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A

Functions of Transcription Factor Oct-2 in Immunoglobulin-Secreting Cells

by

M. Nusrat Sharif

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York

2000

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ABSTRACT

Functions of Transcription Factor Oct-2 in Immunoglobulin-Secreting Cells

by

Nusrat Sharif

Advisor: Dr. Laurel Eckhardt

Oct-1 and Oct-2 belong to the POU homeodomain family of transcription factors that bind the conserved octamer motif - a key regulatory element found in all Ig promoters as well as in immunoglobulin gene enhancers. Oct-1 expression is virtually ubiquitous, implicated in control of genes expressed by all cells, while Oct-2 is predominantly B lineage-restricted. Both Oct-1 and Oct-2 can functionally interact through their POU domains with a B cell-restricted co-activator called OBF-1/BOB-1/OCA-B to mediate strong immunoglobulin promoter activity. Since both Oct-1 and Oct-2 can mediate Ig promoter activity in B cells, there has been some question as to whether these two octamer binding factors serve distinct function in lymphocytes.

We have used the approach of somatic cell fusion to explore the functional differences between the two octamer binding factors. Previous studies in our lab have

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demonstrated that at the immunoglobulin secreting stage of B cell development, Oct-2 plays a critical role in regulating the expression of several B cell-specific genes. The pronounced effect of Oct-2 on the phenotype of plasmacytoma x T lymphoma hybrids established a unique role for this transcription factor in regulating the genetic program of an Ig-secreting cell.

In the studies presented in this thesis, we have exploited the functional differences between the two octamer binding transcription factors using chimeric hOct-1/hOct-2 proteins in our cell fusion experiments. Our findings further demonstrate a unique function for Oct-2 in Ig secreting cells, and show that this function (rescue of Ig and other B cell-specific genes), cannot be achieved by Oct-1, the other octamer binding factor. Our findings thus provide evidence that the C-terminal domain of Oct-2 is critical for the unique functions of Oct-2 in Ig-secreting cells.

Studies in our lab and others have suggested that a tissue-restricted co-factor interacts with the C-terminal domain of Oct-2 to mediate Oct-2 function. We have used yeast two-hybrid approach to identify proteins that can interact with this region of Oct-2. Preliminary results of our screens are also described in this thesis

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I dedicate this work to the loving memory of

Hadhrat Mirza Nasir Ahmad

The Third Successor of Ahmadiyya Movement in Islam

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In the name of Allah the Gracious the Merciful

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Abbreviations

CMV	Cytomegalovirus
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DMF	*N,N*-Dimethyl-formamide
EMSA	Electrophoretic Mobility Shift Assay
ELISA	Enzyme-linked immunoassays
RT-PCR	Reverse transcriptase-polymerase chain reaction
SD medium	Synthetic minimal medium
GAL4 BD	Gal4 DNA-binding domain
GAL4 AD	Gal4 Activation domain

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CHAPTER 1

Introduction

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I. *Overview of the Immune System*

Two main classes of immune reaction are responsible for defending the vertebrate organisms against invasion by foreign pathogens. They are the humoral or the antibody mediated and the cell mediated immune response. The humoral branch of the immune system is at work by the interaction of B lymphocytes with antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. This interaction is mediated by antibody molecules which serve as effectors of this response. The cell-mediated immunity is fulfilled by effector T cells activated in response to antigen. Although the B and the T lymphocytes develop from a common precursor in the bone marrow, the pluripotent hematopoietic stem cell, their diverse functional capacities are determined by the expression of discrete sets of tissue specific genes. Both B and T cells recognize antigen by unique and specialized membrane receptors namely the immunoglobulins on the surface of B cells and the TCR or the T cell receptor on the T lymphocytes. Although the antigen binding T cell receptor is structurally distinct from an immunoglobulin, it does share some common features most notably in the structure of its antigen binding site. Unlike the membrane bound antibody on B cells, the T cell receptor (TCR) does not recognize free antigen. T cells recognize antigen when it is bound by a self molecule encoded by genes in the Major histocompatibility complex (MHC).
The immunoglobulin genes are expressed only in B lineage cells and even within this lineage they are assembled and often reassembled during the process of differentiation into an antibody secreting plasmacyte. The formation of antibody genes and TCR genes is accomplished by the process of DNA rearrangement events.

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Figure 1.

Schematic diagram of structure of immunoglobulin molecule (Immunology, Third Edition by Janis Kuby). (a) Each heavy and light chain in an immunoglobulin molecule contains an amino-terminal variable (V) region that consists of 100-110 amino acids and differs from one antibody to the next. The remainder of the molecule- the constant (C) region- exhibits limited variation that defines the two light-chain subtypes and the five heavy chain subclasses. The amino-terminal domains corresponding to V regions, function in antigen binding; effector functions are mediated by the other domains.

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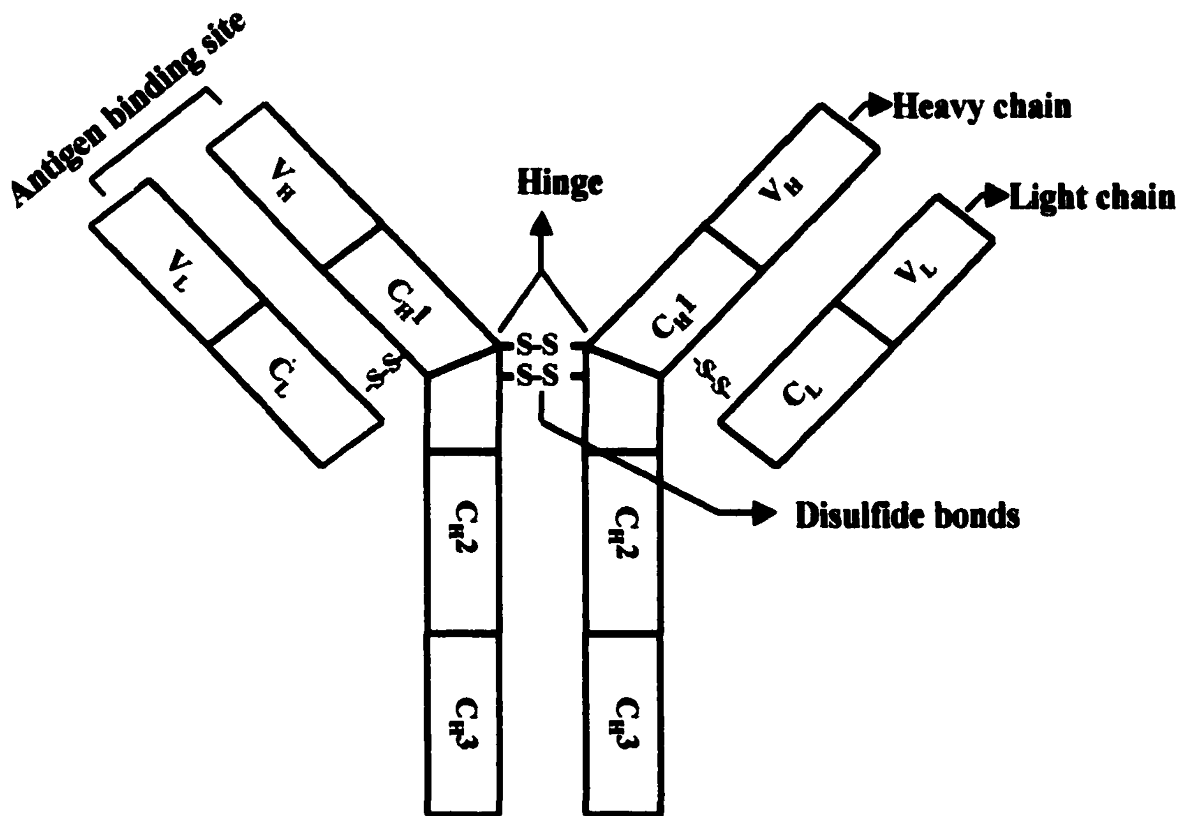

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Each antibody or immunoglobulin (Figure 1) is a Y shaped molecule consisting of two heavy and two light chains, linked together by the interchain disulphide bonds. The first 110 or so amino acids of the amino terminal region of a heavy or light chain varies greatly among antibodies of different specificity. These segments of highly variable sequence are called V regions; V_L in light chains and V_H in heavy. The rest of the antibody molecule is constant in sequences beyond the variable region and is called C region C_L on light chain and C_H on the heavy. The variable region is responsible for the antigen binding capacity, whereas the constant region mediates the physiologic function of the antibody. Immunoglobulins are glycoproteins; with few exceptions, the sites of attachment for carbohydrates are restricted to the constant region.

II. *Immunoglobulin genes in developing B lymphocytes*

The onset of B and T cell development is defined by DNA rearrangement events leading to a unique antigen receptor (Ig) or T cell receptor (TCR), which is controlled by the expression of recombinases RAG 1 and RAG 2 in both B and T cells. The stages of B cell differentiation have been defined as antigen independent (early), including pro-B, pre-B, and mIg^+ B cell stages and antigen dependent (late), including events leading to terminally differentiated plasma cells. The earliest B lineage cells are known as *pro-B cells,* as they are progenitor cells with limited self-renewal capacity. They are derived from pluripotential hematopoietic stem cells and are identified by the appearance of cell surface proteins characteristic of early B lineage cells. A functional heavy chain gene (IgH) during the course of B cell development is assembled from an assortment of V D and J gene segments while a functional light chain gene is assembled from an assortment

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of V and J sequence (Thompson, 1995). The heavy chain locus (H) rearranges first. Rearrangements of D_H gene segment to J_H gene segment occurs in early pro-B cell stages generating late pro-B cells in which a V_H gene segment becomes joined to the rearranged DJ_H. Successful VDJ_H assembly and transcription results in expression of an intact immunoglobulin heavy chain at the cell surface as part of the pre-B cell receptor. Once this occurs the cell is defined as the large pre-B cell which like the progenitor B cells is large and actively dividing. The μ heavy chain in large pre-B cells is expressed transiently on the cell surface in combination with surrogate light chain V pre B and λ5 and Igα and Igβ signal transduction molecules. Large pre-B cells then cease dividing and become small resting pre-B cells in which the μ heavy chain is found inside the cell and in which the light chain genes can be rearranged. Upon successfully assembling a light chain gene, the cell becomes an immature B cell that expresses light chains and μ heavy chains as surface IgM molecules. All the preceding stages of development take place in the bone marrow, independent of antigen; as the surviving B cells now emerge into the periphery, they undergo further differentiation to become mature B cells expressing IgD in addition to IgM. A mature B cell leaves the bone marrow expressing membrane-bound immunoglobulin (mIgM and mIgD) with a single antigenic specificity. These naïve B cells which have not encountered antigen, circulate in the blood and lymph and are carried to the secondary lymphoid organs, most notably the spleen and lymph nodes.

Subsequent steps in B cell development occur in the periphery and require antigen. In the absence of antigen induced activation, naïve B cells in the periphery have a short life span, dying within a few weeks by apoptosis. Antigen-driven activation and clonal selection of naïve B cells leads to generation of plasma cells and memory cells.

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Memory B cells have a longer life span and continue to express membrane-bound antibody with the same specificity as the original parent naïve B cell. Plasma cells do not express membrane-bound antibody; instead they produce the antibody in a form that can be secreted. Although plasma cells live for only a few days, they secrete enormous amounts of antibody during this time. Secreted antibodies are the major effector molecules of humoral immunity.

III. *Transcription Factors affecting B lymphocyte development*

B lymphocyte differentiation can be viewed as an ordered program of gene expression. This developmental process requires changes in transcriptional activity at three critical steps. (a) commitment of a pluripotent hematopoietic stem cell to the B lymphoid lineage, (b) progression from the pre B-cell stage to the mature B cell stage, and (c) terminal differentiation into plasma cells (Staudt and Lenardo, 1991). These changes in gene expression raise two important questions that are relevant to understanding of differentiation programs in many cell lineages. (1) How is tissue specific gene expression achieved? (2) What controls the stable changes in gene expression that occur between developmental stages?

The development of B and T cells from the hematopoietic precursors occurs through a stepwise narrowing of developmental potential that ends in lineage commitment. The key regulators in the commitment process are transcription factors that are both known to be used for the expression of lymphocyte specific genes, and are themselves expressed or activated in a lymphoid specific manner (Figure 2).

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Figure 2.

Diagrammatic representation of B lymphocyte lineage-specific transcription factors. The development of B and T lymphocytes from the hematopoietic precursor occurs in discrete steps and these steps are controlled by lineage specific transcription factors. Transcription factors such as EBF, E2A and Ikaros play an important role in the very early stages of B lymphocyte development, while factors such as Oct-2 have a role in terminal stage of B cell differentiation. Bright is a transcription factor that has a role both in early as well as late stages of B cell differentiation.

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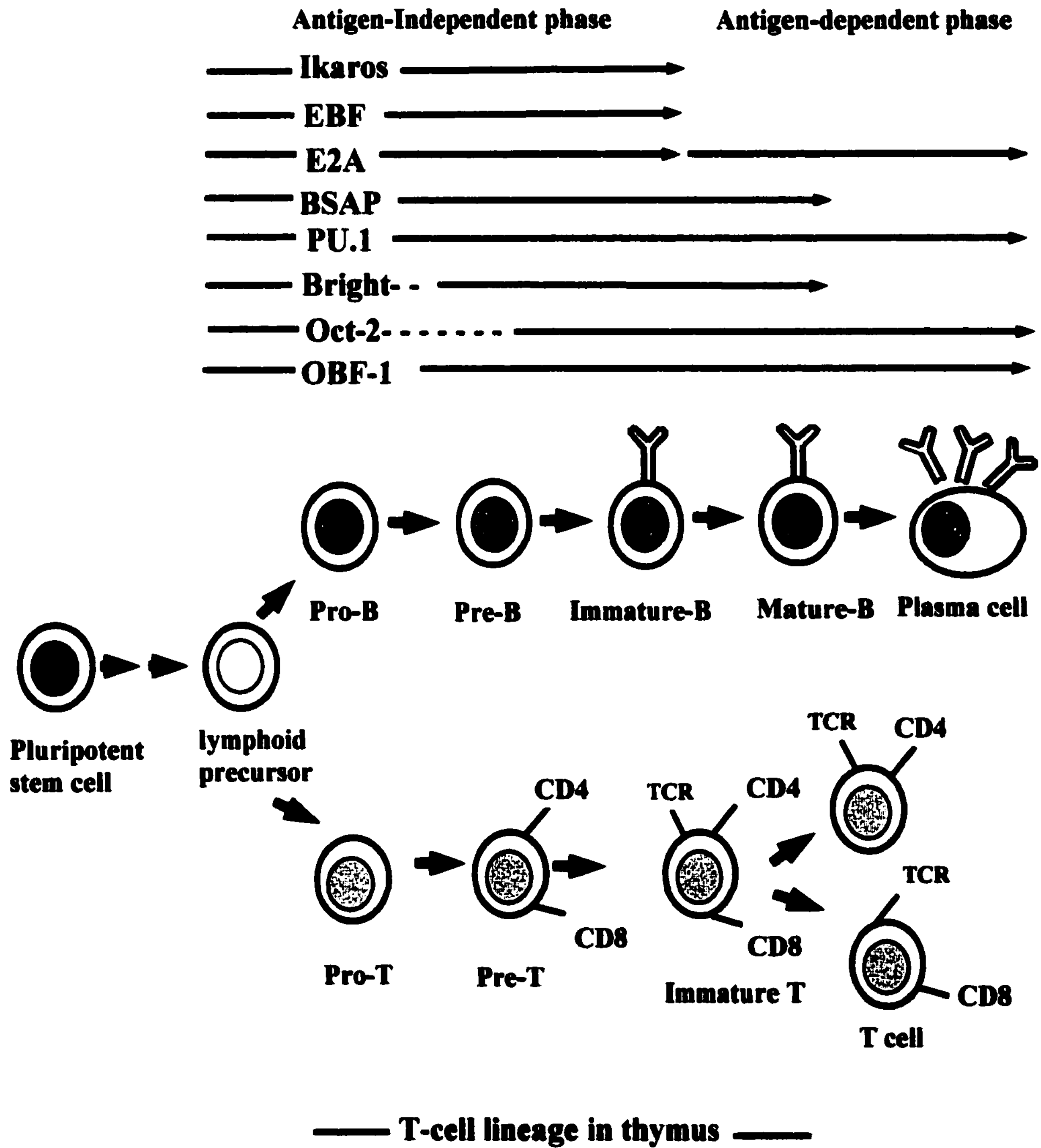

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Some of these transcription factors affect many different hematopoietic lineages, and others affect only a single lineage, such as the developmental pathway that leads to lymphocytes.

One transcription factor that affects multiple lineages is **GATA-2** that recognizes the tetranucleotide sequence GATA in the target genes. It is necessary for the development of the lymphoid, erythroid and myeloid lineages. Animals in which this gene is disrupted die during embryonic development (Clevers and Grosschedl, 1996).

In contrast to GATA-2, another transcription factor, **Ikaros,** is required only for the development of cells of the lymphoid lineage (Figure 2). In the absence of a functional Ikaros gene, the stem cells are diverted into only the erythroid and myeloid lineages (Georgopoulos et al., 1994). Although Ikaros knockout mice do not produce significant numbers of B, T and NK cells, their production of erythrocytes, granulocytes and other cells of myeloid lineage is not impaired (Winandy et al., 1995). Ikaros mice survive embryonic development but are severely compromised immunologically and die of infections at an early age (Wang et al., 1996). Another transcrption factor **PU.1** which is a member of Ets family of transcription factors is important for driving myeloid cell differentiation (Olson et al., 1995). Targeted disruption of the PU.1 locus in mice has revealed a multi-lineage defect in the generation of progenitors for B and T lymphocytes, monocytes and granulocytes (Scott et al., 1997; Scott et al., 1994). With the exception of T cells, evidence for the regulation of tissue and differentiation stage-specific gene expression by PU.1 has been obtained in each of the hematopoietic lineages (Klemsz et al., 1990). PU.1 is essential to early events both in B lymphocytes and monocyte

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development (Simon, 1998) but its functions later in B cell development have not been defined.

The initiation of B cell development critically depends on two transcription factors: the basic helix-loop-helix proteins encoded by the **E2A** gene and **EBF**-early B cell factor. EBF was identified as a novel regulator of the early B cell specific mb-1 (Igα) gene (Hagman et al., 1991). In the absence of either protein (E2A or EBF), B-cell development is aborted at the earliest stage, before D_H-J_H rearrangement of the IgH gene (Bain et al., 1994; Lin and Grosschedl, 1995; Zhuang et al., 1994). Mice that lack E2A or EBF lack RAG1, are unable to make D_H-J_H rearrangement, and fail to express λ5, a critical component of surrogate light chain.

Another transcriptional regulator involved in early B-lymphopoiesis is the B cell-specific activator protein (**BSAP**), (Figure 2) which is encoded by the *Pax5* gene (Li et al., 1996). Within the hematopoietic system *Pax5* is exclusively expressed in the B-lymphoid lineage from the earliest detectable precursor to the mature B cell stage (Adams et al., 1992; Barberis et al., 1989), but not in terminally differentiated plasma cells (Barberis et al., 1990). In the absence of *Pax5*, B cell development is arrested in the fetal liver at a similarly early stage as in *E2A* and *EBF* mutant mice, whereas it proceeds in adult bone marrow up to the early pro-B (pre-B1) cell stage (Urbanek et al., 1994) (Nutt et al., 1997). *Pax-5* deficient pro-B cells undergo D_H-to- J_H recombination at a normal frequency. In contrast, the incidence of V_H-to-D_H J_H rearrangements is reduced ~50 fold compared to wild type pro-B cells (Nutt et al., 1997). Hence these studies show that Pax-5 plays an important role in the developmental pathway controlling the B cell-specific step of the V(D)J recombination process. B-lymphopoiesis in the bone marrow is,

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however, not advanced by expression of a functionally rearranged μ-heavy chain transgene, indicating that the observed developmental block does not solely result from the inability of Pax $5^{-/-}$pro-B cells to undergo V_H-DJ_H rearrangements at the IgH locus (Thevenin et al., 1998).

Binding sites for BSAP are found in the promoter regions of a number of B cell specific genes including Vpre B and λ5, in a number of Ig switch regions and in the 3'Ig heavy chain enhancer (hs1,2). This suggests that BSAP plays a role beyond the early stages of B cell development. Two sites within the 3'αE have been shown to bind BSAP and two groups have presented evidence suggesting that hs1,2 is actively repressed in early B cells by BSAP (Singh and Birshtein, 1996; Singh and Birshtein, 1993).

BSAP functions both as a transcriptional activator and repressor, as it positively controls CD19, mb-1 and N-myc expression and negatively regulates the transcription of PD-1, the gene encoding the cell surface protein (Nutt et al., 1998). As mentioned earlier *mb-1* gene encodes the transmembrane molecule Ig-α, which together with Ig-β (B29) protein, forms a heterodimer mediating signal transduction through the pre-B and the B-cell receptors (Borst et al., 1996). The CD19 protein forms a complex with CD21, CD81 and Leu-13 on the surface of mature B cells, which is known to associate with the B-cell receptor and is important for antigen dependent signaling (Tedder et al., 1997). PD-1 is a member of the immunoglobulin superfamily which has been implicated in signal transduction due to the presence of tyrosine kinase association motifs in its cytoplasmic domain (Ishida et al., 1992). PD-1 is expressed on stimulated B and T cells in different lymphoid organs (Agata et al., 1996). The N-*myc* gene is tightly regulated during B lymphopoiesis, as it is expressed only in pro-B and pre-B cells but not in mature B cells

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(Smith et al., 1992). BSAP is also expressed in the central nervous system and its absence results in severe defects in midbrain development (Urbanek et al., 1994).

In contrast to E2A, EBF and BSAP, that are required for early B cell development, **Bright** is a transcription factor that is tightly regulated both in the early as well as late stages of B cell differentiation, while transcription factor **Oct-2** has an important role in the late stages of B cell differentiation (Figure 2).

The B cell regulator of Ig heavy chain transcription (Bright) is a DNA binding protein that was originally discovered in a mature, antigen-specific B cell line after stimulation with IL-5 and antigen (Webb et al., 1989). The expression of Bright is highly regulated in normal murine lymphocytes and occurs both early (pre B cell) and late (activated mature B lymphocyte) during B cell differentiation (Webb et al., 1998). The first Bright binding sites observed were found 5'of the S107 familyV1 heavy chain promoter, where Bright binding correlated with two to six fold increases in μ heavy chain RNA levels (Webb et al., 1989). Transfectants in which Bright binding sites had been deleted from the V1 gene did not show increased V1 specific heavy chain mRNA levels in response to IL-5 and antigen, implying a role for Bright in heavy chain gene expression. Subsequent co-transfection studies also suggested that Bright may function as a transcription factor when bound to intronic enhancer sequences (Herrscher et al., 1995).

Oct-2 was one of the first transcription factors identified with restricted tissue distribution. Oct-2 is predominantly expressed in B lymphocytes and plays an essential role during the terminal phase of B cell differentiation. The functions of this transcription factor in Ig secreting cells are the focus of this thesis and are discussed in greater detail in this and subsequent chapters.

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IV. *General Mechanisms of Gene Activation by Transcription Factors*

In the early 1980s it became clear that cell type specific DNA rearrangement is not the only factor prohibiting immunoglobulin gene expression in non B cells, because when a fully assembled Ig gene was introduced into cultured non B cells the cells were unable to express it (Eckhardt, 1992). This showed that there were additional control elements that modulated their expression during the course of B cell development so as to ensure their expression only in the appropriate cells in right amount and at the right time. These control elements are promoters and enhancers (Sen and Baltimore, 1986; Staudt and Lenardo, 1991). These regulatory control elements are in turn bound by sequence specific transcription factors, that interact with the *cis* elements and cooperate to regulate Ig gene transcription during the differentiation of B cells.

Transcription factors recognize specific target sequences located in enhancers, promoters or other *cis* regulatory elements that affect a particular target gene. Having bound to DNA, the protein exercises its function by binding to other components of the transcription apparatus. Transcription factors are usually characterized by domains that are responsible for these activities. Often there are separate domains that bind DNA (DNA binding domain) and activate transcription (Activation domain), and which function independently. The main function of the DNA binding domain may be to tether the activating domain in the vicinity of a transcription initiation complex. In principle it requires that the connection between the DNA binding and the activation domain is flexible enough to allow the activation domain to find its protein targets irrespective of the exact site held by the DNA-binding domain in the promoter. The protein target is

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likely to be a basal transcription factor, or a tissue specific co-activator. Experiments with some rather different systems suggest that the principle of independent domains is common in transcriptional activators (Pinkham et al., 1987).

IV A. *DNA-binding and dimerization domains in transcription factors*

Comparisons between the sequences of many transcription factors suggest that common types of motifs can be found that are responsible for binding to DNA. The motifs are usually quite short and comprise only a small part of the protein structure. Motifs have also been identified that are responsible for activating transcription via interactions with proteins of the transcription apparatus. The specific DNA binding activities of several mammalian factors have been localized to relatively small subregions consisting of 60-100 amino acids. These studies have demonstrated that the DNA binding domain is necessary but not sufficient for transcriptional activation (Mitchell and Tijan, 1989). The different types of DNA binding domains found in transcription factors are described.

The zinc finger motif comprises a DNA binding domain. Zinc finger motifs were originally identified as DNA binding structures in the RNA polymerase III transcription factor TFIIIA, which binds to the internal control region of the 5S RNA gene (Miller et al., 1985). TFIIIA like zinc fingers are present in mammalian transcription factor Sp1 (which activates transcription by binding to GC box cis elements) and in a variety of other regulatory proteins. This type of zinc finger motif consists of ~30 amino acids with two cysteine and two histidine residues that stabilize the domain by tetrahedrally coordinating a Zn^{+2} ion. A second class of zinc finger motifs is found in the DNA

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binding domains of steroid hormone receptors. This motif uses two pairs of cysteines rather than the cysteine-histidine arrangement typical of Sp-1.

A second type of DNA binding domain is the homeodomain (HD). This domain which encompases ~60 amino acids was first identified as a conserved protein segment in several regulators of Drosophila embryogenesis, was found in genes of vertebrate organisms as well (Hoey and Levine, 1988). The homeodomain primary sequence is distantly related to the helix-turn-helix DNA binding structures of prokaryotic repressors. Because of the genetically identified regulatory relationships that exist between many of the Drosophila HD-containing proteins that regulate embryogenesis, it was anticipated that HD containing proteins might be DNA binding factors that regulate transcription of genes (Levine and Hoey, 1988). The evidence that HD containing proteins can bind and directly activate transcription of target genes came from biochemical and *in vivo* studies of three mammalian transcription factors, namely Oct-1, Oct-2 (discussed later in this chapter) and Pit-1 (Bodner and Karin, 1987; Clerc et al., 1988; Muller et al., 1988; Scheidereit et al., 1988; Sturm et al., 1988). The DNA binding domains of Pit-1, Oct-1, the lymphoid specific Oct-2 and Unc-86, a developmental regulatory protein of the nematode *Caenorhabditis elegans,* (Finney et al., 1988) constitute a novel subclass within the homeodomain-containing protein family; these factors each contain a conserved bipartite domain of ~160 amino acids, termed the POU domain, which consists of a HD and a second region, the POU box.

The presence of helical regions that bind DNA and the ability of the protein to dimerize are represented in the group of helix-loop-helix proteins (HLH) (Murre et al., 1989). These proteins share a common type of sequence motif; a stretch of 40-50 amino

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acids, containing two amphipathic α helices separated by a linker region (the loop) of varying length. The proteins in this group form homodimers and heterodimers by means of interactions between the hydrophobic residues on the corresponding faces of the two helices. Most HLH proteins contain a region adjacent to the HLH motif itself that is highly basic, and which is needed for binding to DNA. Members of the group with such a region are called bHLH proteins. The members of bHLH group includes two proteins, E12 and E47, that bind to E box elements in the immunoglobulin gene enhancer (in κ and μ intronic enhancer) and the myoD, myogenin and myf 5 transcription factors that are involved in myogenesis (Weintraub et al., 1991). Id (for inhibitor of DNA binding) is a HLH protein that lacks the adjacent basic region, and it inhibits the ability of other HLH proteins to bind DNA when heterocomplexed with them.

