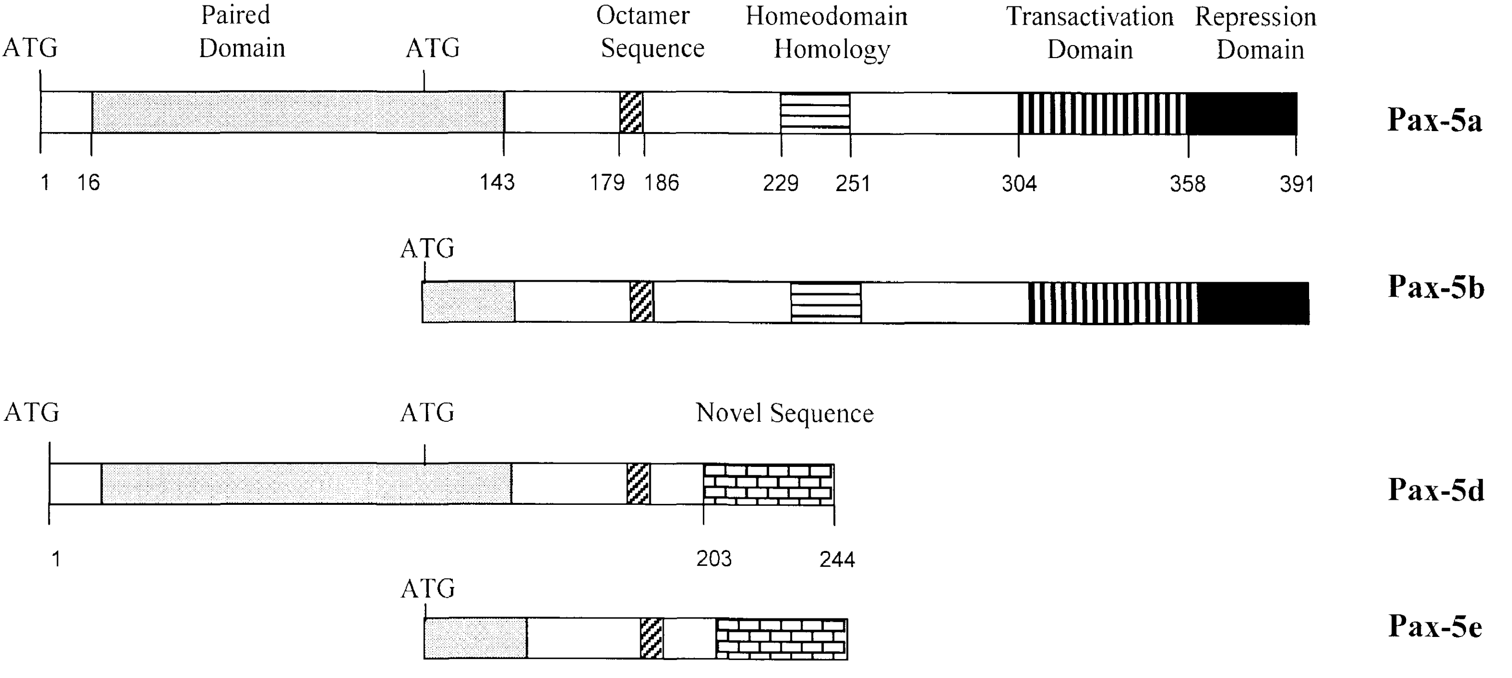


Figure I.4: Pax-5 transcription factor isoforms. The *Pax-5* gene produces four alternatively spliced variants: Pax-5a (53 kD), Pax-5b (41 kD), Pax-5d (31 kD), and Pax-5e (19 kD). *Shaded boxes* indicate the position of the paired DNA-binding domain (aa 16-143); *hatched boxes* indicate the octamer sequence (aa 179-186); *horizontally striped boxes* indicate the homeodomain homology region (aa 229-251); *vertically striped boxes* indicate the transactivation domain (aa 304-358); and *black boxes* indicate the repressor sequence (aa 358-391). Pax-5b and Pax-5e have incomplete paired domains and are unable to bind to DNA. In Pax-5d and Pax-5e isoforms, a region containing the transactivation domain, the repressor sequence, and the homeodomain homology region have replaced with the novel sequence (aas 203-244) of unknown function (*checkered boxes*). All of the *Pax-5* transcripts contain two in-frame translational start sites: one at nucleotide 1 (proximal ATG codon) and another at nucleotide 325 (distal ATG codon).

at the distal ATG start site. While both *Pax-5b* and *Pax-5e* have the proximal ATG codon, translation from that start site creates a termination codon at nucleotide 245 resulting in the expression of a short 3.2-kD (27 aa), peptide (Zwollo *et al.*, 1997). The presence of two alternative start sites on the *Pax-5* transcripts suggests that a single transcript can give rise to two different proteins. The mechanism controlling the switch from one start codon to another may have an important role in regulation of differential expression of Pax-5 isoforms.

3. Regulation of transcription factors

3.1 Combinatorial gene regulation

Complex molecular pathways involved in B cell development and function require cooperation of multiple transcription factors or "combinatorial regulation" of the associated genes (Ernst and Smale, 1995). The diverse patterns of regulation are generated by unique combinations and spatial arrangements of these factors at the promoter and enhancer regions of each specific gene (Chen, 1999). For temporally- and spatially-regulated gene expression, it is essential for a cell to have a particular set of factors present at each stage of the development and differentiation. Hence, the expression and activity of any given factor is tightly regulated by distinct signal transduction pathways in a stage-specific manner.

Four levels of regulation determine the concentration and activity of transcription factors: transcriptional, post-transcriptional, translational, and post-translational (Calkhoven and Geert, 1996). At the level of transcription, the frequency of initiation of transcription and the rate of RNA synthesis determine the expression levels of

transcription factors. At the post-transcriptional level, mRNA stability, transport, and, most importantly, RNA processing events regulate the amount and diversity of the synthesized proteins. At the translational level, the function and availability of a transcription factor can be affected by selection of alternative start-sites, rate of protein synthesis, and by specifics of nuclear transfer associated with that factor. Finally, protein function and activity can be controlled post-translationally by structural modifications and specific interactions with other proteins. In addition, one of the critical factors for the function of transcription factors is their stability. Protein stability can be regulated at any of the mentioned regulatory levels, its changes often induced by various extracellular signals (Pahl and Baeuerle, 1996). Regulatory events occurring at the post-transcriptional and post-translational levels are especially interesting for the work presented in this thesis and will be discussed in more detail in the following sections.

3.2 Post-transcriptional control: alternative RNA splicing

Alternative RNA splicing allows generation of families of transcription factors with diverse and distinct properties (Foulkes and Sassone-Corsi, 1992; López, 1995; Calkhoven and Geert, 1996). Originating from a single gene, alternative mRNA variants may generate polypeptides which differ in their functions, translation efficiency, DNA-binding activity, ability to engage in protein-protein interactions, and stability (López, 1996). The expression levels of alternative isoforms and their proportions are frequently regulated according to developmental stage, tissue specificity, or cell polarity (López, 1996). For example, six alternatively spliced isoforms of *Pax*-8 gene are temporally and spatially regulated during early mouse development (Kozmik *et al.*, 1993). Although

production of alternative isoforms has been reported for a number of transcription factors, the biological consequences and advantages of this phenomenon are not clearly understood in many cases.

Alterations in a DNA-binding domain may result in synthesis of proteins that control distinct sets of genes, as a result of a differential capacity for target sequence binding and recognition. These type of functional variations can be achieved either by truncation of the entire DNA-interaction region, or by generation of isoforms with a different number of DNA-binding domains, different spacing between the motifs, or with variable specificity. For example, alternative insertion of additional amino acid residues affects the paired domains of Pax-6, Pax-3, and Pax-7, resulting in generation of isoforms with various DNA-binding potentials and specificities (Kozmik *et al.*, 1997; Vogan *et al.*, 1996). *Ikaros*, a gene implicated in the control of B- and T-lymphopoiesis, produces eight alternatively spliced isoforms which differ in the number of the N-terminal zinc fingers (Georgopoulos *et al.*, 1997). With at least three zinc fingers required for sequence-specific, high-affinity DNA-binding, only three Ikaros proteins (Ik-1, Ik-2, and Ik-3) contribute directly to transcriptional regulation of their target genes; others are transcriptionally inert.

The most common strategy for the production of activator and repressor isoforms from the same gene involves alterations within the transactivation domain or its complete removal (Foulkes and Sassone-Corsi, 1992). If such isoforms possess identical DNA-binding domains and have similar binding affinities for target sequences, they can compete for DNA binding. This provides a mechanism for differential regulation that depends on the relative concentration of the competing isoforms. The same holds true for

activators of different strengths. For example, Pax-4 and Pax-6 recognize similar sequences and may compete for binding in tissues with overlapping expression (Kalousova *et al.*, 1999). However, because Pax-4 is a weaker activator than Pax-6 (Kalousova *et al.*, 1999), it may actually function as a Pax-6 inhibitor.

Isoforms lacking DNA-binding and/or transactivating domains may exert their inhibitory function by sequestering other factors into inactive complexes (López, 1996). For example, non-DNA-binding Ikaros proteins can play a dominant-negative role in transcription by forming transcriptionally inert heterodimers with active DNA-binding isoforms (Georgopoulos *et al.*, 1997). Alternatively, the inhibitor-isoforms that contain functional protein interaction motifs can inhibit transcription by binding accessory and partner-proteins which are necessary for combinatorial gene regulation.

3.3 Post-translational control: structural protein modifications

Post-translational level of control involves structural modifications which affect the activity and functions of transcription factors. These modifications may include (de)phosphorylation, acetylation, glycosylation, formation of disulfide bonds (redox potential), or any other alterations which may induce conformational changes in a protein. Through exposing, masking, or remodeling a particular functional domain, post-translational modifications may determine the DNA-binding activity and transactivation function of a transcription factor, as well as its nuclear localization, stability, and association with other proteins (Calkhoven and Geert, 1996). Compared to the transcriptional level of control, regulation at the protein level is faster and more readily reversible. Such functional flexibility is important for a great number of transcription

factors which participate in signal-transduction cascades, including NF-κB, c-Jun, p53 (Calkhoven and Geert, 1996). In that context, post-translational modifications provide a sensitive mechanism for a quick and efficient response to specific signaling pathways that lead to activation or inhibition of particular target genes.

Phosphorylation is one of the most common means of functional regulation which affects a wide variety of cellular proteins, including nuclear transcription factors. Easily reversible and abundant, phosphorylation frequently occurs as a step in signal transduction cascades triggered by environmental stress and stimulation of cell-surface receptors (Hunter and Karin, 1992; Calkhoven and Geert, 1996). For instance, multi-site phosphorylation is a major mechanism regulating the activity of p53 in response to various extracellular stimuli (Meek, 1998). This modification can modulate a number of the protein's functions ranging from DNA-binding and transactivation properties to its stability and ability to interact with other factors (Lambert *et al.*, 1998; Meek, 1998; Steegenga *et al.*, 1996). Another example is phosphorylation of c-Jun which is triggered by mitogenic stimulation and stress. The phosphorylation sites of c-Jun are located in the transactivation domain, and their modification results in increased transactivation potential of this transcription factor (Calkhoven and Geert, 1996).

Modification of transcription factors via formation of disulfide bonds has not been studied as extensively as protein phosphorylation. However, several recent studies indicate that modification of redox state is an important mechanism of regulation of transcription factors. Redox regulation occurs through reduction/oxidation of specific cysteine residues and formation of disulfide bonds in different functional domains of a protein (Tell *et al.*, 2000). The process is mediated by reducing enzymes, such as

thioredoxin and Ref-1, and is often triggered by oxidative stress (Hirota *et al.*, 1997; Hirota *et al.*, 1999). Frequently, modification of protein redox state is involved in regulation of DNA binding activity and, as a rule, reduced forms of transcription factors are required for efficient binding to DNA sequences. For example, activity of NF-κB (Schreck *et al.*, 1991; Hirota *et al.*, 1999), AP-1 (Abate *et al.*, 1990; Hirota *et al.*, 1997), HIF-like factor (HLF) (Lando *et al.*, 2000), and Pax-2/5/8 proteins (Tell *et al.*, 1998) depends upon reduced state of their DNA-binding domain. Interestingly, hypoxia-inducible factor 1α (HIF-1α), which is closely related to the HLF, does not require the presence of reducing enzymes for its DNA-binding; however, its transactivation domain is subject to redox control by Ref-1 (Lando *et al.*, 2000). Another facet of redox regulation involves formation of an intermolecular disulfide bond in E2A homodimers (Benezra, 1994), which play an important regulatory role in B lymphocyte development (Murre *et al.*, 1989). In monomeric form, the protein has a very low DNA binding activity, although it can still efficiently heterodimerize with its partner-proteins (Benezra, 1994). Thus, only E2A homodimers can bind to DNA. That interaction is stabilized by a disulfide link between two E2A proteins (Benezra, 1994), and has recently been shown to be regulated by redox-active proteins (Markus and Benezra, 1999).

Glycosylation has long been considered a modification that occurs exclusively on extracellular or lumenal proteins. Recently, however, it has become clear that glycoproteins can also be found in the nucleus (reviewed in Haltiwanger *et al.*, 1997; Comer and Hart, 2000). The best characterized and the most abundant type of glycosylation affecting transcription factors is glycosylation by *O*-linked N-acetylglucosamine (*O*-GlcNAc) (Haltiwanger *et al.*, 1997). This modification involves

addition of a single monosaccharide residue, *O*-GlcNAc, to the side-chain hydroxyl groups of serine and/or threonine. The enzymes responsible for the addition and removal of *O*-GlcNAc moieties to proteins have been identified (Haltiwanger *et al.*, 1990; Dong and Hart, 1994). A uridine diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase (*O*-GlcNAc transferase) is a soluble enzyme with a strong preference for peptides containing a proline residue (Haltiwanger *et al.*, 1997). *O*-GlcNAc transferase has been found in both cytoplasm and nucleus (Haltiwanger *et al.*, 1992; Kreppel *et al.*, 1997; Akimoto *et al.*, 1999), and its gene was cloned from rat, *C. elegans* and human (Kreppel *et al*, 1997; Lubas *et al.*, 1997). A cytosolic and nuclear β-*N*-acetylglucosaminidase with selectivity toward *O*-linked GlcNAc has also been identified and purified (Dong and Hart, 1994).

About fifty different cytoplasmic and nuclear proteins are known to be modified by *O*-glycosylation (Haltiwanger *et al.*, 1997). These include transcription factors Sp1 and Ap1 (Jackson and Tjian, 1988), the tumor suppressor protein p53 (Shaw *et al.*, 1996), nuclear pore complex proteins (Holt *et al.*, 1987), c-Myc (Chou *et al.*, 1995), and various cytoskeletal proteins (for references see Haltiwanger *et al.*, 1997). The levels of glycosylation may vary for different proteins and during specific signaling events, which indicates that this modification is dynamic and reversible (Comer and Hart, 2000). *O*-glycosylation has been hypothesized to play a role in regulation of such processes as cell activation and cell division (Kearse and Hart, 1991; Chou and Omary, 1993), transcriptional regulation (Jackson and Tjian, 1988; Kelly *et al.*, 1993; Chou *et al.*, 1995), and protein synthesis (Datta *et al.*, 1989).

While a strict consensus sequence has not been discerned for *O*-GlcNAc-modified proteins, most glycosylated sites contain a proline residue amino-terminal to the modified serine or threonine (Haltiwanger *et al.*, 1997). These sites are remarkably similar to sites phosphorylated by mitogen-activated and other proline-directed kinases (Kemp and Pearson, 1990). In fact, most *O*-glycosylated proteins are also extensively regulated by phosphorylation, which appears to compete with *O*-GlcNAc for sites on such proteins (Haltiwanger *et al.*, 1997; Comer and Hart, 2000). The interrelation between *O*-GlcNAc-glycosylation and phosphorylation is complex, and the two modifications are probably differentially regulated through distinct signaling pathways, adding infinite diversity to protein function.

4. Significance and goals of the presented research

4.1 Functional significance of Pax-5 isoforms

The *Pax-5* gene plays a central role in B cell development, activation and differentiation. The gene generates at least four alternatively spliced isoforms which have been identified based on the presence of the intact DNA-binding domain and the C-terminal transactivation domain (Zwollo *et al.*, 1997). The Pax-5a isoform, known as B cell-specific activator protein (BSAP), is the most extensively studied product encoded by the *Pax-5* gene, and its transactivation function has been investigated on a number of B cell-specific genes (Hagman *et al.*, 2000). However, the functions and regulatory roles of other Pax-5 isoforms are yet to be determined.

Among the Pax-5 proteins, Pax-5a and Pax-5d are of special interest. These two isoforms are the most abundant in mature B cells and both have an intact DNA-binding

domain that enables them to interact with Pax-5-specific DNA-binding sites. The two isoforms, however, differ dramatically in the composition of their C-terminus. In contrast to Pax-5a, Pax-5d does not have transactivation, repression, or partial homeodomain homology regions, but possesses the novel sequence instead (Zwollo *et al.*, 1997). Although the regulatory role of the novel sequence is not yet known, it is likely to confer a dominant-negative function to Pax-5d isoform. Thus, it is possible that Pax-5a and Pax-5d not only compete for binding, but also have opposing effects on transcription. Consequently, the relative levels of these two isoforms may determine the expression of Pax-5 target genes. Furthermore, it can be speculated that the ratio of the two proteins might change as B lymphocytes progress through the differentiation stages during their development and activation. Such a change may serve as a sensitive switch that determines a rapid activation or repression of particular genes during each stage of B cell differentiation.

While only Pax-5a and Pax-5d can affect transcription through direct promoter-enhancer binding, it is cannot be excluded that Pax-5b and Pax-5e isoforms may also participate in gene regulation (see Figure I.4). Both isoforms possess the octamer sequence implicated in protein-protein interactions and regulation of the transactivation function (see Section ***2.4***). Furthermore, Pax-5b contains the homeodomain and the transactivation domain, which have also been shown to play a role in protein-protein interactions (Eberhard *et al.*, 2000). The presence of three functional domains capable of diverse protein-protein interactions indicates that this isoform may participate in gene regulation as a co-repressor, co-activator, or an inhibitor which sequesters subunits of transcriptionally active complexes. The same model can be applied to the Pax-5e isoform

in which the homeodomain and transactivation region are replaced with the novel sequence. Hence, while having no DNA-binding activity, the Pax-5b and Pax-5e isoforms may retain a capacity for protein-protein interactions, which would allow them to function as dominant-negative variants of Pax-5a and Pax-5d.

Previous studies have shown that the DNA-binding activity of Pax proteins is subject to redox regulation due to the presence of three conserved cysteine residues (Cys 37, Cys 49, and Cys 109) within the paired domain (Tell *et al.*, 1998; Tell *et al.*, 2000). The presence of the two additional cysteine residues in the novel sequence of Pax-5d and Pax-5e presents interesting possibilities for formation of inter- and intra-molecular disulfide bonds within individual proteins and between different isoforms of the Pax-5 family. These may include generation of Pax-5d/Pax-5e homo- and heterodimers, regulation of the novel sequence function via redox-regulated protein folding, as well as formation of transcriptionally-inert complexes between DNA-binding and non-binding isoforms. If such interactions indeed occur, various reducing/oxidizing factors, such as Ref-1, thioredoxin, or reactive oxygen species, may not only affect DNA-binding activity of the paired domain, but may also play a key role in the control of transactivation properties of Pax-5 proteins.

4.2 Research project: major goals

The study presented here pursued two goals. First, the transactivation properties of the Pax-5d isoform were determined *in vivo* using a transient transfection system. Evidence is provided indicating that Pax-5d has a function opposite to that of Pax-5a, and that the two isoforms may compete for binding to Pax-5 recognition sequences. The ratio

of Pax-5a to Pax-5d was, therefore, hypothesized to determine the transcription levels of Pax-5 target genes.

The second goal of this project was to investigate the expression patterns of Pax-5 proteins in resting and LPS-activated B cells. The ratio of Pax-5 isoforms was analyzed in activated and non-activated B cell lines and splenic B lymphocytes. In addition, the DNA-binding activities of Pax-5a and Pax-5d isoforms were assessed in resting and activated normal B cells. The experimental data suggests that activation signals induce a change in the ratio of different Pax-5 proteins and may trigger specific post-translational modifications of the predominant Pax-5a isoform.

4.3 Preliminary studies

In the context of the described research, preliminary investigations were conducted that included identification of concentration requirements for the proposed Pax-5a/Pax-5d binding competition, LPS-activation of B lymphocytes isolated from aged mice, activation of the immature B cell line WEHI-231, and initial characterization of the novel Pax-5_x species. These investigations were designed to provide clarification and/or additional support for the observations of the project described in the previous section. More specifically, the goals of preliminary studies included: a) development of the "staggered" transfection approach for functional studies of Pax-5 proteins; b) comparative analysis of the expression patterns of Pax-5 proteins in young and aged B lymphocytes activated by mitogenic stimulation; c) determination of the ratio of Pax-5 proteins in LPS-activated B cell line WEHI-231; and d) demonstration of a link between Pax-5e isoform and the novel Pax-5_x species.

Summary of specific research aims

1. ***Determination of transactivation properties of isoform Pax-5d. Functional aspects of Pax-5a and Pax-5d DNA-binding competition.*** Non-lymphoid cell lines, NIH 3T3 and COS-1, were transiently co-transfected with different combinations of the Pax-5a and/or Pax-5d effector constructs and an artificial promoter reporter construct that contained three high-affinity Pax-5 DNA-binding site from the murine *CD19* promoter. The transactivating effect of individual isoforms or their combinations was estimated based on the expression of a chloramphenicol acetyltransferase (CAT) reporter gene.

2. ***Analyses of Pax-5 proteins in LPS-activated mature B lymphocytes.*** Mature resting B cells were isolated from spleens of young mice and activated by treatment with bacterial lipopolysaccharide (LPS). Nuclear extracts isolated from resting and activated B cells were assayed for Pax-5 proteins' levels and expression patterns using Western blot analysis. DNA-binding activity of Pax-5a and Pax-5d proteins was determined in nuclear extracts of resting and LPS-activated primary B lymphocytes using electrophoretic mobility shift assay (EMSA).

3. ***Analyses of Pax-5 proteins in B cell lines.*** The protein levels, expression patterns, and the ratio of Pax-5 isoforms were analyzed in B cell lines representing different stages of B cell differentiation using Western blot analysis.

The immature B cell line WEHI-231 was activated by either surface IgM cross-linking or through mitogenic stimulation. Nuclear extracts from activated and non-activated cells were assayed for expression patterns and DNA-binding activity of the Pax-5 isoforms using Western blot analyses and EMSA.

4. ***Analyses of the Pax-5 proteins in LPS-activated B lymphocytes isolated from aged mice.*** Mature resting B cells were isolated from spleens of aged mice and activated by treatment with LPS. Nuclear extracts isolated from resting and activated B cells were assayed for Pax-5 proteins' levels, expression patterns, and DNA-binding activity using Western blot analysis and EMSA. The results were compared with data obtained for young mice (***Aim 2***).

5. ***Development of a "staggered" transfection system.*** A novel transient co-transfection approach was used for detailed examination of Pax-5a/Pax-5d DNA-binding competition requirements.

6. ***Characterization of the novel Pax-5$_x$ species.*** Non-lymphoid cell line NIH 3T3 was transiently transfected with Pax-5e and Pax-5d expression constructs. Nuclear extracts from the transfected cells were analyzed by Western blot to determine the nature of the Pax-5$_x$ band.

Chapter II

Functional Analyses of Alternative Isoforms of the Trancription Factor Pax-5.

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Manuscript in preparation

ABSTRACT

The *Pax-5* gene plays a central role in B cell development, activation and differentiation. At least four different isoforms have been identified. Isoform Pax-5a, known as B cell-specific activator protein (BSAP), is the most extensively studied gene product and its regulatory functions have been investigated for a number of B cell-specific genes. However, potential functions for other Pax-5 isoforms have not yet been reported. In the studies described here, we demonstrate that Pax-5d has a function opposite to that of Pax-5a and, hence, acts as a transcriptional inhibitor of a basal promoter CAT construct containing three Pax-5 binding sites. Furthermore, Pax-5d represses activity of Pax-5a when the two isoforms are expressed simultaneously, suggesting that the ratio of Pax-5a and Pax-5d protein in the nucleus modulates transcriptional activation of target genes. Comparison of resting and LPS-activated mature B lymphocytes as well as B cell lines representing various stages of B cell differentiation, revealed the presence of an unidentified Pax-5 species, Pax-5_x, during the late stages of activation. The Pax-5a/Pax-5_x ratio decreases as mature B cells become

activated, and we hypothesize that the Pax-5_x species acts as a repressor of Pax-5a activity. Moreover, LPS-activation induces post-translational modifications at the C-terminus of Pax-5a affecting the C-terminal repressor domain. Together, our observations suggest that during activation and differentiation of B lymphocytes, Pax-5a function changes, and this is accomplished through post-translational mechanisms as well as through upregulation of the repressor isoforms.

INTRODUCTION

B lymphocytes are the major players of the humoral immune system and are essential to the detection and elimination of pathogens. Their main distinguishing characteristic is the ability to synthesize and secrete specific immunoglobulin (Ig) molecules in response to pathogens. Activation of mature B cells *in vitro* may either be induced by antibody cross-linking of the B cell antigen receptor complex, or through stimulation by specific cytokines and/or B cell mitogens such as bacterial lipopolysaccharide (LPS). Activation signals trigger a cascade of events that result in proliferation, further differentiation of mature B lymphocytes into plasma cells, and Ig secretion (Reya and Grosschedl, 1998).

Spatial and temporal gene expression of B cell-specific transcription factors largely determines the maturation and activation pathways in a B cell, and is a tightly regulated process. Gene expression of transcription factors can be controlled at multiple levels including transcription initiation, alternative RNA splicing, mRNA stability, and translation efficiency (Calkhoven and Geert, 1996). In addition, activity of transcription

factors can be regulated at the post-translational level and may involve phosphorylation, acetylation, proteolysis, and/or regulation of the redox state (Hunter and Karin, 1992; Pahl and Baeuerle, 1996; Calkhoven and Geert, 1996). Post-translational modifications may regulate the DNA-binding activity and transactivation function of a transcription factor, as well as its nuclear localization, stability, and association with other proteins (Calkhoven and Geert, 1996). Modifications of already-expressed transcription factors provide a sensitive and efficient mechanism, allowing for rapid responses to specific signaling pathways during cell differentiation or activation.

A number of transcription factors have now been identified as essential for B cell development and activation (reviewed in Reya and Grosschedl, 1998; Liberg and Sigvardsson, 1999). Among these factors are the products encoded by the *Pax-5* gene which include one of the most critical transcription regulators, the B-cell specific activator protein (BSAP). *Pax-5* is a member of a family of genes involved in development, morphogenesis, and pattern formation (Strachan and Read, 1994; Stuart and Gruss, 1995). The *Pax* family consists of nine members (*Pax-1* through *Pax*-9) all of which share an evolutionarily conserved N-terminal DNA-binding region comprising the paired domain (Walther *et al.*, 1991). The family of Pax proteins is divided into four subclasses based on the presence or absence of specific domains (Adams *et al.*, 1992).

Pax-5 expression is first detected in the developing central nervous system (Adams *et al.*, 1992; Asano and Gruss, 1991). After birth and throughout life, *Pax*-5 transcripts are found in cells of the B-lymphoid lineage and in adult testis of the mouse (Adams *et al.*, 1992). Inactivation of the *Pax-5* gene in mouse results in a complete block of B cell development at the pro-B cell stage, revealing the essential role of this gene in

early B cell lymphopoiesis (Urbanek *et al.*, 1994). Within the B cell lineage, *Pax-5* is expressed during early stages of B cell development up to the mature B-cell, but is greatly downregulated or absent in plasma cells (Barberis *et al.*, 1990).

Pax-5 binding sites have been identified on the promoters of a number of B-cell-specific genes (reviewed in Hagman *et al.*, 2000). Among the positively regulated Pax-5 targets are genes encoding the CD19 co-stimulatory receptor (Kozmik *et al.*, 1992) and the protein tyrosine kinase Blk (Zwollo and Desiderio, 1994). Pax-5 functions as a repressor for the immunoglobulin *J chain* and the Ig 3'α enhancer (Rinkenberger *et al.*, 1996; Singh and Birshtein, 1993; Neurath *et al.*, 1994). In addition to its role in B-lymphopoiesis, Pax-5 has been implicated in activation and proliferation of B lymphocytes since its decreased expression resulted in reduced numbers of cells post-activation (Wakatsuki *et al.*, 1994). However, terminal differentiation of mature activated B cells into plasma cells that are capable of Ig isotype class switching and high antibody production may require downregulation of Pax-5 activity (Usui *et al.*, 1997; Cogne *et al.*, 1994; Stuber *et al.*, 1995).

The *Pax-5* gene produces four isoforms as a result of alternative splicing: *Pax-5a* (full length Pax-5 or BSAP), *Pax-5b*, *Pax-5d*, and *Pax-5e* (Zwollo *et al.*, 1997) (Figure II.1). Of those, only Pax-5a and Pax-5d isoforms are expressed at readily detectable levels in resting B cells, although the levels of *Pax-5d* transcripts are lower than those of *Pax-5a* (Anspach *et al.*, submitted). Both Pax-5a and Pax-5d, but not Pax-5b or 5e, have an intact DNA-binding domain, enabling them to interact with and compete for Pax-5-binding sites on DNA *in vitro* (Figure 1; Zwollo *et al.*, 1997). However, in contrast to

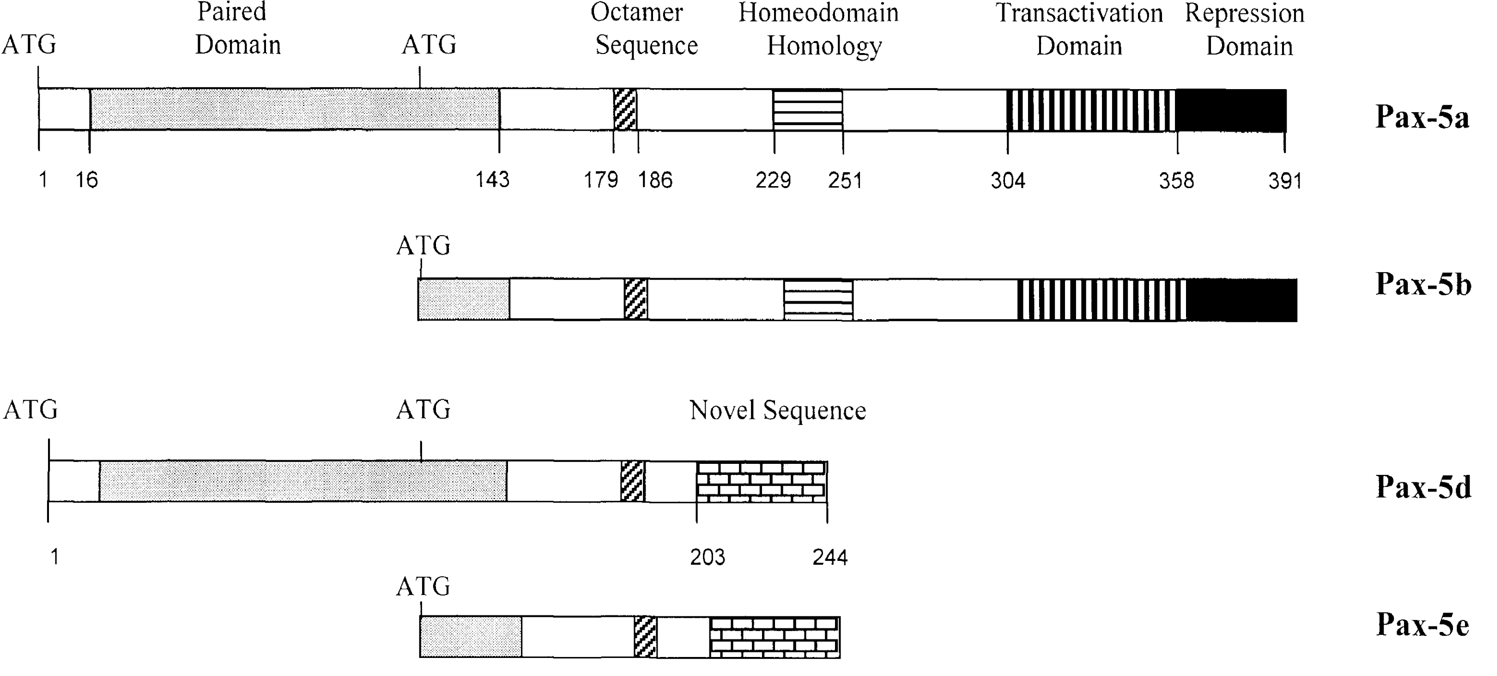


Figure II.1: Pax-5 transcription factor isoforms. The *Pax-5* gene produces four alternatively spliced variants: Pax-5a (53 kD), Pax-5b (41 kD), Pax-5d (31 kD), and Pax-5e (19 kD). *Shaded boxes* indicate the position of the paired DNA-binding domain (aa 16-143); *hatched boxes* indicate the octamer sequence (aa 179-186); *horizontally striped boxes* indicate the homeodomain homology region (aa 229-251); *vertically striped boxes* indicate the transactivation domain (aa 304-358); and *black boxes* indicate the repressor sequence (aa 358-391). Pax-5b and Pax-5e have incomplete paired domains and are unable to bind to DNA. In Pax-5d and Pax-5e isoforms, a region containing the transactivation domain, the repressor sequence, and the homeodomain homology region have replaced with the novel sequence (aas 203-244) of unknown function (*checkered boxes*). All of the *Pax-5* transcripts contain two in-frame translational start sites: one at nucleotide 1 (proximal ATG codon) and another at nucleotide 325 (distal ATG codon).

Pax-5a, Pax-5d does not possess transactivation, repression, or partial homeodomain homology regions at the C-terminus. The region encoded by exons six through ten is replaced in isoform Pax-5d with a 42 amino-acid novel sequence (Zwollo *et al.*, 1997). The role of this sequence is unknown, but it is likely to confer a dominant negative function to Pax-5d protein. Isoform Pax-5e has only a partial DNA binding domain, lacks exons 6 through 10, and shares the same novel C-terminal sequence as Pax-5d (Figure II.1). Based on the DNA binding abilities and expression pattern of Pax-5a and 5d, we hypothesize that the two isoforms compete for binding and have opposite effects on transcription of target genes *in vivo*. Consequently, the relative levels of these two isoforms in the nucleus may regulate expression of target genes.

