Functional Hierarchy of Tissue-Specific Transcription Factors

in Immunoglobulin-Secreting Cells

by

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

The City University of New York

2002

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

9/6/02
Date

Chair of Examining Committee

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Abstract

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B and T lymphocytes arise from a common precursor in the bone marrow but ultimately acquire very different functions. The difference in function is largely attributable to the expression of tissue-specific transcription factors that activate discrete sets of genes. B cells express several tissue-specific transcription factors that bind to enhancer and promoter elements within the immunoglobulin (Ig) and other tissue-restricted genes, activating the B cell program. When an Ig-secreting cell is fused to a T lymphoma, however, genes encoding both tissue-specific transcription factors and tissue-specific structural genes (e.g. Oct-2, PU.1, OCA-B, Ig heavy and light chain and J chain) are extinguished at the transcriptional level. We have previously shown that all tested tissue-specific genes of the Ig-secreting cell are rescued from silencing when Oct-2 expression is artificially expressed. This suggested that the transcription factor Oct-2 plays a central role in maintaining the genetic program of these cells. We have further investigated the role of two other factors that are expressed in the Ig-secreting cell but not in the T lymphoma. One of these factors is the B cell and macrophage-specific

transcription factor, PU.1, and the other is the coactivator of octamer-binding factors, OCA-B. Our results have shown that PU.1 cannot rescue any of the tested B cell genes or reciprocally regulate Oct-2's function. Surprisingly, OCA-B rescues all tissue-specific and structural genes, can reciprocally regulate Oct-2 and it is the exclusive partner of Oct-2 in rescuing the plasmacyte-specific program. In conclusion, transcription factor PU.1 plays a subordinate role to that of Oct-2 and coactivator OCA-B and Oct-2 are at the highest level of the hierarchy in the Ig-secreting cell.

Acknowledgements

I am deeply thankful to my thesis mentor, Dr. Laurel Eckhardt, for her guidance, support, and patience throughout my graduate career. She has allowed me to develop a scientific career at my own pace without pressure allowing me to give the best of myself. She is the one of the best teachers I have encountered in my career, and her example has inspired me to become a teacher as well.

I am thankful to all members of Laurel' lab who provided a cheerful working environment. I also thank Hanna Radomska and Jane Ong for interesting conversations concerning my project.

I am greatly thankful to: Dr. Roeder from the Rockfeller University for providing the OCA-B expression vector; the late Dr. Marian E. Koshland from the University of California for providing the PU.1 expression vector and Dr. Hanna Radomska from Harvard Medical School for providing the PU.1 dependent reporter constructs.

I am also thankful to my good friends Sophie, Violet, and MOM, who have been extremely supportive throughout my graduate education.

I am thankful to my husband, Sergio, who has stood by me through the process of writing, thinking, reading, proofreading and figure-making of my thesis.

CHAPTER 1

Introduction

In 1983, the first transcriptional enhancer, Eμ, was discovered in the mouse IgH locus (1-3). It was soon found that Eμ was not essential to Ig-secreting cell lines (4-6,7, 8). Other enhancers were identified 3' of the IgH locus: 3'α E or Hs1,2 (9, 10) Hs3a (11), Hs3b (12) and Hs4 (13). The presence of these regulatory sequences partially explains the B-lymphoid restricted expression of immunoglobulin genes.

In an attempt to learn why these control elements are only active in B lymphoid cells, we looked for transcription factors that bind to them. Two of the first transcription factors identified were PU.1 and Oct-2 (138, 14, 15). Subsequently, OCA-B, a coactivator of octamer-binding factors Oct-2 and Oct-1, was cloned (16-18). Targeting deletion experiments of PU.1 showed that this factor is needed for committment to the lymphoid lineage (19, 20). Similar experiments of Oct-2 and OCA-B revealed that these factors are needed late in B cell development (21, 22, 23-26, 27). Using the experimental approach of somatic cell fusion hybridization, we have obtained evidence that Oct-2 is essential to B cell genes at the Ig-secreting cell stage (28).

In this thesis, my goals have been to:

1.- Test whether other B cell factors, PU.1 and OCA-B, behave similarly as Oct-2 using the somatic cell fusion approach. These factors are also B cell restricted and have been extinguished in cell fusion studies. Oct-2, when introduced into an Ig-secreting plasmacytoma before fusion to a T lymphoma, is sufficient to prevent the extinction of the plasmacyte's genetic program in the resulting hybrids. I wanted to determine whether

PU.1 and OCA-B could achieve the same rescue function.

2.- Previous work from our laboratory suggested that Oct-2 required a B cell restricted cofactor in order to carry out its gene-rescuing function. This conclusion was obtained due to the difference in rescue function when Oct-2 was maintained in the B cell than in the T cell. When Oct-2 was maintained in the B cell previous to fusion there was 100% rescue of the B cell phenotype and when Oct-2 was maintained in the T cell previous to fusion there was between 20-70 % rescue. The second goal of this thesis project was to test if OCA-B is the coactivator of Oct-2.

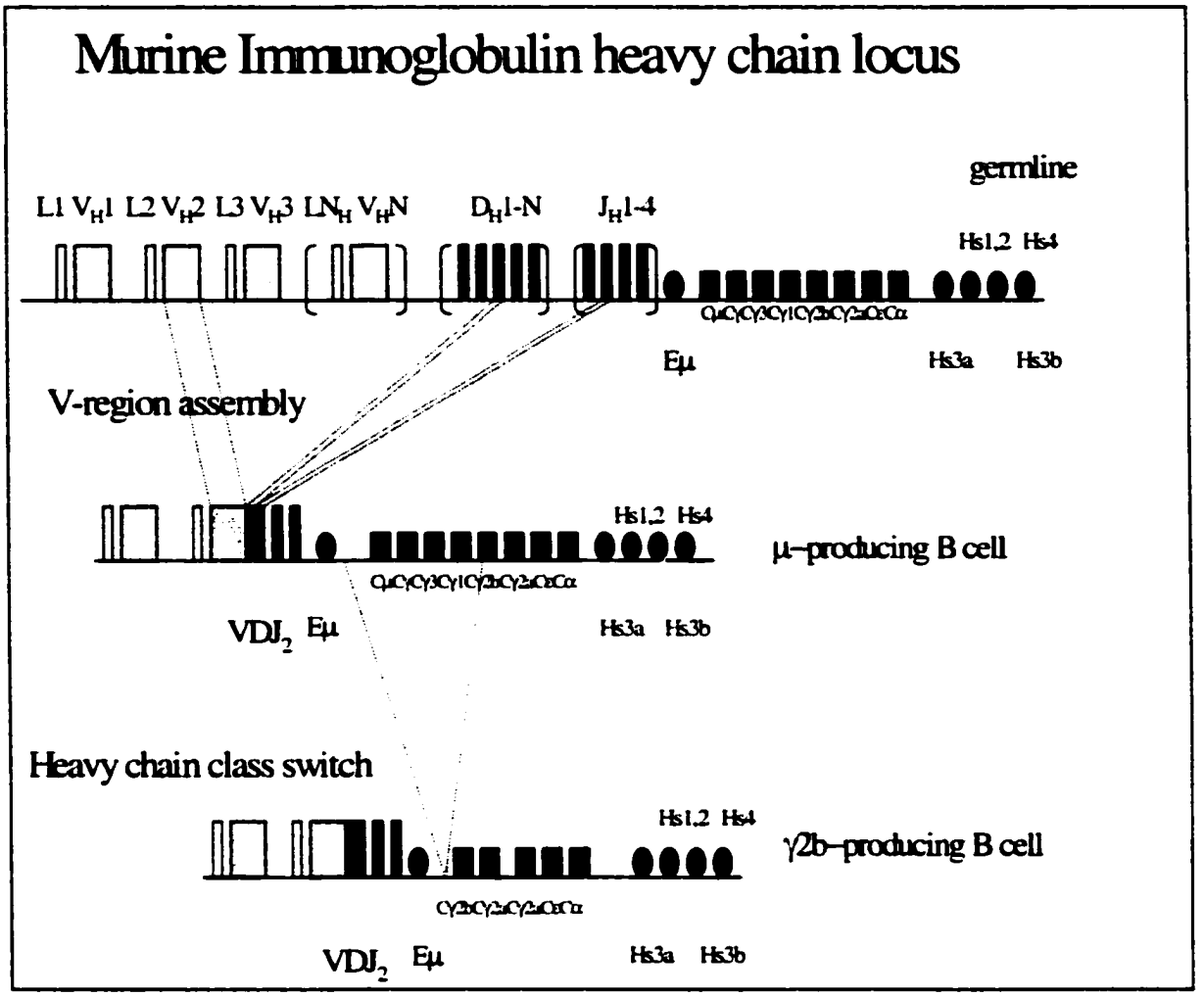
3.- Oct-2, together with a B cell restricted cofactor, sustains the plasmacyte genetic program while Oct-1 alone cannot. A third goal was to test whether addition of OCA-B would enable Oct-1 to carry out this gene-rescuing function.

We have learned that transcription of the IgH locus depends on the activation of IgH regulatory elements by tissue-specific and ubiquitous factors. The murine heavy chain locus, diagrammed in figure 1.1, consists of several V, D and J DNA segments upstream of several IgH constant regions. Lymphocyte recombination enzymes, RAG 1 and RAG 2, recognize the recombination signals sequences flanking the V, D and J DNA segments, spliced them and form a VDJ joining. On a secondary step, a class switch event occurs in which the same VDJ segment combine with a different constant region.

Experiments have shown that the Eμ and the 3' IgH enhancers are required at

different stages of B cell development. Early in a B cell, Eμ is necessary for VDJ assembly and possibly for maintaining IgH expression. As the B cell progresses, there is a shift to the 3' IgH enhancer region for ensuring high level of IgH transcription (29). It is reasonable to infer that transcription factors known to have DNA-binding sites within these regulatory sequences are responsible for changes in IgH transcriptional control as a B cell differentiates. Gene targeting experiments of these B cell factors have allowed us to establish a hierarchy of action at the level of commitment, specification, and differentiation of B cells.

Figure 1.1 Murine heavy chain locus. Assembly of the heavy chain gene involves the splicing of three gene segments (V, D, J) and the deletion of intervening DNA. The first type of heavy chain produced is the IgM, a μ producing B cell. Then the same V region can switch to produce another heavy chain class by a second DNA rearrangement. In the case of the MPC11 used in our studies, there is a class switch to the γ2b segment. In this process there is a duplication of the γ2a constant region.



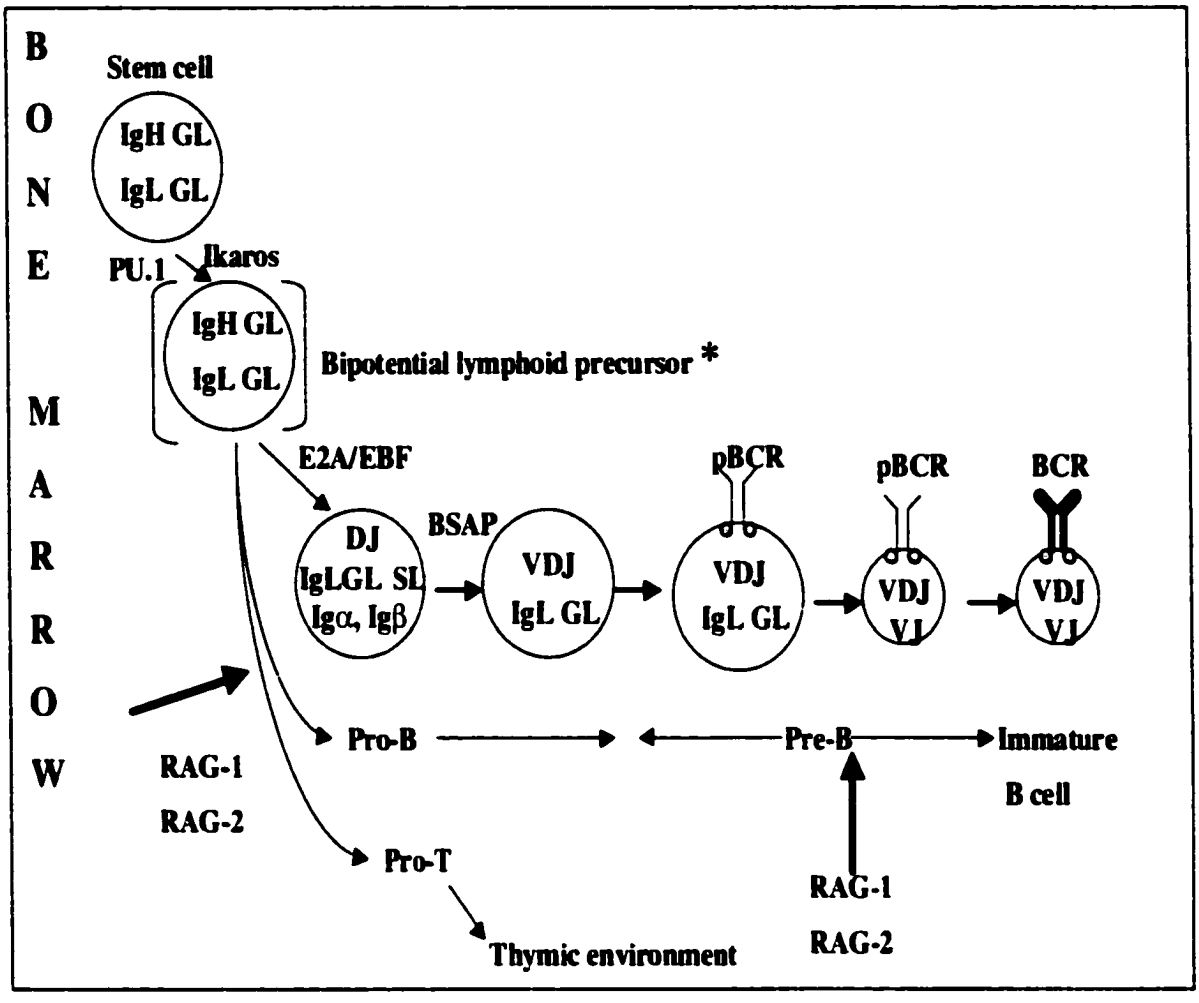
Abbreviations: L, V, D, J_H : leader peptide, variable region, diversity and joining protein coding segments, respectively; VDJ_2: joined Variable, diversity and joining segment 2, typical rearrangement that occurs in MPC11; Eμ: intronic enhancer; Hs3a, Hs1,2, Hs3b and Hs4: 3' IgH enhancers; Cμ Cγ Cγ3 Cγ1 Cγ2b Cγ2a Cε Cα : Immunoglobulin heavy chain constant region coding segments.

Lymphoid lineages are made of B and T lymphocytes which arise from hematopoietic stem cells (HSCs). HSCs are self-renewing, pluripotent stem cells that also give rise to other blood cell lineages: the erythrocytes, the megakaryocytes, the mast

cells, the granulocytes, the monocytes, the osteoclasts and the neutrophils. Lymphocytes are produced in the fetal and in the adult bone marrow. B cells continue to mature in the bone marrow but T lymphocytes exit the bone marrow and mature in the thymus.

B cells undergo a series of DNA rearrangements starting at the pro-B cell stage (Figure 1.2). It is at the pro-B cell stage that DJ rearrangement starts followed by VDJ rearrangement giving rise to an assembled variable heavy chain gene. In a series of targeting deletion experiments, E2A, EBF and BSAP have been identified as transcription factors essential to this B cell stage (30, 31) (Figure 1.2). The next stage is the pre-B cell, in which the Ig light chain loci undergoes variable region gene assembly. Heavy and light chain proteins migrate to the cell surface forming the surface IgM^+ receptor. Immature naive B cells $sIgM^+$ IgD^+ exit the bone marrow and they migrate to the periphery lymphoid organs. Upon encountering antigen, B cells undergo a series of changes at the germinal centers. Finally, B cells can differentiate into plasmacytes or memory cells. Other targeting deletion experiments have identified transcription factors that are needed at later stages of B cell development. These are Oct-2, OCA-B, Spi-B, NF-κB, Blimp1 and XBP-1 (Figure 1.3).

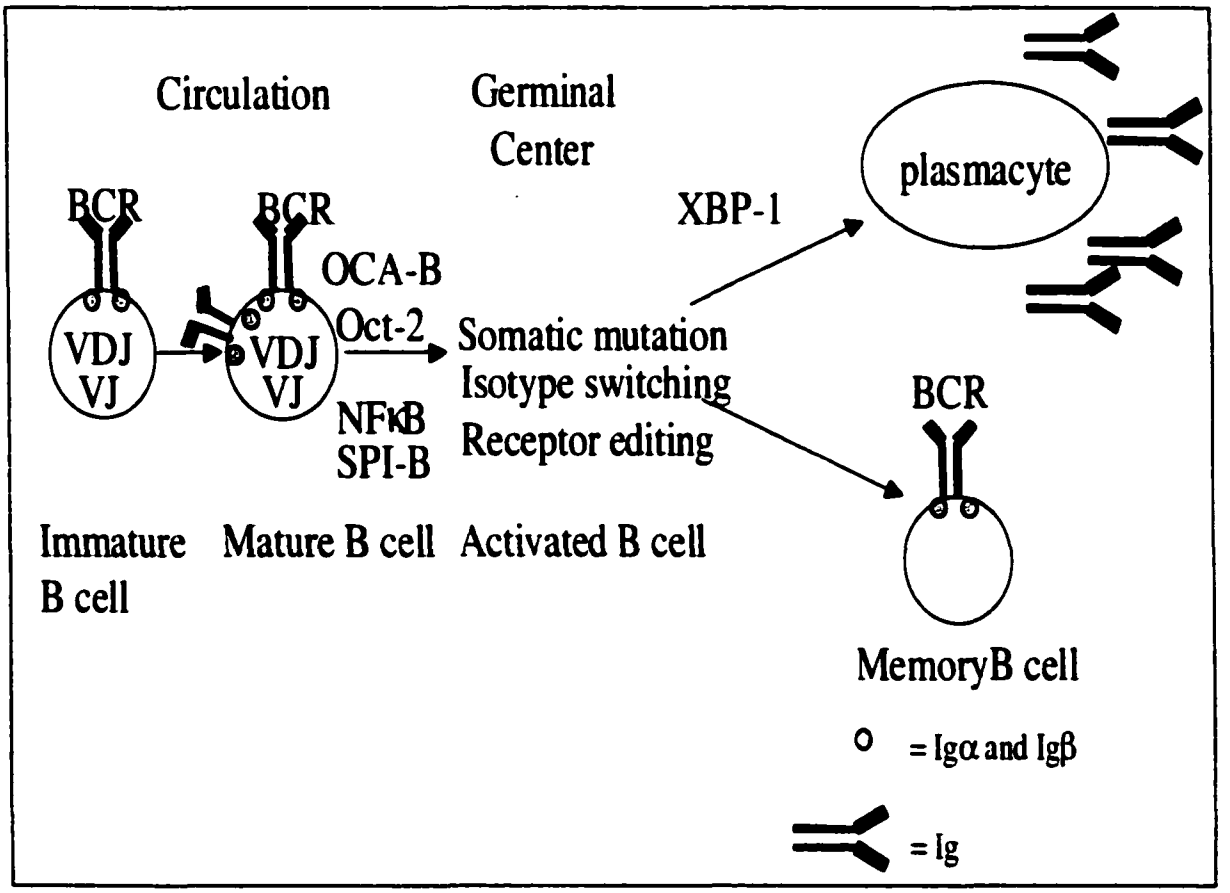
Figure 1.2 Antigen-independent stages of B cell development. B cells are derived either directly from a stem cell or from a putative bipotential lymphoid precursor. The first well known precursor of a B cell is the pro-B cell where DJ rearrangement occurs followed by the VDJ rearrangement of the IgH locus. A productive rearrangement produces a functional heavy chain protein which associates with the surrogate light chain protein and together migrates to the plasma membrane where association with accesory proteins constitutes the pre-B-Cell Receptor (BCR). This is the first check point of the B cell development. Upon productive heavy chain rearrangement, then the light chain starts rearrangement at the pre-B cell stage. The productive heavy and newly rearranged light chains migrate to the cell surface where they become the BCR. This stage corresponds to the immature B cell stage. Exit to the periphery allows maturation of the B cell.



Abbreviations: IgH GL: IgH locus in germline configuration; IgL GL: IgL locus in germline configuration; DJ: joining of one diversity region gene segment to a J region segment in the IgH locus; VDJ: joining of one variable region promoter to a rearranged DJ of the IgH locus; SL: surrogate light chain; RAG-1 and RAG-2: recombination enzymes responsible for rearrangement D to J and V to DJ; Igα and Igβ: accesory proteins responsible for assembly of Ig at the cell surface and for signal transduction of the immunoglobulin receptor; Pre-BCR: Pre-B Cell Receptor complex: IgM heavy chain together with the surrogate light chain and accesory proteins Igα and Igβ; BCR complex : B Cell Receptor IgM heavy chain together with the light chain and accesory proteins Igα and Igβ;

* this existence of this committed precursor has not been clearly proved as yet. PU.1 and Ikaros knockouts experiments showed lack of lymphoid lineages; E2A/EBF knockouts experiments showed arrest before DJ rearrangement; BSAP knockout experiments showed arrest after DJ rearrangement.

Figure 1.3 Antigen-dependent stages of B cell development. Mature naïve B cell has IgM and IgD cell surface expression at the cell surface. Upon encountering antigen and with the help of T cells, B cells get activated and clonally expand in regions called the germinal centers where several events take place that ultimately change in IgH constant region by the process of class switch and introduce point mutations within the rearranged Variable region by somatic hypermutation.



Abbreviations: VDJ: joining of one Variable, one Diversity and one joining segment on one of the IgH locus allele; VJ: joining of one variable and one joining segment of the IgL chain; BCR: B-Cell Receptor.

Knockout of OCA-B, Oct-2, NF-kB and Spi-B resulted in lack of germinal centers; XBP-1 knockout experiments showed lack of plasma cells .

Early events in lymphopoiesis are not well defined. The idea that lymphocytes are directly descendants of HSCs or, alternatively, arise from an intermediate with a bipotential lymphoid- restricted precursor is not completely resolved (Figure 1.2). B and T lymphocytes are the only cells that can rearrange their loci to generate the antigen receptor Ig or the TCR, respectively. Both of these cells share a recombination machinery responsible for random V(D)J rearrangement. The tissue-specific components of this machinery are the enzymes RAG-1 and RAG-2. Experiments have shown that lymphocyte restriction or commitment occurs before the activation of this recombination machinery (32-34). Therefore, even though the expression of recombination factors is required for lymphocyte maturation, it does not represent the earliest event in the development of the lymphoid system. The expression of sterile transcripts from the Ig and TCR loci prior to rearrangement revealed that these loci have an open chromatin configuration prior to expression of the recombination factors. The idea is that there may be some master transcription factors that control the initial stages of commitment, including tissue-specific opening of the chromatin within the Ig and TCR in developing B and T cells, respectively. In spite of the numerous transcription factors identified in hematopoiesis, little is known about which are responsible for commitment to the B versus the T lymphoid lineages. Targeting experiments have shown PU.1 and Ikaros as putative controllers of B lymphocyte differentiation (35).

PU.1 is a member of the ETS family of transcription factors (36). This protein is the product of the Spi-1 proto-oncogene found as a consequence of the common integration site by the spleen focus-forming provirus (SFFV) in induced eythroleukemias

(138, 14, 37, 38). PU.1 is expressed at high levels in both the B and monocytic lineages. Two PU.1 targeting deletion experiments provided evidence that this gene is specifically required for these lineages since neither lineage was present in PU.1 deficient mice. One of the targeting mutations caused late embryonic lethality (19) and in the other, mice died shortly after birth (20). The latter mice developed a T cell compartment postnatally and produced B cells with aberrant phenotype. αβ TCR cells (αβ T cells express a T cell receptor consisting of α and β chains) were detected in these mice although their maturation was not normal. Mckercher et al. claimed that there was no PU.1-DNA binding activity in these mice. However, there were no analyses done to determine whether or not a truncated version of the protein was produced.

Chimeric mice were generated by introducing PU.1 $^{-/-}$ mutant cells from fetal liver or PU.1 $^{-/-}$ embryonic stem (ES) cells into irradiated mice. In these mice, no T cells, B cells, monocytes or neutrophils were generated (40). However, erythroid progenitors and megakaryocytes were produced in normal numbers. These experiments suggested that this gene is required for the formation of a multipotential lymphoid-myeloid progenitor or its differentiation. Consistent with this belief, in vitro differentiation experiments using hematopioetic stem cells (HSC)s showed the existence of a precursor in the fetal liver with bipotential functions, myeloid and lymphoid, and this population was dramatically reduced in the fetal liver of PU.1 mutant mice (40, 41). Moreover, these bipotential precursors were unable to differentiate into pro-B cells in response to signaling by interleukin-7 and stromal cell contact. One hypothesis is that the mutant progenitors may lack the interleukin-7 receptor required for interaction with the stromal layer. Consistent

with this idea, it was shown that PU.1 regulates the expansion of progenitors by controlling the expression of the IL-7Rα gene that is the receptor for IL-7 (42).

In view of the discrepancy in phenotype between original knockout experiments, Spain et al. reexamined the PU.1$^{-/-}$ fetal thymus and found that the phenotype of the major PU.1$^{-/-}$ population corresponded to the most immature thymic progenitors before commitment to the T lineage. There were also some rare thymocytes expressing markers of T cell commitment. In conclusion, they demonstrated that PU.1 was not absolutely required for T cell development but does play a role in efficient commitment and /or early differentiation of most common T progenitors (39).

We are interested in the function of PU.1 in Ig-secreting B cells. PU.1 is detected throughout B cell development (14, 43, 44, 45) suggesting a role for this factor at all stages in B cell development. We cannot assess the role of PU.1 in late stage B cells by the conventional knockout strategy since knockout animals do not make B cells because of the essential role of PU.1 in the commitment toward the B lymphoid lineage.

Another key transcription factor (46, 47) identified to be needed for specification of lymphoid cells is encoded by the Ikaros gene. The Ikaros gene encodes a family of six zinc-finger containing proteins that are produced through alternative mRNA splicing. Ikaros protein binds the CD3δ gene enhancer, and this activity prompted the cloning of the Ikaros gene. CD3 is a multi-protein complex required for antigen-specific signalling of T cells. Ikaros is expressed in the yolk sac, fetal liver, and fetal thymus. It is

expressed at varying levels in B cells, T cells, natural killer (NK) cells, and antigen presenting cells (APC). Disruption of Ikaros causes a loss of B cell differentiation and severe defects in fetal and adult T cell lineages (47). There were no effects within the erythroid, megakaryoid, and myeloid lineages, but there were effects on NK cells. Although Ikaros mutants showed a complete lack of fetal lymphocytes, the postnatal thymus developed a T cell compartment entirely consisting of αβ T cell lineage and lacking the γδ T cells (T cells that express a TCR consisting of γδ chains). This is similar to the T cell phenotype encountered in the PU.1 gene targeting experiments. There was a severe reduction of the more mature T cell stages suggesting that Ikaros is essential for progression beyond the early stages of T cell development. Ikaros null mice lack inguinal, axial, cervical, and mesenteric lymph nodes, Peyer's patches and lymphoid follicles in the gastroinstestinal track. A dominant-negative mutation of the Ikaros gene produces worse consequences than the Ikaros null mice (47). It does not allow either T or B cells to develop postnatally. It was concluded that Ikaros acts in concert with another factor (s) that is destroyed in the presence of the negative mutant. Consistent with these ideas, several homologues to Ikaros (Aiolos, Helios) have been identified (48, 49). It is clear that Ikaros is not absolutely required for T cell development but does play a role in efficient commitment and /or early differentiation of most common T progenitors (39).

Both PU.1 and Ikaros-deficient mice lack T and B cell precursors in their fetal lymphoid organs. However, postnatally they develop αβ T cells and therefore, neither of these factors is absolutely essential for commitment to the αβ T cell lineage.