A motif found in several transcription factors and involved in both homo and heteromeric interactions is the leucine zipper (Landschulz et al., 1988; McKnight and Yamamoto, 1992). Leucine zippers consist of a stretch of amino acids with a leucine residue in every seventh position. A leucine zipper in one polypeptide interacts with the zipper in another polypeptide to form a dimer. Adjacent to each zipper is a stretch of positively charged residues that is involved in binding to DNA. The mammalian enhancer binding protein C/EBP consists of a highly conserved stretch of ~30 amino acids with a net basic charge followed by a leucine zipper (Johnson et al., 1987), while other factors including Myc and Oct-2 contain leucine repeat motifs without the conserved basic region found in C/EBP factors. The leucine region in Myc is required for tetramerization and for transformation of primary cells (Dang et al., 1989).

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Oct-2 has a leucine zipper-like structure with four leucines (at seven-residue intervals) in the described periodic array in the C-terminal domain of the protein (between amino acids 373 and 394) (Muller et al., 1988; Scheidereit et al., 1988). Compared with the previously described zipper domains (Landschulz et al., 1988), it is unusual in its amino acid content, containing only one charged amino acid. It could nonetheless form an amphipathic α helix, with eleven serine and threonine residues localized on the side opposite the leucine array. Although the functional significance of this zipper, for dimerization, remains to be proven, Oct-2 has been shown to bind as a dimer to two distinct functional elements (heptamer and octamer) in the heavy chain immunoglobulin promoter (Poellinger et al., 1989).

IV B. ***Activation domains in transcription factors***

Transcriptional activation functions of DNA binding factors depend on regions of as few as 30-100 amino acids that are separate from the DNA binding domain. The first defined activation regions in eukaryotic transcription factors were identified by the studies of the yeast factors, GAL4 and GCN4 (Brent and Ptashne, 1985; Ma and Ptashne, 1987) (Ma and Ptashne, 1987a). The activation domain of these factors consist of short stretches of amino acids with significant negative charge and can form amphipathic α-helical structures. The transcriptional activation domains of glucocorticoid receptor also consist of an acidic α-helix. It has been proposed that acidic activation domains may facilitate transcription initiation by interacting in a relatively non specific manner with a general component of the initiation complex, such as TATA binding TFIID or with pol II itself.

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The Sp-1 transcription factor has four separate regions that contribute to transcription activation (Courey et al., 1989; Courey and Tjian, 1988). The potent activation domains consist of ~25% glutamine residues and these glutamine rich regions are also found in other transcription factors such as Oct-1 and Oct-2, Jun, AP-2 and SRF.

A third type of activation domain is a proline rich domain identified in the transcription factor CTF/NF-1 (Mermod et al., 1989). A proline rich domain (20-30%) in the COOH terminus of CTF activates transcription when linked to various DNA binding domains including the zinc fingers of Sp-1.

The three different activation domains (acidic, glutamine rich and proline rich) are likely to represent regions that function by binding other proteins. The idea that all activation domains directly contact and stabilize the binding of the same general transcription factor, such as TATA binding TFIID, seems unlikely, unless TFIID can accommodate multiple interaction surfaces. At least five general transcription factors are required for initiation by pol II (Saltzman and Weinmann, 1989), and, as described above transcription factors have different types of activation domains to contact different general factors or different subunits of pol II.

The regulatory activity of a transcription factor is influenced both by the context of the binding site in the promoter or enhancer and by cell type specific factors and co-activators (Roberts and Green, 1995). Therefore it is important to study the activity of the factor in the context of the appropriate cell type. A common approach used to distinguish the regulatory activities of related transcription factors involves over-expression of individual family members in an appropriate cell type. Targeted disruption of genes

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encoding individual family members represents a powerful tool of analyzing their function and is widely used in the study of functions of a specific transcription factor. It was through this experimental approach that the several transcription factors described earlier (BSAP, EBF, E2A etc.) were shown to have functions in B lymphocyte development.

V. *Transcription factor binding motifs within the regulatory sequences of the IgH locus*

The immunoglobulin heavy chain (IgH) intronic enhancer or Eμ is a well characterized regulatory element that lies between the J_H gene segment cluster and the μ constant region sequence (Figure 3) (Banerji et al., 1983; Gillies et al., 1983; Neuberger, 1983). Like the viral enhancers, the intronic enhancer functions in either oritentation, however unlike viral enhancers previously identified, it shows tissue specificity (Banerji et al., 1983; Grosschedl and Baltimore, 1985). Originally identified by virtue of its effects on the transcription of cloned genes introduced into B-lineage cells, Eμ has been shown to play an important role in V_H gene assembly as well (Chen et al., 1993; Serwe and Sablitzky, 1993). The role of Eμ in the late stage of B cell development was however questionable as a number of cell lines lacking this element continued to produce IgH chains at high levels (Eckhardt and Birshtein, 1985; Klein et al., 1984; Zaller and Eckhardt, 1985). The intronic enhancer functions *in vivo* by interacting with trans-acting factors (Ephrussi et al., 1985). B-cell specific proteins have been shown to bind to the octamer, and μB motif.

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Figure 3.

A. Schematic representation of murine IgH locus. The IgH locus is not drawn to scale but shows the relative locations of regulatory elements involved in the control of heavy chain gene expression. The intronic enhancer, Eμ, is located within the intron between the last J_H segment and Cμ. Four 3'IgH enhancers, Hs3a, Hs1,2, Hs3b, Hs4, are located at the far 3'end of the locus. They span ~33 kb region over 170 kb from the J_H region.

B. A diagram of protein binding sites within the intronic enhancer (Eμ). Tissue-specific factors to their cognate binding sites are indicated. C1,C2 and C3 symbolize "core"sequences which have homology to the SV40 enhancer element.

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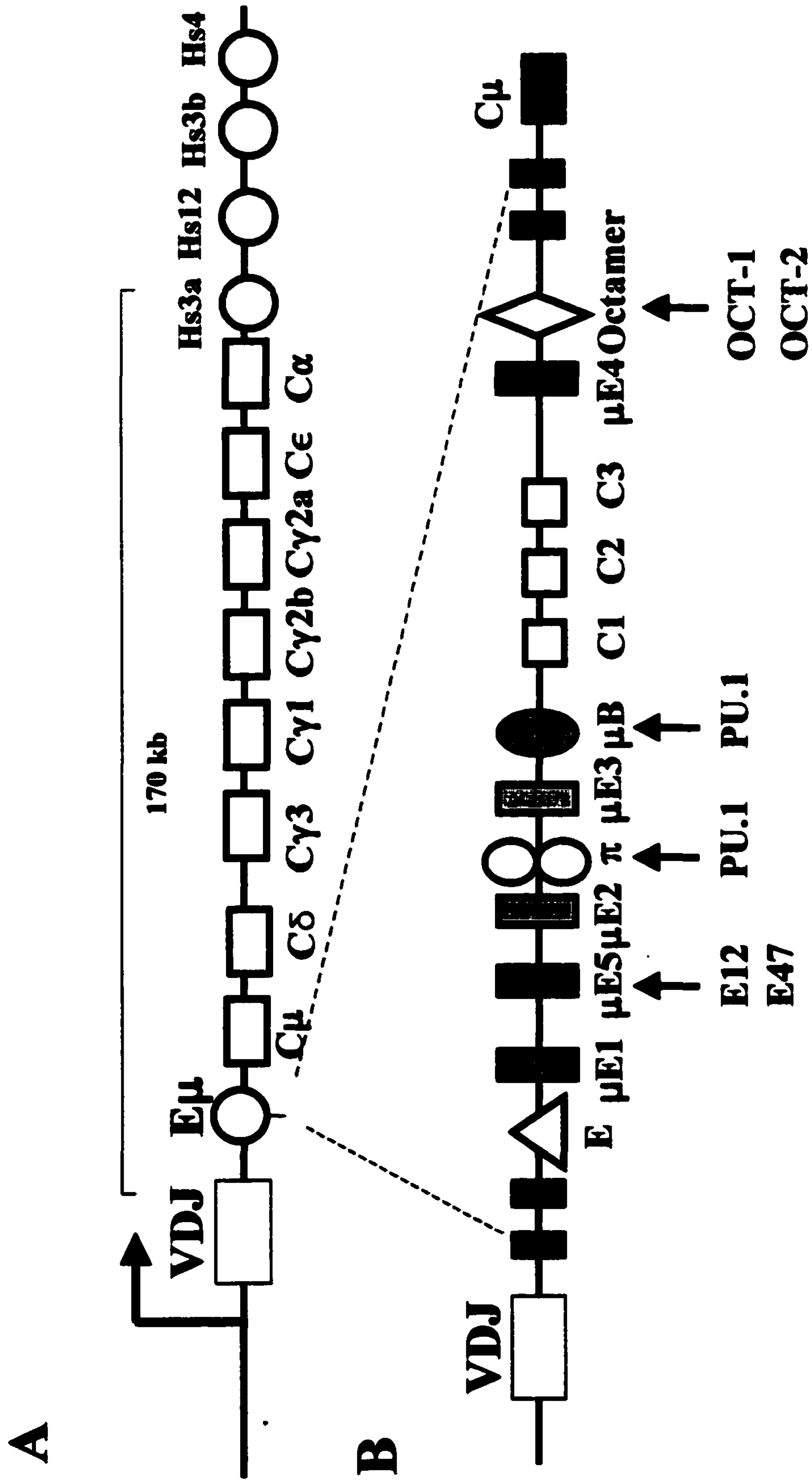

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μE1, μE2, μE3 and μE5 motifs within the intronic enhancer were shown, by *in vitro* binding assays to bind ubiquitous proteins. The intronic enhancer is negatively regulated in non lymphoid cells (Kadesch et al., 1986; Lenardo et al., 1987; Weinberger et al., 1988; Zaller et al., 1988). Thus the tissue specificity of this enhancer is dually controlled by positive and negative influences (Staudt and Lenardo, 1991).

V A. *E-box motifs in the intronic enhancer*

The μE motifs (μE1-μE4) (Figure 3) which share the consensus (CAGGTGGC) are also found in Kappa light chain intronic enhancer (κE1-κE3) (Eckhardt, 1992), and bind a variety of transcription factors (Sen and Baltimore, 1986). Full activity of both Eμ and iEκ was shown to be dependent on these E box elements, suggesting that enhancer activity would also be dependent on the E protein family (Kiledjian et al., 1988). E motifs also govern regulatory elements restricted to other tissues, notably the muscle-specific creatine kinase enhancer (Araki et al., 1988) and the pancreatic β-cell insulin promoter (Libermann et al., 1990). Although the μE1, μE2, μE3 and μE5 sites share the same consensus sequence, they have been shown to bind distinct proteins, all of which belong to the basic helix-loop- helix family of transcription factors (Murre et al., 1989).

The bHLH proteins have been categorized into different classes based on dimerization specificity and tissue distribution.

The class I bHLH proteins bind to E-motifs with high affinity as homodimers or heterodimers with one another. Members of this class are ubiquitously expressed. Two proteins of this class E12 and E47 are derived from the E2A locus by differential splicing

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of E12 and E47 specific, basic-HLH (bHLH) encoding exons (Murre et al., 1989; Sun and Baltimore, 1991). The sequence similarity between these two spliced products and their identical expression patterns raised the question of whether they performed distinct functions during B lymphopoiesis. As noted earlier, inactivation of the E2A locus, through gene targeting results in a complete block in B cell differentiation. The block to B cell differentiation occurs prior to immunoglobulin gene $D_H J_H$ rearrangement and the expression of B-lineage specific marker B220. Analysis of E2A-deficient mice expressing transgenes for either E12 alone and/or E47 alone reveal distinct roles for the two proteins at the pro B to pre B cell transition. Although the expression of the E12 transgene in the E2A deficient background promoted development of pro-B cells, E12, in the absence of E47 was unable to support the development of mature-B lymphocytes (Bain et al., 1997).

The class II HLH proteins comprises MyoD, myogenin, NeuroD, and members of the achaete-scute complex in Drosophila melanogaster, and display a tissue restricted expression pattern and bind DNA only as heterodimers with the class I HLH proteins (Murre et al., 1991). Class II HLH proteins cannot interact efficiently with DNA as homodimers but do bind as heterodimers with class I HLH proteins.

In B cells, there do not appear to be any class I bHLH proteins. Nevertheless, one or more protein complexes are seen to occupy the μE5 site of the IgH intronic enhancer in electrophoretic mobility shift assays (Murre et al., 1991). These complexes (BCF-1 and BCF-2) are believed to consist of oligomers of E12 and E47 proteins. Although these E2A proteins are present in non B cells they cannot form these BCF-1 and BCF-2 complexes in a non B cell environment (Murre et al., 1991). One possible explanation for this observation is that the non B cells contain HLH inhibitor proteins that can dimerize

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with E12 and E47 creating complexes incapable of binding DNA. One such inhibitor of bHLH proteins is called Id1. Id1 has a HLH domain but not the adjacent basic region, and can dimerize with bHLH proteins resulting in non-DNA binding heterodimers (Id+E12 and Id+E47) or heterotrimers (Id+E47-E12) of E12/E47 proteins (Benezra et al., 1990).

It is found that Id1 mRNA is present at varying levels in all cells tested, making unlikely a simple correlation between the presence of Id and inactivity of enhancer motifs dependent on HLH proteins. A more likely interpretation is that the positive and negative control of these enhancer sequences is achieved in part through a fine tuning of the ratio of Id like proteins to positively-acting HLH transcription factors (Eckhardt, 1992).

As noted earlier, inactivation of the E2A locus, through gene targeting results in a complete block in B cell differentiation. The block to B cell differentiation occurs prior to immunoglobulin gene D_HJ_H rearrangement and the expression of B-lineage specific marker B220.

A role for E proteins in late events in B cell differentiation, specifically in heavy chain class switching has been demonstrated using a cell line which undergoes spontaneous and inducible isotype switching. Over-expression of Id1 in this cell line blocked both spontaneous and, to a lesser extent, cytokine-inducible class switching (Goldfarb et al., 1996).

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V B. ***The μB motif in Eμ***

Another motif found in the intronic enhancer and active only in B cells is the *μB* motif (TTTGGGGAA) (Nelson et al., 1990) (Figure 3). When the μB motif was destroyed, there was a significant (50-60%) reduction in the activity of the intronic enhancer in mature B and plasmacytoma cell lines and an even greater reduction (60-95%) in pre-B cell lines (Libermann and Baltimore, 1993; Nelson et al., 1990). In vitro footprinting experiments with nuclear extracts made from B- lymphoid cell lines showed a strong foot-print over the μB element (Libermann and Baltimore, 1993; Nelson et al., 1990). A subfragment of the IgH intronic enhancer, a 70bp fragment containing μB and adjacent μE3 sites behaved as a transcriptional enhancer in B cells. When either of these sites was destroyed or mutated, it resulted in a reduction of this activity (Libermann et al., 1990). It has been shown that PU.1, (Figure 3) a macrophage and B cell-restricted transcription factor, binds this motif (Nelsen et al., 1993; Rivera et al., 1993). As described earlier mice lacking this factor show a multilineage defect in the generation of progenitors for B and T lymphocytes, monocytes and granulocytes.

V C. ***Octamer Motif***

The octamer motif -ATTGCAAT- is a highly conserved regulatory element that is found in all Ig heavy (H) and light (L) chain gene promoters (Falkner and Zachau, 1984; Parslow et al., 1987) as well as in the intronic enhancer (Eμ) (Banerji et al., 1983; Gillies et al., 1983) (Figure 3). The octamer motif is also found in the various enhancers at the 3' end of the IgH locus (Dariavach et al., 1991; Lieberson et al., 1991; Matthias and Baltimore, 1993; Pettersson et al., 1990) as well as in promoters of several other B cell

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restricted genes (B29 and J-chain genes). The octamer element also functions as an important regulatory element in the expression of certain ubiquitously expressed genes. It is involved in the cell cycle regulation of histone H2B gene (LaBella et al., 1988) and in the transcription of small nuclear RNA's (snRNA) (Dahlberg and Lund, 1987).

The functional significance of the octamer element for Ig promoter function and B cell activity was first demonstrated by transfection experiments using truncated versions of Ig V_H or V_L promoter constructs (Bergman et al., 1984; Falkner and Zachau, 1984; Mason et al., 1985). The constructs were inactive when truncations eliminated the octamer element. Synthetic promoters combining the TATA box and the octamer element were active only in B cells thereby showing that the octamer motif was sufficient to mediate B lymphocyte specific expression of immunoglobulin genes (Gerster et al., 1987; Lenardo et al., 1987; Wirth et al., 1987).

Studies with transgenic mice have revealed that mutation of the octamer within an IgH transgene promoter reduced expression of the transgene in B lymphocytes 30 fold (Jenuwein and Grosschedl, 1991). It was found that multimerization of a 51bp sub fragment of Eμ that contained the octamer resulted in a potent and tissue-specific enhancer. Further evidence for a crucial role for the octamer site in Ig gene transcription came from somatic cell fusion experiments (described later in this chapter under Oct-2 function as revealed by somatic cell fusion experiments).

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VI. *Octamer binding factors Oct-1 and Oct-2, Coactivator OBF-1, and the postulated functions of these proteins in B lymphocytes*

The two best characterized octamer binding proteins are transcription factors Oct-1 and Oct-2. Oct-1 protein is ubiquitously expressed, while the expression of Oct-2 is largely B-lineage restricted (Landolfi et al., 1986; Staudt et al., 1986). Oct-1 and Oct-2 belong to a novel family of transcription factors that share a well conserved DNA binding domain called the POU domain (Herr et al., 1988). POU domain transcription factors have been shown to be critical regulators of various developing cell lineages (Rosenfeld, 1991). The POU domain is a region of sequence similarity between three mammalian transcription factors, Pit-1, Oct-1 and Oct-2 and the product of nematode gene *unc86* (Bodner and Karin, 1987; Herr et al., 1988). The POU domain is a 150 amino acid long bipartite domain which comprises a novel POU specific domain linked to a variant homeodomain (Sturm and Herr, 1988). Oct-1 and Oct-2 proteins are very related in their POU domains displaying 87% identity over the entire POU domain. The POU specific region consists of 75 amino acid residues and is separated by a short non conserved linker DNA (15-20 amino acids) from the POU homeobox (60 amino acids) (Sturm and Herr, 1988). Comparison of the nucleotide sequence of Oct-1 (Sturm et al., 1988) and Oct-2 (Clerc et al., 1988) genes reveals that the POU specific regions of the two proteins are 99% identical at the amino acid level but not at the nucleotide level, because the nucleotide sequence at the silent positions has diverged greatly. The POU domains of Oct-1 and Oct-2 differ by one residue in the 75 amino acid long POU specific domain and by seven residues in the 60 amino acid long POU homeodomain.The POU specific

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domain recognizes the 5'portion of the octamer (Verrijzer et al., 1992) and is required for high affinity binding of the octamer binding factors to DNA (Rosenfeld, 1991). The POU homeodomain contacts the 3' portion of the octamer (Verrijzer et al., 1990) and is required for DNA binding. Without the POU specific domain, the POU homeodomain is capable of low affinity DNA binding. The POU homeodomain has a helix-turn-helix region that is important for high affinity DNA binding of the intact POU domain (Sturm and Herr, 1988).

Octamer transcription factors seem to represent a novel class of transcriptional activators that strongly depend on cell type-specific cofactors to perform their activities. This dependence might represent a novel strategy to achieve tissue specific gene expression. The POU domains of Oct-1 and Oct-2 proteins are capable of making unique and specific protein-protein interactions. The POU domain of Oct-1 can bind to the herpes simplex virus transcription factor VP16 and the resulting complex can stimulate transcription from a TAATGARAT containing site that responds to neither Oct-1 nor VP16 alone (Cleary et al., 1993). A single glutamic residue at position E22 in the first helix of the homeodomain of Oct-1 (POU_{H1}) was shown to be the single critical residue responsible for this transactivation. In Oct-2, POU homeodomain (POU_{H2}) this position is a divergent alanine, and does not allow for complex formation with VP16.

MAT1 was identified as a POU domain interacting factor by a yeast two hybrid screen of a human B cell cDNA library using POU domain of Oct-1 as a bait (Inamoto et al., 1997). MAT1 is a subunit of CDK activating kinase (CAK). CAK consists of MAT1 along with CDK7 and cyclin H; MAT1 acts to assemble the latter two subunits to form a stable active kinase containing all three subunits. MAT1 was shown to interact with the

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POU domains of Oct-1, Oct-2 and Oct-3 *in vitro* in a DNA independent manner. Some populations of CAK exist as part of general transcription factor TFIIH and MAT1 containing TFIIH was also shown to interact with POU domains of Oct-1 and Oct-2. (Inamoto et al., 1997). MAT1 not only binds the POU domains of Oct factors directly, but consequently enhances their phosphorylation by CAK.

The RNA polymerase pol II and pol III specific multi-protein transcriptional coactivator SNAPc/PTF interacts, through the largest of its four subunits, (SNAP190), with the POU specific domains of both Oct-1 and Oct-2 (Mittal et al., 1996; Murphy et al., 1992). $SNAP_c$/PTF itself binds to a conserved proximal sequence element found in close proximity to the octamer site found in many small nuclear RNA (snRNA) promoters. This binding is independent of the SNAP 190 interaction with the POU domain of Oct-1 and Oct-2, but it is enhanced approximately 8-10 fold in the presence of this interaction, demonstrating cooperative binding of these two factor complexes to snRNA promoters (Mittal et al., 1996).

The POU domains of Oct-1 and Oct-2 can also interact functionally with a B cell specific coactivator protein referred to as Bob-1, OBF-1, or OCA-B to generate strong IgH promoter function (Gstaiger et al., 1995; Luo and Roeder, 1995; Strubin et al., 1995). Amino acid residues L6 and E7 in the POU specific domain (POU_S) and residues K155 and 1159 in the POU homeodomain (POU_H) are found to be critical in *vitro* and in *vivo* for selective interaction with OBF-1 (Sauter and Matthias, 1998).

On either side of the centrally located and highly conserved POU domain, Oct-1 and Oct-2 share little homology with each other in their N and C-terminal domains. The N terminal region of Oct-1 is 80 amino acids longer than the N terminal domain of Oct-2

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and the two N-terminal domains show 28.5% amino acid sequence homology (Clerc et al., 1988; Herr et al., 1988; Sturm et al., 1988; Tanaka and Herr, 1990). They have in common a high concentration of glutamine residues, however, a feature shared by the transactivation regions of many transcription factors. The carboxyl terminal regions of Oct-1 and Oct-2 also show very little homology in size and sequence (~12% homology) but again there is some similarity as both have stretches rich in serine, threonine, and proline residues (Clerc et al., 1988; Herr et al., 1988; Sturm et al., 1988; Tanaka and Herr, 1990).

Many early studies suggested that the tissue specific transcription factor Oct-2 played a central role in the lymphoid specific expression of immunoglobulin genes. This was because Oct-2 had the capacity to trans-activate co-transfected reporter plasmids in non B cells in an octamer site dependent manner (Gerster et al., 1990; Muller et al., 1988; Tanaka and Herr, 1990). The same transgenes were inactive in non-B cells in the absence of Oct-2. Since Oct-1 was ubiquitously present in these cells, it was thought that it was not capable of trans-activating these Ig like promoters but instead was responsible for ubiquitous function of the octamer element in the cell cycle regulation of the histone H2B gene and in the transcription of snRNA.

Transient cotransfection experiments using reporter genes with octamer (promoter or enhancer position) in non B cells identified both the N and C-terminal domains of Oct-2 as having transactivation function. The two transactivation domains were apparently redundant (i.e. could have only one or the other) with respect to transactivation of an octamer-containing promoter (Gerster et al., 1990; Muller-Immergluck et al., 1990). The two trans-activation domains differed with respect to transactivation from a distance,

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however, the C-terminal domain of Oct-2 was crucial for efficient enhancer transactivation (Annweiler et al., 1992; Annweiler et al., 1994; Pfisterer et al., 1994). Interestingly, enhancer activation by Oct-2 was observed only in B cells, suggesting a requirement for an additional B-cell restricted component.

In one study involving co-transfections of Oct-1 or Oct-2 transgenes into non-B cells, it was found that Oct-2 and not Oct-1 could trans-activate a synthetic octamer-dependent promoter. The functional difference between Oct-1 and Oct-2 was localized to the C-terminal transactivation domain and correlated to differential phosphorylation (Tanaka and Herr, 1990). On the other hand, several studies, suggested that Oct-1 and Oct-2 were functionally interchangeable within lymphoid cells (Johnson et al., 1990), (LeBowitz et al., 1988; Luo et al., 1992; Pierani et al., 1990). Both the ubiquitously expressed Oct-1 and the B cell restricted Oct-2 were able to activate an octamer dependent promoter in otherwise octamer binding factor-depleted B cell extracts, but this required their association with a B cell restricted coactivator protein OBF-1/OCA-B/BOB-1 mentioned earlier (Gstaiger et al., 1995; Luo and Roeder, 1995; Strubin et al., 1995).

As discussed earlier, OCA-B/OBF-1 / Bob 1 (subsequently referred to as OBF-1 in this thesis) is a B cell-specific cofactor that directly interacts with Oct-1 and Oct-2. OBF-1 also makes specific protein-base contacts in the major groove of the conserved octamer element (Cepek et al., 1996). OBF-1 can activate octamer dependent reporter genes in transient transfections or in vitro transcription assays, by being recruited to the octamer sequence through interaction with Oct-1 or Oct-2 (Gstaiger et al., 1995; Luo and Roeder, 1995; Strubin et al., 1995). In these assays, while the Ig promoter was well

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activated by Oct-1 or Oct-2 and OBF-1, addition of OBF-1 only weakly augmented histone H2B promoter activity, suggesting that *trans*-activation by OBF-1was promoter specific (Luo and Roeder, 1995; Strubin et al., 1995). In addition, OBF-1 augments Oct-1/Oct-2 transactivation of octamer-dependent promoters but not enhancers (Pfisterer et al., 1995; Schubart et al., 1996). The exact mechanism of transcription co-activation by OBF-1 is not understood, but can partly be explained by the presence of a strong transcription activation domain in the C-terminal domain of OBF-1(Pfisterer et al., 1995). The N terminal region of OBF-1 is involved in interactions with the Oct factors (Gstaiger et al., 1996; Strubin et al., 1995). OBF-1 is highly selective for the POU domain it binds. Although it binds the POU domains of Oct-1 and Oct-2, it does not bind the POU domain of Oct-4, Oct-6 or Pit 1 factors. OBF-1 expression is highly cell specific and is found in B lymphocytes of all developmental stages (Schubart et al., 1996; Strubin et al., 1995) as well as transiently in T lymphocytes upon activation (Sauter and P, 1997; Zwilling et al., 1995). The induction of OBF-1 in T lymphocytes is correlated with phosphorylation of Ser ^{184} of OBF-1, leading to a transcriptionally active form of the protein (Zwilling et al., 1997). OBF-1 appears to be phosphorylated on the same residue in B cells, and hence it has been postulated that in B cells, the OBF-1 kinase is constitutively active as compared to T lymphocytes. It was suggested that in T cells, OBF-1 contributes to the activity of octamer binding sites important for transcription of inducible T cell growth factor genes such as IL-2, IL-5 and others.

Evidence for unique roles played by Oct-2 and by OBF-1 in the terminal stage of B cell differentiation was revealed by targeted knock-outs of loci encoding both proteins in mice.

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Genetic inactivation of *Pou2f2* (murine locus encoding Oct-2) in a B cell line as well as in embryonic stem cells from which mice were further generated revealed that Oct-2 was not essential for Ig gene assembly nor for Ig gene transcription in early-stage B cells (Corcoran et al., 1993; Feldhaus et al., 1993). Ig+ spleen cells from the Oct-2 deficient mice, however appeared to be blocked from further differentiation, suggesting that Oct-2 might serve an essential function in generating and /or maintaining late stage B cells (Corcoran and Karvelas, 1994; Humbert and Corcoran, 1997). The complication in studying this issue further was that the animals died soon after birth. The cause of neonatal lethality has yet to be determined but may be related to the fact that Oct-2 is expressed in a subset of brain cells.