Although a number of studies have shown clear functional significance of isoform Pax-5a (BSAP) (Zwollo *et al.*, 1998; Kozmik *et al.*, 1992; Fitzsimmons *et al.*, 1996), no work has yet characterized the function of isoform Pax-5d. Thus the first goal of our studies was to determine the transactivation properties of Pax-5d. Here we show, using a transient transfection system, that Pax-5d has a function opposite to that of Pax-5a. These results are in agreement with the hypothesis that the ratio of Pax-5a to Pax-5d affects transcription of Pax-5 target genes.

Our second goal was to investigate the regulatory function of Pax-5a and Pax-5d isoforms in LPS-activated, normal B cells. Our experimental data suggest that LPS-activation signals induce significant changes at the protein, but not RNA, level of both isoforms. Unexpectedly, we found that LPS activation resulted in a loss of Pax-5d protein concomitant with a dramatic increase in the levels of a novel species Pax-5_x during the late stages of activation. Finally, comparison of resting and LPS-activated B

lymphocytes revealed the induction of a slower migrating Pax-5a species which differed in the structure of the C-terminus. We hypothesize that activation of B-cells triggers post-translational protein modification(s) that allow stabilization of Pax-5a and prevent the removal of the C-terminal repressor domain.

In summary, data presented here suggest that during B cell activation, the transcriptional activity of Pax-5a may be regulated through sensitive post-translational mechanisms that either inhibit Pax-5a function directly, and/ or through upregulation of the repressor isoform(s).

MATERIALS AND METHODS

Cell Lines. Murine B-lymphoid cell lines KEFTL-1 (pro-B), HAFTL-1 (pro-B), PD31 (pre-B), 70Z/3 (pre-B), A20/2J (mature B), B17.10 (mature), and CH12 (pre-secretor B) were gifts from Dr. Steve Desiderio (The Johns Hopkins University School of Medicine, MD). WEHI-231 (immature B), 2PK3 (mature B), and Sp2/0 (plasma cell) cell lines were purchased through ATCC. Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Bio-Whitaker, Inc.), 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 mM β-mercaptoethanol. The COS-1 cell line (ATCC), a transformed African green monkey kidney cell line, was maintained in DMEM medium containing 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. NIH 3T3 (ATCC), an embryonic mouse fibroblast cell line, was grown in DMEM medium supplemented with 10% calf serum (Gibco BRL Life Technology), 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

DNA Constructs. The construction of the p(-191)*blk*CAT2mE reporter construct has been described elsewhere (Zwollo and Desiderio, 1994). This construct contains the murine *blk* promoter (nucleotides –191 to +134) that drives expression of the chloramphenicol acetyltransferase (CAT) coding region, and two copies of the intronic Ig μE downstream of CAT. The γ42(3i)AS-CAT reporter construct was created using the pγ42CassI CAT reporter (Wallin *et al.*, 1998) (Figure II.2A). Expression of CAT is driven by the truncated rat γ-fibrinogen promoter (-54 to +36), which includes a TATA box and a single Sp-1 binding site. Three copies of the high-affinity Pax-5 binding site from the *CD19* promoter (5'-CAGACACCCATGGTTGAGTGCCCTCCAG-3') were inserted into the polylinker upstream of the γ42-fibrinogen promoter. Recombinant constructs were sequenced to determine copy number and orientation of Pax-5 binding sites. The effector constructs pcDNA5a and pcDNA5d were made by cloning the full-length cDNA sequences of either Pax-5a or Pax-5d isoform into *Not*I restriction sites of the expression vector pcDNA3 (Invitrogen). The pcDNA3 construct was used as a negative control effector construct, and the HBIICAT construct (Zwollo and Desiderio, 1994) was used as a control for transfection efficiency.

Transient Transfections and Chloramphenicol Acetyltransferase Assay. Transient transfections of A20/2J, PD31, and 703/Z B cell lines were performed by either the DEAE-dextran (Grosschedl and Baltimore, 1985) or SuperFect (Qiagen) method. Non-lymphoid cell lines COS-1 and NIH 3T3 were transfected using LipofectAMINE.Plus (Gibco BRL) according to the manufacturer's protocols. For B-lymphoid cell lines, $1X10^6$ (SuperFect) or $2X10^7$ (DEAE-dextran) cells in logarithmic growth phase were transfected with 3-5 μg of total plasmid DNA. Non-lymphoid cell

lines were transfected with 1-6 μg of total plasmid DNA. Cell extracts were prepared 43-48 hr after transfection and assayed for CAT activity as described previously (Lansford *et al.*, 1992). Briefly, cell extracts containing the same amount of total protein were combined (in a 150 μl volume) with the reaction mixture containing 470 mM Tris, pH 8.0, 3.3 nM ^{14}C-chloramphenicol (Amersham Pharmacia Biotech), and 0.5 mM acetyl CoA (Sigma). Following 5-8 hr incubation at 37^{0}C, the samples were extracted with 500 μl ethyl acetate and the organic phase was collected and concentrated on a vacuum centrifuge (SpeedVac® Plus, Savant). The reaction products were chromatographically separated on a silica gel plate (EM Science) using a chlorophorm-methanol (95:5) mixture. The plate was exposed to a Kodak X-OMAT-AR film for 2-10 days. The obtained data were quantified using an NIH Image software analysis program (**www:http://rsb.ihfo.nih.gov/nih-image/**). The relative CAT conversion was determined by calculating the ratio of values for acetylated and uncetylated chloramphenicol as follows: %CAT conversion=[Acetylated-Mock]/[(Acetylated-Mock)+(Unacetylated-Mock)], where "Mock" is the activity of the mock (transfection without DNA) transfection. The values were normalized to the maximum value of relative CAT conversion within each experiment set at 100%. Protein concentration of cell lysates was determined by the Bradford assay (Bio Rad Laboratories).

Isolation of Cell Fractions and Activation of Small Resting B Cells. Splenic B cells were obtained from 3-6 months old BALB/c mice (bred at The College of William and Mary). Teased spleen suspensions from BALB/c mice were collected through a 40 μm nylon cell strainer in complete RPMI-1640 medium supplemented as described above, washed and re-suspended in Hank's balanced salt solution. Small resting B cells

(SRBs) were isolated from a 70% Percoll gradient (Amersham Pharmacia Biotech) according to the manufacturer's instructions and re-suspended in complete RPMI-1640 medium at $1x10^7$ cells per ml. The 70% Percoll layer contained approximately 25% T cells; however, the complement lysis purification step was omitted due to possibility of partial activation of B cells by reactions with anti-Thy.1, anti-CD4, and anti-CD8 (Zwollo, unpublished observations). Approximately 10^7 cells from the obtained population were processed immediately to obtain nuclear extracts (as described below). The remainder of the population was activated by culturing in complete RPMI-1640 medium (supplemented as above) in the presence of 20 μg/ml bacterial lipopolysaccharide (LPS) (Sigma) for the required period.

Nuclear Extract Preparation. Cells were collected at specified times and processed for nuclear extracts as described elsewhere (Wallin *et al.*, 1999). Briefly, 10^6-10^7 cells were treated with lysis buffer (200 μl) containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, pH 8.0, and 0.4% Nonidet P40. Following 15 min incubation, the cytoplasmic fractions were collected, and the nuclear pellet was resuspended in 100 μl extraction buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, pH 8.0) and incubated with agitation for 15 min. Nuclear extract aliquots (10 μl) were collected and stored at -80^0C. All buffers contained the following mixture of protease inhibitors: 0.5 nM phenylmethylsulphonyl fluoride, 0.5 mM dithiothreitol, aprotinin (10 units/ml), leupeptin (5 mg/ml), and pepstatin A (5 mg/ml). Procedures for nuclear extract preparation were carried out on ice in a cold room at 6^oC. Total protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories) according to the supplier's recommendations.

RNA Isolation and RNase Protection Assay. Total cellular RNA was isolated from Percoll-purified SRBs or LPS-activated B cells using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Anti-sense radiolabeled probes were prepared as described (Zwollo *et al.*, 1997). Briefly, the plasmid pBS-10.1, containing the complete *Pax-5d* sequence, was linearized using the restriction enzyme *Bsr*FI and transcribed using T7 RNA polymerase in the presence of [α-^{32}P]-CTP (800 Ci/mmol). The resulting anti-sense *Pax-5* riboprobe 10.1 contained exons 4 and 5 (nts 447-607) plus the novel sequence of *Pax-5d* (nts 608-735), and was used for detection of the *Pax-5a* and *Pax-5d* transcripts. A β-tubulin-specific probe was similarly synthesized from the *Bam* HI-linearized form of the plasmid pμ 5, which contains nucleotides 170-263 of the murine β-tubulin gene. RNase protection assays were performed as described (Zwollo *et al.*, 1997). For each sample, the RNA probe (5x10^5 cpm) was annealed to 1-5 μg of total cellular RNA for 5 hr at 55^{0}C. After digestion with RNAse A (40 μg/ml) and RNAse T1 (2 μg/ml) for 30 min at 15^{0}C, proteinase K and sodium dodecyl sulfate were added to 150 μg/ml and 1%, respectively, and the reactions were incubated for additional 15 min at 37^{0}C. Products were extracted with phenol/chloroform, precipitated with ethanol in the presence of 10 μg of tRNA, and resuspended in sample buffer containing 80% formamide. Products were fractionated by electrophoresis on a 5% polyacrylamide gel containing 7M urea and detected by autoradiography. Radioactive RNA size markers were prepared using linearized pBluescript vectors and T3 and T7 RNA polymerases, which resulted in labeled RNA transcripts ranging in size form 50 to 291 nucleotides.

To obtain an optimal internal control, the riboprobe 10.1 and a β-tubulin-specific probe were incubated simultaneously with each RNA sample.

Western Blot Analysis. Nuclear extracts and cytoplasmic fractions from SRBs, LPS-activated B cells, or cell lines were separated on 12% denaturing SDS-polyacrylamide gels and electrophoretically transferred onto nitrocellulose filters (Schleicher and Schuell) as described previously (Zwollo *et al.*, 1998). Antibody probing was performed as described previously (Zwollo *et al.*, 1998). Filters were first incubated with 1 hr in blocking solution (5% milk in PBS), followed by a 1-2 hr incubation with a primary antibody in blocking solution (Table I). Next, filters were incubated for 1 hr with a horseradish peroxidase-conjugated secondary antibody in blocking solution (see next section). Filters were developed with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech), and bands visualized on Eastman Kodak X-OMAT-AR film. The density of Pax-5a and Pax-5d bands was quantified using an NIH Image software analysis program (**www:http://rsb.ihfo.nih.gov/nih-image**/).

Anti-Pax Antibodies. Information about isotype-specific Pax-5 antibodies used in this study is summarized in Table I. Pax-5d/Pax-5e-specific mouse monoclonal antibody 6G11, recognizing the C-terminal "novel" sequence, was generated in our lab (Anspach *et al.*, submitted). 6G11 supernatants were used at a 1:30 dilution and detected with a horseradish peroxidase-conjugated goat-anti-mouse IgG secondary antibody (Zymed). ED-1 antiserum (Zwollo *et al.*, 1998) was used at a 1:2000 dilution. Pax-5/N-19 and Pax-5/C-20 were used at a 1:400 dilution and detected with a horseradish peroxidase-conjugated rabbit anti-goat IgG (Zymed). OC-1 was used at 1:1000. Rabbit polyclonal antiserum to the transcription factor TFIID (Santa Cruz Biotechnology) was

used at a dilution 1:200. The ED-1, OC-1, and anti-TFIID antibodies were detected with a horseradish peroxidase-conjugated donkey-anti-rabbit IgG secondary antibody (Amersham Pharmacia Biotech).

Table 1: List of antibodies used in the protein studies.

Antibody	Type	Source or Reference	Specificity	Recognized Proteins
anti-TFIID	Rabbit polyclonal	Santa Cruz Biotechnology	N-terminal domain of TFIID (TBP) p36	TFIID (TBP) p36
ED-1	Rabbit polyclonal	Zwollo *et al.*, 1998	Paired domain aa 13-159	Pax-5a, Pax-5b, Pax-5d, Pax-5e
6G11	Mouse monoclonal	Anspach *et al.*, submitted	Novel sequence aa 218-235	Pax-5d, Pax-5e
N-19	Goat polyclonal	Santa Cruz Biotechnology	N-terminal domain of Pax-5 aa 2-20	Pax-5a, Pax-5d
C-20	Goat polyclonal	Santa Cruz Biotechnology	C-terminal domain of Pax-5 aa 370-391	Pax-5a, Pax-5b
OC-1	Rabbit polyclonal	Zwollo *et al.*, 1997	Homeodomain homology region aa 234-255	Pax-5a, Pax-5b

Electrophoretic Mobility Shift Assays. Standard binding assays were carried out for 20 min at 30^0C in 10-15 μl reactions containing 60 mM KCl, 12 mM HEPES, pH 7.9, 4 mM Tris-Cl, pH 7.9, 1 mM EDTA, 1 mM DTT, 30 ng of BSA, 12% glycerol, 1 μg of nuclear extract, 2-4 fmol of ^{32}P-labeled DNA probe, and 2 μg poly(dI•dC) (Zwollo and Desiderio, 1994). The double-stranded oligonucleotide CD19/BSAP probe (5'-CAGACACCCATGGTTGAGTGCCCTCCAG-3') was labeled with [^{32}P]α-dCTP as

described previously (Zwollo and Desiderio, 1994). The ratio of nuclear extract to poly(dI•dC) (in μg) was kept constant at 1:2 in all experiments. In antibody supershift/competition EMSAs, nuclear extracts were pre-incubated in the presence of 1 μl of antibody without the probe for 10 min at 30°C. Products were separated by electrophoresis on 5% non-denaturing polyacrylamide gel in buffer containing 33 mM Tris-HCl, 33 mM boric acid, and 0.74 mM EDTA. Gels were dried and exposed to Eastman Kodak X-OMAT-AR film.

In vitro Transcription and Translation of Pax-5 Isoforms. The plasmids (pBluescript) containing the isoform Pax-5a (pBS.1.2) or Pax-5d (pBS.10.1) were transcribed in sense direction with T3 or T7 RNA polymerase respectively, as described previously (Zwollo *et al.*, 1997). Translation was carried out using rabbit reticulocyte lysate (TnT; Promega) according to the manufacturer's directions.

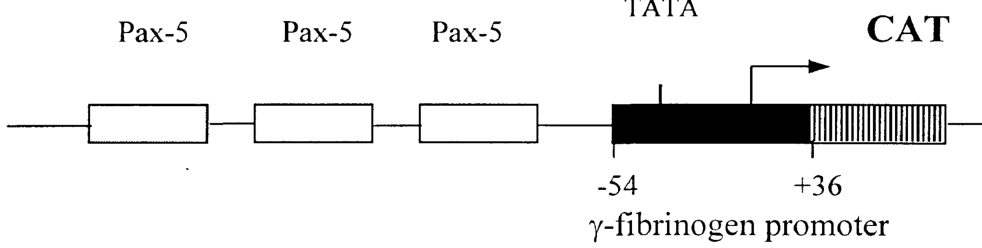
RESULTS

Functional analysis of Pax-5d *in vivo*.

The transactivation function of isoform Pax-5a had previously been investigated in our lab using the *blk* promoter as the target sequence (Zwollo *et al.*, 1998). However, the regulatory role of Pax-5d is not yet understood. To determine the transactivation properties of isoform Pax-5d *in vivo*, we initially used a *blk*CAT reporter construct (-191blkCAT2mE) that contains 325 nt of *blk* promoter sequence driving expression of the CAT reporter gene (Zwollo and Desiderio, 1994). The expression of the *blk* gene is similar to that of *Pax-5* in cells of the B-lymphoid lineage and has been shown to be positively regulated by Pax-5a (Dymecky *et al.*, 1992; Zwollo and Desiderio, 1994).

Transient co-transfections with DEAE-dextran were performed in the B cell lines A20/2J, 70Z/3 and PD31, and the non-lymphoid cell line COS-1, using the *blk*CAT reporter construct and different combinations of Pax-5a and Pax-5d effector constructs, pcDNA5a and pcDNA5d, respectively. Due to low transfection efficiency in B cell lines combined with low activity of the *blk* promoter (Zwollo and Desiderio, 1994), we were unable to determine reliable quantitative differences between experimental samples. As an alternative approach, we created an artificial promoter containing high affinity Pax-5 DNA binding sites. Dörfler and Busslinger (1996) had previously used an artificial promoter system containing three Pax-5 binding sites 5' of the TATA box of the β-globin gene, and showed that this provides an excellent system for assessment of Pax-5 regulatory activity. Three copies of a double-stranded oligonucleotide containing the Pax-5 DNA binding site from the murine *CD19* promoter were cloned in sense or anti-sense orientation upstream of the TATA element of the truncated rat γ42-fibrinogen promoter driving expression of the *CAT* gene. Both sense and anti-sense reporters gave similar promoter activities and the anti-sense construct, named γ42(3i)AS-CAT, was used in all subsequent experiments (Figure II.2A).

To verify that the reporter construct γ42(3i)AS-CAT was expressed in the presence, but not absence, of endogenous Pax-5 protein, transfections were performed in the mature B cell line A20/2J and the plasma cell line SP2/0. As expected, CAT expression was detected in the Pax-5 positive A20/2J line, but not in the Pax-5 negative SP2/0 line (result not shown). Because of low transfection efficiencies in B cell lines, subsequent analysis was carried out in two highly transfectable, non-lymphoid cell lines,



γ42(3i)AS-CAT

Figure II.2A: The γ42(3i)AS-CAT reporter construct. The γ42(3i)AS-CAT reporter construct used for transient transfections contains three copies of the high-affinity Pax-5 binding site form the murine *CD19* promoter (Kozmik *et al.*, 1992) inserted in anti-sense orientation upstream of the TATA element of the rat γ-fibrinogen promoter driving the expression of the *CAT* gene.

COS-1 and NIH 3T3. In all experiments, a pcDNA3 vector without insert was used as a negative control and was also added to some of the samples to maintain equal amounts of transfected DNA per sample. The HBIICAT construct, which drives high and ubiquitous CAT expression, was used to evaluate transfection efficiency within each experiment (Zwollo and Desiderio, 1994).

Co-transfections of γ42(3i)AS-CAT with the effector constructs expressing Pax-5a or Pax-5d were first performed in NIH 3T3 cells. The reporter gene was expressed at high levels in the presence, but not absence, of Pax-5a (Figure II.2B) confirming a positive transactivating function of this isoform in the regulation of an artificial promoter construct. In contrast, isoform Pax-5d was unable to activate the reporter gene, yielding only basal levels of transcription that were similar to those produced in the presence of the control plasmid pcDNA3 (Figure II.2B).

Next, we used the COS-1 cell line to further assess Pax-5d function. COS-1 cells do not express Pax-5, but since they are derived from kidney cells, they express the closely related Pax-8 protein. Both Pax-5 and Pax-8 belong to the same subclass of Pax proteins and both recognize the Pax-5 DNA binding sequence from the human *CD19* promoter (Walther *et al.*, 1991; Kozmik *et al.*, 1993). We first confirmed the presence of Pax-8 in COS-1 cells by EMSA using the CD19/BSAP probe and a variety of anti-Pax antibodies (Table I). We were able to show that the upper protein-DNA complex, which was expected to contain Pax-8, was absent in the presence of anti-paired domain antiserum Ed-1, and reduced using the OC-1 antiserum (Figure II.2C). The latter is due to partial sequence homology within this region between Pax-5 and Pax-8 (Kozmik *et al.*, 1993). In contrast, two Pax-5-specific antibodies, Pax-5/N-19 and Pax-5/C-20, were

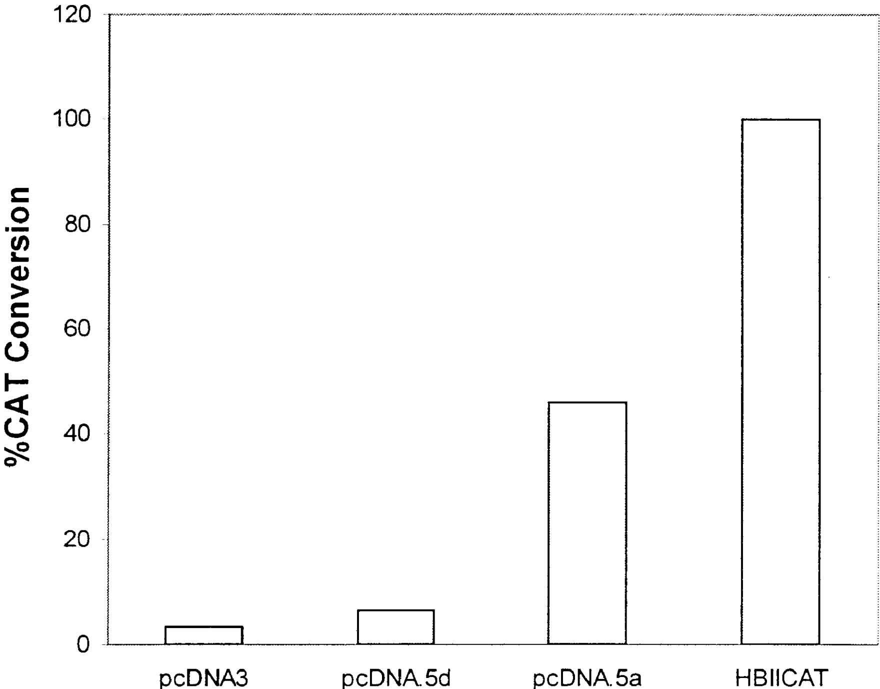


Figure II.2B: Activation of the reporter gene by exogenous Pax-5. The γ42(3i)AS-CAT reporter construct (1μg) was transiently co-transfected with various effector constructs (2μg) into NIH 3T3 cell line, as indicated. HBIICAT construct was used to evaluate transfection efficiency. The pcDNA3 expression vector was used as a negative control. Percent conversion of unacetylated to acetylated chloramphenicol was normalized to the value for HBIICAT transfection set at 100%. This chart represents a single experiment with 3 μg of total transfected DNA.

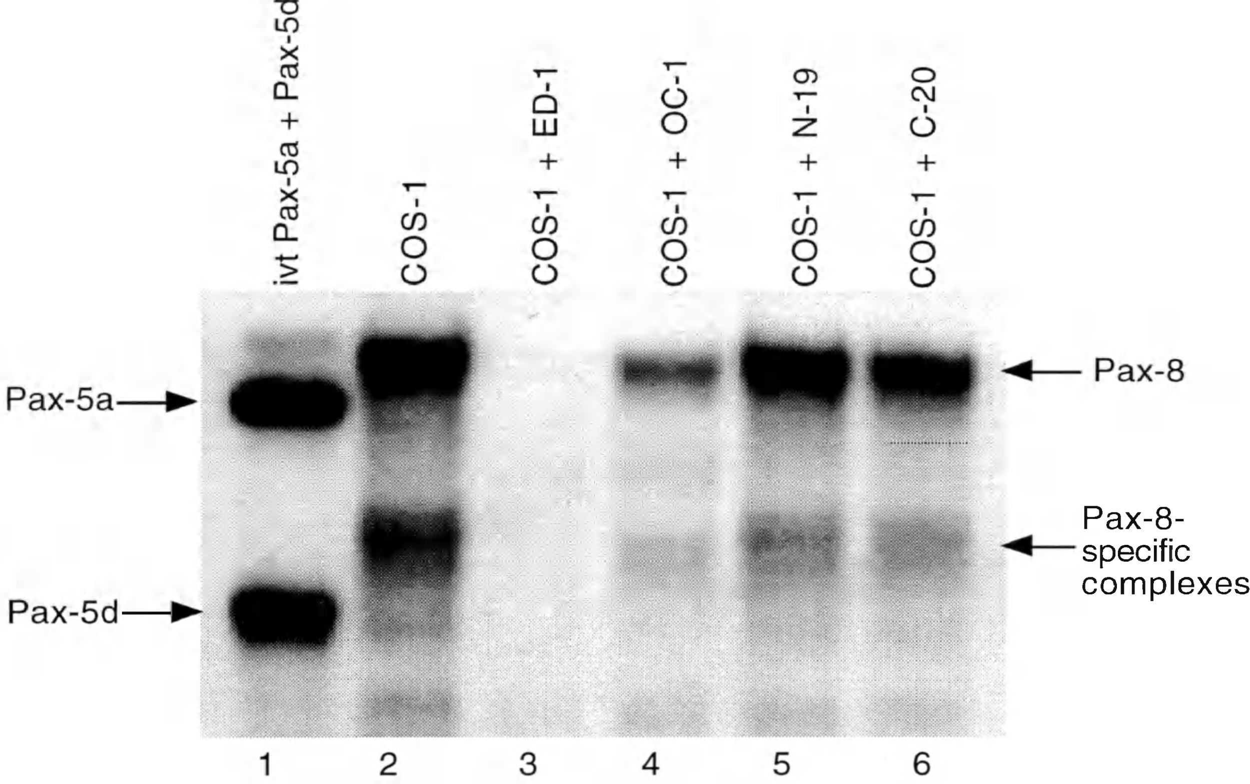


Figure II.2C: EMSA of nuclear extracts from non-lymphoid cell line COS-1. COS-1 nuclear extracts (1 μg) were assayed for the presence of Pax-8 protein by EMSA using the CD19/BSAP probe. Prior to assay, the samples were pre-incubated with antibodies directed against various regions of Pax-5 (see Table I). Lane 1: ivt Pax-5a (1μl lysate) and ivt Pax-5d (2 μl lysate); lane 2: COS-1 nuclear extract; lane 3: COS-1+ED-1 (anti-paired domain antibody); lane 4: COS-1+OC-1 (homeodomain-specific antibody); lane 5: COS-1+N-19 (Pax-5 N-terminus-specific antibody); lane 6: COS-1+C-20 (Pax-5 C-terminus-specific antibody). ivt, *in vitro* translated protein.

unable to remove the complex. Together, the data provided the necessary evidence to confirm that COS-1 cells express Pax-8.

Because of the similarities in specificity of Pax-5 and Pax-8, the COS-1 cell line provided a useful tool for examination of inhibitory and/or competition functions of isoform Pax-5d. First, endogenous Pax-8 was able to transactivate CAT reporter expression in COS-1 cells in the absence of Pax-5 effector constructs (Figure II.3A, pcDNA alone). Second, when increasing amounts of the Pax-5d effector construct were co-transfected, a dose-dependent decrease in CAT activity was observed (Figure II.3A). Addition of increasing amounts of pcDNA5d DNA correlated with the increase in the levels of expressed Pax-5d proteins, as determined by Western blot analysis of the transfected samples (Figure II.3C). In contrast, adding increasing amounts of Pax-5a did not have a significant effect on promoter activity, possibly because endogenous Pax-8 already has a saturating transactivating effect on the reporter (result not shown).

To confirm an activating role for Pax-5a, the γ42(3i)AS-CAT construct was co-transfected with increasing amounts of Pax-5a construct in the NIH 3T3 cell line, and this resulted in increased CAT activity (Figure II.3B). In this system, increasing amounts of the transfected DNA also correlated with an increase in the levels of corresponding proteins (result not shown). Together, these results show that isoform Pax-5d, but not Pax-5a, is able to suppress activity of a Pax-5 sensitive promoter.

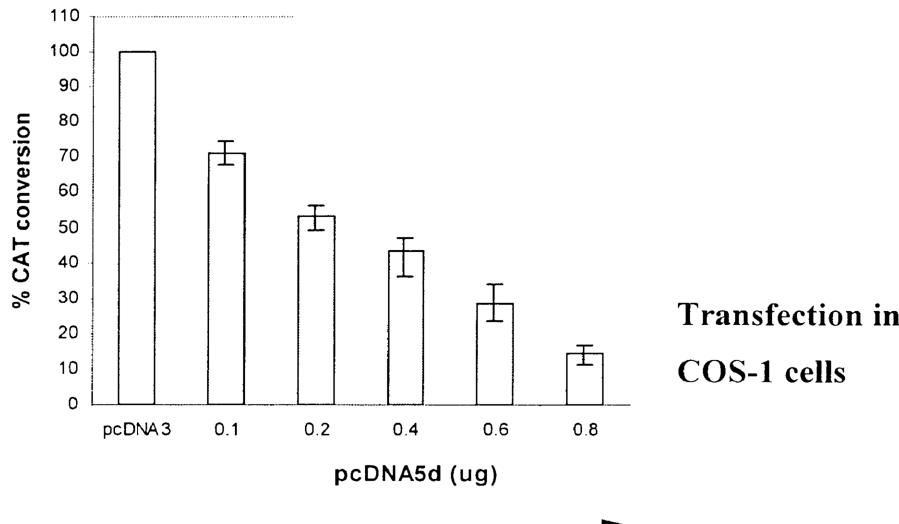


Figure II.3A: Pax-5a and Pax-5d have opposite effect on transcription: Pax-5d inhibits transcription of the reporter gene. The γ42(3i)AS-CAT reporter construct (0.5 μg) was transiently co-transfected with various concentrations of the pcDNA5d effector construct into COS-1 cell line. The concentrations of the pcDNA5d construct (in μg) are designated at bottom of the chart. In each experiment, a pcDNA3 vector without insert was used as a negative control and added to maintain equal amounts of transfected DNA in each sample. Error bars show the mean ± S.E. (*n=3*). Percent CAT conversion was determined as described in "Materials and Methods" and normalized to the maximum percent conversion value within each experiment.

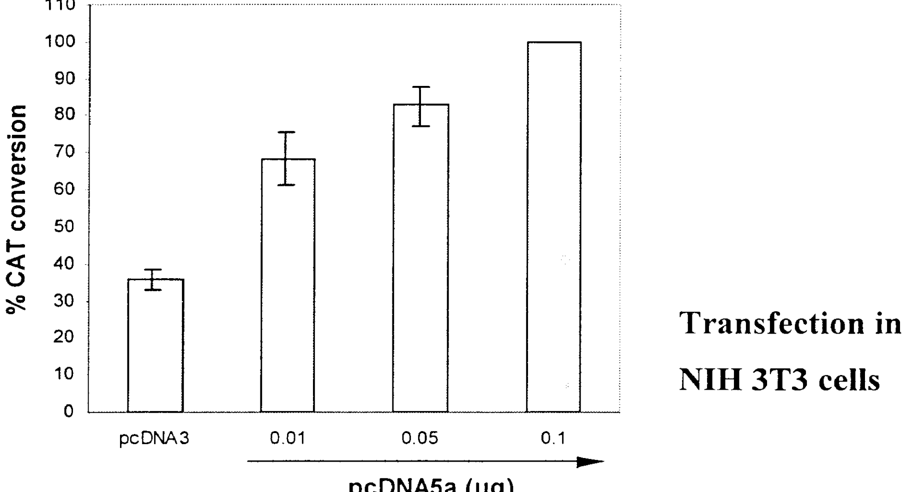


Figure II.3B: Pax-5a and Pax-5d have opposite effect on transcription: Pax-5a activates transcription of the reporter gene. The γ42(3i)AS-CAT reporter construct (0.5 μg) was transiently co-transfected with various concentrations of the pcDNA5a effector construct into NIH 3T3 cell line. In each experiment, a pcDNA3 vector without insert was used as a negative control and added to maintain equal amounts of transfected DNA in each sample. The concentrations of the pcDNA5a construct (in μg) are designated at bottom of the chart. Error bars show the mean ± S.E. (*n=3*). Percent CAT conversion was determined as described in "Materials and Methods" and normalized to the maximum percent conversion value within each experiment.

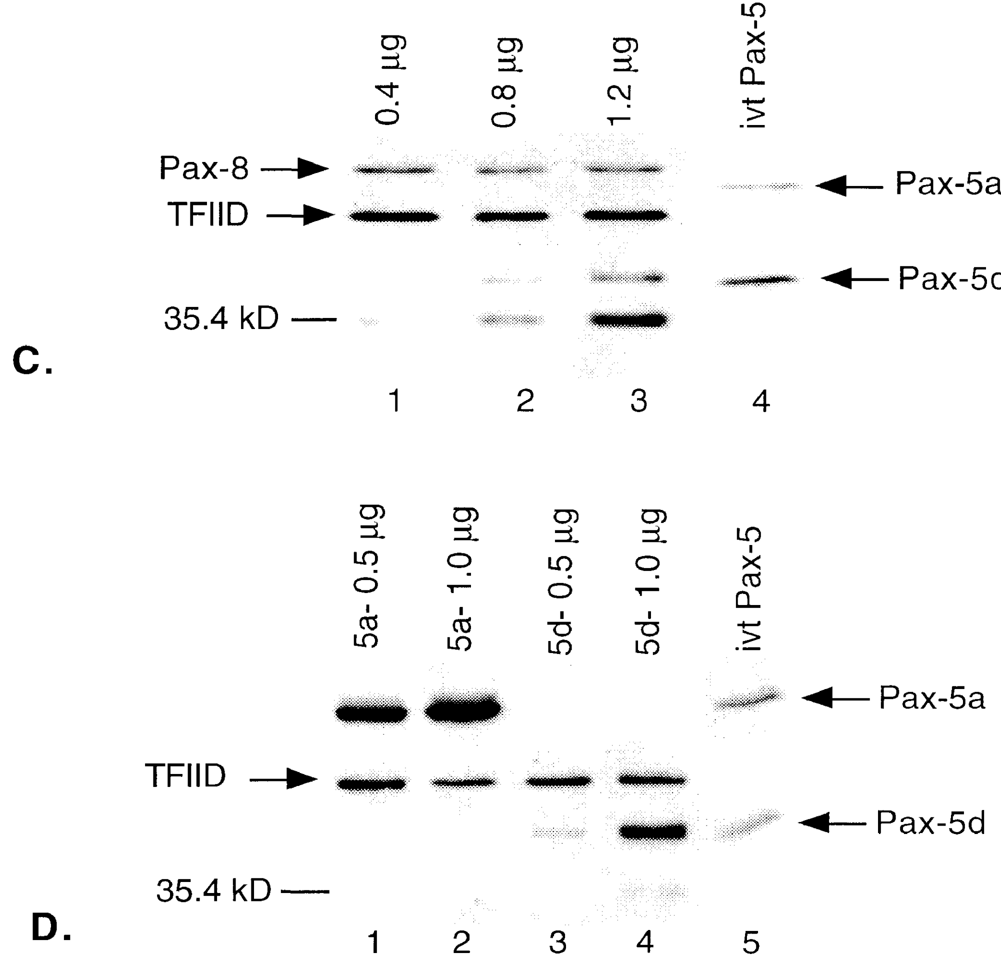


Figure II.3C and II.3D: Correlation between the amount of transfected DNA and expression of Pax-5 proteins. Nuclear extracts from COS-1 and NIH 3T3 transfected samples were analyzed by Western blot using the paired domain-specific antibody ED-1. The blot were simultaneously probed with anti-TFIID serum to monitor the total amount of loaded protein. Positions of Pax-5 isoforms are indicated. **(C)** The increasing amounts of Pax-5d expression construct (indicated above the lanes) were transiently transfected into a non-lymphoid cell line, COS-1. The nature of the species detected at 35.4 kD is unknown, but may be related to Pax-5x protein discussed later in text. **(D)** NIH 3T3 cell line was transiently transfected with different amounts of either Pax-5a or Pax-5d expression construct (indicated above the lanes).