Additionally, PU.1 mutant embryos continue to express the Ikaros gene, so we can say that the loss of lymphoid cells in the former is not due to the lack of Ikaros. Further, these results suggest that Ikaros is not sufficient for specification towards the lymphoid cells when the PU.1 factor is missing. In conclusion, Ikaros and PU.1 have overlapping but not identical effects.

Other factors limited to the B cell lineages have been identified. The E2A gene, initially identified as a gene encoding immunoglobulin enhancer-binding proteins, codes for two members (E47 and E12) of the bHLH family of transcription factors which bind the E-box elements present in the intronic IgH and Igκ enhancers (50). E47 and E12 are produced from alternatively spliced mRNAs from the E2A locus, and they differ in their homo-heterodimerization potentials. These proteins have a basic domain that is necessary for DNA binding and a helix-loop-helix structure that allows for dimerization with other bHLH proteins. Specifically in B cells, E47 forms a homodimer BCF1 (51, 52). It is still a mystery as to why E47, a ubiquitous protein, forms this homodimer only in B cells.

E47 may be one of the earliest acting proteins specifying the B cell phenotype. $E2A^{-/-}$ mutant mice are defective with respect to B cell development. T cells and other non lymphoid hematopoietic cells are present in normal numbers (30, 31). The block of B cells is before Ig-rearrangement. RAG1, as well as Iμ sterile transcription are absent. Consistent with a role for E2A in Ig gene rearrangement was the observation that overexpressed E47 in a pre-T cell led to the production of sterile μ transcripts from the

unrearranged T cell IgH locus (53, 54). When the E2A proteins associate with Id proteins, the activity of the E2A proteins is inhibited. It is believed that at the time of B cell commitment, Id is complexed with E2A. Upon further differentiation, the E2A proteins are set free and then IgH rearrangement occurs (55).

A second factor, EBF1, has been shown to be restricted to B cell development. It was defined as an early B-cell factor and identified in pre-B cells and B cells as a protein that recognizes an element in the promoter of mb1, which encodes Igα, one of the signal transducers of the B cell receptor (membrane-bound Ig). EBF1 contains an HLH-like dimerization domain. Targeted deletion of this locus resulted in failure of early B cell development while T and myeloid lineages were unaffected (56). The arrest on B cell development was prior to D-J rearrangement of the IgH locus which is similar to the $E2A^{-/-}$ mutation. $B220^{+}$ cells that carry the Ig loci in germline configuration were detected, suggesting that EBF1 regulates an early step in B cell development but it is not needed for commitment to the B cell lineage.

The third factor is BSAP encoded by PAX5 a member of the family of paired-box genes (57, 58). It recognizes an extended nucleotide sequence through its bipartite paired domain. It was identified as a pre-B and B-cell specific protein that regulates CD19, mb-1 and N-myc genes. Within the hematopoietic system, BSAP expression is restricted to the B cell lineage, but it is also present in brain and testis. It is not expressed in differentiated plasma cells. Disruption of this gene resulted in arrest at the pro-B cell stage of B cell development after D-J rearrangement. Other cell lineages were unaffected

(59). PAX-5 appears to act downstream of EBF1 since its expression is not detected in EBF1$^{-/-}$ mutant B -lineage progenitors (56).

Targeting experiments have identified other factors that affect late B cell development. As mentioned earlier, Oct-2 was the first tissue-restricted transcription factor identified in B lymphocytes. A surprising finding was that Oct-2 was not needed for Ig-promoter activity. Recent studies by our laboratory and others suggested that its essential role takes place late in B cell development and differentiation. Oct-2 is a POU-domain transcription factor that binds the octamer motif, a highly conserved motif present in all IgH/L promoters and enhancers. This factor is predominantly expressed in B cells and it is closely related to the ubiquitous Oct-1 factor. Targeting disruption of the Oct-2 gene resulted in neonatal death for unknown reasons. B lymphopoiesis was found to be normal up to the membrane IgM^{+} stage, but splenic B cell numbers were reduced. Oct2$^{-/-}$ B cells were less prone than wild-type B cells to secrete Ig upon mitogenic LPS stimulation in vitro (21). Reconstitution experiments with Oct 2$^{-/-}$ fetal liver cells into immunodeficient (SCID or RAG$^{-/-}$) recipients were generated to obtain adult B cells. These showed that the early stages of B cell development were not affected, but terminal differentiation to Ig-secreting cells was severely impaired. In particular, Go to G1b cell cycle transition upon LPS or anti-μ stimulation was impaired blocking differentiation into antibody forming cells (AFC). However, proliferation and differentiation in response to signals from activated Th cells or to CD40L did not appear to require Oct-2 (23). Evidence for an essential function for Oct-2 in Ig-secreting B cells also has come from somatic cell fusion studies (28). Moreover, studies in the M12 cell line, a nonsecreting Ig

early plasma cell line, have shown that Oct-2 but not Oct-1 is needed to activate the IgH 3 'enhancers in transient transfection experiments (60).

Originally, the lymphoid -specific expression of Ig-genes was attributed to Oct-2. However, there is evidence that another B cell factor is collaborating for Ig-expression. This factor is OCA-B, a coactivator of octamer-binding proteins, Oct-1 and Oct-2. It potentiates the activity of immunoglobulin heavy and light chain promoters in association with these factors and is expressed exclusively in the B cell lineages. For this reason, it was suggested that OCA-B rather than Oct-2, would be essential to Ig promoter function. As with Oct-2, however, gene knockout studies showed that this was not the case. Targeting mutation of the OCA-B gene resulted in a defect in the antigen-dependent maturation of B cells but not in the development of B cells per se. These mice lacked germinal centers (which are the sites of antigen-specific B cell proliferation and differentiation) and had abnormally low levels of non-IgM isotypes (61, 27). The latter defect was not in the switching process but appeared to be due to low expression of the switched isotypes by switching B cells. Consistent with a role of OCA-B late in B cell development is the result that OCA-B is needed in T cell mediated activation of the IgH 3' enhancer regulatory sequences (62). Sharp et al. also have shown the need for OCA-B to synergise with Oct-2 factor in the M12 cell line (60). In an attempt to clarify possible redundancy between Oct-2 and OCA-B, recent reconstitution experiments that lack simultaneously Oct-2 and OCA-B were done. Surprisingly, double OCA-$B^{-/-}$/Oct-$2^{-/-}$ mutant fetal liver cells gave rise to B cells up to the IgM^{+} stage and transcription of the Ig gene was unaffected. However, both factors are essential for germinal center formation

and for the maturation of B cells (25).

Other members of the ETS family of transcription factors also have been shown to be needed late in B cell development. Spi-B is a member of the ETS family of transcription factors closely related to PU.1. It is expressed exclusively in lymphoid cells. A null mutation at the Spi-B locus generated mice that were viable, fertile and possessed mature B and T lymphocytes (63). Spi-B splenic cells respond normally to LPS stimulation, but they proliferate poorly and die in response to B cell receptor cross linking, display abnormal T-dependent antigenic responses in vivo, and produce low levels of certain secondary isotypes. Consistent with this defect, there is a dramatic defect in germinal center formation.

Other factors also shown to participate in late B cell development are NF-κB proteins. These are transcription factors involved in various immune responses (64). They can form homo or heterodimer complexes whose activities are modulated by association with the inhibitory IκB family of proteins. NF-κB is a heterodimer composed of p50 and p65 subunits, was shown to be an important regulator of the κ light chain intronic enhancer in transcriptional assays (65). The p50 subunit of NF-κB lacks a transcriptional activation domain and can form a heterodimer with p65 (Rel A), c-Rel or Rel B. Pre-B cells express mainly p50 and p65, mature B cells express p50 and c-Rel and plasmacytoma lines express p52 and Rel B along with others. Targeted mutation of NF-κB1, the locus encoding p50, resulted in animals with normal B cell numbers but defects in B cell activation, Ig secretion and Ig heavy chain class switching (66). NF-κB

binding sites have been identified within the downstream IgH enhancers hs1.2 and hs 4 (67). NF-κB binding activity contributes to the activation of the hs1.2 at the plasma cell stage; in B cells, NF-κB binding participates in repression of this enhancer. For hs 4, a complex containing p50 possibly regulates positively hs 4 activity at multiple stages of B cell development.

Blimp-1 or B lymphocyte-induced maturation protein expression factor is crucial for B cell differentiation. It is considered to be a master regulator of terminal B cell development because it was sufficient to trigger differentiation in the surface Ig^+ BCL1 lymphoma cell line. Its expression is limited to mature or terminally differentiated B cells (68).

Lastly, XBP-1, has been recently cloned and also found to allow differentiation of BCL1 cell line to the plasma cell stage. Targeting deletion of this gene does not prevent the formation of B cells or their activation. However, the development to the plasma cell stage is blocked (69).

Homologous recombinant techniques have proven useful to understand the roles and hierarchy of transcription factors in the expression of B cell genes at different stages of B cell development. To summarize, null mutation of the transcription factor PU.1 has shown abrogation of the myeloid and the lymphoid pathway suggesting a multipotential common progenitor for both of these lineages in hematopoiesis. Similar experiments with the Ikaros gene showed a role of this factor in determining the B cell lineages. Null

mutation experiments of the E2A, EBF, BSAP/Pax5 showed an important role in commitment to the B cell pathway. Finally, null mutation of Oct-2 and OCA-B revealed a role for these factors late in B cell development.

The outcome of targeting deletion experiments define when a transcription factor first takes an essential action, but they do not allow to ask when and whether the transcription factor has activity after that point.

The focus of this thesis is on the terminal cell in B cell development: the Ig-secreting cell. In order to study the transcriptional factors critical to these cells, we made use of the somatic cell fusion approach. In general when one fuses an Ig-secreting line (myeloma) to a T cell (or fibroblasts), extinction at the transcriptional level of B-cell factors and B-cell structural genes results. The process of extinction of IgH and IgL expression has been well documented in fusions between a myeloma and fibroblast (70-74). The fact that extinction involves transcriptional silencing of the Ig genes and that the Ig promoter and Eu enhancers are molecular targets for the silencing machinery has been shown in myeloma and T lymphoma fusions (75, 76, 77). A study analyzing both Ig-extinguished and the rare Ig-expressing hybrids of myeloma and T lymphoma fusions led to the finding that multiple myeloma-specific genes were coordinately regulated in these cell fusions. Among the myeloma-specific genes coordinately silenced with the Ig were Oct-2, PU.1 and OCA-B (74, 76, 78, 28, Salas, unpublished results).

In earlier experiments, we asked whether providing Oct-2 back to the Ig-

extinguished hybrid lines could reactivate Ig-specific expression (28). We found that if we prevented the loss of Oct-2 at the time of cell fusion, the Ig genes were not silenced in the resulting hybrids. In fact, all other myeloma-specific genes assayed were rescued from silencing in these hybrids, including the genes encoding OCA-B and PU.1 (28, Salas, unpublished results). In these experiments, we prevented the loss of Oct-2 by providing the myeloma cell with a cloned Oct-2 gene under viral (cytomegalovirus) promoter control. We concluded that Oct-2 plays a central role not only in maintaining Ig expression but also in regulating the entire genetic program of Ig-secreting cells.

In the experiments described in this thesis, I used this cell fusion system to ask how two of the other myeloma-specific transcription factors, OCA-B and PU.1 contribute to gene function in Ig-secreting cells. Whereas gene knockout studies have shown that PU.1 function precedes that of Oct-2 and OCA-B during B cell development, the functional hierarchy of these factors once cells reach the Ig-secreting cell stage is not known. Progression to the Ig-secretion is critical to the development of a full-fledged antibody response to infection. Understanding the molecular events that underlie this progression and that ensure proper functioning of these Ig-secreting cells, therefore, is of obvious medical importance.

As described in Chapters 3 and 4, I have tested the effects of ectopic expression of PU.1 and OCA-B, respectively, in fusions between a myeloma and T lymphoma cell line. Since the genes encoding both of these transcription factors, like the one encoding Oct-2, are usually silenced in myeloma and T lymphoma hybrids, I asked whether ectopic

expression of PU.1 or OCA-B, like extopic expression of Oct-2, could rescue the myeloma genetic program from silencing-fusion mediated. In related experiments, I tested the notion that OCA-B serves as a necessary co-factor in Oct-2' s gene-rescuing function (Chapter 4 C).

CHAPTER 2

Materials and Methods

Cell lines

BW5147.G.1.4.OUAR was obtained from the American Type culture Collection (Rockville, Maryland, ATCC CRL 1588). It is a variant subline of the AKR/J mouse thymoma-derived cell line, BW5147, and is resistant to 10^{-4} M 6-thioguanine and to 10^{-3} ouabain (79). 45.6.2.4. is a γ2b/κ-producing myeloma cell line derived form the BALB/c mouse tumor, MPC11 (80). In the present study, we refer to these two cell lines as BW5147 (BW) and MPC11 (MP), respectively. MPC11 cells grow in hypoxanthine, aminopterin, and thymidine-containing medium (HAT) but die in medium containing ouabain. Both cell lines were maintained in 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, 100ug/ml streptomycin (GIBCO BRL, Grand Island, New York, catalog number 15140-015), and 0.1 mM nonessential amino acids (GIBCO BRL, Grand Island, New York, catalog number 11140-019)(complete DMEM).

Plasmid Constructions

pCGN-OCA-B is a flu epitope-tagged eukaryotic expression vector that produces human OCA-B (Accession # Z47550) (18), kindly provided by Dr. Roeder's laboratory from the Rockefeller University. The vector has a cytomegalovirus (CMV) promoter that drives the expression of hOCA-B cDNA. Sequencing of this vector with a flu-primer showed that the flu-epitope codons are followed by the OCA-B cDNA that includes all but the start codon (Met) of the OCA-B coding sequence. This vector was co-transfected with

the pSV2-hisD or pSV2-Neo vectors (81, 82) for selection.

BCMGSNEO-PU.1 is an expression vector that produces the murine PU.1 which sequence has been reported by Klemsz (14). This PU.1 expression vector was kindly provided by the late Dr. Koshland from the University of California, Berkeley. It has a CMV □promoter, and PU.1 cDNA was inserted into the vector as a Xho1 / Not1 fragment. The PU.1 sequence was excised to generate the BCMGSNEO- "empty vector", a control in transactivation studies. The 5' and 3' ends of the expression vector were sequenced using specific primers taken from the published sequence (14). This expression vector is a derivative of the expression vector BMGNeo which has been used by others (83).

For the transactivation studies on PU.1, the c-fms promoter sequence (-417 to +71) upstream of the PXP2 luciferase reporter vector was used. The c-fms gene encodes the receptor of the macrophage colony-stimulating factor (M-CSF) which is required for maturation of monocytic phagocytes. The c-fms PXP2 reporter vector was obtained from Dr. Daniel Tenen's laboratory, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts, but it was originally developed in Dr. Koshland's laboratory, University of California, Berkeley. This reporter has been shown previously to respond to PU.1 in HeLa cells (non-B human cell line) (84). The PXP2-luciferase vectors have been described (85). The c-fms promoter was excised from the reporter vector c-fms-PXP2 using the BamHI-Bgl II to create a promoterless construct.

pCGNOct-2 is a flu epitope-tagged eukaryotic vector that produces human Oct-2 (86). This vector has a cytomegalovirus (CMV) promoter that drives the expression of hOCt-2 cDNA. The pCGNOct-2 vector was covalently linked to the hisD transcription unit of pSV2 to generate pCGNOct-2his (28).

Stable Transfections

Transfections were as previously described (28). Briefly, 10^7 cells in 1 ml of complete DMEM (without serum) were transfected with 10 ug of linearized plasmid DNA by electroporation in a 0.4 cm cuvette. For the co-transfecting agent, about 1ug, was used per transfection. An electric pulse of 250 V and 960 uF was delivered by the Bio-Rad Gene Pulser electroporator with Capacitance Extender (Hercules, California). The cells were resuspended in 12 ml of complete DMEM (with 20% serum) and plated into 96-well plates at 10^4 cells/well. Forty-eight hours later, the medium was replaced with the appropiate drug-selection medium. For stable transfections, BCMGSNEO-PU.1 was linearized with the Pvu I enzyme. To select for stable PU.1 transfectants, 2mg/ml G418 was used in MPC11 transfections (GIBCO BRL, Grand Island, New York, catalog number 860-1811IJ) and 3mg/ml G418 in BW5147 transfections. For stable transfections, pCGN-OCA-B was linearized with DraIII, and the other co-transfected vector (pSV2 hisD or pSV2 neo) was linearized with EcoRI. MPC11OCA-B clones (co-transfected with pSV2 hisD) were selected in 5mM histidinol media. BWOCA-B clones (co-transfected with pSV2 neo) were selected in 3 mg/ml of G418 media. Drug-resistant clones were further confirmed by Western Blots for expression of flu-tagged OCA-B with an apparent molecular weight in SDS-PAGE of 35 kD.

Transient Transfections (Transactivation of PU.1 dependent promoter in BW5147 cells)

4 x 10^6 BW5147 cells (per transfection) were transfected by electroporation at 280 V and 960 uF using the Bio-Rad Gene Pulser electroporator with Capacitance Extender (Hercules, California). Cells were transfected with 18 ug of reporter plasmid (c-fms-PXP2 luciferase), 8 ug of either BCMGS-neo PU.1 or BCMGS-neo-empty vector and 2 ug of β -galactosidase expression vector. DNA was dispensed first into the 0.4 cuvette and then 4 x 10^6 cells in 400 ul of complete DMEM were added. β-galactosidase expression vector (pSV-β galactosidase) was added to normalize for transfection efficiency. The cells were then placed in a 37°C incubator with 7 to 8% CO_2. Cells were harvested 21 hours posttransfection and assayed for β-galactosidase and luciferase activities. A comercially available kit was used to assay for luciferase (luciferase assay kit, catalog no. E1501, Promega). Cell pellets from each transfection were lysed in 200 ul of lysis buffer and 10 ul of the lysate was used in the assay. Luminescence was measured with a luminometer (1LA-911-optocomp1, Tropix, Bedford, MA). Duplicate samples were assayed and the average taken. β-galactosidase assays were performed using the β-galactosidase assay kit from Promega (catalog no. E2000). 150 ul of the β-galactosidase reagent was added to 150 ul of the cell lysates, and the mixtures were incubated at 37° C for 20 minutes. The reaction was stopped by adding 500 ul of Na_2CO_3. The mixture was transferred to a disposable cuvette (Bio-Rad, catalog no.223-9955) and absorbance read at 420 nm (spectrophotometer DU series 600, Beckman).

Cell fusions

The method of somatic cell fusion in an electroporator has been described in (87). In brief, 5 x 10^6 cells of each fusion partner were mixed and washed twice in complete DMEM without serum. Cells were resuspended in 0.1 ml of complete DMEM without serum and then the mixture placed in a 0.2cm cuvette and centrifuged at low speed for 5 minutes. Cells were then subjected to a single electric pulse (250 V, 960 uF) in Bio-Rad Gene Pulser apparatus with Capacitance Extender (Hercules, California). After 30 minutes of incubation at 37° C in 7.5% CO_2/air atmosphere, cells were gently resuspended in 12 ml of complete DMEM supplemented with 20% serum and plated into 96-well plates at 10^5 cells/well. About 48 hours postfusion, hybrids were selectively grown in complete DMEM containing HAT and ouabain (10^{-4} M hypoxanthine, 4 x 10^{-6} M aminopterin, 1.6 x 10^{-4} M thymidine, and 10^{-3} M ouabain). The clones that survived were transferred into HT medium and then DMEM/10% serum and then frozen. In all fusions, growing cells were recovered in <30% of the wells and, therefore, represented single fusion events.

Southern Blot Analyses

DNA blot analyses were performed essentially has been described earlier (28). About 10 ug of BamHI-digested genomic DNA were size-fractionated on 1% agarose gels. DNA was transferred to Nytran (Schleicher and Schuell, Incorporated, Keene, New Hampshire), blots were baked for 2 hours at 80°C, and then prehybridized (2 hours) and hybridized at 65° C in buffer containing 7.5 x Denhardt's, 3 X SSC, 100 ug/ml sonicated salmon sperm DNA, and 0.5% SDS. Probes were labeled by random primer method

using Amersham Mega Prime Labeling Kit (Arlington Heights, Illinois). After 18-24 hours hybridization, the blots were washed twice for 30 minutes each in 0.1x SSC and 0.1% SDS at 65° C. The B cell-and T cell-derived IgH loci were detected with a 1.8 kb BamHI-EcoR I fragment from pJ11 (88), which contains the JH3 and JH4 coding sequences and IgH intron enhancer sequences. This probe only detects the γ2b producing locus of MPC11 (139).

Electrophoretic Mobility Shift Assay (EMSA)

For octamer-binding proteins, nuclear extracts were prepared as described (89). Binding reactions and gel retardation were performed as described previously (76). In brief, 10 ug of nuclear extracts were incubated for 20 minutes at room temperature with 10^4 cpm of end-labeled 51 bp fragment from the IgH enhancer (76). The 51bp fragment has the following sequence: TCAGC AAAAC ACCAC CTGGG TAATT TGCAT TTCTA AAATA AGTTG AGGATT (octamer in bold). The reaction mixture contained 2 ug of poly dI-dC, 10mM Tris-HCl (pH 7.5), 50mM NaCl, 1mMDTT, 1mM EDTA, and 5% glycerol in total volume of 25 ul. Unbound fragment and protein-bound fragments were separated from one another by electrophoresis through 4% polyacrylamide gels (acrylamide: bisacrylamide ratio 30:1) in Tris-glycine buffer (50mM Tris-base, 380 mM glycine, 2mM EDTA) at 200 V for three hours at 4°C.

For PU.1 EMSAs, the method was similar. In this case, however, 10 ug of nuclear extracts were incubated for 15 min on ice with 2.5 x10^4 cpm of end-labeled PU.1 box probe in a total volume of 25 ul. The binding assay was done in 10mM Hepes, pH

Abstract

Functional Hierarchy of Tissue-Specific Factors in Immunoglobulin-Secreting cells

by

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B and T lymphocytes arise from a common precursor in the bone marrow but ultimately acquire very different functions. The difference in function is largely attributable to the expression of tissue-specific transcription factors that activate discrete sets of genes. B cells express several tissue-specific transcription factors that bind to enhancer and promoter elements within the immunoglobulin (Ig) and other tissue-restricted genes, activating the B cell program. When an Ig-secreting cell is fused to a T lymphoma, however, genes encoding both tissue-specific transcription factors and tissue-specific structural genes (e.g. Oct-2, PU.1, OCA-B, Ig heavy and light chain and J chain) are extinguished at the transcriptional level. We have previously shown that all tested tissue-specific genes of the Ig-secreting cell are rescued from silencing when Oct-2 expression is artificially expressed. This suggested that the transcription factor Oct-2 plays a central role in maintaining the genetic program of these cells. We have further investigated the role of two other factors that are expressed in the Ig-secreting cell but not in the T lymphoma. One of these factors is the B cell and macrophage-specific

7.5, 50mM KCl, 5mM MgCl2, 1mM DTT, 1mM EDTA, and 5% glycerol and indicated amounts of competitor. Unbound fragment and protein-bound fragments were separated from one another by electrophoresis through 5% polyacrylamide gels in 0.25 TBE buffer at 200 V for three hr at 4° C. (1 x TBE = 0.089 M Tris, 0.089 M Boric acid, 2.5mM EDTA (pH 8.3). The PU.1 box probe had the following sequence: GATCCTGAAA**GAGGAA**CTTGGTA (sequence in bold show core PU.1 box). A mutant PU.box was used for the competition experiments (GATCCTGAAA**GA<u>CC</u>AA**CTTGGTA, underlined bases have been mutated). For supershifts, 1 ul of an antibody to the N-terminal domain of PU.1 (1297 rabbit antisera, kindly provided by Richard Maki unpublished), was added to the nuclear extracts and preincubated on ice for 15 minutes. The radiolabeled probe was then added and the reaction incubated for an additional 15 minutes. Gels were dried onto Whatmann 3MM paper and exposed to film at room temperature.

For competition studies: 140 ng of labelled PU.1 probe (specific activity 1.0 x10^7 cpm/ng (2.5 x 10^4 cpm/ul) and cold competitor were added to the nuclear extracts immediately prior to the addition of the radioactive probe. Competitor was added in 50, 100 and 500 fold-molar excess.

Northern Blot Analyses

Total cytoplasmic RNA was isolated by the Trizol protocol (Gibco catalog #15596). Briefly, 10^7 cells were resuspended in 1 ml of Trizol reagent and RNA was extracted as per protocol provided. Typical yields were 150 ug to 250 ug. Approximately 20 ug of RNA was denatured in formamide and fractionated on 1% agarose-formamide gels (91).

RNA was transferred from the gels to Nytran as previously described (75). Hybridizations were for 24 hours at 37° C in 50% formamide, 2X x Denhardt's, 5 x SSC, 50mM $NaPO_4$ (pH 6.8), 50 ug/ml sonicated salmon sperm DNA, 0.1% SDS and 50 ug/ml of poly [A]. Blots were washed in 2XSSC, 0.2% SDS for 30 minutes at 37° C. A 1.2kb cDNA (Jc21) was employed to identify J chain mRNA (92). To ensure the uniform levels of RNA on each gel, blots were stripped (15 minutes of boiling 0.1XSSC, 0.01% SDS) and rehybridized to murine GAPDH (catalog # 7330; Ambion).

RT/PCR

First strand cDNA was prepared from 2 ug of total RNA, using 1 ug of an antisense oligonucleotide complementary to mouse PU.1 sequences following the Promega AMV Reverse Transcriptase protocol (catalog # M501, lot # 82680). The antisense primer "746 bp": 5' GACGAGAACTGGAAGGTCCA 3' corresponds to bp 726-746 of the published sequence (14). The "746" primer together with specific forward primers to the endogenous and exogenous PU.1 were used to differentiate between both mRNAs. Specific forward exogenous PU.1primer: 5' CTCGAGGTCGAGGGTATCGA 3' is vector sequence obtained from the sequenced BCMGSNeoPU.1 expression vector and corresponds to a region 21-41 bp upstream of the PU.1 sequence. The PU.1 sequence in this vector begins with the bp designated 117 in the published sequence. Specific endogenous forward PU.1: 5' CAGGCCTGAGCCCTGCGTCT3' correspond to bp 10-30 in the published sequence (14). As noted above, this portion of the PU.1 gene (non-translated region) is not included in BCMGSNeo-PU.1. A common forward control primer to detect the presence of PU.1 mRNAs was used. Primer "117 bp" corresponds to

117-137 bp of the published sequence (14): 5'CCTGGAGCTCAGCTGGATGT3'. PCR cycles (35) were as follows: denaturation of 1 min at 94° C, 1 min annealing at 48° C, and 1 min extension at 72° C with an extra extension time at 72° C for 7 min. The specific exogenous product was 670 bp, the specific endogenous product was 736 bp and the control that picked up both exogenous and endogenous was 629 bp. PCR products were size fractionated on 0.8% agarose gels and blotted to nylon filters. Blots were hybridized with a 800 bp Sac fragment from BCMGSneoPU.1.

Enzyme-linked immunoassays (ELISAs)

Cytoplasmic lysates were made and ELISA performed as described earlier (143). In brief, microtiter plates (Dynatech Laboratories, Incorporated, Chantilly, Virginia) were coated with 2 ug/ml of affinity-purified Fc fragment-specific rat antimouse IgG2b (Cat# 02041D; BD PharMingen, San Diego, CA). Aliquots (50 ul) of undiluted Nonidet P-40 cytoplasmic lysates were then incubated with the coated wells. γ2b heavy chains were detected with alkaline phosphatase-conjugated rat antimouse γ2b antibody (cat# 02033E; BD PharMingen 1:1000 dilution) and enzyme substrate (cat# 104-105; Sigma Diagnostics, St.Louis, MO). For κ light chain assays, plates were coated with 10 ug/ml of affinity-purified goat anti-mouse κ antibody (cat# 1050-01; Fisher Biotech, Pittsburgh, Pennsylvania). Bound κ chain was detected with biotinylated goat anti-mouse κ antibody (dilution factor 1:3000, cat# 1179; Amersham International, Little Chalfont Buckinghamsphire, U.K.) and subsequent incubation with alkaline phosphatase-avidin conjugate (dilution factor 1:3000, cat# 62-253-1; Miles Scientific, Napierville, Illinois).