Because *Pou2f2*$^{-/-}$ mice die at birth, further studies were done by reconstitution of SCID or RAG$^{-/-}$ mice with *Pou2f2*$^{-/-}$ fetal liver cells (Corcoran and Karvelas, 1994). In such chimeras, the B and T cell compartments developed essentially normally, indicating that B cell development and Ig transcription could proceed in the absence of Oct-2. In these mice serum Ig levels were reduced, in particular IgM, IgG1, IgG3 and IgG2b. In a proliferation assay, splenic B cells from such chimeric mice failed to respond to stimulation by LPS or anti-μ antibody (T cell independent B cell activators). This defect was manifested by an arrest in the G1 phase of the cell cycle (Corcoran and Karvelas, 1994). Further studies with RAG$^{-/-}$mice reconstituted with *Pou2f2*$^{-/-}$ lymphoid cells have shown that these chimeric mice do not respond to TI or to T cell dependent (TD) antigens (Humbert and Corcoran, 1997). Thus, although initial Ig gene transcription, as well as B cell development, proceed normally in the absence of Oct-2, late steps of immune response are dependent on this transcription factor.

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Like the *Pou2f2*$^{-/-}$ knock-out mice, OBF-1$^{-/-}$knock-out mice produced surface Ig positive B cells, suggesting that neither Oct-2 nor OBF-1 is essential for the activation of Ig genes or for early events in B cell development (Corcoran et al., 1993; Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996). It remains untested whether they are functionally redundant at this early stage in B cell development. Both types of knock-out mice, however, showed defects in events that commonly occur after B cell activation (i.e. post-antigenic challenge), (Humbert and Corcoran, 1997; Kim et al., 1996; Nielsen et al., 1996) (Qin et al., 1998). In the OBF-1 knock-out mice there was a B cell defect as evidenced by the lack of germinal centers and severe reduction in the serum levels of IgA, IgG2a, IgG2b, IgG3 and IgE (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996). The development of plasma cells was not affected, however (Qin et al., 1998 1001). Rather cells that have the switched isotype appear to express the new isotype at reduced levels. While the phenotypes of OBF-1$^{-/-}$ and *Pou2f2*$^{-/-}$ mice are not identical, the overlap is great enough to suggest that Oct-2 and OBF-1 function together in multiple genetic events occurring subsequent to antigen-stimulation.

VII. *Oct-2 Function as revealed in Somatic Cell Fusion Experiments: prelude to this thesis*

It was known in the early 1970s that when two different cell types were fused together to generate a stable hybrid, genes that were active in one of the parents were frequently shut off, a phenomenon referred to as "gene extinction." A well known example of extinction is the silencing of immunoglobulin gene expression when an immunoglobulin producing myeloma is fused to a non B cell such as a fibroblast or a T

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lymphoma (e.g. Zaller et al., 1988). The targets for cell fusion mediated gene extinction were identified to be the Ig heavy and light chain gene promoters and the intronic enhancer (Junker et al., 1988; Zaller et al., 1988). Somatic cell fusion experiments have revealed a crucial role for the octamer element in Ig gene transcription. In these assays reporter constructs under the control of Ig gene regulatory elements (e.g. a heavy chain gene promoter) were first stably integrated into B cell lines. These reporter cell lines were then fused either to fibroblasts or to a T lymphoma line (BW5147) and expression of the reporter gene was subsequently measured in the hybrids (Junker et al., 1988; Junker et al., 1990; Yu et al., 1989). It was found that expression of the reporter gene was rapidly silenced as was the expression of the endogenous Ig gene. Replacement of a kappa promoter octamer site by an Sp1 or NF1 binding site rendered the promoter resistant to extinction (Junker et al., 1990). Reciprocally the octamer element was shown to be sufficient to mediate gene repression in somatic cell hybrids (Shen et al., 1993). Thus it appeared that the octamer element, either as a natural element of Ig promoters or as an artificial component of chimeric promoters or synthetic enhancers was able to mediate B cell specific gene transcription.

When an immunoglobulin producing plasmacytoma was fused with a T lymphoma, hybrids ceased expression not only of Ig genes but also of other plasmacytoma-specific genes (Yu et al., 1989; Zaller et al., 1988), expressing instead the genes of the T cell parent (Hines et al., 1998; Lieberman et al., 1993). It was found that in rare hybrids that continued to express Ig, other plasmacytoma genes also remained active while the T cell specific genes were silenced (Hines et al., 1998; Lieberman et al., 1993).

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The coordinate silencing and expression of the plasmacyte-specific genes in these two types of hybrids suggested that these genes were under the control of a common regulator.

Several transcription factors with restricted tissue-distribution were silenced in the plasmacytoma x T lymphoma and plasmacytoma x fibroblast hybrids (eg PU.1,Oct-2, BCF-1,OBF-1) (Bergman et al., 1990; Junker et al., 1990; Yu et al., 1989). It was possible that that one of these factors was the postulated common-regulator. This regulator (perhaps working in opposition to a similarly pleiotropic negative regulator derived from the T lymphocyte) might work directly on each of the remaining transcription factor genes and the plasmacytoma-specific structural genes (eg Ig). Alternatively it might sit at the apex of a regulatory cascade, driving the expression of a subset of downstream transcription factor genes that, in turn, were each required for expression of subsets of plasmacytoma-specific structural genes. To test these hypotheses, one of the silenced transcription factors (Oct-2) was re-introduced into the plasmacytoma x T lymphoma hybrids and its ability to rescue endogenous Ig gene expression was determined (Radomska et al., 1994). Four such hybrids (resulting from plasmacytoma x T lymphoma fusions) were transfected with an Oct-2 expressing plasmid (pCGNOct-2). Despite high levels of hOct-2 (human Oct-2) in the transfected hybrid lines, none of the lines produced endogenously-encoded Ig $\gamma 2b$ or κ chains (Radomska et al., 1994). In a similar reconstitution experiment in plasmacytoma x fibroblast hybrids, transient expression of Oct-2 in the hybrid lines led to low level expression of a formerly silent immunoglobulin promoter driven reporter gene, but endogenous gene expression was not assayed (Junker et al., 1990).

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In previous studies done in our lab, it was found that transcriptional silencing resulted in changes in chromatin structure in the vicinity of the affected genes (Zaller et al., 1988). This suggested that while one event might initiate the silencing of a gene, its reversal might not re-establish gene expression. For example, a repressive transcription factor might terminate transcription and lead to subsequent methylation of the silenced locus. Loss of the repressor would not necessarily be sufficient to "reactivate" (demethylate) the same locus. Thus the provision of Oct-2 to an Oct-2 deficient hybrid was not sufficient to re-activate extinguished myeloma specific genes. In order to determine whether preventing loss of Oct-2 at the time of fusion would affect the outcome, hOct-2 under the influence of a viral promoter and enhancer was introduced into plasmacytoma cell lines (Radomska et al., 1994). The stable transformants constitutively expressing hOct-2 (hOct-2 that could not be silenced) were then fused with the T lymphoma fusion partner.

Remarkably, it was found that if the expression of Oct-2 was maintained in the plasmacytoma cell line prior to fusion with the T lymphoma, Ig and all other B cell-specific genes (J-chain, endogenous Oct-2 and transcription factor PU.1) assayed were simultaneously rescued from silencing (Radomska et al., 1994). This showed that Oct-2's role in Ig secreting cells is a critical one and is required for the continued expression of numerous tissue-specific genes. This places Oct-2 at a central or primary position in a hierarchy of gene function.

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Overview of Thesis Study

We have used the technique of somatic cell fusion experiments to extend the findings described above. In particular we undertook a structure-function study of Oct-2 within this system in order to better understand how it functioned to sustain the genetic program of Ig secreting cells.

In plasmacytoma x T lymphoma hybrids, while *Pou2f2* (locus encoding Oct-2) was normally silenced *Pou2f1* (locus encoding Oct-1) remained active. It would appear, therefore that Oct-1 cannot substitute for Oct-2 in preserving the genetic program of the plasmacyte. In an effort to explore the functional differences between these two octamer binding transcription factors, we have used chimeric hOct-2/hOct-1 proteins in our cell fusion experiments as a means for identifying the specific protein domain(s) of Oct-2 that is responsible for its unique function in Ig-secreting cells. This work is described in Chapter 3 of this thesis and is a manuscript submitted for publication.

When hOct-2 was constitutively maintained in the plasmacytoma prior to cell fusion with a T lymphoma, it preserved immunoglobulin gene expression in all of recovered hybrid lines (Radomska et al., 1994). This was true for three independent plasmacytoma transformants expressing differing amounts of hOct-2. When hOct-2 was introduced into the T lymphoma prior to fusion with the plasmacytoma, the percentage of hybrids with Ig+ phenotype was 20% for one transformant and 72% for another. There appeared to be a dose dependent effect, since the latter transformant produced roughly three times the amount of hOct-2 as produced by the former.

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Our hypothesis to explain the dose dependent effect as well as our experimental strategy of *yeast two hybrid system* to isolate a cofactor interacting with the C-terminal domain of Oct-2 comprise Chapter 4 of this thesis.

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CHAPTER 2

Materials & Methods

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Materials and Methods described in this chapter include those for structure-function studies using chimeric hOct-1/hOct-2, as well as for the studies to identify a potential interacting factor of Oct-2 (yeast two-hybrid system).

I. EXPERIMENTAL PROCEDURES FOR CHIMERIC PROTEIN STUDIES

Plasmid Constructions

PCGNOct-1 is a flu epitope-tagged eukaryotic vector that produces hOct-1 (Tanaka and Herr, 1990). This vector has a cytomegalovirus (CMV) promoter that drives expression of hOct-1 cDNA. The Oct-1 coding sequences begin at the first Oct-1 AUG codon described previously (Sturm et al., 1988) and encode a 743 amino acid protein. PCGNOct-1neo, a derivative of pCGNOct-1 containing the bacterial neo^r gene as selectable marker, was constructed by inserting a 2.4kb EcoR1-BamH1 fragment from pKOneo (Van Doren et al., 1984) into the Pvu 1 site of pCGNOct-1.

The pCGNOct 1.2.2, pCGNOct 2.1.2, pCGNOct 2.2.1, and pCGNOct 1.1.2 vectors encoding hOct-2/hOct-1 chimeric proteins (Tanaka and Herr, 1990) were covalently linked to *his D* transcription unit from pSV2his (Hartman and Mulligan, 1988) to generate pCGNOct 1.2.2.his, pCGNOct 2.1.2.his, pCGNOct 2.2.1.his, and pCGNOct 1.1.2.his respectively.

Cell lines

BW5147.G.1.4 OUA^R.1 (T-lymphoma) was obtained from the Amersham Type Culture Collection (Rockville, Maryland, ATCC CRL 1588). This cell line is resistant to 10^{-3} M Ouabain and 10^{-4}M 6-thioguanine. It is derived from AKR/J mouse thymoma, 45.6.2.4 is

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a γ 2b/κ producing plasmacytoma cell line derived from the BALB/c mouse tumor MPC11 (Laskov and Scharff, 1970). We refer to this cell line as MPC11. MPC11 cells grow in medium containing hypoxanthine, aminopterin and thymidine (HAT), but die in medium containing Ouabain.

These cell lines were maintained in "complete DMEM" which consists of Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, catalog number 12100-061) containing 10% bovine calf serum (Hyclone laboratories, Incorporated, Logan, Utah, catalog A-2151-L), 100U/ml penicillin,100μg/ml streptomycin (GIBCO BRL, Grand Island, New York, catalogue number 15140-015 and 0.1mM non-essential amino acids (GIBCO BRL, Grand Island, New York, catalogue number 11140-019).

Transfections

Transfections were as previously described (Radomska et al., 1994). Breifly 10^7 cells were spun in 50ml falcon tube in an IEC centrifuge at 3 K for 3. The supernatant was aspirated out and cell pellet was resuspended in 10-15 ml DMEM (without serum) and spun again. Cell pellet was resuspended in 1ml DMEM in a (cuvette) and transfected with 10μg linearized plasmid by electroporation (0.25 kV, 960μF). Cells were plated at 10^5 cells /well in 96-well plates and forty eight hours later, appropriate drug-selection medium was added. BW5147 cells transfected with pCGNOct-1neo were selected in 1mg/ml G418-containing DMEM (G418 from GIBCO BRL, Grand Island, New York, catalog number 860-18111J). MPC11 cells transfected with *his D*-containing vectors were placed in 3mM –L-histidinol-containing DMEM. Stable transformants usually appeared on the 14th day of transfection and were frozen at -80^0 C.

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Cell Fusions

Cell fusions were performed in an electroporator as previouly described (Bio-Rad Gene Pulser with Capacitance Extender, Hercules California, (Radomska and Eckhardt, 1995). After electro-fusion, cells were incubated at 37^0C, 30-45 minutes. Cells were then plated at a density of 10^5 cells /well in a 96-well plate and selective medium was added 48 hours later. In cell fusions between BO1(16)neo and MPC11, selection of hybrids was in complete DMEM supplemented with 10^{-3} M ouabain and 1mg/ml G418. When BW5147 was fused with MPC11 transformants where the exogenous genes were covalently linked to *his D,* selection of hybrids was in complete DMEM supplemented with 10^{-3} M ouabain and 3mM L-histidinol.

In all fusions, growing cells were recovered in less than 30% of the wells and, therefore, likely represent independent fusion events. Two weeks from the date of fusion, growing clones were transferred to 12-well plates and cultured for an additional week before being frozen as stable hybrids. Clones were further analyzed by genomic Southern to test for retention of plasmacytoma-derived and T lymphoma-derived IgH and IgL loci before they were designated informative hybrids.

Genomic Southern Blots

Southern blots were carried out as previously described (Radomska et al., 1994). Approximately 15μg of genomic DNA were digested with BamH1 restriction endonuclease, the digested DNA size-fractionated by electrophoresis in a 0.7% agarose gel, and the DNA then transferred to nylon membranes (Nytran, Schleicher and Schuell, Inc., Keene, NH or Magna NT, MSI, Westboro, MA). Ig loci were detected with a 1.8 kb

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BamH1-EcoR1 fragment from pJ11 that contains J_{H3}-J_{H4} coding sequences and IgH intron enhancer sequences (Marcu et al., 1980). This probe detects the γ2b-producing locus of MPC11 but not the aberrantly rearranged IgH locus of this cell line. The Igκ loci were detected with a 1.8 kb genomic Xba1-BamH1 fragment spanning Cκ. In MPC11 there are two rearranged Igκ loci only one of which produces a functional light chain (Seidman and Leder, 1980). When MPC11 DNA is digested with BamH1, the 7.7 kb fragment detected with the Cκ probe corresponds to the functional locus. A 0.83 kb PvuII- Hinc II fragment isolated from the pSV2his was used to detect *His D* sequences (Hartman and Mulligan, 1988).

Northern Blots

Total cytoplasmic RNA was extracted using an RNA Isolation kit (Stratagene, La Jolla, CA Cat #200345). Northern blots were as previously described (Radomska et al., 1994). Twenty micrograms RNA were used for each sample analyzed. J-chain mRNA was identified with a 1.2 kb cDNA fragment from plasmid Jc21 (Cann et al., 1982). PU.1 mRNA was identified with 0.4 kb Sac 1 cDNA fragment derived from pBSKS-PU.1 (Klemsz et al., 1990). To normalize amounts of RNA in each sample, RNA blots were erased (by treating for 15 minutes with 1 liter of boiling 0.1 X SSC, 0.01% SDS) and then rehybridized with a β-actin probe (0.28 kb EcoR1-HindIII fragment from plasmid pSp6-β actin: Ambion, Austin, Texas Cat # 7315).

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Electrophoretic Mobility Shift Assays (EMSAs)

Binding reactions and electrophoretic mobility shift assays were performed as previously described (Radomska et al., 1994; Yu et al., 1989). Approximately 15μg of protein (nuclear extract) were incubated with 10^4 cpm end-labelled 51 bp fragment from the IgH intron (Eμ) enhancer (Yu et al., 1989). The sequence of the 51 bp fragment containing the octamer element is A ATCCT CAACT TATTT TAGAA ***ATGCAAAT*** TA CCCAG GTGGT GTTTT GCTCA (octamer italicized and emboldened). In experiments involving antibody, anti-flu tag antibody (12CA5 hybridoma culture supernatant at 1:8 dilution) (Field et al., 1988) was added to the incubation mix before adding the radioactive probe.

Enzyme-linked immunoassays (ELISAs)

Cytoplasmic lysates were made for ELISAs as previously described (Yu and Eckhardt, 1986). For detection of γ2b heavy chains, ELISA plates (Dynatech Laboratories, Inc: Chantilly, Virginia) were coated with purified anti-mouse γ2b (Rat IgG1) (Pharmingen, 10975 Torreyana Road, San Diego, CA.92121,Cat # 02041(D). Coated wells were then incubated with cell lysates and γ2b (Pharmingen, Cat. #02033E) and enzyme substrate (Sigma Diagnostics, St. Louis, MO, 63178, Cat # 104-105). For κ light chain assays, the coating antibody was affinity-purified goat anti-mouse κ antibody (Fisher Biotech, Pittsburgh, PA, Cat # 1050-01). The detecting antibody was biotinylated goat anti-mouse κ antibody (Amersham International, Little Chalfont Buckinghamshire, England Cat # 1179), used together with an avidin-conjugate alkaline phosphatase (Miles Scientific, Naperville, IL Cat # 62-253-1).

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Reverse transcriptase –polymerase chain reaction (RT-PCR)

RT-PCR was as described by Radomska et al. (Radomska et al., 1994) except that 2 μg starting RNA was used. A pair of primers unique for murine Oct-2 mRNA was used to amplify cDNA derived from endogenous murine *Pou2f2* (forward primer: 5' – GCCAC AGGCA CAGCA GAGTCAG –3' GenBank accession X57936, nucleotides 450-471 ; reverse primer: 5'CCAGA ATTCT AAGGG GCAGG GTTCC ACCA – 3'. Accession X57936, nucleotides 1371-1390). RT-PCR products were size-fractionated on 0.8% agarose gels and blotted to nylon filters. Blots were hybridized with a HindIII-BglII fragment from pCGNOct-2. The expected RT-PCR product was 0.9 kb.

BW x MP-hOct 1.1.2 hybrids were also analyzed by PCR for expression of chimeric hOct 1.1.2 mRNA. The primers used were the same reverse primer shown above and a forward primer specific for human Oct-1 (5'AGCCA AGCCA GCCAA GCCAG CCTTC CCAGC A3', accession X53468, nucleotides 307-329).

II. *EXPERIMENTAL PROCEDURES FOR YEAST TWO-HYBRID SYSTEM*

Construction of bait plasmids pBDOct-2POU+C and pBDOct-2C

The bait plasmid pBDOct-2POU+C (containing the POU and the C-terminal domain of Oct-2 linked to the GAL4 binding domain) was constructed as follows: The 872 bp DNA fragment containing the POU and the C terminal domains of Oct-2 was isolated from the plasmid pCGNOct-2 (provided to us by Dr. Winship Herr, Cold Spring Harbor Laboratories) by restriction digest with Xho1 and BamH1 enzymes. The fragment was gel purified by electro-elution, and Klenowed to create blunt ends. The blunt ended fragment was ligated to pBDGAL4 Cam phagemid vector (6.5kb) which was linearized with Srf-1

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and CIP treated prior to ligation. The positive clones containing the insert (Oct-2 POU +C) ligated to the phagemid vector were identified by restriction digest with Hind III restriction enzyme. The nucleotide sequence of the DNA insert was determined by sequencing using GAL4 BD primers (5'-GTGCGACATCATCATCGGAAG-3') made by Hunter Sequencing Facility, and verified that it would be expressed as a fusion protein with GAL4 BD.

The second pBDOct-2 C containing the C terminus of Oct-2 linked to the GAL4 binding domain was constructed by the same strategy as mentioned above, except that the C terminus of Oct-2 was isolated from the plasmid pCGNOct 1.1.2 (also provided by Dr. Winship Herr, Cold Spring Harbor Laboratories) by restriction digest with BamH1 and Sal I enzyme. A large-scale preparation of both the bait plasmids was made by using Qiagen Maxi Prep kit.

Invivo Mass Excision of cDNA Library of MPC11

The cDNA library of MPC11 (plasmacytoma) was custom made by Stratagene (11011 North Torrey Pines Road, LA Jolla, California 92037) in a lambda phage vector called HYBRIZAP-2.1 vector (Short and Sorge, 1992). The library had to be converted into a phagemid vector pAD-GAL2.1 before transformation into the YRG-2 yeast strain. A single colony of XL-1 Blue MRF' cells was inoculated from a freshly streaked bacterial plate containing LB +10mg/ml tetracycline into 50mls of L-Broth containing 10mg/ml tetracycline. The liquid culture was grown overnight with vigorous shaking at 37^0 C. The OD_{600} of the culture was determined after 12-16 hours and was found to be between 1.4-1.6. A 1:50 dilution of the culture was made in a sterile flask by adding 1ml of cell

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culture in 49mls of LB-tetracycline medium. It was placed in the shaker at 37^0C vigorously shaking for approximately 90-95 minutes until an OD_{600} of 0.3-0.4 was reached. At this point, care was taken to ensure that the OD_{600} did not exceed 0.4. Cells were quickly harvested by spinning the culture at 500 x g (1800 rpm) in a Sorvall Centrifuge (Kendro Laboratory Products, Model #RC5B, S/N 8302367) in a SS-34 rotor for 15 minutes. The supernatant was then discarded and the cell pellet resuspended in 10mM MgSO4, in an approximate volume of 1.0ml such that the OD_{600} was 5.0. (It was assumed that this OD_{600} gave an approximate final concentration of 4 x 10^9 cells.) (Stratagene HybriZAP-2.1 Two Hybrid Libraries Instruction Manual #838401-13).

In a sterile 125 ml conical flask, the excision reaction was set up by combining a portion of the amplified library stock with the XL-1 Blue MRF' cells at a MOI of 1:10 lambda phage-to-cell ratio. It was recommended by Stratagene that we excise 10 to 100 fold more lambda phage than the size of the primary library to ensure statistical representation of the excised clones. Since our library stock contained 10^6 members, we excised 10^7- 10^8 pfu from the amplified library stock. ExAssist helper phage was added at a 10:1 helper phage-to-cells ratio to ensure that every cell was co-infected both with lambda phage and helper phage.

Working out the conditions for mass excision of cDNA library, it was found that the best excision reaction was obtained when we combined 2.5 x 10^9 cells = 625µl (of OD_{600} =5.0) , with 2.5 x 10^8 Library phage (11µl) and 2.5 x10^{10} ExAssist helper phage (100µl). The titer of the library was provided by Stratagene. The helper phage titer was determined as described below. The excision reaction was incubated at 37^0C for 15

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minutes undisturbed. After 15 minutes, 20ml of pre-warmed L-Broth was added to the excision reaction and incubated with gentle shaking at 37^0 C for 3 hours.

The excision reaction was placed in a 70^0 C water bath for exactly 20 minutes. This was to lyse the lambda phage particles and the XL-1 Blue MRF'cells. The culture was spun in a Sorvall Centrifuge (Kendro Laboratory Products, Model# RC5B, S/N 8302367) at 500 x g (1800 rpm SS-34 rotor) for 10minutes to pellet the cell debris. The supernatant was collected in a sterile falcon tube. This contained the excised phagemids, which were stored at $+4^0$C for about a month.

Determination of Titer of ExAssist helper phage

Inoculate a single colony of XL-1 Blue MRF' cells into 50mls of L-broth containing 10mg/ml tetracycline. Next morning a 1:50 dilution of this culture was made by inoculating 1ml of XL-1 Blue MRF' cells into 49 mls of L-broth containing 10mg/ml tetracycline and grown with vigorous shaking at 37^0C for about 4-5 hours. Spin the culture at 500 x g (1800 rpm in a SS-34 rotor) for 10 minutes. The pellet containing XL-1 Blue MRF' was resuspended in 10mM $MgSO_4$ to an $OD_{600} = 1.0$. 100µl of cells was then aliquoted aseptically into a series of sterile glass tubes and placed on ice. A series of serial dilutions of ExAssist helper phage (10^2, 10^4, 10^6, 10^8) were made in sterile 1X TE buffer in sterile glass tubes and also placed on ice.10µl of each phage dilution was delivered into the appropriately labeled glass tube containing the bacterial cells using sterile capillary tubes. The tube with its contents was gently flicked to mix the cells with the phage particles. All the tubes were then placed *undisturbed* at 37^0C for 15 minutes to allow the phage to attach to cells. After 15 minutes 3 ml of NZY top agar (melted and

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cooled to 48^0C) was then poured immediately on pre-warmed NZY agar plates. The plates were incubated overnight at 37^0C. ExAssist plaques had a cloudy appearance. The titer of ExAssist helper phage was determined by counting the plaques on 10^8 dilution plate which had roughly about 175 plaques. The titer of helper phage was calculated as follows: 175 x 10^8 x 100µl = 1.75 x 10^{12} pfu/ml. The expected titer of helper phage as provided by Stratagene was 1.0 x 10^{10} pfu/ml.

Determination of Titer of Excised Phagemids

The titer of excised phagemid was determined by inoculating a single colony of XLOLR cells from a freshly streaked bacterial plate into 50mls of L-broth containing 10mg/ml tetracycline. The liquid culture was grown overnight with vigorous shaking. After 12-16 hours OD_{600} was found to be between 1.4-1.8. A 1:50 dilution of this culture was made in a sterile 250ml conical flask and incubated at 37^0 C with vigorous shaking for approximately 3 hours until an OD_{600} = 0.3-0.4 was reached. The liquid culture was spun in a Sorvall centrifuge at 500 x g (1800 rpm; SS-34) rotor for 15 minutes and cell pellet was resuspended in 4-5 ml of 10mM MgSO4 to an OD600=1.0. A series of serial dilutions of excised phagemids (10^2, 10^4, 10^6, 10^8, 10^{10}) were made in sterile TE buffer and placed on ice. 200µl of XLOLR cells (OD_{600} =1.0) were then aliquoted into a series of separate sterile glass tubes one for each respective serial dilution of phagemid. They were also placed on ice. 10µl of each serial dilution of the phagemid was added into the corresponding tube containing XLOLR cells for that dilution. The mixture was then poured onto LB agar plates and spread with a glass rod containing 50µg/ml of ampicillin.

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The plates were incubated at 37^0 C overnight. The titer of excised phagemid (cfu/ml) was determined as follows:

{Number of colonies (cfu) x dilution factor/Volume of phagemid plated(μl)} x 1000μl/ml.

700×10^4 / 10μl x 1000

7×10^8 pfu /ml

The calculated titer for excisions was, in most cases, in the range of $7\text{-}8 \times 10^8$ pfu/ml

20 individual colonies from excision plates were cross-picked and inoculated into 3mls of L-broth containing 50μg/ml ampicillin. They were placed in a 37^0 shaker vigorously shaking overnight. Next day, mini-prep DNA was isolated by Rapid Boiling prep and size of the library inserts was determined by restriction digest with HindIII restriction enzyme. The insert size for cDNA library (of MPC11) was found to lie in the range of 0.8-3.0kb. This was in accordance with the insert size that was sent to us by Stratagene (11011 North Torrey Pines Road, LA Jolla California 92037).

Amplification of Excised Phagemid Library

The cDNA library now in the phagemid vector was amplified so that large amounts of plasmids representing the library could be prepared by any convenient plasmid DNA preparation. A single colony from a freshly streaked XLOLR bacterial plate was inoculated into 50mls of L-Broth containing 10mg/ml tetracycline, and incubated overnight at 37^0 C with vigorous shaking. A 1:50 dilution of the XLOLR culture was made in 4 separate 2 liter flasks each flask having 300mls of L-broth and containing

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50µg/ml of ampicillin. The cultures were placed at 37^0 C for approximately 3 hours with vigorous shaking until OD_{600} was between 0.3-0.4. The cells were spun in 50ml Falcon tubes in a swing bucket rotor (185mm rotor in CCR4 centrifuge) at 500 x g (1600 rpm) for 10 minutes. The cell pellet in each flask was resuspended in 7-8 mls of 10mM MgSO4. The contents from different flasks were pooled together. OD_{600} of the culture was determined which was approximately equal to 1.0.