In earlier EMSA studies, we were able to show that *in vitro* translated forms of Pax-5a and 5d have a similar affinity for Pax-5 DNA binding sites on the *blk* promoter (Zwollo *et al.*, 1997). Based on the inhibitory effect of Pax-5d on promoter activity (Figure II.3A), we next sought to investigate whether Pax-5d competes directly for binding with Pax-5a. To test this, the reporter construct γ42(3i)AS-CAT was transiently co-transfected with various ratios of pcDNA5a and pcDNA5d into NIH 3T3 cells, as shown in Figure II.3E. This experiment showed that Pax-5d suppresses Pax-5a-dependent activity of the reporter. However, at least 20 times the amount of pcDNA5d DNA was needed, as compared to pcDNA5a DNA, to detect significant decreases in Pax-5a activity (Figure II.3E). This suggested either that Pax-5d protein was present at much lower levels than Pax-5a, or that Pax-5a had a much higher affinity for Pax-5 binding sites in this artificial promoter system, as compared to Pax-5d. Interestingly, Western blot analysis of nuclear extracts from NIH 3T3 cells transfected with the same amounts of either Pax-5a or Pax-5d expression constructs showed that the Pax-5d construct generates less protein per μg DNA transfected as compared to Pax-5a (Figure II.3D). The exact cause of this phenomenon is unknown at this time; but such a discrepancy in the amount of produced protein may reflect the differences in either transcriptional/translational efficiencies or protein stabilities of the two Pax-5 isoforms. It is also possible that Pax-5d needs a B cell specific factor that modulates its DNA binding affinity, which is absent in NIH 3T3 cells. Western blot analysis of cytoplasmic extracts isolated from the transfected samples revealed no Pax-5-specific bands, excluding the possibility that Pax-5d protein is retained in the cytoplasm.

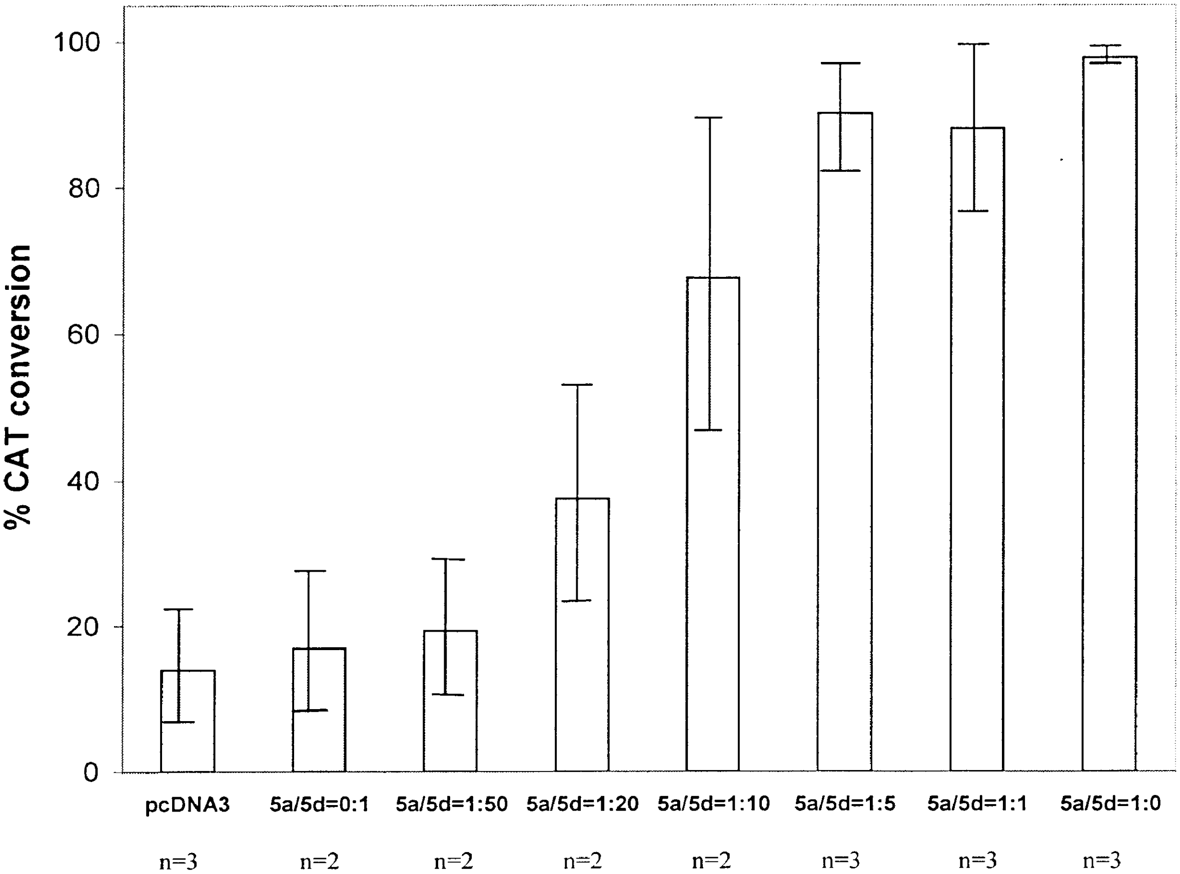


Figure II.3E: Pax-5a and Pax-5d compete for DNA binding.
The γ42(3i)AS-CAT reporter construct was transiently co-transfected into NIH 3T3 cells with the pcDNA5a and pcDNA5d effector constructs mixed in various ratios. In each experiment, a pcDNA3 vector without insert was used as a negative control and added to maintain equal amounts of transfected DNA in each sample. The Pax-5a/Pax-5d ratios are indicated at the bottom of the chart. Error bars indicate the mean ± S.E. with the number of replicates (*n*) indicated below each bar. Percent CAT conversion was determined as described in "Materials and Methods" and normalized to the maximum percent conversion value within each experiment.

In summary, our functional assays demonstrate that Pax-5d can suppress activity of an artificial promoter containing Pax-5 binding sites, and this effect is opposite to the observed Pax-5a activation on the same promoter. In addition, we show that, when Pax-5d is co-expressed with Pax-5a, it can suppress activity of Pax-5a in a dose-dependent manner. Thus, the ratio of Pax-5a to Pax-5d in the nucleus may provide a mechanism for regulation of expression of Pax-5 target genes.

LPS-activation of mature B cells affects the ratio of Pax-5 proteins.

Our transfection studies suggested that the Pax-5a/Pax-5d ratio may regulate Pax-5a function. This was investigated by activating mature B cells with bacterial lipopolysaccharide (LPS) and measuring Pax-5a and 5d levels before and after activation. Nuclear extracts and total cellular RNA were prepared from samples collected before and at various times after LPS treatment and analyzed using Western blot analyses and RNAse protection assays.

First, we determined if LPS activation induced a shift in transcript levels of isoforms *Pax-5a* and *Pax-5d.* The antisense *Pax-5* riboprobe 10.1 (Zwollo *et al.*, 1997) was used to detect isoform *Pax-5a* as well as the novel sequence of isoform *Pax-5d.* Probe design and expected protected RNA fragments are indicated in Figure II.4A. Riboprobe 10.1 detects exons 4 (partial) and 5 plus the novel sequence (nucleotides 447-735) of *Pax-5d*, resulting in a 288-nucleotide protected Pax-5d specific fragment. The probe detects *Pax-5a* transcripts as a 160-nucleotide fragment corresponding to part of exon 4 and exon 5 in the absence of the novel sequence (Figure II.4A). A control tubulin (pμ) riboprobe was used for monitoring overall levels and quality of RNA.

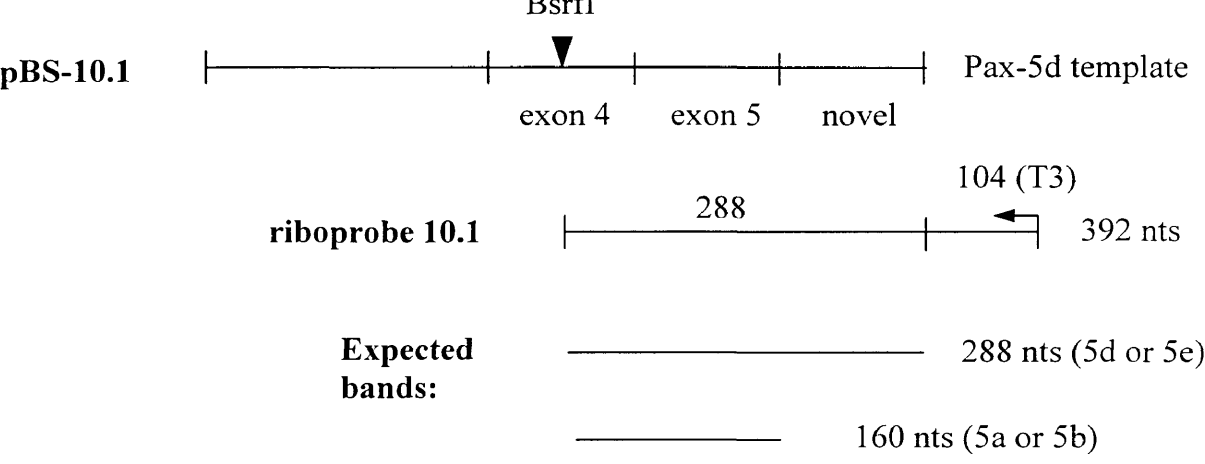


Figure II.4A: Overview of the plasmid template pBS-10.1 (*Pax-5d*), riboprobe 10.1, and expected protected regions on *Pax-5a* and *Pax-5d* mRNA, as used in the RNAse protection assay. See "Materials and Methods" for details. The position of the novel sequence unique to Pax-5d and Pax-5e (nts 607-735) is indicated.

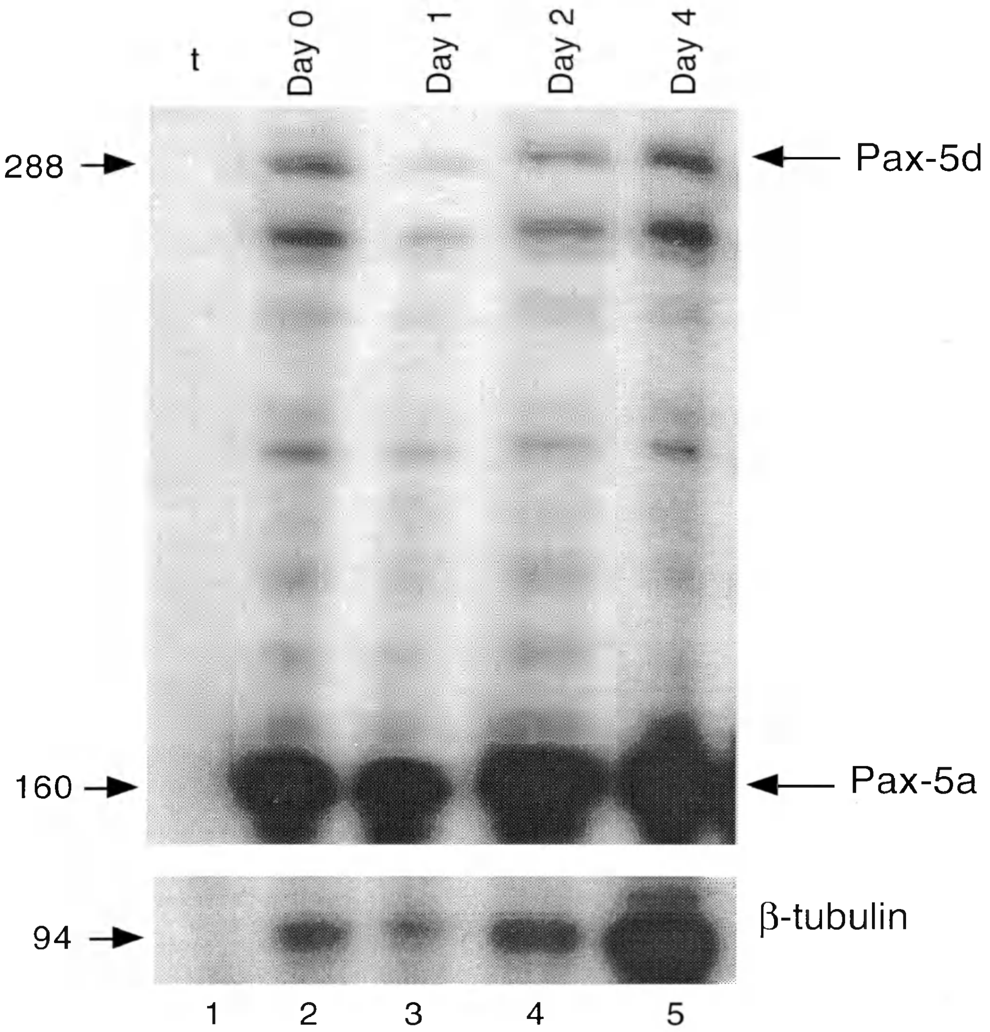


Figure II.4B: RNAse protection assay on total RNA from small resting and LPS-activated B lymphocytes. Small resting B cells (SRBs) were activated with bacterial lipopolysaccharide (LPS) for specified periods of time, as indicated, and analyzed for levels of *Pax-5a* and *Pax-5d* RNA using the 10.1 probe (see Figure II.4A). The 288 nt band corresponds to *Pax-5* transcripts which contain sequence from exon 5 as well as the novel sequence, i.e. *Pax-5d*. The 160 nt band corresponds to the *Pax-5a* transcripts which share exon 5 but not the novel sequence. Sizes of protected fragments are indicated on the left (nts). t, tRNA used as a negative control. The internal control RNA probe pμ100 detects a 94 nt β-tubulin transcript.

SRBs were activated with LPS and samples were collected at day 0, 1, 2, 4 (Figure II.4B), 7, 8, and 9 after stimulation (results not shown). From RNAse protection assay data, it appears that there was no significant increase in the overall levels of *Pax-5a* and *Pax-5d* transcripts. Interestingly, the ratio of the 288 (*Pax-5a*) to 160 (*Pax-5d*) protected bands remained unchanged for the entire nine days after LPS activation, and this ratio was comparable to that in resting B cells (Figure II.4B, lane 2). In addition, no new bands were seen in activated samples. This indicated that mitogenic stimulation of B cells by LPS did not induce changes at the level of alternative *Pax-5* splicing.

Next, we determined whether activation of B cells by LPS changes the protein levels of Pax-5a and Pax-5d in the nucleus. Western blot analyses of nuclear and cytoplasmic extracts were performed using anti-Pax-5 antibodies ED-1 or 6G11. The polyclonal antiserum ED-1 is specific for the paired domain and detects all four Pax-5 isoforms (Table I). 6G11 is a monoclonal antibody that recognizes a C-terminal sequence unique to Pax-5d (Table I; Anspach *et al.*, submitted). We used *in vitro* translated Pax-5a and Pax-5d as controls and monitored total nuclear protein levels by probing the filters simultaneously with an antibody that detects the basal transcription factor TFIID. As expected, no Pax-5 bands were detected in cytoplasmic fractions from either resting or activated B lymphocytes.

Results from Western blot analyses showed that nuclear Pax-5a protein levels increased as B cells proliferated in response to LPS activation, peaking at day three and four after stimulation, as shown in Figure II.5A. After day four, Pax-5a levels decreased and by day six had returned to levels similar to those of resting B cells (day 0). Interestingly, nuclear extracts from SRBs appeared to contain two bands in Pax-5a

position, which we named 5a.1 and 5a.2. The estimated size difference of these bands is ~ 2.5 kD, which corresponds to approximately 20 amino acids. In contrast, only the Pax-5a.1 band was present in late activated B cells. This observation was confirmed when samples were subjected to extended SDS-PAGE separation in the presence of high levels (4%) of SDS (Figure II.5B). Further comparison of Pax-5 protein patterns between resting and activated B cell samples revealed that resting B cell nuclear extracts displayed an extensive banding pattern of lower molecular weight species (<50 kD), as shown in Figure II.5A (Lane 3). This is likely the result of protein degradation, as it does not correlate with the patterns of existing splice variants of Pax-5. The number of degradation bands decreased with time in activated samples. By day two, the fragments were almost undetectable (Figure II.5A). The observed change in banding pattern indicates that Pax-5a protein may become stabilized upon LPS activation. It should be noted that levels of TFIID were undetectably low in the late-activation samples (day 6-day 8). The underlying cause and significance of this phenomenon are unclear.

Using the anti-Pax-5d antibody 6G11 (Figure II.5C, lower panel), we unexpectedly discovered that levels of the full-length Pax-5d protein were undetectable in activated B cells. Instead, the novel sequence-specific 6G11 detected a new, yet unidentified band at 27 kD. The new species, which we termed Pax-5_x, occurred at very low levels in nuclear extracts from resting B cells (day 0) and during the first 2 days (48 hrs) of LPS treatment (Figure II.5C, Lane 2-4). However, starting at day four after LPS stimulation, we observed a dramatic increase in the intensity of the Pax-5_x band (Figure II.5C, Lane 4-6). The nature of the Pax-5_x species is unknown at this time. It may represent either an N-terminal degradation product of Pax-5d or isoform Pax-5e

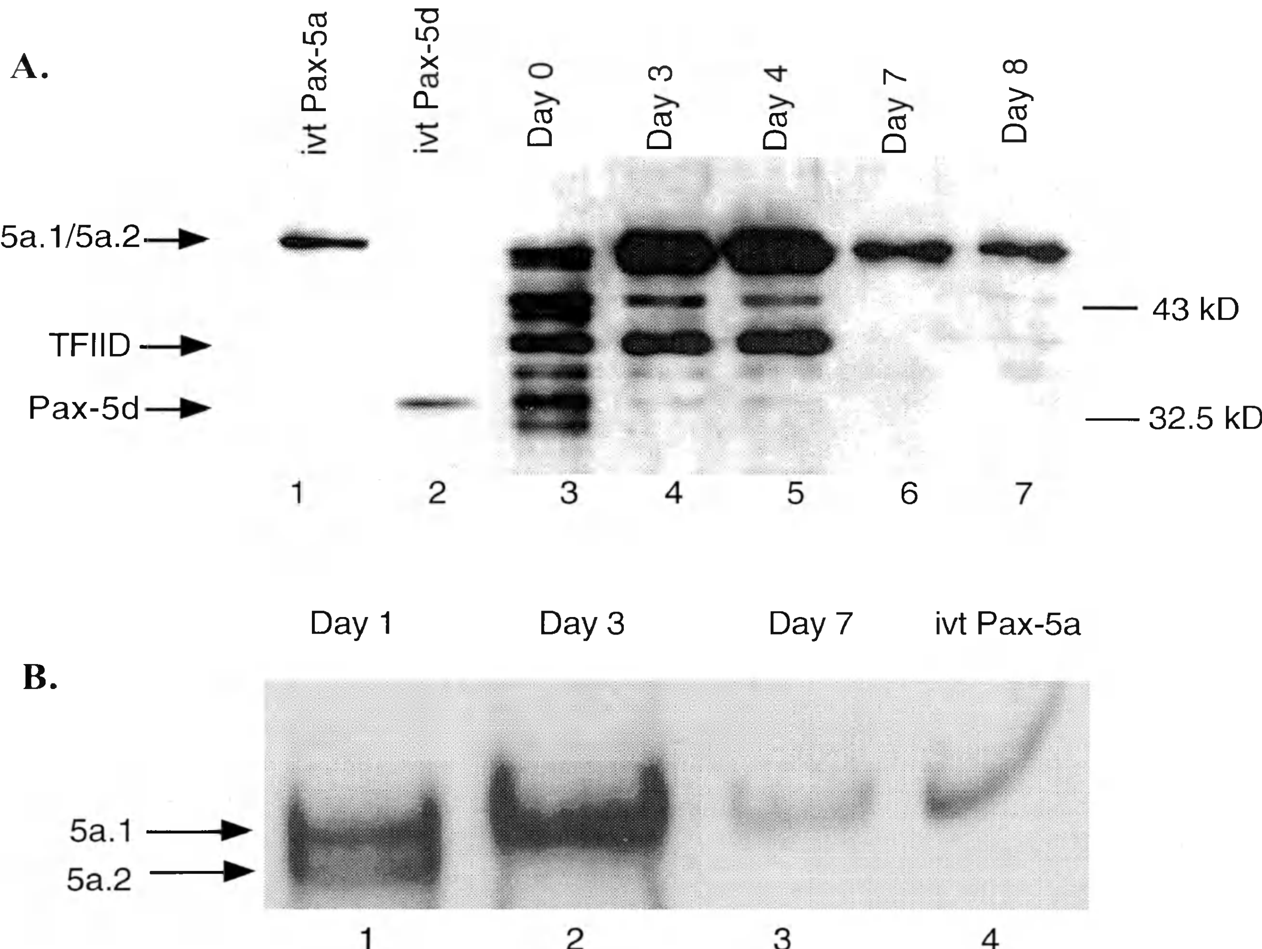


Figure II.5: Detection of Pax-5 proteins in resting and LPS-activated B cells using the anti-paired domain serum ED-1. Nuclear extracts from SRBs and LPS-activated B lymphocytes were analyzed by Western blot using the anti-paird domain serum ED-1. Days of LPS treatment are indicated. **(A)** Nuclear extracts from resting (Day 0) B cells are characterized by extensive degradation. Pax-5 protein is stabilized in activated samples where 5a.1 is a predominant species. **(B)** Distinct 5a.1 and 5a.2 bands are clearly visible in a sample from partially activated B cells (Day 1) after an extended electrophoresis in 4% SDS (see text). Anti-TFIID antibody was used to monitor the amount of total protein in the samples. The positions of Pax-5a.1, Pax-5a.2, Pax-5d and TFIID are indicated on the left. Molecular weight markers in kilodaltons are indicated on the right. ivt, *in vtro* translated protein.

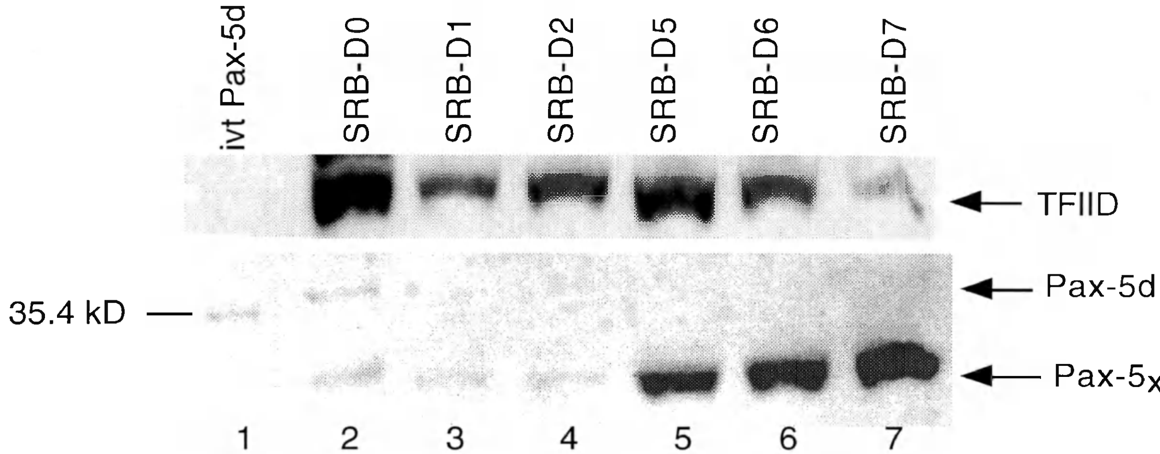


Figure II.5C: Detection of Pax-5 proteins in resting and LPS-activated B cells using the novel sequence-specific antibody 6G11. Nuclear extracts from SRBs and LPS-activated B lymphocytes were analyzed by Western blot using 6G11 antibody which recognizes the novel sequence present on Pax-5d and Pax-5e. Days of LPS treatment are indicated above each lane. The blot was probed with anti-TFIID antibody to assess the levels of total protein in the samples. The positions of Pax-5d and Pax-5_X species are indicated on the right. ivt Pax-5d, *in vitro* translated Pax-5d.

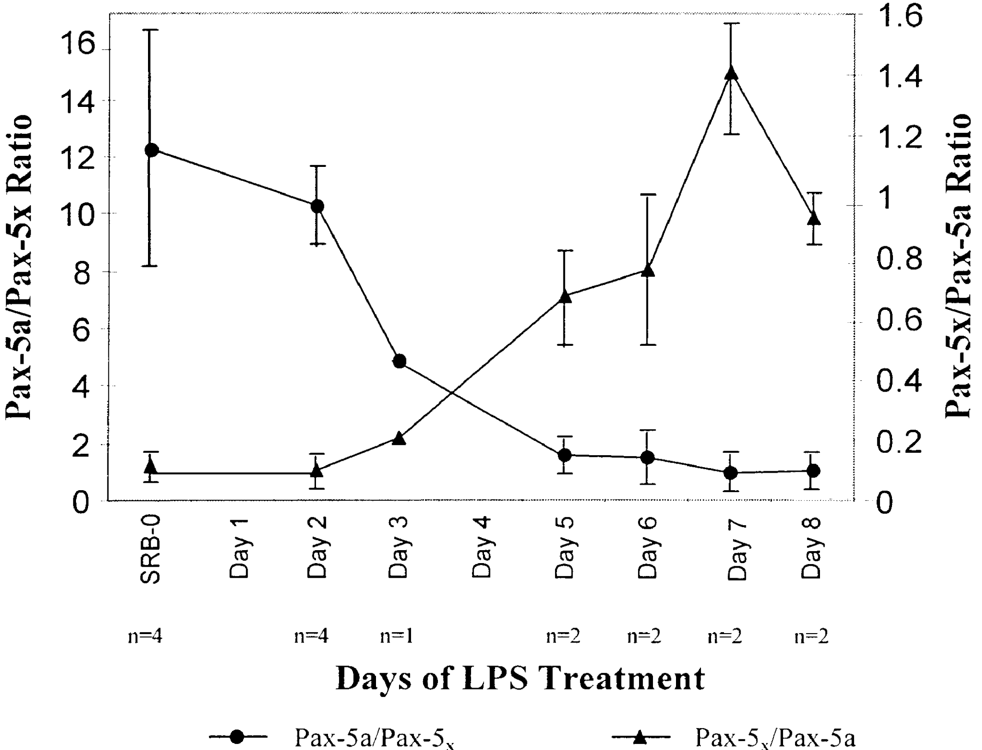


Figure II.5D: Assessment of the Pax-5a to Pax-5$_x$ ratio in nuclear extracts from resting and LPS-activated B cells. Nuclear extracts from resting and activated B cells were analyzed by Western blot. Pax-5a and Pax-5$_x$ proteins were detected using ED-1 and 6G11 antibodies respectively. The Pax-5a/Pax-5$_x$ (indicated at the left) and Pax-5$_x$/Pax-5a (indicated on the right) ratios were determined using the values of Pax-5a and Pax-5$_x$ band density obtained as described in "Materials and Methods". The calculated ratios refer to the intensity of the corresponding bands measured on two independent blots, and not to the molar ratio of the two proteins in the samples. Days of LPS-treatment are indicated on the bottom of the chart. Error bars show the mean ± S.E. with the number of replicates (*n*) indicated below the chart. No measurements were available for days 1 and 4 of LPS activation.

(although the band runs much higher in the gel then would be expected for the 19 kD Pax-5e protein). This is not a degradation product of Pax-5a, as it contains the novel sequence unique to Pax-5d and 5e.

We were unable to use the ED-1 antiserum to determine relative amounts of Pax-5a, Pax-5d and Pax-5_x proteins, due to the presence of three strong bands in the region around 35-25 kD in nuclear extracts from resting B cells (Figure II.5A), which made it impossible to differentiate between Pax-5a degradation products and other Pax-5 proteins. Furthermore, ED-1 did not recognize the Pax-5_x band efficiently, unless greater amounts of total nuclear protein were loaded on the gel (not shown). This might be an indication of an incomplete paired domain, such as the one present in Pax-5e isoform.

In summary, LPS induced a dramatic upregulation of Pax-5_x protein levels concomitant with a corresponding drop in Pax-5a.2 levels. This resulted in a 15.6 (+/-3.6) fold decrease in the ratio of Pax-5a to Pax-5_x protein after resting B cells were activated with LPS (Figure II.5D). It should be noted that the ratio of Pax-5a to Pax-5_x was calculated based on the relative intensities of the corresponding bands ("see "Materials and Methods"), and does not refer to the molar ratios of the two proteins in samples. The molar ratios could not be estimated because Pax-5a and Pax-5_x protein species were detected using two different antibodies with unknown affinities, which made it impossible to determine the precise stoichiometry of antibody binding.

Presence of specific Pax-5 protein species in B cell lines.

Our observation that LPS induced a shift in the Pax-5a/ Pax-5_x ratio in primary B lymphocytes led us to further examine this phenomenon in B cell lines representing

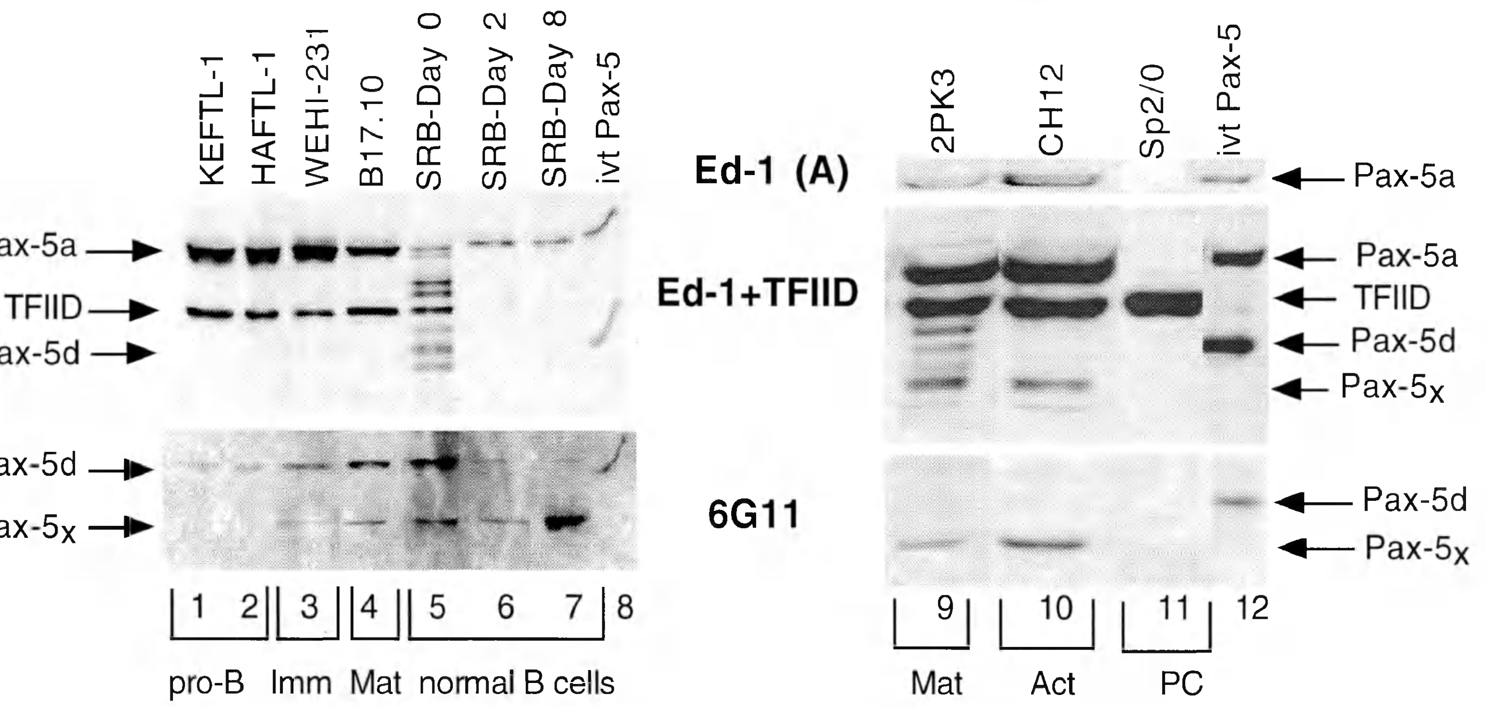


Figure II.6A: Nuclear protein levels of Pax-5 isoforms change during B cell differentiation. The levels of Pax-5 proteins were analyzed by Western blot using nuclear extracts from B cell lines representing different stages of B cell differentiation. Nuclear extracts of SRBs (lane 5) and LPS-activated B lymphocytes (lane 6,7) were included for comparison. The top panel shows blots probed simultaneously with ED-1, the paired domain-specific antiserum, and anti-TFIID antibody. Panel ED-1(A) shows a shorter exposure of Pax-5a bands. The same blots probed with the novel sequence-specific antibody 6G11 are shown on the bottom panel. Positions of different Pax-5 species are indicated. Pro-B, pro-B cell stage; Imm, immature B cell stage; Mat, mature B cell stage; Act, pre-secretor/activated B cell stage; PC, plasma cell stage.