Western Blot Analyses

Whole cell lysates were obtained by the freeze and thaw method. Briefly, about 10^7 cells were washed in 1X PBS and resuspended in extraction buffer (20mM Hepes ph 7.9, 20% glycerol, 400mM KCl, 0.5mM EDTA, 0.5mM EGTA, 0.025% NP40, 0.5mMDTT). This extract was freeze-thawed 6 times in liquid nitrogen, spun for 5 minutes, and supernatants isolated. Extracts corresponding to 50 ug of total protein (Bradford Assay; catalog # 500-0006; Bio-Rad) were size-fractionated by electrophoresis through an 8% SDS-polyacrylamide gel and then proteins were electrophoretically transferred to nitrocellulose membranes (Pure-Nitrocellulose transfer membrane # WP2HYA0010) Bio-Rad Transblot apparatus) as described (93). The transfer buffer was 0.025 M Tris, 0.25M Glycine, 20% methanol. The membrane was incubated in 6% carnation milk, 40mM Tris-HCl pH 8, 200mM NaCl, 0.1% Tween 20 for two hours at room temperature or in the cold room overnight to block nonspecific binding of proteins. All blots were incubated with the respective antibody concentration in blocking buffer, washed three times in the same buffer for 30 minutes total and incubated with a secondary antibody in the same buffer.

Flu-tagged proteins (OCA-B and Oct-2) were detected with anti-flu epitope mouse monoclonal antibody, HA.11 (Covance MMS-101P/ lot number:# 142030001; Berkeley Antibody, Richmond, CA). This antibody was used at a concentration of 1:1000. For the secondary step, an HRP-conjugated goat antimouse IgG antibody was used for 45 minutes at a 1:10000.

Oct-2 was detected with: rabbit polyclonal anti-Oct-2 (C-20)X (SC-233X: Santa Cruz Biotechnology, Santa Cruz, CA) at 1:600 dilution followed by HRP-conjugated donkey anti-rabbit whole Ig at 1:7500 (NA934; Amersham Pharmacia Biotech, Piscataway, NJ).

Oct-1 was detected with rabbit polyclonal anti-Oct-1 (C-21)(SC-232: Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 and followed by HRP-conjugated donkey anti-rabbit whole Ig at 1:2000 (NA934; Amersham Pharmacia Biotech, Piscataway, NJ)

OCA-B was detected with polyclonal rabbit anti-OCA-B (kindly supplied by R.Roeder, The Rockefeller University, New York, NY) at 1: 5000 and HRP-conjugated donkey anti-rabbit whole Ig at 1:5000 NA934; Amersham Pharmacia Biotech, Piscataway, NJ).

Heavy and light chain proteins were detected with HRP-conjugated rabbit antimouse whole IgG at 1:1000. Blots were developed with Super Signal chemiluminescent substrate (Pierce), and chemiluminescence was visualized by various exposures to Kodak (Rochester, NY) X-OMAT film.

CHAPTER 3

The role of transcription factor PU.1 in Ig-secreting cells

Introduction

All promoters and some enhancers of the Ig heavy and light chain loci contain the well-conserved octamer motif that binds transcription factors Oct-1 and Oct-2. A less well-conserved motif found within a subset of these regulatory elements is the Ets-binding site (EBS) recognized by members of the ETS-family of transcription factors. PU.1 is the only member of this family that is expressed exclusively in B and not in T lymphoid cells. Other members of the ETS- family, such as Ets-1 and Spi-B, are present in both of these lymphoid sublineages. PU.1 is also found in monocytes and is present in all developmental stages of this and the B lymphoid lineage (94).

The promoter of the PU.1 gene has itself been well characterized. It contains an octamer site and PU.1 and Sp1 binding sites, whereas it lacks a TATA motif. Transient transfection experiments have shown that PU.1 can regulate its own promoter suggesting that it participates in an autoregulatory loop. Moreover, the coactivator OCA-B augments PU.1 promoter activity in association with octamer binding factors Oct-1 and Oct-2 (95, 96).

PU.1 has been implicated in the regulation of Ig heavy and light chain genes and of the gene encoding the Ig-polymerizing protein, J chain (97). The role of PU.1 within B lymphocytes has not been completely ascertained, however, since PU.1-deficient mice die as embryos (19, 20). Even experiments using PU.1-deficient bone marrow to reconstitute lethally-irradiated $PU.1^{+/+}$ animals proved uninformative with respect to PU.1 function within the B cell pathway. These mice, like $PU.1^{-/-}$ mice, lack the

lymphoid and myeloid lineages. Although both types of experiments clearly show that PU.1 is required for early commitment to the lymphoid and myeloid lineages, they provide no information with regard to PU.1's function at subsequent stages of B cell development. Since PU.1 is expressed throughout B cell development, it is likely to serve a function in several, if not all, developmental stages of this lineage.

We have used somatic cell fusion as a method for studying the role of transcription factors in Ig-secreting cells. In general, when an Ig-secreting plasmacytoma is fused to a T lymphoma, plasmacytoma-specific genes are extinguished at the transcriptional level (75, 98). We showed previously that by maintaining expression of the tissue-specific transcription factor Oct-2 during cell fusion, other tissue-specific genes such as PU.1, OCA-B, J chain and Ig were rescued from fusion-mediated silencing (28). The dramatic effect on phenotype suggests that Oct-2 plays a central role in determining the genetic program of the Ig-secreting cell, but it remains unclear how this transcription factor mediates this function.

In the present study, we have used a similar approach to study the role of transcription factor PU.1 in Ig-secreting cells. PU.1, like Oct-2, is expressed in the Ig-secreting plasmacytoma MPC11 but not in the T lymphoma BW5147. We asked whether PU.1 was also able to sustain the plasmacytoma-specific program in hybrids between these two cell lines. Alternatively, PU.1 might act downstream of Oct-2 unable to rescue Oct-2 gene activity but capable of rescuing a subset of the genes rescued by Oct-2. Another possibility was that PU.1, acting downstream of Oct-2, would not be able to

rescue expression of any other tissue-specific genes. This would follow if these genes required both PU.1 and Oct-2 for their activity. Using this experimental system, therefore, we could begin to unravel the hierarchy of transcription factors mediating the functional program of Ig-secreting cells.

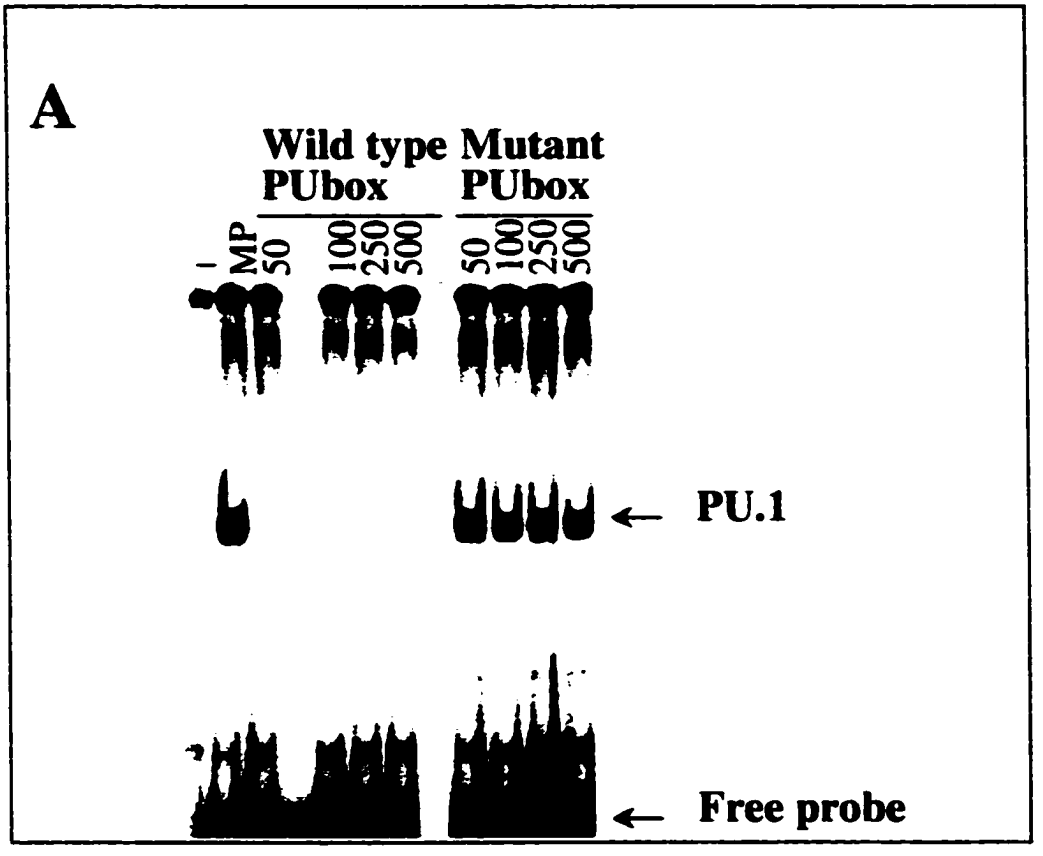
Results

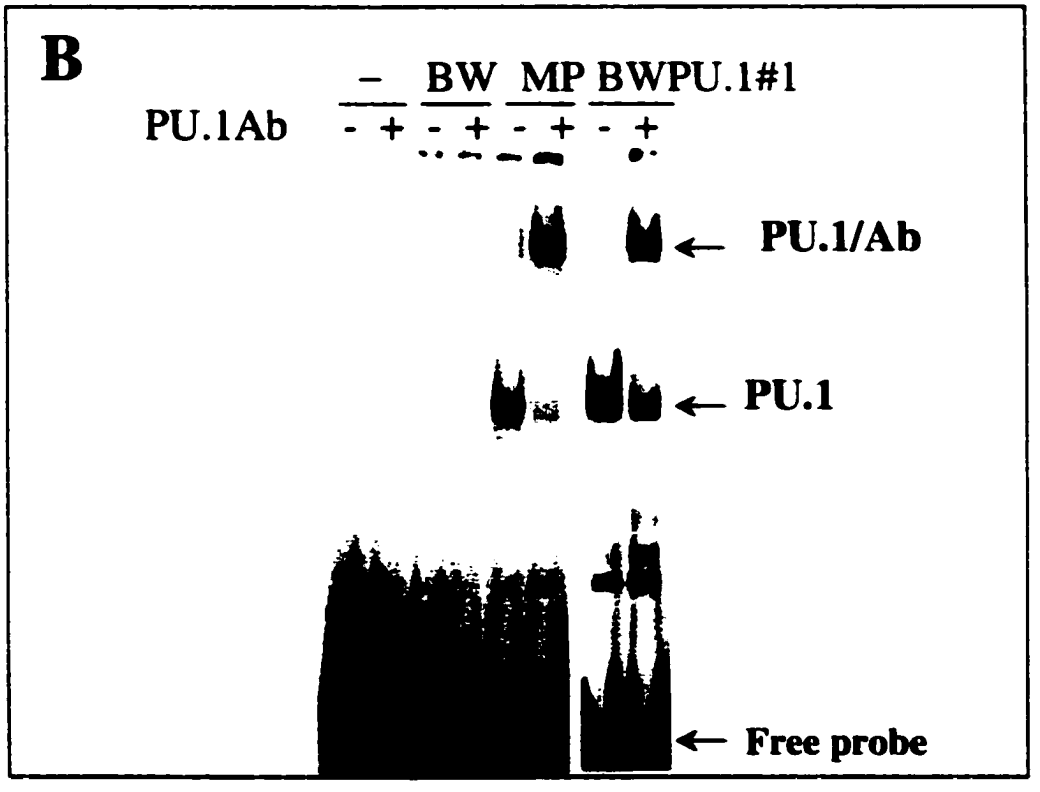
Development of a PU.1-expressing T cell lymphoma

A PU.1 expression vector, BCMGS-Neo-PU.1 (kindly provided by the late Dr. Marian Koshland, University of California, Berkeley) was used to stably transform the PU.1-negative BW5147 (T lymphoma) cell line. Electrophoretic mobility shift assays (EMSA) were used to identify the functional PU.1 protein by virtue of its ability to bind a previously identified binding site derived from the SV40 enhancer (14, 99). Nuclear extracts from the plasmacytoma cell line, MPC11, were used to show that endogenous murine PU.1 is able to bind specifically to this PU.1 box (Figure 3-1A). An oligonucleotide with mutant PU.1 binding site, when added in 50-fold molar excess, had no effect on the formation of the complex. Fifty-fold molar excess of an oligonucleotide with wild type PU.1 binding site, however, effectively competed for the complex, showing that PU.1 binds specifically to its target. An antibody to the N-terminus of the PU.1 protein was used to confirm that the protein contained in this complex was PU.1. As shown in Figure 3.1B, addition of this antibody led to the formation of a supershifted complex.

Figure 3.1 A) EMSA (Electrophoretic Mobility Shift Assay) of PU.1 protein in the plasmacytoma MPC11 (MP) cell line. MP nuclear extracts were incubated with a P^{32}-radiolabelled PU. box. Competition analysis was performed using 140ng of end-labelled PU. box. Cold competitor oligonucleotides were present at 50,100, and 500-fold molar excess. PU.1 = wild type 23 bp sequence; mut PU.box = mutant 23bp sequence.

B) EMSA of PU.1 protein in nuclear extracts from BW (T cell), MP (B cell), and BW5147-PU.1 clone 1 (BW-PU.1#1). In these incubations of probe with extract an antibody to the N-terminal domain of PU.1 was used. PU.1/Ab indicates the supershifted complex of DNA + PU.1 +anti-PU.1 antibody.





As expected, EMSAs using nuclear extracts from the PU.1-negative BW5147 cell line did not result in a PU.1-DNA complex (Figure 3.1B). BW5147 cells transformed with the BCMGS-Neo-PU.1 vector, however, did express PU.1 as determined by EMSA (Figure 3-1B).

We tested the transactivation function of the PU.1 protein encoded by this expression vector by transient assay. BW5147 cells were co-transfected with the BCMGS-Neo-PU.1 expression vector and a reporter construct with c-fms promoter driving the luciferase gene. The c-fms promoter (-417 to +71) has been shown previously to respond to PU.1 in Hela cells (non-B human cell line) (84). As shown in Figure 3.2, addition of the c-fms promoter to the promoterless luciferase vector PXP2 did not have an effect in luciferase expression in the T lymphoma line BW5147 (compare PXP2 vs. c-fms-PXP2; see also Table1). The experiments were done four times in duplicates, with at least three different BCMGSneo-PU.1 plasmid isolations. Standard deviation bars are shown. Addition of the PU.1 expression vector, however, augmented activity of c-fms-PXP2 twenty-fold, consistent with previous studies (84). As expected, PU.1 did not increase activity of the promoterless vector (Figure 3.2 and Table I).

Figure 3.2 Transactivation potential of PU.1. PU.1-negative BW5147 cell line was transfected by electroporation with 18 ug of promotorless luciferase construct PXP2 or the wild type c-fms receptor construct (c-fmsPXP2). These constructs were transfected with and without BCMGS-neo PU.1 expression vector. Luciferase activities were measured after 21 hours and normalized for transfection efficiency with the cotransfected B-galactosidase plasmid. Fold enhancement was calculated by setting the value obtained by transfecting the c-fmsPXP2 construct without PU.1 to 1. The data represents 4 different experiments done in duplicate using three different BCMGS-neoPU.1 plasmid isolations. The standard errors of the means are indicated by the error bars.

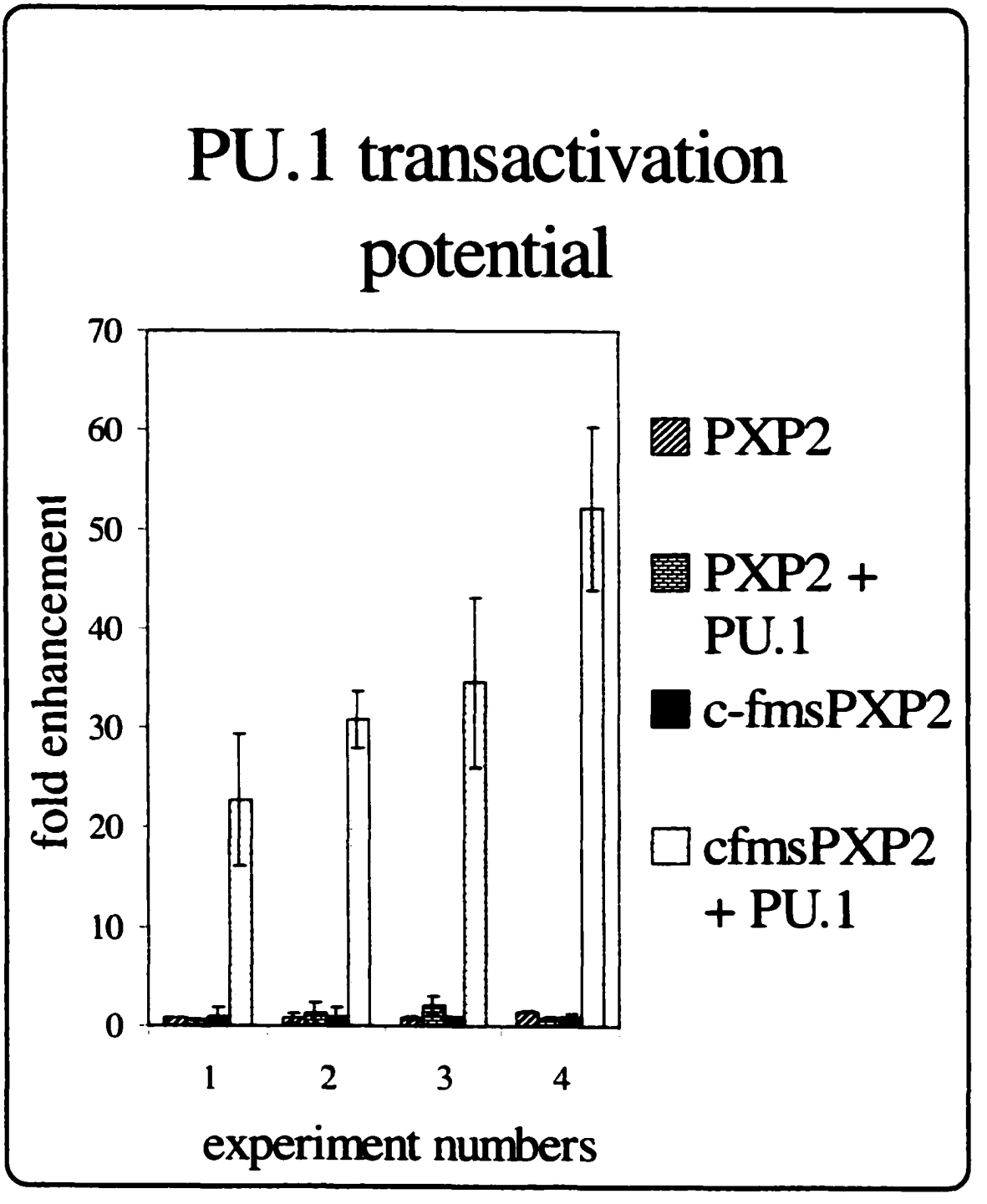


Table I. Transactivation potential of PU.1

Fold enhancement of luciferase activity

(ratio to c-fmsPXP2)

	Exp # 1	Exp # 2	Exp # 3	Exp #4
PXP2	0.78	0.75	0.816	1.5
PXP2 + PU.1	0.6	1.2	2.04	0.85
c-fmsPXP2	1	1	1	1
c-fmsPXP2 + PU.1	22.7	30.8	34.6	52.1

Luciferase values were normalized for transfection efficiency by dividing each by the amount of activity obtained from the β-galactosidase reporter gene control. The fold enhancement was calculated by setting the luciferase/B-galactosidase value for the PU.1 dependable promoter c-fmsPXP2 to 1.

Constitutive expression of mPU.1 in a T cell previous to cell fusion is unable to rescue expression of plasmacytoma-specific genes

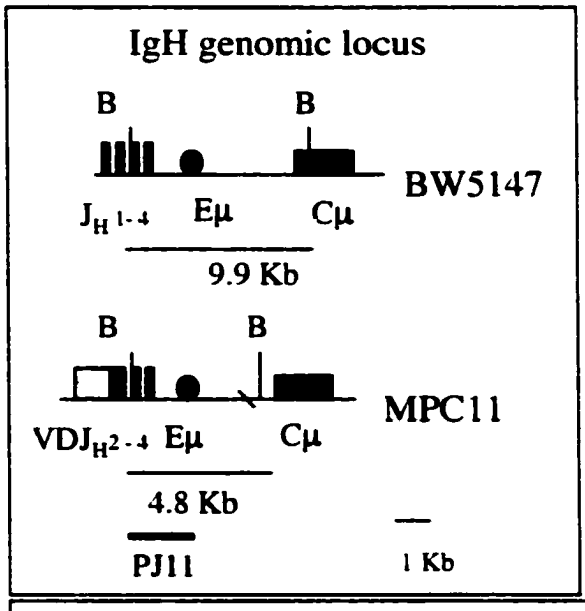
A T lymphoma transformant, BWPU.1#1, expressing PU.1 was fused to the IgG2b-secreting plasmacytoma, MPC11. Hybrid cell lines were selected in medium containing HAT/Ouabain as described in Material and Methods. Genomic DNA samples isolated from individual hybrids were analyzed by genomic Southern to assay for the presence of plasmacytoma and T cell-derived IgH loci. This was done to confirm the hybrid nature of these cells.

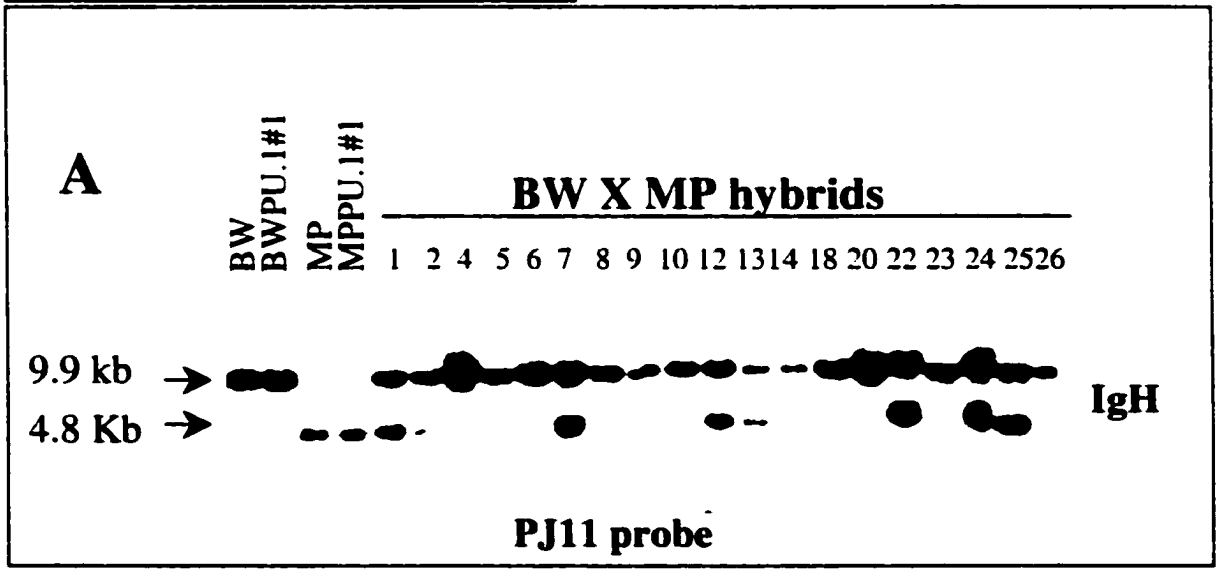
Figure 3.3A shows the results of IgH gene analyses in hybrids derived from BW5147 (no PU-1) and MPC11 control hybrids. Figure 3.3B shows comparable Southern blots for BW-PU.1#1 x MPC11 fusions. A DNA probe derived from the Ig-JH region (pJ11) hybridizes to a 9.9kb BamHI fragment in BW5147 genomic DNA while it hybridizes to a 4.8kb BamHI fragment in MPC11 DNA. The BamHI fragment in MPC11 DNA is derived from the productively rearranged and expressed γ2b gene in this cell line (139). Due to random chromosome loss, some of the hybrid lines lack the IgH loci contributed by either the plasmacytoma or the T lymphoma line (figure 3.3A). These analyses also served to reveal, therefore, which hybrid lines were informative with respect to IgH expression.

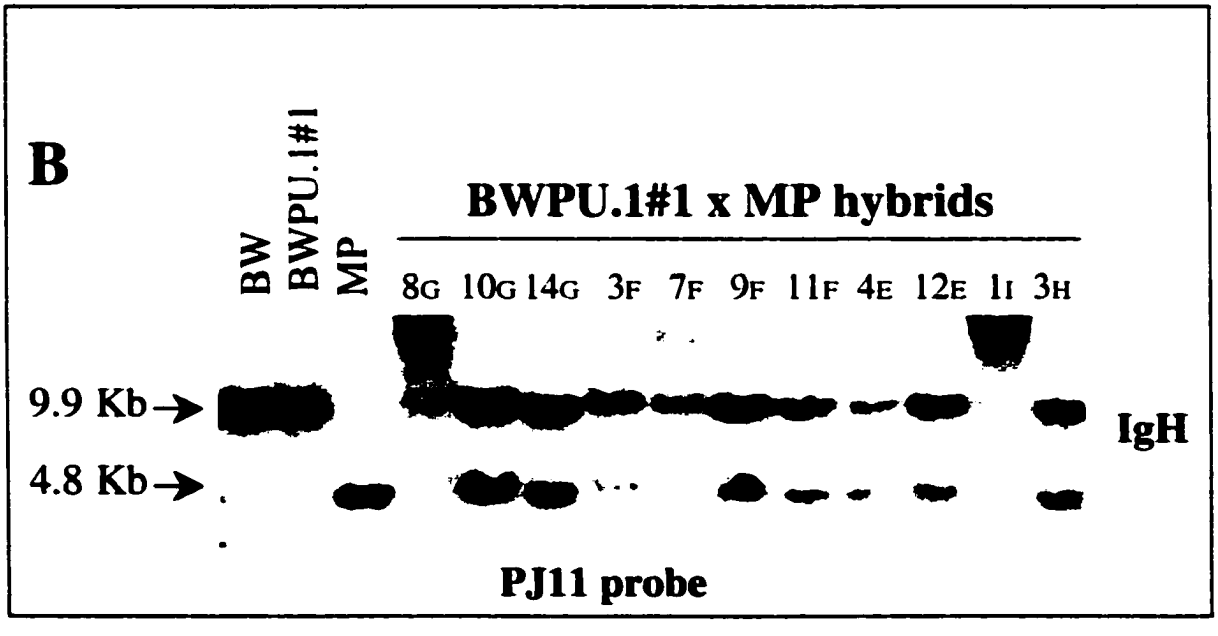
Figure 3.3 Genomic Southern blots of BW and MP derived IgH loci in hybrids.

A) Genomic DNA from parental and BW x MP hybrids was digested with BamHI size-fractionated by gel electrophoresis, transferred to Nytran and the resulting Southern Blot probed with pJ11 DNA probe. This probe hybridizes to the functionally rearranged IgH locus of MP (4.8 kb) and to the germline locus of BW (9.9 kb). Hybrids retaining the 4.8 kb and the 9.9 kb bands were selected for further analyses.