For amplification, Stratagene suggested that we combine XLOLR cells with a portion of the excision supernatant in a two-liter flask at a minimum cells-to-phagemid ratio of 10:1. (This was with the assumption that OD_{600} of 1.0 was equal to a cell concentration of 8 x 10^8 cells). Based on the calculated titer of our excised phagemid (7-8 x 10^8 pfu/ml) as well as from the library size (2.5 x 10^6) we calculated the amount of phagemid as well as the volume of XLOLR cells needed for amplification of our excised phagemids. The amplification reaction was set up as follows: We combined 27mls of XLOLR cells (OD600=1.0) with 2.5mls of phagemid in a sterile 2 liter flask and gently swirled the flask to mix the contents. For maximum amplification, we set up four such flasks at a time. The XLOLR cells and the phagemid were placed at 37^{0}C for 15 minutes. Pre-warmed L-broth (625 ml) containing 50µg/ml ampicillin was then added to each amplification reaction. The liquid culture was then subjected to vigorous shaking at 37^0 C for 3 hours, and OD_{600} was recorded after every hour. When OD600 was approximately 0.3, the culture was taken out from the incubator and the cells harvested by spinning in a Sorvall centrifuge at 6000 x g for 15 minutes at 4^{0}C in a Sorvall GSA rotor {6000 rpm). Plasmid DNA was isolated by using a Qiagen Plasmid Maxi Prep Protocol.

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Plasmid DNA representing the cDNA library of MPC11 amplified in this manner gave yields as high as 2.4μg/ μl, the total yield being 300-400μg in one round of amplification.

Preparation of yeast competent cells

YRG-2 yeast competent cells were first transformed with the bait plasmids (pBD-GAL4 Oct-2 POU+C or pBDOct-2 C). The stable transformants of YRG-2, expressing either pBD-GAL4 Oct-2 POU+C or pBD-GAL4 Oct-2C, were then transformed by the cDNA library (Durfee et al., 1993).

The first step in transformation procedure as given in "Stratagene Instruction Manual" (#6195350034) called for making competent yeast cells prior to each transformation. Yeast competent cells were prepared by inoculating 1ml of sterile YPAD medium in a 1.5ml sterile microfuge tube with approximately 2-4 yeast colonies from a freshly streaked yeast YPAD plate. The colonies were well resuspended in the medium by vortexing the culture vigorously until no clumps were visible. This 1ml culture was then transferred into a 250ml flask containing 50ml YPAD broth. The culture was then incubated overnight at 30^0 C for 18-24 hours with shaking at 225-250 rpm.

Yeast cells were made competent only if OD_{600} of the YRG-2 culture was above 1.2. The culture was discarded if the OD_{600} was < 1.2 even after 20 hrs., and a fresh culture started. The 50ml yeast culture was then added to 300ml YPAD broth in a 1 liter flask and incubated for 3 hours at 30^0 C with shaking at 225-250 rpm. Yeast cells were harvested by centrifugation at 1000 x g (2200 rpm); GS3 rotor in a Sorvall centrifuge) for 5 minutes at room temperature. The supernatant was discarded and the cells resuspended

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in 1.5ml of freshly prepared TE-LiAc solution. Yeast cells made competent in this manner were further used for yeast library µg transformations, and yielded as high as 0.8-1.0 x10^4 colonies/µg of DNA

Transformation of competent YRG-2 cells with control plasmids and the bait plasmids

The competent cells thus prepared were then transformed with a set of control plasmids such as pGAL4 (expressing the full length GAL4 protein); p53 and pSV40 (co-transformation); and plaminC (encoding amino acid residues 67-230 of human plaminC) and pSV40 (Co-transformation), according to the procedure described in "Stratagene Instruction Manual" (#6195350034).

The expectation was that p53 interacts with pSV40 and would serve as positive control when tested for lacZ expression using the filter lifts assay. PlaminC does not intract with pSV40, so we would expect no blue color when tested for lacZ expression. They would thus serve as a negative control in a filter lift assay. A similar method was used to transform yeast cells with the bait plasmids (pBDOct-2POU+C and pBDOct-2C).

Transformation of competent yeast cells with the control plasmids was done by adding 100µg of carrier DNA (Salmon sperm, 100µl) to each transformation tube containing 100µl of yeast competent cells. Yeast competent cells were always aliquoted using *wide bore* pipet tips {blue tips were cut for large-scale or library transformations while yellow tips were cut and used for small-scale transformations involving control plasmids as well as the baits}. Carrier DNA (Cat # 201190 from Stratagene) was boiled for 20 minutes and immediately transferred to ice just prior to use in transformations. About 100ng of the desired plasmid was added to each tube. For co-transformations,

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200ng of each plasmid (e.g. p53 and pSV40 or plaminC and pSV40) were added, for a total of 400ng of plasmid DNA in each microfuge tube. In setting up transformations with the bait plasmids, 100ng of each bait plasmid was added to100µl of yeast competent cells. 600µl of freshly prepared TE-LiAc-PEG solution was added to each microfuge tube and the contents were mixed by vortexing. The samples were then incubated at 30^0 C for 30 minutes with shaking at 200 rpm in a floor-model platform shaker. 70µl of DMSO was added to each tube and the contents mixed gently. The samples were heat shocked at 42^0 C (in a water-bath previously set for the purpose) for 15 minutes. The samples were then quickly placed in ice for 10 minutes. This was followed by centrifugation of samples at 3000 rpm in a microfuge for 10 seconds to pellet the cells. The supernatant was then thoroughly discarded and the tubes were re-spun for a few seconds to get rid of any residual supernatant. The cell pellet was then resuspended in 0.5ml of 1x TE buffer and vortexed to resuspend the cells. When the cells were completely resuspended, they were then plated on the appropriate SD-selective plates i.e. cells transformed with pGAL4 plasmid were plated on –leu plates, those that were transformed with the appropriate bait plasmids were plated on –trp plates while cells co-transformed with p53 and pSV40 as well as plaminC and pSV40 were plated on –leu –trp plates. All plates were incubated at 30^0 C for 2-4 days until colonies appeared. Incubation of plates at 30^0C was continued for about a week until the colonies were 1-2 mm in diameter.

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Transformation of Library DNA into competent yeast cells containing pBDGAL4 Oct-2 POU+C (Library screening)

YRG-2 yeast cells containing the bait plasmid pBDGAL4Oct-2 POU+C were made competent by the procedure described above. The competent yeast cells containing the bait plasmid were transformed with the library DNA. The procedure for large scale transformation of yeast cells with library DNA was similar to small scale transformation of yeast by the control and the bait plasmids except for a few modifications. 1ml of SD medium lacking Trp was inoculated with 15-20 yeast colonies containing the bait plasmid, and vortexed to resuspend the yeast cells completely in the medium. This culture was then inoculated into a flask containing 50mls of SD-Trp medium and placed in a shaker at 30^0 C. The culture was allowed to shake at 225-250 rpm for 12-18 hours until an OD_{600} = 1-6-1.8 was attained.

The cells were harvested and made competent in exactly the same manner as described earlier. Competent yeast cells containing the bait plasmid pBDGAL4 Oct-2 POU+C were then transformed with the library DNA. Typically 1ml of bait competent cells were taken in a 50ml sterile Falcon tube and 2mg of carrier DNA {200µl of 10mg/ml} was added. The carrier DNA was prepared prior to transformation by boiling for 20 minutes and then immediately chilling it on ice. 40µg of target DNA (library DNA) was then added followed by 6ml of TE-LiAc-PEG solution and the contents vortexed so that they were uniformly resuspended. The transformation tube was placed at 30^0 C and left shaking at 225-230 rpm for exactly 30minutes. 700µl of DMSO was added and the cells were heat-shocked for 15 minutes in a 42^0 C water bath. The tube was placed on ice for 10 minutes. This was followed by centrifugation of sample at 3000 rpm {swing

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bucket centrifuge JOAUN, in 185mm rotor} for 10 seconds to pellet the cells. The supernatant was completely removed and the cells were re-centrifuged to get rid of any residual supernatant. Cells were resuspended in 10mls of sterile 1 x TE buffer and plated on selective plates. 10μl aliquots were plated in duplicate on SD-Leu –Trp plates for determination of efficiency of transformation reaction, and the remaining volume of cells was spread on SD-Leu-Trp-His plates (150mm thickness) 500μl per plate for selection of potential interacting clones. SD-Leu-Trp plates were placed at 30^0 C for 2-3 days whereby the plate was covered with colonies. The colonies were counted and transformation efficiency was calculated. SD-Leu-Trp-His plates were incubated at 30^0 C for 8-10 days until colonies were 1-2 mm in diameter.

Lac Z Filter Lift Assay for His$^+$ colonies

His$^+$colonies were screened for β- gal activity using a filter lift assay (Breeden and Nasmyth, 1985) (Durfee et al., 1993). The conditions for the Lac Z Filter Lift Assay were initially worked out with the control plasmids that were provided by Stratagene HybriZAP Two-Hybrid Library Kit (cat#978000), and used in a similar way for screening potential interacting clones from the cDNA library. SD-Leu- Trp-His plates containing His$^+$colonies were considered ready for filter lift assay (to test the presence of β-galactosidase activity) when the colonies were 1-2mm in diameter. For the control plasmids, typically, colonies from a plate stored at 4^0 C could be spotted or streaked (with wire loop) on the desired selective plate and incubated at 30^0 C for 48 hours before performing this assay. In this way transformants containing the control plasmids could be

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stored at 4^0C, for 2-3 weeks and restreaked 48 before performing filter lift assay after each screening procedure. In library screens, the His+ clones on the original screening plate were grown to 1-2mm diameter and filter lifts were done on this plate.

The Z buffer with X-gal was prepared fresh each time by mixing 98ml of Z buffer, 0.27 ml of β-mercaptoethanol and 1.67 of X-gal stock solution (20mg/ml). 5mls of Z buffer with X-gal was added to the bottom of a sterile, empty 150mm petri-plate. A sterile qualitative filter paper (VWR brand, size 12.5cm, Cat #28310-106) was placed in the plate, ensuring that the filter paper was uniformly wetted. A dry sterile 0.45 µm supported Nitrocellulose transfer and immobilization membrane (obtained from Schleicher & Schuell, Item # 10439148) and 137mm in diameter was placed on the surface of the plate containing colonies. Any air bubbles were carefully removed by using the sterile forceps to even out the surface, and the filter paper was firmly pressed on the surface of the plate with the help of two sterile forceps. The orientation on the filter paper was marked by punching orientation holes with a sterile,18mm gauge needle. The filter was left in contact with the colonies for about 3 minutes. The Nitrocellulose membrane was then carefully lifted from the plate, using sterile forceps, and placed colony-side up in a styrofoam dish containing liquid nitrogen. The Nitrocellulose membrane was placed in liquid nitrogen for 30 seconds, removed, and then placed on a new qualitative filter paper to thaw. The thawed nitrocellulose membrane was then carefully placed colony side up onto the filter paper soaked in the Z buffer with X-gal. Any air bubbles trapped between the two pieces of filter paper were carefully removed by using the forceps to even out the surface. The petri dishes containing the nitrocellulose membranes were covered with their tops and then placed in re-sealable bags and

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incubated at 30^0 C for 3 hours to overnight. The plates containing the NC membranes were monitored for the production of blue color indicating ß-galactosidase activity.

Colonies containing the pGAL4 control plasmid turned blue after 30 minutes of incubation of plates at 30^0 C, while colonies containing p53 and pSV40 (positive controls) turned blue after 3 hours. Colonies containing the negative control plasmids plaminC and pSV40 turned pale blue after 24 hours of incubation yet could be distinguished easily from the positive controls that were intensely blue. For library screening experiments, all colonies that turned blue after 3 hours to overnight were considered His^+lacZ+. These were once again re-streaked on fresh SD-leu-trp-his plates and again assayed for lacZ gene expression by the filter lift assay.

Isolation of plasmid DNA from His$^+$ and Lac Z$^+$ yeast colonies

The plasmid DNA from the His+ and Lac Z+ colonies was isolated by the procedure outlined in "Stratagene Yeast DNA Isolation System," (Instruction manual Catalog #200052). The His+lacZ+clones were streaked on a fresh SD-leu-trp-his plate and placed at 30^0C for 2 days. Yeast DNA was isolated from single colonies by gently picking up the colony using a sterile toothpick and then placing it in 20µl of lysis solution. The yeast cells were evenly dispersed in the lysis solution by stirring with the toothpick. The lysis reaction was then placed in a dry ice methanol bath for 20 seconds, and thawed at 37^0 C for 1-2 minutes. The lysis reaction was heated at 95^0 C for 5 minutes, and spun in microfuge for 30 seconds at 14 K. The supernatant was transferred into a new tube and placed on ice.

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The super-competent XL-1 Blue MRF' cells obtained with Yeast DNA Isolation System (cat # 200052 Stratagene 11011 North Torrey Pines Road, LA Jolla, California 92037) were thawed on ice and 75μl aliquoted into 3, 15ml conical tubes (Corning Costar), pre-chilled on ice. The β-mercaptoethanol also obtained with the same kit, was also thawed on ice. 1.3μl of β-mercaptoethanol was added to each tube containing the super-competent cells and contents swirled gently. The transformations were then incubated on ice for 10 minutes, swirling gently every 2 minutes. After 10 minutes 2μl of lysate was added to each experimental tube and 1μl of p53 DNA was added into the control tube. All reactions were placed on ice for 22 minutes. The reactions were heat-pulsed at 42^0 C for 40 seconds exactly and then placed on ice for 2 minutes. 0.4ml of NZY-broth was added to each reaction tube and placed in 37^0 C shaker at 225 rpm for 1 hour. The transformation reaction was spread on ampicillin plates (50μg/ml) 0.4ml per plate. The plates were incubated at 37^0 C overnight.

10 colonies were cross-picked from each experimental plate and mini-prep plasmid DNA was isolated and restricted with HindIII enzyme to determine the insert size.

Sequence Analysis

Plasmid DNA with different insert sizes was purified by Qiagen Midi prep Procedure and sequenced using a GAL4 AD primer (Operon Technologies Inc. 1000 Atlantic Avenue Suite 108 Alamceda, CA 94501) (5'AGGGATGGTTTAATACCACTAC-3'). Sequencing was done by the 'DNA Sequencing Lab' (The Rockefeller University, 1230

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York Ave, NY, NY 10021). Sequence analysis and homology searches were done using BLAST search for DNA and protein sequence.

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CHAPTER 3

Unique function for carboxyl-terminal domain of Oct-2 in immunoglobulin-secreting cells

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ABSTRACT

The activity of immunoglobulin gene promoters and enhancers is regulated by two related transcription factors, Oct-1 (ubiquitous) and Oct-2 (B-lineage specific), that bind the octamer motif (ATTTGCAT) present in these elements. As immunoglobulin promoter-binding factors, Oct-1 and Oct-2 each work together with a B lymphocyte-specific co-factor OBF-1/OCA-B/Bob-1 that interacts with them through their POU (DNA-binding) domains. Since both can mediate Ig promoter activity in B cells, there has been some question as to whether these two octamer-binding factors serve distinct functions in lymphocytes. We have shown previously that the silencing of B-lymphocyte specific genes in plasmacytoma x T-lymphoma hybrids can be prevented by preserving Oct-2 expression. The pronounced effect of this transcription factor on the phenotype of plasmacytoma x T-lymphoma hybrids established a critical role for Oct-2 not only in maintaining Ig gene expression, but in maintaining the overall genetic program of Ig-secreting cells.

In the present study we have explored the functional differences between Oct-1 and Oct-2 using chimeric Oct-1/Oct-2 proteins in cell fusion assays. Our results provide further evidence for an essential role for Oct-2 in Ig-secreting cells and identify the C-terminal domain of Oct-2 as responsible for its unique function in these cells.

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INTRODUCTION

Oct-1 and Oct-2 are POU homeodomain proteins that recognize the same sequence, the octamer motif (ATGCAAAT), but are differentially expressed in vivo. Oct-1 is a ubiquitous transcription factor implicated in the control of genes expressed by all cells, while Oct-2 is largely B-lineage restricted and has been implicated in the control of B lymphocyte-specific gene expression (Landolfi et al., 1986; Rosales et al., 1987; Scheidereit et al., 1987; Singh et al., 1986; Staudt et al., 1986; Sturm and Herr, 1988). Both proteins are members of the POU family of transcription factors, having in common a bipartite DNA-binding domain that consists of an N-terminal POU-specific domain and a C-terminal homeodomain separated by a flexible linker region (Herr and Cleary, 1995; Herr et al., 1988; Rosenfeld, 1991). Although the POU domains of Oct-1 and Oct-2 are highly homologous (87% amino acid identity), there is very little sequence homology between these two proteins on either side of this centrally located DNA-binding domain (Ko et al., 1988; Sturm and Herr, 1988).

The notion that Oct-2 and Oct-1 differed in their ability to drive B cell-specific gene expression was initially a widely held assumption (Muller et al., 1988; Scheidereit et al., 1988; Staudt et al., 1986; Wirth et al., 1987). Oct-2 cDNA was cloned by virtue of its ability to encode a factor that could trans-activate an immunoglobulin (Ig) promoter in non-lymphoid cells, something the endogenously expressed Oct-1 was unable to do (Clerc et al., 1988; Muller et al., 1988; Scheidereit et al., 1988). The functional difference between these two octamer-binding proteins, when assayed with octamer-containing reporter genes in non-lymphoid cells, mapped to their carboxyl-terminal domains (Tanaka and Herr, 1990).

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Several studies, however, have suggested that Oct-1 and Oct-2 are functionally interchangeable within B lymphoid cells (Johnson et al., 1990; LeBowitz et al., 1988; Luo et al., 1992; Pierani et al., 1990). The tissue-restricted activity of Ig promoters has been attributed not to Oct-2 but, instead, to the B lineage-restricted, co-activator OBF-1 (OCA-B, Bob-1) (Gstaiger et al., 1995; Luo and Roeder, 1995; Strubin et al., 1995). This co-activator significantly augments Ig promoter activity through its interaction with either Oct-1 or Oct-2, suggesting that it is the limiting factor prohibiting Ig promoter activity in non-B lymphoid cells.

Gene knock-out studies involving the *Pou2f2* (murine locus encoding Oct-2) and *OBF-1* loci have not resolved this issue. Both *Pou2f2*$^{-/-}$ and *OBF-1*$^{-/-}$ knock-out mice produce surface Ig-positive B cells, suggesting that neither Oct-2 nor OBF-1 is essential for the activation of Ig genes nor for early events in B cell development (Corcoran et al., 1993; Kim et al., 1996; Schubart et al., 1996). Both types of knock-out mice, however, show defects in events that commonly occur after B cell activation (i.e. post-antigenic challenge) (Corcoran and Karvelas, 1994; Humbert and Corcoran, 1997; Kim et al., 1996; Nielsen et al., 1996; Qin et al., 1998). These results could mean either that Oct-2 and OBF-1 only function late in B cell development/differentiation or that they function early in B cell development but are redundant at this stage (e.g. Oct-1+ OBF-1 = Oct-2), taking on unique functions only in late-stage cells.

In the present study, we provide additional evidence for a unique and essential function for Oct-2 in Ig-secreting cells. In earlier studies, we showed that B cell-specific genes were uniformly and coordinately silenced when Ig-secreting cells (plasmacytomas) were fused to a non-B lineage cell line (Lieberman et al., 1993). *Pou2f2* was one of the

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genes silenced. We found that if we prevented the loss of Oct-2 expression in the hybrids, however, *Pou2f2* and all other assayed, B cell-specific genes were simultaneously rescued from silencing (Radomska et al., 1994). We have interpreted this to suggest that Oct-2's role in Ig-secreting cells is a critical one and is required for the continued expression of numerous tissue-specific genes. We noted that while *Pou2f2* was normally silenced in the plasmacytoma x T cell hybrids, *Pou2f1* (the locus encoding Oct-1) remained active. It would appear, therefore, that Oct-1 can not substitute for Oct-2 in preserving the genetic program of the plasmacyte. We have used chimeric hOct-2/ hOct-1 proteins in cell fusion assays as a means for identifying specific protein domain(s) required for Oct-2's unique function in Ig secreting cells. As detailed below, we find that the carboxyl-terminal region of Oct-2 is critical to this activity.

MATERIALS AND METHODS

Plasmid constructions

pCGNOct-1 is a flu epitope-tagged eukaryotic vector that produces hOct-1 (Tanaka and Herr, 1990). This vector has a cytomegalovirus (CMV) promoter that drives expression of hOct-1 cDNA. The Oct-1 coding sequences begin at the first Oct-1 AUG codon described previously (Sturm et al., 1988) and encode a 743 amino acid protein. pCGNOct1neo, a derivative of pCGNOct1 containing the bacterial *neo*^{r} gene as selectable marker, was constructed by inserting a 2.4kb EcoRI-BamHI fragment from pKOneo (Van Doren et al., 1984) into the PvuI site of pCGNOct1.

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The pCGNOct 1.2.2, pCGNOct 2.1.2, pCGNOct 2.2.1, and pCGNOct 1.1.2 vectors encoding hOct-2/ hOct-1 chimeric proteins (Tanaka and Herr, 1990) were covalently linked to the *his D* transcription unit from pSV2his (Hartman and Mulligan, 1988) to generate pCGNOct 1.2.2.his, pCGNOct 2.1.2 his, pCGNOct 2.2.1his, and pCGNOct 1.1.2his respectively.

Cell lines

BW5147.G.1.4 OUA^{R}.1 (T-lymphoma) was obtained from the American Type Culture Collection (Rockville, Maryland, ATCC CRL1588). This cell line is resistant to 10^{-3} M Ouabain and 10^{-4} M 6-thioguanine. It is derived from an AKR/J mouse thymoma. 45.6.2.4 is a γ2b/κ producing plasmacytoma cell line derived from the BALB/c mouse tumor MPC11 (Laskov and Scharff, 1970). We refer to this cell line as MPC11. MPC11 cells grow in medium containing hypoxanthine, aminopterin and thymidine (HAT), but die in medium containing Ouabain. S194 plasmacytoma (IgA-secreting plasmacytoma derived from BALBL/c) was a gift from Dr. B.K. Birshtein, Albert Einstein College of Medicine (Bronx, NY).

These cell lines were maintained in "complete DMEM" which consists of Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, catalog number 12100-061) containing 10% bovine calf serum (Hyclone laboratories, Incorporated, Logan, Utah, catalog A-2151-L), 100U/ml penicillin, 100µg/ml streptomycin (GIBCO BRL, Grand Island, New York, catalogue number15140-015) and 0.1 mM non-essential amino acids (GIBCO BRL, Grand Island, New York, catalogue number 11140-019).

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Transfections

Transfections were as previously described (Radomska et al., 1994). Briefly, 10^7 cells were transfected with 10μg linearized plasmid by electroporation (0.25 kV, 960μF). Cells were plated at 10^5 cells/well in 96-well plates and forty-eight hours later, appropriate drug-selection medium was added. BW5147 cells transfected with pCGNOct-1neo were selected in 1mg/ml G418-containing DMEM (G418 from GIBCO BRL, Grand Island, New York, catalog number 860-1811IJ). MPC11 cells transfected with *his D*-containing vectors were placed in 3mM-L-histidinol-containing DMEM.

Cell fusions

Cell fusions were performed in an electroporator, as previously described (Bio-Rad Gene Pulser with Capacitance Extender, Hercules, California) (Radomska and Eckhardt, 1995) After electrofusion, cells were plated at a density of 10^5 cells/well in a 96-well plate and selective medium was added 48 hours later. In cell fusions between B01(16)neo and MPC11, selection of hybrids was in complete DMEM supplemented with 10^{-3} M ouabain and 1mg/ml G418. When BW5147 was fused with MPC11 transformants where the exogenous genes were covalently linked to *his D,* selection of hybrids was in complete DMEM supplemented with 10^{-3} M ouabain and 3mM L-histidinol.

In all fusions, growing cells were recovered in less than 30% of the wells and, therefore, likely represent independent fusion events. Two weeks from the date of fusion, growing clones were transferred to 12-well plates and cultured for an additional week before being frozen as stable hybrids. Clones were further analyzed by genomic Southern

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to test for retention of plasmacytoma-derived and T lymphoma-derived IgH and IgL loci before they were designated informative hybrids.

Genomic Southern blots

Southern blots were carried out as previously described (Radomska et al., 1994). Approximately 15μg of genomic DNA were digested with BamH1 restriction endonuclease, the digested DNA size-fractionated by electrophoresis in a 0.7% agarose gel, and the DNA then transferred to nylon membranes (Nytran, Schleicher and Schuell, Inc., Keene, NH or Magna NT, MSI, Westboro, MA). Ig loci were detected with a 1.8 kb BamHI -EcoRI fragment from pJ11 that contains J_{H3}- J_{H4} coding sequences and IgH intron enhancer sequences (Marcu et al., 1980). This probe detects the γ2b-producing locus of MPC11 but not the aberrantly rearranged IgH locus of this cell line. The Igκ loci were detected with a 1.8 kb genomic Xba1- BamHI fragment spanning Cκ. In MPC11 there are two rearranged Igκ loci only one of which produces a functional light chain (Seidman and Leder, 1980). When MPC11 DNA is digested with BamHI, the 7.7kb fragment detected with the Cκ probe corresponds to the functional locus. A 0.83kb PvuII- HincII fragment isolated from pSV2his was used to detect *HisD* sequences (Hartman and Mulligan, 1988).

Northern blots

Total cytoplasmic RNA was extracted using an RNA Isolation kit (Stratagene, La Jolla, CA; Cat# 200345*)*. Northern blots were as previously described (Radomska et al., 1994). Twenty micrograms RNA were used for each sample analyzed. J-chain mRNA was

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identified with a 1.2kb cDNA fragment from plasmid Jc21 (Cann et al., 1982). PU.1 mRNA was identified with a 0.4kb Sac1 cDNA fragment derived from pBSKS- PU.1 (Klemsz et al., 1990). To normalize amounts of RNA in each sample, RNA blots were erased (by treating for 15 minutes with 1 liter of boiling 0.1X SSC, 0.01% SDS) and then rehybridized with a β-actin probe (0.28kb EcoRI- HindIII fragment from plasmid pSP6-β actin; Ambion, Austin, Texas Cat# 7315).

Electrophoretic Mobility Shift Assays (EMSAs)

Binding reactions and electrophoretic mobility shift assays were performed as previously described (Radomska et al., 1994; Yu et al., 1989). Approximately 15μg of protein (nuclear extract) were incubated with 10^4 cpm end-labeled 51 bp fragment from the IgH intron (Eμ) enhancer (Yu et al., 1989). In experiments involving antibody, anti-flu tag antibody (12CA5 hybridoma culture supernatant at 1:8 dilution) (Field et al., 1988) was added to the incubation mix before adding the radioactive probe.

Enzyme-linked immunoassays (ELISAs)

Cytoplasmic lysates were made for ELISAs as previously described (Yu and Eckhardt, 1986). For detection of γ2b heavy chains, ELISA plates (Dynatech Laboratories, Inc.; Chantilly, Virginia) were coated with purified anti-mouse γ2b (Rat IgG1) (Pharmingen, 10975 Torreyana Road, San Diego, CA. 92121, Cat.# 02041D). Coated wells were then incubated with cell lysates and γ2b chains detected with alkaline phosphatase-conjugated (Rat IgG_{2a}) anti-mouse γ2b (Pharmingen, Cat. #02033E) and enzyme substrate (Sigma Diagnostics, St. Louis, MO,63178, Cat# 104-105). For κ light chain assays, the coating

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antibody was affinity-purified goat anti-mouse κ antibody (Fisher Biotech, Pittsburgh, PA, Cat# 1050-01). The detecting antibody was biotinylated goat anti-mouse κ antibody (Amersham International, Little Chalfont Buckinghamshire, England Cat # 1179), used together with an avidin-conjugated alkaline phosphatase (Miles Scientific, Naperville, IL Cat# 62 -253-1).

Reverse transcriptase - polymerase chain reaction (RT-PCR)

RT-PCR was as described by (Radomska et al., 1994) except that 2μg starting RNA was used. A pair of primers unique for murine Oct-2 mRNA was used to amplify cDNA derived from endogenous murine *Pou2f2* (forward primer: 5' - GCCAC AGGCA CAGCA GAGTCAG - 3', GenBank accession X57936, nucleotides 450-471 ; reverse primer: 5' CCAGA ATTCT AAGGG GCAGG GTTCC ACCA-3', accession X57936, nucleotides 1371-1390). RT-PCR products were size-fractionated on 0.8% agarose gels and blotted to nylon filters. Blots were hybridized with a HindIII-BglII fragment from pCGNOct-2. The expected RT-PCR product was 0.9kb.

BW x MP-*hOct1.1.2* hybrids were also analyzed by PCR for expression of chimeric *hOct1.1.2* mRNA. The primers used were the same reverse primer shown above and a forward primer specific for human Oct-1 (5'AGCCA AGCCA GCCAA GCCAG CCTTC CCAGC A3', accession X53468, nucleotides 307-329).