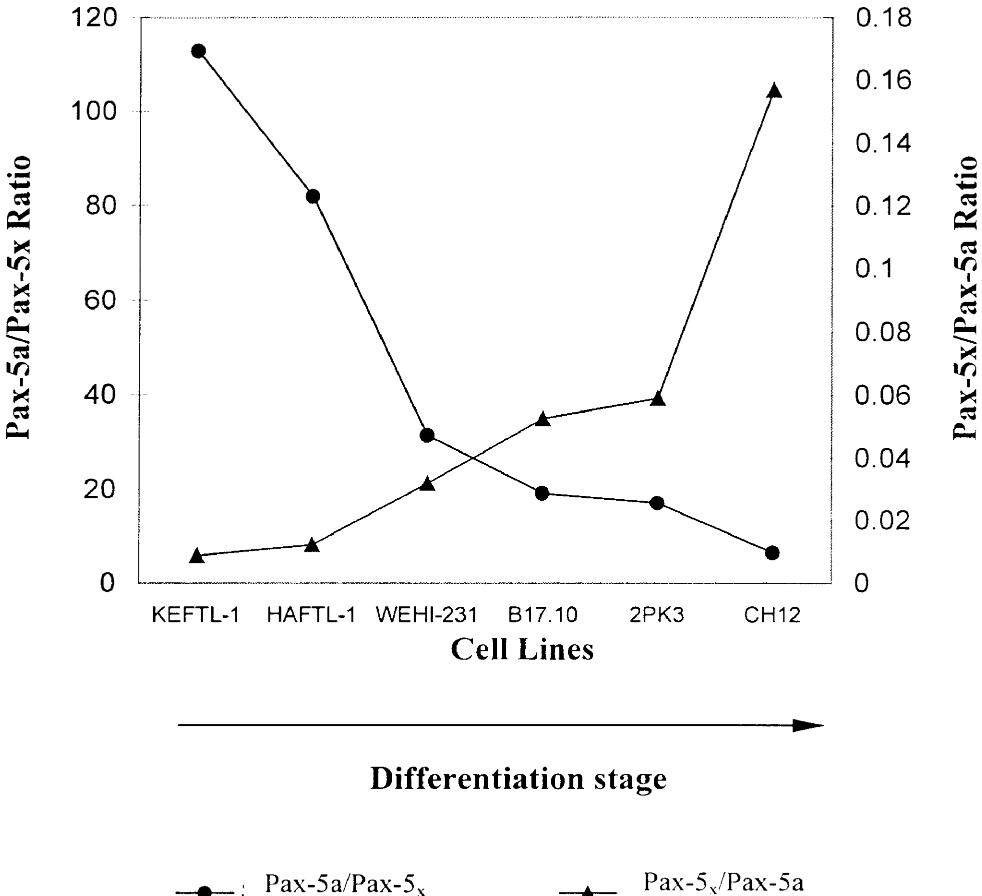


Figure II.6B: The Pax-5a/Pax-5$_x$ ratio changes during B cell differentiation. Pax-5a and Pax-5$_x$ nuclear protein levels were analyzed by Western blot using nuclear extracts of B-lymphoid cell lines representing different stages of B cell differentiation. Pax-5a and Pax-5$_x$ proteins were detected using ED-1 and 6G11 antibodies, respectively. The Pax-5a/Pax-5$_x$ (indicated on the left) and Pax-5$_x$/Pax-5a (indicated on the right) ratios were calculated using relative intensities of the corresponding bands, which were determined by image analysis ("Materials and Methods"). *n=1.* KEFTL-1, HAFTL-1- pro-B cell stage; WEHI-231- immature B cell stage; B17.10, 2PK3- mature B cell stage; CH12- pre-secretor (activated) B cell.

various stages of late B cell differentiation. Previous studies had already shown that the transcript levels of *Pax-5a* and *Pax-5d* remain unchanged during B cell development until both isoforms cease to be expressed in plasma cells (Zwollo *et al.*, 1997). Relative levels of Pax-5a.1, Pax-5a.2, Pax-5d, and Pax-5_x proteins were determined in nuclear extracts from pro-B, immature B, mature B, pre-secretor B, and plasma cell lines using ED-1 and 6G11 antibodies (Figure II.6A).

Using Western blot analysis, we observed that the nuclear levels of Pax-5a isoform do not change significantly in different B cell lines (Figure II.6A, ED-1/TFIID panel). In addition, the degradation pattern seen in resting B cells was absent from most B cell lines (except the mature B cell line 2PK3). In contrast to Pax-5a isoform, the nuclear levels of Pax-5_x species were undetectable at the pro-B stage, but later increased in a stage-dependent manner (Figure II.6A, panel 6G11). Isoform Pax-5d was present at very low levels in pro-B cell lines (Figure II.6A, panel 6G11, lanes 1,2), and was undetectable during late stages of B cell development (Figure II.6A, 6G11 panel, lanes 9-11). Interestingly, as in SRBs, Pax-5d and Pax-5x are expressed at the comparable levels in the immature and early mature B cell lines (WEHI-231 and B17.10, respectively). However, two highly differentiated B cell lines *en route* to the plasma cell stage (2PK3 and CH12) possess the Pax-5_x, but not Pax-5d species, similar to the pattern in LPS-activated normal B cells. The plasma cell line Sp2/0, as expected, had no detectable levels of any Pax-5 proteins.

The relative intensity of the bands was quantified (see Methods), and the ratio of nuclear Pax-5a to Pax-5_x was determined (Figure II.6B). As in the previous experiments, the estimated values reflect not the molar ratios of the two proteins, but the relative

intensity of the corresponding bands. A significant decrease in the Pax-5a to Pax-5_x ratio correlates with differentiation state of the cell lines, and this is in agreement with our LPS-activation experiments using normal B cells. The results suggest that specific Pax-5_x protein species play an important regulatory role during B cell differentiation.

Changes in Pax-5a DNA binding after LPS activation.

Activities of transcription factors can be regulated by a variety of mechanisms. One common post-translational means of regulation is alteration of a factor's DNA-binding activity through structural modifications of its DNA-binding domain (Steegenga *et al.*, 1996; Tell *et al.*, 1998). Hence, our next goal was to determine whether LPS-activation of mature B cells results in a change in DNA-binding activity of Pax-5 proteins, using EMSA. Previous EMSA experiments using SRB nuclear extracts had identified the presence of two distinct, Pax-5a-like complexes, which were named 5a.1 and 5a.2 (Anspach *et al.*, submitted).

Comparison of nuclear extracts from resting and LPS-activated B cells showed a shift in the relative amount of the 5a.1 and 5a.2 complexes (Figure II.7A). In nuclear extracts from SRBs, the faster migrating species (5a.2) was a predominant band. Interestingly, after LPS treatment, the relative intensity of the two bands reversed, shifting toward the slower migrating species (5a.1) until 5a.1 became the predominant band in the late-activation samples (Figure II.7A, lanes 8-10). The EMSA results confirmed our earlier observation that Pax-5a protein is more susceptible to degradation in SRBs, as compared to late stage LPS-activated samples. Several molecular species (all lower than Pax-5a) were present in nuclear extracts from SRBs and from early activated

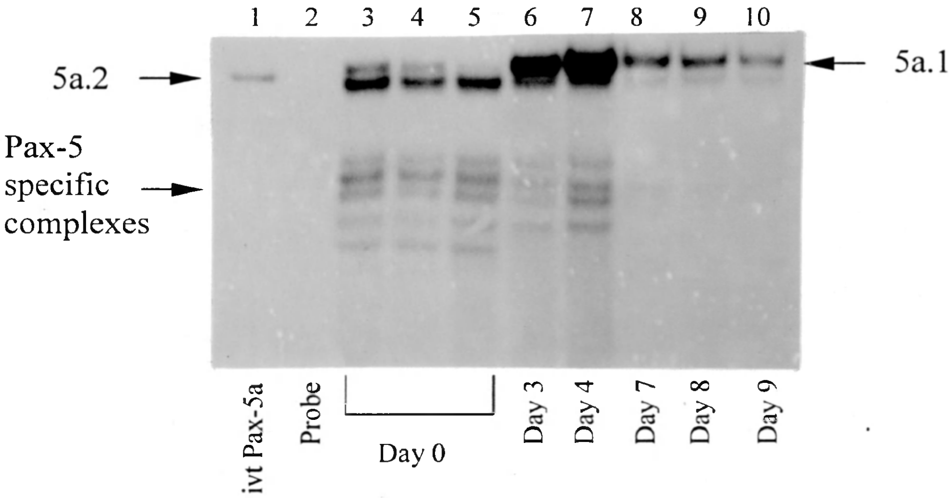


Figure II.7A: LPS activation induces a shift from Pax-5a.2 to Pax-5a.1 species. Nuclear extracts (1 μg) from resting and LPS-activated B lymphocytes were analyzed using EMSA and CD19/BSAP probe. Days of LPS treatment are indicated. Pax-5a isoform is represented by two distinct species: Pax-5a.1 and Pax-5a.2 (indicated). The two bands occur at different ratios in resting and activated B cells, with Pax-5a.1 being a predominant species during late activation stages (Day 7-9). The faster migrating bands represent Pax-5-specific complexes, which include Pax-5d isoform and Pax-5a degradation products. Lane 1: *in vitro* translated Pax-5a (1 μl lysate); lane 2: probe alone; lanes 3-5: different samples of non-activated B cells (SRBs).

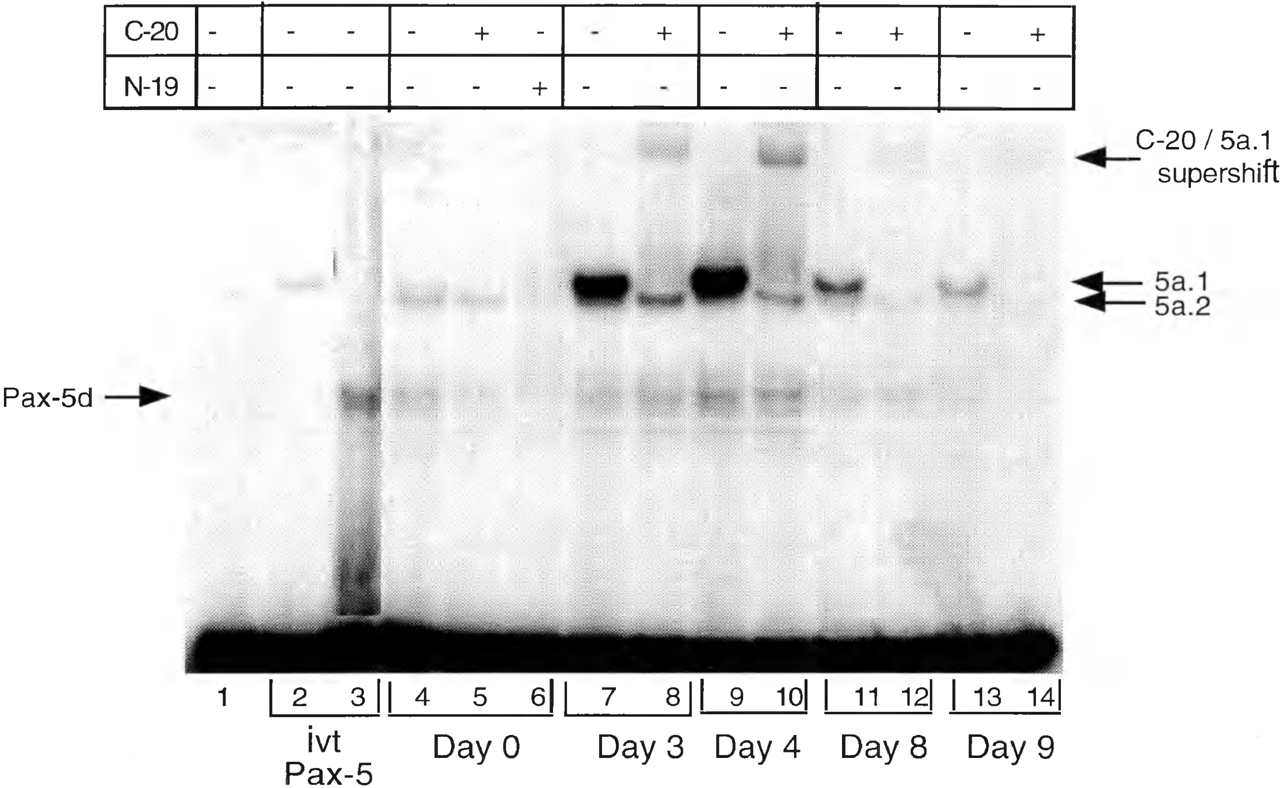


Figure II.7B: Pax-5a.1 and Pax-5a.2 species differ in the composition of the C-terminus. Nuclear extracts (1 µg) from resting and LPS-activated B cells were analyzed by EMSA using CD19/BSAP probe. Prior to the assay, the samples were incubated with or without C-20 (Pax-5 C-terminus-specific) and N-19 (Pax-5 N-terminus-specific) antibodies. Supershift with C-20 antibody occurs only with Pax-5a.1 band, whereas N-19 interacts with all Pax-5 molecules containing N-terminal amino acids 1-20. Supershift with N-19 is shown only for SRBs, but the results were identical for all samples. Lane 1: probe alone; lane 2: ivt Pax-5a (1µl lysate); lane 3: ivt Pax-5d (2 µl lysate); this lane was taken from a longer exposure of the same gel to better define the position of Pax-5d band.

samples (Figure II.7A, Days 0-4, lanes 3-7), but absent from the late activation stage (Figure II.7A, Days 7-9).

Next, nuclear extracts from resting and LPS-activated B cells were pre-incubated with one of the following antibodies: ED-1, OC-1, Pax-5/N19, or Pax-5/C-20 (for specificities, see Table I), followed by incubation in the presence of the probe. Earlier studies have shown that all Pax-5 species that are capable of DNA-binding can interact with the paired domain-specific antibody ED-1 (Zwollo *et al.*, 1998). Our data demonstrated that these species also interact with Pax-5/N-19 antibody (Figure II.7B, Lane 6). This indicated that all the DNA-protein complexes detected in SRB samples shared not only the paired domain sequence, but also the N-terminal 19 amino acids which are detected by the Pax-5/N-19 antiserum. In contrast, neither the C-terminus-specific Pax-5/C-20 antiserum (Figure II.7B, Lanes 5, 8, 10) nor the partial homeodomain-specific OC-1 antiserum (result not shown) was effective in inhibiting formation of the lower molecular weight DNA-protein complexes. Together, these results suggest that all the lower molecular weight Pax-5-DNA complexes contain the C-terminally truncated Pax-5 proteins that possess the paired domain sequence and share an intact N-terminal region.

The Pax-5/C-20 antibody recognizes the 22 most C-terminal amino acids of Pax-5a. In supershift EMSAs, C-20 recognized and supershifted the 5a.1 band, which represents the most abundant Pax-5a species in activated cells (Figure II.7B, Lanes 8, 10, 12, 14). Significantly, Pax-5/C-20 was unable to supershift the slightly lower migrating 5a.2 band present in resting and early activated cells (Figure II.7B, lanes 5, 8, 10, 12). Together with the results of ED-1 and Pax-5/N-19 supershift EMSAs, this observation

suggests that 5a.1 and 5a.2 proteins differ in structure at the C-terminus, but not the N-terminus. This is particularly interesting given the presence of a repressor domain within the last 33 amino acids of Pax-5a (Dorfler and Busslinger, 1996). Whether the C-terminus of 5a.2 is modified, thereby masking the epitope recognized by C-20, or whether it is absent altogether, could not be determined using this method. However, the results of Wetern blot analysis (Figure II.5A,B) suggested that 5a.2 may have lost the C-terminal region of about 20 amino acids. If the Pax-5a C-terminus is indeed absent, this could be either the result of specific proteolysis, or alternatively, the result of alternative RNA splicing. In conclusion, the presence of the C-terminal repressor sequence on Pax-5a.1 appears to correlate with B cell activation.

To test the possibility that the 5a.2 protein species represents a novel, alternatively spliced Pax-5 variant, we performed RNase protection assays using an anti-sense riboprobe pBS.1.2 (Zwollo and Desiderio, 1994) that covers a region between exons 8 and 10 of *Pax-5a*. Using RNA prepared from SRBs and LPS-activated B cells, we found no evidence for the existence of alternatively spliced isoforms that were missing either exon 8, 9, or 10 (data not shown). This is in agreement with earlier experiments, including the screening of a spleen cDNA library, which also showed no evidence for expression of isoforms lacking the C-terminal repressor domain coding region (Zwollo *et al.*, 1997).

Lastly, the EMSA approach could not be used to assess levels of Pax-5d- and Pax-5_x-containing complexes. We were unable to specifically identify Pax-5d protein in SRBs, due to Pax-5a degradation products running with similar mobility to Pax-5d on denaturing gels. This problem could not be resolved due to the unavailability of a

suitable antibody. The anti-Pax-5d monoclonal 6G11 does not recognize the native form of Pax-5d efficiently (Anspach *et al.*, submitted) and, hence, could not be used for EMSAs. Analysis of LPS-activated samples by EMSA detected neither Pax-5d- nor Pax-5_x-specific complexes. The absence of Pax-5d bands is not surprising since Pax-5d protein was not detected in the activated samples (as determined by Western blot). The lack of DNA-binding activity in Pax-5_x species is probably due to its incomplete paired domain, in agreement with the hypothesis that Pax-5_x represents Pax-5e.

In summary, EMSA analysis revealed that LPS-activation of normal mature B cells induced post-translational changes of isoform Pax-5a. This resulted in a significant decrease in levels of a Pax-5a species (5a.2) in which the C-terminal repressor domain has been either lost or modified. Simultaneously, LPS activation correlated with an increase in Pax-5a species with an intact repressor domain, namely Pax-5a.1. Post-translational changes of the repressor domain of Pax-5a may modulate its function in LPS-stimulated B lymphocytes.

DISCUSSION

In this study we analyzed the function of Pax-5d in relation to Pax-5a activity. Among the Pax-5 isoforms generated by alternative splicing, Pax-5a and Pax-5d present a special interest because they are the two most abundant isoforms in mature B cells, are both capable of binding to DNA, and are dramatically different in the composition of their C-termini. Studies on the related Pax-8 gene had shown previously that it expresses at least six alternative isoforms that are developmentally expressed and display

differential tranactivating potential (Kozmik *et al.*, 1993). Similarly, Pax-5 isoforms may differ in transactivating potential as well.

Our results demonstrated that, opposite to Pax-5a, Pax-5d functions as a repressor of transcription. The two isoforms compete for binding to Pax-5-specific sites on DNA, Contrary to our initial hypothesis, the ratio of Pax-5a to Pax-5d does not seem to change significantly during B cell differentiation due to very low protein levels of Pax-5d in normal B lymphocytes, as well as in different B cell lines. However, the ratio of Pax-5a to Pax-5e, the novel, Pax-5e-like species, decreases dramatically during late stages of B cell differentiation, suggesting an important role for the Pax-5_x protein in regulation of transcription during B cell activation. In addition, we showed that activation signals induce stabilization of Pax-5a isoform leading to the retention of the C-terminal repressor sequence. Together, our data indicates that, in activated B lymphocytes, the transcriptional activity of Pax-5a (BSAP) transcription factor may be regulated through translational and post-translational mechanisms. The pathways involved in these mechanisms are directed to inhibition of Pax-5a activity, which is achieved either through structural modifications Pax-5a protein or by upregulation of the alternative Pax-5 isoform(s) with dominant-negative functions.

Pax-5d is a repressor of Pax-5a activity.

In the first set of results, we demonstrate that Pax-5d has a transactivation function opposite to that of Pax-5a and acts as a transcriptional suppressor. In addition to repressing the activity of endogenous Pax-8 in the kidney cell line COS-1 in a dose-dependent manner, more significantly, we also show that Pax-5d can similarly repress

activity of Pax-5a in NIH 3T3 cells. Thus Pax-5a and Pax-5d have an opposite regulatory function *in vivo*. Together, our functional studies suggest that Pax-5d functions as a transcriptional repressor that regulates activity of Pax-5a and affects expression of Pax-5 target genes.

The observed repressor function of Pax-5d is most likely the result of the absence of a transactivating domain, although this needs further investigation. The lack of both the transactivating domain and the adjacent, extreme C-terminal repressor domain, which are present on Pax-5a, prevents interaction of Pax-5d with the basal initiation complex. While different at the C-terminal regions, the two isoforms share an intact paired domain and the octamer sequence. The latter has been shown to interact with the co-repressor Groucho4 although it is unclear what the functional significance of this interaction is in the B cell (Eberhard *et al.*, 2000). Both the octamer motif and the partial domain are capable of interacting with the Retinoblastoma protein and the TATA-box binding protein (Eberhard and Busslinger, 1999), but it remains to be determined whether Pax-5d would interact with such proteins as well, as the Pax-5a specific partial homeodomain may also be necessary for this interaction (Eberhard and Busslinger, 1999). The presence of two protein-protein interaction regions, identical to those found in Pax-5a isoform, may allow Pax-5d bind to the partner-proteins that can be involved in interactions with Pax-5a. The shared specificity for binding partners may result in a competition between Pax-5a and Pax-5d for binding not only to Pax-5 recognition sites on DNA, but also for certain accessory factors and partner-proteins.

The ratio of Pax-5 proteins changes as mature B cells become activated.

Our data clearly show that regulation of Pax-5a and Pax-5d activity during B cell activation by LPS treatment does not occur at the transcriptional level. The ratio of Pax-5a/Pax-5d mRNA remains unchanged during the entire activation period of 9 days. In contrast, a number of changes were observed at the protein level. An initial increase in Pax-5a levels in nuclear extracts was observed at days 2-4 after LPS stimulation corresponding with active B cell proliferation, followed by a decrease to pre-LPS levels by day 5 or 6, as reported previously by Wakasuki *et al.* (1994). Contrary to our hypothesis about the role of the Pax-5a/Pax-5d ratio in regulation of Pax-5a function, the nuclear levels of Pax-5d were very low in resting B lymphocytes and undetectable in activated cells. However, we detected a new band at ~27 kD which represents a yet unidentified protein species named Pax-5_x. The intensity of Pax-5_x band increases dramatically in nuclear extracts during the late activation stages (days 5-9), concomitantly with the decrease in the Pax-5a levels. Together, these changes lead to a significant decrease in the 5a/5x ratio as B cells become activated by LPS. The Pax-5_x band has also been detected in nuclear extracts of various B cell lines, where its intensity was higher in cell lines representing late stages of differentiation. Thus, the ratio of Pax-5a to Pax-5_x proteins decreases during activation and differentiation of B lymphocytes.

The exact nature of Pax-5_x species is unknown at this time. Western blot of resting and activated SRBs shows that the 27 kD band can interact with the novel sequence-specific antibody 6G11, but is undetectable using the paired domain antiserum ED-1. These results indicate the presence of the novel sequence and suggest that the paired domain is either incomplete or absent. The fact that Pax-5_x does not possess any

detectable DNA-binding activity suggests that the Pax-5_x band may represent the Pax-5e isoform. However, the expected size of Pax-5e is 19 kD. One possible explanation for the ~8 kD difference in size between Pax-5e and Pax-5_x is that the band represents a modified form of the Pax-5e protein. Pax-5e might be modified through such post-translational modifications as *O*-glycosylation or formation of disulfide bonds. These, however, requires further investigation. Finally, we cannot exclude the possibility that Pax-5_x band represents an N-terminal degradation product of Pax-5d. Identification of Pax-5_x species is the focus of our current research.

B cell activation corresponds with an increase in Pax-5a.1 protein.

The protein patterns of Pax-5a changed during the early stages of activation (days 1-4), and this correlated with activation-induced proliferation of B cells (Wakatsuki *et al.*, 1994). Most strikingly, activation signals triggered an increase in the relative level of 5a.1 and concomitant decrease in 5a.2 molecular species. In our experiments, we have shown that the emergence of a slower migrating 5a.1 complex was due to a modification at the C-terminus of Pax-5a protein.

We hypothesize that specific proteases remove the C terminal sequence from Pax-5a.1 in resting, but not activated/proliferating B cells. Proteases also apparently cause multiple degradation bands in resting B cell nuclear extracts. The observed effect is unlikely to be a result of nuclear extract preparation, because all the buffers used for this procedure contained several protease inhibitors (see "Materials and Methods"). Moreover, the identical patterns were observed in several independent experiments in which samples originating from different sources were used (i.e. cell lines, different

strains of mice). It is unclear how Pax-5a in activated B cells and proliferating cell lines is "protected" from specific proteolysis. To confirm this hypothesis, the half-life of Pax-5a protein has to be determined in resting and LPS-activated B cells, which can be achieved using pulse-chase assays.

In support of a functional role for C-terminus-initiated degradation, is the presence of a potent repressor domain in this region (see Figure II.1; Döefler and Busslinger, 1996). Since both 5a.1 and 5a.2 forms are present in resting B cells, but only the repressor-containing form is present in proliferating B cells, this may define the set of Pax-5 target genes during each of the two developmental stages. Nutt *et al.* (1998) showed that a subset of Pax-5 target genes, including those for which Pax-5 functions as a recruiter (e.g. *mb-1*), can be regulated by the paired domain alone. In contrast, other target genes require the complete Pax-5a sequence including the C-terminal transactivating domain (e.g. *CD19*). We speculate that, in resting B cells, specific C-terminal proteolysis generates an array of shorter Pax-5a with distinct transactivating activities.

Proteolysis is a common mechanism that quickly and irreversibly regulates transcription factor function. This means of regulation is particularly useful when cells need to respond rapidly to changes in their environment. Several eukaryotic transcription factors, including NF-κB, p53, c-Myc, and c-Jun, have been shown to be regulated by proteolytic events, most of which involve the ubiquitin-proteasome pathway (reviewed in Peters, 1994; Pahl and Baeuerle, 1996). Proteolytic degradation may occur either in cytoplasm (e.g., NF-κB (Thanos and Maniatis, 1995)) or in the nucleus (e.g., HIF-1α

(Salceda and Caro, 1997)), indicating that this process is mediated by the specifically localized proteasome systems.

The lability of many regulatory proteins is critically important under normal conditions. The disruption of proper steady-state levels of these proteins may have deleterious consequences for a cell (Treier *et al.*, 1994). However, in some cases, protein stability changes drastically when cells are subjected to a stressful stimulus. Wild-type p53, for instance, is a short-lived nuclear transcription factor which becomes stabilized and activated following cellular stress or DNA damage (Pahl and Baeuerle, 1996). The exact mechanism of this stabilization is not clearly understood, but thought to be regulated by multi-site phosphorylation (Steegenga *et al.*, 1996; Meek, 1998). Another example is the hypoxia-inducible factor-1α (HIF-1α), a transcriptional activator complex involved in regulation of several hypoxia-regulated genes (Salceda and Caro, 1997). Under normoxic conditions, HIF-1α is continuously degraded in the nucleus by the ubiquitin-dependent pathway. The lack of oxygen, or hypoxia, induces stabilization and accumulation of HIF-1α followed by subsequent activation of its target genes (Salceda and Caro, 1997). The signals triggering stabilization of the HIF-1α protein are currently unknown; however, some evidence suggests that redox-mediated changes might be involved (Salceda and Caro, 1997).

Thus, stress-induced stabilization of transcription factors is a well-documented phenomenon which may take place in activated B lymphocytes. It remains to be determined whether Pax-5a degradation, occuring in resting B cells, involves the ubiquitin-proteasome pathway. Further investigations are needed to identify the factors

which contribute to susceptibility of Pax-5a to degradation, as well as to its stabilization during activation.

Effects of changes in Pax-5 proteins on Pax-5/BSAP target genes

The increased levels of Pax-5_x induced during B cell activation may inhibit Pax-5a function through competition for various accessory factors, thereby aiding in down-regulation of certain Pax-5 target genes during this stage. One possible effect of such competition is the relief of Pax-5a repressor function. Many of the genes negatively regulated by Pax-5a, such as *J chain* and Ig heavy chain genes, are not expressed until the late stages of B cell differentiation (pre-secretor to mature plasma cell). Their expression indicates an inhibition of negative transcriptional control exerted by Pax-5a, although Pax-5a is clearly still present in the nucleus during this time. Although the mechanism of de-repression is currently unclear, it is possible that Pax-5_x plays an important role in attenuating Pax-5a activity during transition of B cells to the plasma cell stage.

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REFERENCES

Adams, B., Dorfler, P., Agguzi, A., Kozmik, P., Urbanek, P., Maurer-Fogy, I., and Busslinger, M. (1992) *Pax-5* encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev.*, **6**, 1589-1607

Anspach,J., Poulsen, G., Kaattari, I., Pollock, R., and Zwollo, P. (2000) Reduction in DNA binding activity of the transcription factor Pax-5a in B lymphocytes of aged mice. Submitted.

Asano, M., and Gruss, P. (1991) Pax-5 is expressed in the midbrain-hindbrain boundary during mouse development. *Mech. Dev.*, **39**, 29-39

Barberis, A., Widenhorn, K., Vitelli, L., and Busslinger, M. (1991) A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. *Genes Dev.*, **22**, 37-43

Calkhoven, F. and Geert, A.B. (1996) Multiple steps in the regulation of transcription factor level and activity. *Biochem. J.*, **317**, 329-342

Cogné, M., Lasford, A., Bottaro, A., Zhang, J., Gorman, J., Young, F., Cheng, H-L., and Alt, F.W. (1994) A class switch control region at the 3' end of the immunoglobulin heavy chain locus. *Cell*, **77**, 737-747

Dörfler, P. and Busslinger, M. (1996) C-terminal activating and inhibitory domains determine the transactivation potential of BSAP (Pax-5), Pax-2, and Pax-8. *EMBO J.*, **15**, 1971-1982

Dymecki, S.M., Zwollo, P., Zeller, K., Kuhadja, F.P., and Desiderio, S.V. (1992) Structure and developmental regulation of the B-lymphoid tyrosine kinase gene *blk*. *J. Biol. Chem.*, **267**, 4815-4823

Eberhard, D., Jimenez, G., Heavey, B., and Busslinger, M. (2000) Transcriptional repression by Pax-5 (BSAP) through interaction with corepressors of the Groucho family. *EMBO J.*, **19**, 2292-2303

Fitzsimmons, D., Hodsdon, W., Wheat, W., Maira, S.M., Wasylyk, B., and Hagman, J. (1996) Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. *Genes Dev.*, **7**, 2198-2211

Grosschedl, R., and Baltimore, D. (1985) Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. *Cell*, **41**, 885-897

Hagman, J., Wheat, W., Fitzimmons, D., Hodson, W., Negri, J., and Dizon, F. (2000) Pax-5/BSAP: regulator of specific gene expression and differentiation in B lymphocytes. *Curr. Top. Microbiol. Immunol.*, **245**, 169-194

Hunter, T. and Karin, M. (1992) The regulation of transcription by phosphorylation. *Cell*, **70**, 375-378

Kozmik, Z., Kurzbauer, R., Dorfler, P., and Busslinger, M. (1993) Alternative splicing of *Pax-8* gene transcripts is developmentally regulated and generates isoforms with different transactivation properties. *Mol. Cell. Biol.*, **13**, 6024-6035

Kozmik, Z., Wang, S., Dorfler, P., Adams, B., and Busslinger, M. (1992) The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol. Cell. Biol.*, **12**, 2662-2672

Lansford, R.D., McFadden, H.J., Siu, S.T., Cox, J.S., Cann, G.M., and Koshland, M.E. (1992) A promoter element that exerts positive and negative control of the interleukin 2-responsive J-chain gene. *Proc. Natl. Acad. Sci. USA*, **89**, 5966-5970

Liberg, D. and Sigvardsson, M. (1999) Transcriptional regulation in B cell differentiation. *Curr. Rev. Immunol.*, **19**, 127-153

Neurath, M.F., Strüber, E.R., and Strober, W. (1995) BSAP: a key regulator of B cell development and differentiation. *Immunology Today*, **16**, 564-569

Nutt, S.L., Morrison, A.M., Dörfler, P., Rolink, A., and Busslinger, M. (1998) Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. *EMBO J.*, **17**, 2329-2333

Pahl, H.L. and Baeuerle, P.A. (1996) Control of gene expression by proteolysis. *Curr. Opin. Cell Biol.*, **8**, 340-347

Peters, J.-M. (1994) Proteasomes: protein degradation machines of the cell. *Trends Biochem. Sci.*, **19**, 377-382

Reya, T. and Grosschedl, R. (1998) Transcriptional regulation of B-cell differentiation. *Curr. Opin. Immunol.*, **10**, 158-165

Rinkenberger, J.L., Wallin, J.J., Johnson, K.W., and Koshland, M.E. (1996) An interleukin-2 signal relieves BSAP (Pax-5)-mediated repression of the immunoglobulin J chain gene. *Immunity*, **5**, 377-386

Salceda, S and Caro, J. (1997) Hypoxia-inducible factor 1α (HIF-1) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. *J. Biol. Chem.*, **272**, 22642-22647

Singh, M. and Birshtein, B.K. (1993) HF-HB (BSAP) is a repressor of the murine immunoglobulin heavy-chain 3'α enhancer at early stages of B-cell differentiation. *Mol. Cell. Biol.*, **13**, 3611-362

Stüber, E., Neurath, M., Calderhead, D., Fell, H.P., and Strober, W. (1995) Cross-linking of OX-40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. *Immunity*, **2**, 507-521

Tell, G., Scaloni, A., Pellizari, L., Formisano, S, Pucillo, C., and Damante, G. (1998) Redox potential controls the structure and DNA binding activity of the paired domain. *J. Biol. Chem.*, **273**, 25062-25072

Urbaneck, P., Wang, Z.Q., Fetka, I., Wagner, E.F., and Busslinger, M. (1994) Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell*, **79**, 901-912

Usui, T., Wakatsuki, Y., Matsunaga, Y., Kaneko, S., Kosek, H., and Kita, T. (1997) Overexpression of B cell-specific activator protein (BSAP/Pax-5) in a late B cell is sufficient to suppress differentiation to an Ig high producer cell with plasma cell phenotype. *J. Immonol.*, **158**, 3197-3204

Wakatsuki, Y., Neurath, M., Max, E.E., and Strober, W. (1994) The B-cell specific transcription factor BSAP regulates B cell proliferation. *J. Exp. Med.*, **179**, 1099-1108

Wallin, J.J., Rickenberger, J.L., Rao, S., Gackstetter, E.R., Koshland, M.E., and Zwollo, P. (1999) B cell-specific activator protein prevents two activator factors from binding to the immunoglobulin J chain promoter until the antigen-driven stages of B cell differentation. *J. Biol. Chem.*, **274**, 15959-15965

Walther, C., Guinet, J.-L., Simon, D., Deutsch, U., Jostes, B., Goulding, M. D., Plachov, D., Balling, R., and Gruss, P. (1991) Pax: a murine multigene family of paired box-containing gene. *Genomics*, **11**, 424-434

Zwollo, P., Rao, S., Wallin, J.J., Gackstetter, E.R., and Koshland, M.E. (1998) The transcription factor NF-kB/p50 interacts with the *blk* gene during B cell activation. *J. Biol. Chem.*, **273**, 18647-18655

Zwollo, P., Arrieta, H., Ede, K., Molinder, K., Desiderio, S., and Pollock, R. (1997) The Pax-5 gene is alternatively spliced during B-cell development. *J. Biol. Chem.*, **272**, 10160-10168

Zwollo, P. and Desiderio, S.V. (1994) Specific recognition of the *blk* promoter by the B-lymphoid transcription factor BSAP. *J. Biol. Chem.*, **269**, 15310-15317

Chapter III

Preliminary Studies of the Activity of Pax-5 isoforms *in vivo* and *in vitro*

The studies presented in this chapter aimed to address various aspects of Pax-5 function using alternative experimental approaches. These included development of a novel transfection system for the analysis of Pax-5a/Pax-5d competition (Section 1), examination of B cell activation in aged B lymphocytes (Section 2), and activation studies in the immature B cell line WEHI-231 (Section 3). The experiment described in Section 4 is a part of an ongoing study that deals with identification of Pax-5_x species.