B) Genomic Southern of selected BWPU.1 #1 x MP experimental hybrids. Genomic DNA from parental and BWPU.1#1 x MP hybrids was digested with BamH1, size-fractionated, transferred to Nytran and probed with the pJ11 probe. All hybrids showed the IgH-derived loci from both, MPC11 and BWPU.1 #1 parental cell lines. These hybrids were selected for further studies.







In the examples shown, some of the control hybrids (BW x MP) retained BamHI fragments derived from the IgH loci of both parental lines (e.g. clones 1, 2, 7) while others lacked the plasmacytoma-derived BamHI fragment (e.g. clones 4, 5, 6).

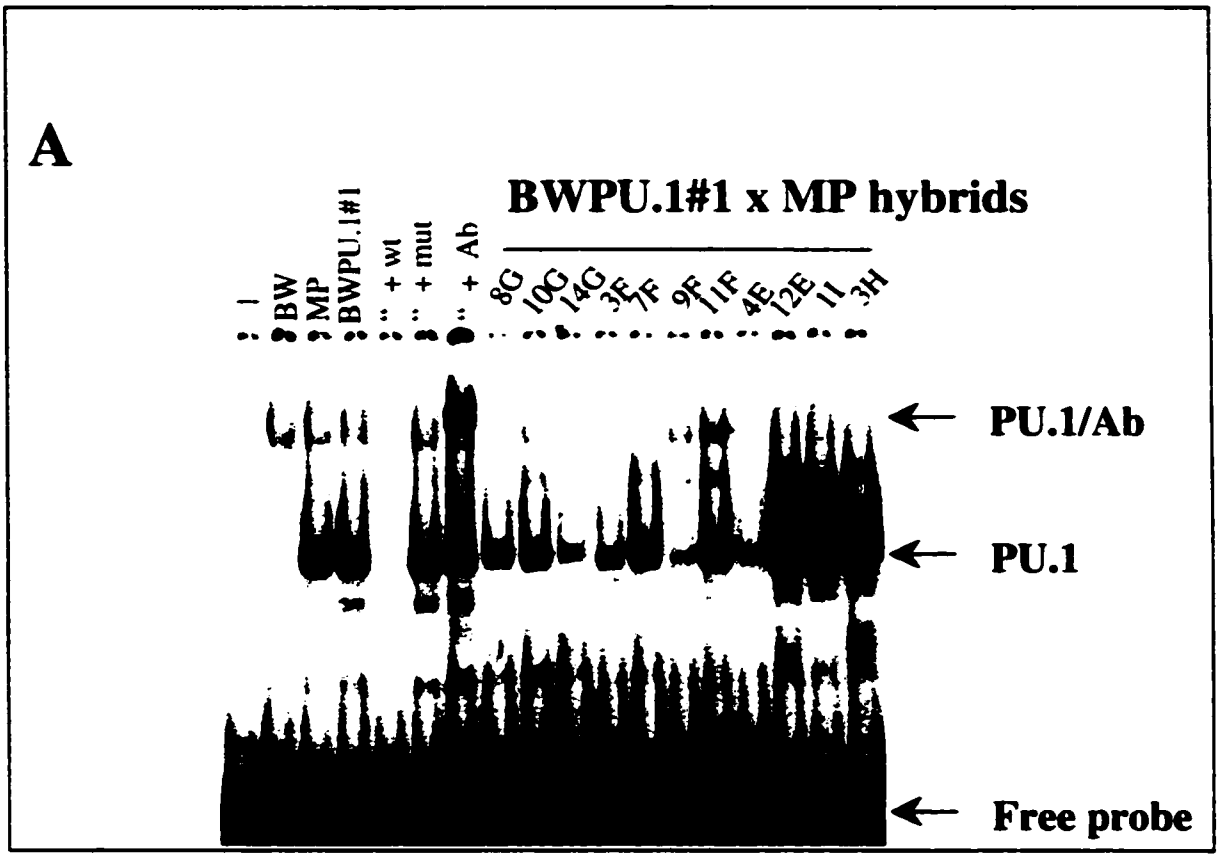
EMSA was used to confirm continued expression of exogenous PU.1 in these hybrid lines. The majority of the BW-PU.1 #1 x MPC11 hybrids expressed PU.1 (representative data, Figure 3.4 A). In contrast, only one out of six BW5147 x MPC11 hybrids (no exogenous PU.1) expressed PU.1 (Figure 3.4B: EMSA of control hybrids). In the latter hybrids, therefore, the endogenous PU.1 has been silenced as is typical of plasmacytoma-specific genes in such cell fusions (28). The one PU.1-expressing hybrid recovered in these control fusions was consistent with previous studies in which it was found that "exceptional" hybrids displaying the plasmacytoma phenotype arise at a low frequency (between 0%-3%) (28).

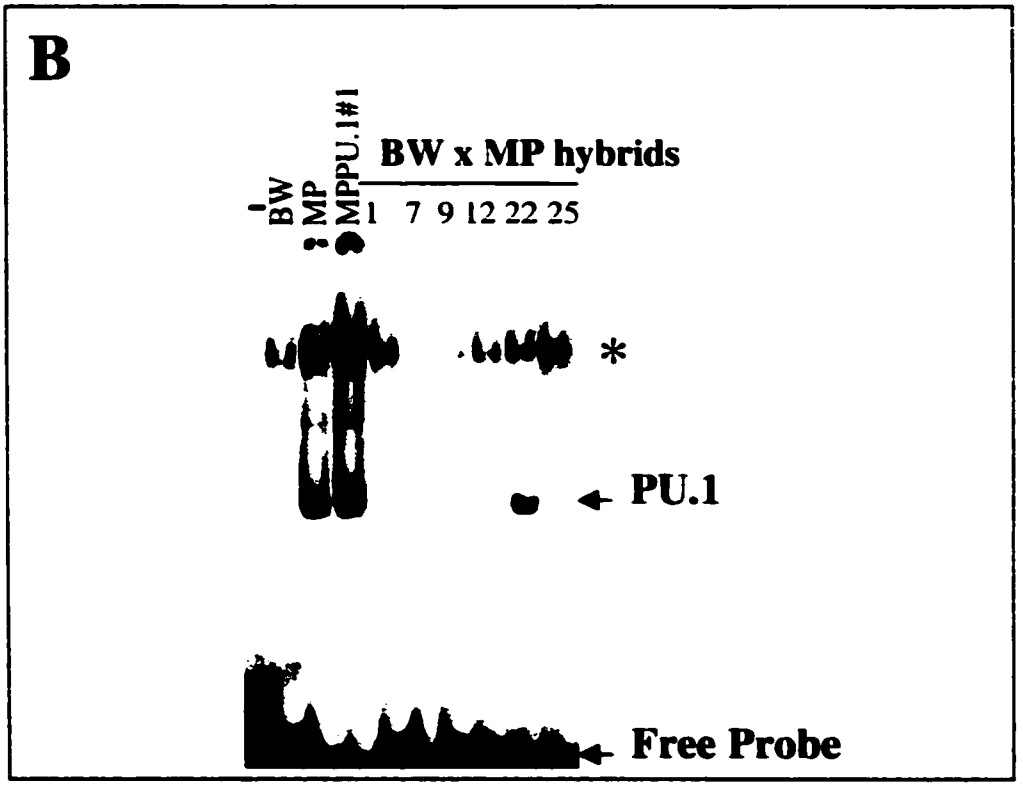
We concluded, therefore, that the PU.1 expressed in the BW-PU.1 #1 x MPC11 hybrids is that encoded by the BCMGS-Neo-PU.1 transgene. Because the protein encoded by the endogenous PU.1 gene was indistinguishable from that encoded by this transgene, we were unable to determine whether the endogenous gene was also active.

Figure 3.4 EMSA of PU.1 protein in BWPU.1 #1 x MP and BW x MP hybrids.

A) 10 ug of nuclear extracts from parental and selected BWPU.1 #1 x MP (8, 10 and 14 from fusion G ; 3, 7, 9 and 11 from fusion F; 4, 12 from fusion E, 1 from fusion I and 3 from fusion H) were incubated with radiolabelled PU.Box probe DNA. Competition was done using 140 ng of end-labelled PU.Box probe and 100 x fold excess of cold competitor (wt : wild type PU. Box DNA probe; mut: mutant PU box DNA probe. Supershift analyses were done using an antibody to the N-terminal domain of PU.1 protein (+ AB)

B) 10 ug of nuclear extracts from parental and selected BW x MP hybrids were incubated with radiolabelled PU.Box probe DNA. The asterisks reveals a non-specific protein binding.



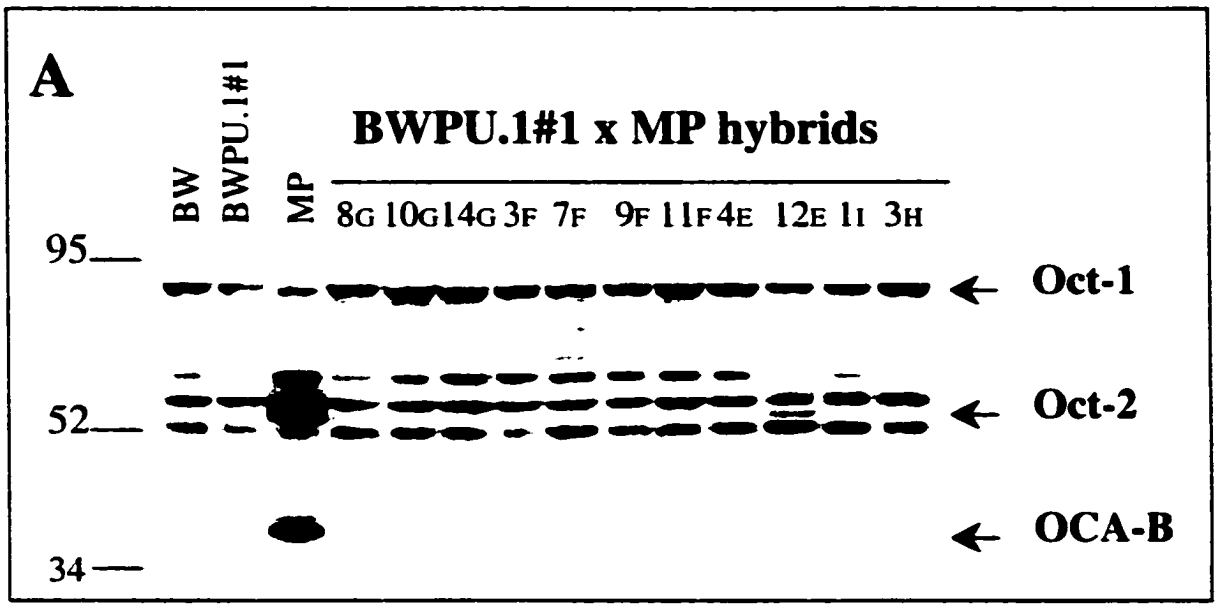


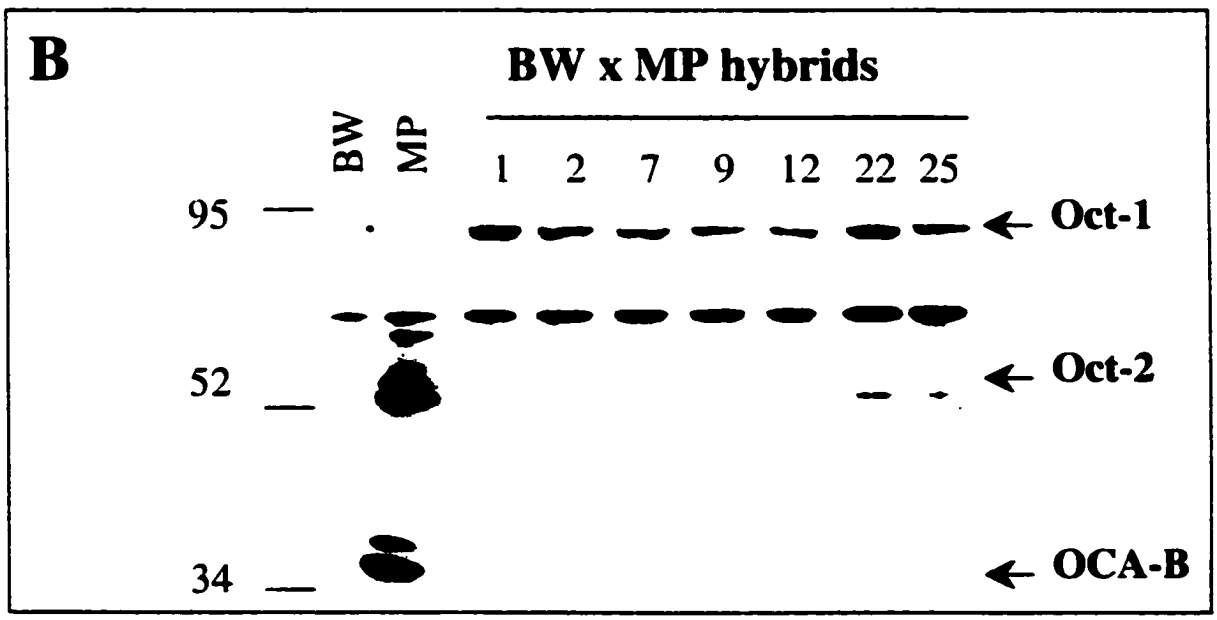
The Oct-2 transcription factor was lacking in both the control hybrids (Figure 3.5B) and BWPU.1#1 x MP hybrid lines (Western blots, Figure 3.5A). The expected Oct-2 molecular weights are from 55-60 Kd. Our antibody picks up crossreacting bands that are present in the T cell (does not make Oct-2). Similarly, the Oct-1/Oct-2 co-activator OCA-B was absent in both types of hybrids. Constitutive expression of PU.1, therefore, was unable to rescue expression of these two plasmacytoma-specific transcription factors.

Figure 3. 5 Western Blot analysis of BWPU.1 #1 x MP and BW x MP hybrids.

A) 50 ug of total cell lysates prepared form parental and BWPU.1#1 x MP hybrids were size-fractionated in an 8% SDS-PAGE electophoresis, transferred to nitrocellulose and then probed with the respective antibodies.

B) 50 ug of total cell lysates prepared from parental and BW x MP hybrids were size-fractionated and probed with the respective antibodies.

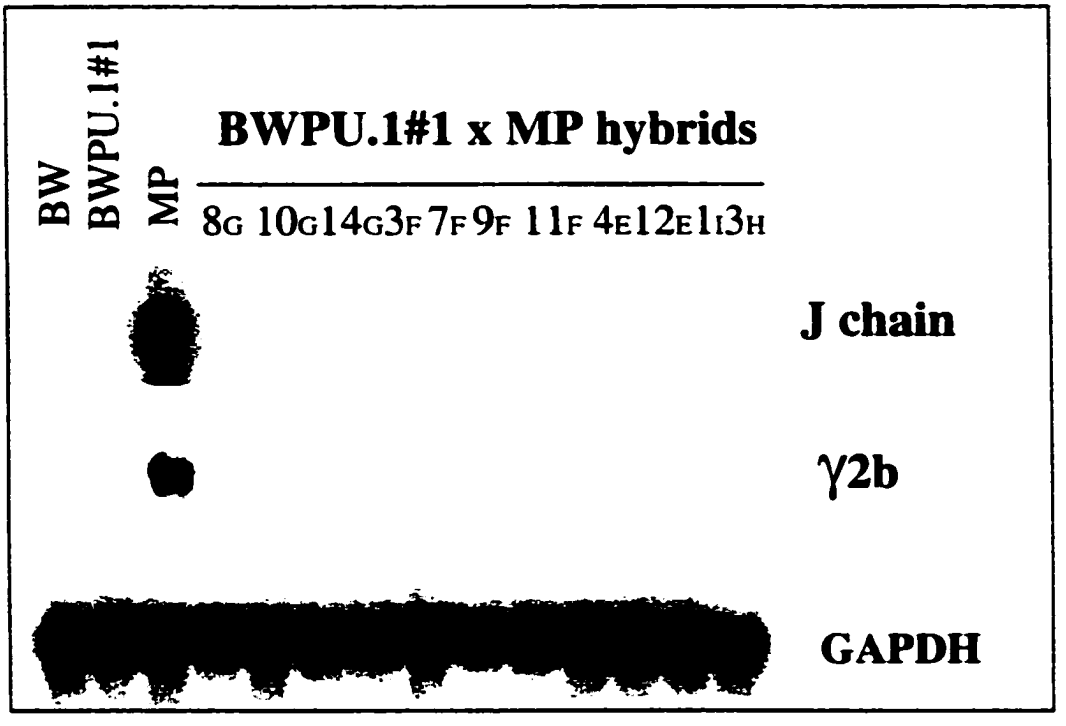




As noted earlier, PU.1 has been implicated in the regulation of the gene encoding J chain polypeptide (99). This gene becomes activated when mature B cells contact antigen and are stimulated by T cell cytokines. Since PU.1$^{-/-}$ knockout mice do not produce B lymphocytes, these mice provide no means of assessing the effect of PU.1 on J chain gene expression. We analyzed the BWPU.1#1 x MP hybrids for J-chain expression as an alternate means of approaching this question. As shown in Figure 3.6, the MPC11 parental line produced large amounts of J-chain mRNA while hybrids between this line and the T lymphoma BW5147 produced none. BWPU.1#1 x MP hybrids, constitutively producing PU.1 transcription factor, were also negative for J-chain mRNA. The presence of PU.1 alone, therefore, is not sufficient to sustain expression of the J-chain gene.

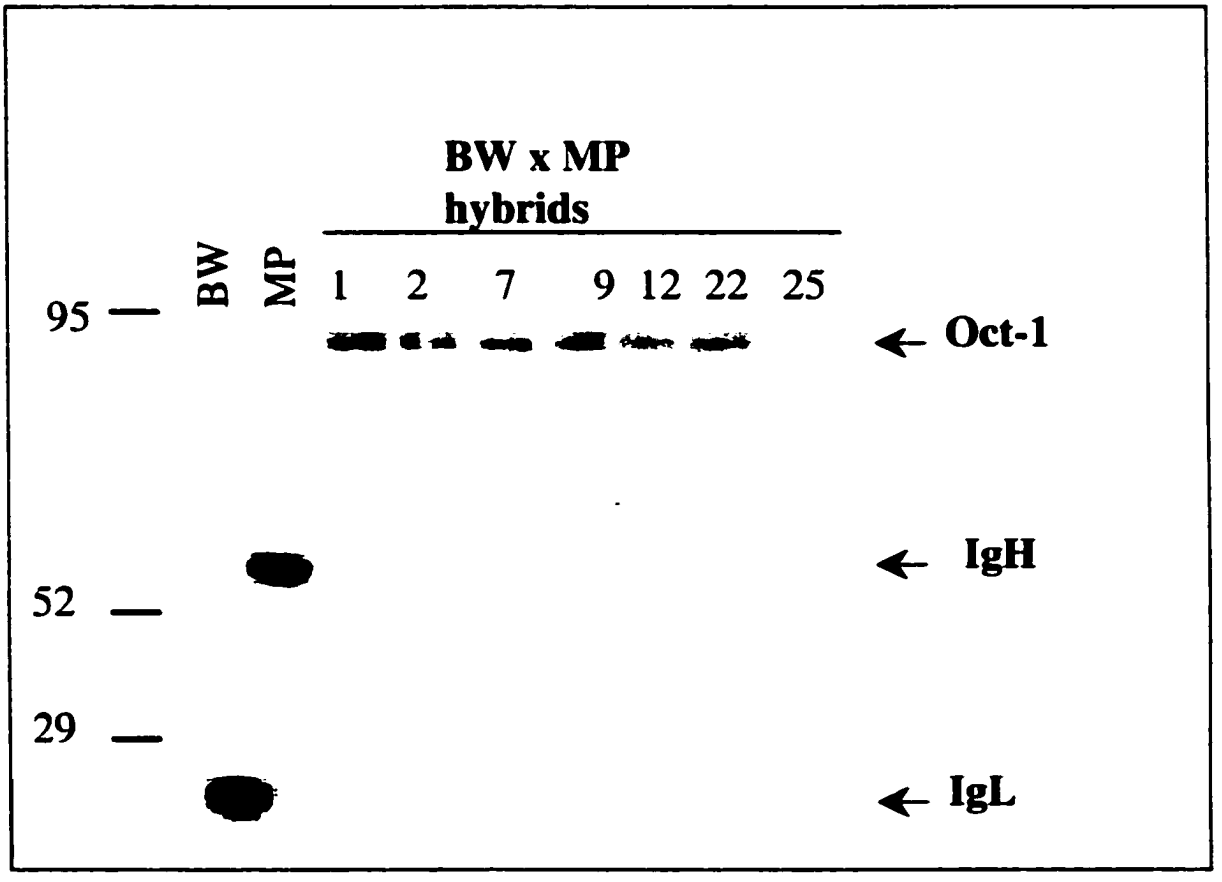
Figure 3. 6 PU.1 cannot rescue Immunoglobulin and J chain expression.

Northern blots of BWPU.1 #1 x MP hybrids. Immunoglobulin heavy chain and J chain mRNA expression in BWPU.1 #1 x MP hybrids. Total RNA isolated from the parental cell lines and hybrids were sequentially hybridized with GAPDH, γ2b and J chain probes.



Finally, the PU.1-expressing hybrids were examined for Ig heavy γ2b mRNA by Northern blot. As shown in Figure 3.6 none of the hybrids produced γ2b chain (ELISA was negative for heavy and light chains were done on 8G, 10G and 14G data not shown). Constitutive expression of PU.1 in the BW5147 T lymphoma, therefore, did not result in rescue of other plasmacytoma-specific genes (γ2b, Oct-2, OCA-B, J-chain) when this cell line was fused to the Ig-secreting cell line MPC11. These PU.1 expressing hybrids did not look any different from control hybrids shown in Western Figure 3-7 that IgH has not been produced. Figure 3.7 shows the lack of IgH/L of the control hybrids.

Figure 3.7 Expression of IgH/L chain in control hybrids. Western Blot analysis of control hybrids (no exogenous PU.1 expression). 50 ug of total cell lyzates were run on an 8% gel SDS-PAGE. The gel was then blotted with the respective antibodies. The result shows lack of expression of the heavy chain as well as light chain.



Constitutive expression of mPU.1 in the plasmacytoma prior to cell fusion does not lead to plasmacytoma gene rescue

Previous somatic cell fusion experiments with the transcription factor Oct-2 showed a differential effect on the rescue of plasmacytoma-specific genes when Oct-2 was introduced into the plasmacytoma as compared to the T lymphoma parental line (28). When a constitutively active Oct-2 gene was introduced into the plasmacytoma prior to cell fusion with BW5147 (T lymphoma), all hybrids expressed all plasmacytoma-specific genes assayed. When the same gene was introduced into the T lymphoma prior to cell fusion, a significant subset (but clearly not all) of the hybrids expressed plasmacytoma-specific genes. We have suggested that this difference be due to Oct-2's requirement for a plasmacytoma-specific co-factor in mediating its gene rescue function (28). Reasoning that PU.1 might similarly require such a factor, we tested the effect of introducing a gene expressing PU.1 into the plasmacytoma prior to cell fusion.

BCMGS-Neo-PU.1 was used to transform MPC11 cells and transformants were grown in G418 media and further screened by polymerase chain reaction for presence of the neo^r gene. As mentioned earlier, the PU.1 protein produced from this expression vector was indistinguishable from the PU.1 produced from MPC11's endogenous PU.1 gene. As a result, expression of exogenous PU.1 was only inferred from the fact that the transformants carried and expressed the selectable neo^r gene. Also Genomic Southern confirmed the presence of PU.1 cDNA.

Two MPC11-PU.1 transformants were individually fused to the BW5147 T lymphoma line. Hybrids were first selected in HAT/OUAB medium, and then in medium containing 2mg/ml G418 (selects for neo^r gene). We reasoned that any PU.1 expression in the resulting hybrids was derived from the exogenously introduced PU.1 cDNA since we had previously shown that the endogenous PU.1 gene is silenced in control MPC11 X BW5147 hybrids (Figure 3.4B). While there are exceptions, these exceptional hybrids (expressing endogenous PU.1 gene) arise at a low frequency (0-3%). In contrast, hybrids derived from the MPC11-PU.1 x BW5147 fusions expressed PU.1.

We collected nine hybrids (5 fusions total) that both maintained PU.1 expression and maintained the plasmacytoma-derived IgH gene from MPC11 (Table II, Figure 3.7 PU.1 gel shifts). As mentioned earlier, our criteria for selection of hybrids was that they carried the IgH loci derived from both parental cell lines (data not shown). Western Blots done with total cell lysates prepared from the hybrid lines revealed that both Oct-2 and OCA-B were lacking in most of the hybrids (Figure 3.8A). Interestingly, one hybrid (#8) produced a protein that reacted with the anti-Oct-2 antibody but that was significantly larger than the Oct-2 protein produced by MPC11 or the parental MPC11-PU.1 line. In any case this, and all of the other hybrids lacked γ2b and Igκ chains (Figure 3.8B). PU.1 expression in plasmacytoma x T lymphoma hybrids, therefore, was not sufficient to maintain expression of other plasmacytoma-specific genes. This was true whether the gene constitutively expressing PU.1 was introduced into the T lymphoma or into the plasmacytoma prior to cell fusion.

Table II. Characterization of MPPU.1#1 x BW hybrids

#	hybrids	PJ11	γ2b	Kappa	Oct-2	PU.1	OCA-B
1	8A	+	-	-	+*	+	-
2	22A	+	-	-	-	+	-
3	24A	+	-	-	-	+	-
4	3B	+	-	-	-	+	-
5	10B	+	-	-	-	+	-
6	21B	+	-	-	-	+	-
7	17C	+	-	-	-	+	-
8	16D	+	-	-	-	+	-
9	3E	+	-	-	-	+	-

*diff isoform

PJ11 was the DNA probe used to detect the IgH-derived loci from both of the parental cell lines, MPC11 and BW5147.

* There are several Oct-2 isoforms and it is the Oct-2A that has shown to rescue .

Figure 3.8 EMSA of nuclear extracts from MPPU1 #1 x BW : EMSA of PU.1 protein in MPPU1 transformants and MPPU1#1 x BW hybrids. 10 ug of nuclear extracts from parental and selected MPPU.1 #1 x BW were incubated with labelled PU probe and run on a 4% non denaturing gel.