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RESULTS

Constitutively expressed hOct-1 does not prevent immunoglobulin gene silencing in plasmacytoma x T-lymphoma hybrids

By preventing the silencing of *Pou2f2*, it is possible to rescue from silencing all other tested plasmacytoma genes in T lymphoma x plasmacytoma cell fusions (Radomska et al., 1994). As noted above, the endogenous *Pou2f1* locus was never silenced in T lymphoma x plasmacytoma fusions, suggesting that Oct-1 was unable to achieve the same effect. Our means of maintaining Oct-2 expression in fusion experiments, however, involved use of a cloned gene expressing Oct-2. The Ig-secreting plasmacytoma was stably transformed with human Oct-2 cDNA *(hOct-2)* under the control of a cytomegalovirus promoter (CMV promoter) before cell fusion. Because the CMV promoter was not subject to T lymphoma-mediated silencing, the *hOct-2* gene remained active in hybrid lines. The expressed hOct-2 carried an influenza epitope (16 amino acids) at the N-terminus and could be distinguished from endogenously-encoded murine Oct-2 by use of an anti-flu antibody (Radomska et al., 1994; Tanaka and Herr, 1990).

We considered the possibility that differences in the levels or patterns of expression of the transfected *hOct-2* gene and endogenous *Pou2f1* gene might explain the ability of the former, but not the latter, to preserve plasmacytoma gene expression in the cell hybrids. To test this hypothesis, we made use of an expression vector encoding hOct-1. The vector backbone was the same as that used for the *hOct-2* experiments and appended an influenza epitope at the amino-terminus of hOct-1. The vector also included a selectable marker (pSV2neo^r) that allowed transformants to be growth-selected in

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G418-containing medium. Nuclear extracts made from G418^r clones were then assayed by electrophoretic mobility shift assay (EMSA) in the presence of anti-flu antibody (12CA5, ref. Tanaka and Herr, 1990) to test for expression of the transfected *hOct-1* gene.

Four out of five BW5147 transformants produced flu-tagged hOct-1 (four transformants shown, Figure 4A). One of these, BO1(16)neo, was subsequently fused to the Ig-secreting plasmacytoma MPC11. Hybrid clones were growth-selected in HAT/ouabain-containing medium and then tested for G418-resistance as a measure of their retaining the transfected *hOct-1* gene. Eighteen hybrids that met these criteria also retained Igγ2b and/or Igκ genes from the plasmacytoma parent as well as Ig loci from the T lymphoma parent (data not shown). Retention of Ig loci from both parental lines was evidence that the lines were truly hybrid in nature. The presence of Ig γ2b and/or Igκ loci from the plasmacytoma parent also made these hybrids informative with respect to Ig gene silencing.

EMSAs performed with nuclear extracts from these hybrids identified 16 that continued to produce hOct-1 from the transfected *hOct-1* gene (representative data, Fig. 4B). When tested by ELISA for the production of γ2b and κ chains, only one of the sixteen hybrids was producing Ig (Table I). One Ig-expressing hybrid out of 16 is comparable to the frequency of "exceptional" hybrids normally recovered in fusions between MPC11 and BW5147 (Lieberman et al., 1993; Radomska et al., 1994). There was no indication, therefore, that hOct-1 was having an impact on the silencing of plasmacytoma-specific genes in the MPC11 x BW5147-*hOct-1* hybrids.

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Figure 4.

Expression of transfected hOct-1 in T cell transformants and hybrids

A) EMSA of octamer-binding proteins in BW5147 (BW) and its hOct-1 transformants (#13-16). ^{32}P labeled DNA probe is a 51bp fragment from the IgH intronic (Eμ) enhancer that contains the octamer motif. DNA/protein complexes formed by endogenous Oct-1 (Oct-1) and exogenous Oct-1 (hOct-1) co-migrate, but a "super-shifted" complex containing flu-tagged hOct-1 is visible with anti-flu antibody (hOct-1/Ab).

B) Nuclear extracts from the plasmacytoma parent (MPC11) and T lymphoma parent (hOct-1 expressing BO1) and from their hybrids were analyzed as in Figure 1A. Representative data for six hybrids are shown. The Oct-2 complex generated from MPC11 extracts is indicated.

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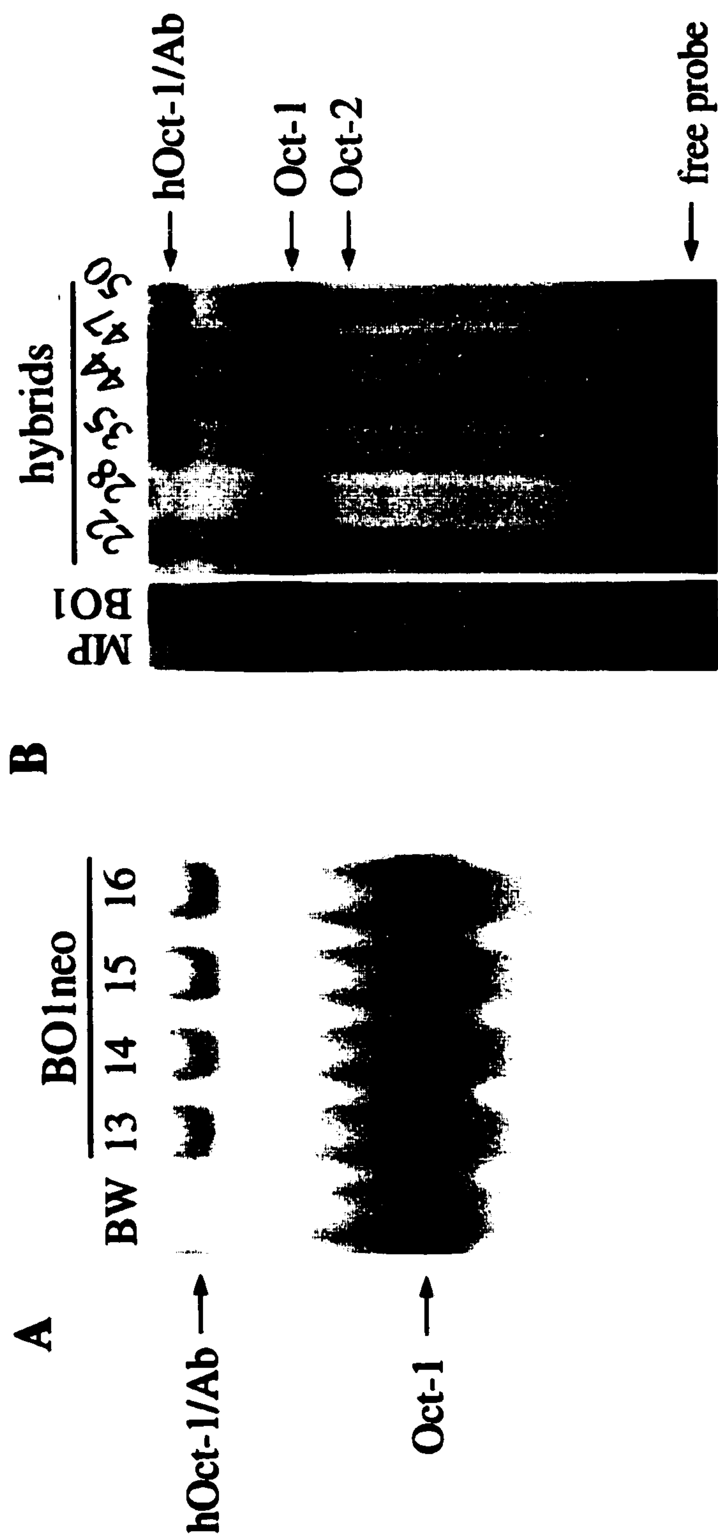

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Table I.

Data obtained in somatic cell fusion experiments is summarized in the table.

Hybrids resulting from fusion of MPC11 x BO1(16)neo (T-lymphoma expressing hOct-1) as well as those resulting from fusions of MPC11 (expressing chimeric Oct-1/Oct-2) with T lymphoma are indicated. # Hybrids indicates hybrids that were confirmed by genomic southern analysis for expressing both the heavy and light chain gene loci derived from both parental lines.

These hybrids were considered informative and were used for further analysis by EMSA and ELISA assays.

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Table I

Plasmacytoma fusion partner	T lymphoma fusion partner	# hybrids[a]	Ig-producing hybrids	% Ig-producing hybrids
MPC11	BO1(16)neo[b]	16	1	6
MPC11-hOct1.2.2	BW5147	10	10	100
MPC11-hOct2.1.2	BW5147	18	18	100
MPC11-hOct2.2.1	BW5147	10	0	0
MPC11-hOct1.1.2	BW5147	45	35	78

[a] Hybrids analyzed that retained Ig loci from both parental lines and continued expression of chimeric hOct gene
[b] T lymphoma transformant constitutively expressing hOct-1

In these experiments, the hOct-1-expressing plasmid was introduced into the T lymphoma parent before fusion with the Ig-secreting plamacytoma MPC11. In previous studies we found that the phenotype of hybrids differed depending upon which parental line was transfected with the cloned *hOct-2* gene prior to cell fusion [Radomska, 1994 #797]. MPC11-*hOct-2* transformants, upon fusion with BW5147, gave rise to hybrids that were uniformly Ig-positive and that expressed all tested plasmacytoma-specific genes. This was true in fusions using three different MPC11-*hOct-2* transformants and was independent of the amount of hOct-2 produced in the individual transformants (Radomska et al., 1994). When *hOct-2* was introduced into the T-lymphoma parent before fusion, however, a lower fraction of hybrids expressed Ig and this fraction varied directly with the amount of hOct-2 produced in the T lymphoma transformant. The higher the levels of hOct-2 produced in the T lymphoma parent, the greater the number of hybrid lines retaining plasmacytoma-specific gene expression (range of 20-72%, \Radomska et al., 1994).

The frequency of Ig-expressing hybrids in BW-*Oct-1* x MPC11 hybrids (6%) was well below that seen in any BW-*hOct-2* fusions. Given the more pronounced effect of hOct-2 expressed in MPC11, however, we also made attempts to produce hybrids with comparable MPC11-*hOct-1* lines. While MPC11-*hOct-1* transformants were readily produced, we were unable to isolate hybrids that both retained plasmacytoma-derived Ig genes and continued to express hOct-1. This was in contrast to the ease with which hybrids retaining plasmacytoma Ig genes and expressing *hOct-2* were obtained [Radomska et al., 1994 and see below]. In both types of cell fusions, therefore, hOct-1

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behaved very differently from hOct-2, supporting the hypothesis that the two were not functionally equivalent in Ig-secreting cells.

A modified hOct-2 protein with the POU domain of Oct-1 is able to preserve Ig gene expression in hybrid cells.

The centrally located POU domains of Oct-1 and Oct-2 are 87% identical (not including the "linker" region between POU-specific and homeodomains) (Clerc et al., 1988; Herr et al., 1988; Sturm et al., 1988). Despite this near identity, there are functional differences between these two POU domains. For example, the POU domain of Oct-1 binds the viral transcription factor VP16 while that of Oct-2 cannot. This functional difference maps to one of seven amino acid differences between the two POU-homeodomains (Lai et al., 1992). The N-terminal regions of Oct-1 and Oct-2 differ both in size and sequence (N-terminal region of Oct-1 is 80 amino-acids longer than that of Oct-2, 28.5% amino-acid sequence homology, Clerc et al., 1988; Herr et al., 1988; Sturm et al., 1988; Tanaka and Herr, 1990). They have in common, however, a high concentration of glutamine residues, a feature shared by the transactivation regions of many transcription factors. The carboxyl-terminal regions of Oct-1 and Oct-2 also show very little homology either in size or sequence (~12% homology; C-terminal domain of Oct-1 is 185 amino-acids longer than that of Oct-2) but again, there is some similarity in that both have stretches rich in serine, threonine, and proline (Clerc et al., 1988; Herr et al., 1988; Sturm et al., 1988; Tanaka and Herr, 1990)

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Figure 5.

Diagrams of hOct-1, hOct-2, and chimeric proteins used in cell fusion experiments. N, POU and C refer to the amino-terminal region, the central DNA-binding domain, and the carboxyl-terminal regions of the proteins, respectively. hOct-2-derived domains are shaded.

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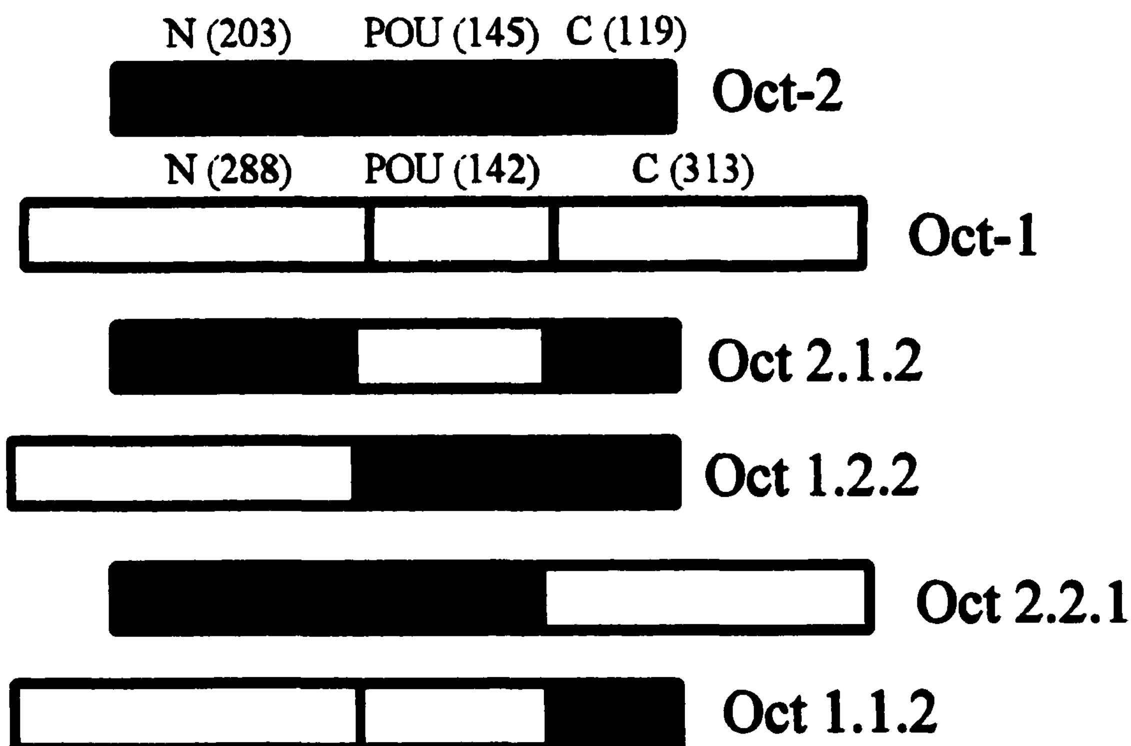

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In order to map the protein domain(s) responsible for Oct-2's unique function in Ig secreting cells, we made use of a series of expression vectors encoding flu-tagged Oct-1/Oct-2 chimeric proteins (Tanaka and Herr, 1990). The vectors were modified to include the selectable marker gene *his D* (structures diagramed in Figure5). Vectors encoding chimeric proteins were introduced into the MPC11 plasmacytoma. L-histidinol-resistant clones were examined by EMSA for DNA/protein complexes characteristic of the chimeric proteins.

Expression of the chimeric proteins was further confirmed in some cases by the addition of anti-flu antibody to the DNA-protein binding reaction which resulted in a "super-shifted" complex in EMSAs (Fig 6A-6C). Two to three independent transformants for each of the chimeric genes were subcloned and then fused with the BW5147 T-lymphoma by electrofusion. Hybrids were growth-selected in medium supplemented with ouabain and L-histidinol to select hybrid lines that retained the transfected chimeric gene (Materials and Methods). The hybrids were then analyzed by genomic Southern to test for retention of plasmacytoma and T-lymphoma derived IgH and IgL loci (representative data for BWxMP1.1.2 hybrids, Figure 7).

To test whether differences in the POU region were responsible for Oct-2's unique ability to preserve B cell specific gene function, we tested the activity of hOct2.1.2 in our cell fusion assay (for structure, see Figure 5). MPC11 transformants expressing hOct2.1.2 were isolated (Fig. 6A). Two of these, MPOct2.1.2 #6 and MPOct2.1.2#7, were fused with BW5147 and the resulting hybrids analyzed by genomic Southern. Eighteen hybrids that retained Ig loci from both parental lines were also expressing hOct2.1.2 by EMSA (data not shown). All eighteen also expressed Ig (Table).

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Figure 6.

Expression of chimeric Oct-binding proteins in MPC11 transformants.

Assays were as described in Figure 4A. Anti-flu epitope antibody was added to all binding reactions shown.

A) hOct 1.2.2, intermediate in size to Oct-1 and Oct-2, is seen as a distinct band between the Oct-1 and Oct-2-containing complexes. hOct 2.1.2, approximately the same size as endogenously-encoded, murine Oct-2, co-migrates with it. Expression of hOct 1.2.2 and hOct 2.1.2 was confirmed by formation of an anti-flu antibody "super-shifted" complex (hOct+Ab).

B) hOct 2.2.1 comigrates with Oct-1. It is best visualized in a complex with anti-flu antibody (hOct2.2.1/Ab)

C) hOct 1.1.2, intermediate in size to Oct-1 and Oct-2, forms a discrete band between Oct-1 and Oct-2-containing complexes. It is also visualized in a complex with anti-flu antibody (hOct1.1.2/Ab).

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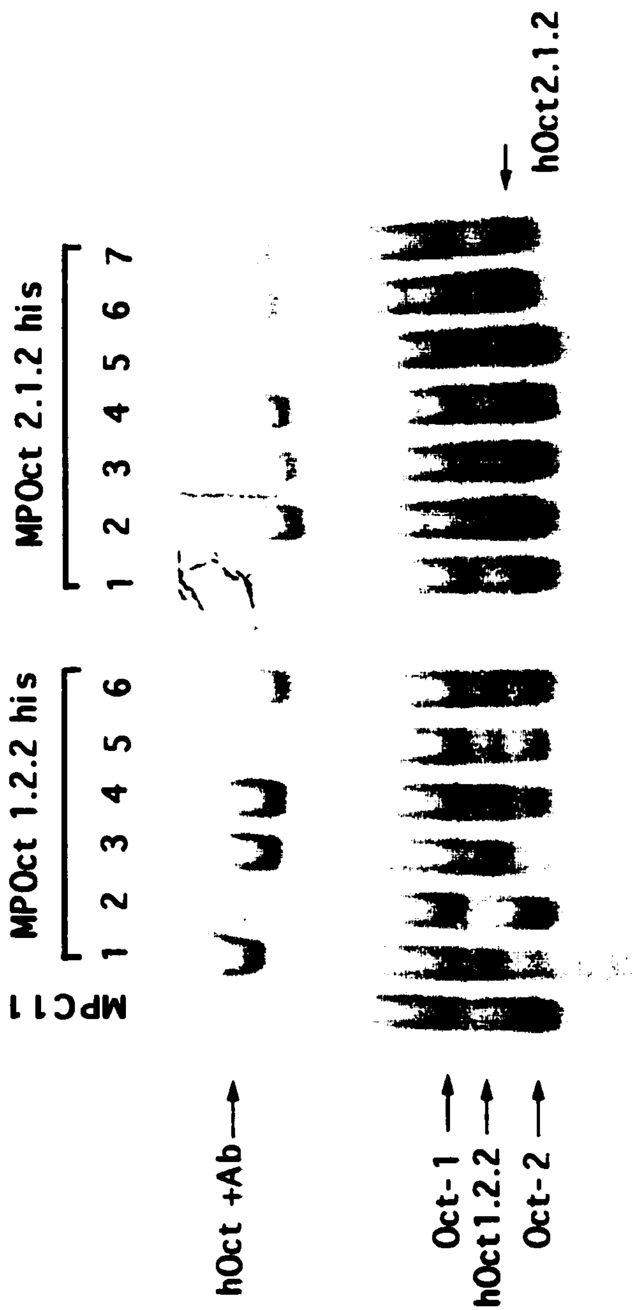

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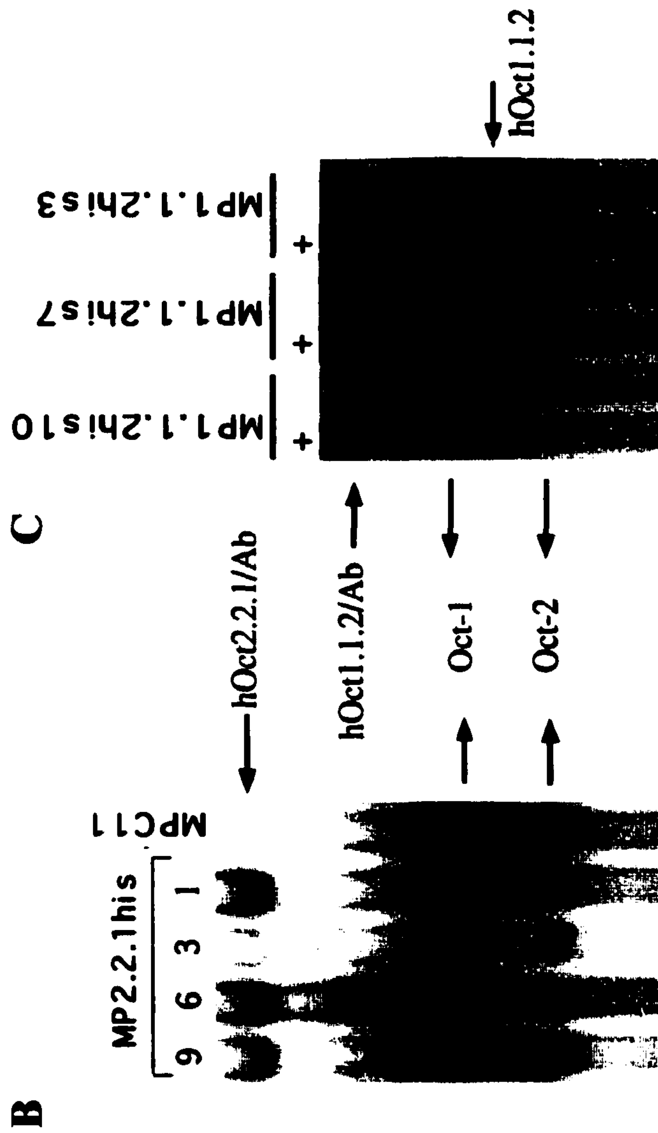

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Figure 7.

Genomic Southern blot showing the presence of plasmacytoma and T-lymphoma derived genes in BW5147 x MPOct 1.1.2 hybrids.

A) Genomic DNA digested with BamHI and hybridized with ^{32}P-labelled pJ11 probe to detect IgH genes (IgH). DNA from the hOct 1.1.2-expressing plasmacytoma parent (MP1.1.2his), the T lymphoma parent (BW5147), and their hybrids (data for ten representative1.1.2 hybrids shown) was analyzed. Clones retaining both the 4.8kb and 9.9kb bands from the parental lines were considered hybrids and were included in further analyses.

B) Southern blots of BamHI-digested DNA from the same cell lines in Figure 4A, hybridized with Cκ probe to detect Igκ genes. (see Materials and Methods). The two fragments detected with MPC11 DNA correspond to the functionally rearranged Igκ locus of MPC11 (7.7kb BamHI fragment) and an aberrantly rearranged Igκ locus (3.0kb).

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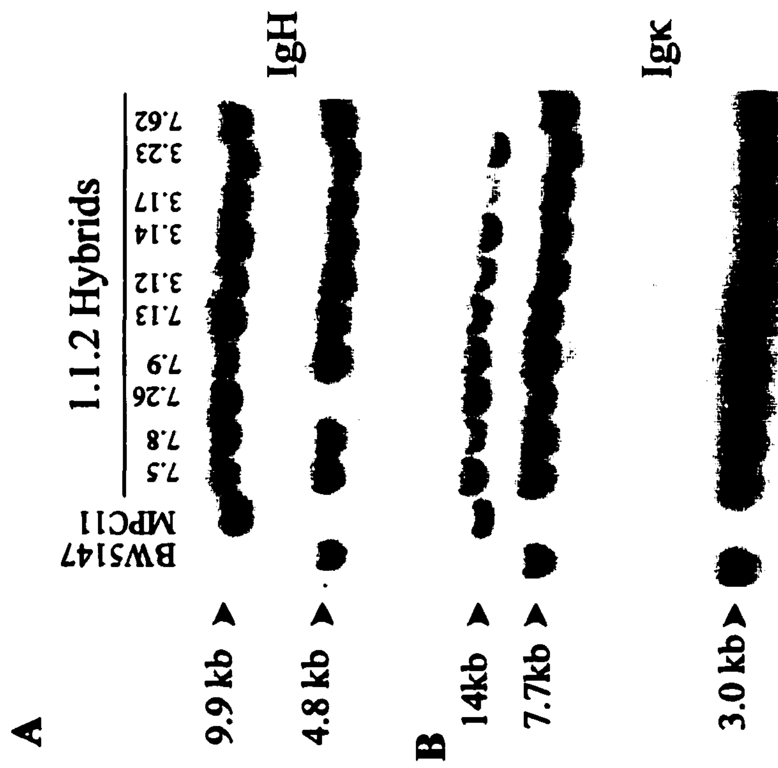

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Figure 8.

PU.1, J-chain, and γ2b expression in BW x MPOct2.1.2 hybrids.

Northern blot analyses of total cytoplasmic RNA isolated from BW5147, MPC11 and eight representative hybrid lines. A single Northern blot was hybridized successively with PU.1, J-chain, γ2b and β-actin probes. β-actin mRNA levels were used to normalize RNA amounts.

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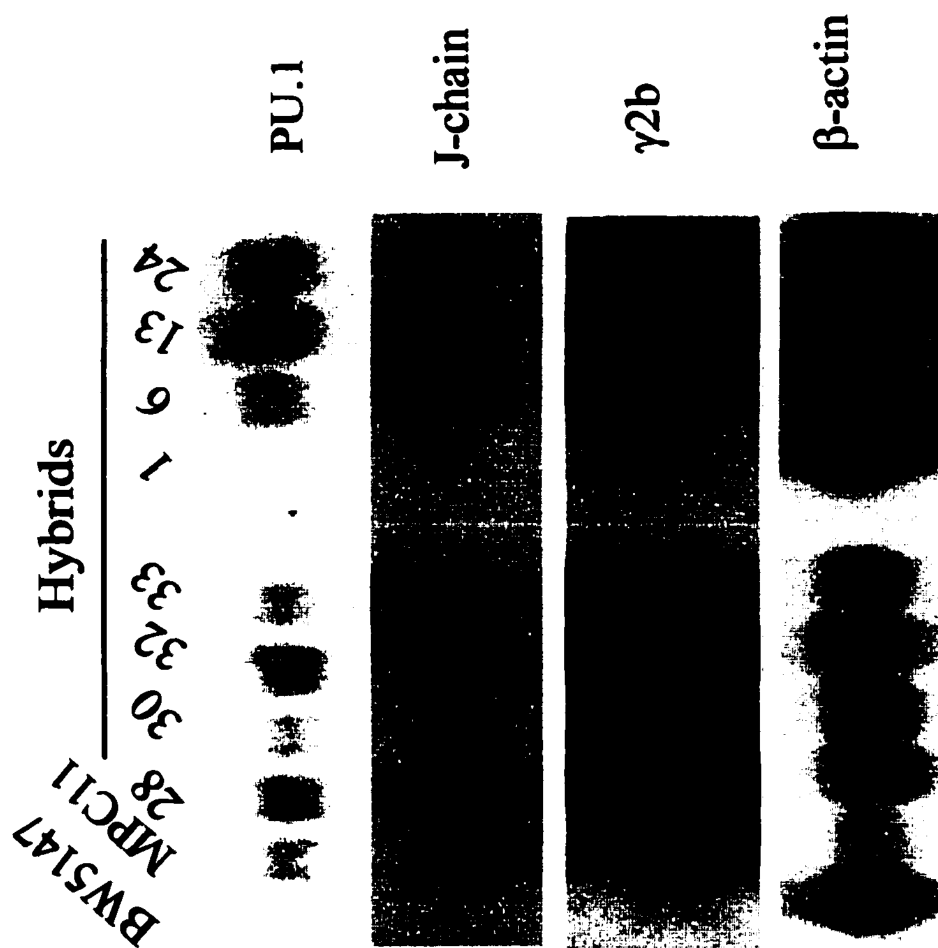

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Given our previous observation that the Ig loci behaved coordinately with other plasmacytoma-specific genes in plasmacytoma x T cell hybrids (Lieberman et al., 1993; Radomska et al., 1994), we tested several of the hybrids for PU.1 and J-chain gene expression by northern blot (representative data, Fig.8). As expected from the ELISA data, all examined hybrids expressed γ2b mRNA, although the level varied among hybrids. Hybrid clone #1 expressed particularly low levels of γ2b, but even this level of expression is not seen in "Ig-silenced" hybrids where the Ig locus is not only transcriptionally inactive (as determined by nuclear run-on assays) but also becomes *de novo* methylated (Zaller et al., 1988). J-chain and PU.1 mRNA levels in the hybrids paralleled that of γ2b, with PU.1 mRNA being the least abundant and, therefore, not detectable in hybrid clone #1. This variation in gene-expression levels was also seen previously in hybrids involving MP11-*hOct-2* transformants (Radomska et al., 1994). This suggests that while Oct-2 ensures that the tissue-specific genes of the plasmacytoma remain "on" in all hybrids, there are other regulatory factors that influence levels of gene expression and that are not found in constant amounts among the hybrids. It is interesting to note that the direct correlation between PU.1 mRNA and J-chain mRNA levels seen in the hybrids is consistent with the hypothesized role of PU.1 as a regulator of J-chain gene expression (Shin and Koshland, 1993).