1. Staggered Transfections

In Pax-5d, the entire transactivation domain and the homeodomain homology region have been replaced with a novel sequence of unknown function (Zwollo *et al.*, 1997). As stated previously, the absence of an important C-terminal module may significantly change the activation function Pax-5 (Anspach *et al.*, submitted). Thus, while the Pax-5d isoform can efficiently bind to DNA, it fails to interact with the transcription initiation complex and can have a dominant-negative effect on transcription. This hypothesis is supported by transient transfections studies presented here, which revealed that Pax-5d and Pax-5a isoforms have the opposite effects on the transcription of an artificial minimal promoter construct containing Pax-5 binding sites from the *CD19*

promoter (Chapter II). Functional studies also indicated that the two isoforms (Pax-5a and Pax-5d) may compete for binding to Pax-5 DNA binding sites (Chapter II). Intriguingly, in our competition experiments, the activity of Pax-5a was inhibited only when very high levels of Pax-5d expression construct were trasnsfected, and the Pax-5a/Pax-5d ratio (based on the amount of the transfected DNA) was less than 1/10. Three possibilities could explain these findings. First, Pax-5d protein may be degraded more rapidly than Pax-5a (at least in the chosen cell line). Second, Pax-5d may have a lower affinity for Pax-5 binding sites *in vivo*, as compared to Pax-5a. As a result, Pax-5d may be unable to bind to DNA in the presence of Pax-5a. Finally, it cannot be excluded that the two isoforms differ in the rate and efficiency of transcription and/or translation.

To rule out the possibility of differential affinity, a "staggered" transient transfection system was developed. In this system, the reporter construct γ42(3i)AS-CAT was first co-transfected with the Pax-5d expression construct followed, 20-24 hr later, by a second transfection on the same cells with the Pax-5a expression construct alone. Cells were incubated for additional 20-24 hr and then processed as cell lysates (for CAT assays) and nuclear extracts (for Western blot analyses and EMSA) according to standard methods (Chapter II). A control co-transfection with HBIICAT and pcDNA3 (Chapter II) was performed to determine the effect of "staggered" transfections on transfection efficiency. Additional controls included a panel of samples transfected at either first or second day only. These controls were designed to assess the levels of protein expression at 24 and 48 hr after introduction of the constructs (Figure III.1A).

The rationale behind this approach was based on the assumption that if the pcDNA5d effector construct was introduced into the system prior to Pax-5a expression,

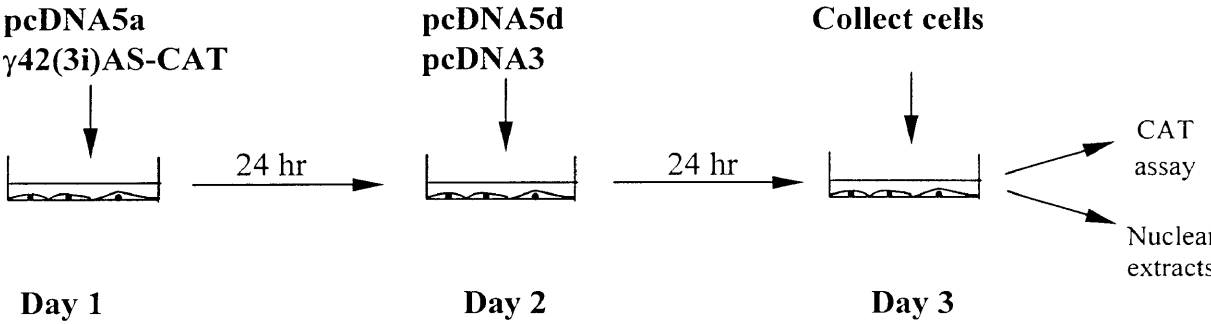


Figure III.1A: "Staggered" transfection method. Various combinations of constructs were transiently co-transfected into NIH 3T3 cell line in a "staggered" fashion: the reporter construct γ42(3i)AS-CAT was first co-transfected with the Pax-5d (or Pax-5a) expression construct followed, 20-24 hr later, by a second transfection on the same cells with the Pax-5a (or Pax-5d) expression construct alone. A pcDNA3 vector without insert was used to maintain equal amounts of transfected DNA. After total 48 hr of incubation, the samples were collected, and cell extracts were analyzed by CAT assay, Western blot, and EMSA.

the Pax-5d isoform may have a chance to occupy the binding sites and, later, prevent Pax-5a from binding. It was, therefore, hypothesized that binding competition could occur at low Pax-5d concentrations if this transcription factor had a sufficient time to be synthesized and to bind to a target site on DNA without competition from Pax-5a.

CAT assay results from the pilot experiments provided some evidence for that hypothesis (Figure III.1B, see "Samples"). With the Pax-5a/Pax-5d ratio of 1/5 (according to the amount of transfected DNA), we observed a three fold decrease in the relative CAT conversion for samples transfected with the Pax-5d effector construct first followed by the Pax-5a construct transfection on the second day, as compared to the sample with the reversed order of the introduced constructs (compare 55.6% for [5a+5d] to 18% for [5d+5a]). However, a more careful experimental design, which included additional controls, proved our initial results unreliable due to significant differences in the protein levels present in the samples after 24 and 48 hours of transfection (Figure III.1B, see "Controls"). For example, the CAT expression level in the Pax-5a control samples collected 48hr post-transfection was 10.6 fold higher than in those samples which were incubated only for 24hr (81.7% for [5a-day 1] vs. 7.7% for [5a-day 2]). Likewise, the length of incubation had an effect on the CAT activity induced by the HBIICAT construct (compare 100% for [HBIICAT-day 1] to 79.4% for [HBIICAT-day 2]) and in the samples co-transfected with Pax-5a and Pax-5d simultaneously (compare 28.5% for [5a/5d-day 1] to 7.2% for [5a/5d-day 2]). After a thorough analysis of these data, we concluded that the difference in CAT activity observed in the "staggered" samples is not based on Pax-5a/Pax-5d binding competition, but is more likely to be

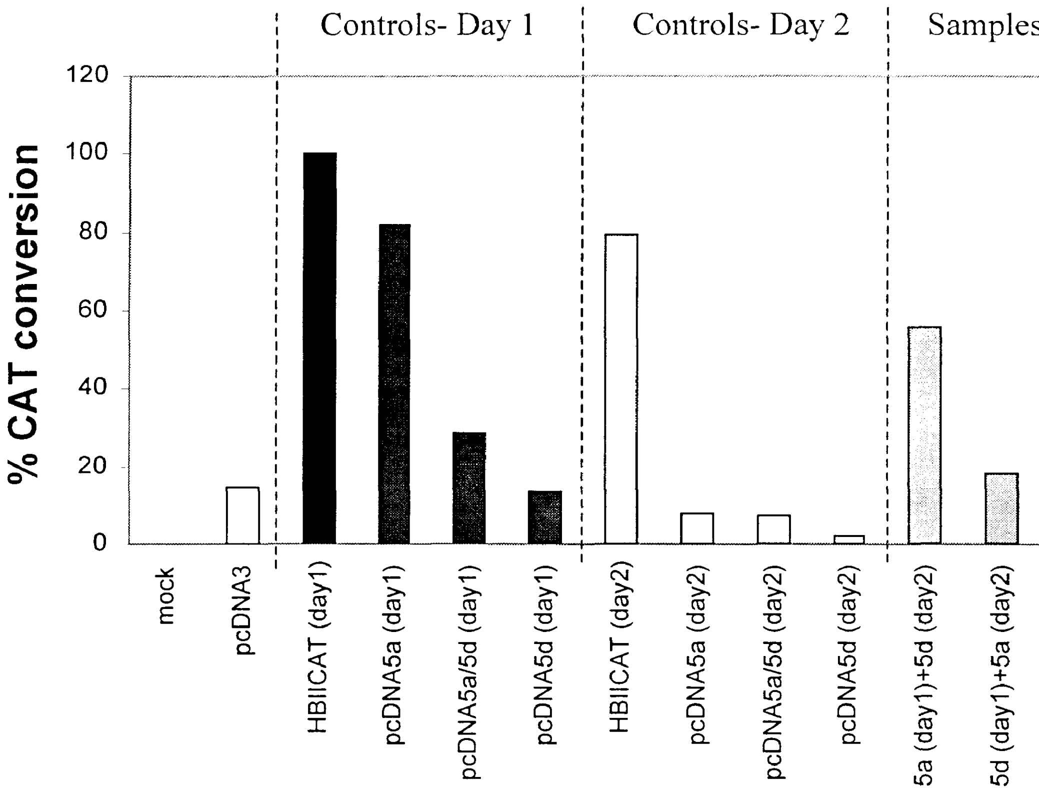


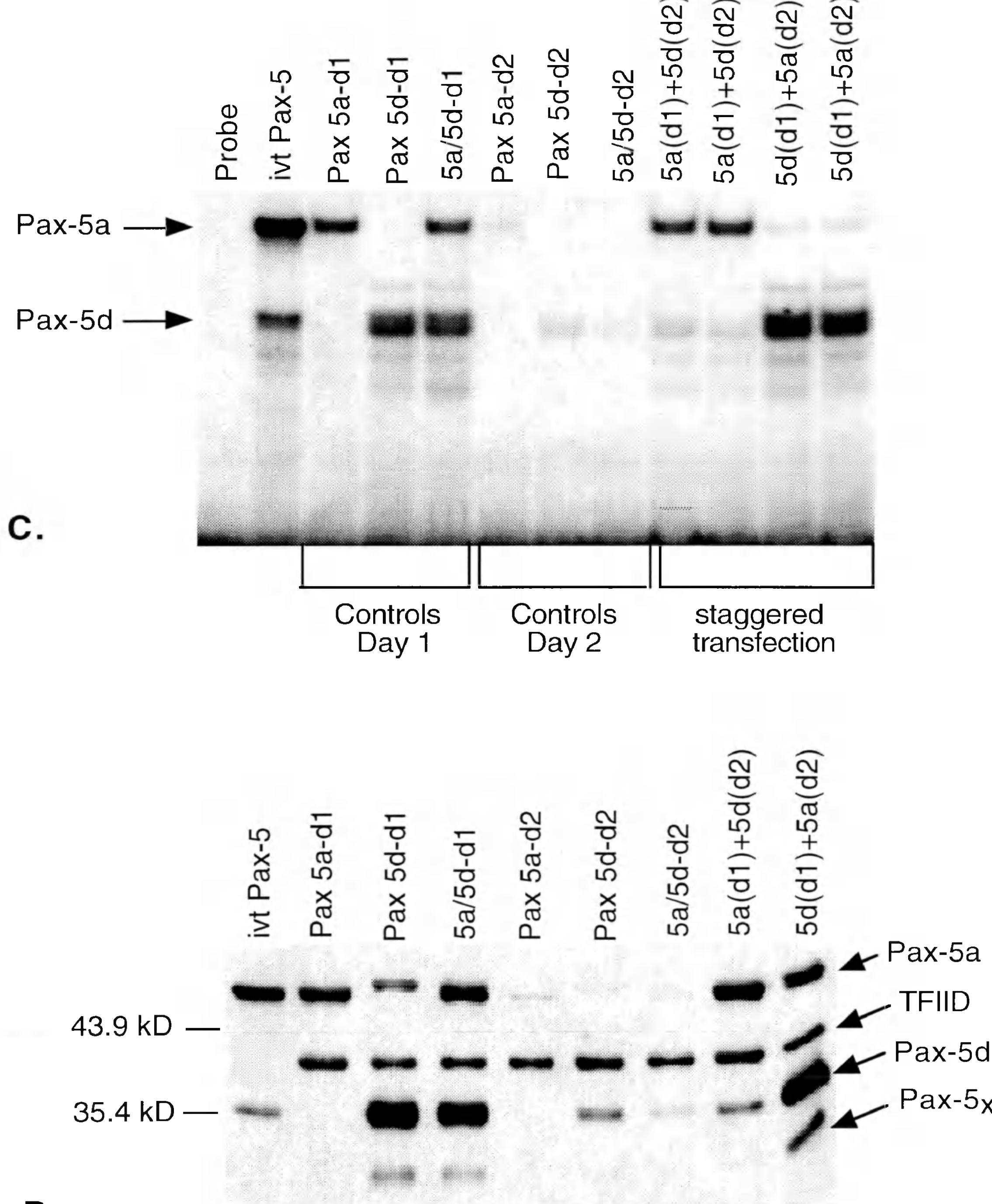
Figure III.1B: CAT assay of staggered transfection samples. The effector constructs, pcDNA5a (0.1μg) and pcDNA5d (0.5μg), were cotransfected into NIH 3T3 cell line in combination with the γ42(3i)AS-CAT reporter construct (0.5μg). The effectors were introduced in a staggered fashion to assimilate a situation in which Pax-5d binds to DNA without competition from Pax-5a (see text for details). Controls included samples transfected with one or both constructs at either day 1 or day 2 of the experiment. Percent CAT conversion was determined as described in "Materials and Methods" (Chapter II) and normalized to the percent conversion value for "HBIICAT- day1" sample set at 100%.

determined by different concentrations of Pax-5a present in the samples at any particular time-point.

To confirm conclusions that were made based on the experimental data, nuclear extracts from the transfected samples were analyzed using Western blot and EMSA as described (see "Materials and Methods", Chapter II). Pax-5a and Pax-5d protein levels were assessed and DNA-binding patterns determined using isoform-specific antibodies (ED-1 and 6G11) (Figure III.1D) and the CD19/BSAP probe (Figure III.1C). The results of these experiments do indeed indicate that, after 24 hr post-transfection, the protein levels of both isoforms are barely detectable (Figure III.1D), which consequently results in a low signal from the bound probe in EMSA (Figure III.1C). In contrast, after 48 hr incubation, the protein levels are readily detectable allowing for more accurate data analysis.

Although Western blot analyses and EMSA were used for an assessment of the protein levels in the transfected samples, it is important to note that the levels of Pax-5a and Pax-5d in the nuclear extracts are not necessarily equivalent to those that drive the expression of the CAT gene measured by CAT assay (Figure III.1E). The measurements of Pax-5a and Pax-5d levels obtained by Western blot for each time-point correspond with CAT activity at some undetermined time in the future, after transcription and translation of CAT protein ("delay factor"). Thus, the main complication with using the "staggered" transfection approach is that it is difficult to correlate the levels of Pax-5 isoforms at a given time-point with the activity of the target promoter at that time. This problem can be solved by determining the currently unknown "delay factor". This will involve: 1) measuring protein levels of Pax-5a and Pax-5d at several points within the

Figure III.1 (see next page): Analyses of expression levels and DNA-binding activities of Pax-5a and Pax-5d proteins in "staggered" transfection samples. NIH 3T3 cell line was transfected with Pax-5a and Pax-5d expression constructs using the "staggered" transfection approach (see text). Control samples were used to determine the relative levels of the expressed proteins after 24 (day 1) and 48 (day 2) hr post-transfection. **(C)** Nuclear extracts (1 μg) from the "staggered" transfection samples were analyzed by EMSA using CD19/BSAP probe (see Chapter II, "Materials and Methods"). ivt, *in vitro* translated Pax-5a (1 μl lysate) and Pax-5d (2 μl lysate). **(D)** Nuclear extracts from the "staggered" transfection samples were analyzed by Western blot using the paired-domain-specific antibody ED-1. The blot was simultaneously probed with anti-TFIID antibody to monitor the amount of total protein in the samples. The positions of Pax-5a proteins and TFIID are indicated by arrows. The band at ~27 kD represents Pax-5_x species (for more detail, see Chapter II and Section 4).


Probe
ivt Pax-5
Pax 5a-d1
Pax 5d-d1
5a/5d-d1
Pax 5a-d2
Pax 5d-d2
5a/5d-d2
5a(d1)+5d(d2)
5a(d1)+5d(d2)
5d(d1)+5a(d2)
5d(d1)+5a(d2)
Pax-5a
Pax-5d
C.
Controls
Day 1
Controls
Day 2
staggered
transfection
ivt Pax-5
Pax 5a-d1
Pax 5d-d1
5a/5d-d1
Pax 5a-d2
Pax 5d-d2
5a/5d-d2
5a(d1)+5d(d2)
5d(d1)+5a(d2)
Pax-5a
TFIID
43.9 kD
Pax-5d
35.4 kD
Pax-5x
D.

staggered transfection time-period to establish the patterns and dynamics of their expression; 2) measuring CAT activity over a time range in the samples transfected with each individual isoform, as well as their combinations; and, finally, 3) determining how long does it take from binding of Pax-5 transcription factors to the promoter of the γ42(3i)AS-CAT DNA construct, to expression of CAT protein. In these investigations, assaying the system for CAT transcripts using RNAse protection assay may provide additional information about the dynamics of CAT transcription/translation.

In conclusion, the "staggered" transfection approach did not provide unequivocal data that would help to resolve the problem of concentration disparity in Pax-5a/Pax-5d binding competition. It is still unclear what conditions and requirements have to be satisfied in order for such competition to occur. Determination and comparison of Pax-5a and Pax-5d protein half-lives may help in better understanding of the interactions between the two isoforms. Based on the results of those experiments, it might possible to predict whether Pax-5a/Pax-5d binding competition is physiologically relevant. Although the "staggered" transfections did not provide conclusive results at this time, the approach can be potentially developed into a useful tool for studies of protein-protein interactions between Pax-5 isoforms and for investigation of their cooperative effect on gene transcription.

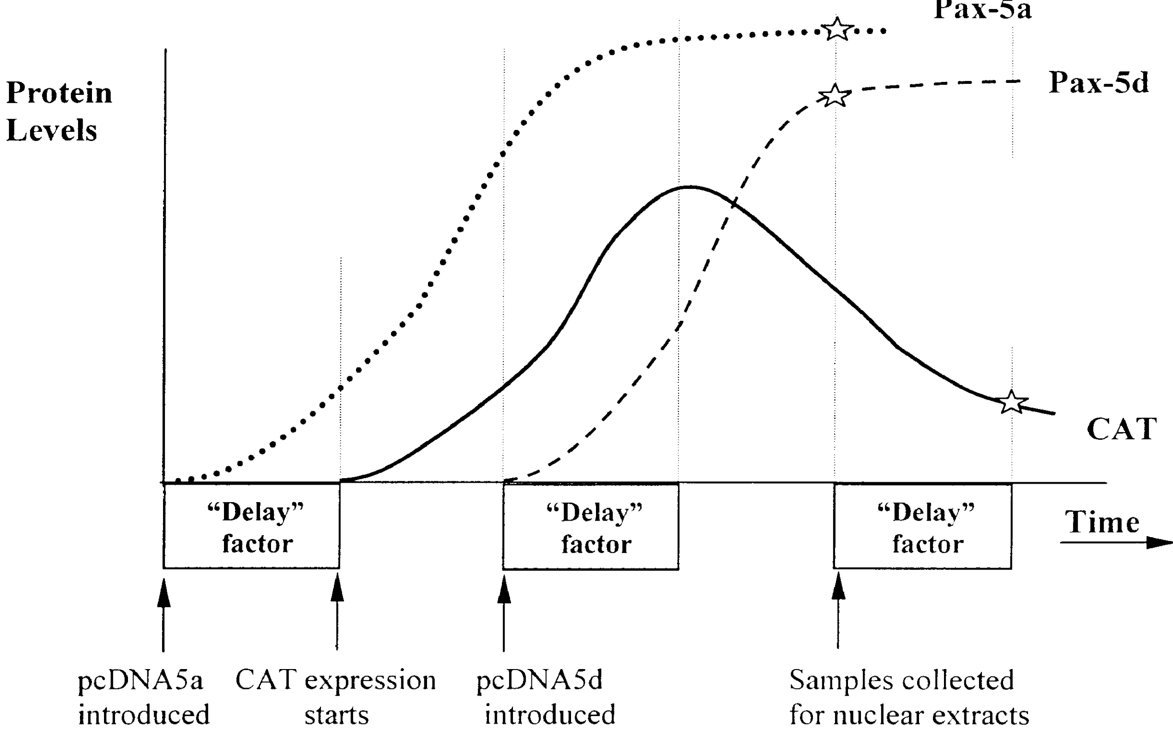


Figure III.1E: Hypothetical diagram of expression patterns of Pax-5a, Pax-5d, and CAT proteins in "staggered" transfection experiments. When pcDNA5a and γ42(3i)AS-CAT constructs are co-transfected into NIH 3T3 cell line, expression of the CAT protein is delayed by an unknown factor which reflects the time necessary for transcription/translation of Pax-5a and its interaction with the CAT promoter. Subsequent introduction of the pcDNA5d construct will inhibit the expression of CAT, however this event will also be delayed. Consequently, the transactivating effect exerted by a combination of Pax-5a and Pax-5d that is present in the system at any particular time-point (such as indicated by a star) can only be assessed by measurements of CAT activity at the later time-point. Therefore, the protein levels of Pax-5a and Pax-5d determined using Western blot and EMSA do not reflect the concentrations of these proteins effective in the regulation of the CAT promoter.

2. LPS-activation of SRBs isolated from aged mice

Aging has been associated with profound changes in the immune system including elevated susceptibility to infections, increased incidence of autoimmune disorders, and decrease in quality of humoral immune response (Ghia *et al.*, 2000). Age-associated decline of humoral immunity results from altered B lymphopoesis, reduced production of B cells, and an impaired antibody response. The immunoglobulins generated by the aging immune system often possess autoreactive properties in addition to their decreased diversity and low affinity (Song *et al.*, 1997; Ghia *et al.*, 2000). While no experimental evidence is available at this time, it would not be surprising if the dramatic changes in the characteristics of the B cell could be correlated with alterations in the expression or activity of transcription factors involved in development and differentiation of B lymphocytes. This has already been demonstrated for senescent T lymphocytes which display age-related decline in the expression of genes encoding such products as c-Fos, c-Jun, c-Myc, and c-Myb (Pieri *et al.*, 1992; Song *et al.*, 1992). Alteration of gene transcription occurring in aged cells inevitably involves changes in the associated regulatory molecules. Consequently, age-related deregulation of hematopoietic transcription factors may have a powerful impact on the functioning and health of the senescent immune system.

Recent investigations in our lab have focused on identification of age-related changes in B cell-specific transcription factor Pax-5. Resting mature B cells of young (2-4 month old) and aged (18-23 month old) mice were used for comparative analyses of Pax-5a and Pax-5d isoforms, their expression patterns and DNA-binding activities (Anspach *et al.*, submitted). It was found that, while the protein levels of the two

isoforms remain constant in both experimental groups, the DNA-binding activity of Pax-5a, but not Pax-5d, is significantly reduced in aged B cells. This decline in DNA-binding has been shown to correspond to changes in the expression of at least two Pax-5 target genes, both of which are associated with Ig genes and negatively regulated by Pax-5 (Anspach *et al.*, submitted). In that work, one of the proposed hypothesis stated that an age-associated decrease in Pax-5a DNA-binding activity may result in deregulated class switching and attenuated proliferative capacity of activated B lymphocytes. Thus, one of the projects presented here aimed to analyze mitogenically stimulated B cells obtained from aged mice, and investigate the effect of LPS treatment on the expression and DNA-binding patterns of Pax-5 proteins.

Splenic small resting B lymphocytes were obtained from six 21 month old BALB/c mice and cultured in LPS-containing medium as described (Chapter II). It is important to note that, while in these experiments the isolated B cells were pooled for convenience, this practice should be avoided in the future due to the potentially high variability in biochemical and physiological characteristics among aged animals. The samples of LPS-treated cells were collected at 0 (Day 0), 48 (Day 2), 96 (Day 4), 144 (Day 6), and 192 (Day 8) hours post-stimulation, and processed for nuclear extracts, which were further used for Western blot analyses and EMSA. Curiously, the experimental cultures contained a high proportion of adherent lymphocytes which had not been seen in the previous studies with SRBs from young mice. This phenomenon could be a result of the increased proportion of cells expressing high levels of leukocyte function-associated antigen-1 (LFA-1). LFA-1 belongs to the integrin family of cell surface molecules which have an important role in cell adhesion and are expressed at

moderate levels on all subsets of leukocytes (Hogg and Berlin, 1995). Typically, effector and memory lymphocytes are characterized by elevated levels of LFA-1 expression which amplifies their capacity for migration into inflamed tissues (Hogg and Berlin, 1995). Age-associated changes in lymphocyte adhesion have been reported for the senescent SAM-P/8 and SAM-R/1 mice, and are attributed to the increase in the relative number of memory cells expressing high levels of LFA-1 (Powers *et al.*, 1992).

Western blot analysis of LPS-activated aged samples revealed no significant changes in either Pax-5a or Pax-5_x protein levels (Figure III.2A). This is in contrast to activated young SRBs in which variable levels of the two protein species were observed at different time-points of LPS stimulation, with the Pax-5a/Pax-5_x ratio decreasing gradually in activated B lymphocytes (see Chapter II, Figure II.5A-D). The Pax-5a.2 band was not clearly defined in resting aged B lymphocytes, although, similarly to young SRBs, these samples showed an extensive Pax-5 degradation pattern which was absent from the nuclear extracts of activated lymphocytes (Figure III.2A, ED-1/TFIID panel). In contrast to young SRBs, the Pax-5d band was not detectable at any time-point (Figure III.2A, 6G11 panel).

Next, nuclear extracts from LPS-activated aged SRBs were analyzed by electrophoretic mobility shift assay using a CD19/BSAP probe (Chapter II). Prior to the assay, nuclear extracts were pre-incubated without the probe with Pax-5/C-20 antibody (Table I) to distinguish between Pax-5a.1 and Pax-5a.2 species identified in our earlier studies (Anspach *et al.*, submitted; Chapter II). In agreement with data on young mice (Anspach *et al.*, submitted), the nuclear extracts of aged resting B cells also had no detectable Pax-5a bands, indicating a dramatic decrease in the amount of Pax-5a protein

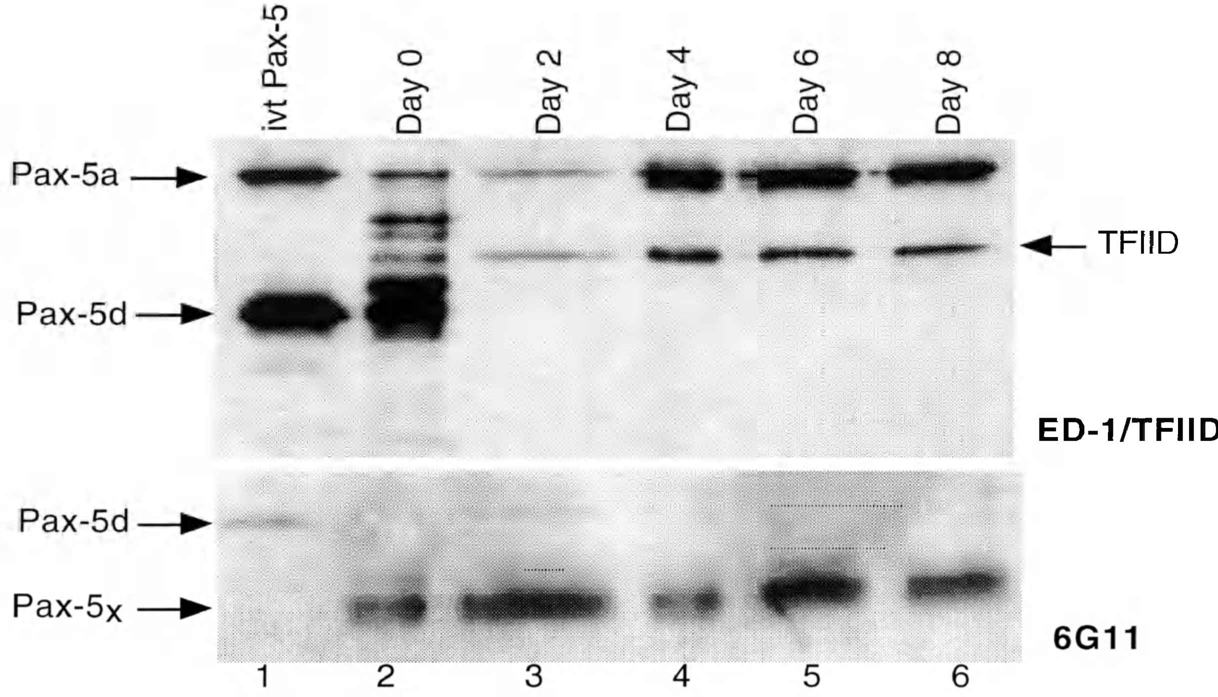


Figure III.2A: Western blot of LPS-activated SRBs from aged mice. Nuclear extracts from LPS-activated aged (from 21 month old mice) B lymphocytes were separated on SDS-PAGE, and filters were probed with anti-paired domain serum ED-1 (top panel) and the novel sequence-specific antibody 6G11 (bottom panel). Anti-TFIID antibody was used simultaneously with ED-1 to monitor the total amount of protein in the samples. The positions of Pax-5 isoforms and TFIID are indicated by arrows. Duration of LPS treatment in days is indicated above the lanes. "Day 2" sample (lane 3) was partially lost due to a defect in the gel and may not represent a true result for that time point.

bound to the CD19/BSAP probe (Figure III.2B, lane 3-6). Consequently, the DNA-binding activity of the Pax-5d isoform could be enhanced through availability of more DNA binding sites. The presence of two distinct bands running close to Pax-5d position provides evidence for this model. However, due to the lack of a suitable Pax-5d-specific antibody, it cannot be excluded that these bands represent paired domain-containing Pax-5a degradation products which retained their DNA-binding ability. Interestingly, these bands disappeared after LPS treatment of B cells, similarly to the effect observed for young activated SRBs (Figure II.7A, Chapter II). Furthermore, the Pax-5a band emerged on day 4 of LPS-activation (Figure III.2B, lane 7, 9, 11) and became progressively stronger by day 8. Based on its interaction with the C-terminus-specific antibody C-20, the observed band has been previously hypothesized to represent Pax-5a.1 species which contain an intact C-terminal regulatory module, consisting of the transactivation domain and the adjacent repressor sequence. Activation-induced stabilization/modification of the Pax-5a C-terminal region has been reported in the experiments with SRBs from young mice (Chapter II), which suggests that Pax-5a.1 species have an important function during activation and differentiation of B lymphocytes in response to mitogenic stimulation.

In summary, activation experiments with SRBs isolated from aged mice have demonstrated two important differences from young mice. Aged SRBs have no detectable Pax-5a.2 band, and the ratio of Pax-5a to Pax-5_x proteins remains constant in all activated aged samples, in contrast to its dramatic decrease observed during activation of SRBs from young mice. Nevertheless, the samples from both age groups show activation-associated stabilization of the Pax-5a isoform, with the ensuing accumulation

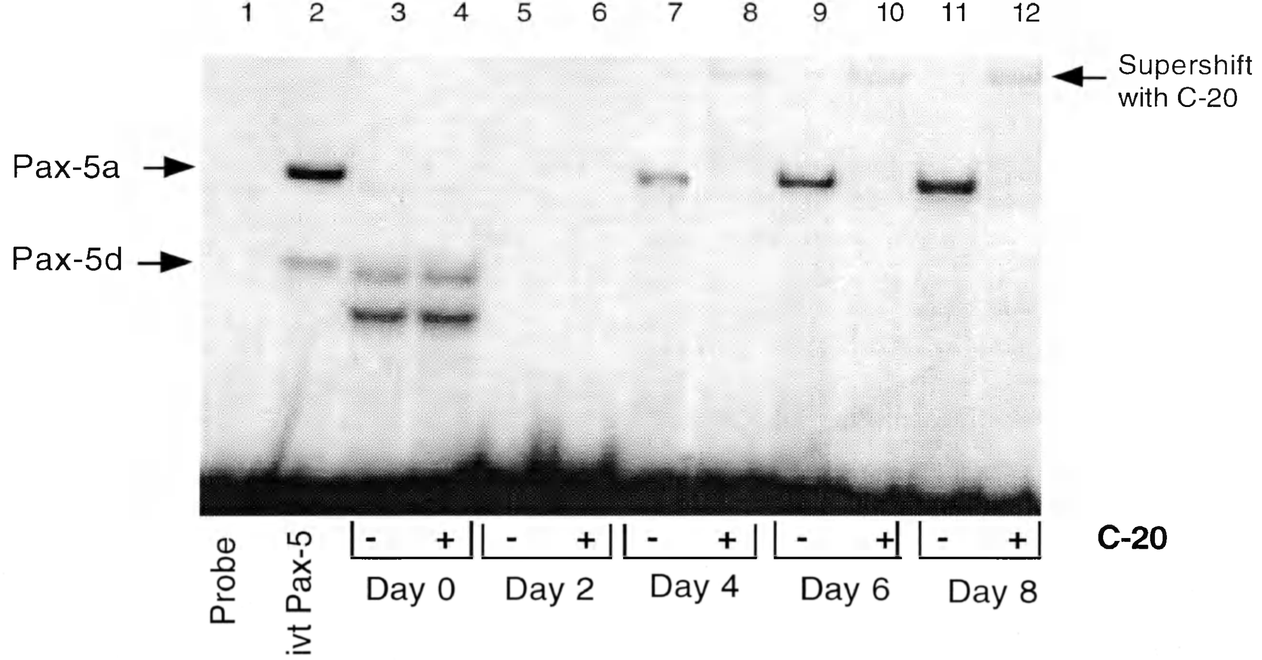


Figure III.2B: EMSA of LPS-activated SRBs from aged mice. Nuclear extracts (1μg) from LPS-activated aged SRBs were analyzed by EMSA using CD19/BSAP probe. Some samples (lanes 4, 6, 8, 10, 12) were pre-incubated without the probe for 15 min at room temperature with Pax-5a C-terminus-specific antibody, C-20. The resulting supershift complex between the full length Pax-5a (see text) and C-20 is indicated on the right. Duration of LPS treatment in days is shown at the bottom. Positions of Pax-5a and Pax-5d complexes are indicated on the left. Lane 1: CD19/BSAP probe alone; Lane 2: *in vitro* translated Pax-5a (1μl lysate) and Pax-5d (2μl lysate).

of the full-length Pax-5a.1 protein containing the repressor sequence. The experimental data have also confirmed age-related reduction of Pax-5a DNA-binding activity in senescent non-stimulated SRBs, as well as the loss of Pax-5d DNA-binding in the activated samples, previously determined by aging and activation studies conducted in our lab. As for SRBs from young animals, the physiological significance of the observed events has yet to be determined. However, it is clear that, while fundamentally similar, the activation processes in young and aged lymphocytes display age-specific differences that trigger distinct patterns of Pax-5a and Pax-5d protein expression and DNA-binding. These differences may contribute, at least partially, to the age-related decline of humoral response to mitogenic challenge and affect the quality of the antibodies produced by aged B lymphocytes.