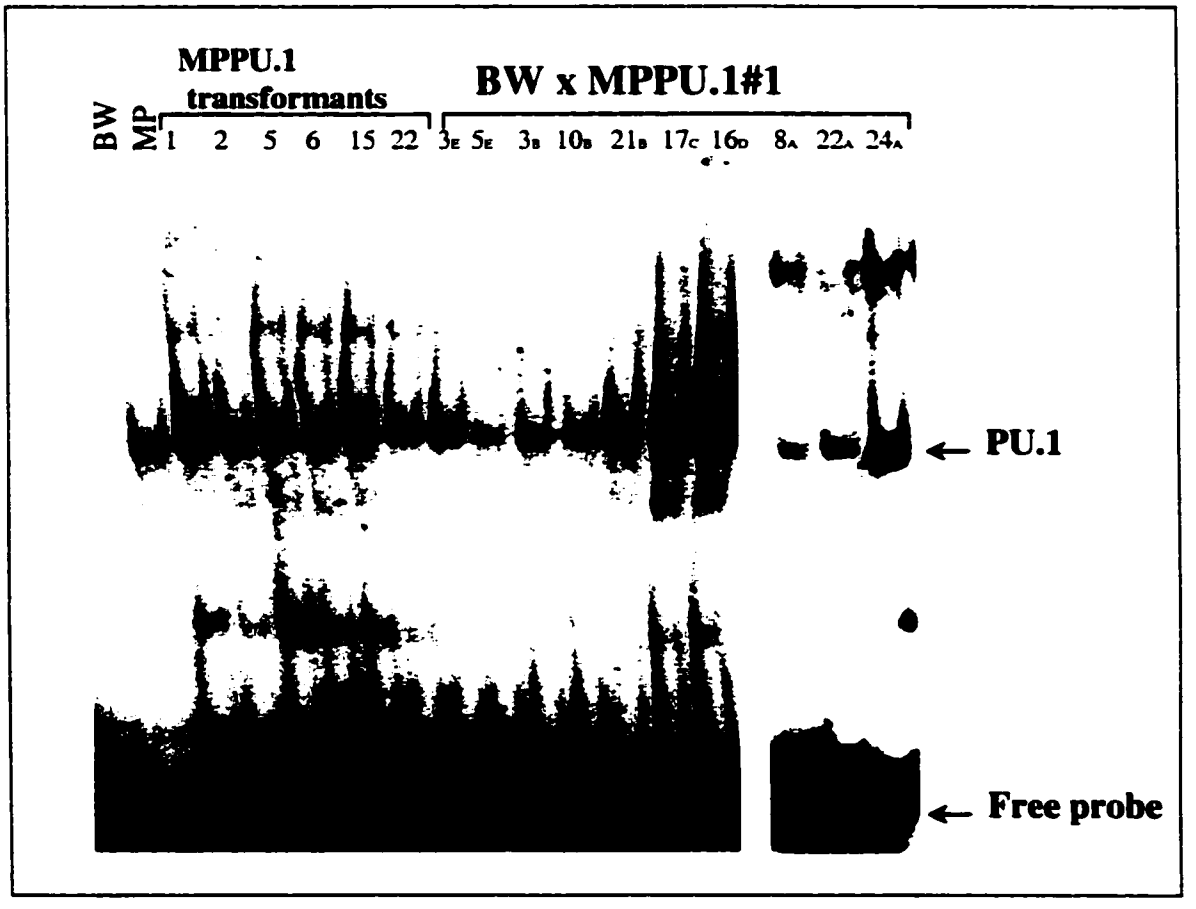
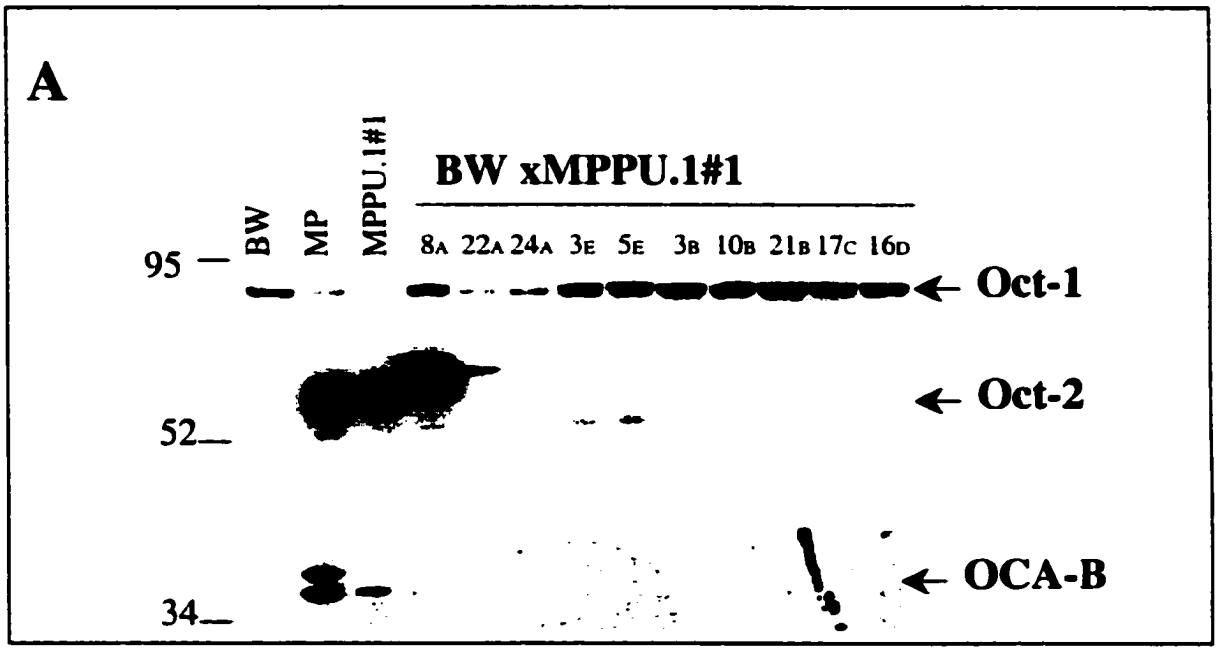
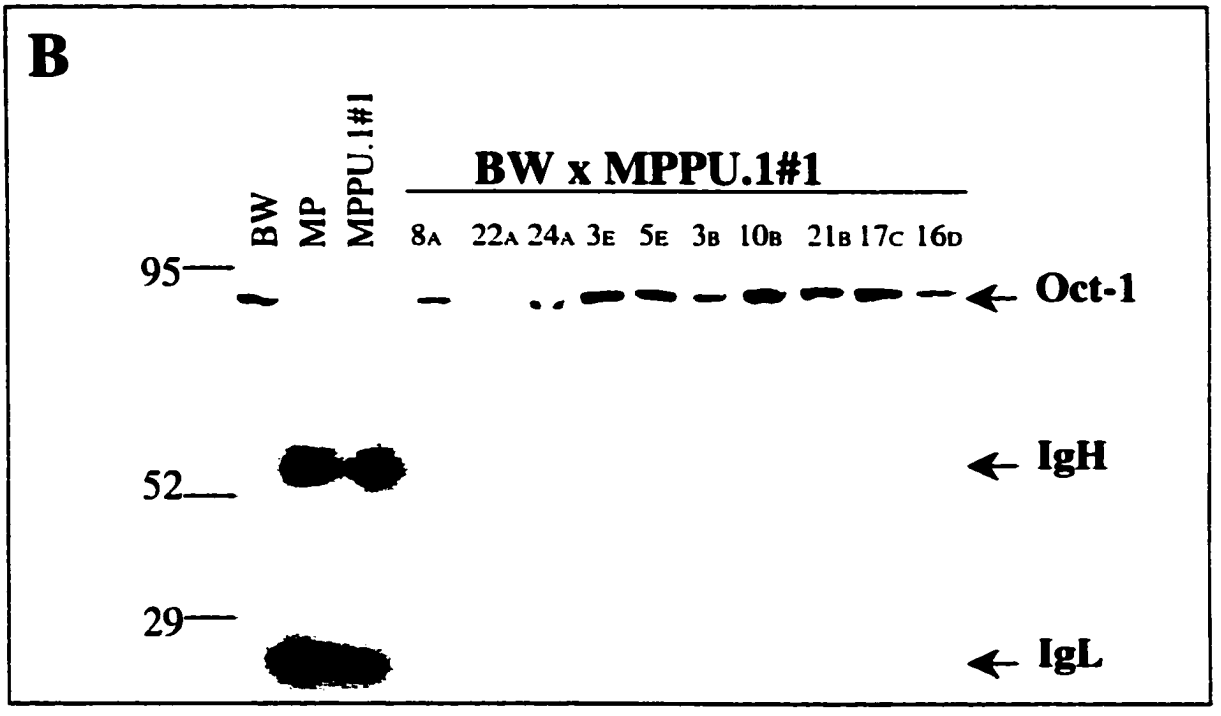


Figure 3.9 MP-PU.1 #1 x BW expressing hybrids do not rescue immunoglobulin heavy or light chains, Oct-2 or OCA-B expression.

A) Total cell lysates of hybrids were size-fractionated in an 8%SDS-PAGE and probed with the respective antibodies.

B) Total cell lysates of hybrids were size-fractionated in an 8% SDS-PAGE and probed with the respective antibodies.





Discussion

The expression vector used for PU.1 studies coded for a murine form of the protein. Since our MPC11 has endogenous PU.1, we needed to establish a means to differentiate between the endogenous and exogenous PU.1.

In an attempt to identify the exogenous versus the endogenous PU.1 in our cell hybrids, an RT/PCR assay was established using an antisense primer to the PU.1 and then specific forward primers to the BCNGSneo PU.1 vector backbone and to the untranslated endogenous PU.1 sequences. Although I was able to obtain a specific product for the exogenous and endogenous as well as a control that picks up both, at the time when this assay was about to start, some of the cell hybrids that had the selectable marker did not continue to be resistant to the drug.

Using the somatic cell fusion approach, it was found that PU.1, unlike Oct-2, was not able to rescue the B cell phenotype when it was maintained prior to fusion in either the myeloma or T-lymphoma cell. I further found that PU.1 responded to OCA-B (discussed in Chapter 4) and Oct-2 (previous experiments done in the laboratory) but could not reciprocally regulate the genes encoding either one of these factors. Therefore, PU.1 was not sufficient to rescue the B cell phenotype and played a subordinate role to OCA-B/Oct-2 in the maintenance of the B cell program at the Ig-secreting cell. However, the outcome of these experiments did not say that PU.1 was not necessary to the Ig-secreting B cell.

This technique provided with helpful information that we would not otherwise obtain since knockouts of PU.1 could not provide any precursors to the lymphoid lineages, therefore precluding any further analysis, particularly, to the B cell pathway. Moreover, we could use the somatic cell fusion approach for finding out about other genes that have been reported to be under the regulation of PU.1 at this stage of a B cell. For instance, the J chain gene was transcribed when a B cell was activated, and it was found that overexpression of PU.1 was not able to overcome extinction of the J chain gene. PU.1 is not alone in the category of factors that are not able to rescue the B cell program. Oct-1 has similar behavior. Similar experiments with Oct-1 showed that it was not able to rescue B cell expression (102). It has also been shown that repression of liver-specific genes in hepatoma x fibroblast hybrids is also accompanied by silencing of transcription factors known to transactivate those genes (141). It was shown in these fusions that ectopic expression of HNF-1 (silent in these hybrids) either before or after fusion, could not rescue gene expression. Therefore PU.1's behavior is characteristic of most tissue-specific transcription factors and is likely to reflect its position within a hierarchy of gene-controlling factors.

My report is consistent with another report on somatic cell fusion between plasmacytoma and embryonal carcinoma (EC) cells where transcription factor PU.1 is also extinguished (140).

CHAPTER 4

The role of coactivator OCA-B in Ig-secreting cells

Introduction

One of the best characterized motifs within promoters and enhancers of the IgH/L locus is the octamer motif. In B cells, there are two octamer-binding proteins, members of the POU family of homeodomains, Oct-1 and Oct-2. Oct-2 is predominantly B-cell specific and Oct-1 is ubiquitous. OCA-B/OBF-1/Bob1 is a coactivator of Oct-1 and Oct-2 transcription factors and is found exclusively in cells of the B lymphocyte lineage. OCA-B potentiates the activity of immunoglobulin heavy and light chain promoters in an in vitro IgH transcription assay when supplemented or Oct-2 is also present (16, 100). OCA-B was initially thought to be responsible for lymphoid-restricted expression of Ig. However, the early stages in B cell development take place normally in OCA-$B^{-/-}$ mice, and IgM expression appears normal in these cells (27, 101, 26). There is a defect in B lymphocytes from these mice which becomes evident only after antigen-stimulated maturation of the cells. The mice make dramatically reduced amounts of the non-IgM isotypes (e.g. IgG2a), not because the B cells are unable to switch class (by class-switch recombination = CSR), but because the transcription rate of the IgH genes in these mutant cells appear to decrease after heavy-chain class-switching (27).

The phenotype of OCA-$B^{-/-}$ mice pointed to a unique role for this coactivator late in B cell development. It was similarly concluded from the phenotype of B cells from Oct-2-/- mice and from the results of cell fusion experiments that Oct-2 served a unique and essential function at or after B cell activation and in Ig-secreting cells (21, 23, 24, 28). Since OCA-B and Oct-2 take on critical functions at the later stages of B cell development and are known to be able to work together as co-factors, it was natural to

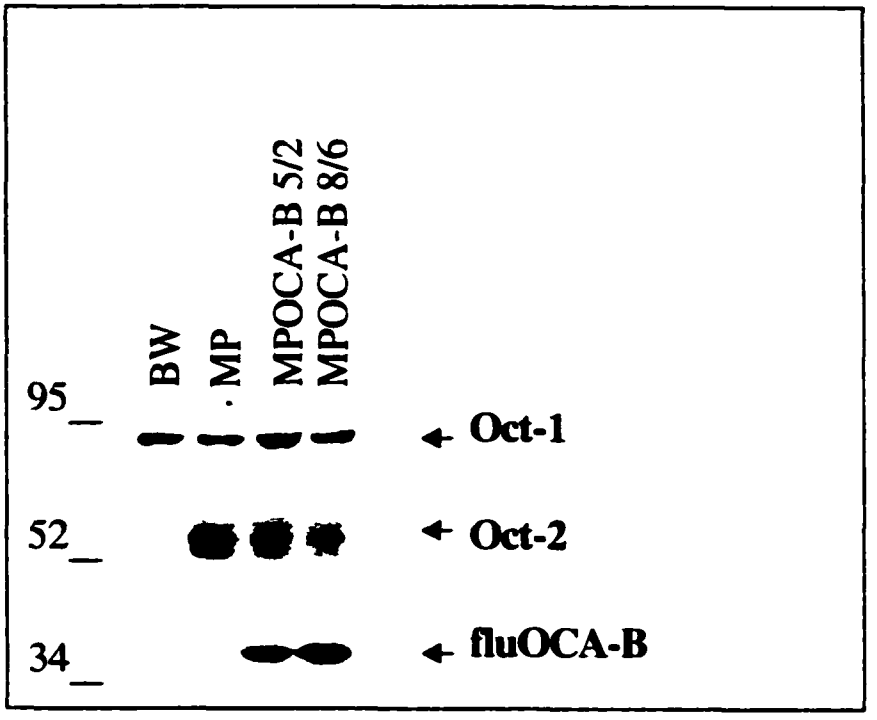
ask whether these two transcription factors acted in a mutually dependent fashion. We were interested as well in knowing whether they were able to regulate one another's expression. To approach these questions, we again turned to the somatic cell fusion system.

Results

hOCA-B, when expressed constitutively in an Ig-secreting plasmacytoma, rescues the Ig-secreting cell's genetic program from silencing by a T lymphoma

Since we had previously found that sustained expression of hOct-2 in plasmacytoma x T lymphoma hybrids simultaneously sustained expression of endogenously-encoded OCA-B, we asked whether the reciprocal was true. MPC11 cells (IgG2b-expressing plasmacytoma) were transformed, by electroporation with the pCGN-hOCA-B expression vector in order to establish cell lines that constitutively expressed this factor under the control of a ubiquitously-active promoter (vector kindly provided by Dr. R. Roeder, The Rockefeller University, New York, NY). A Western blot confirming expression of the flu-tagged hOCA-B protein in the transformants is shown in Figure 4.1. One of these transformants, MPOCA-B(8/6) was fused to BW5147 (a T lymphoma).

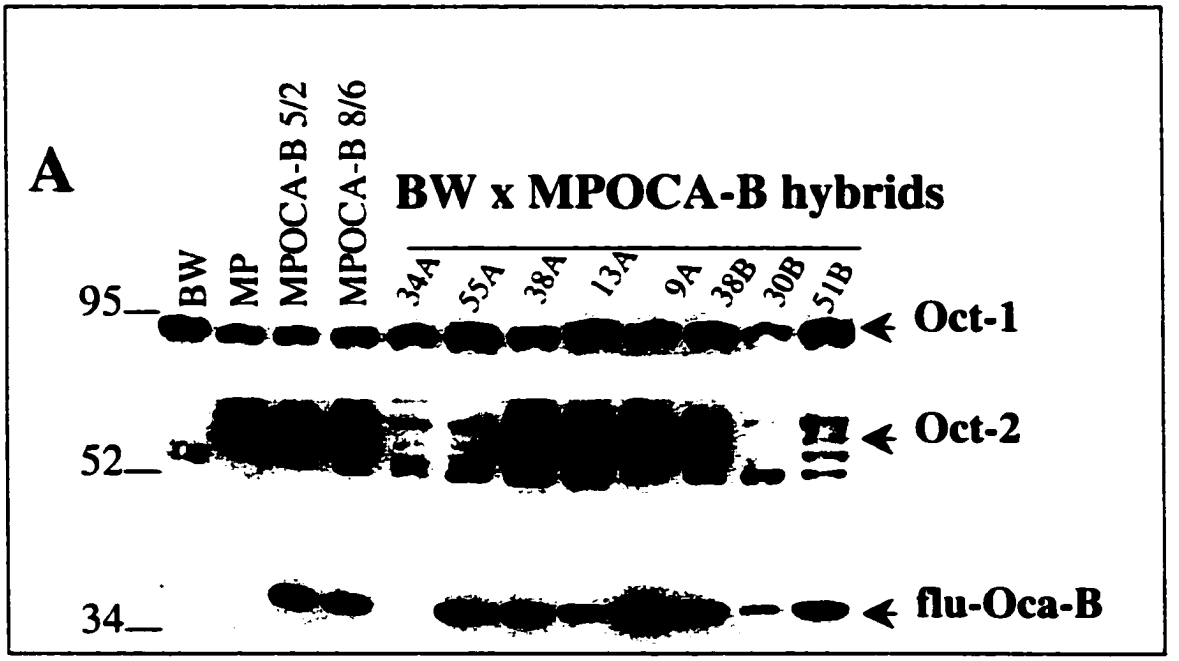
Figure 4.1 Western Blots of flu-tagged OCA-B-MPC11 transformants. 50 ug of total cell lysates were loaded in an 8% SDS-PAGE gel, transferred to nitrocellulose and probed with the respective antibodies to detect the presence of Oct-1, used for normalization purposes, Oct-2 and flu- tagged OCA-B protein. Two OCA-B transformants, MP OCA-B 5/2 and MPOCA-8/6 are shown. Both of these were fused to the BW cell line and it is the product of the MPOCA-8/6 x BW fusion that is presented in this work.



Seventeen of the hybrids recovered in selective media (see Materials and Methods, Table III) retained IgH loci derived from both the plasmacytoma and T lymphoma parents and continued to express flu-hOCA-B. These were selected for further study. Almost all of these hybrids expressed endogenously-encoded Oct-2. This was demonstrated by both Western blot (Figure 4.2A) and by electrophoretic mobility shift assay (EMSA, Figure 4.2B). As shown, some of the hybrids expressed Oct-2 at levels that approximated that of MPOCA-B (8/6) transformant (e.g. hybrids 38A , 13A, 9A, 38B) while others expressed Oct-2 at much lower levels (e.g. hybrids 51B and 55A). The variation in expression levels for rescued genes was previously observed in experiments involving hOct-2 as the rescuing transcription factor (28, 102). As was seen in the latter studies, rescuing factor levels and rescued gene expression levels did not co-vary (e.g. compare hOCA-B and Oct-2 levels in hybrids 55 and 38A, Figure 4. 2A). This is discussed below. There was one hybrid among the 17 analyzed that did not express detectable Oct-2 (clone 21, data not shown). Gel shift using the octamer probe was consistent with the Western Blots on levels of expression of Oct-2. EMSA to detect PU.1 showed that this factor was present in almost all hybrids (Representative data, Figure 4.3). The finding that hOCA-B expression could rescue that of the PU.1 locus is consistent with previously-published transient transactivation experiments in which it was shown that OCA-B could transactivate a reporter gene that contains PU.1 promoter (95).

Figure 4. 2 Expression of mOct-2 and mOCA-B proteins in parental and selected BW x MPOCA-B8/6 hybrid cell lines.

A) Western Blots 50 ug of total cell lysates were loaded in an 8% SDS-PAGE gel and transferred into nitrocellulose. The respective antibodies were used to detect the presence of Oct-1 used for normalization purposes, the levels of mOct-2 and flu-tagged OCA-B. B) Oct-2 EMSA. Nuclear extracts of parental and MPOCA-B 8/6 x BW hybrids were incubated with a P^{32} labeled 51 bp from the IgH intronic (Eμ) enhancer. This fragment includes an octamer motif to which two octamer -binding proteins, Oct-1 and Oct-2, can bind. DNA/protein complexes formed by endogenous mouse Oct-1 and mouse Oct-2 are shown.



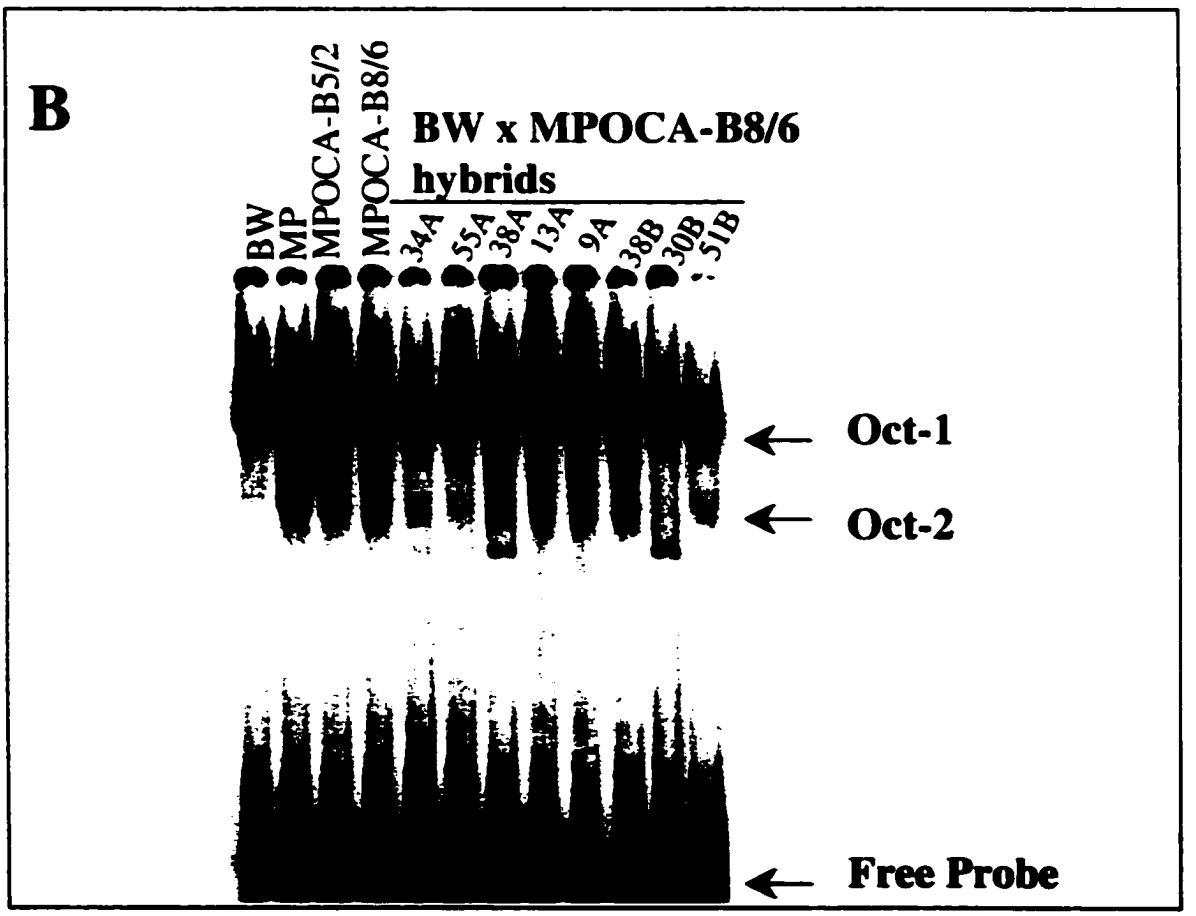
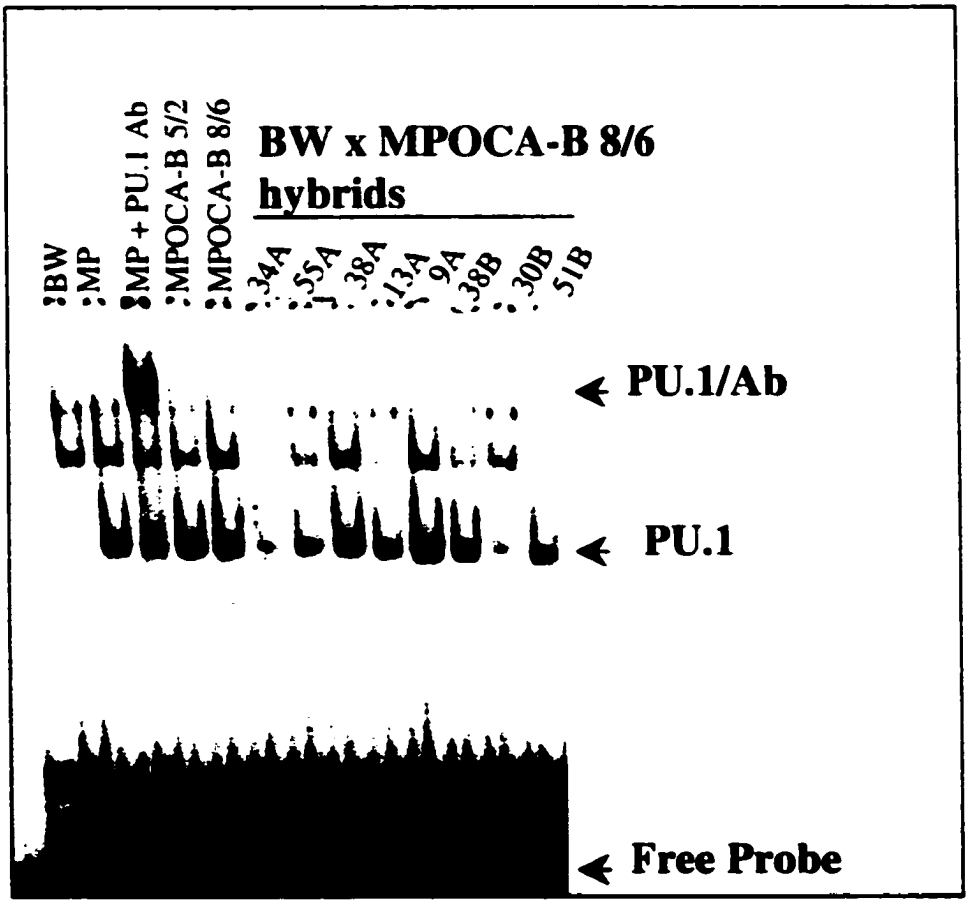


Table III: MPOCA-B8/6 x BW hybrids

Hybrids	PJ11	g2b	K	Oct-2	hOCA-B	PU.1	Oct-1	mRNAg2b	mRNA Jchain
9A	+	+	+	+	+	+	+	+	+
13A	+	+	+	+	+	+	+	+	+
34A	+	+	+	+	-	+	+	very low	low
38A	+	+	+	+	+	+	+	+	+
55A	+	+	+	+	+	+	+	+	+
30B	+	+	+	+	+	+	+	Undetectable*	+
38B	+	+	+	+	+	+	+	+	+
51B	+	+	+	+	+	+	+	+	+
21B	+	-	-	-	+	nd	+	-	-
4A	+	+	+	+	+	nd	+	nd	nd
15A	+	+	+	+	+	nd	+	nd	nd
31A	+	+	+	+	+	nd	+	nd	nd
2B	+	+	+	+	+	nd	+	nd	nd
14B	+	+	+	+	+	nd	+	nd	nd
32B	+	+	+	+	+	+	+	nd	nd
36B	+	+	+	+	+	nd	+	nd	nd
41B	+	+	+	+	+	nd	+	nd	nd

nd: not determined *** undetectable by Northern Blots**

Figure 4.3. BW x MPOCA-B 8/6 hybrids rescued PU.1 expression. 10 ug of nuclear extracts from parental and selected hybrid cell lines were incubated with a radiolabelled PU. box. A supershifted complex of DNA/PU.1/ Ab is shown when an antibody to the N-terminal domain of the protein is incubated with the extracts and the PU. Box probe.