In summary, hOct-2's ability to rescue plasmacytoma-specific genes from T-lymphoma-mediated silencing was not detectably affected by replacement of the protein's POU domain with that of hOct-1.

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The N-terminal domains of hOct-1 and hOct-2 are interchangeable with respect to preserving Ig gene expression in plasmacytoma x T-lymphoma hybrid cell lines

In the chimeric protein hOct 1.2.2, the N-terminal domain of Oct-2 is replaced by that of Oct-1, resulting in a chimeric protein with a molecular weight intermediate to that of Oct-1 and Oct-2 (Fig. 5A). Two MPC11-*hOct1.2.2* transformants were independently fused to BW5147. 10 hybrids arising from the two fusions both expressed chimeric hOct 1.2.2 and retained Ig loci from both the plasmacytoma and T lymphoma parent (Fig. 9 and data not shown). When tested by ELISA, all of the hybrids were Ig positive (Table). These hybrids also expressed PU.1 and J-chain mRNA, again at varying levels (data not shown). EMSAs not only confirmed that hOct1.2.2 was being expressed in these hybrids but also revealed that the endogenous *Pou2f2* gene (usually silenced) remained active (Fig.9).

In summary, expression of hOct1.2.2 was fully capable of preserving immunoglobulin gene expression in MPC11-*hOct1.2.2* x T lymphoma fusions and also had a pronounced effect on mOct-2, PU.1, and J-chain expression in the resulting hybrids. In these respects, the effects of hOct1.2.2 were indistinguishable from those of intact hOct-2.

The C-terminal domain of Oct-2 is required for its unique function

Given that the activity of hOct-1 in the fusion assays was clearly distinguishable from that of hOct-2 and from that of the hOct1.2.2 and hOct2.1.2 chimeric proteins, the most likely interpretation was that the C-terminal domain of Oct-2 was responsible for this transcription factor's unique function. This domain was shown previously to

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Figure 9.

Oct-2 and hOct1.2.2 expression in BW x MPOct1.2.2.hybrid lines.

EMSA of nuclear extracts made from parental and hybrid lines. Anti-flu tag antibody confirmed that the intermediate complex contained flu-tagged hOct1.2.2 (data not shown). Methods as described in Figure 4A.

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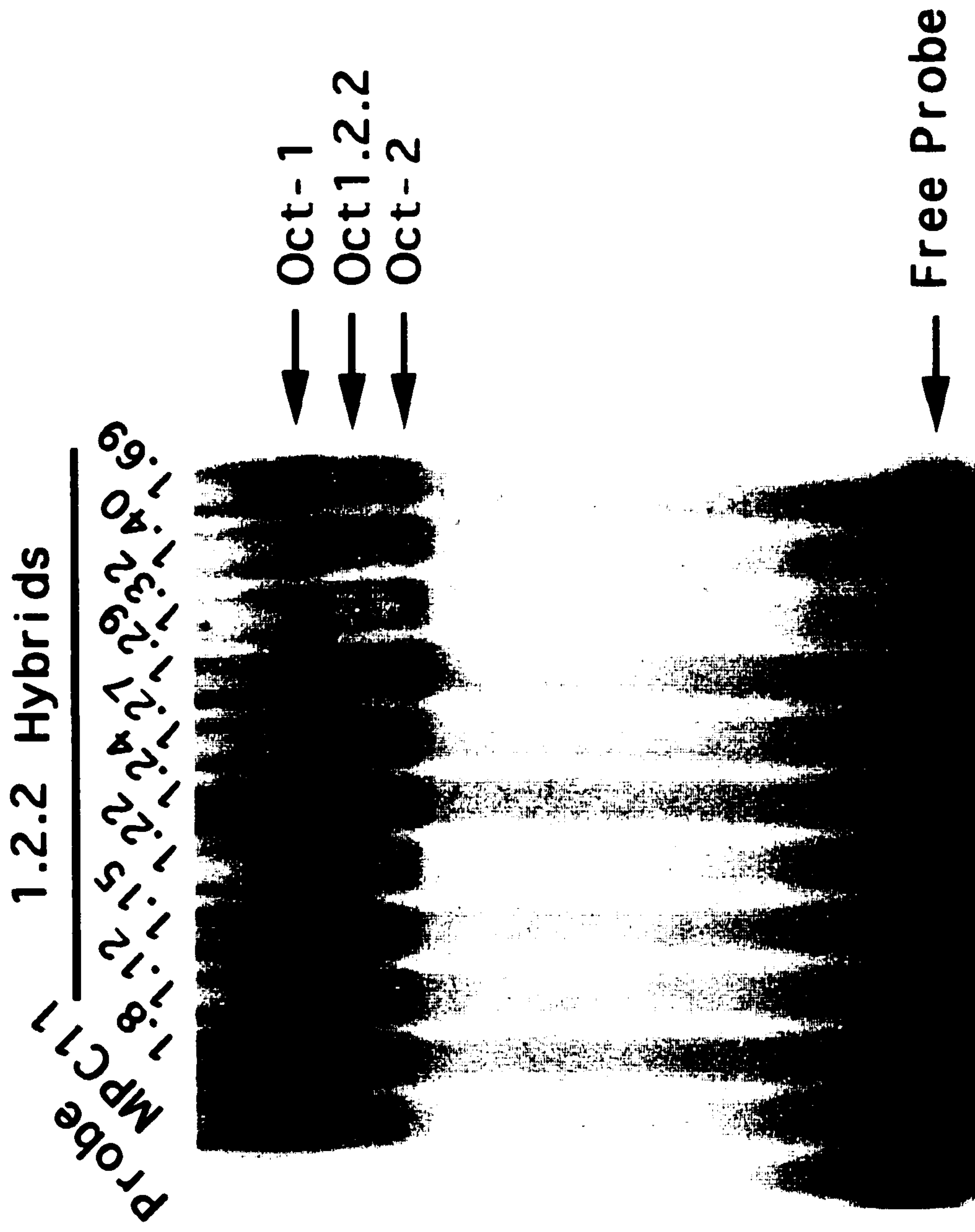

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distinguish between Oct-1 and Oct-2 in transactivation studies of a synthetic, octamer-dependent promoter (Tanaka and Herr, 1990). We were interested to determine if the more global effect of Oct-2 on the natural, endogenous genes of the plasmacyte was similarly dependent upon this domain.

Two chimeric, octamer-binding proteins were used to test this hypothesis: a chimeric protein exchanging the C-terminal region of Oct-1 for that of Oct-2 (hOct2.2.1) and a reciprocal, chimeric protein consisting of the N-terminal and POU domains of Oct-1 and the C-terminal domain of Oct-2 (hOct1.1.2).

A gene encoding the chimeric protein hOct 2.2.1 (Figure5) was introduced into MPC11 and stable transformants growth-selected in histidinol-containing medium. Sixteen of 18 histidinol-resistant clones expressed flu-tagged hOct2.2.1 as determined by EMSA (representative data, Figure 6B). Two transformants, MPOct2.2.1#1 and MPOct2.2.1#9, were fused to the T lymphoma BW5147. Ten clones arising from the fusion were confirmed hybrids by genomic Southern analysis, expressed the chimeric Oct2.2.1 protein, and retained functional Ig loci from MPC11. None of the hybrids expressed either Ig heavy or Ig light chain (data summarized in Table). The hybrids were also negative for mOct-2 as determined by EMSA (Figure 10A) and for J-chain gene expression, as determined by Northern (Figure 10B).

In summary, hOct2.2.1 was unable to preserve Ig, endogenous mOct-2, or J-chain gene expression in the plasmacytoma x T lymphoma hybrids, supporting the notion that these "rescue" functions require the C-terminal domain of Oct-2.

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Figure 10.

J-chain mRNA and Oct-2 protein are absent in BW x MPOct2.2.1 hybrids

A) EMSA of Oct 2.2.1 hybrids. Extracts from MPC11, a hOct2.2.1-expressing transformant, MP2.2.1#1 (MP2.2.1), and five representative hybrid lines. Methods were as described in Figure 4A except that both binding reactions with (+) and without anti-flu antibodies were included. In the absence of anti-flu antibody, hOct 2.2.1 migrates close to Oct-1, resulting in a dense complex (compare hybrid extracts with and without antibody).

B) Northern blot of total RNA from BW5147, MPC11, a hOct2.2.1-producing transformant of MPC11 (MP2.2.1), and four representative BW x MP2.2.1 hybrids. Northern blots were probed with a J-chain probe and then, for normalization, with a β-actin probe.

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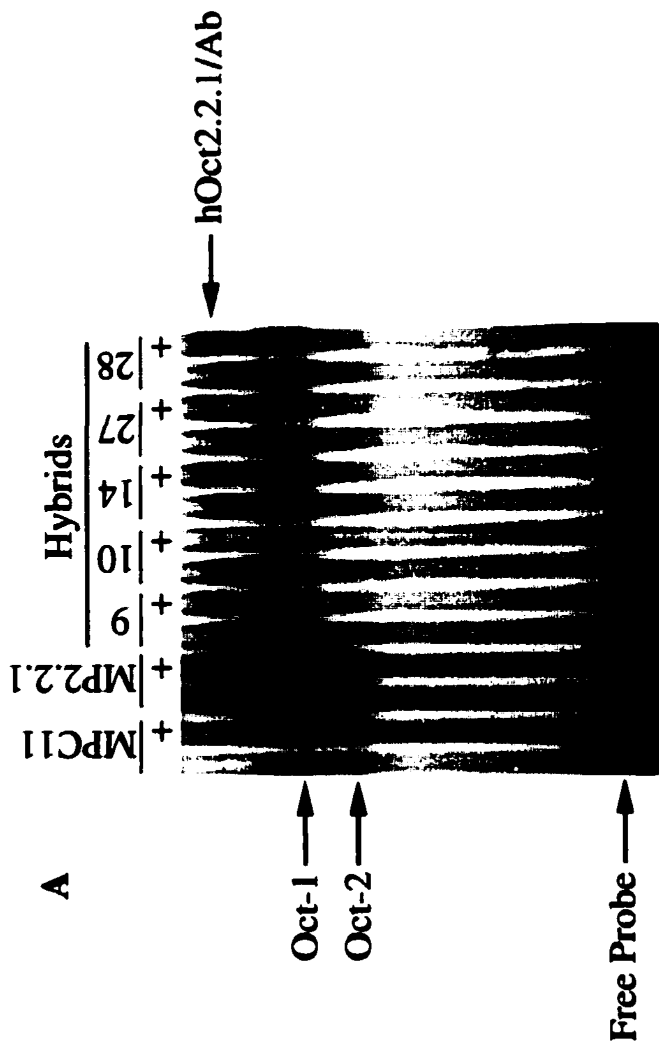

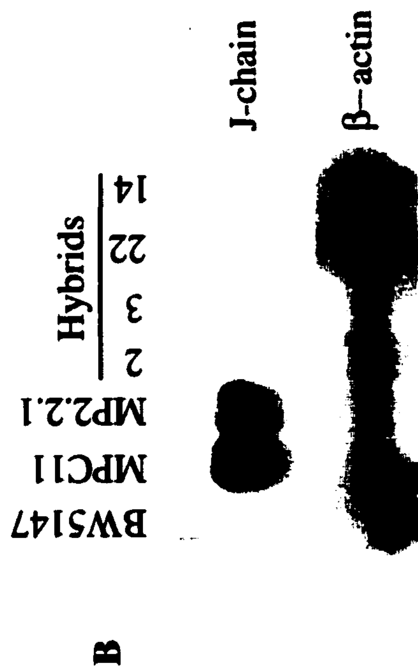

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A reciprocal chimeric protein, hOct1.1.2, was used to ask whether the C-terminal domain of hOct-2 was sufficient to convert hOct-1 into a form that could preserve B cell- specific gene expression in hybrid cells (structure shown in Fig 5). Two MPOct1.1.2his stable transformants (MPOct1.1.2his#3 and MPOct1.1.2.his#7, Figure 6B) were fused to BW5147, and forty-five informative hybrid clones were recovered. The Ig phenotype of these hybrids was mixed, however. Thirty-five continued to express Ig while ten did not. Several of the Ig-expressing and Ig-negative hybrids were also analyzed for PU.1 and J-chain expression. The Ig-expressing hybrids also expressed PU.1 and J-chain mRNA (Fig. 11A) while the Ig-negative hybrids were negative for J-chain mRNA (PU.1 not tested, Figure 11B). Similarly, only the Ig-expressing hybrids also expressed endogenous mOct-2 (as measured by EMSA and RT-PCR, Figure 12). Although efficiency appears compromised in the hOct1.1.2 protein (78% instead of 100% of hybrid clones expressed Ig), much of the activity of the hOct-2 protein is transferred to the hOct-1 protein through this single domain exchange.

DISCUSSION

The aberrant behavior of Ig-positive B cells in *Pou2f2*$^{-/-}$ mice suggests that this factor has essential functions at a late stage in B cell development (Corcoran and Karvelas, 1994; Humbert and Corcoran, 1997). In our previous studies, we have shown that Oct-2 is essential to the preservation of the Ig-secreting cell's genetic program in the context of a cell fusion system (Radomska et al., 1994). When an Ig-secreting plasmacytoma was fused to the T lymphoma BW5147, all tested genes expressed in the plasmacytoma were

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Figure 11.

Coordinate expression of plasmacyte-specific genes in BW x MPOct1.1.2 hybrids

A) Northern blots of total RNA from MPC11, an Oct1.1.2-expressing transformant of MPC11 (MPOct1.1.2), BW5147, and 10 representative Ig-expressing BW x MPOct 1.1.2 hybrids. The same blot was sequentially hybridized with PU.1, J-chain, and β-actin probes.

B) Northern blots of five representative Ig-negative BW x MPOct 1.1.2 hybrids. Blots were hybridized successively

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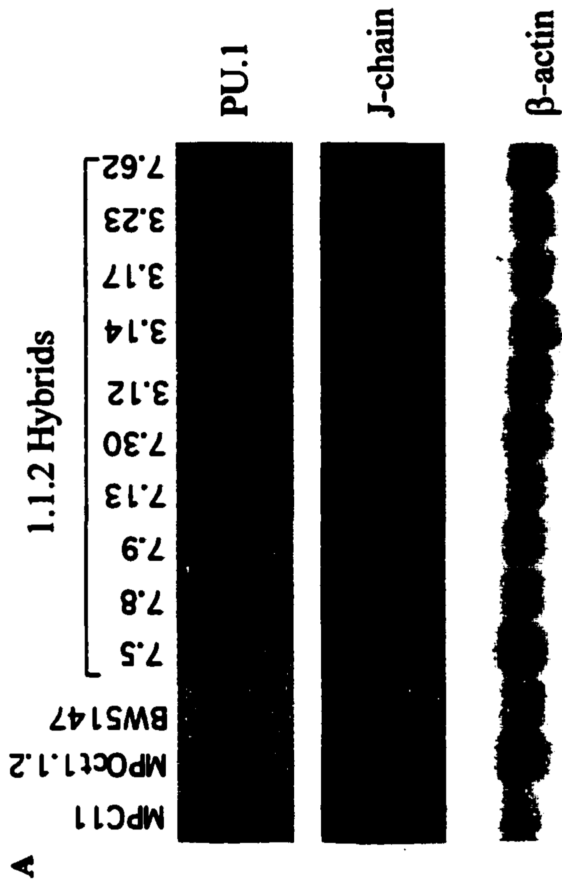

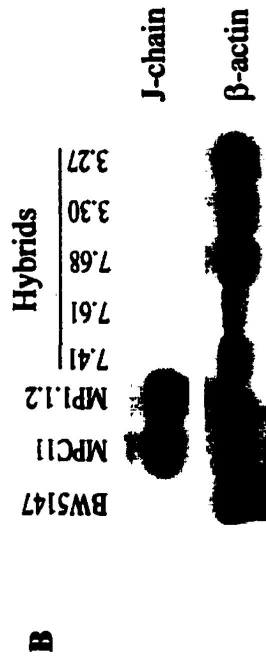

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Figure 12.

EMSA and RT-PCR analysis of Oct 1.1.2 hybrids.

A) EMSA of Oct 1.1.2 hybrids showing the expression of chimeric Oct 1.1.2. Chimeric Oct 1.1.2 is seen as a complex between Oct-1 and Oct-2 based on its molecular size. The * shows the presence of a non-specific complex which is sometimes evident in the EMSA's.

B) RT-PCR analysis of Oct 1.1.2 hybrids showing the presence of mOct-2.

A pair of primers unique for murine Oct-2 mRNA was used to amplify cDNA derived from endogenous murine *Pou2f2* (forward and reverse primers as described in Materials and Methods).RT-PCR products were size-fractionated on 0.8% agarose gels and blotted on nylon filters. mOct-2 was detected by HindIII-BglI fragment from pCGNOct-2. The expected RT-PCR product was 0.9 kb

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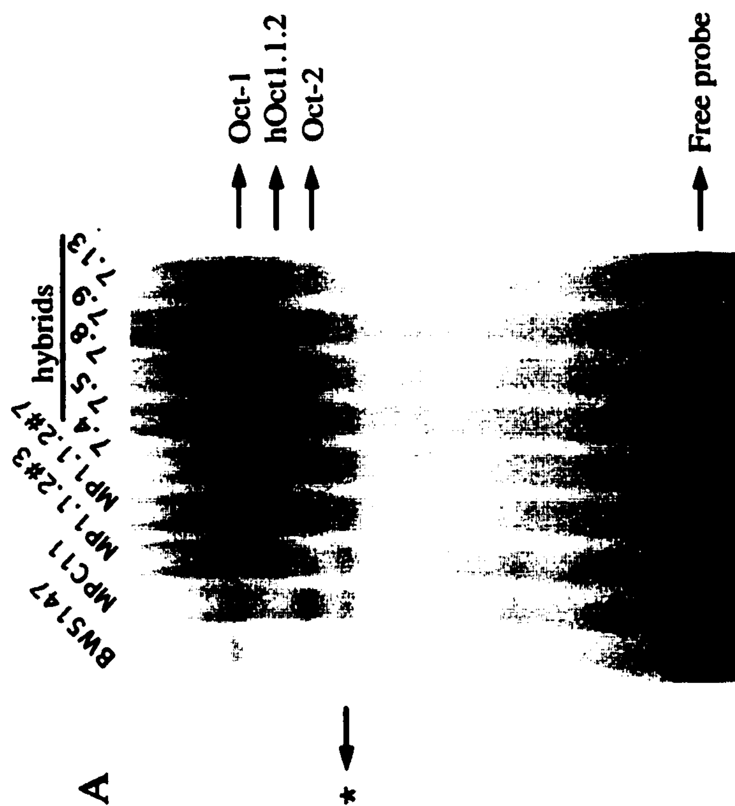

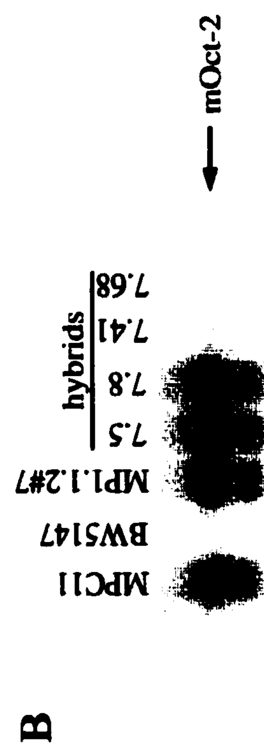

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silenced in the resulting hybrids. If we preserved the expression of only one of the only one of the silenced gene products, Oct-2, all other plasmacytoma genes were rescued from silencing. This strongly supports a central role for Oct-2 in the normal functioning of tissue-specific genes in Ig-secreting cells.

In the present study, we have shown that this is an activity that cannot be achieved by Oct-1, the other octamer-binding factor common to both the plasmacytoma and the T lymphoma. We also show that the C-terminal domain of Oct-2 is critical to its program-preserving activity. If it is replaced by the comparable domain of Oct-1, the resulting protein is unable to dictate plasmacytoma-specific gene expression in the cell hybrids.

We have previously postulated the involvement of another B cell-restricted factor in the program-sustaining activity of Oct-2 (hypothetical B cell accessory factor BAF, Radomska et al., 1994). When hOct-2 was introduced into the plasmacytoma prior to fusion with T lymphoma BW5147, all resulting hybrids displayed the phenotype of the plasmacytoma parent. If, however, hOct-2 was introduced into the T lymphoma before fusion, there was a dose-dependent effect on the percentage of hybrids with an Ig+ phenotype. If hOct-2 must work together with another B cell-restricted factor (BAF) to preserve plasmacytoma gene expression, it is in the presence of that factor in MPC11-*hOct-2* transformants even before cell fusion. In BW5147-*hOct-2* transformants, however, BAF would not be available. Upon fusion with the plasmacytoma, hOct-2 must find and form a complex with BAF before the gene encoding BAF is silenced by T-lymphoma-derived mechanisms. This implies, of course, that the Oct-2/BAF complex not only positively regulates plasmacyte-specific genes, but also serves to inhibit T cell-derived repressor(s) function. The levels of hOct-2 at the time of fusion would influence

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the likelihood that the necessary functional interaction with BAF would take place and, therefore, the percentage of hybrids that escaped plasmacytoma gene-silencing.

OBF-1 would appear to be a candidate for this tissue-restricted, cooperating factor. OBF-1 interacts with both Oct-2 and Oct-1, however, and does so through their POU domains. As we show here, Oct-1 cannot achieve the effects on gene expression seen with Oct-2. Simple recruitment of OBF-1 by Oct-1 to an octamer site, therefore, must not be sufficient to achieve the broad effects on plasmacytoma-specific gene expression that we see.

Given the importance of the C-terminal domain to Oct-2's activity in the cell fusion studies, we think that a better candidate for Oct-2's functional "partner" is one that would either bind to or have effects on this region of the Oct-2 protein. Transient transfection studies in which octamer-dependent enhancers were introduced into B lymphocytes have similarly suggested a dependence upon a B cell-restricted factor(s) and the C-terminal domain of Oct-2 for Oct-2's effects on enhancer (as distinct from promoter) activity (Annweiler et al., 1992; Pfisterer et al., 1994; Seipel et al., 1992). The latter studies used synthetic, octamer-dependent enhancers, however, and it was not clear how the results translated to effects on endogenous genes. This was particularly difficult in view of the fact that, when measured in transient assays, natural, octamer-containing enhancers (e.g. the IgH intronic enhancer Eμ) often retained significant activity after octamer site-mutation (Kiledjian et al., 1988; Lenardo et al., 1987). In the present study, we were able to perform a structure-function assay of Oct-2 in which we were measuring effects on the natural target genes of this transcription factor. Again, we found a critical requirement for the C-terminal domain of Oct-2.

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An immediate goal is to identify the functional "partner" for Oct-2 and relevant genes subject to Oct-2/BAF regulation. The fact that hOct-2 and, similarly, hOct1.1.2 rescue the expression of endogenous, murine Oct-2 suggests that *Pou2f2* is one locus subject to this regulation (Radomska et al., 1994 and present study). Because the chimeric protein rescues endogenous Oct-2 expression, it is formally possible that only the *Pou2f2* locus can be activated directly by this modified protein (lacking Oct-2's N-terminal and POU domains) and that other genes rescued by expression of Oct1.1.2 are actually dependent upon the endogenous and intact Oct-2 for their expression. In any case, these experiments place the C-terminal domain of Oct-2 at the apex of the regulatory cascade.

As shown in these and our previous studies, hOct-2 rescues the expression of PU.1 (Radomska et al., 1994 and present study). While gene knockout studies suggest that PU.1 expression is not dependent upon Oct-2 in early B cells (compare phenotypes, reviewed in Matthias, 1998; Simon, 1998), it is not surprising that transcriptional regulation of this and other genes would change as B cells are induced to differentiate into Ig-secreting plasmacytes. This is certainly the case for the IgH locus (Arulampalam et al., 1997; Ong et al., 1998). One approach to identifying the hierarchy of action of B cell-specific genes within Ig-secreting cells is the cell fusion approach described here (study the effects of "rescuing" other transcription factor genes from silencing - e.g. PU.1).

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CHAPTER 4

Studies to identify a factor interacting with the C-terminal domain of Oct-2 in Ig secreting cells.

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INTRODUCTION

The POU transcription factors Oct-1 and Oct-2 bind the conserved octamer motif (ATGCAAAT) found in all Ig promoters as well as in the enhancers of immunoglobulin genes ((Hatzopoulos et al., 1990; Singh et al., 1986; Staudt et al., 1988; Staudt and Lenardo, 1991; Staudt et al., 1986). Oct-1 is a ubiquitous factor expressed in most cell types, while Oct-2 is a mostly lymphoid cell restricted protein (Sturm et al., 1987; Sturm et al., 1988) (Clerc et al., 1988; Muller et al., 1988; Scheidereit et al., 1988). Both Oct-1 and Oct-2 share significant homology in their DNA binding domains called POU domain (Herr et al., 1988; Rosenfeld, 1991). In addition to mediating –specific DNA binding, the POU domain is also involved in multiple protein-protein interactions (Herr and Cleary, 1995) (Pfisterer et al., 1995; Wirth et al., 1995; Zwilling et al., 1995). As contrasted to the POU domain, the N and the C termini of the two proteins are highly divergent (Ko et al., 1988; Sturm and Herr, 1988).

Several studies suggested that both Oct-1 and Oct-2 were functionally interchangeable within B-lymphoid cells (Johnson et al., 1990; LeBowitz et al., 1988; Luo et al., 1992; Pierani et al., 1990). Both Oct-1 and Oct-2 can directly interact with OBF-1(OCA-B or Bob-1) which is a B cell-specific co-activator protein and can activate an octamer element driven reporter gene in a B cell specific manner (Gstaiger et al., 1995; Luo et al., 1992; Luo and Roeder, 1995; Strubin et al., 1995). This suggested a functional overlap between Oct-1 and Oct-2 proteins. However, more defined analysis also demonstrated functional differences between Oct-1 and Oct-2 proteins (Annweiler et al., 1992; Corcoran et al., 1993; Feldhaus et al., 1993; Pfisterer et al., 1994). Genetic evidence for unique roles for Oct-2, were demonstrated by gene targeting either in a B

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cell line (Feldhaus et al., 1993) or in embryonic stem cells, from which mice were subsequently generated (Corcoran et al., 1993). Both types of studies revealed that Oct-2 was not essential for immunoglobulin gene transcription in early-stage B cells. Importantly, Oct-2 deficient B cells showed severe defects in the terminal phase of B cell development resulting in a great reduction in the numbers of antibody secreting plasma cells (Corcoran and Karvelas, 1994).