3. WEHI-231 activation

Western blot analysis of Pax-5 proteins in B cell lines representing various stages of B cell differentiation revealed that the patterns of the alternatively spliced isoforms change in a stage-specific manner (see Chapter II). Furthermore, the isoform levels and the respective protein ratios found in the immature B (WEHI-231) and early mature B (B17.10) cell lines were most similar to those seen in partially activated normal B cells, whereas the cell lines representing later stages were comparable to activated B lymphocytes (Figure II.6A, Chapter II). Based on this observation, we sought to investigate whether activation of the immature B cell line WEHI-231 *in vivo* can induce processes similar to those occurring during activation of normal B lymphocytes. More

specifically, this project aimed to determine whether activation of the WEHI-231 cell line triggers similar changes in Pax-5 isoforms, their protein levels, and DNA binding patterns.

WEHI-231 is a murine B cell line with the IgM^{+}/IgD^{+} surface phenotype (Gottschalk and Quintans, 1995). Although this phenotype is typical for mature B cells, WEHI-231 is commonly used to study immature B lymphocytes because it shares several characteristics of the immature B cell stage, such as the ability to undergo apoptosis through pathways similar to those used in negative selection (Gottschalk and Quintans, 1995). Moreover, this cell line is frequently exploited for B cell activation studies, as WEHI-231 cells display unique sensitivity to antigenic stimulation. Depending on the source of a subline and the nature of an antigen, WEHI-231 cells may respond to stimulation by either undergoing growth arrest and apoptosis, or by proliferation and further differentiation into antibody secreting cells (Gottschalk and Quintans, 1995; Aoki *et al.*, 1995). This responsiveness was utilized for activation studies in which the WEHI-231 cell line was stimulated by either IgM cross-linking or by culturing in the presence of bacterial lipopolysaccharide (LPS). Typically, $2\text{-}5\times10^{7}$ cells were either treated with 25 mM IgM $F(ab')_2$ fragment for 3 min (Aoki *et al.*, 1995), or activated by LPS as described (Chapter II). The cells were incubated for specified periods of time and then processed for nuclear extracts according to standard methods (Chapter II).

The results of Western blot analyses of activated WEHI-231 samples showed that the protein level of the Pax-5a isoform remains unchanged regardless of the type of antigen used in the experiment (Figure III.3A; ED-1/TFIID panel). The levels of Pax-5d were relatively low in all samples and did not change significantly during 48 hr of IgM

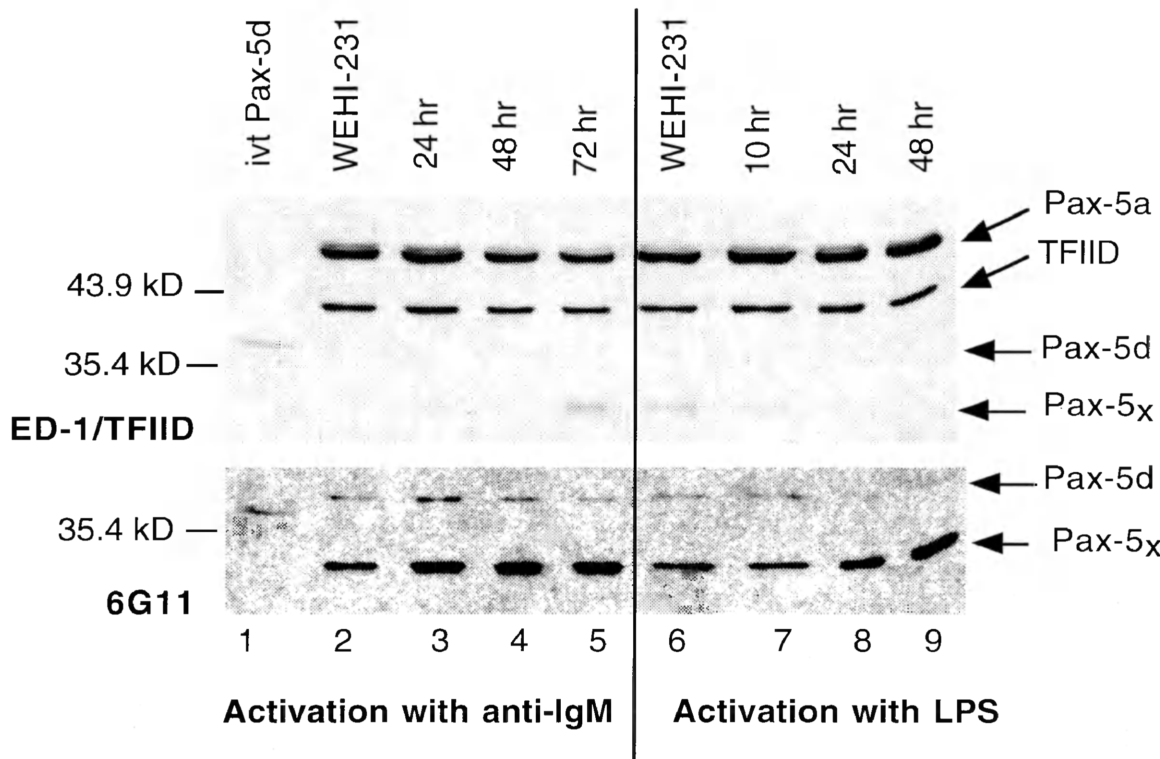


Figure III.3A: Western blot of activated WEHI-231 samples. WEHI-231 cells were activated by either IgM cross-linking or treatment with LPS. Nuclear extracts were collected at times indicated above the lanes, and patterns of Pax-5 proteins were analyzed using antibodies specific for Pax-5 functional domains. Paired domain-specific ED-1 can recognize all Pax-5 isoforms; the novel sequence-specific 6G11 detects only Pax-5d and Pax-5e. Anti-TFIID antibody was used to monitor the amount of total protein in the samples.

cross-linking and in the first 10 hr of LPS-activation (Figure III.3A; 6G11 panel). Eventually, the intensity of Pax-5d band decreased in both anti-IgM- and LPS-treated samples (at 72 hr and 24 hr, respectively), although the reduction was not as noticeable for the cross-linked samples (Figure III.3A, 6G11 panel, lanes 5, 8, 9).

Similarly to splenic B lymphocytes, WEHI-231 cells contain the unidentified Pax-5_x species migrating at ~27 kD (compare to Figure II.5C, Chapter II). The intensity of the Pax-5_x band increased when WEHI-231 cells were activated by either IgM cross-linking (at 72 hr) or LPS stimulation (at 24 hr) (Figure III.3A, 6G11 panel). The observed change, though not very dramatic, is similar to the pattern seen in LPS-activated SRBs (compare to Figure II.5C, Chapter II), which suggests that this might be a widely occurring phenomenon having a functional significance during B lymphocyte activation.

Analysis of WEHI-231 nuclear extracts by EMSA revealed the presence of three distinct Pax-5 complexes in non-activated cells: a slower-migrating Pax-5a band (5a.1) and two faster-migrating complexes running close to Pax-5d position (Figure III.3B, lane 3). Since, according to Western blot data, the protein levels of Pax-5d were very low in the experimental samples, the lower bands are likely to represent either Pax-5a degradation products or other Pax-5-specific complexes (rather than the Pax-5d isoform). In agreement with the Western blot data, analyses of nuclear extracts from WEHI-231 activation samples by EMSA showed no significant change in Pax-5a binding pattern for resting and activated WEHI-231 cells (Figure III.3B). The observed Pax-5a band corresponded to the previously identified Pax-5a.1 species, which is able to interact with the C-terminal-specific antibody, C-20 (supershift with C-20 is not shown) (Anspach *et al.*, submitted; Chapter II).

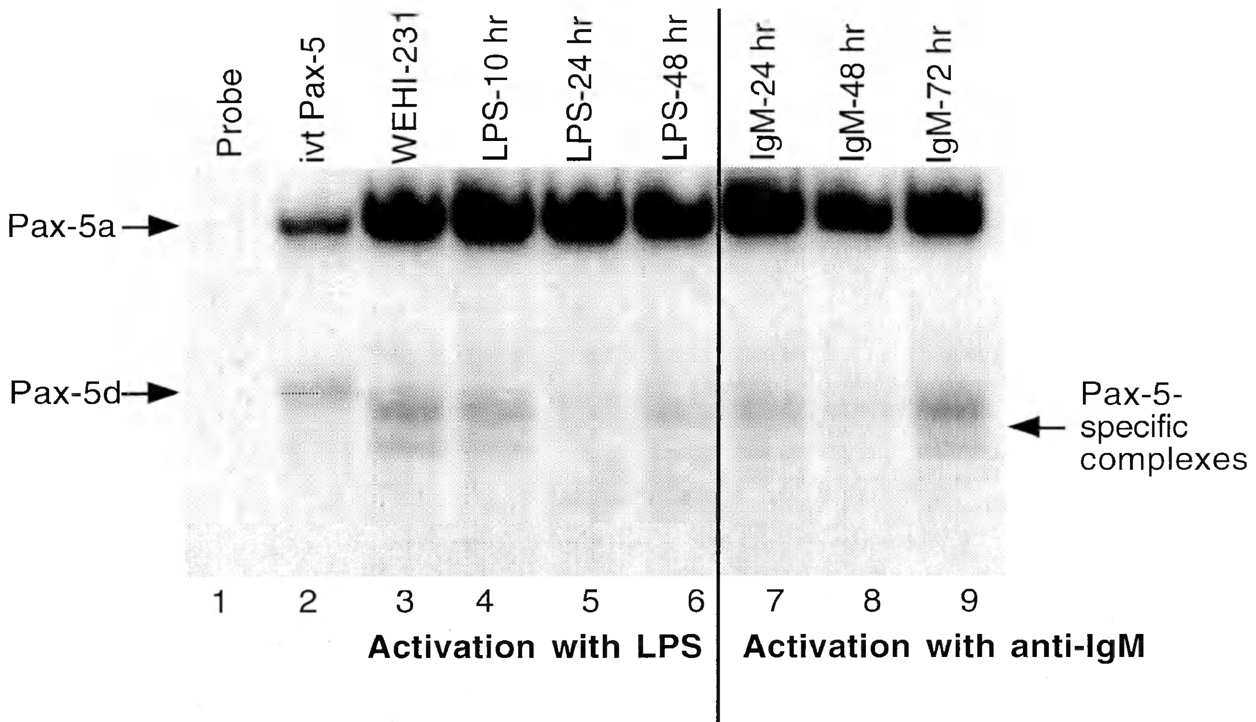


Figure III.3B: EMSA of activated WEHI-231 samples. Cells from WEHI-231 B cell line were stimulated with either LPS or $F(ab')_2$ fragment of anti-IgM antibody. The duration of treatments is indicated above lanes. Nuclear extracts from activated WEHI-231 cells (1µg) were analyzed by EMSA using CD19/BSAP probe. Positions of Pax-5a and Pax-5d complexes are indicated on the left. The complexes observed in the Pax-5d position represent various Pax-5 species. Lane 1: CD19/BSAP probe alone; lane 2: *in vitro* translated Pax-5a (1µl lysate) and Pax-5d (2µl lysate).

In conclusion, activation of WEHI-231 cells by either IgM cross-linking or LPS treatment had no effect on either expression or DNA-binding patterns of the Pax-5a isoform. This is not surprising as Pax-5a (BSAP) has been implicated in cell proliferation (see Chapter I, Section 2.2), a function which is unlikely to be altered when a mitogen is added to a continuously dividing B cell line. In contrast, activation-induced changes were observed in the protein expression of the Pax-5_x species, which suggested a functional role for this protein (or complex of proteins) in activated B lymphocytes. Further characterization and comparative analyses of WEHI-231 activation system may provide a reasonable alternative to SRBs when used for studies of B cell activation.

4. Characterization of Pax-5_x species

A novel Pax-5_x species was detected in nuclear extracts of B cell lines and splenic B cells analyzed by Western blot using the novel sequence-specific antibody 6G11 (see Figures II.5C, II.6A, and III.3A). Since the band was poorly detectable with ED-1 anti-paired domain serum and no Pax-5_x-specific complexes were observed in nuclear extracts analyzed by EMSA (Figures II.5A, II.7A, and III.3A), it is likely that the novel Pax-5 protein possesses an incomplete DNA-binding domain. It was, therefore, hypothesized that Pax-5_x band represents Pax-5e, the novel-sequence-containing isoform with the truncated paired domain (Figure I.4). However, the biggest challenge of this hypothesis was to explain the discrepancy between the size of the newly detected Pax-5_x band (27 kD) and the estimated size of Pax-5e isoform (19 kD).

To confirm that Pax-5_x is a Pax-5e-specific band, Pax-5e expression construct was transiently transfected into a non-lymphoid cell line, NIH 3T3, as described (see

"Materials and Methods", Chapter II). Nuclear extracts from the transfected cells were analyzed by Western blot using Pax-5-specific antibodies, ED-1 and 6G11 (Table I). The transfected samples were compared to nuclear extracts from the immature B cell line WEHI-231 and from resting normal B lymphocytes (SRBs).

The novel sequence-specific antibody 6G11 detected a single band at ~27 kD present in samples transfected with the Pax-5e expression construct (Figure III.4A; 6G11 panel). This band corresponded with the Pax-5_x species that had been previously observed in nuclear extracts of WEHI-231 cells and SRBs (Figure III.4A, lanes 1-3). The novel Pax-5_x protein present in the transfected NIH 3T3 samples could also interact with the paired domain-specific antibody ED-1 (Figure III.4A, ED-1 panel). Interestingly, the intensity of Pax-5_x bands detected with ED-1 varied depending on the source of nuclear extracts (Pax-5e-transfected samples, WEHI-231 cells or SRBs). This could be explained by the differences in relative levels of Pax-5_x in the assayed samples. Thus, based on the results of this experiment, Pax-5_x can be characterized as a Pax-5e-like species.

In addition to the Pax-5_x band, nuclear extracts from Pax-5e-transfected samples contained two other protein species (Figure III.4A, ED-1 panel, lane 1). A fast migrating band at ~21 kD is likely to represent Pax-5e species that have been neither modified nor complexed with other proteins. The nature of the second band (at ~45 kD) is unclear; however, it is clearly Pax-5e-specific and may represent either a Pax-5e homodimer or extensively modified Pax-5e protein molecules. Curiously, a similar, high-running band was also observed in the samples of NIH 3T3 cells transfected with Pax-5d expression construct (Figure III.4A, ED-1 panel, lane 7). Both alternatively spliced variants, Pax-5e and Pax-5d, possess the novel sequence containing two cysteine residues. Although

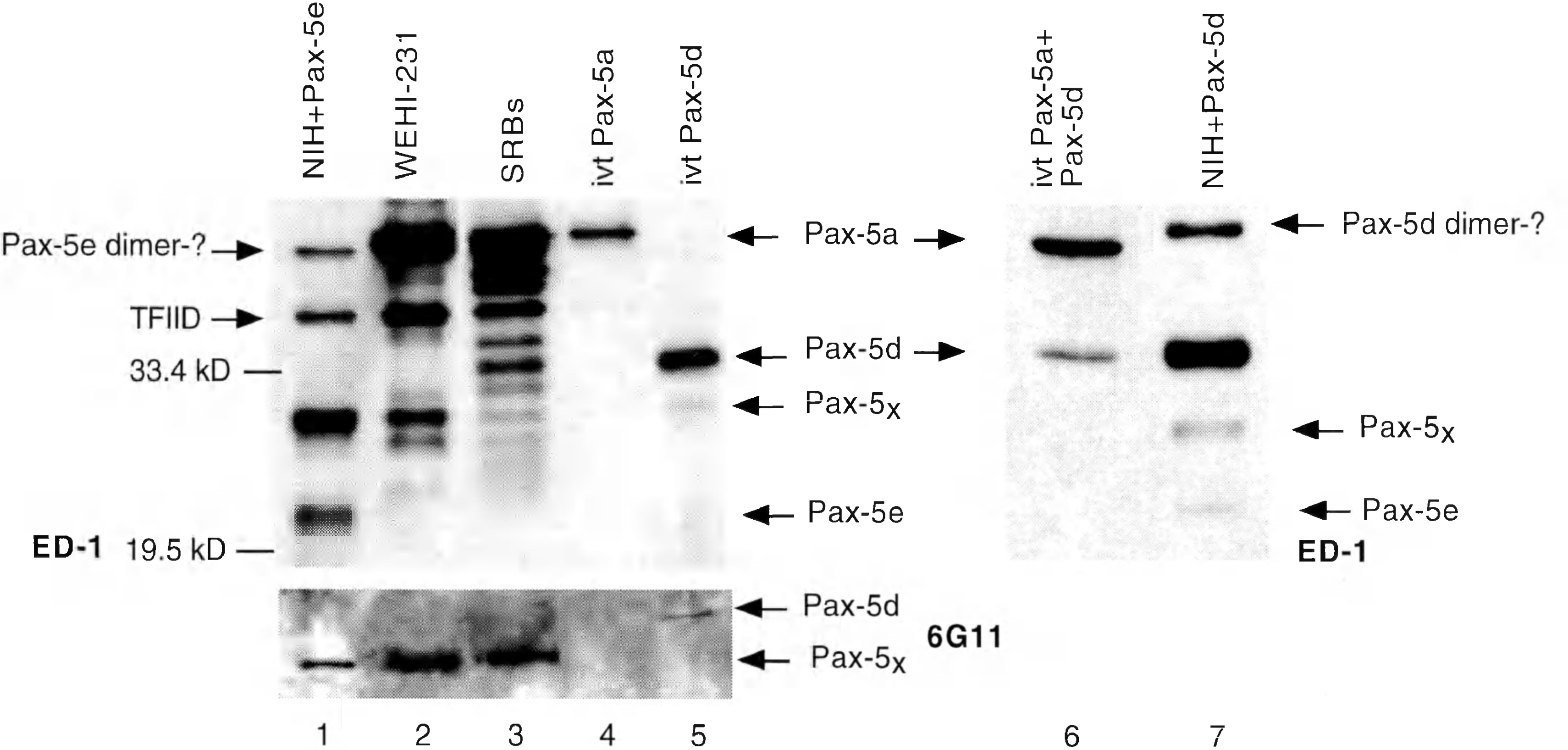


Figure III.4: Identification of Pax-5e-specific bands. NIH 3T3 cells were transfected the Pax-5d and Pax-5e expression constructs. Nuclear extracts from the transfected samples were analyzed by Western blot using the anti-paired domain serum ED-1 and the novel-sequence-specific antibody 6G11 (not shown for NIH+Pax-5d sample). The banding patterns were compared to those observed in WEHI-231 and SRB nuclear extracts. The blot on the left was probed with anti-TFIID antibody to monitor the amount of total protein in the samples. Pax-5-specific bands are indicated.

dimerization of either isoform has not been reported, it would not be surprising if these proteins could form intermolecular disulfide bonds (see Chapter I, Section ***4.1***). If the 45 kD band indeed represents a Pax-5e homodimer, it is likely that the bonds that form between two Pax-5e monomers are highly reactive, and tend to reform during electrophoresis. As a result, a fraction of Pax-5e molecules remains dimerized despite the denaturing conditions of SDS-PAGE. If the same logic is applied to Pax-5_x species, detection of the 27 kD band could be explained by the presence of a heteromeric complex of Pax-5e with some ubiquitous factor. In this case, a simple modification of the electrophoresis conditions (such as pre-assay treatments of the samples with certain reducing or denaturing agents) may help to reduce the number of observed complexes and promote the formation of a "true", 19-21 kD, Pax-5e band (see Discussion for more details). Alternatively, the bands that run above 21 kD may represent modified forms of Pax-5e protein. However, since the exact function and properties of this isoform remain unknown at this time, it is difficult to predict accurately what types of post-translational modifications might affect the Pax-5e molecule.

Analysis of nuclear extracts from NIH 3T3 cells transfected with Pax-5d expression construct provided curious clues pointing to a possible origin of Pax-5_x species in activated normal B cells. Due to the presence of two alternative start sites, the *Pax-5d* transcript can generate either Pax-5d or Pax-5e protein, with Pax-5d being a predominant translation product. This phenomenon is illustrated by the presence of Pax-5e band in [NIH+Pax-5d] samples analyzed by Western blot using ED-1 antibody (Figure III.4A; ED-1 panel, lane 7). Intriguingly, [NIH+Pax-5d] nuclear extracts also contained the Pax-5_x band. According to the results described in Chapter II, the protein levels of

the novel Pax-5_x species increase dramatically during B cell activation, concomitantly with a decrease in the levels of Pax-5d isoform. The absence of activation-induced changes at the RNA levels indicated that any change in the expression patterns of Pax-5 proteins occurs at either translational or post-translational level (see Discussion). Thus, it is conceivable that activation of B lymphocytes may trigger the use of an alternative (distal) start codon on the *Pax-5d* transcripts, giving a rise to Pax-5_x species. This, however, requires further experimental prove.

Chapter IV

Discussion

In the studies presented in this thesis, the functions of alternative Pax-5 isoforms were explored using *in vivo* and *in vitro* assays that measured the transcriptional and DNA-binding activities of the isoforms and determined their protein expression patterns in resting and activated B cells. More specifically, the described project focused on analyses of transactivating function of the Pax-5d isoform in relation to the activity of Pax-5a, determination of relative ratios of Pax-5 proteins in resting and LPS-activated B lymphocytes, and investigations of activation- and differentiation-stage-specific changes in the expression patterns and DNA-binding activities of Pax-5 isoforms occurring in splenic B cells from young and aged mice and in various B cell lines.

The existence of alternatively spliced variants with different DNA-binding activities and variable functional domains suggests that Pax-5 isoforms may have different, perhaps opposing, transactivating functions and play distinct roles during development and differentiation of B cells. Previous studies of the related *Pax-8* gene have shown that it generates at least six developmentally regulated isoforms that display differential transactivation potentials (Kozmik *et al.*, 1993). Other investigations have revealed that alternative splicing affects the paired domains of Pax-8, Pax-6, Pax-3, and Pax-7 resulting in generation of isoforms with various DNA-binding activities and specificities (Kozmik *et al.*, 1997; Vogan *et al.*, 1996). Thus, the isoforms of Pax-5 may also have different transactivation properties and variable regulatory capacities, including

the ability to interact with and sequester other factors and compete for sites on target genes.

4.1 Pax-5d is a repressor of Pax-5a activity

The results of the functional studies demonstrated that, in contrast to Pax-5a, Pax-5d acts as an inhibitor of transcription. In addition, Pax-5d not only inhibits the activity of endogenous Pax-8 in the kidney cell line COS-1, but can also repress activity of Pax-5a when the two isoforms are expressed simultaneously in NIH 3T3 cells. Thus, it can be concluded that Pax-5a and Pax-5d have opposite regulatory functions *in vivo*. Moreover, in certain circumstances, Pax-5d may function as a repressor that controls Pax-5a activity either through DNA-binding competition or by sequestration of accessory factors required for regulation of Pax-5 target genes.

Pax-5a and Pax-5d proteins share two functional domains including an intact paired domain and the octamer sequence; however, they differ in the composition of their C-termini (see Chapter I, Figure I.4). Pax-5d lacks the homeodomain homology box and the entire C-terminal regulatory module consisting of the transactivation domain and repressor sequence. In place of these regions, Pax-5d possesses a novel sequence of unknown function (Zwollo *et al.*, 1997). The observed repressor function of Pax-5d is likely to be due to the absence of the transactivating domain; however, this needs to be examined further. The opposite transactivating potential of Pax-5a and Pax-5d coupled with their similar DNA-binding activities suggests a possible mechanism for regulation of Pax-5a function. Since the two isoforms appear to compete for binding, the presence of excess Pax-5d in a system may reduce the Pax-5a binding to its target sites on DNA.

Consequently, the Pax-5a/Pax-5d ratio may be a key determinant of Pax-5 function in regulation of target genes.

Another consideration of Pax-5d function comes from the presence of the octamer sequence shared by all Pax-5 proteins. This region has been shown to bind the co-repressors from the Groucho family, and that interaction has been hypothesized to inhibit the transcriptional activity of Pax-5a (Eberhard *et al.*, 2000). It remains to be determined whether such contact can occur between the Groucho corepressors and other Pax-5 isoforms, including Pax-5d. However, it is conceivable that, at high enough concentrations, the alternative variants of the *Pax*-5 gene could increase Pax-5a activity by "absorbing" the Groucho co-repressors and, hence, preventing their interaction with Pax-5a. Alternatively, interactions between Pax-5d and various accessory proteins may inhibit Pax-5a-regulated gene transcription through sequestration of factors required for efficient transcription of target genes. Therefore, we cannot exclude the possibility that the observed inhibition of Pax-5a activity stems from Pax-5a/Pax-5d competition for binding to accessory partner-proteins rather than to Pax-5-specific DNA-sequences.

4.2 Pax-5a and Pax-5d differ in protein stability

The data from the competition experiments indicate that inhibition of Pax-5a activity can only be detected when the Pax-5a/Pax-5d ratio of the transfected effector DNA is very low (less then 1/10). Difference in protein stabilities has been hypothesized to be one of the possible reasons for the disparity in concentrations of the two isoforms. Supporting, albeit indirect, evidence for this prediction is provided by the analysis of

Pax-5a and Pax-5d protein levels in nuclear extracts from NIH 3T3 cells transfected with either Pax-5a or Pax-5d expression construct. The results of Western blot analysis revealed that, for equal amounts of the transfected plasmid DNA, nuclear extracts from the transfected samples contained approximately three times less Pax-5d than Pax-5a protein. However, this observation does not exclude the possibility of differences between the two isoforms at the level of transcription and/or translation (e.g., transcriptional/translational efficiency, rate of protein synthesis, etc.). Therefore, only direct measurements of the proteins' half-lives can accurately determine whether the issue of protein stability has relevance for activity of Pax-5 isoforms.

The amino acid sequences of many rapidly degraded eukaryotic proteins contain one or more regions rich in proline (P), glutamic acid (E), serine (S), and threonine (T) (Rogers *et al.*, 1986). These regions, named "PEST" sequences, often represent sites of constitutive and signal-induced phosphorylation, which can affect the intrinsic stability of a protein. For example, activation-induced phosphorylation of serine and threonine residues within the PEST domain of IκB (the NF-κB inhibitor) has been shown to target this protein for degradation (Lin *et al.*, 1996). An extensive PEST sequence has also been found in the p53 protein (Rogers *et al.*, 1986). Phosphorylation of specific residues regulates a number of the p53 properties, from the protein's stability and conformation states to its activity and interaction with other proteins (Meek, 1998). While no research has been done so far to demonstrate the correlation between regulatory phosphorylation of p53 and the existence of the PEST domain, the link has been suggested by the comparative analysis of p53 with other PEST-containing proteins (Rogers *et al.*, 1986).

Curiously, PEST sequences are strikingly homologous to the sites of *O*-GlcNAc glycosylation, which is one of the most ubiquitous and abundant post-translational modifications found in nuclear proteins (see Chapter I, Section ***3.3***; Haltiwanger *et al.*, 1997). It has been postulated that the major regulatory function of *O*-glycosylation is to compete with phophorylation sites on proteins, thus adding another level of control to signal transduction cascades inside the cells (Haltiwanger *et al.*, 1997; Comer and Hart, 2000). It is possible that the reciprocal relation between protein phosphorylation and *O*-glycosylation has a role in regulation of protein stability. Supporting this notion is a study in which the total levels of *O*-glycosylation have been correlated with the stability of the transcription factor Sp1 against proteasome degradation (Han and Kudlow, 1997). Together with earlier reports, these investigations have demonstrated that hypoglycosylated Sp1 is more susceptible to proteolytic degradation, whereas extensive *O*-glycosylation of this protein results not only in its stabilization but also in an increased transcriptional activity (Jackson and Tjian, 1988; Han and Kudlow, 1997).

Based on sequence analysis, isoform Pax-5a (as well as Pax-5b) contains three PEST consensus sequences (S/T-X-X-E/D) in a region immediately C-terminal from the homeobox homology domain (aa 251-306). The presence of the PEST domains may indicate susceptibility of Pax-5a protein to degradation. Isoform Pax-5d (and Pax-5e) has no such regions due to alternative splicing; however, its stability may be regulated through the novel sequence by mechanisms that have yet to be determined. For example, the novel sequence of Pax-5d contains three lysine residues. This amino acid is often modified by ubiquitination in proteins that are destined for degradation (Pahl and Baeuerle, 1996). Alternatively, PEST sequences may function as sites for *O*-

glycosylation, in which case Pax-5a could be marked by a longer half-life. In contrast, the lack of PEST domains in Pax-5d would render this protein more susceptible to proteolytic degradation. Examination of the role of the PEST sequences in Pax-5 protein stability may help to better understand pathways that regulate the processes occurring during B cell activation.

4.3 B cell activation induces stabilization of Pax-5a isoform

Differentiation of mature B lymphocytes into plasma cells is a complex, antigen-dependent process that consists of multiple steps involving B cell activation, proliferation, and expression of stage-specific sets of genes. In this process, the activation signals initiate a variety of pathways which lead to immunoglobulin isotype switching and antibody secretion. Previously, Pax-5 (BSAP) has been shown to control the switch recombination events through regulation of Ig gene rearrangement (reviewed in Hagman, 2000). Pax-5 also plays an important role in B cell proliferation which is necessary for initiation of Ig isotype class switching, somatic mutations, and formation of germinal centers (Wakatsuki *et al.*, 1994; Neurath *et al.*, 1995). However, while Pax-5 is an integral part of an efficient immune response, terminal differentiation of B lymphocytes into plasma cells and Ig secretion require downregulation of Pax-5 expression (Usui *et al.*, 1997; Stüber *et al.*, 1995).

The mechanism which downregulates the expression and activity of Pax-5 in activated B lymphocytes is not clearly understood. However, our experimental data clearly show that initial control occurs at the protein level. The ratio of Pax-5a to Pax-5d mRNA remains unchanged during LPS stimulation of B cells, indicating that neither rate

of RNA synthesis nor RNA stability is affected by the activation signals and onset of differentiation. This finding confirms previous observations by Anderson *et al.* (1996) who reported that BSAP (Pax-5a) mRNA levels stay constant in resting and LPS-activated primary B lymphocytes.

In contrast, a number of changes were detected at the protein levels of Pax-5 isoforms, when nuclear extracts from resting and activated B lymphocytes were analyzed by Western blot and EMSA. In the previous studies, two Pax-5a species have been detected in resting mature B cells, Pax-5a.1 and Pax-5a.2 (Anspach *et al.*, submitted). Slightly different migration rates of these species under non-denaturing conditions (EMSA) suggested that the representative proteins are likely to possess different conformations or structures. In the study reported here, evidence is provided that Pax-5a.1 and Pax-5a.2 differ in the composition of the C-terminus, based on their interaction with Pax-5 N- and C-terminus-specific antibodies. The most likely explanation of these data is that the Pax-5a.2 protein lacks the repressor sequence, whereas Pax-5a.1 species represent a full length Pax-5a protein with the intact C-terminal regulatory module. Significantly, during B cell activation, the relative ratio between the two species shifts toward Pax-5a.1, reflecting a modification at the C-terminus, followed by an overall stabilization of the Pax-5a isoform in activated samples. Interestingly, the stabilization of Pax-5a and formation of Pax-5a.1 species during B cell activation coincides with an increase in the relative protein levels of Pax-5a isoform in nuclear extracts of activated samples. That finding is in agreement with the results of a previous study in which an increase in BSAP/Pax-5a levels has been correlated with the time of active proliferation of B cells in response to LPS-activation (Wakatsuki *et al.*, 1994). Perhaps, this explains

why the patterns of Pax-5a isoform do not change during activation of WEHI-231 B cell line: cells that divide continuously presumably should have only those Pax-5a species that support B cell proliferation, namely Pax-5a.1. This is also supported by the results of an independent experiment in which B cell lines, representing different stages of B cell differentiation, were analyzed by EMSA (result not shown). That data revealed that nuclear extracts from all assayed samples possess Pax-5a.1 only, which is in agreement with the suggested role of this species in B cell proliferation.

The three most 3'-terminal exons of *Pax-5a* has been shown to be unaffected by alternative splicing, thus eliminating a possibility that Pax-5a.2 band represents a novel Pax-5 isoform. Alternatively, 5a.2 may represent either a dephosphorylated form or a degradation product of 5a.1. Site-specific phosphorylation is a common trigger of proteolytic degradation that plays an important role in regulation of NF-κB, p53, and a wide variety of nuclear and cytoplasmic proteins (Hunter and Karin, 1992). In many cases, phosphorylation of specific residues allows recognition of the modified protein by the E3 ubiquitin-protein ligase which catalyses the binding between the ubiquitin and a protein substrate (Pahl and Baeuerle, 1996). This mechanism has not unequivocally been ruled out as a factor in generation of Pax-5a.1 and Pax-5a.2 molecular species, although the phosphatase treatment of nuclear extracts from resting B cells had no effect on either protein expression or DNA-binding patterns of the two Pax-5a species (data not shown). Another alternative is that Pax-5a.2 species is a product of degradation of the full-length Pax-5a protein (Pax-5a.1). It is possible that, in resting but not activated/proliferating B lymphocytes, specific proteases remove the C-terminal sequence of Pax-5a.1 and reduce stability of this protein, causing multiple degradation bands. The degradation products

could include the Pax-5a.2 species which contain all of Pax-5a functional domains, except the repressor sequence. During B lymphocyte activation, the alterations in the cell's environment may either specifically inactivate these proteases or, alternatively, promote certain protein modifications that stabilize/protect Pax-5a.1 from C-terminal degradation. The exact mechanism of these modifications is not understood at this time, and its elucidation may require determination of Pax-5a half-life in resting and activated B cells, which is the focus of our current research.

In support of a functional role for C-terminus-initiated degradation is the presence of a potent repressor domain in this region (see Chapter I, Figure I.4). In the full-length Pax-5a protein, the C-terminal transactivation domain is flanked by a 33-amino acids repression sequence which has been shown to inhibit Pax-5-dependent transcription of a minimal promoter in transient transfection experiments (Dörfler and Busslinger, 1996). Specific proteolysis of the extreme carboxy-terminal repression module may produce a highly active Pax-5a species with a potent transactivation function. In contrast, stabilization of the protein, followed by subsequent retention of the repressor sequence, may yield a non-truncated Pax-5a version with attenuated capacity for activation of transcription. It is possible that the functions of the full length Pax-5a *in vivo* involve mostly the control of cell proliferation and the maintenance of only a small subset of B cell-specific genes; however, this needs further investigation.