We next assayed the hybrids for expression of Ig. Sixteen of the 17 hybrids expressed both γ2b heavy chains and κ light chains as determined by Western blot (Figure 4.4; summarized in Table III). RNAs from a subset of the hybrids were analyzed for γ2b mRNA by Northern Blot. Hybrid clone 21B produced no detectable γ2b mRNA (Figure 4.5). γ2b mRNA was barely detectable in clone 34A and was undetectable in clone 30B. As judged by Western, however, clone 30B was producing γ2b protein, albeit at very low levels. Clones 30B produced low amounts of Oct-2 and PU.1 as well (Figures 4.2 & 4.3). While expression of the rescued genes, therefore, did not co-vary with the rescuing protein (hOCA-B), the rescued genes did correlate with one another. That is, a clone that produced low amounts of Oct-2 also produced low amounts of PU.1 and Ig. Clones expressing high levels of Oct-2 also expressed high levels of PU.1 and Ig. This was also seen with respect to J-chain gene expression. As shown in Figure 4.5, J-chain mRNA was clearly present in six of the clones. Clone #21 produced no detectable J-chain mRNA.

Figure 4.4. BW x MPOCA-B hybrids rescued Immunoglobulin heavy and light chain expression. Western Blots of parental and BW x MPOCA-B 8/6 hybrids. 50 ug of total cell lysates were size-fractionated in an 8% SDS-PAGE electrophoresis, transferred to nitrocellulose and probed with the respective antibodies.

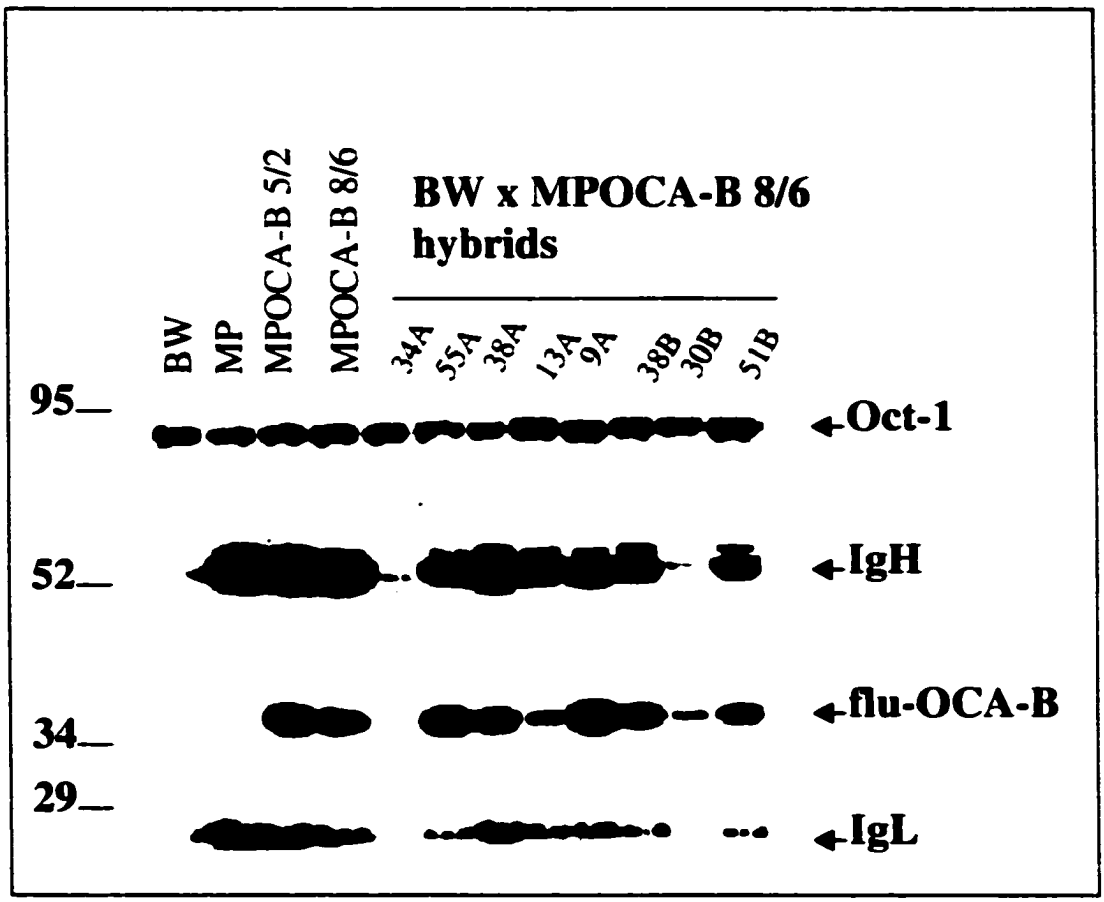
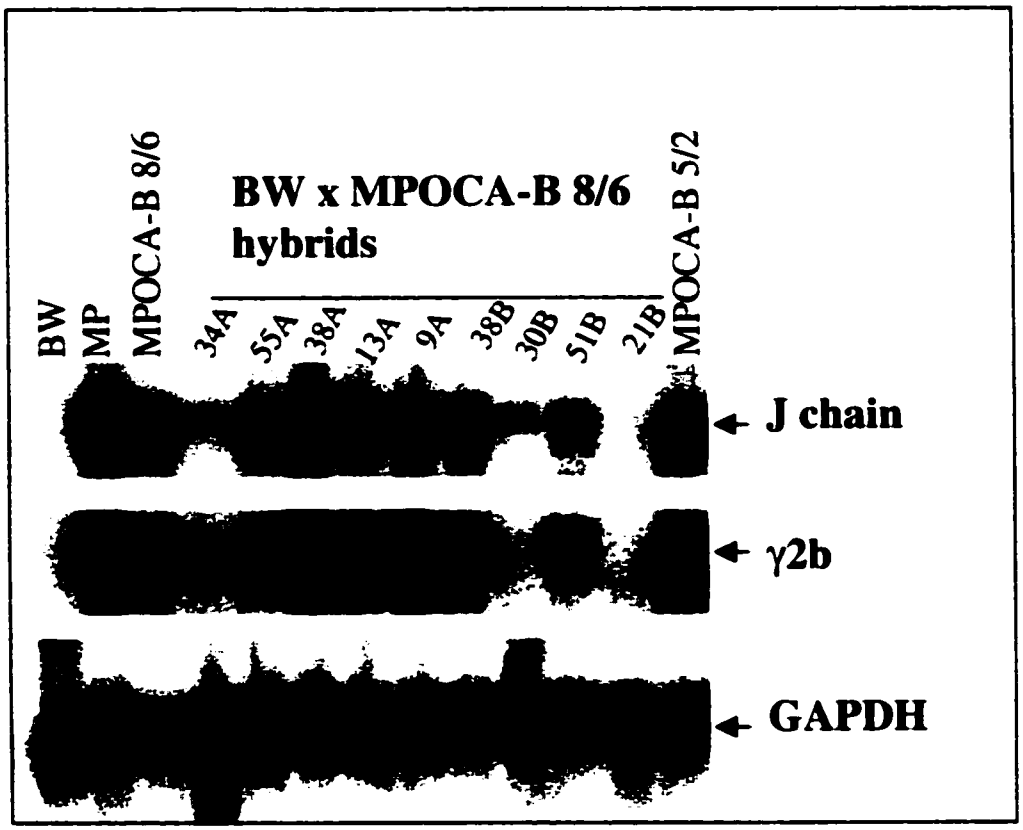


Figure 4.5. BW x MPOCA-B 8/6 hybrids rescued J chain mRNA expression. Northern Blots of parental and OCA-B experimental hybrids. Total RNA was size-fractionated and sequentially probed for GAPDH, γ 2b and J chain probe.



In summary, when MPOCA-B (8/6) was fused to the T lymphoma BW5147, 16/17 hybrids (94%) expressed genes unique to the Ig-expressing plasmacytoma parent (Oct-2, PU.1, IgH and IgL). As shown in Chapter 3, these genes are normally silenced in fusions between MPC11 (no exogenous flu-OCA-B expression) and BW5147. This ability to sustain the Ig-secreting cell's genetic program resembles that of Oct-2. Notably, another transcription factor, PU.1 is rescued from silencing by both Oct-2 and OCA-B, cannot serve this rescuing function (Chapter 3). We conclude that OCA-B and Oct-2 each serves a critical function in Ig-secreting cells and that they regulate one another's expression in a reciprocal manner. Moreover, since both factors rescue expression of other regulatory and structural genes unique to the B cell program, we propose that they are at the apex of a regulatory chain responsible for the Ig-secreting cell phenotype.

As previously noted, OCA-B functions as a co-activator with the Oct-1 and Oct-2 transcription factors. In plasmacytoma x T lymphoma fusions, Oct-1 expression is never silenced. When either Oct-2 or OCA-B is expressed in the plasmacytoma under the control of a promoter that cannot be silenced, the ectopically expressed protein (e.g. flu-OCA-B) is co-expressed in the resulting hybrids along with the endogenous partner (e.g. mOct-2). As a result, it is difficult to determine whether or not OCA-B function in Ig-secreting cells is dependent upon co-expression of mOct-2. Alternatively, OCA-B may function independently of mOct-2, working efficiently with Oct-1, yet one of its side-effects is rescue of Pou2f2 expression (locus encoding Oct-2). Similarly, when hOct-2 is used to rescue the plasmacytoma genetic program in such fusion experiments, it may

function independently of OCA-B and only incidentally rescue OBF-1 expression (locus encoding OCA-B).

Previous work from our laboratory suggests that hOct-2 function in Ig-secreting cells, in any case, requires a tissue-specific (plasmacytoma-specific) co-activator. Stable expression of a hOct-2-expression vector in the MPC11 plasmacytoma prior to fusion with the T lymphoma BW5147 resulted in hybrids that uniformly expressed Ig and other plasmacytoma-specific gene products. As noted earlier, the level of Ig expression varied among the hybrids, but all hybrids expressed these uniquely B lineage genes. When the same hOct-2 expression vector was introduced into the T lymphoma prior to cell fusion, the outcome was quite different. One BW-hOct-2 transformant, when fused to MPC11, yielded 23% hybrids with the plasmacytoma phenotype. Another transformant, producing roughly three times as much hOct-2 as the other, yielded 75% hybrids with the plasmacytoma phenotype. When hOct-2 was expressed in the T lymphoma parent prior to cell fusion, therefore, it appeared to have a dose-dependent effect on hybrid phenotype.

The fact that the nuclear environment prior to cell fusion affected hOct-2's function suggested that Oct-2 required a plasmacytoma-specific co-factor for maximal activity. One interpretation of the data was that hOct-2 formed an association with its co-factor prior to cell fusion when the hOct-2-expression vector was introduced into the plasmacytoma. When introduced into the T lymphoma, however, hOct-2 could only form an association with its co-factor after cell fusion. In this case, it might rarely be able to do so before the gene encoding that co-factor had been silenced by T cell-derived

factors. In the relatively rare instance where hOct-2 was able to form an association with the co-factor prior to gene-silencing, the whole plasmacytoma-specific program would be rescued from silencing. The likelihood of the latter would be increased as the concentration of one or both of the interacting partners increased, thereby explaining how a T lymphoma expressing more hOct-2 than another could yield a higher proportion of hybrids with the plasmacytoma phenotype.

One possibility is that OCA-B is the plasmacytoma-specific co-factor required for Oct-2 function. If this were true, we would expect that introducing both Oct-2 and OCA-B into the parental T lymphoma line before cell fusion would uniformly yield hybrids with the plasmacytoma phenotype. In this instance, Oct-2 and OCA-B could form the necessary association to prevent silencing of any part of the plasmacytoma program when the T lymphoma and plasmacytoma were fused.

To test this hypothesis, we first introduced OCA-B into the T lymphoma (BW5147) before fusion with the plasmacytoma (MPC11). In this case, we were asking whether OCA-B, like Oct-2, would demonstrate a requirement for a plasmacytoma-derived partner. If so, we expected to find a lower percentage of hybrids with the plasmacytoma phenotype than had been seen in fusions involving the plasmacytoma-flu-OCA-B transformant, MPOCA-B(8/6).

Constitutive expression of hOCA-B in the T lymphoma does not rescue efficiently the Ig-secreting cell phenotype in hybrids

Two T cell-OCA-B transformants expressing OCA-B were isolated (see Figure 4. 6, (BWOCA-B #5 and BWOCA-B# 10). The transformant expressing higher amounts of OCA-B was fused to the Ig-secreting plasmacytoma MPC11. Seven hybrids were recovered that both expressed the OCA-B transgene and retained Ig loci from the T lymphoma and the plasmacytoma parent line (Figure 4.7A and data not shown). A Western blot of whole cell extract obtained from six of these hybrids is shown in Figure 4.7A and 4.7B. As shown, each of the hybrids continued expressing the OCA-B transgene (flu-OCA-B), albeit at lower levels than the parental transformant. One of the seven hybrids also expressed endogenous Oct-2 (Figure 4.7A). When cell extracts were examined by Western blot for IgG2b and Igκ chain, all except one hybrid, hybrid #14, were negative for these proteins (Figure 4. 7B). Six of the hybrids, therefore, lacked Oct-2 and Ig expression and one (hybrid #14) expressed both Oct-2 and Ig (14% rescue). While the number of hybrids analyzed is relatively low, it is clear that the effect of OCA-B, when expressed in the T lymphoma, does not approach that of OCA-B when expressed in the plasmacytoma before cell fusion (14% rescue, compared to 94% rescue, respectively). Like Oct-2, therefore, OCA-B appears to require a partner(s) within the plasmacytoma nuclear environment in order to efficiently maintain the genetic program of the plasmacyte. As we know that Oct-1 is present in both the parental lines and the hybrids (the Pou2f1 locus is not silenced), it is clear that OCA-B does not work with Oct-1 to mediate this function.

Figure 4.6. Western Blots of OCA-B transformants and Oct-2 -OCA-B transformants: OCA-B MPC11 transformants, OCA-B-T cell transformants and OCA-B-Oct-2-T cell transformants are shown. 50 ug of total cell lysates was loaded in an 8% SDS-PAGE gel and transferred into nitrocellulose. The respective antibodies were used to detect the presence of Oct-1 used for normalization purposes. Oct2 and flu-tagged OCA-B. Both BWOCA-B #5 and BWOCA-B #10 transformants were fused to the MP cell line. It is the characterization of the BWOCA-B#10 x MP fusion that is described in this work.

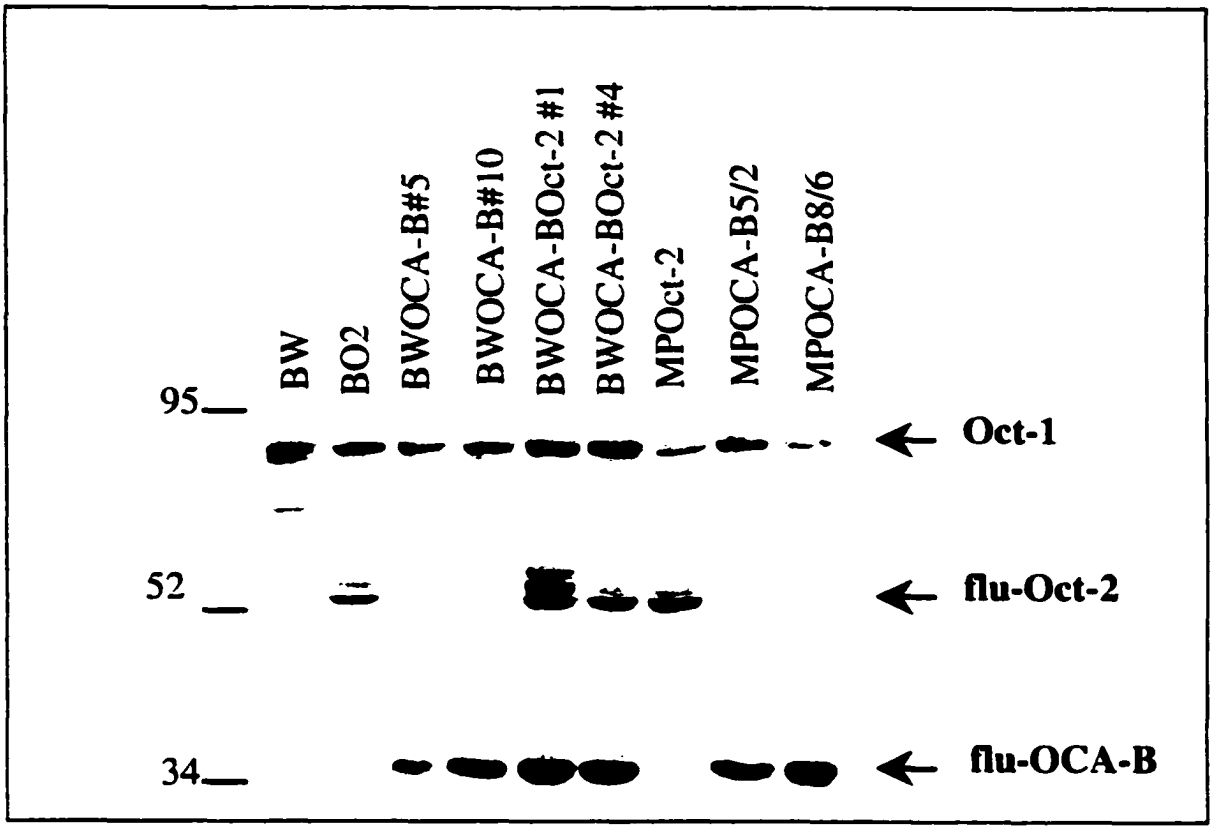
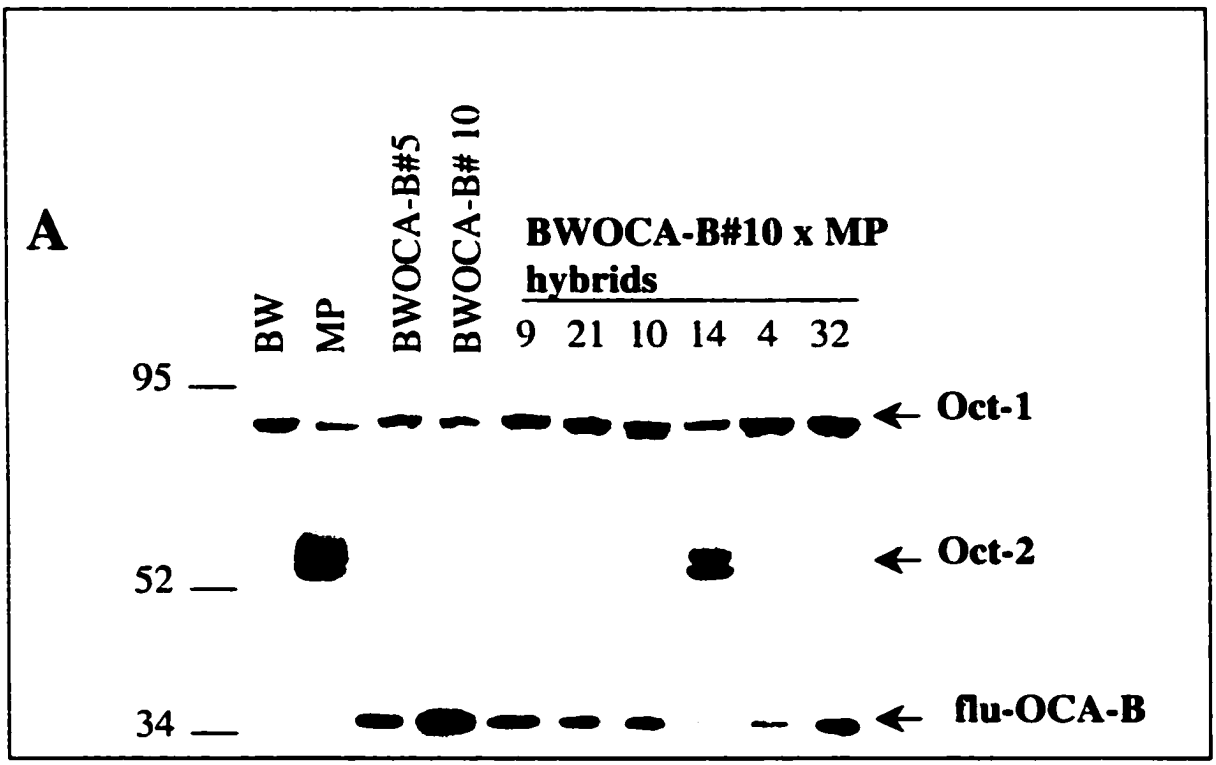
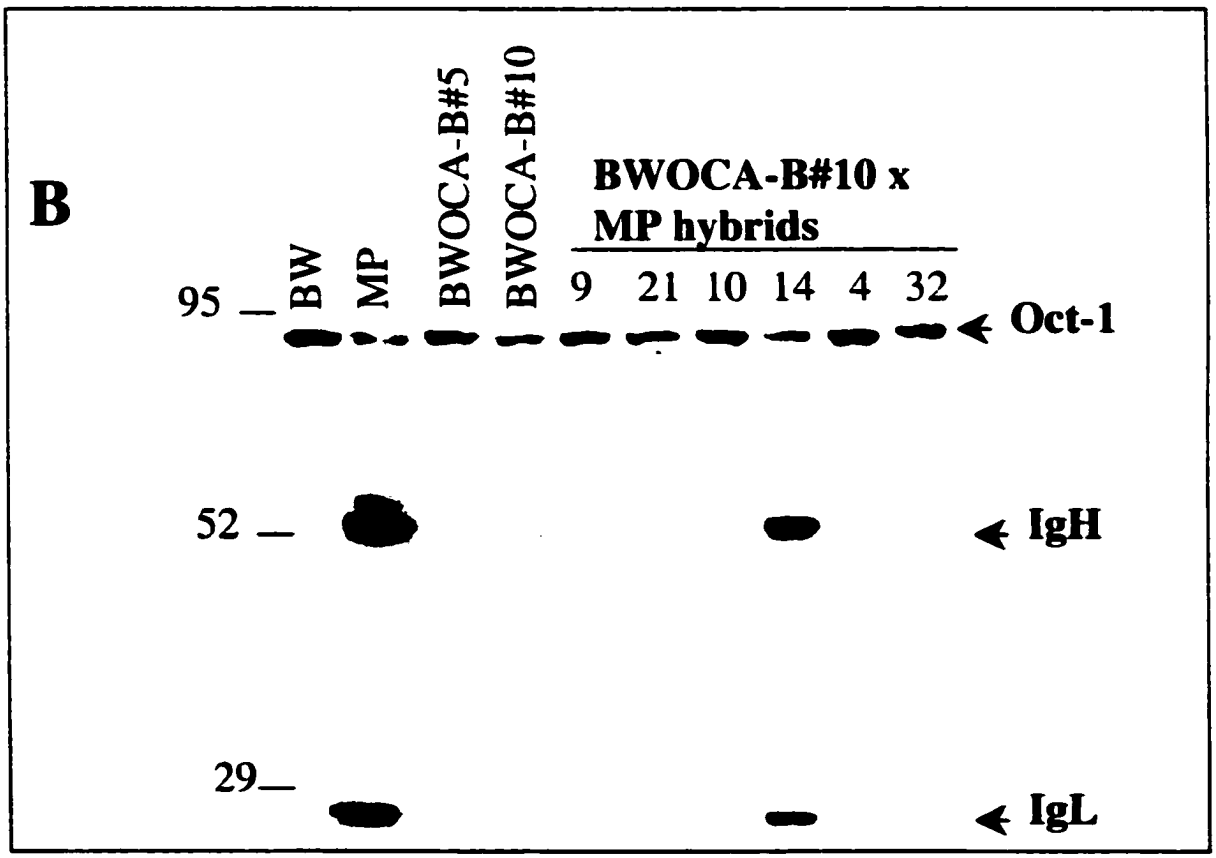


Figure 4.7. Endogenous mOct-2 and IgH/L expression are not rescued in BWOCA-B#10 x MP hybrids.

A) Western Blots of BWOCA-B#10 x MP hybrids. 50 ug of total cell lysates were size-fractionated in an 8% SDS-PAGE electrophoresis, transferred to nitrocellulose and probed with the respective antibodies to detect the presence of Oct-1 used for normalization purposes, Oct-2 and flu-tagged OCA-B.

B) Western Blots of BW OCA-B#10 x MP hybrids. Levels of Oct-1 and Ig heavy and light chain expressions are shown. 50 ug of total cell lysates were loaded in an 8 % SDS-PAGE gel and transferred into nitrocellulose. The respective antibodies were used to detect the presence of Oct-1 used for normalization purposes, and IgH/L chain expressions.





Simultaneous, constitutive expression of OCA-B and Oct-2 in the T lymphoma before fusion results in synergistic rescue of the Ig-secreting cell program

The BWOCA-B#10 clone was transfected with a flu-tagged Oct-2 expression vector (pCGN-Oct-2his, (28)) to produce clones that were expressing both flu-tagged OCA-B and flu-tagged Oct-2. A Western blot of nuclear extract from two such clones is shown in Figure 4.6 (BWOCA-BOct-2#1, BWOCA-BOct-2#4). Both transformants made equivalent amounts of OCA-B, while one (clone #1) made more Oct-2 than the other (clone #4). Notably, clone #4 made Oct-2 at a level similar to a BWOct-2 transformant (BO2) that we had previously shown gave rise to ~23% hybrids with an Ig-secreting cell phenotype. As described above. the original BWOCA-B#10 clone gave rise to ~14% hybrids with an Ig-secreting cell phenotype.

When clones BWOCA-BOct-2#1 and BWOCA-BOct-2#4 were fused to MPC11, four hybrids from the fusions with clone #1 and five hybrids from the fusions with clone #4 were selected for further analyses. All of these hybrids retained Ig genes from both parental lines, and all continued to express the flu-tagged OCA-B and Oct-2 transgenes (Figure 4.8). Western blots revealed that all of these hybrids produced both γ2b heavy chain and light chain (Figure 4. 9). One exception for Igκ was hybrid clone 7D.

EMSAs showed that all of the hybrids also produced the transcription factor PU.1 (Figure 4.10) and Northern blots showed that all produced J-chain mRNA, and consistent with the Western data, all produced γ2b mRNA (Figure 4.9). When

expressed together in the T lymphoma, flu-hOCA-B and flu-hOct-2 worked synergistically to effect rescue of the Ig-secreting cell phenotype in all hybrids. Together, in the T lymphoma, they behaved like either one alone in the plasmacytoma.

Interestingly, the level of expression of the rescued genes in the BWOCA-BOct2 x MPC11 hybrids very closely approximated that of the same genes in the plasmacytoma parent, MPC11. The Northern blots allowed quantitative comparisons of γ2b and J-chain mRNA levels (Figure 4.11). Unlike the MPOCA-B x BW5147 hybrids or MPOct-2 x BW5147 hybrids, these hybrids showed relatively little variation in Ig and J-chain expression levels, and these expression levels were not significantly different from those in MPC11.

Figure 4.8. Western blots of BWOCA-B-Oct-2 x MP hybrids: Levels of flu-OCA-B/flu-Oct-2 are shown. 50 ug of total cell lysates was loaded in an 8 % SDS-PAGE gel and transferred into nitrocellulose and an antibody to the flu-epitope contained N-terminal to the proteins was used to detect the flu-tagged OCA-B and Oct-2 proteins. An antibody to Oct-1 was used for normalization purposes.