Gene knock out experiments involving the OBF-1 locus have shown that like the Oct-2 locus knock-outs {*Pou2f2*$^{-/-}$}, in mice, early stages of B cell development in the bone marrow are not affected in OBF-1$^{-/-}$ mice. However there was a B cell defect as evidenced by the lack of germinal centers and severe reduction in the serum levels of IgA, IgG1, IgG2a, IgG2b, IgG3 and IgE. (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996). The development of plasma cells was however not affected (Qin et al., 1998). More recent studies on OBF-1$^{-/-}$ mice have shown that OBF-1 is required for the development of B cells at the time when they exit the bone marrow and enter the peripheral lymphoid organs (Schubart et al., 2000). This was evident from the FACS analysis of wild type and OBF-1 knockout mice when labelled with mAb493 antibody (labels a protein of 135 kd on B cells representing early stages of development). There was a complete absence of mAb493^{+} B cells (immature B cells) from the spleen of OBF-1 deficient mice as compared to the wild type mice, although their precursors in the bone marrow were normal, thus showing that in the absence of OBF-1, the transit of immature B cells from the bone marrow to the spleen was greatly impaired. This observation was further confirmed by the analysis of animals deficient in both OBF-1 and Btk which showed normal B cell development in the bone marrow, and a complete lack of B cells in

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the peripheral lymphoid organs (Schubart et al., 2000). Thus although the phenotypes of *Pou2f2*$^{-/-}$ and OBF-1$^{-/-}$ mice are not identical, there is enough evidence to show that both Oct-2 and OBF-1 function together in the terminal differentiation of B cells (i.e. in steps occuring after antigen activation of B cells).

In independent studies involving somatic cell fusion experiments we obtained evidence that Oct-2 played a central role in regulating the expression of Ig and other tissue-specific genes in Ig secreting cells (the terminal stage of B cell differentiation) (Radomska et al., 1994). Our recent studies as described in chapter 3 of this thesis show that the C-terminal domain of Oct-2 is crucial for the program-preserving activity of Oct-2 seen in Ig secreting cells (Sharif et al.,). When the C-terminus of Oct-2 was replaced by the corresponding C-terminal domain of Oct-1 (as in Oct 2. 2.1), the resulting protein could not rescue Ig gene expression in the hybrid cells. We have previously postulated the involvement of another B cell specific factor in the program sustaining activity of Oct-2 (hypothetical BAF,\Radomska,). This is because of a difference we observed when hOct-2 was introduced into the T lymphoma rather than into the plasmacytoma prior to cell fusion. When hOct-2 was constitutively expressed in the plasmacytoma prior to fusion with a T lymphoma, all resulting hybrids were Ig producing. This was true regardless of the amount of transfected hOct-2 expressed in the plasmacytoma prior to fusion with the T lymphoma. Three independent Oct-2 transformants expressing different levels of hOct-2 resulted in hybrids that all maintained immunoglobulin gene expression. When hOct-2 was introduced in the T lymphoma prior to cell fusion, however the percentage of hybrids with Ig+ phenotype was 20% for one transformant and 72% for another. This appeared to

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be a dose-dependent effect since the latter transformant produced roughly three times the amount of hOct-2 produced by the former.

We could explain the dose-dependent effect of hOct-2 by the following model (Figure 13). We postulate that Oct-2 plays a critical role in regulating the genetic program of a plasmacyte but it requires the presence of a B cell restricted factor (BAF/B cell accessory factor) for this function. When hOct-2 is expressed in the plasmacytoma, then it is able to form a complex with BAF even before the fusion of T lymphoma with the plasmacytoma. Upon fusion of the plasmacytoma with the T lymphoma, the hOct-2/ BAF complex is able to protect other target B cell specific genes (endogenous Oct-2, and BAF PU.1 and J-chain) from being silenced. When hOct-2 is introduced in the T lymphoma, then it is expressed in the absence of BAF. Upon fusion of the T lymphoma with the plasmacytoma, hOct-2 would have to find BAF and form a functional complex with it before the repressor from the T lymphoma silences the gene encoding BAF. In hybrids (Ig^+ hybrids) where hOct-2 is able to form a complex with BAF before the gene for BAF is silenced, the result is an Ig producing hybrid. In hybrids where the activity of hOct-2 lags behind the repressor, it results in silencing of BAF, so that hOct-2 cannot associate with it and as such there is a silencing of plasmacytoma specific genes. In the fusions involving T lymphoma transformants, the higher the level of hOct-2 in the T lymphoma prior to fusion the greater the likelihood that a functional interaction between hOct-2 and BAF will take place and therefore the greater the number of hybrids that escape Ig gene silencing. Thus the interaction between hOct-2 and BAF is concentration dependent with regard to hOct-2 in the T lymphoma prior to fusion with the plasmacytoma.

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Figure 13.

Diagram of model to explain the dose-dependent effect when Oct-2 is expressed in T lymphoma vs plasmacytoma.

BAF refers to postulated B cell accessory factor, R refers to a repressor or a repressive factor coming from T lymphoma.

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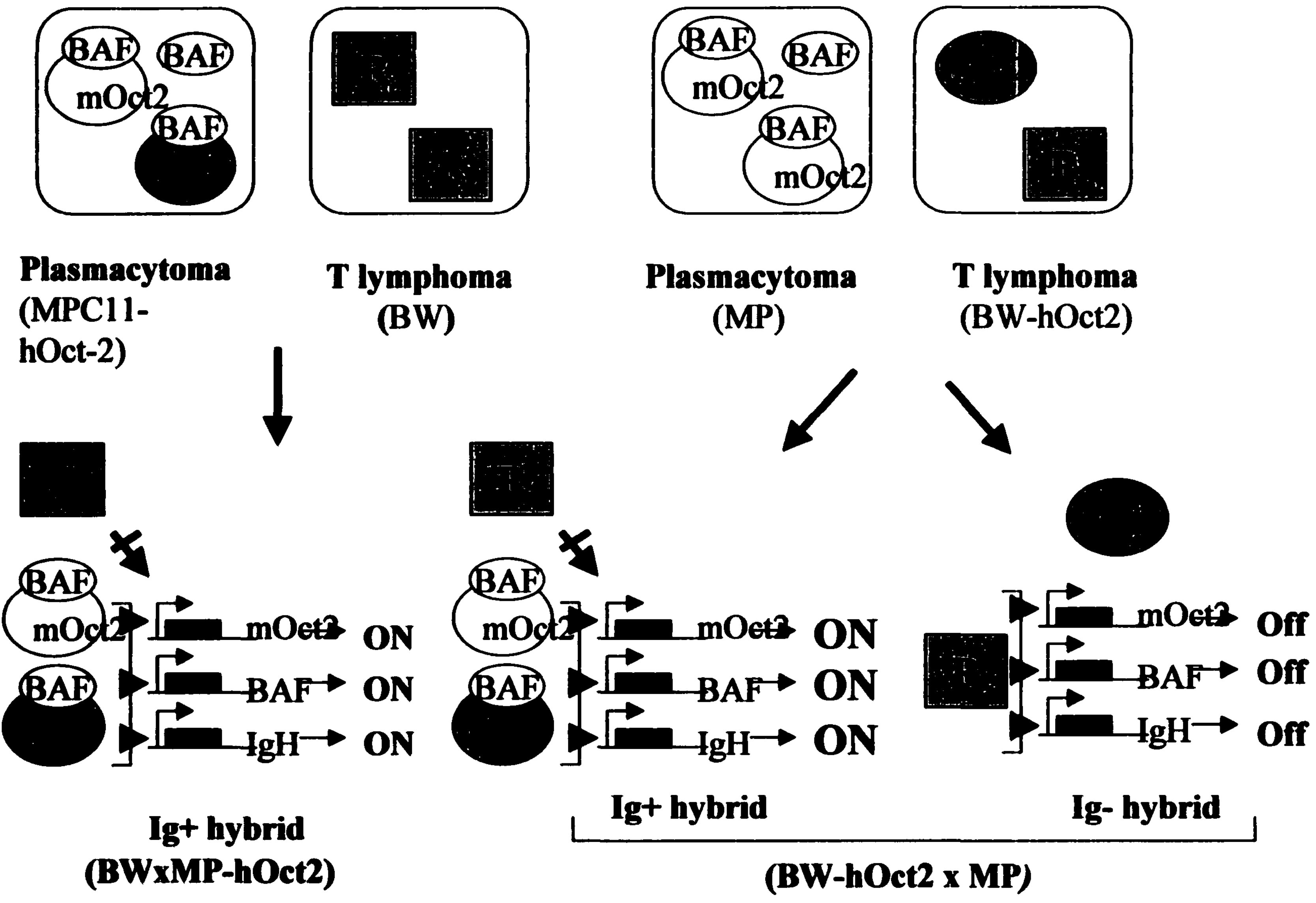

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Based on our finding that the C-terminal domain of Oct-2 is critical for the unique functions of Oct-2 Ig secreting cells, one possibility is that this factor (BAF) interacts with the C-terminus of Oct-2.

Evidence of a tissue restricted co-factor interacting with Oct-2 has also been shown by transient transfection studies. In these studies, synthetic octamer-dependent reporter plasmids containing an octamer in an enhancer position were introduced into B lymphocytes as well as into non B cells. Transactivation of the reporter gene from the distal enhancer position was observed only in B cells and not in non B cells, thereby suggesting the involvement of a B cell specific activity for octamer function from an enhancer position (as distinct from the promoter position). In these experiments, transactivation of the reporter gene from octamer dependent enhancers in B cells required the C-terminal domain of Oct-2 (Annweiler et al., 1992; Annweiler et al., 1994; Pfisterer et al., 1994; Seipel et al., 1992). These experiments were done in S197 cell line, which has low levels of endogenous Oct-2. Despite these low levels the multimerized enhancer yielded significant octamer dependent activity, in these cells. Cotransfection of Oct-2 expression vectors resulted in a clear stimulation of baseline activity. The same experiments using deletion mutants of Oct-2 (lacking N or the C-terminus), have shown that the C-terminal domain is essential for enhancer activity observed in these cells.

Although in these studies synthetic octamer dependent enhancers were used rather than any known B cell restricted gene enhancer, these findings together with ours support the notion of an important interaction between Oct-2 and a B cell restricted co-factor. OBF-1 would appear to be a possible candidate for this tissue restricted cooperating factor. This is based on the evidence that both Oct-2 and OBF-1 have an important

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function in the late stage of B cell development. Somatic cell fusion experiments to test the notion that OBF-1 is the possible cooperating factor interacting with Oct-2 in Ig secreting cells are currently in progress in the lab.

While OBF-1 may prove to be the tissue-specific cooperating factor required for Oct-2 function in Ig secreting cells, there is evidence of a requirement of one additional co-factor (Matthias, 1998). Our recent findings have shown that the C-terminal domain of Oct-2 is critical to its function in Ig secreting cells. This implies interaction of this region of Oct-2 with other regions of the transcriptional machinery. We know that OBF-1 does not interact with the C-terminal domain of Oct-2. It interacts with the POU domain of Oct-2 and also of Oct-1. It appears therefore that the interaction involving the C-terminal domain of Oct-2 does not involve OBF-1.

In an effort to determine the mechanism by which Oct-2 functions in Ig secreting cells, we have used the technique of *yeast two hybrid system* to screen for proteins that interact with the C-terminal domain of Oct-2. A GAL4 Activation Domain (AD) tagged cDNA library of the plasmacytoma (MPC11) was screened in yeast cells using two different types of "bait" constructs. One of them has the POU and the C terminal domain of Oct-2 (POU+C) linked to the GAL4 DNA Binding Domain (BD). The expectation was that this bait would capture atleast the cDNA for OBF-1 since OBF-1 binds the POU domain of Oct-2. This bait could therefore be used to test the representational nature of the cDNA library as well as the efficiency of the screen. Our idea was that this bait would also be able to capture any other plasmacytoma protein that is able to bind the POU domain of Oct-2 (besides OBF-1) or the factor (BAF) that can bind the C-terminal domain of Oct-2.The second bait construct has only the C-terminal domain of Oct-2

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linked to the GAL4 BD and is considered more selective than the POU+C bait as we expected that this bait would be able to capture any plasmacytoma cDNAs specifically interacting with the C-terminal domain of Oct-2 alone. Although this bait could prove highly selective in capturing any plasmacytoma proteins interacting with the C-terminal domain of Oct-2 alone, there was the possibility that this domain alone would not be able to adopt the proper protein confirmation needed to bind the factor that we were looking for. We have used protein domains (POU+C) or (C-terminal domain of Oct-2) rather than intact Oct-2 protein since several studies have shown that isolated domains can bind protein coactivators (Inamoto et al., 1997; Murphy et al., 1992; Sauter and Matthias, 1998 ; Stern et al., 1989). For example OBF-1 can bind both the isolated POU specific as well as the POU homeodomain and can tether them to the octamer sequence in the promoter of a reporter gene thereby leading to its transactivation (Sauter and Matthias, 1998). The results of our screens with the POU+C bait revealed four potential interacting clones. Partial sequences of these clones have been obtained and are described and discussed below.

RESULTS

To successfully screen for cDNAs (from the plasmacytoma MPC11) encoding protein(s) able to interact with the C-terminal domain of Oct-2, we employed the general scheme as outlined in (Figure 14). As a first step, two sets of fusion proteins were constructed; the first one generates a hybrid between sequences for the DNA-binding domain (BD) of the yeast transcription factor GAL4 (amino acids 1-147) and a portion of Oct-2 protein (designated as "bait" constructs). As described above, we made two

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Figure 14.

Schematic representation of yeast two-hybrid interaction system.

The Bait plasmid (POU+C or C alone) was linked in frame with the yeast GAL-BD to create GAL-BD-POU+C fusion protein. The cDNA library of the plasmacytoma MPC11 was linked to the GAL-AD to generate the AD-tagged cDNA library (Stratagene).

A) When transfected into yeast cells (YRG-2), GAL-BD domain tethers to the UAS (GAL binding sequences) present upstream of the reporter gene LacZ and *HIS3* which are present in the yeast strain. It however cannot activate the reporter genes by itself.

B) When the target protein (present in the Activation Domain tagged cDNA library) and the bait (POU+C) interact, the GAL-AD and the GAL-BD are brought close to each other and act together with the bound transcription factors to initiate transcription of the lacZ and *HIS3* reporter genes.

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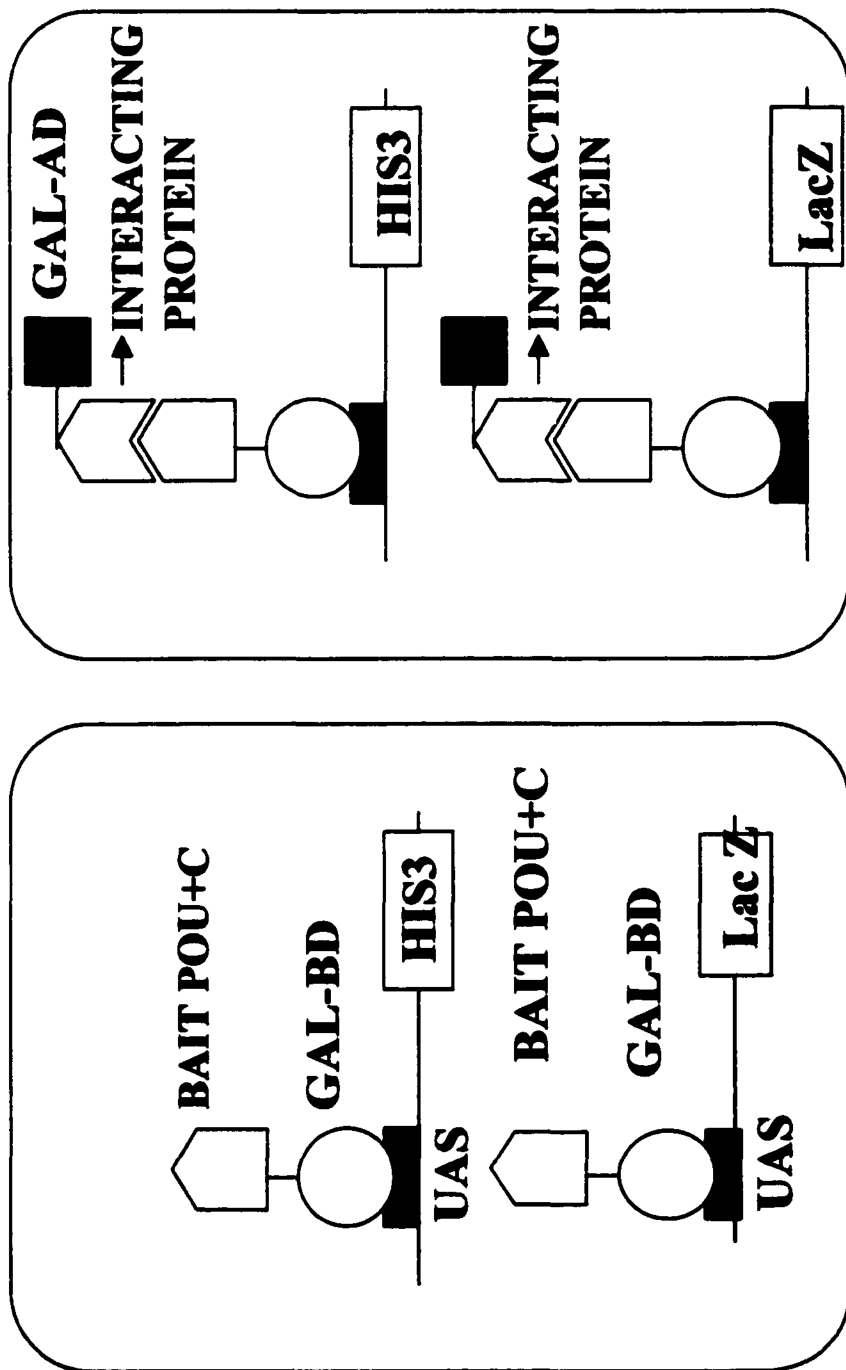

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different bait constructs (POU+C; and C-only) to screen for plasmacytoma proteins able to interact with the C-terminal domain of Oct-2.

A second expression plasmid has the cDNA library generated from murine plasmacytoma (MPC11) fused to the GAL4 Activation Domain (AD) (amino acids 761-788).The cDNA library was constructed by Stratagene™ (La Jolla, California) in a phage vector HybriZAP-2.1™ and has a complexity of $\sim 10^6$ clones. The strategy of yeast two-hybrid was actually first demonstrated with Gal4 and Gal80 interactions (Ma and Ptashne, 1987a) and later generalized (Fields and Song, 1989). If the two proteins expressed in yeast are able to interact, the resulting complex will regain the ability to activate transcription from promoters containing GAL4 binding sites (UAS-Upstream Activating Sequences).

A new yeast strain (YRG-2) has been constructed and provided to us by Stratagene™ (La Jolla, California), and provides a dual selection system to efficiently screen cDNA libraries for clones interacting with the protein of interest. This yeast strain (YRG-2) has both a lacZ reporter gene and a *HIS3* gene with upstream activating sequence (UAS) already present in the genome of the yeast. To screen for associated proteins, YRG-2 cells expressing POU and the C-terminal domains of Oct-2 or (only the C-terminal domain of Oct-2) fused to the GAL4 binding domain were transformed with Activation Domain tagged cDNA library (MPC11). When the POU and the C-terminal domains of Oct-2 become tethered to the lacZ and *HIS3* promoters through the binding of GAL4-BD to the UAS, the lacZ and *HIS3* genes will only be activated if a protein encoded by the cDNA library binds to either Oct-2 POU or C-terminal domain bringing the Activation Domain of GAL4 to the same promoter. It was possible that the "bait"

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encoding only the C-terminal domain (pBDOct-2C) could activate the reporter genes by itself as this domain of Oct-2 serves a transcription activation function in lymphoid cells. We tested this possiblility by transforming yeast cells with the bait constructs alone (both POU+C and C-terminal domain) and then testing for activation of transcription of lacZ reporter using filter lifts to test forβ-galactosidase activity. We found that the C-terminal domain could not by itself activate the lacZ reporter gene in yeast cells. Having confirmed this, we used these baits for our yeast two hybrid screens. Yeast cells expressing the bait constructs (either POU+C or the C-terminal domain of Oct-2) were then transformed with the cDNA library of the plasmacytoma and any interacting hybrids were isolated by first screening for *His*$^+$ prototrophs and then for β-galactosidase activity using the filter lift assay (Breeden and Nasmyth, 1985). The advantage of *HIS3*/lacZ screen combination is that it virtually eliminates the appearance of false positives (Durfee et al., 1993).

•

Screening for plasmacytoma proteins that interact with either the POU or the C-terminal domain of Oct-2

To screen for plasmacytoma proteins able to interact with this portion of Oct-2 , YRG-2 yeast cells were subjected to a two step sequential transformation procedure. In the first step YRG-2 cells were stably transformed with bait constructs (either POU+C or C-terminal domain) and in the second step yeast cells stably expressing the bait constructs were transformed with the cDNA library of the plasmacytoma.Transformants stably expressing the bait plasmids (POU+C or C alone) were selected on SD-trp plates and were transformed with the cDNA library. A portion of the resulting double transformants were selected on SD-leu-trp plates as a measure of the transformation frequency. The

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remainder were plated on the Synthetic minimal (SD) medium lacking Histidine, Leucine and Tryptophan amino acids (SD-His-Leu-Trp) to select for only those clones capable of activating the *HIS3* reporter gene. Only the His+ transformants were assayed forβ-galactosidase activity by the filter lift assay.

A total of ~ 1.3 million transformants (for data see table II and tableIII) were screened cDNA with the POU+C bait. Of these 1.3 million transformants, 5 x 10^4 (~ 4%) grew into colonies within 3-5 days on –His-Leu-Trp plates. Many colonies were very small but were nevertheless included in the count. These colonies were allowed to grow for 6-7 days before filter lifts were performed

These His+ transformants were then screened for their ability to produce β-galactosidase, using a filter lift assay (Breeden and Nasmyth, 1985). Eleven His^+ colonies were also blue in this assay (Table II). These His^+ / $lacZ^+$colonies were considered positive in the intial screen and were used for additional studies.

Using the second bait construct pBDOct-2C (expressing the C-terminal domain of Oct-2 as a fusion protein with the GAL binding domain) we screened a total of 2 x 10^5 transformants (TableIII). Out of these, 9000 (4.5%) were His^+ and 4 were both $His^+$and $lacZ^+$.

Characterization of the positive clones isolated with the bait plasmid containing Oct-2 POU and C-terminal domains

Four out of the eleven His^+lacZ^+ colonies were confirmed positive on further restreaking and testing for β-galactosidase activity. Plasmid DNA was isolated from these colonies

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Table II.

Results of cDNA library screens using POU+C bait

This table shows number of transfections performed in yeast using POU+C bait, number of His+ clones obtained in each transfection as well as the number of His+ LacZ+ clones obtained.

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Table II

# Of Transfections	Efficiency of transformation	His^+ colonies	His+ $LacZ^+$ colonies
1	66880	11800	2
2	136000	9280	1
3	320000	5160	1
4	150000	7200	2
5	290000	10000	1
6	220000	5000	2
7	320000	6700	2

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Table III.

Summary data of Library Screens.

This table is a representation of data obtained when plasmacytoma cDNA library was screened with the two baits (POU+C; C-only).

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Table III

Bait used	No of clones Screened Leu+ Trp+	His^+ colonies	His+ $LacZ^+$ colonies
pBDOct-2POU+C	1.3×10^6	55140	11
pBDOct-2C	2.0×10^5	9000	4

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using the Yeast DNA Isolation System obtained from Stratagene™ (La Jolla, California). Plasmids isolated were then used to transform YRG-2 either alone or in combination with the bait pBDOct-2 POU+C. Transformants were assayed for β-galactosidase activity. All four plasmids induced expression of lacZ in the presence of pBDOct-2POU+C, but not in its absence. Plasmid DNA representing the above colonies was sequenced. Two of the clones had an insert size of 1.0kb while the other two had an insert size of 1.6kb. Sequence analysis of the first two (1.0kb) showed them to be identical and revealed homology to Nrfl mRNA (see sequence Q0025329). The region of maximum homology with Nrfl was within the N terminal domain of Nrfl which is rich in serine/threonine residues (see attached results for blast search). It is possible that these residues are involved in protein-protein interactions with the C-terminal domain of Oct-2. Surprisingly, one of the two 1.6kb insert clones showed multiple termination codons in all three reading frames (see sequence Q0025328) with different reading frames. The sequence of the fourth clone has not yet been determined.

DISCUSSION

We successfully developed the yeast two-hybrid interaction trap to capture any plasmacytoma specific proteins capable of interacting with the C-terminal domain of Oct-2. Our results, though preliminary at this stage, reveal interaction of Oct-2 with a protein that is homologous to the ubiquitous transcription factor Nrfl found in humans (Chan et al., 1993). Nrfl is a member of the basic leucine-zipper protein family that activates transcription via the consensus (TGAGTCA) NF-E2 /AP1 binding site. Whether it is the POU or the C-terminal domain that is involved in this interaction has yet to be

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Table IV.

Sequences of interacting clones isolated by yeast two-hybrid screens are placed here. Sequence Q0025329 is ~ 1 kb sequence that shows homology with transcription factor Nrf-1. Homology search was done using Blast 2 Sequences. The maximum region of homology is with the N-terminal domain of Nrf-1 (280 bp sequence).The N-terminus of Nrf-1 is rich in serine/threonine- a feature common of activation domains.

Sequence Q0025328 is a 1.6 kb insert got in the library screens. Sequence of this clone reveals multiple stop codons in all three reading frames.