Another interesting possibility is based on the recent study which demonstrated that the paired domain polypeptide was in some cases sufficient for reconstitution of endogenous Pax-5 target gene expression in Pax-5-deficient pre-BI cells (Nutt *et al.*, 1998). I propose that, in resting B cells, proteolysis at the C-terminus of Pax-5a does not

only produce species with a higher transactivating potential (such as Pax-5a.2), but also generates an array of truncated polypeptides that have no transactivation domain but contain an intact paired domain. These shorter versions of Pax-5a protein may play a significant role in regulating a subset of Pax-5 target genes which do not require the complete Pax-5a sequence, including those where Pax-5 functions as a docking protein (e.g. *mb-1*). Activation-induced stabilization of Pax-5a rapidly reduces the number of regulatory fragments, and, during the late stages of LPS-activation, full-length Pax-5a (with the repressor domain) is the only molecular species available for transcriptional regulation. Consequently, the transactivating effect of Pax-5a on its target genes may be modified as soon as the activation signal is received, allowing for a quick change in gene expression. Thus, different length Pax-5a fragments may have distinct transactivation potentials, ranging from a weakly active protein to a potent regulator to a polypeptide with a recruiter capacity. In combinatorial regulation of Pax-5 target genes, a particular function of each fragment would likely to be determined by the specific regulatory sequence context.

This hypothesis could explain the expression pattern of the *mb-1* gene which encodes a component of BCR, Ig-α molecule. The *mb-1* gene is expressed during all stages of B lymphocytes development, but not in late activated B and plasma cells (Fitzsimmons *et al.*, 1996). According to our model, the gene is positively regulated by a set of truncated Pax-5a fragments in non-activated B cells. Following antigenic challenge, Pax-5a is stabilized and the full-length, repressor sequence-containing form of the protein (5a.1) starts to accumulate in activated B lymphocytes. The stabilized Pax-5a is no longer able to function as a potent transcriptional activator, and transcription of the

positively regulated *mb-1* gene is inhibited (in activated B cells) and, subsequently, shut off (in plasma cells).

Proteolysis is a common mechanism that quickly and irreversibly regulates transcription factor function. This means of regulation is particularly useful when cells need to respond rapidly to changes in their environment. Several eukaryotic transcription factors, including NF-κB, p53, c-Myc, and c-Jun, have been shown to be regulated by proteolytic events, most of which involve the ubiquitin-proteasome pathway (reviewed in Peters, 1994; Pahl and Baeuerle, 1996). Proteolytic degradation may occur either in cytoplasm (e.g., NF-κB (Thanos and Maniatis, 1995)) or in the nucleus (e.g., HIF-1α (Salceda and Caro, 1997)), indicating that this process is mediated by the specifically localized proteasome systems. The lability of many regulatory proteins is critically important under normal conditions, and disruption of proper steady-state levels of these proteins may have deleterious consequences for a cell (Treier *et al.*, 1994). However, in some cases, protein stability changes drastically when cells are subjected to a stressful stimulus. Wild-type p53, for instance, is a short-lived nuclear transcription factor which becomes stabilized and activated following cellular stress or DNA damage (Pahl and Baeuerle, 1996). The exact mechanism of this stabilization is not clearly understood, but thought to be regulated by multi-site phosphorylation (Steegenga *et al.*, 1996; Meek, 1998). Another example is the hypoxia-inducible factor-1α (HIF-1α), a transcriptional activator complex involved in regulation of several hypoxia-regulated genes (Salceda and Caro, 1997). Under normoxic conditions, HIF-1α is continuously degraded in the nucleus by the ubiquitin-dependent pathway. The lack of oxygen, or hypoxia, induces stabilization and accumulation of HIF-1α followed by subsequent activation of its target

genes (Salceda and Caro, 1997). The signals triggering stabilization of the HIF-1α protein are currently unknown; however, some evidence suggests that redox-mediated changes might be involved (Salceda and Caro, 1997).

Thus, stress-induced stabilization of transcription factors is a well-documented phenomenon which may take place in activated B lymphocytes. It remains to be determined whether Pax-5a degradation, occuring in resting B cells, involves the ubiquitin-proteasome system. Further investigations are also needed to identify the factors which contribute to susceptibility of Pax-5a to degradation, as well as to its stabilization during activation.

4.4 The ratio of Pax-5 proteins changes after LPS stimulation

To investigate whether the suppressor activity of Pax-5d has functional significance during B cell activation, relative protein levels of Pax-5a and Pax-5d isoforms were analyzed in mature resting and LPS-activated B cells. Contrary to the preliminary hypothesis about the role of the Pax-5a/Pax-5d ratio in regulation of Pax-5a function, the levels of Pax-5d were very low in resting B lymphocytes and undetectable in nuclear extracts of activated cells. However, a new band was detected at ~27 kD that represents a yet unidentified protein species named Pax-5_x. The intensity of Pax-5_x band increases dramatically during the late activation stages concomitantly with the decrease in the Pax-5a levels. It is noteworthy that the Pax-5_x band has also been detected in nuclear extracts of various B cell lines, including those from anti-IgM- and LPS-stimulated cells of the WEHI-231 cell line. In agreement with the results of SRB

activation, the intensity of the Pax-5_x band was higher in cell lines representing late stages of differentiation and in activated WEHI-231 cells.

The exact nature of Pax-5_x species is unknown at this time. Western blot of resting and activated SRBs shows that the band can interact with the novel sequence-specific antibody 6G11, but is undetectable using the paired domain antiserum ED-1. These results indicate the presence of the novel sequence and suggest that the paired domain is either incomplete or absent in the novel species. The fact that Pax-5_x does not possess any detectable DNA-binding activity suggests that the Pax-5_x band may represent the Pax-5e isoform. However, the expected size of Pax-5e is 19 kD, and, while the Pax-5_x band has been shown to be Pax-5e-specific, the reason for such a discrepancy in size between Pax-5e and Pax-5_x remains unclear. One possible explanation is that the band represents a modified form of the Pax-5e protein. The configuration of the modified protein might be such that it prevents the efficient interaction of ED-1 with the epitopes in the paired domain of Pax-5e, which, coupled with the reduced size of the region, could result in a poor response of Pax-5_x species to this antibody. Alternatively, the levels of the paired domain epitopes interacting with ED-1 may simply be too low in normal B cells. In support of this explanation is the data from WEHI-231 activation experiments. The levels of Pax-5 proteins are much higher in WEHI-231 cell line compared to splenic B lymphocytes. Although detection of the Pax-5_x species by ED-1 was still poor in these samples, the band could be seen on overexposed films (not shown).

The patterns for the Pax-5_x band were very similar in LPS-stimulated SRBs, in activated WEHI-231 cells, and in B cell lines representing different stages of B cell differentiation (all determined using 6G11 antibody). The protein levels of Pax-5_x

species increase markedly during late stages of B cell differentiation in all experimental systems, suggesting a common mechanism involved in response of B lymphocytes to antigenic challenge and in their progression toward the plasma cell stage. Since no bands corresponding to Pax-5_x species were observed in data from EMSAs, it can be concluded that these species lack either the entire DNA-binding domain or a part thereof, which is in agreement with the Pax-5e-specific nature of the Pax-5_x protein. Consequently, Pax-5_x cannot compete for DNA-binding with Pax-5a isoform; however, it may participate in protein-protein interactions that can take place within the octamer and/or the novel sequence regions. It is conceivable that, at high enough levels, Pax-5_x may be able compete with other isoforms for certain co-factors such as, for example, co-repressors of the Groucho family implicated in regulation of Pax-5a activity (Eberhard *et al.*, 2000). Thus, the increase in levels of Pax-5_x observed in activated B lymphocytes provide additional support for the important regulatory role of Pax-5_x species and suggest a functional significance for the Pax-5a/Pax-5_x ratio during late stages of B cell differentiation. Therefore, while the original model suggesting the role for the Pax-5a/Pax-5d ratio may be incorrect, regulation of Pax-5a activity via change in the relative levels of other isoforms is likely to be important, but will require more experimental support.

4.5 The elusive Pax-5_x: speculations on the origin of a species

Identification of Pax-5_x species is a focus of our current investigations. As discussed above (Section **4.4**), the position of the band on SDS-PAGE gel (~27 kD) suggests that Pax-5_x may represent a modified form of Pax-5e protein. Possible

modifications that could account for an ~8 kD discrepancy in size may include *O*-glycosylation or formation of disulfide bonds.

Pax-5e, which contains three cysteine residues, may be able to form homo- and heterodimers, as well as intramolecular disulfide bonds. In support of this hypothesis, is the presence of a higher molecular weight band (~50 kD) observed in nuclear extracts of NIH 3T3 cells transfected with the Pax-5e expression construct. Failure of this band to interact with the novel sequence-specific antibody 6G11 suggests that the novel sequence is either masked (as in dimmers interacting through that region) or modified (e.g., by internal disulfide bonds). In the latter case, Pax-5e protein could fold on itself creating a very compact molecule that would have a much lower rate of migration in a polyacrylamide gel, resulting in a slower moving band. It is possible that disulfide bonds forming during such interactions are highly reactive and can reform during electrophoresis despite the presence of β-mercaptoethanol (reducing agent) in sample buffer, although no precedence for this was found in the literature. To prevent the cysteines from participating in bond re-formation, the samples could be treated with a strong oxidizing agent such as performic acid, which will cause irreversible cleavage of existing disulfide bonds (Lou *et al.*, 1987). Perhaps, such stringent conditions will help to keep all the proteins in nuclear extracts as separate, fully denatured entities, easily separable and individually detectable.

Another possible modification involves *O*-glycosylation of Pax-5e protein. Addition of *O*-GlcNAc residues usually occurs at the sites containing a proline residue N-terminal to the modified serine or threonine (see Chapter I, Section *3.3*). Curiously, Pax-5e (as well as Pax-5d) contains four P-S/T regions in its amino acid sequence. Although

it is not known whether these regions are subject to glycosylation, it is tempting to speculate that the retarded migration rate of Pax-5e (at ~27 kD instead of the expected 19 kD on denaturing gels) is caused by the addition of bulky sugar moieties. Unfortunately, it is difficult to estimate the possible number of putative *O*-GlcNAc residues (MW= 0.2 kD) based on the difference in the bands' size (~ 8 kD) because the correlation is not necessarily linear. However, it might be interesting to examine the effect of various deglycosylating enzymes and glycosylation inhibitors (see Future Directions) on the Pax-5e banding pattern. The notion of glycosylation of Pax-5 proteins is supported by the analyses of *O*-glycosylation in activated T lymphocytes. These studies demonstrated that mitogenic stimulation of T cells induces a rapid increase in *O*-GlcNAc-modified nuclear proteins concomitantly with the decrease in the levels of glycosylated proteins in the cytoplasm (Kearse and Hart, 1991).

So far, I have not considered the case in which the Pax-5_x band is composed of a Pax-5d degradation product. The protein would have to be degraded from the N-terminus (since it still can interact with 6G11 antibody), with the paired domain being affected to such an extent that it is no longer able to interact with either Pax-5-specific sites on DNA or ED-1 antibody. This possibility is the one that is difficult to address using the methods employed in this project (i.e., Western blot and EMSA). The N-terminus-specific antibody N-19 (see Chapter II, Table I) does not recognize Pax-5 proteins in their denatured form and, hence, could not be used for Western blot detection of Pax-5_x species. Likewise, these species could not be analyzed by EMSA due to the lack of DNA-binding activity. Thus, at this time, it is virtually impossible to distinguish between Pax-5d N-terminal degradation products and Pax-5e isoform, unless microsequencing

techniques are used. However, it is useful to consider the following observations. First, a gradual increase of Pax-5_x levels in late activated SRB samples does not correspond with a sudden fall in the intensity of the Pax-5d band to undetectable levels immediately following LPS activation. Furthermore, since the RNA levels of the Pax-5a and Pax-5d isoforms have been shown to remain constant during B lymphocyte activation/development, the degradation of Pax-5d N-terminus would most likely occur at the protein level through specific proteolysis. In this case, it is not clear how and why the identical region on Pax-5a protein is protected from the proteolytic effect of proteases involved in the process. Lastly, the Pax-5_x band was observed in nuclear extracts of NIH 3T3 cell line transfected with Pax-5e expression construct. This result confirmed that the Pax-5_x band is Pax-5e-specific, and is more likely to represent a post-translationally modified form of Pax-5e protein than a degradation product of Pax-5d.

What is the source of the Pax-5_x species in activated B cell samples? Based on the results of RNAse protection assays, the increase in Pax-5_x protein levels is not a result of transcriptional changes, but a product of translational and post-translational regulation. The presence of two translational start sites on the *Pax-5d* mRNA transcripts allows generation of either Pax-5d or Pax-5e from each single transcript. In fact, when non-lymphoid cell line NIH 3T3 is transfected with the Pax-5d cDNA construct, four species are produced: a largely predominant Pax-5d protein (at 35 kD), Pax-5_x species (27 kD), the Pax-5e isoform (~19 kD), and an additional molecule (or protein complex) of unknown nature (~ 50 kD). The bands for Pax-5e and Pax-5_x correspond to those seen in nuclear extracts from NIH 3T3 cells transfected with the Pax-5e-containing construct. Therefore, based on these observations, I hypothesize that, during LPS activation, B

lymphocytes receive a signal that initiates a preferential use of the distal ATG codon on the *Pax-5d* transcripts resulting in the increased production of Pax-5e protein. In increased concentrations, Pax-5e may be able to bind to or sequester various factors, some of which might be important for regulation of transcriptional activity of Pax-5a isoform (e.g., Groucho co-repressors). Importantly, I hypothesize that isoform Pax-5d is unable to fulfill this role because its upregulation would result not only in sequestration of accessory factors, but also in DNA-binding competition with Pax-5a. The resulting inhibition of Pax-5a activity might be detrimental for Pax-5a function exerted on a subset of target genes in activated B cells. It is unclear whether activating stimuli have a similar effect on other Pax-5 isoforms. The experimental data do not show protein levels of Pax-5b isoform changing in a manner identical to that of Pax-5e, which suggest that the proposed mechanism is likely to be very specific. This, however, requires further investigation.

4.6 Effects of aging on B cell activation: the case of Pax-5 proteins

Currently, the experimental evidence demonstrating the effects of age on B cell responses to various antigens remains fragmentary. The progress in this field has been slow in part because it is difficult to determine whether age-related changes in B lymphocytes function are caused by biochemical modifications within B cells, or by alterations in the activity of T cells. Several studies that utilize B cell mitogens suggest that the magnitude of B lymphocyte function, defined by cell proliferative response, decreases with age (Andersson *et al.*, 1977; Schulze *et al.*, 1992; Powers *et al.*, 1992). Although the exact mechanism of this decline is unknown, it is conceivable that Pax-5

proteins, among other transcription factors, may be partially responsible for impaired proliferation of activated B lymphocytes. This hypothesis is supported by experimental findings which have demonstrated that reduced levels of Pax-5a expression lead to decreased response to LPS (Wakatsuki *et al.*, 1994).

While no quantitative measurements of B cell proliferation were conducted on either young or aged lymphocytes, the previously determined lack of Pax-5a DNA-binding (Anspach *et al.*, submitted) implies that, in aged mice, the proliferative response to LPS stimulation can be expected to be lower than in young animals. In support of this prediction, a characteristic initial rise in Pax-5a protein levels, corresponding to the peak of activation-induced proliferation of B lymphocytes, was not observed during activation of aged B cells, suggesting an attenuated growth of LPS-activated cultures. Interestingly, in the studies described here, a gradual return of Pax-5a DNA-binding was observed during late stages of LPS activation. Specifically, the observed band represents Pax-5a.1 species which has been correlated with the activation-induced B cell proliferation in young mice (Chapter II). This result confirms the functional significance of the full-length (i.e., Pax-5a.1) Pax-5a protein in the process of B cell activation and proliferation, and in the development of humoral immune response in young, as well as aged, animals.

In the discussion of Pax-5a.1 and Pax-5a.2 species (Section **4.3**), I proposed that instability of Pax-5a isoform observed in resting B lymphocytes may have a function as a mechanism of generating a variety of Pax-5a proteins with distinct transactivating properties. The Pax-5a.2 protein was suggested to be of a particular importance, as it represents a highly active form of the Pax-5 transcription factor possessing all functional domains except the repressor sequence. In aged SRBs, the Pax-5a.1 species were absent,

5a.2 significantly decreased, and the overall Pax-5a degradation pattern displayed fewer DNA-binding fragments compared to young SRBs. Curiously, the relative levels of Pax-5a and Pax-5_x proteins remained constant in resting and activated aged SRBs. If the Pax-5a/Pax-5_x ratio has a functional significance in B cell differentiation, then such invariability in the relative proportions of the two proteins may be a sign of inadequate regulation and/or impaired signaling occurring during activation of aged B lymphocytes. Perhaps, a high concentration of the Pax-5_x species in resting B cells is the reason for a reduction in DNA-binding activity of various Pax-5a fragments, including the important Pax-5a.2 protein. Moreover, constantly high levels of Pax-5_x might be responsible for excessive sequestration of critical accessory factors, which may result in either inappropriate activation of Pax-5a or unregulated competition with other Pax-5 proteins.

Age-associated decreases in DNA-binding properties have been reported for several transcription factors including Sp-1, NFAT, and Ap-1 (Ammendola *et al.*, 1992; Pahlavani *et al.*, 1995; Pahlavani *et al.*, 1996). Sp-1 is a general transcription factor that activates a wide variety of genes and is present in all mammalian cells. Independent studies of this protein have shown that its transcriptional activity in normal, non-senescent tissues can be regulated by post-translational modifications including *O*-linked glycosylation, phosphorylation of serine residues, and multimerization of Sp1 on its DNA-binding sites (Ammendola *et al.*, 1992). In aged rat brain and liver tissues, Sp1 is characterized by a very low (60 fold decrease compared to young tissues) binding efficiency, although the expression of the *Sp1* gene is not affected (Ammendola *et al.*, 1992). The observed change in DNA-binding activity was correlated with the decreased expression of at least one of the Sp1 target genes and proposed to be a result of age-

related defects in post-translational modifications (Ammendola *et al.*, 1992). Another example is the T-cell-specific transcription factor NFAT that plays an important role in the regulation of IL-2 transcription. One of the T cell secreted cytokines, IL-2 provides important signals for T cell proliferation and antibody production and, hence, plays a central role in induction and development of the immune response (Arai *et al.*, 1990). The production of IL-2 declines with age (Rea *et al.*, 1996), which coincides with a decrease in NFAT DNA-binding (Pahlavani *et al.*, 1995). Interestingly, the reduction of NFAT binding activity is also thought to be caused by age-related alterations in post-translational regulatory mechanisms (Pahlavani *et al.*, 1996).

Thus, the experimental data described in this thesis, together with reports from other labs, support the hypothesis that aged animals display an age-associated decline of B cell responsiveness to mitogenic stimulation, as reflected in decreased proliferative capacity of B lymphocytes. This reduction in proliferative ability of activated B lymphocytes may be related to alterations in DNA-binding activities and relative levels of Pax-5 proteins. Furthermore, age-induced changes in characteristics and properties of Pax-5 isoforms may be triggered by altered regulatory mechanisms occurring at the translational and/or post-translational levels. The lack of Pax-5a DNA-binding in resting B cells, coupled with the unchanging Pax-5a/Pax-5_x ratio in activated B lymphocytes, may contribute to inhibition of genes that trigger cell division and regulate antibody production, ultimately leading to poor humoral immunity in senescent organisms.

4.7 Model of Pax-5 function

The models regarding function and regulation of the Pax-5 transcription factor that have been proposed in this work and by other research groups can be summarized in the following key points (Figure IV.1A):

- The *Pax-5* gene generates four alternatively spliced products: Pax-5a, Pax-5b, Pax-5d, and Pax-5e. The four isoforms differ in DNA-binding activities and transactivation properties, and display distinct expression patterns during different stages of B cell development and differentiation. The differentiation stage-dependent regulation of Pax-5 proteins occurs at the translational and/or post-translational, but not transcriptional, level(s).

- The predominant isoform Pax-5a can function as an activator, a repressor, or a docking protein, depending on the target gene. The less abundant isoform Pax-5d is unable to interact with the basal initiation complex due to the absence of the transactivation domain, and hence functions as a repressor of transcription. Isoforms Pax-5b and Pax-5e lack DNA-binding activity due to incomplete DNA-binding domain, and thus can only function as either co-repressors or co-activators in transcriptional regulation, via protein-protein interaction. In addition, Pax-5b and Pax-5e may act as dominant-negative inhibitors of Pax-5a and Pax-5d, respectively, as they share several functional domains, including those that have been implicated in protein-protein interactions.

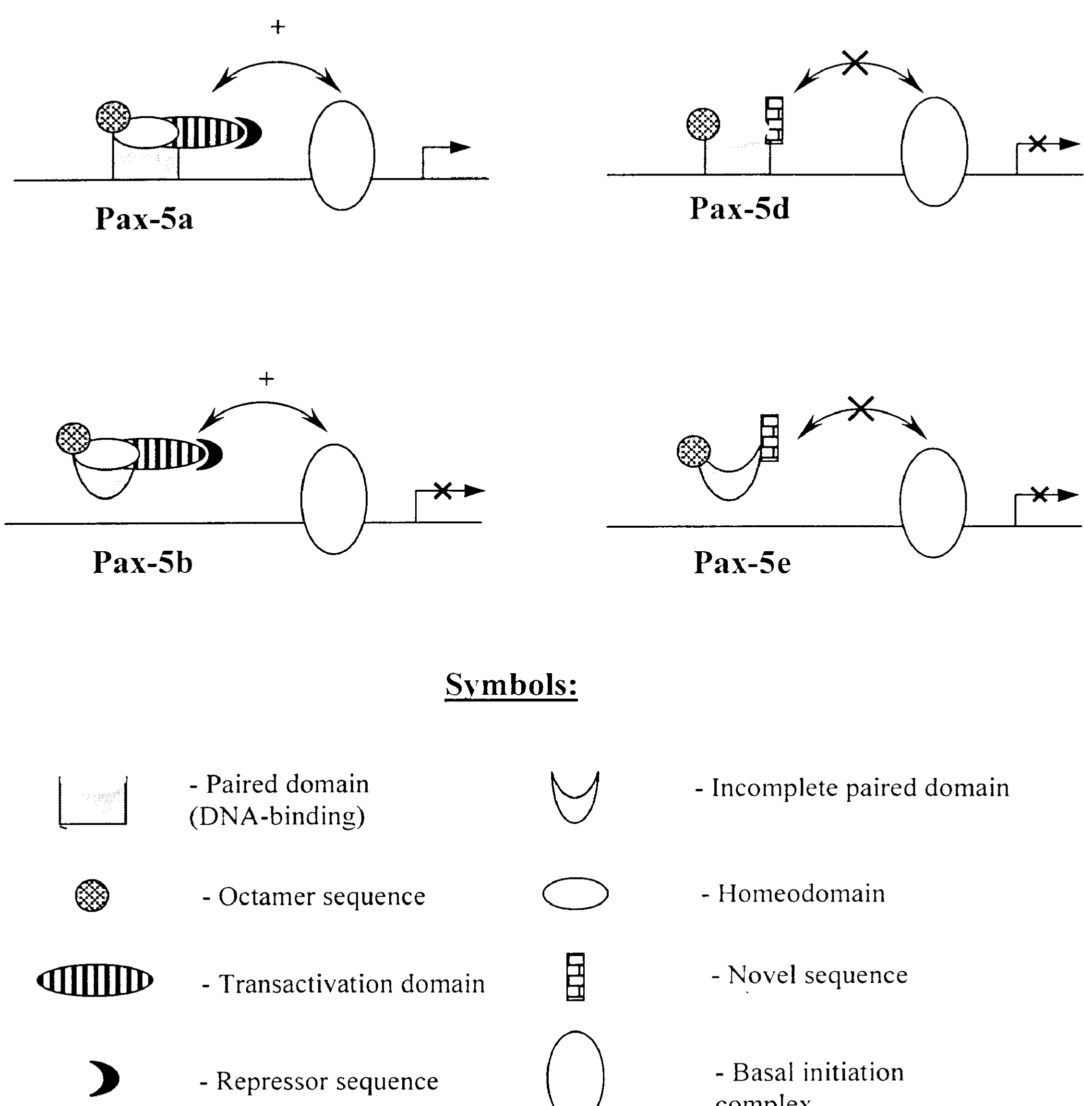


Figure IV.1A: Functions of alternative isoforms of the transcription factor Pax-5. Pax-5 isoforms have different DNA-binding and transactivating properties. Only isoforms Pax-5a and Pax-5d can efficiently bind to Pax-5-specific sequences on DNA. Due to the absence of DNA binding activity, Pax-5e and Pax-5b can only have a dominant-negative or co-repressor function. Pax-5e and Pax-5d possess a novel sequence in place of a region containing the transactivation domain and homeodomain. The presence of the novel sequence confers a repressor function to isoform Pax-5d. In Pax-5a, the activity of the transactivation domain is regulated by the adjacent C-terminal repressor sequence. The functions of Pax-5 isoforms are shown on a hypothetical positively regulated Pax-5 target gene. See text for details.

- Transactivation function of the Pax-5a isoform may be regulated by at least two different mechanisms: a) by other isoforms, via competition for either DNA-binding sites or accessory factors;
  b) through post-translational modifications that increase Pax-5a protein stability and allow retention of the repressor sequence.

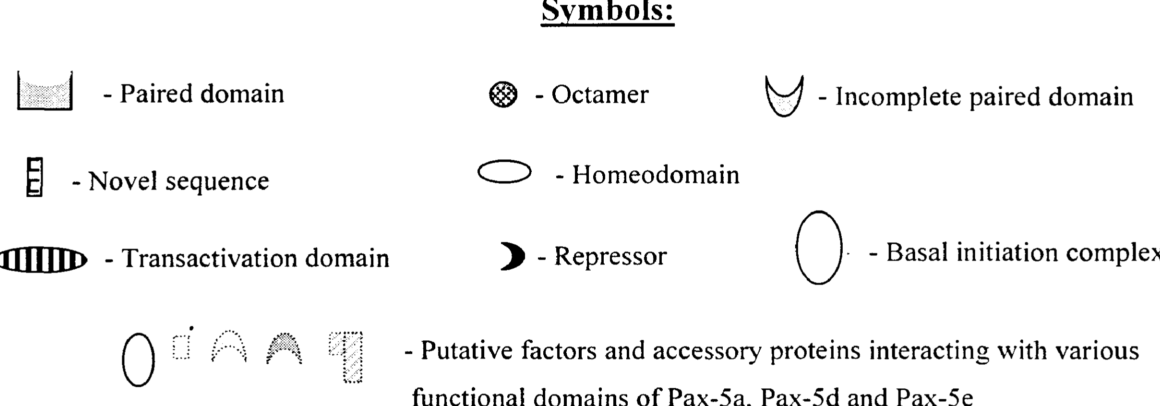
Based on these key points, I would like to propose the following model for regulation of Pax-5 proteins in resting and activated B lymphoctes (Figure IV.1B). In resting B cells, isoform Pax-5a is highly susceptible to C-terminal proteolytic degradation. Proteplytic degradation generates a variety of paired domain-containing Pax-5a fragments. These truncated polypeptides have distinct transactivation properties which ensure differential regulation for a wide range of Pax-5 target genes. Activity of some fragments may be modulated by DNA-binding competition with the Pax-5d isoform, which will exert an opposite effect on transcription of genes affected by such competition. In addition, both Pax-5d and Pax-5e may compete with Pax-5a fragments for a variety of yet unidentified co-factors and partner-proteins. However, since the levels of these two isoforms are very low in resting B cells, their effect might be either negligible or very specific. Pax-5e and Pax-5d may also form homo- and heterodimers through interactions between the novel sequences.

Activation of B lymphocytes results in a rapid stabilization of Pax-5a, followed by the subsequent accumulation of a full-length Pax-5 protein containing the C-terminal repressor sequence (5a.1). In activated B cells, the functions of the Pax-5a isoform are limited to control of B cell proliferation and regulation of a small subset of target genes.

Since DNA-binding competition with Pax-5a fragments is no longer needed, the Pax-5d isoform is either degraded or used to produce more Pax-5e (through alternative use of the proximal start site during translation). Without interfering with Pax-5a DNA-binding, Pax-5e sequesters certain accessory proteins or regulatory factors, inhibiting basal Pax-5a activity, other than that necessary for B cell proliferation and maintenance of target "housekeeping" genes which are independent of B cell development. As activated B lymhpocytes progress to the plasma cell stage, the majority of positively regulated Pax-5 target genes are turned off or downregulated simultaneously with the relief of Pax-5 repressor function on its negatively regulated target genes. Finally, upregulation of Pax-5e isoform may have a function independent of other Pax-5 proteins, such as, for example, regulation of B cell apoptosis during late stages of B cell differentiation. In this model, the role of isoform Pax-5b is still unclear, but may involve a dominant-negative function during late stages of B cell activation and in the plasma cell stage.

This model needs to be refined by further experimental evidence and by clarification of the mechanisms involved in the proposed regulatory events. However, it can be used as a "template" for future investigations that will focus on a wide variety of pertinent issues, including the aspects suggested in the next section.

Figure IV.1B (see next page): A model of function and regulation of Pax-5 proteins in resting and activated B lymhpocytes. See text for details.



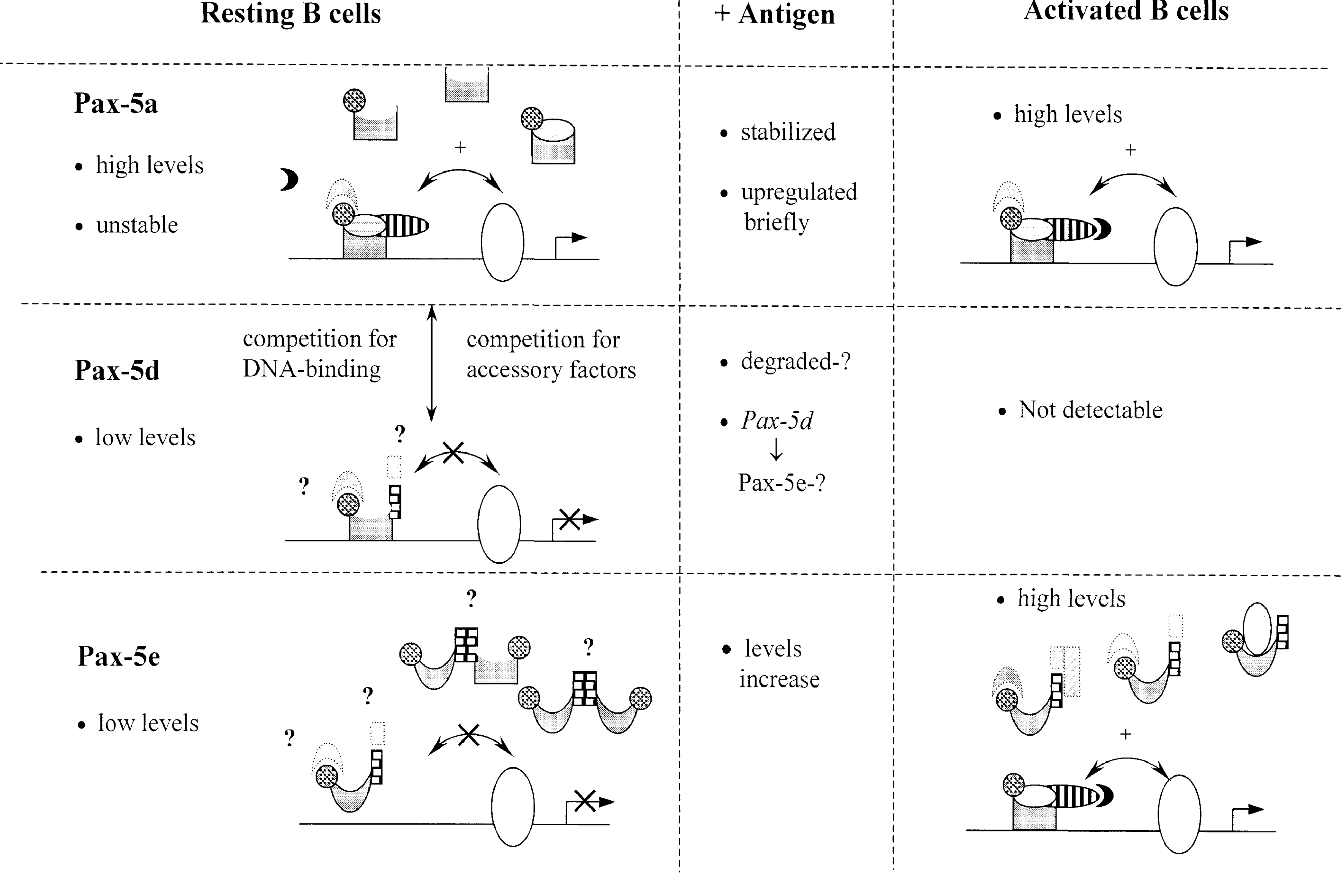


Figure IV.1B

4.8 Future directions.

Identification of Pax-5$_x$ species. Elucidation of the nature and origin of Pax-5$_x$ species will have great implications for further understanding of the activation process in B lymphocytes. Currently, the main focus of our research is to determine whether the novel Pax-5$_x$ band represents a modified form of Pax-5e protein. To solve this problem, we first have to optimize experimental conditions in ways that will favor detection of individual, fully denatured proteins. These will involve determining whether inter- and intramolecular disulfide bonds form in Pax-5e molecules and whether they can be re-established during electrophoresis. To address this question, nuclear extracts will be subjected to a wide range of pre-assay treatments including incubations with strong reducing/oxidizing agents and modifying enzymes and prolonged boiling at high temperatures. Another important aspect of these investigations will aim to determine whether the Pax-5$_x$ species represent an *O*-glycosylated form of Pax-5e. This will be done through examination of effects of deglycosylating enzymes on the Pax-5e/Pax-5$_x$ banding patterns. Alternatively, we could use the inhibitors of glycosylation, such as PUGNAC (Comer and Hart, 2000), to promote hyperglycosylation of Pax-5 proteins and determine how this will affect their rates of migration on denaturing gels. Finally, monoclonal antibodies directed against *O*-GlcNAc (Turner *et al.*, 1990) may be a useful tool for detection of glycosylated Pax-5 proteins. In all the experiments mentioned above, the experimental samples will consist of nuclear extracts from activated and non-activated WEHI-231 cells, cell lines transfected with various Pax-5 DNA constructs, and, finally, resting and activated normal B lymphocytes.

After the nature of the Pax-5_x band has been discerned, investigations can proceed to either further identification of the partner protein(s) or to functional analysis of the Pax-5e isoform. The latter will be conducted using the approach described for the functional studies of Pax-5d and will involve transient co-transfections of lymphoid or non-lymphoid cell lines with the Pax-5e effector construct and an artificial promoter reporter construct (Chapter II). These studies may also include competition experiments targeting the question of whether transcriptional activity of Pax-5a can be regulated by overexpression of the Pax-5e isoform. Identification of possible Pax-5e binding partners may involve co-immunoprecipitation of the Pax-5_x complex using 6G11 antibody. In these experiments, Pax-5_x species will be precipitated from nuclear extracts of activated B lymphocytes, and then analyzed by Western blot in order to determine what factors might be sequestered in this complex by Pax-5e.