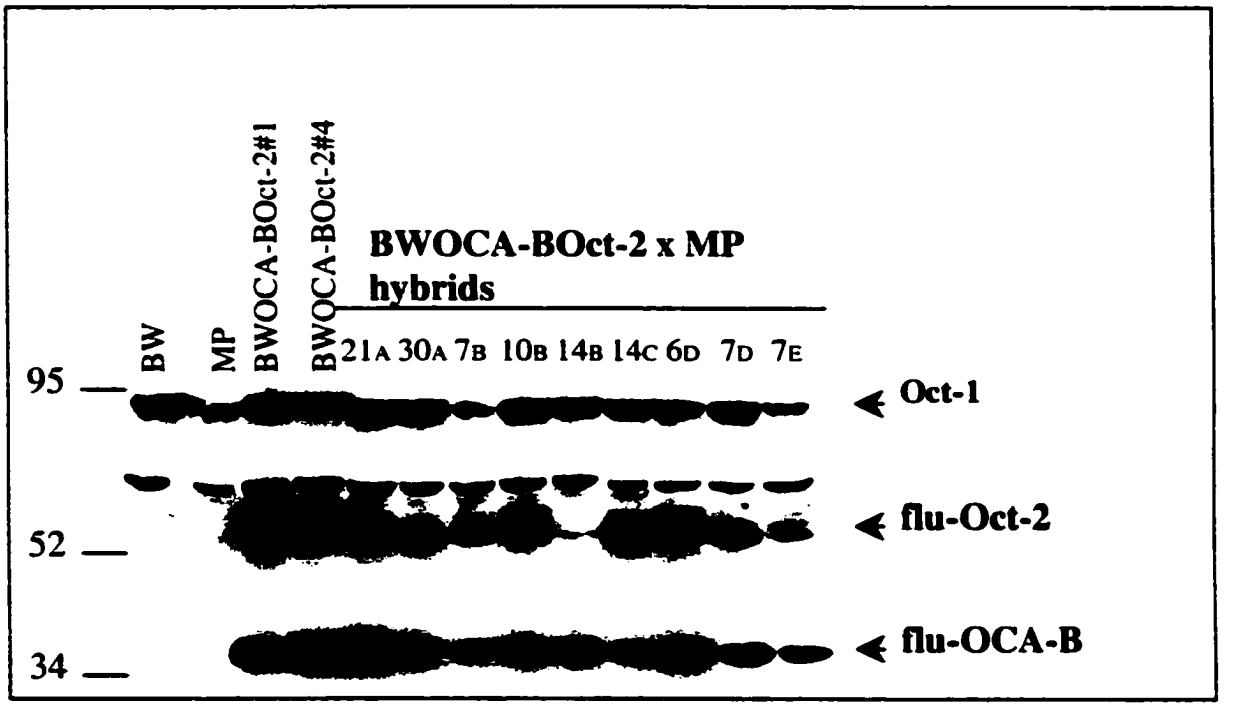


Figure 4.9. BWOCA-B-Oct-2 x MP hybrids rescue Immunoglobulin heavy and light chain expression. Western blots show levels of Oct-1 and Ig heavy and light chain expressions. 50 ug of total cell lysates were loaded in an 8% SDS-PAGE gel and transferred into nitrocellulose. The respective antibodies were used to detect the presence of Oct-1, used for normalization purposes, and IgH/L chain expressions.

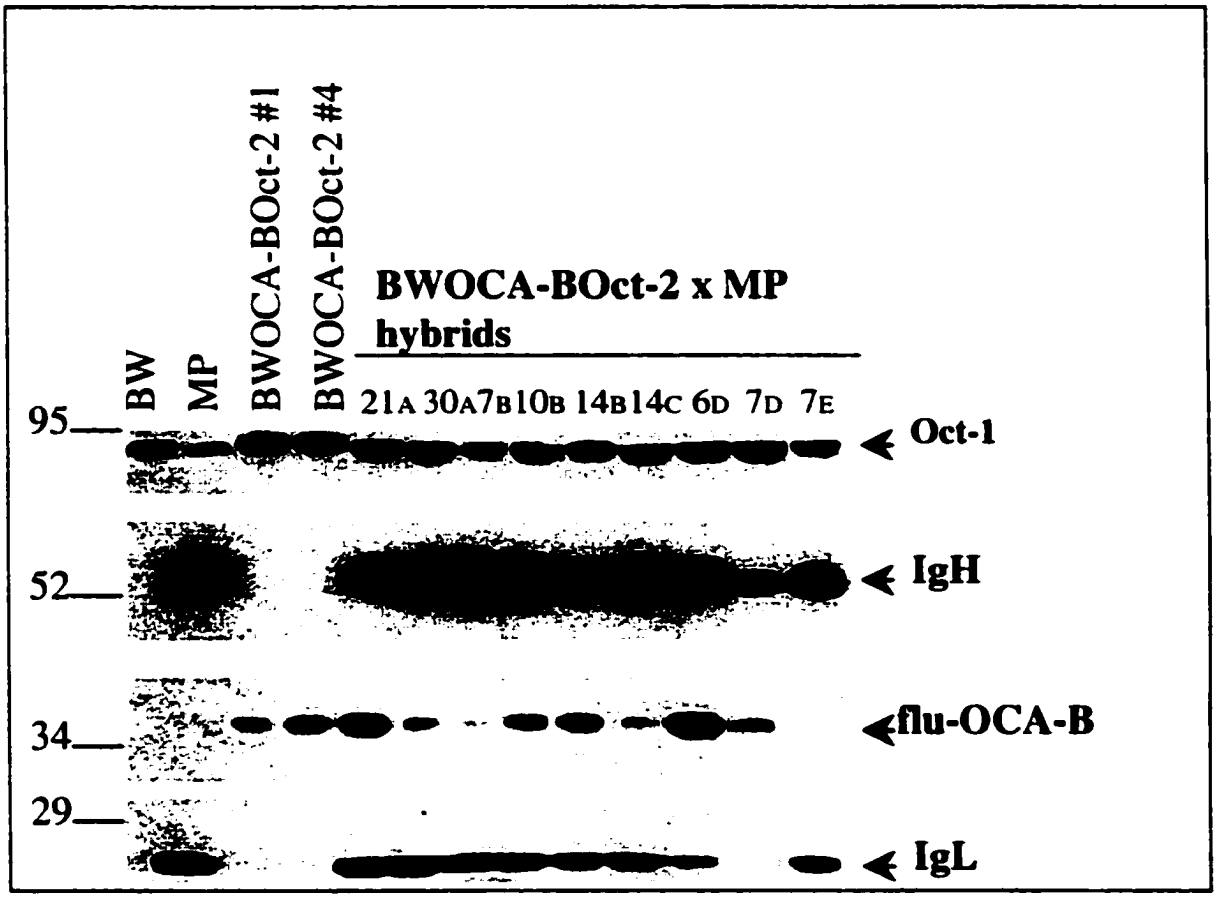


Figure 4.10. BWOCA-B-Oct-2 x MP hybrids rescue PU.1 expression. PU.1 EMSA was done for parental and selected hybrids. 10 ug of nuclear extracts were incubated with radiolabelled PU.box and size-fractionated. A supershift complex was shown when an antibody against the N-terminal domain domain was added to the extracts and probe.

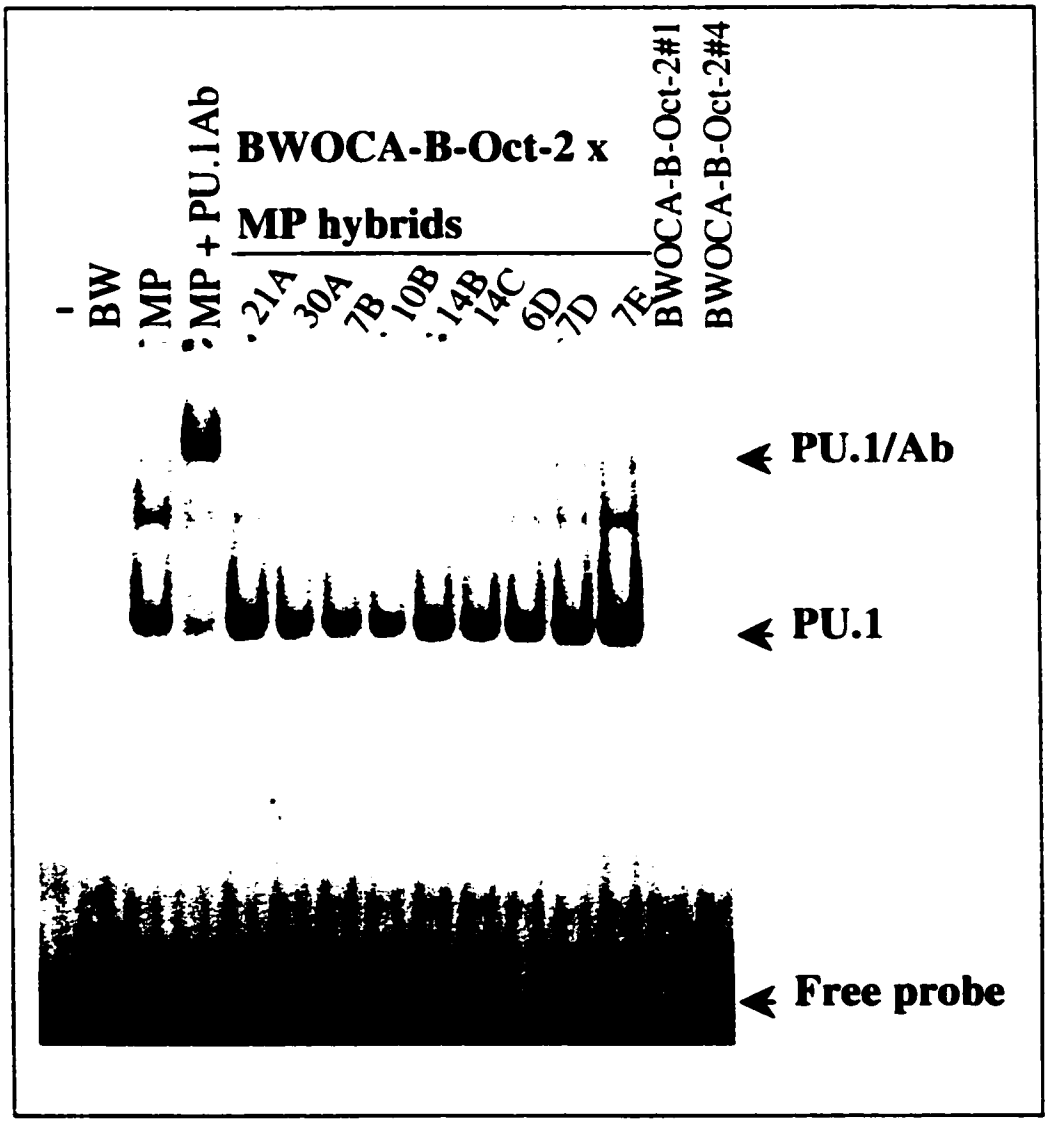
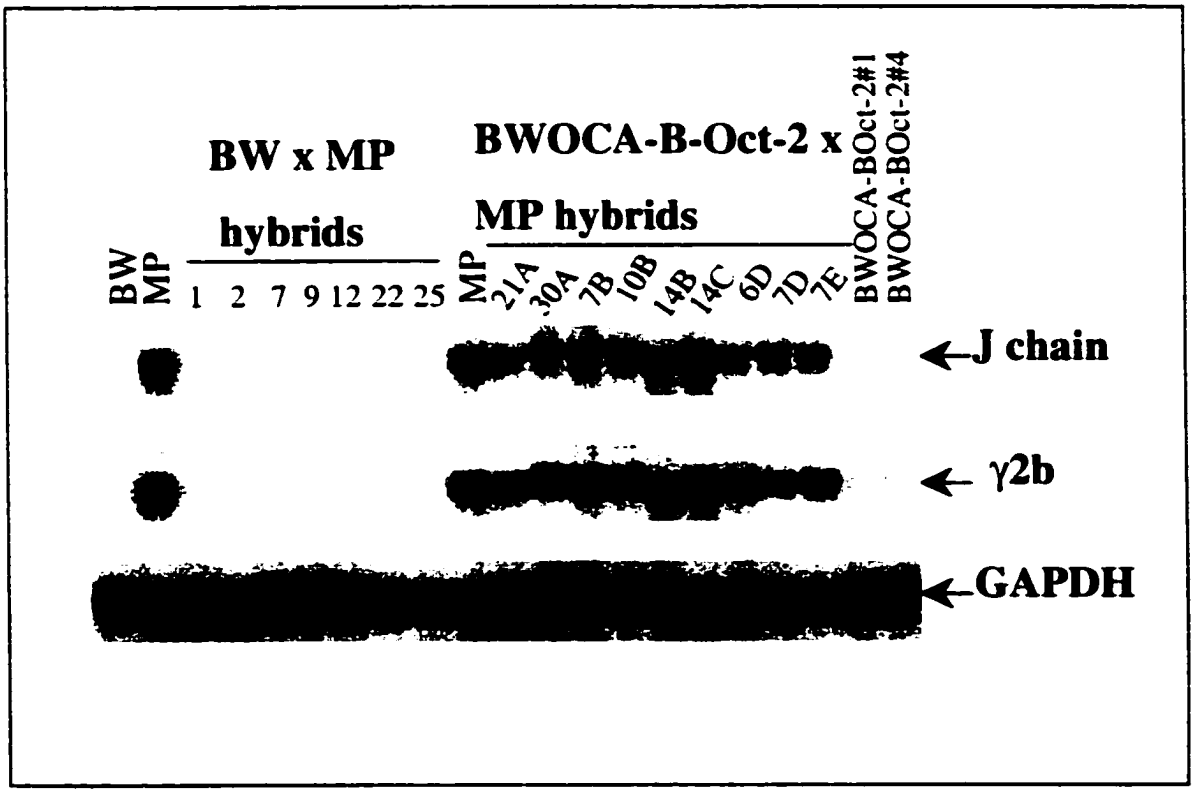


Figure 4.11. Comparison of BW x MP and BWOCA-BOct-2 x MP hybrids in levels of γ2b and J chain mRNA expression. Northern Blots of BW x MP selected hybrids and BWOCA-BOct-2 x hybrids. Total RNA was size-fractionated, transferred to nytran and probes sequentially with GAPDH, γ2b and J chain probes.



Discussion

We have learned that OCA-B behaves similar to Oct-2 in maintaining the B cell program in somatic cell fusions. Each factor can reciprocally regulate the expression of the other one and are therefore at the apex of the regulatory cascade in the Ig-secreting cell. The rescuing function of these factors is very exclusive as we have seen in Chapter 3. When OCA-B/ Oct-2 are expressed together in a T-lymphoma before fusion, the rescue is the same or better than when OCA-B is expressed in the myeloma before fusion. I found that OCA-B is the partner for Oct-2 in the rescuing events. Oct-1, the other octamer binding factor than was shown to interact with OCA-B, does not rescue in collaboration with OCA-B.

CHAPTER 5

Discussion/Future Studies

Immunoglobulin (Ig) gene transcription occurs only in B lineage cells. Critical to B cell development is the specific regulation of promoter and enhancer sequences within the IgH/L locus. One of the best characterized motifs within these regulatory sequences is the octamer motif which has been shown to be relevant has been shown by in vitro transcription, cell transfection and transgenic mice (103, 104, 105, 106, 107). Members of the POU family of proteins with homeodomains, Oct-1 and Oct-2, bind this well conserved octamer motif. Oct binding factor (OBF)-1, (also known as OCA-B or Bob1), a coactivator of octamer binding factors, enhances the activity of either Oct-1 or Oct-2 when bound to immunoglobulin promoters. While Oct-2 is predominantly B-cell specific, Oct-1 is ubiquitous and OCA-B is found exclusively in cells of the B lymphocyte lineage. Thus, OCA-B has the ability of turning an otherwise ubiquitous factor such as Oct-1 into a tissue specific factor when the OCA-B/Oct-1 complex acts on Ig promoters. Whereas the histone H2B promoter (ubiquitously active) is also dependent on octamer-binding factors, OCA-B does not participate in the transactivation of this gene. Although OCA-B does not directly bind DNA, it binds Oct-1 and Oct-2 augmenting the transcriptional activity of these octamer-binding factors when bound to specific promoter sequences. Therefore OCA-B is a B cell, promoter and factor specific coactivator.

It was hence believed that OCA-B is the factor responsible for lymphoid-restricted expression of Ig. However, knockout OCA-B experiments showed that this gene is not needed for the early stage of B cell development such as the initial

rearrangement, transcription of the Ig locus, and for the progression to the immature stage of B cells up to the IgM stage (27, 26, 101). However, B lymphocytes have dramatically reduced amounts of the non-IgM isotypes (e.g. IgG2a). This defect is due not because these B cells are unable to switch class (by class-switch recombination = CSR), but because the transcription rate of the IgH genes in these mutant cells appears to decrease after heavy-chain class-switching (27). Consistent with the defect in isotype production, $OCA^{-/-}B$ mice do not develop germinal centers and are unable to mount an immune response to T-dependent antigens.

The phenotype of OCA-$B^{-/-}$ mice suggested that another B cell factor would be responsible for Ig-specific expression in the early stages of B cell development. Similar knockout experiments also had already discarded Oct-2 as the other factor responsible for Ig-specific expression since early stages of B cell development were unaffected. Either knockout develops normal amounts of B cells yet with a defect in maturation. Since neither factor seems to be responsible for Ig-specific expression, one notion is that OCA-B and Oct-2 may be functionally redundant at early stages, yet both genes have unique functions later on. In Oct-$2^{-/-}$ mice, OCA-B interacts with Oct-1 to promote B-cell specific expression. In the OCA-$B^{-/-}$ mice, Oct-2 promotes B cell-specific expression. If OCA-B is redundant with Oct-2 then one or the other must be present to achieve B-cell restricted expression of Ig in developing B cells. Then double knockout experiments of both OCA-B and Oct-2 would yield a mouse with no B cells (unable to express Ig). When OCA-$B^{-/-}$Oct-$2^{-/-}$ mice were generated, however, early stages in B cell development were normal, demonstrating that B cell restricted expression of Ig is entirely independent

of both of these factors in developing B cells. These results suggested that a B cell coactivator other than OCA-B interacts with Oct-1 to promote B cell-specific activity at early stages of B cell development. The phenotype of these mice with respect to mature and activated B cells was more drastic than either single knockout revealing that in these later-stage cells, OCA-B and Oct-2 have some overlapping functions. It was found that levels of peripheral B cell pool were decreased even more when both genes were deleted. In the present studies, I provide additional information regarding Oct-2/OCA-B function at later stages of Ig-secreting cells. I show that OCA-B interacts exclusively with Oct-2 in the rescue of the Ig-secreting cell program using the somatic cell fusion approach.

Another less well conserved motif within the IgH/L locus is the EBS motif, bound by the ETS family of transcription factors. PU.1/ Spi-1 proto-oncogene is a member of this family and found predominantly in B cells and macrophages. The Spi-1 proto-oncogene (for SFFV proviral integration) was originally found as a consequence of integration of the spleen focus forming virus (SFFV) into the genome of erythroblasts. PU.1 was isolated while screening for a nuclear factor that binds the CCAAT sequence of the MHC class II I-Aβ gene promoter. It was later found to be identical to NF-JB, a nuclear protein that mediates the positive regulatory activity of the JB element of the J chain promoter (14) (99). PU.1 has also been implicated in the regulation of the Ig heavy and light chain genes and the genes encoding the signaling molecules associated with the Ig (mb-1 and B29) (45, 108, 109, 110, 111, 112). However, a direct role for PU.1 in B cell development and function has not been possible due to the lack of lymphoid progenitors in PU.1$^{-/-}$ mice. PU.1 expression is present throughout B cell development

suggesting roles for this factor at all stages.

In fusions between the MPC11 myeloma line and the T lymphoma BW5147 (as well as fibroblasts), B cell factors OCA-B, Oct-2 and PU.1 are silenced along with many myeloma-specific genes, including those encoding the immunoglobulin heavy and light chains and J chain (28, 98, 113). This phenotype was interpreted as a consequence of the loss of one or more essential positively-acting transcription factors (through T cell-mediating silencing). To our surprise, we found that when Oct-2 expression was preserved in these somatic cell fusions, Oct-2 was able to rescue expression of PU.1 and OCA-B as well as other B cell-specific genes. Oct-2 was the first B cell factor shown, therefore, to be essential to the preservation of the Ig-secreting cell's genetic program (28). It was then natural to ask whether other B cell factors, such as OCA-B and PU.1, behaved similarly to Oct-2 at this particular cell stage and could reciprocally regulate Oct-2's expression. OCA-B and PU.1, like Oct-2, are expressed in the Ig-secreting cell, MPC11, but not in the T lymphoma BW5147. The function of OCA-B and PU.1 was then investigated by introducing each one of the cDNAs in a form that cannot be subjected to silencing and then ask whether there was rescue of the plasmacyte-specific program after fusion. In this way, we studied the effects of transcription factors PU.1 and OCA-B on the expression of B cell-specific genes Oct-2, J chain and IgH/L. We learned from these studies where in relation to Oct-2 these factors lie in the hierarchy of B-cell factors acting at the Ig-secreting stage.

We thought that if either one of these factors had an effect on B cell genes, its

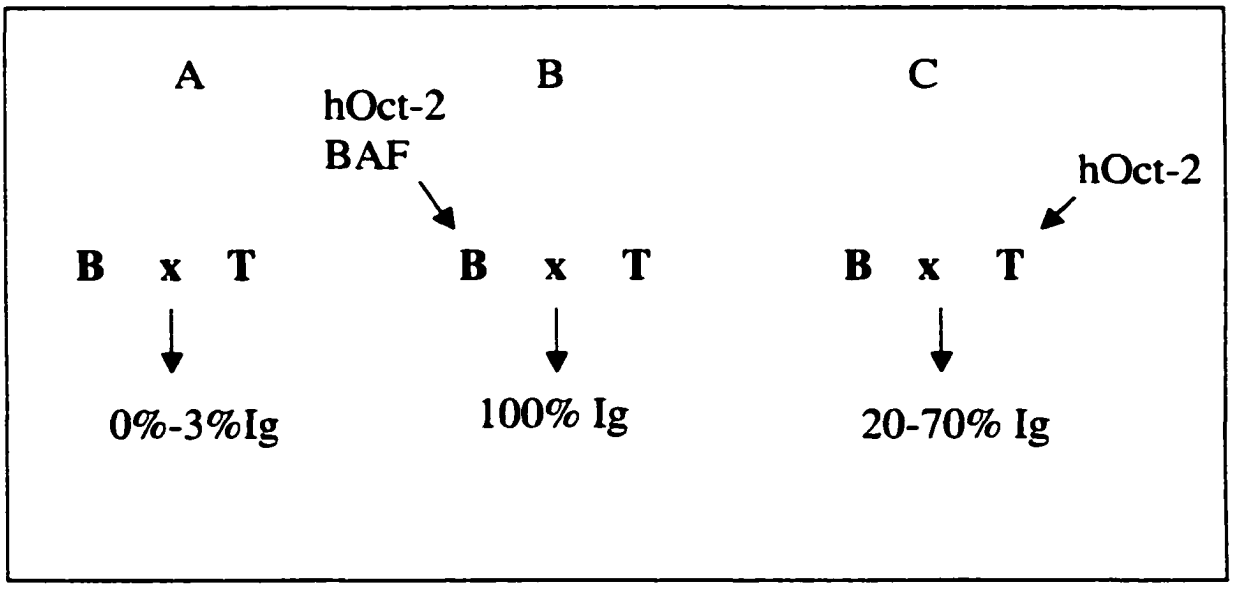
overexpression, using the somatic cell fusion approach would allow us to rescue at least one of these genes: Oct-2, PU.1, J chain, or IgH/L. Based on the somatic cell studies presented here, we established an essential role for OCA-B in the continued expression of tissue-specific genes specifically at the Ig-secreting cell stage. A subordinate role was attributed to PU.1 since it responded to OCA-B and Oct-2 but could not reciprocally regulate the genes encoding either one of these factors. Therefore, one can build a hierarchy of B cell factors where OCA-B and Oct-2 are at the apex of the regulatory chain in the Ig-secreting cell stage and PU.1 is downstream of OCA-B and Oct-2.

When OCA-B was preserved in the Ig-secreting cell line before fusion, there was a significant proportion of hybrids "escaping" myeloma-specific gene silencing. This percentage approached 94 %, a dramatically high percentage when compared to control fusions in which there is no rescue. Simply ensuring uninterrupted expression of OCA-B was enough to maintain the loci encoding immunoglobulin heavy and light chains, J chain, and mOct-2 and PU.1 in a transcriptionally active state. Without OCA-B or Oct-2, all of these loci fell silent. OCA-B is required then, for transcription of these myeloma-specific genes in Ig-secreting cells.

Somatic cell fusions studies done by Radomska et al. postulated that Oct-2 collaborates with a B cell associated factor, BAF (B Associated Factor), to rescue the plasmacyte-specific program (Figure 5.1B). This idea came forward due to the differential effects on B cell-specific expression when the Oct-2 vector was introduced into the myeloma cell or, alternatively, into the T cell previous to fusion (Figure 5.1B and

Figure 5.1C). In the former case, all hybrids expressed the plasmacyte program while the latter case, between 20-70% hybrids had silenced this program. In normal fusions, a very small percentage of rescue is observed (Figure 5.1A)

Figure 5.1.- Schematic representation of hybrids cell lines

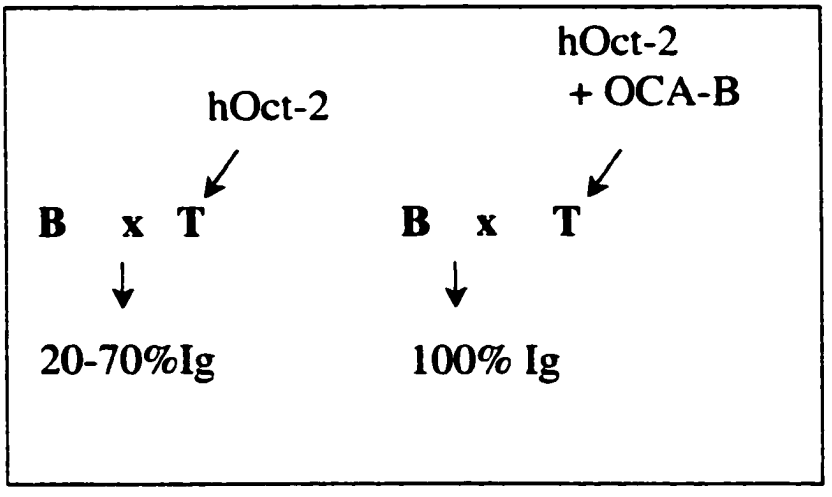


The same Oct-2 transgene was expressed in hybrids in both cases. Therefore, the only explanation for the different outcome was that expression of the Oct-2 transgene in the myeloma prior to fusion was qualitatively different from its expression in the T lymphoma prior to fusion. Either the T cell environment was handicapping Oct-2 or the myeloma environment was uniquely assisting Oct-2 function. It was hypothesized that Oct-2 and this unique factor called BAF associate before fusion and inhibit the activity of the T-cell derived repressor after fusion so that the B cell genetic program is favored.

Since OCA-B is a B cell restricted coactivator of octamer- binding factors, we wanted to find out if it might be the factor uniquely assisting Oct-2 in these cell fusions.

We reasoned if OCA-B is all that is needed to assist Oct-2 in the rescue of B cell-specific gene expression, overexpression of both factors in the T lymphoma before fusion would lead to 100% rescue in the resulting hybrids. This was indeed the case (Figure 5.2 D). When OCA-B and Oct-2 were introduced simultaneously into the T lymphoma, we observed synergy in the rescue to levels comparable to those seen expressing either factor alone in the MPC11 line (Figure 5.2 C).