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Q0025328

GGNTGNAGTNTATAACTATNCTNTTNCGNAGNAGNAANAACCCCACCAAACCCAAAAAAGNGNATCGAATTAGGATCCTC

TGCTAGCAGGAGNAATTCGGCACGAGCTAGNAGNAGTTTTGACAGTAGCATAATCAAATCCTCATCTTTGTCCATATCTA

CTATTTACAAGCTGTCAGAGAAATGGGTGACATGGCCAGTGGCTTATAGCAGGCTGGTGTCATTTAGACTTGTGTGATTT

TAATTCTCTGCCTGGCGTTTGGTATGTTGGAATATTTCAGGTTCTGTAATAGTCTGTAGTTACCGGCACAGGCATTACTT

ATACTTGGACACACTTTGGTTTTCACCAGCTCTGATCCTCTGATCAGTTAGAAGATTCCAGACCTTCTTATTCTACTGAA

CAGAGAAAACCCTAACCTTGATTCTTGAGTTAATGTTTCATGGTGAATGTTTCATAAGTAAGAATTTACCTTAGAGTATT

AATATATAAAATACAGTAGATGTTAATTGTTTTATATCTTTAATTTTAAAAATGTATCTTTAGAGGAGAGGAGGTAGCTG

GAGGTGAGTAGACAACCTCCAGGAGTCTCTCCTTTCCTTCTGCCATGTGGGTTCAGGGATTAAACACTGGTCAGCAAGCT

TGGTGGCAAGCATCTCTACCCACTGAGCCATCTTGTTAGGCCTTTTCTTTTAATATCTTTTTACTTCTACATAATTTTTT

TATCATGAGATCTACTGGGCGCATTATAGAAATCTGTATGTTGNGTTTTTTGGTTTTGGATCATCAANATAGGTACTTTT

TCATATATTTTAATACAAATTTGGGGTATGAAGTCTTATGAAAAAAGTCCCTTTGGGCCTTATTTACCCGGAGTTC

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Q0025329

GGNTGNNGTNTATAACTATNCTATTNCGNGNTGNAANAACCCCACCAAACCCAAAAAAANANATCGAATTAGGATCCTCT

GCTAGCAGNANAATTCGGCACGAGNAAACATTCTTGGCAAATGCTTTCGCTCTGGTCCGTCTTGCGCCGGTCCAAGAATT

TCACCTCTAGCGGCGCAATACGAATGCCCCCGGCCGTCCCTCTTAATCATGGCCTCAGTTCCGAAAACCAACAAAATAGA

ACCGCGGTCCTATTCCATTATTCCTAGCTGCGGTATCCAGGCGGCTCGGGCCTGCTTTGAACACTCTAATTTTTTCAAAG

TAAACGCTTCGGGCCCCGCGGGACACTCAGCTAAGAGCATCGAGGGGGCGCCGAGAGGCAAGGGGGGGGGGACGGTTTNC

CNNNNTCCNCCNTTTTTTTTNTNCTNCCTTTTTGNTNTNTNNTNCNNNCATTTCCATTCCCTNCCNNTTTTCTTATNTCT

TTTTTTCCCNTTAGTTNTTNNTTNTNTCNNNAAATTNCTTCNCNTNTTCCTCCCNTNTCTTNCNTAATTNTTNNCACCNN

NCCNNCANNNNTNNTNCCANTTAANNTTGTTNNNNCGNCGNCNTNNTTNAATTNANANGTTCNNTNTTGTANNNACTTTN

TATNCCCCCNCNNTTCNTTTNNCCCNNTTTTTNCCCCCCTCCGCNCNCGNNNGCNCCNACCCCNTTTTNTCCCCANNCCC

CCANNCTTTNTCTTTNTNNNTTNNCCCCAATTNNANTNNNCATTTTTACTACCTTTNTTCACCCNNNCNNNNGCCCCCGN

TCNNNTNNGNNGNANATCCCNNNTTTAAAAGCTTNNCATNATCCGNNTANTTTNNNCANNCNTNNTCNTNNTANACGNCA

AACGTGNTANTTTCNTCNNTNTNNNTTTNCNCCNNCCT

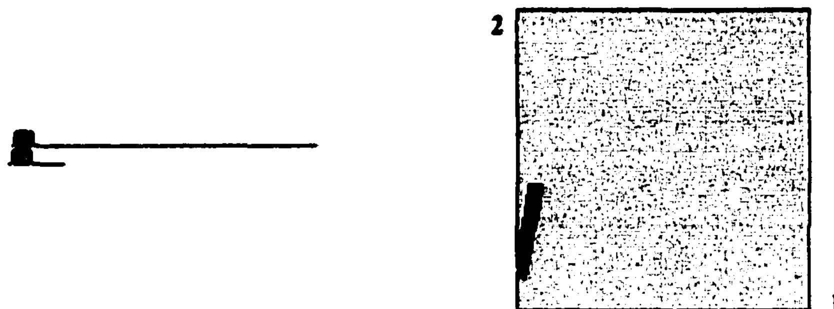
Blast 2 Sequences results

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.0.14 [Jun-29-2000]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 50 expect: 10.0 wordsize: 11 Filter ✓ Align

Sequence 1 gi 438646 Homo sapiens NRF1 protein (NRF1) mRNA. **Length** 4992 (1 .. 4992)

Sequence 2 lcl|seq_2 **Length** 918 (1 .. 918)



NOTE The statistics (bitscore and expect value) is calculated based on the size of nr database

```
Score =  519 bits (270), Expect = e-144
Identities = 277/278 (99%), Positives = 277/278 (99%), Gaps = 1/278 (0%)
```

```
Query: 48  aaacattcttggcaaatgctttcgctctggtccgtcttgcgccggtccaagaatttcacc 107
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 106 aaacattcttggcaaatgctttcgctctggtccgtcttgcgccggtccaagaatttcacc 165

Query: 108 tctagcggcgcaatacgaatgcccccggccgtccctcttaatcatggcctcagttccgaa 167
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 166 tctagcggcgcaatacgaatgcccccggccgtccctcttaatcatggcctcagttccgaa 225

Query: 168 aaccaacaaaatagaaccgcggtcctattccattattcctagctgcggtatccaggcggc 227
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 226 aaccaacaaaatagaaccgcggtcctattccattattcctagctgcggtatccaggcggc 285

Query: 228 tcgggcctgctttgaacactctaatttttcaaagtaaacg-ttcgggccccgcgggaca 286
           |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||
Sbjct: 286 tcgggcctgctttgaacactctaatttttcaaagtaaacgcttcgggccccgcgggaca 345

Query: 287 ctcagctaagagcatcgagggggcgccgagaggcaagg 324
           ||||||||||||||||||||||||||||||||||||||
Sbjct: 346 ctcagctaagagcatcgagggggcgccgagaggcaagg 383
```

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```
CPU time:     0.13 user secs.      0.00 sys. secs          0.13 total secs

Gapped
Lambda     K      H
    1.33    0.621     1.12


Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 5
Number of Sequences: 0
Number of extensions: 5
Number of successful extensions: 2
Number of sequences better than 10.0: 1
length of query: 4992
length of database: 706,884,452
effective HSP length: 25
effective length of query: 4967
effective length of database: 703,344,377
effective search space: 3493511520559
effective search space used: 3493511520559
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 20 (39.1 bits)
```

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determined. From protein sequence analysis, the N termini of the two identical clones isolated show homology to the N terminus of Nrf1 which is rich in acidic residues flanked by multiple serine and threonine residues. Nrf1 was initially isolated from a human erythroleukemia cell line (K562) and could activate transcription from the tandem NF-E2 /AP-1 repeat present in HS2 of the β-globin gene LCR. It was found that co-transfection of Nrf1 with reporter plasmids into K562 cells increased the expression of a reporter gene in an NF-E2 /AP-1 motif dependent manner. A marked reduction in the expression of the reporter was seen when the N terminal region of Nrf1 was truncated, showing that activation by Nrf1 requires the residues in the N terminal domain (Chan et al., 1993). The exact mechanism of transactivation by Nrf1 as well as its role in β-globin gene expression has yet to be determined. The presence of NF-E2 /AP-1 binding sites in the promoters of several genes involved in the synthesis of heme and iron storage protein ferritin suggest that Nrf1 has a role in these genes expression (Andrews et al., 1993).

I would suggest the following studies for pursuing further the functional importance of the gene we have isolated.

As a first test of whether the cDNA we isolated actually encodes a protein that interacts with the C-terminal domain of Oct-2, it could be tested for interaction with our second bait construct which contains only the C-terminal domain of Oct-2. If it interacts with the C-terminal domain of Oct-2 alone, its tissue distribution should then be determined. This could be done by Northern blot analysis of total RNA from different murine tissues and cell lines. It is possible that the essential function of the C-terminal domain of Oct-2 involves interaction with either a tissue-specific or a ubiquitous protein.

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If we find that the interacting protein is tissue-specific, this would directly explain both the requirement for a tissue-specific co-factor and the C-terminal domain of Oct-2 for the critical functions of Oct-2 in Ig secreting cells

Further characterization would depend on the tissue specificity results. If the cloned gene encodes a known transcription factor, then a co-immunoprecipitation assay could be done with available antibodies, so as to determine physiological interactions *in vivo*. If the cloned gene does not show similarity to any known transcription factors but is B lymphocyte restricted, then we could further confirm its interaction with Oct-2 by doing transient assays. . For this a full-length cDNA clone needs to be isolated, cloned into a eukaryotic expression vector and used in co-transfection experiments in non B cells with Oct-2 along with an octamer dependent reporter gene (containing the octamer in an enhancer position). The goal would be to see if there was trans-activation of the reporter from the enhancer position.

If the protein interacts with the C-terminal domain of Oct-2 but is actually a ubiquitously-expressed protein, then we would look for possible protein-protein interaction motifs (using a specialized DNA analysis programs designed for the purpose). It is possible that the gene encodes a protein that contains functional motifs (e.g. a kinase motif or a serine/threonine rich domain, a second protein-protein interaction domain) that provide insight into its function. It is possible that the essential function of the C-terminal domain involves its interaction with a ubiquitous factor that is a component of the transcriptional machinery.

We expected that the POU-C bait in the yeast two-hybrid screens would capture cDNA for OBF-1, since OBF-1 binds to the POU domain of Oct-2. We screened a total

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of 1.3x10^6 double transformants (-leu-trp) with the POU+C bait, but still did not capture OBF-1 in our library screens. It is possible that we need to screen our plasmacytoma cDNA library further to be able to capture OBF-1. One of groups who cloned the gene for OBF-1 (Matthias, 1998) by a genetic selection system related to the yeast-two hybrid system .had discovered OBF-1 in a minimum of 2 x 10^6 double transformants, whereby they recovered three independent cDNA inserts from the same cellular mRNA.

Despite further screening of the cDNA library in yeast, if we are still unable to recover OBF-1 then we might want to modify our strategy of yeast two hybrid similar to that used by others (Strubin et al., 1995). In screening for a coactivator protein capable of interacting with Oct-1 and Oct-2, Strubin *et al* constitutively expressed full length Oct-1 in yeast cells along with a *HIS3* reporter gene that had 6 copies of an octamer element inserted upstream of *HIS3* regulatory sequences. The cDNA library from Namalwa cells (human B cell line) was tagged with the viral transactivator VP16. Hybrid proteins capable of interaction with DNA-bound Oct-1 would be recruited to the *HIS3* promoter, thereby activating transcription of *HIS3*. This would probably work better, if the protein that we are looking for can only interact with DNA bound Oct-2. Although OBF-1 was isolated by the strategy described above, *in vitro* binding assays have shown that isolated POU domains of Oct-1/Oct-2 can bind OBF-1, even in the absence of DNA.

Alternatively we could clone the cDNA encoding OBF-1 in pADGAL4 vector so that it is expressed as a fusion protein with GAL4 AD and then test for interaction with the bait pBDOct-2POU+C by cotransformation of YRG-2 cells.The reason we chose to work with isolated domains of Oct-2 rather than the intact protein itself was because of evidence (as described in the introduction) that isolated domains of Oct-2 could bind

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protein partners. It has been shown by transient transfection experiments with octamer dependent reporters that OBF-1 can be tethered to the DNA by a POU domain that has been artifically separated into two halves in the middle of the linker region. Even under these conditions OBF-1 causes transactivation of the reporter gene (Sauter and Matthias, 1998). While OBF-1 may prove to be the tissue-specific partner required for Oct-2 function in Ig secreting cells, there is evidence (as described earlier) for requirement of atleast one additional co-factor (Matthias, 1998). It is possible that the function of Oct-2 in Ig secreting cells requires OBF-1 as well as another tissue specific factor interacting with the C-terminal domain of Oct-2. The strategy of yeast two hybrid system will help us identify any protein interacting with the C-terminal domain of Oct-2 and thereby help us understand its mechanism of action in Ig secreting cells.

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CHAPTER 5

Discussion/ Future Studies

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Transcriptional regulation of immunoglobulin heavy chain gene expression by the two octamer binding transcription factors Oct-1 and Oct-2 is a topic of great interest. The identical binding properties but differing distributions of the two Oct factors Oct-1 (ubiquitous) and Oct-2 (B-lineage restricted), have led researchers to evaluate the contribution of these factors to immunoglobulin gene expression.The precise role played by each of these factors for transcription activation through the octamer motif is still not completely understood. The work embodied in this thesis provides additional evidence for a unique and essential function for Oct-2 in Ig secreting cells, and serves to identify a critical structural element (C-terminal domain) for this function.

While the N-terminal and the POU domains of Oct-1 can functionally substitute for those of Oct-2 when tested individually, the C-terminal domain cannot. This allows us to map the unique function of Oct-2 (with regard to rescue of Ig and other B cell specific genes) to its C-terminal domain. The rescue of Ig producing hybrids when the chimeric protein Oct 1.1.2 was used in the cell fusion experiments was 78% as compared to a complete rescue (100%) observed when intact hOct-2 was used. This suggests that although chimeric Oct 1.1.2 does not behave exactly like intact hOct-2, however, the bulk of the rescue activity of Oct-2 resides in its C-terminal domain. It is possible that inter-domain interactions are accountable for the differences seen in the activity of this chimeric protein vs intact Oct-2. Interestingly, chimeric Oct 1.2.2 (which has the N-terminal domain of Oct-2), behaved like wild type Oct-2, resulting in 100% Ig-expressing hybrids when fused to BW5147 (T lymphoma). Oct 1.1.2 and Oct 1.2. 2 differ only in the POU domains which, in turn, have only 8 amino acids differences. Further mutational analysis of the POU domain of Oct 1.2.2 might provide insight into the functional

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difference between Oct 1.1.2 and Oct 1.2.2 proteins. It should also be noted, however, that Oct 2.1.2, like Oct 1.2.2 yielded 100% Ig positive hybrids. If 1.1.2 is somewhat deficient in its activity (relative to intact Oct-2), therefore, this deficiency can be overcome through protein motifs (interacting proteins) within Oct-2's N-terminal or Oct-2's POU domain.

The fact that hOct-2 and, similarly, hOct 1.1.2 rescues the expression of endogenous, murine Oct-2 suggests that *Pou2f2* is one locus subject to Oct-2 mediated regulation. Because the chimeric protein rescues endogenous Oct-2 expression, it is formally possible that only the *Pou2f2* locus can be activated directly by this modified protein (lacking Oct-2's N-terminal and POU domains) and that other genes rescued by the expression of Oct 1.1.2 are actually dependent upon endogenous Oct-2 for their expression. One way to test this hypothesis would be by rescuing *Pou2f2*$^{-/-}$ mice with an Oct-2 vs Oct 1.1.2 expressing transgene. The primary defect in *Pou2f2*$^{-/-}$ mice is in the stimulation of IgM positive B cells to Ig secreting plasma cells, and expression of an Oct-2 transgene should be able to remedy this defect. Expression of an Oct 1.1.2 transgene in *Pou2f2*$^{-/-}$ mice, would not be able to upregulate Oct-2 expression (since the Pou2f2 locus is disrupted in these mice), and would allow us, therefore a means of clearly testing functional differences between Oct 1.1.2 and Oct-2.

Given our results in cell fusion studies, we predict that loss of Oct-2 from an Ig secreting plasmacyte would have pronounced effects on the expression of Ig and other tissue specific genes. However, because our method of silencing Oct-2 involves introduction of a T cell-derived repressor(s) into an Ig secreting cell, it is possible that the repressor is responsible for many of the effects we see. For example, it is possible that

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Oct-2 is simply required to inactivate a T-cell derived repressor in the cell fusion system. It is not needed to positively regulate B cell specific genes but is required only to prevent them from being silenced. One test of this notion involves another cell fusion system. As described earlier, Oct-2 k/o experiments in both mice and a pre-B cell line have shown that Oct-2 is not required for Ig expression in either pre-B or surface Ig+ B cells. When a pre-B cell line is fused with BW5147 (the T lymphoma of our experiments), IgH expression ceases as does Oct-2 expression. If the repressor inactivation model were correct, we would predict that ectopic expression of Oct-2 in the pre B cell cell line would preserve immunoglobulin gene expression in the pre B cell x BW hybrids, similar to similar to plasmacytoma x T lymphoma hybrids. However, unpublished data from our lab have shown that Oct-2 does not rescue pre-B cell x BW hybrids from Ig gene silencing. These fusion experiments, therefore, like the knock-out experiments show that Oct-2 has very different functions in pre B vs Ig-secreting cells and are consistent with a model in which Oct-2 does not simply inactivates a T cell-derived repressor.

A direct knock-out of the Oct-2 locus within Ig-secreting cells is the most direct method for determining the function of this transcription factor. The *Pou2f2* locus knock-out in mice results in neonatal lethality and B cells cultured from these mice do not produce Ig-secreting cells, providing no means of directly studying the function of Oct-2 in Ig-secreting cells (Corcoran et al., 1993).

While a gene knock-out in Ig secreting cells is one possibility, other strategies could be helpful towards achieving the same goal. One approach would be to design specific truncated versions of Oct-2 that might serve a dominant negative function with respect to Oct-2's activities, when over-expressed in Ig-secreting cells.

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An expression vector encoding the C-terminal domain of Oct-2 can be over-expressed in an Ig secreting cell line, and its effects on endogenous gene expression measured. The hypothesis here is that this domain (Oct 0.0.2) might bind a factor (which we have already proposed), that is in limiting quantities in the cell, so that when the C-terminal domain is present in large quantities, it sequesters this required co-factor, leaving none available to bind to full length Oct-2 when it is bound to an octamer site.

The expression vector encoding the C-terminal domain of Oct-2 has already been constructed, and its expression as a peptide has been confirmed in T-lymphoma cells (BW5147), by transient transfections of these cells. The next step would be to stably transfect the putative dominant negative into Ig-secreting cells, and then measure its effects on gene expression (endogenous *Pou2f2* locus, Ig and J-chain loci etc). Currently experiments involving the use of an inducible gene expression system (commercially obtained from Stratagene) to induce expression of the putative dominant negative in the Ig secreting cell line (Oct 0.0.2) are being worked out in the lab.

We know that the POU domain of both Oct-1 and Oct-2 can bind the B cell specific coactivator protein OBF-1/OCA-B/Bob-1, and can mediate IgH promoter function (Gstaiger et al., 1995) (Luo et al., 1992) (Strubin et al., 1995). A second possible way in which Oct-2 function can be eliminated in Ig secreting cells is by abrogating the binding of OBF-1 to the POU domain of Oct-2, while still maintaining the ability of Oct-2's POU domain to bind the octamer sequence. This is based on the rationale that over-expression of a mutant POU domain might successfully compete with full length Oct-2 for octamer binding sites, thus displacing both endogenous Oct-2 and tethered OBF-1 from their normal target sites. This mutant POU domain (POU^{mut}) would bind the

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octamer sequence on the Oct-2 regulated target genes in an Ig secreting cell line, but would be unable to trans-activate these genes, as it lacks the transactivating domains (N and the C-terminal domain), and the means for recruiting the transactivation domain of OBF-1. Consequently, a down-regulation of Oct-2 regulated genes (endogenous *Pou2f2*, Ig, J-chain etc), would be expected.

Work by others have shown that point mutations in the POU specific domain of Oct-1/Oct-2 at residues L53A and N59A are affected most dramatically in complex formation with OBF-1 as evidenced by the electrophoretic mobility shift assays (Gstaiger 1996). The L53A mutant bound OBF-1 at 11% wild type levels, while the N59 mutant bound it at 6% wild type levels, while both bound the octamer site at wild type levels (Gstaiger et al., 1996). In a similar way, these point mutations can be made in the POU domain of Oct-2 (to generate a mutant POU domain) and tested for their ability to behave as putative negatives for Oct-2 function in Ig secreting cells.

A third protein that could be used for this study is a truncated version of Oct-2 that lacks the C-terminal domain of Oct-2 (Oct 2.2.0). The rationale here is that OBF-1 would still be able to bind the POU domain in Oct 2.2.0, however functions that require the C-terminal trans-activating domain of Oct-2 would be expected to show an effect. This protein would be expected to have a more limited effect than the protein with the mutant POU domain.

In the cases of POU^{mut} and Oct 2.2.0 where a DNA-binding competition with wild type Oct-2 is expected, expression vectors encoding these proteins could be transiently transfected into BW5147 cells that already express the flu tagged hOct-2. Competition for binding to an octamer sequence could be assessed by electrophoretic mobility shift assay

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(EMSA). If, as predicted there were decreasing Oct-2/octamer complexes when in the presence of increasing amounts of POU^{mut} peptide for example, the next goal would be to measure the effect of this competition on the expression of genes in Ig-secreting cells. As mentioned earlier, an inducible gene expression system (Stratagene) could be used to induce expression of these putative dominant negatives in an Ig secreting cell and their effects on gene expression could be measured.

Our data are consistent with studies of immunoglobulin gene expression which provide evidence for a shift in the array of transcription factors mediating gene control as B cells develop and differentiate. While in early stages of B cell development, Eμ is essential for efficient IgH gene assembly (V-D-J joining) and transcription, this transcription element is dispensable in Ig-secreting cells, probably because additional enhancers at the 3'end of the IgH locus become active at the surface Ig+ stage (Ong et al., 1998). In recent studies of $OBF\text{-}1^{-/-}$ mice, it has been shown that the observed immune defect in $OBF\text{-}1^{-/-}$ mice as evidenced by their reduced ability to secrete the switched isotypes is possibly due to deficiencies in the function of 3'-IgH enhancer elements (Stevens et al., 2000). These studies have shown that OBF-1 is required for cytokine-induced function of the 3'-IgH enhancer elements, as the 3'enhancer elements become important, they depend upon OBF-1 consistent with problems of Ig expression in $OBF\text{-}1^{-/-}$mice. It is possible that the 3'enhancers are specifically dependent upon Oct-2 for their activity. The 3' IgH enhancer contains four octamer sites, and to determine whether the activity of these elements was dependent on Oct-2, these octamer motifs were mutated in the 3'enhancers of the reporter vectors, which were transiently transfected into M12 (mature B) cells (Tang and Sharp, 1999). These experiments showed that enhancer

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activity was directly regulated by Oct-2 and that phosphorylation of Oct-2 was an important aspect of this regulation. There was also a synergistic activation of Oct-2 by OBF-1, suggesting that Oct-2 and OBF-1 interact with the 3'enhancer in regulation of the IgH locus.

We have previously postulated the involvement of another B cell-restricted factor in the program-sustaining activity of Oct-2 (hypothetical B cell accessory factor BAF) (Radomska et al., 1994). This was based upon the dose-dependent effect observed when hOct-2 was expressed in the T-lymphoma prior to fusion with the plasmacytoma. Given the importance of the C-terminal domain of Oct-2 in the somatic cell fusion experiments described in this thesis, we think that a possible candidate for Oct-2's functional "partner"is one that would either bind to or have effects on this region of the Oct-2 protein. Thus our findings together with the results from other groups (Annweiler et al., 1992) (Pfisterer et al., 1994) (Seipel et al., 1992) strongly support the notion of an important interaction between Oct-2 and one or more tissue restricted co-factor.

Our data regarding the interaction of Oct-2 with a cofactor are still preliminary and require further characterization of the putative interacting clones isolated by this system. Preliminary data from our studies however, reveal interaction of Oct-2 with a protein that is homologous to ubiquitously expressed protein called Nrfl (Chan et al., 1993). Nrfl is a member of the basic leucine-zipper protein family that activates transcription via the consensus (TGAGTCA) NF-E2/AP1 binding site (described in detail in chapter four of this thesis). The maximum homology of this potential interacting clone is with the N-terminus of Nrf-1.

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Our finding that the C-terminal domain of Oct-2 is critical to its function in Ig secreting cells, implies interaction of this domain with other components of the transcriptional machinery. Although we expected to capture a tissue-specific factor interacting with Oct-2 in our yeast two hybrid screens, we did not rule out the possibility of a ubiquitously expressed factor interacting with the C-terminal domain of Oct-2. The requirement for a tissue-specific factor, and the requirement for the C-terminal domain of Oct-2, may be independent of one another. For example the tissue specific factor required might bind another domain (e.g. OBF-1 binding the POU domain of Oct-1/Oct-2), while the C-terminal domain might be required for its ability to uniquely associate with a protein that is found in all cells. It is also possible that OBF-1+Oct-2 is enough to get rid of the dose dependent effect in T-lymphocytes and, therefore, OBF-1 is the only missing tissue-specific component for Oct-2 rescuing activity. Cell fusion experiments to test this notion are currently in progress in the lab. In these experiments stable transformants of BW5147 already expressing hOct-2 are transformed with an expression vector encoding flu-tagged hOBF-1 prior to fusion with the plasmacytoma MPC11. If hOct-2 and OBF-1 are mutually-dependent transcription factors involved in the sustained expression of the plasmacyte program, they may give similar results when provided individually to the plasmacytoma (yielding uniformly Ig+ hybrids) or the T-lymphoma (dose-dependent increases in number of Ig+ hybrids). Together in the T-lymphoma, however, they could synergize to yield uniformly Ig+ hybrids. Alternatively, OBF-1 might lie below Oct-2 in the regulatory hierarchy-its gene (OBF-1) capable of being rescued by hOct-2 but the hOBF-1 protein incapable of rescuing Oct-2 expression from the Pou2f2 locus. In this case hOBF-1 would be expected to have little or no effect on its own when introduced

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either into the plasmacytoma or T-lymphoma prior to fusion. It might, nevertheless, synergize with hOct-2 in the T-lymphoma if it is the partner hOct-2 requires for its plasmacytoma-sustaining effects. The results of these experiments should provide significant insight into the interplay of these two transcription factors and their effects on the genes expressed by the Ig-secreting cells.

We expected to capture cDNA encoding OBF-1 while using the bait POU+C, since OBF-1 interacts with the POU domain of Oct-2. We isolated four putative interacting clones using the POU+C bait. Sequence analysis of these putative interacting clones however, did not identify any sequence resembling or identical to OBF-1. Since we knew from other evidence that OBF-1 binds isolated POU domains of Oct-1 and Oct-2 (Sauter and Matthias, 1998), we expected to trap the cDNA encoding OBF-1 using this system. A possibility worth consideration in this regard is that it might be useful to screen the plasmacytoma cDNA library further (roughly twice the library size), since the groups who cloned the B cell co-activator OBF-1/OCA-B/Bob-1 (Gstaiger et al., 1995) (Luo and Roeder, 1995) (Strubin et al., 1995), discovered this co-activator in a total of 2×10^6 double transformants. The reason we wanted to capture OBF-1 by this strategy was because this would test both the representational nature of the cDNA library as well as the efficiency of the screen. The POU+C bait was meant to serve both as a positive control for our cDNA library screens (capture OBF-1) and as a means for capturing a C-terminal domain interacting protein. The cDNA library should also be screened using the second bait which has only the C-terminal domain of Oct-2 linked to the GAL4-DNA binding domain. A consideration in using this bait is that this domain might not be able to adopt the proper protein configuration required for binding the factor we seek (hence parallel

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use of the POU-C vector). On the other hand, strong interactions with the POU domain or interactions with POU that involve a relatively abundant factor might mask a more weakly binding or less abundant protein interacting with the C-terminal domain.

The four cDNA clones isolated from the plasmacytoma cDNA library using the bait POU+C should also be tested for their ability to interact with the C-terminal domain alone. If they do interact with the C-terminal domain, the next step would be to determine the tissue specificity of their expression (e.g. by Northern blot using RNA from various murine tissues and cell lines), and also to confirm physiological interactions in vivo by co-immunoprecipitation assay, using available antibodies. A full length cDNA clone could be isolated and used in *in vitro* interaction assays, with radiolabelled protein (of the above clone) prepared by in vitro translation, and immobilized GST fusion protein (GST-C-terminus), either on filters or by means of glutathione-agarose beads. Interactions could then be detected by autoradiography.

If however, these clones do not interact with the C-terminal domain of Oct-2, then it is possible that these clones interact with the POU domain, since they were captured by the POU+C bait, and such interactions also need to be cofirmed in vivo by co-immunoprecipitation assays. Also the tissue specificity results will provide information that could be useful for further analysis. Suggestions for further characterization and analysis of these clones were provided in chapter four of this thesis.

Our overall goal is to understand how Oct-2 functions in Ig secreting cells and also to know whether this effect is unique only to Oct-2. This could be determined by studying the hierarchy of transcription factor function in Ig secreting cells. This question is currently being addressed in the lab.

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It should be noted that other B lymphocyte-restricted transcription factors that we have analyzed (e.g. PU.1) are part of the family of genes protected by Oct-2 from silencing. PU.1 is a transcription factor expressed in macrophages and B lymphocytes (Klemsz et al., 1990) and is essential to early events both in lymphocyte and monocyte development (Simon, 1998). It has been shown that PU.1 binds the J-chain gene promoter and can transactivate this promoter in transient transfection assays. This suggests that a putative downstream target of PU.1 in Ig secreting cells is the J-chain locus (Shin and Koshland, 1993). There is also an octamer site in the J-chain gene promoter so that this locus might be regulated by a combination of Oct-2/PU.1 factors (Matsuuchi et al., 1986). The experimental approach that is currently being used in the lab to determine the hierarchy of transcription factor function in Ig secreting cells, is also somatic cell fusions, whereby the expression of PU.1 is constitutively maintained during the fusion process. The question here is whether this gene, like the Oct-2 expressing gene, can rescue Ig genes from silencing (there are PU.1 binding sites in the IgH Eμ enhancer).

These experiments will not only tell us the role played by PU.1 in Ig secreting cells, but will also tell us whether PU.1 has an effect on Oct-2 expression or PU.1 is strictly downstream of Oct-2 in Ig secreting cells. While gene knock-out experiments suggest that PU.1 expression is not dependent upon Oct-2 in early-stage B cells (Matthias, 1998; Simon, 1998) this may not be true in later stage cells.

In conclusion, the studies presented in this thesis have contributed to a better understanding of transcriptional regulation of genes specifically expressed in Ig secreting cells by the octamer binding transcription factor Oct-2. The mechanism of transcriptional regulation by Oct-2 is clearly complex. The ultimate goal of future studies would be to

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determine the mechanism by which Oct-2 regulates the development and function of Ig-secreting cells as these cells are of great importance in humoral immunity. The information gained from these studies, as well as the experimental strategies proposed for studying Oct-2 function in Ig secreting cells, could be applied to further understanding how this and other transcription factors ensure gene expression in differentiated cells.

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