Other investigations may aim to determine the role of Pax-5e in apoptosis and to examine the possibility of Pax-5e regulation through the use of alternative start sites on the *Pax-5d* transcript. To address the question of whether the Pax-5e isoform is involved in regulation of apoptosis, the Pax-5e protein will be overexpressed in a B cell line, followed by an assay for the expression of defined apoptotic markers. To confirm the hypothesis about a possible origin of Pax-5e in activated B lymphocytes (see Section **4.5**), the distal start codon of *Pax-5d* will be mutated by site-directed mutagenesis. The expression construct containing the mutant Pax-5d coding region will then be transiently transfected into NIH 3T3 cell line. Nuclear extracts from the transfected samples will be analyzed by Western blot using ED-1 antibody, and the results will be compared to the data obtained for the wild type Pax-5d (see Chapter III, Section **4**).

Determination of the role of the novel sequence. The function of the novel sequence is currently unknown. Previous research seems to indicate that this region does not display transactivating properties and, in fact, may confer repressor characteristics to Pax-5d isoform. However, at this time it is unclear whether the inhibitory function of the novel sequence is determined by the absence of the transactivation domain, or by the presence of a yet unidentified motif with the repressor function. It will be interesting to investigate the properties of truncated Pax-5d and Pax-5e mutant-proteins containing incomplete novel sequence. A Pax-5d mutant lacking the entire novel sequence (Pax-5dΔNS) has already been cloned in our lab, and will be used for functional studies in the near future. It would not be surprising if this mutant possesses functions of a weak activator as it is identical to a truncated Pax-5a protein with an intact paired domain. The transactivating properties of such fragments were discussed in detail in Section **4.3**.

The presence of two additional cysteine residues in the novel sequence presents unique opportunities for formation of inter- and intramolecular disulfide bonds (see Chapter I, Section ***4.1***). In this context, it will be interesting to explore the effect of targeted point mutations of the selected cysteines on the behavior and interactions of Pax-5 proteins. In the scope of these investigations, a panel of mutant Pax-5 proteins will be created by site-directed mutagenesis and cloned into an expression vector. The mutations will target either both or individual cysteine residues in the novel sequence and, possibly, one or two selected cysteines in the paired domain. The mutant-containing expression vectors can first be co-transfected into non-lymphoid cell lines to examine whether mutations of cysteine residues will affect the folding of the proteins, as well as their DNA-binding activity, and stability. Furthermore, similar co-transfections can be

performed in a B cell line (i.e., WEHI-231), followed by subsequent activation of the transfected cells. Nuclear extracts from activated and non-activated cells will then be analyzed to determine the levels and expression patterns of the mutant proteins. The results will be compared to the observations reported for normal Pax-5 isoforms.

Protein stability studies. Our previous research provides fragmentary evidence suggesting the important role of protein stability in the function and activity of Pax-5 proteins. The protein stability of Pax-5a seems to be greatly affected during B cell activation (see Chapter II and Section **4.3**), and the difference in half-lives of Pax-5a and Pax-5d might be a key consideration in determining experimental conditions for Pax-5a/Pax-5d competition studies (see Section **4.2**). Thus, future studies should focus on determination of the isoforms' half-lives. These investigations can be conducted using pulse chase assays in resting and activated normal B cells and in cell lines transfected with DNA constructs expressing the isoforms of interest.

Protein stability studies may also encompass issues dealing with identification of mechanisms and factors regulating stability of Pax-5 proteins in resting and activated B lymphocytes. If my hypothesis concerning Pax-5a degradation in resting B cells is correct, it might be possible to stabilize this isoform by treating SRB cultures with specific inhibitors of the ubiquitin-proteasome system. Alternatively, the problem of proteolytic degradation might be addressed by looking for a correlation between the presence of PEST sequences and protein half-lives. Recently, a Pax-5a mutant lacking PEST sequences has been created in our lab. It will be interesting to examine the effects

of this mutation on the stability of the Pax-5a isoform, and to compare protein half-life of the mutant to that of normal Pax-5d protein.

Study of the alternative B cell activation pathways. The conclusions and hypotheses presented in this thesis are based mostly on the data obtained for LPS-activated normal B lymphocytes. However, it cannot be excluded that the activation pathways occurring in B lymphocytes are antigen-specific, and may involve variable mechanisms of Pax-5 regulation. One of the future projects may aim to investigate Pax-5 protein expression and DNA-binding patterns in normal SRBs stimulated by the B cell receptor cross-linking. In this system, simultaneous addition of a CD40 ligand alone with the specific cytokines (e.g. IL-4, IL-5, IL-6) may assimilate interaction of B cells with thymus-dependent antigens (see Chapter I, Section 1). Any differences from the patterns observed in this study will indicate that the model of Pax-5 protein regulation proposed here is accurate only for humoral response triggered by the challenge with thymus-independent antigens.

Conclusions

Several major observations of this project are summarized as follows:

1. Isoform Pax-5d has a function opposite to that of Pax-5a. Activity of Pax-5a may be regulated by Pax-5d via competition for binding either to target sites on DNA or to accessory/regulatory factors.
2. The ratio of Pax-5 proteins changes in activated B lymphocytes, reflecting a shift toward higher concentrations of the isoforms which may inhibit activity of Pax-5a. Isoform Pax-5e is likely to play an important role in regulation of Pax-5a function in activated B lymphocytes.
3. Activity of Pax-5a appears to be regulated at the post-translational level through modulation of its stability. Data suggests that Pax-5a is highly susceptible to C-terminal proteolytic degradation in resting B cells, but becomes stabilized in activated B lymphocytes.
4. The ratio of Pax-5 proteins changes during activation of the immature B cell line WEHI-231 in a pattern similar to that observed for mature B lymphocytes. The changes in ratio of Pax-5 proteins can also be correlated with the developmental stages of B lymphocytes (as assessed through analyses of B cell lines representing different stages of B cell differentiation).
5. Expression patterns and DNA-binding properties of Pax-5 proteins are altered in resting and activated B cells isolated from aged mice.
6. Pax-5a and Pax-5d proteins are likely to have different half-lives.

Literature Cited:

Abate, C., Patel, L., Rauscher, F.J., and Curran, T. (1990) Redox regulation of Fos and Jun DNA-binding activity in vitro. *Science*, **249**, 1157-1161

Adams, B., Dorfler, P., Agguzi, A., Kozmik, P., Urbanek, P., Maurer-Fogy, I., and Busslinger, M. (1992) *Pax-5* encodes the transcription factor BSAP and is expressed in B lymphocytes, the developing CNS, and adult testis. *Genes Dev.*, **6**, 1589-1607

Agematsu, K., Hokibara, S., Nagumo, H., and Komiyama, A. (1999) CD27: a memory B cell marker. *Immunol. Today*, **21**, 204-206

Akimoto, Y., Kreppel, L.K., Hirano, H., and Hart, G.W. (1999) Localization of the O-linked N-acetylglucosamine transferase in rat pancreas. *Diabetes*, **48**, 2407-2413

Ammendola, R., Mesuraca, M., Russo, T., and Cimino, F. (1992) Sp1 DNA binding efficiency is highly reduced in nuclear extracts from aged rat tissues. *J. Biol. Chem.*, **267**, 17944-17948

Andersson, J., Coutinho, A., and Melchers, F. (1977) Frequencies of mitogen-reactive B cells in the mouse. Distribution in different lymphoid organs from different inbred strains of mice at different ages. *J. Exp. Med.*, **145**, 1511-1530

Andersson, T., Neurath, M.F., Grant, P.A., and Pettersson, S. (1996) Physiological activation of the IgH 3' enhancer in B lineage cells is not blocked by Pax-5. *Eur. J. Immunol.*, **26**, 2499-2507

Anspach, J., Poulsen, G., Kaattari, I., Pollock, R., and Zwollo, P. (2000) Reduction in DNA binding activity of the transcription factor Pax-5a in B lymphocytes of aged mice. Submitted.

Aoki, Y., Kim, Y.-T., Stillwell, R., Kim, T.J., and Pillai, S. (1995) The SH2 domains of Src family kinases associate with Syk. *J. Biol. Chem.*, **270**, 15658-15663

Arai, K.I., Lee, F., Miyajima, A., Miyatake, S., Arai, N., and Yokota, T. (1990) Cytokines: coordinators of immune and inflammatory response. *Annu. Rev. Biochem.*, **59**, 783-836

Arpin, C., Dechanet, J., Van Kooten, C. (1995) Generation of memory B cells and plasma cells in vitro. *Science*, **268**, 720-722

Asano, M. and Gruss, P. (1991) Pax-5 is expressed in the midbrain-hindbrain boundary during mouse development. *Mech. Dev.*, **39**, 29-39

Barberis, A., Widenhorn, K., Vitelli, L., and Busslinger, M. (1990) A novel B-cell lineage-specific transcription factor present at early but not late stages of differentiation. *Genes Dev.*, **22**, 37-43

Benezra, R. (1994) An intermolecular disulfide bond stabilizes E2A homodimers and is required for DNA binding at physiological temperatures. *Cell*, **79**, 1057-1067

Busslinger, M. and Urbanek, P. (1995) The role of BSAP (Pax-5) in B-cell dvelopment. *Curr. Opin. Genet. Dev.*, **5**, 595-601

Busslinger, M., Klix, N., Pfeffer, P., Graninger, P.G., and Kozmik, Z. (1996) Deregulation of *PAX-5* by translocation of the Eμ enhancer of the *IgH* locus adjacent to two alternative *PAX-5* promoters in a diffuse large-cell lymphoma. *Proc. Natl. Acad. Sci. USA*, **93**, 6129-6134

Calkhoven, F. and Geert, A.B. (1996) Multiple steps in the regulation of transcription factor level and activity. *Biochem. J.*, **317**, 329-342

Chen, L. (1999) Combinatorial gene regulation by eukaryotic transcription factors. *Curr. Opin. Struct. Biol.*, **9**, 48-55

Chou, T.-Y., Dang, C.V., and Hart, G.W. (1995) Glycosylation of the c-Myc transactivation domain. *Proc. Natl. Acad. Sci. USA*, **92**, 4417-4421

Chou, C.F. and Omary, M.B. (1993) Mitotic arrest-associated enhancement of O-linked glycosylation and phosphorylation of human keratins 8a and 18. *J. Biol. Chem.*, **268**, 4465-4472

Cogné, M., Lasford, A., Bottaro, A., Zhang, J., Gorman, J., Young, F., Cheng, H-L., and Alt, F.W. (1994) A class switch control region at the 3' end of the immunoglobulin heavy chain locus. *Cell*, **77**, 737-747

Comer, F.I. and Hart, G.W. (2000) *O*-glycosylation of nuclear and cytoplasmic proteins. *J. Biol. Chem.*, **275**, 29179-29182

Czerny, T., Schaffner, G., and Busslinger, M. (1993) DNA sequence recognition by Pax proteins: bipartite structure of the paired domain and its binding site. *Genes Dev.*, **7**, 2048-2061

Dahl, E., Koseki, H., and Balling, R. (1997) *Pax* genes and organogenesis. *Bioessays*, **19**, 755-765

Datta, B., Ray, M.K., Chakrabarti, D., Wylie, D.E., and Gupta, N.K. (1989) Glycosylation of eukaryotic peptide chain initiation factor 2 (eIF-2)-associated 67-

kDa polypeptide (p^{67}) and its possible role in the inhibition of eIF-2 kinase-catalyzed phosphorylation of the eIF-2 α-subunit. *J. Biol. Chem.*, **264**, 20620-20624

Dong, D.L.-Y. and Hart. G.W. (1994) Purification and characterization of an *O*-GlcNAc selective *N*-acetyl-β-D-glucosaminidase from rat spleen cytosol. *J. Biol. Chem.*, **269**, 19321-19330

Dörfler, P. and Busslinger, M. (1996) C-terminal activating and inhibitory domains determine the transactivation potential of BSAP (Pax-5), Pax-2, and Pax-8. *EMBO J.*, **15**, 1971-1982

Dutton, R.W., Swain, S.L., and Bradley, L.M. (1999) The generation and maintenance of memory T and B cells. *Immunol. Today*, **20**, 291-293

Dymecki, S.M., Zwollo, P., Zeller, K., Kuhadja, F.P., and Desiderio, S.V. (1992) Structure and developmental regulation of the B-lymphoid tyrosine kinase gene *blk*. *J. Biol. Chem.*, **267**, 4815-4823

Eberhard., D. and Busslinger, M. (1999) The partial homeodomain of the transcription factor Pax-5 (BSAP) is an interaction motif for the retinoblastoma and TATA-binding protein. *Cancer Res. (suppl.)*, **59**, 1716s-1725s

Eberhard, D., Jimenez, G., Heavey, B., and Busslinger, M. (2000) Transcriptional repression by Pax-5 (BSAP) through interaction with corepressors of the Groucho family. *EMBO J.*, **19**, 2292-2303

Epstein, J., Cai, J., Glaser, T., Jepeal, L., and Maas, R. (1994) Identification of a *Pax* paired domain recognition sequence and evidence for DNA-dependent conformational changes. *J. Biol. Chem.*, **269**, 8355-8361

Ernst, P. and Smale, S.T. (1995) Combinatorial regulation of transcription I: general aspects of transcriptional control. *Immunity*, **2**, 311-319.

Fitzsimmons, D., Hodsdon, W., Wheat, W., Maira, S.M., Wasylyk, B., and Hagman, J. (1996) Pax-5 (BSAP) recruits Ets proto-oncogene family proteins to form functional ternary complexes on a B-cell-specific promoter. *Genes Dev.*, **7**, 2198-2211

Foulkes, N.S. and Sassone-Corsi, P. (1992) More is better: activators and repressors from the same gene. *Cell*, **68**, 411-414

Georgopoulos, K., Winandy, S., and Avitahl, N. (1997) The role of the Ikaros gene in lymphocyte development and homeostasis. *Ann. Rev. Immunol.*, **15**, 155-176

Ghia, P., Melcherws, F., and Rolink, A.G. (2000) Age-dependent changes in B lymphocyte development in man and mouse. *Exp. Gerontol.*, **35**, 159-165

Gottschalk, A.R. and Quintans, J. (1995) Apoptosis in B lymphocytes: the WEHI-231 perspective. *Immunol. Cell Biol.*, **73**, 8-16

Grosschedl, R., and Baltimore, D. (1985) Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. *Cell*, **41**, 885-897

Hagman, J., Wheat, W., Fitzimmons, D., Hodson, W., Negri, J., and Dizon, F. (2000) Pax-5/BSAP: regulator of specific gene expression and differentiation in B lymphocytes. *Curr. Top. Microbiol. Immunol.*, **245**, 169-194

Haltiwanger, R.S., Holt, G.D., and Hart, G.W. (1990) Enzymatic addition of O-GlcNAc to nuclear and cytosolic proteins. Identification of a uridine diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase. *J. Biol. Chem.*, **265**, 2563-2568

Haltiwanger, R.S., Blomberg, M.A., and Hart, G.W. (1992) Glycosylation of nuclear and cytoplasmic proteins. Purification and characterization of a uridine diphospho-*N*-acetylglucosamine:polypeptide β-*N*-acetylglucosaminyltransferase. *J. Biol. Chem.*, **267**, 9005-9013

Haltiwanger, R.S., Busby, S., Grove, K., Li, S., Mason, D., Medina, L., Moloney, D., Philipsberg, G., and Scartozzi, R. (1997) *O*-glycosylation of nuclear and cytoplasmic proteins: regulation analogous to phosphorylation? *Biochem. Biophys. Res. Commun.*, **231**, 237-242

Han, I. and Kudlow, J.E. (1997) Reduced *O*-glycosylation of Sp1 is associated with increased proteasome susceptibility. *Mol. Cell. Biol.*, **17**, 2550-2558

Hertz, M. and Nemazee, D. (1998) Receptor editing and commitment in B lymphocytes. *Curr. Opin. Immunol.*, **10**, 208-213

Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997) AP-1 transcriptional activity is regulated by a direct association between thoredoxin and Ref-1. *Proc. Natl. Acad. Sci. USA*, **94**, 3633-3638

Hirota, K., Murata, M., Sachi, Y., Nakamura, H., Takeuchi, J., Mori, K., and Yodoi, J. (1999) Distinct roles of thioredoxin in the cytoplasm and in the nucleus. *J. Biol. Chem.*, **274**, 27891-27897

Hodgkin, P.D. and Basten, A. (1995) B cell activation, tolerance, and antigen-presenting function. *Curr. Opin. Immunol.*, **7**, 121-129

Hogg, N. and Berlin, C. (1995) Structure and function of adhesion receptors in leukocyte trafficking. *Immunol. Today*, **16**, 327-330

Holt, G.D., Snow, C.M., Senior, A., Haltiwanger, R.S., Gerace, L., and Hart, G.W. (1987) Nuclear pore complex glycoproteins contain cytoplasmically disposed O-linked N-acetylglucosamine. *J. Cell Biol.*, **104**, 1157-1164

Hunter, T. and Karin, M. (1992) The regulation of transcription by phosphorylation. *Cell*, **70**, 375-378

Jackson, S.P. and Tjian, R. (1988) *O*-glycosylation of eukaryotic transcription factors: implications for mechanisms of transcriptional regulation. *Cell*, **55**, 125-133

Jun, S. and Desplan, C. (1996) Cooperative interactions between paired domain and homeodomain. *Development*, **122**, 2639-2650

Kalousova, A., Beneš V., Pačes, J., Pačes, V., Kozmik, Z. (1999) DNA binding and transcativating properties of the paired and homeobox protein Pax-4. *Biochem. Biophys. Res. Comm.*, **259**, 510-518

Kearse K.P. and Hart, G.W. (1991) Lymphocyte activation induces rapid changes in nuclear and cytoplasmic glycoproteins. *Proc. Natl. Acad. Sci. USA*, **88**, 1701-1705

Kelly, W.G., Dahmus, M.E., and Hart G.W. (1993) RNA polymerase II is a glycoprotein. *J. Biol. Chem.*, **268**, 10416-10424

Kemp, B.E. and Pearson, R.B. (1990) Protein kinase recognition sequence motifs. *Trends in Biochem. Sci.*, **15**, 342-346

Klinman, N.R. (1997) The cellular origins of memory B cells. *Semin. Immunol.*, **9**, 241-247

Kozmik, Z., Wang, S., Dörfler, P., Adams, B., and Busslinger, M. (1992) The promoter of the CD19 gene is a target for the B-cell-specific transcription factor BSAP. *Mol. Cell. Biol.*, **12**, 2662-2672

Kozmik, Z., Kurzbauer, R., Dorfler, P, and Busslinger, M. (1993) Alternative splicing of *Pax-8* gene transcripts is developmentally regulated and generates isoforms with different transactivation properties. *Mol. Cell. Biol.*, **13**, 6024-6035

Kozmik, Z., Sure, U., Rüedi, D., Busslinger, M., and Aguzzi, A. (1995) Deregulated expression of *PAX-5* in medulloblastoma. *Proc. Natl. Acad. Sci. USA*, **92**, 5709-5713

Kozmik, Z., Czerny, T., and Busslinger, M. (1997) Alternatively spliced insertions in the paired domain restrict DNA sequence specificity of Pax-6 and Pax-8. *EMBO J.*, **16**, 6793-6803

Krenacs, L., Himmelmann, A.W., Quintanilla-Martinez, L., Fest, T., Riva, A., Wellman, A., Bagdi, E., Kehrl, J.H., Jaffe, E.S., and Raffeld, M. (1998) Transcription factor B-cell-specific protein (BSAP) is differentially expressed in B cells and subsets of B-cell lymphomas. *Blood*, **92**, 1308-1316

Kreppel, L.K., Blomberg, M.A., and Hart, G.W. (1997) Dynamic glycosylation of nuclear and cytoplasmic proteins. Cloning and characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. *J. Biol. Chem.*, **272**, 9308-9315

Kruisbeek, A. and Storb, U. (1994) Lymphocyte development. *Curr. Opinion Immunol.*, **6**, 199-202

Lambert, P.F., Kashanchi, F., Radonovich, M.F., Shiekhattar, R., and Brady, J.N. (1998) Phosphorylation of p53 serine 15 increases interaction with CBP. *J. Biol. Chem.*, **273**, 33048-33053

Lando, D., Pongratz, I., Poellinger, L., and Whitelaw, M.L. (2000) A redox mechanism controls differential DNA binding activities of hypoxia-inducible factor (HIF) 1α and the HIF-like factor. *J. Biol. Chem.*, **275**, 4618-4627

Lansford, R.D., McFadden, H.J., Siu, S.T., Cox, J.S., Cann, G.M., and Koshland, M.E. (1992) A promoter element that exerts positive and negative control of the interleukin 2-responsive J-chain gene. *Proc. Natl. Acad. Sci. USA*, **89**, 5966-5970

Lechner, M.S. and Dressler, G.R. (1996) Mapping of Pax-2 transcription activation domains. *J. Biol. Chem.*, **271**, 21088-21093

Li, Y.-S., Hayakawa, K., and Hardy, R.R. (1993) The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. *J. Exp. Med.*, **178**, 951-960

Liberg, D. and Sigvardsson, M. (1999) Transcriptional regulation in B cell differentiation. *Curr. Rev. Immunol.*, **19**, 127-153

Lin, R., Beauparlant, P., Makris, C., Meloche, S., and Hiscott, J. (1996) Phosphorylation of IκB in the C-terminal PEST domain by casein kinase II affects intrinsic protein stability. *Mol. Cell. Biol.*, **16**, 1401-1409

López, A.J. (1995) Developmental role of transcription factor isoforms generated by alternative splicing. *Dev. Biol.*, **172**, 396-411

Lou, M.F., Poulsen, L.L., and Ziegler, D.M. (1987) Cellular protein-mixed disulfides. *Meth. Enzym.*, **143**, 124-129

Lowen, M.A. and Zwollo, P. Functional analyses of alternative isoforms of the transcription factor Pax-5. In preparation.

Lubas, W.A., Frank, D.W., Krause, M., and Hanover, J.A. (1997) O-linked GlcNAc transferase is a conserved nucleocytoplasmic protein containing tetratricopeptide repeats. *J. Biol. Chem.*, **272**, 9316-9324

Luo, R.X., Postigo, A.A., and Dean, D.C. (1998) Rb interacts with hystone deacetylase to repress transcription. *Cell*, **92**, 463-473

Mahmoud, M.S., Huang, N., Nobuyoshi, M., Lisukov. I.A., Tanaka, H., and Kawano, M.M. (1996) Altered expression of Pax-5 gene in human myeloma cells. *Blood*, **87**, 4311-4315

Markus, M. and Benezra, R. (1999) Two isoforms of protein disulfide isomerase alter the dimerization status of E2A proteins by a redox mechanism. *J. Biol. Chem.*, **274**, 1040-1049

Meek, D.W. (1998) Multisite phosphorylation and the integration of stress signals at p53. *Cell. Signal.*, **10**, 159-166

Melchers, F., Rolink, A., Grawunder, U., Winkler, T.H:, Karasuyama, H., Ghia, P., and Andersson, J. (1995) Positive and negative selection events during B lymphopoiesis. *Curr. Opin. Immunol.*, **7**, 214-227

Murre, C., McCaw, P.S., and Baltimore, D. (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and Myc proteins. *Cell*, **56**, 777-783

Neurath, M.F., Strüber, E.R., and Strober, W. (1995) BSAP: a key regulator of B cell development and differentiation. *Immunology Today*, **16**, 564-569

Neurath, M.F., Strober, W., and Wakatsuki, Y. (1994) The murine immunoglobulin 3'α enhancer is a target site with repressor function for the B-cell lineage-specific transcription factor BSAP (NF-HB, Sα-BP). *J. Immunol.*, **153**, 730-742

Nornes, H.O., Dressler, G.R., Knapik, E.W., Deutsch, U., and Gruss,P. (1990) Spatially and temporally restricted expression of Pax-2 during muring neurogenesis. *Development*, **109**, 797-809

Nutt S.L., Urbanek, P., Rolink, A., and Busslinger, M. (1997) Essential functions of Pax-5 (BSAP) in pro-B cell development, difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. *Genes Dev.*, **11**, 476-491

Nutt, S.L., Morrison, A.M., Dörfler, P., Rolink, A., and Busslinger, M. (1998) Identification of BSAP (Pax-5) target genes in early B-cell development by loss- and gain-of-function experiments. *EMBO J.*, **17**, 2329-2333

Nutt S.L., Heavey, B., Rolink, A., and Busslinger, M. (1999) Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. *Nature*, **401**, 556-562

Okabe, T., Watanabe, T., and Kudo, A. (1992) A pre-B and B cell-specific DNA binding protein, EBB-1, which binds to the promoter of the VpreB1 gene. *Eur. J. Immunol.*, **22**, 37-43

Pahl, H.L. and Baeuerle, P.A. (1996) Control of gene expression by proteolysis. *Curr. Opin. Cell Biol.*, **8**, 340-347

Pahlavani, M.A., Harris, M.D., and Richardson, A. (1995) The age-related decline in the induction of IL-2 trabscription is correlated to changes in the transcription factor NFAT. *Cell. Immunol.*, **165**, 84-91

Pahlavani, M.A., Harris, M.D., and Richardson, A. The age-related decline in the induction of DNA binding activity of the transcription factors AP-1 and NFAT is correlated to changes in the expression of $p21^{ras}$/MAP kinase signaling pathway. In: First International Conference on Immunology and Aging; 1996 Jun 16-19; Bethesda (MD): NIH; 1996. p.S-13 (Abstract).

Peters, J.-M. (1994) Proteasomes: protein degradation machines of the cell. *Trends Biochem. Sci.*, **19**, 377-382

Pieri, C, Recchioni, R., Moroni, F., Marcheselli, F., and Lipponi, G. (1992) Phytohemagglitinin induced changes of membrane lipid packing, c-myc and c-myb encoded protein expression in human lymphocytes during aging. *Mech. Ageing Dev.*, **64**, 177-187

Plachov, D., Chowdhury, K., Walther, C., Simon, D., Guenet, J.L., and Gruss, P. (1990) *Pax-8*, a murine paired box gene expressed in the developing excretory system and thyroid gland. *Development*, **110**, 643-651

Powers, D.C., Morley. J.E., and Flood, J.F. (1992) Aged-related changes in LFA-1 expression, cell adhesion, and PHA-induced proliferation by lymphocytes from senescence-accelerated mouse SAM-P/8 and SAM-R/1 substrains. *Cell Immunol.*, **141**, 444-456

Rea, I.M., Stewart, M., Campbell, P., Alexander, H.D., Crockard, A.D., and Morris, T.C. (1996) Changes in lymphocyte subsets, interleukin 2, and soluble interleukin 2 receptor in old and very old age. *Gerontology*, **42**, 69-78

Reimold, A.M., Ponath, P.D., Li, Y.-S., Hardy, R.R., David, C.S., Strominger, J.L., and Glimcher, L.H. (1996) Transcription factor B cell-lineage-specific activator protein regulates the gene for human X-box binding protein 1. *J. Exp. Med.*, **183**, 393-401

Reya, T. and Grosschedl, R. (1998) Transcriptional regulation of B-cell differentiation. *Curr. Opin. Immunol.*, **10**, 158-165

Rinkenberger, J.L., Wallin, J.J., Johnson, K.W., and Koshland, M.E. (1996) An interleukin-2 signal relieves BSAP (Pax-5)-mediated repression of the immunoglobulin J chain gene. *Immunity*, **5**, 377-386

Rogers, S., Wells, R., and Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science*, **234**, 364-368

Rolink, A. and Melchers, F. (1996) B-cell development in the mouse. *Immunol. Lett.*, **54**, 157-161

Rudin, C.M. and Thompson, C.B. (1998) B cell development and maturation. *Sem. Oncol.*, **25**, 435-446

Salceda, S. and Caro, J. (1997) Hypoxia-inducible factor 1α (HIF-1) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. *J. Biol. Chem.*, **272**, 22642-22647

Scaffidi, C., Kirchhoff, S., Krammer, P., and Peter, M.E. (1999) Apoptosis signaling in lymphocytes. *Curr. Opin. Immunol.*, **11**, 277-285

Schreck, R., Rieber, P., and Baeuerle, P.A. (1991) Reactive oxygen intermediates as appearently widely used messengers in the activation of NF-kappa B transcription factor and HIV-1. *EMBO J.*, **10**, 2247-2258

Schulze, D.H., Mancillas, P., Kaushik, A., Bona, C., and Kelsoe, G. (1992) Mitogen-induced V_H and V_κ expression is similar in young adult and aged mice. *Aging: Immunol. Infect. Dis.*, **3**, 127-134

Shaw, P., Freeman, J., Bovey, R., and Iggo, R. (1996) Regulation of specific DNA binding by p53: evidence for a role for O-glycosylation and charged residues at the carboxy-terminus. *Oncogene*, **12**, 921-930

Singh, M. and Birshtein, B.K. (1993) HF-HB (BSAP) is a repressor of the murine immunoglobulin heavy-chain 3'α enhancer at early stages of B-cell differentiation. *Mol. Cell. Biol.*, **13**, 3611-3622

Song, L., Stephens, J.M., Kittur, S, Collins, G.D., Nagel, J.E., Pekala, P.H., and Adler, W.H. (1992) Expression of c-fos, c-jun and jun B in peripheral blood lymphocytes from young and elderly adults. *Mech. Ageing Dev.*, **65**, 149-156

Song, H., Price, P.W., and Cerny, J. (1997) Age-related changes in antibody repertoire: contribution from T cells. *Immunol. Rev.*, **160**, 55-62

Steegenga, W.T., van der Eb, A.J., and Jochemsen, A.G. (1996) How phosphorylation regulates the activity of p53. *J. Mol. Biol.*, **263**, 103-113

Strachan, T. and Read, A.P. (1994) Pax genes. *Curr. Opin. Genet. Dev.*, **4**, 427-438

Stuart, E.T., Kioussi C., Aguzzi, A., and Gruss, P. (1995a) *PAX-5* expression correlates with increasing malignancy in human astrocytomas. *Clin. Cancer Res.*, **1**, 207-214

Stuart, E.T., Haffner, R., Oren, M., and Gruss, P. (1995b) Loss of p53 function through PAX-mediated transcriptional repression. *EMBO J.*, **14**, 5638-5645

Stüber, E., Neurath, M., Calderhead, D., Fell, H.P., and Strober, W. (1995) Cross-linking of OX-40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. *Immunity*, **2**, 507-521

Thanos, D. and Maniatis, T. (1995) NF-κB: a lesson in family values. *Cell*, **80**, 529-532

Tell, G., Scaloni, A., Pellizari, L., Formisano, S, Pucillo, C., and Damante, G. (1998) Redox potential controls the structure and DNA binding activity of the paired domain. *J. Biol. Chem.*, **273**, 25062-25072

Tell, G., Zecca, A., Pellizzari, L., Spessotto, P., Colombatti, A., Kelley, M.R., Damanter, G., and Pucillo, C. (2000) An "environment to nucleus" signaling system operates in B lymphocytes: redox status modulates BSAP/Pax-5 activation through Ref-1 nuclear translocation. *Nucl. Acids Res.*, **28**, 1099-1105

Tian, J., Okabe, T., Miyazaki, T., Takeshita, S., and Kudo, A. (1997) Pax-5 is identical to EBB-1/KLP and binds to the VpreB and lambda5 promoters as well as the KI and KII sites upstream of the Jkappa genes. *Eur. J. Immunol.*, **27**, 750-755

Treier, M., Staszewski, L.M., and Bohmann, D. (1994) Ubiquitin-dependent c-Jun degradation in vivo is mediated by the δ domain. *Cell*, **78**, 787-798

Turner, J.R., Tartakoff, A.M., and Greenspan, N.S. (1990) Cytologic assessment of nuclear and cytoplasmic O-linked N-acetylglucosamine distribution by using anti-streptococcal monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*, **87**, 5608-5612

Urbanek, P., Wang, Z.Q., Fetka, I., Wagner, E.F., and Busslinger, M. (1994) Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. *Cell*, **79**, 901-912

Urbanek, P., Fetka, I., Meisler, M.H., and Busslinger, M. (1997) Cooperation of *Pax-2* and *Pax-5* in midbrain and cerebellum development. *Dev. Biol.*, **94**, 5703-5708

Usui, T., Wakatsuki, Y., Matsunaga, Y., Kaneko, S., Kosek, H., and Kita, T. (1997). Overexpression of B cell-specific activator protein (BSAP/Pax-5) in a late B cell is sufficient to suppress differentiation to an Ig high producer cell with plasma cell phenotype. *J. Immonol.*, **158**, 3197-3204

Vogan, K.J., Underhill, D.A., and Gros, P. (1996) An alternative splicing event in the Pax-3 paired domain identified the linker region as a key determinant of paired domain DNA-binding activity. *Mol.Cell. Biol.*, **16**, 6677-6686

Wakatsuki, Y., Neurath, M., Max, E.E., and Strober, W. (1994) The B-cell specific transcription factor BSAP regulates B cell proliferation. *J. Exp. Med.*, **179**, 1099-1108

Wallin, J.J., Rickenberger, J.L., Rao, S., Gackstetter, E.R., Koshland, M.E., and Zwollo, P. (1999) B cell-specific activator protein prevents two activator factors from binding to the immunoglobulin J chain promoter until the antigen-driven stages of B cell differentation. *J. Biol. Chem.*, **274**, 15959-15965

Walther, C., Guinet, J.-L., Simon, D., Deutsch, U., Jostes, B., Goulding, M. D., Plachov, D., Balling, R., and Gruss, P. (1991) Pax: a murine multigene family of paired box-containing gene. *Genomics*, **11**, 424-434

Weintraub, S.J., Chow, K.N., Luo, R.X., Zhang, S.H., He, S., and Dean, D.C. (1995) Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature*, **375**, 812-815

Xu, W., Rould, M.A., Jun, S., Desplan, C., and Pabo, C.O. (1995) Crystal structure of a paired domain-DNA complex at 2.5 Å resolution reveals structural basis for Pax developmental mutations. *Cell*, **80**, 639-650

Zwollo, P. and Desiderio, S.V. (1994) Specific recognition of the *blk* promoter by the B-lymphoid transcription factor BSAP. *J. Biol. Chem.*, **269**, 15310-15317

Zwollo, P., Arrieta, H., Ede, K., Molinder, K., Desiderio, S., and Pollock, R. (1997). The Pax-5 gene is alternatively spliced during B-cell development. *J. Biol. Chem.*, **272**, 10160-10168

Zwollo, P., Rao, S., Wallin, J.J., Gackstetter, E.R., amd Koshland, M.E. (1998) The transcription factor NF-κB/p50 interacts with the *blk* gene during B cell activation. *J. Biol. Chem.*, **273**, 18647-18655

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