Figure 5.2- Schematic representation of hybrid cell lines



As reported earlier for flu-Oct-2 (28, 102), expression of flu-OCA-B in MPC11 before fusion rescued numerous B cell-specific genes from T-lymphoma-mediated

silencing, but the levels of gene expression varied among hybrids. The levels of exogenous flu-hOCA-B (or flu-hOct-2) did not correlate with rescued gene expression levels, but all B cell genes were rescued coordinately. In flu-hOCA-B rescued hybrids, mOct-2, PU.1, IgH/L and J chain expression levels correlated in individual hybrids. In some hybrids expression of all four rescued genes was low, in others all were highly expressed (Figure 4.2, 4.3, 4.4, 4.5 Chapter 4). We reasoned that the difference in levels of rescue in individual hybrids was due to the fact that only one of the partner genes was maintained as a transgene, the amount of endogenous factor either OCA-B or Oct-2, was limiting (limiting the amount of functional complex). Consequently, the degree of rescue in each hybrid depends on this limiting endogenous factor. In the experiments where both transgenes (flu-hOct-2 and flu-OCA-B) were maintained, there were higher levels of functional complex available in all hybrids. From these experiments, it is clear that OCA-B together with Oct-2 rescued B cell expression to a greater extent in all hybrids than was achieved by either factor transgene by itself.

Based on the somatic cell fusion results presented here, OCA-B and Oct-2 have similar and essential roles at the Ig-secreting cell stage. Similar experiments with transcription factor PU.1, on the other hand, yielded very different results. Although OCA-B with Oct-2 were sufficient to rescue B cell expression, PU.1 was not sufficient to ensure maintenance of the B cell program since upon its preservation there was not rescue of J chain, IgH/L, mOct-2 or mOCA-B. Reciprocally, we reasoned that all genes tested PU.1, J chain and IgH/L were dependent on the expression of these two factors, OCA-B and Oct-2. However, these B cell genes were not dependent on PU.1 expression.

In conclusion, our somatic cell fusion studies OCA-B and Oct-2 factors were sufficient to get rescue of B cell-specific genes.

I already mentioned that in hybrids that maintained PU.1 expression there was no "escaping" from silencing of B cell-specific genes. This phenotype was similar when PU.1 was preserved either in the MPC11 or the BW5147 cell line before fusion. Possibly the lack of effects of PU.1 in B cell-specific expression is not due to the need of a coactivator of PU.1 that is missing from the fusions. A most likely explanation is that PU.1 is not sufficient to rescue, and there are other B cell factors missing that are responsible for B cell-specific expression. Importantly, in hybrids that maintained PU.1 expression, OCA-B and Oct-2 expression were turned off. It is reasonable to conclude that the lack of these two factors was the cause of the silencing of B cell expression in these hybrids.

One of the B cell genes turned off was the polymerizing protein J chain that is normally expressed in MPC11. Two factors, PU.1 and the myocyte enhancer factor 2 (MEF2)- related nuclear factor B-MEF2, have been shown to transactivate the J chain promoter (99, 114). Although members of the MEF2 family have been described to be muscle-specific factors, B-MEF2 is specifically expressed at all B cell stages with an upregulation in activated B cells which correlates with J chain expression. I would expect that this particular factor, B-MEF2, due to its tissue-specificity, should be turned off as other B cell genes are in these fusions and not able to activate the J chain promoter. The J chain promoter also contains an octamer motif which in these fusions can only be

bound by Oct-1 since Oct-2 is off. I do not think that Oct-1 can participate in activating this promoter since OCA-B is not on (115). My conclusion is, therefore, that the J chain promoter, normally activated through binding of PU.1, B-MEF2, and Oct-1 or Oct-2/OCA-B, now has only PU.1 available in these cell hybrids. Apparently, PU.1 alone is not sufficient to sustain activity of this promoter.

We can reason similarly for the inability of PU.1 alone to sustain Ig gene expression in the hybrids. PU.1 has been implicated to the activation of some Ig promoters (116) and in the activity of the Ig heavy chain (E μ) and Ig light chain (k and λ) enhancers in transient transfection assays (45, 108, 109). Particularly, mutation of the μB element within an Eμ enhancer subfragment (μB is bound by PU.1) reduced completely the activity of this enhancer subfragment in transient expression experiments. Moreover, the MPC11 cell line used in our studies has a Vκ promoter that contains an imperfect octamer and a pyrimidine motif bound by octamer-binding proteins and PU.1, respectively. Also the 3' enhancer regions of the IgH locus known to become active in surface IgM^+ B cells (12, 117,118) contain multiple octamer motifs, as well as other binding sites for proteins such as ETS and the E-box binding proteins. It is quite reasonable to assume that due to the presence of numerous octamer sites, these are essential to enhancer function and the lack of Oct-2 and its necessary cofactor, OCA-B explains for this IgH gene's silence.

I would expect that the endogenous PU.1 will not be rescued in hybrids expressing ectopic PU.1. The PU.1 promoter contains an octamer, PU.1 and Sp1 binding

sites (95). In B cells, the octamer was shown to be the most important functional motif in the PU.1 promoter by mutational analysis. In myeloid cells, the PU.1 site was shown to be the most important, and PU.1 was shown to activate its own promoter in a positive autoregulatory loop (95). OCA-B with octamer-binding factors were shown to transactivate the PU.1 promoter in transient experiments (95). Since Oct-2 and OCA-B are not on and the octamer motif is critical to promoter function in B lineage cells, I expect that endogenous PU.1 is silent in these hybrids. We can discard the participation of Oct-1 in the hybrids since its collaborator (OCA-B) is not on. As explained in the result section, we were not able to test this hypothesis directly because the hybrids overexpressing PU.1 did not maintain the exogenous PU.1 gene after several passages in the resistant drug media. While PU.1 may be necessary for expression for a particular gene at the Ig-secreting cell stage, it is not sufficient to rescue B cell gene expression in somatic cell fusions. Rather, Oct-2 and OCA-B must be present to ensure rescue.

As a consequence of the OCA-B fusion experiments, it was found that, contrary to many published observations, OCA-B does not collaborate with Oct-1 in the maintenance of B cell-specific expression. The percent hybrids rescued when only OCA-B was expressed in the BW parental cell line before fusion was 14 % compared to 100% when both OCA-B and Oct-2 were maintained in this parental line. This is actually the first instance in which OCA-B has been shown to collaborate exclusively with Oct-2 in an experiment where these transcription factors are acting on natural promoter targets (endogenous Ig, J-chain genes). The other report showing the exclusive interaction of Oct-2 and OCA-B was in the full activation of the 3' enhancer elements by an altered

DNA-binding specificity system (60).

Our somatic cell fusion results agreed with a crucial role for OCA-B at the Ig-secreting cell stage, consistent with the phenotype of OCA-$B^{-/-}$ mice. It was similarly concluded from the phenotype of B cells from Oct-$2^{-/-}$ mice and from the results of cell fusion experiments that Oct-2 served a unique and essential function at or after B cell activation and in Ig-secreting cells (21, 23, 24, 28). Maintaining the expression of either one of these factors can rescue the B cell phenotype. Most importantly, each factor can reciprocally regulate the expression of the other. We showed that the coactivator, OCA-B participates with Oct-2 in rescue of the B cell phenotype. What makes Oct-2/OCA-B so unique to a B cell is still unknown since early stages of B cell development can occur without Oct-2 or OCA-B. Yet definitely, late stages of B cell development require both OCA-B and Oct-2.

The conclusion is that OCA-B and Oct-2 are the only tissue-specific factors necessary for the rescue of the B cell-specific program. Both factors were mutually dependent and together they give rescue of the plasmacyte program to levels comparable to MPC11. Recent published results on structure-function analyses on Oct-2/Oct-1 showed that the majority of the rescue activity by Oct-2 in cell fusion assays was concentrated at its C-terminal end (102). This evidence, together with the stronger effects observed when Oct-2 was expressed constitutively in the myeloma rather than in the T cell, implied that Oct-2 needs a tissue-specific cofactor and Oct-2 needs its C-terminal domain in order to rescue the plasmacyte program. One possibility was that these two

issues were related, suggesting that the C-terminal domain is the domain that works with the tissue-specific factor. This suggested that a different coactivator other than OCA-B, is responsible for the high level of rescue since OCA-B tethers octamer-binding factors through the POU domain of Oct-2/ Oct-1(not the C-terminal domain). On the other hand, these two issues may be unrelated such that the transcription cofactor required by Oct-2 is OCA-B and Oct-2' s C-terminal domain is required because of its interactions with other, perhaps not tissue-specific transcription factors. The latter might be factors necessary for the basal transcriptional machinery such as PolII and TFIID. I suggest this latter explanation to be the most accurate for two reasons. First, from our experiments, OCA-B with Oct-2 gave 100% rescue of the B cell phenotype, a similar phenotype obtained when Oct-2 alone is preserved in the Ig-secreting cell, suggesting that we have supplied Oct-2 with its necessary cofactor and it is OCA-B. Second, original studies on the transactivation potential of Oct-2 showed the C-terminal domain as one of its important activation domains (119, 120, 121). The other is the N-terminal domain of Oct-2. The fact that OCA-B needs another domain in addition to the POU-domain of Oct-2 to fully transactivate does not contradict with this model since OCA-B could be using the N-terminal domain of Oct-2 leaving Oct-2' s C-terminal domain to interact with the basal transcriptional machinery (100).

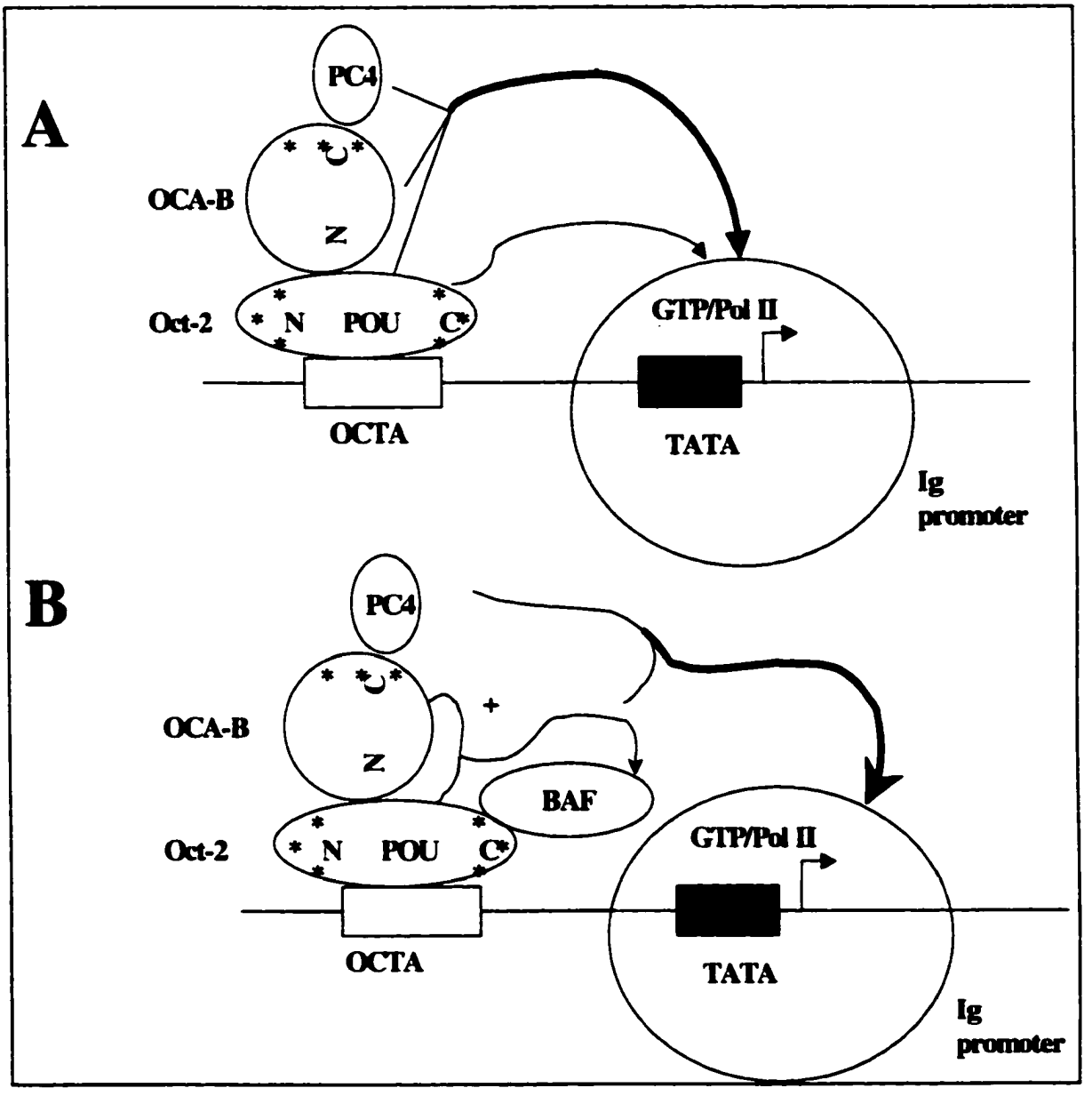
In another model, OCA-B and Oct-2 could be maintaining transcription by activating other B cell gene (s). We can suggest that overexpression of OCA-B and Oct-2 in the BW cell line can induce another B cell factor, BAF, that is able to interact with the C-terminal domain of Oct-2. This would suggest that there is a hierarchy of action

where Oct-2 and OCA-B turn on a set of genes including BAF and OCA-B/Oct-2/BAF then turn on other sets of genes. We would then expect that in the transformant overexpressing both OCA-B and Oct-2, this factor BAF is also on.

Figure 5.3- Model of activation mechanism for Ig promoter. Activation domains of Oct-2 and OCA-B are shown by asterisks.

A) According to the model, N-terminal domain of OCA-B is recruited to the octamer motif of the Ig promoter through the POU domain of Oct-2. The C-terminal domain or activation domain of OCA-B interacts with general coactivator PC4 (122) thus OCA-B serves as an adaptor between Oct-2 and the basal transcription machinery. Since OCA-B needs another domain for full transactivation, a possibility that the N-terminal domain of OCA-B interacts with the N-terminal domain of Oct-2 is shown. The C-terminal transactivation domain of Oct-2 may be needed to interact with factors needed for basal transcription machinery.

B) In this model, OCA-B and Oct-2 induced the activity of a factor BAF and this BAF and the C-terminal domain or activation domain of OCA-B interacts with general coactivator PC4 (122) to promote immunoglobulin gene transcription.



OCA-B appears to function in collaboration with Oct-2 but also with other general coactivators. It has been shown that USA general coactivators such as PC4 and PC2, potentiate the activity of an IgH reporter gene with OCA-B and Oct-1/Oct-2 (122) (USA: upstream stimulatory activity is a crude fraction that strongly stimulated transcription by activators. Fractionation of USA led to the discovery of several positive co-factors; one of them is PC4). In this way, we can view OCA-B, as a class of eukaryotic factors that work in an adaptor-like fashion by creating a bridge between octamer-site bound transcription factors to some component of the basal transcriptional machine. Consistent with this, pull-down experiments using GST-OCA-B have shown association of OCA-B with the general transcription factors, TBP, TFIIA, TFIIB and TAFII105, a TFIID-associated factor, and RNA polymerase II (123,124, 61, 125).

The somatic cell fusion experiments pointed out a unique requirement for OCA-B and Oct-2 in regulating the B cell genetic program at the Ig-secreting cell stage. We can envision that OCA-B and Oct-2 become important regulators of the 3' enhancers of the IgH locus since the enhancers become activated at later stages (12, 117, 118). This idea suggests that OCA-B and Oct-2 may have a role in activating octamer-dependent enhancer elements, contrary to much of the published literature on OCA-B that stated that this factor could only work at promoter-proximal octamer sites with either one of the octamer-binding proteins, Oct-1 or Oct-2. In one such study, it was suggested that a different coactivator worked with the C-terminal domain of Oct-2 at distal-enhancer positions (126, 61, 127, 128, 129). There are other recent reports that are consistent with the notion that OCA-B regulates activity of the 3' enhancers elements. Transient

experiments were done, using reporter constructs with a minimal LBK promoter (liver/bone/kidney alkaline phosphatase) in combinations with the Eμ enhancer or the 3' enhancer elements. These constructs were introduced into spleens from normal and OCA-$B^{-/-}$ mice. When spleen cells from normal mice were used, IL4 and CD40 stimulation led to a dramatic increase in reporter gene expression. This increase was seen with both the Eμ and with the 3' enhancers genes. When OCA-$B^{-/-}$ spleen cells were used, there was no IL4 or CD40 induced increase in reporter gene expression when the 3' IgH enhancers were used, yet there was induction when the Eμ □enhancer was present. OCA-B is required, therefore, for T cell- mediated simulation of the 3' IgH enhancers (62).

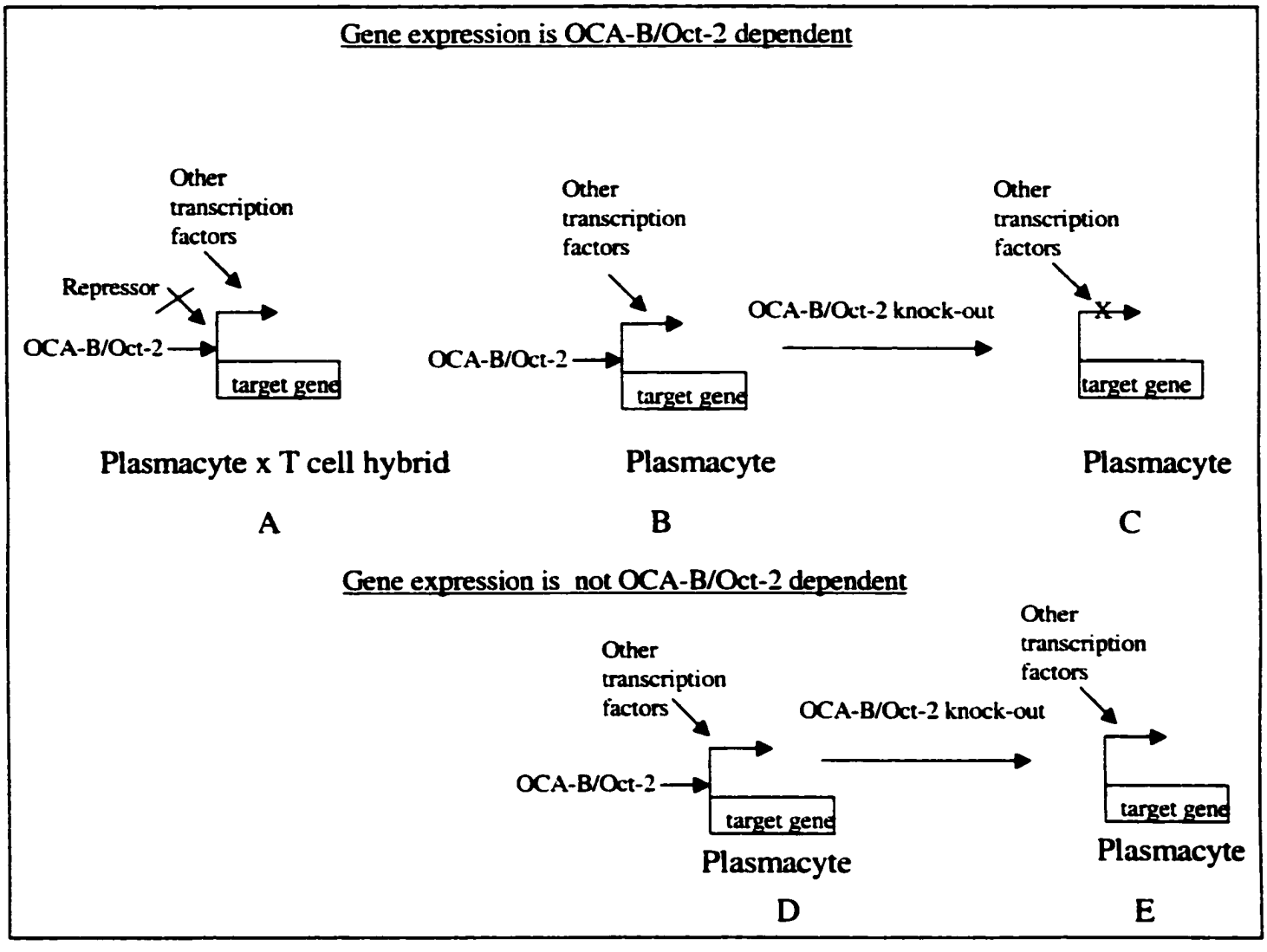
The other evidence is the establishment of an enhancer reporter system that is responsive only to Oct-2 protein by using an altered DNA-binding specificity mutant of Oct-2. It was found that only Oct-2 (and not Oct-1) directly regulates the 3' enhancer elements with OCA-B in transient transfection assays (60).

We have mainly dealt with the notion that in the somatic cell fusion studies there are dominant transcription factors responsible for maintenance of the B cell genes. We have addressed the issue that upon preserving the factor that positively activates the B cell program, we maintain B cell-specific expression. That was the case for Oct-2 and OCA-B factors but not for PU.1. Therefore, I predict that the loss of the OCA-B and Oct-2 but not of PU.1in Ig-secreting cells would have pronounced effects on Ig expression. However, since in the fusions there are dominant transcription factors that inactivate or "repress" the target genes, it is possible that Oct-2/OCA-B are required in

order to inactivate such repressor factors, not in order to drive expression of B-cell specific factors. If this were true, loss of OCA-B/Oct-2 in Ig-secreting cells (where there is no T cell-derived repressors) might have no effect on expression. In order to conclusively know what Oct-2 or OCA-B are doing at the Ig-secreting cell stage, we need to do targeting mutation of both genes at this cell stage (Figure 5.5).

We think that the mechanism by which OCA-B with Oct-2 effect the rescue is by competively inhibiting the repressor (s) from binding the target B cell genes. In the case of the PU.1 hybrids, the repressor is dominant to PU.1 positive action. Experimental attempts to identify the T cell-derived repressor (s) have been unsuccessful (130). Reintroduction of Oct-2 into hybrids was not sufficient to rescue plasmacyte gene expression. Therefore, the conclusion was that Oct-2 does not have a role in the initial activation of the Ig and other B cell genes (28). We do not know whether OCA-B and Oct-2, if reintroduced into an Ig-silenced hybrids would reactivate the locus. If so, then we could definitively conclude that OCA-B and Oct-2 were able to activate B cell specific expression. If the answer were no, then we could only say that they were responsible for the maintenance of the program.

Figure 5.4. Schematic results obtained when OCAB/Oct-2 are overexpressed in hybrids. Since in fusions, a T-mediating activity or repressor may influence the outcome of our somatic cell experiments, we need to ask whether in physiological circumstances, OCA-B/Oct-2 may be essential to Ig-expression. In A, OCA-B/Oct-2 rescue expression of the target gene in plasmacyte x T cell. The repressor is not able to bind to target gene. In B, if the OCA-B/Oct-2 complex is needed for target gene expression in the plasmacyte, then upon knockout of these genes, there would be no expression of target gene afterwards. If gene expression is not OCA-B/Oct-2 dependent (D and E), then upon knockout of OCA-B/Oct-2, target gene expression would continue.



The fact that OCA-B with Oct-2 showed such a dramatic effect on the expression of B cell genes, together with the fact that PU.1 did not behave equivalently to Oct-2 allowed us to establish a hierarchy of action of B cell factors at the Ig-secreting cell stage. PU.1 was not alone in this hierarchy since a close relative to Oct-2, Oct-1, ubiquitously expressed, was also unable to rescue plasmacyte-gene expression (102).

It has been previously shown that DNA methylation is correlated with a lack of gene expression and a closed chromatin structure (131). On the other hand, an open chromatin configuration suggested a gene that was being transcribed. In the case of MPC11 used in our fusion studies, the IgH-derived locus was transcribed and this locus was unmethylated. Characterization of the expressed IgH gene (γ2b) of MPC11 in MPC11 x BW5147 hybrids revealed that if was both silenced and methylated, whereas the IgH loci of BW5147 were methylated before and after fusion (75). In those exceptional hybrids that expressed Ig, the MPC11-derived IgH locus remained unmethylated, and the BW5147-derived IgH locus lost methylation. Another indicator for opening of the chromatin in a B-cell pathway is the appearance of sterile RNAs that emanate from the J-Cμ intron (132, 133). We then can look for the presence of these sterile μ transcripts that correlate with the opening of the IgH locus. Future experiments will be required to look at those hybrids that escaped silencing for changes in methylation state and the presence of sterile μ transcripts. We would expect that in the hybrids that showed rescue (e.g. T lymphoma overexpressing OCA-B and Oct-2 factors simultaneously) there is lack of methylation within both the plasmacytoma and T lymphoma –derived Ig loci and the presence of sterile μ transcripts emanating from the T

cell-derived locus. Whereas in hybrids using the T lymphoma expressing either OCA-B or Oct-2 or PU.1, there is not rescue of Ig2b or J chain, Ig/L, PU.1. I would expect that there would not be any μ transcripts. These results should be equivalent to those observed in control fusions.

OCA-B and Oct-2 together must not be all that is needed for a BW cell to become a B cell since overexpression of these factors did not rescue PU.1 in the BW-OCA-B-Oct-2 transformants. These transformants did not show a PU.1 binding DNA complex by gel shift assay. However, preliminary experiments done in our laboratory showed that the BW cell line expressing both OCA-B and Oct-2 cDNAs was able to drive a VH promoter suggesting that both B-cell factors are sufficient to drive an otherwise tissue-specific VH promoter (Yi Yung, Eckhardt, unpublished results). The latter results suggest it would be interesting to look for a correlation between sterile μ transcripts and the presence of both factors. The presence of sterile μ transcripts is thought to precede rearrangement of the IgH heavy chain locus. Therefore if OCA-B and Oct-2 have a role in opening the chromatin surrounding the IgH locus, we would expect the presence of the sterile transcripts. Transient transfection into fibroblasts were done with OCA-B, Oct-2 and PU.1 factors, tested individually. There was no correlation between sterile μ transcript and the overexpression of these factors. The E2A proteins were the only factors able to show such a correlation (54). I predict we may see a correlation when I test the combination of OCA-B and Oct-2 in these transfections. I suggest that OCA-B and Oct-2 can induce formation of the BCF1 complex (composed of homodimers of E2A proteins) which in turn can induce the activity of the IgH locus (measured in a T cell as

sterile μ □transcripts). BCF1 complex was reported when the Oct-2 gene alone was preserved in the BW cell line. When E2A proteins were introduced into fibroblasts, BCF1 was formed and sterile μ transcripts were produced.

Overall, our aim was to understand the role of tissue-specific factors, OCA-B, Oct-2, and PU.1 at the Ig-secreting cell stage using the somatic cell fusion approach. We could apply the same approach to other stages of B cell development and obtain a more complete picture on the effects of these B cell factors throughout B cell development. This technique could be extremely helpful since most knockout experiments cannot get past a specified stage of development. Sometimes, as in the case of PU.1, we cannot even obtain any progenitors to the lymphoid or to the myeloid lineages. In some other cases, the knockout result is lethal to the mice. One cannot analyze the phenotype unless one uses adoptive transfer of fetal liver knockout cells into irradiated hosts or more specifically uses the RAG complementation assay. This would ensure that lymphocytes are derived from the knockout ES stem cells. Even in those cases, we cannot get past a particular stage. In preliminary studies, we have already explored the role of Oct-2 at the pre-B cell stage. When a pre-B cell line was fused to the BW5147 T lymphoma, we saw transcriptional silencing of the pre-B cell-derived μ gene. Results showed that the presence of hOct-2 before fusion did not rescue the μ gene from silencing (unpublished observations). The impact of hOct-2 at this pre-B cell stage is much different than at the Ig-secreting cell stage, consistent with a role for Oct-2 at later stages of B cell development. Similarly, we could use the same PU.1 vector used in these studies to see if there is an effect on B cell-specific expression at this pre-B cell stage. This would be

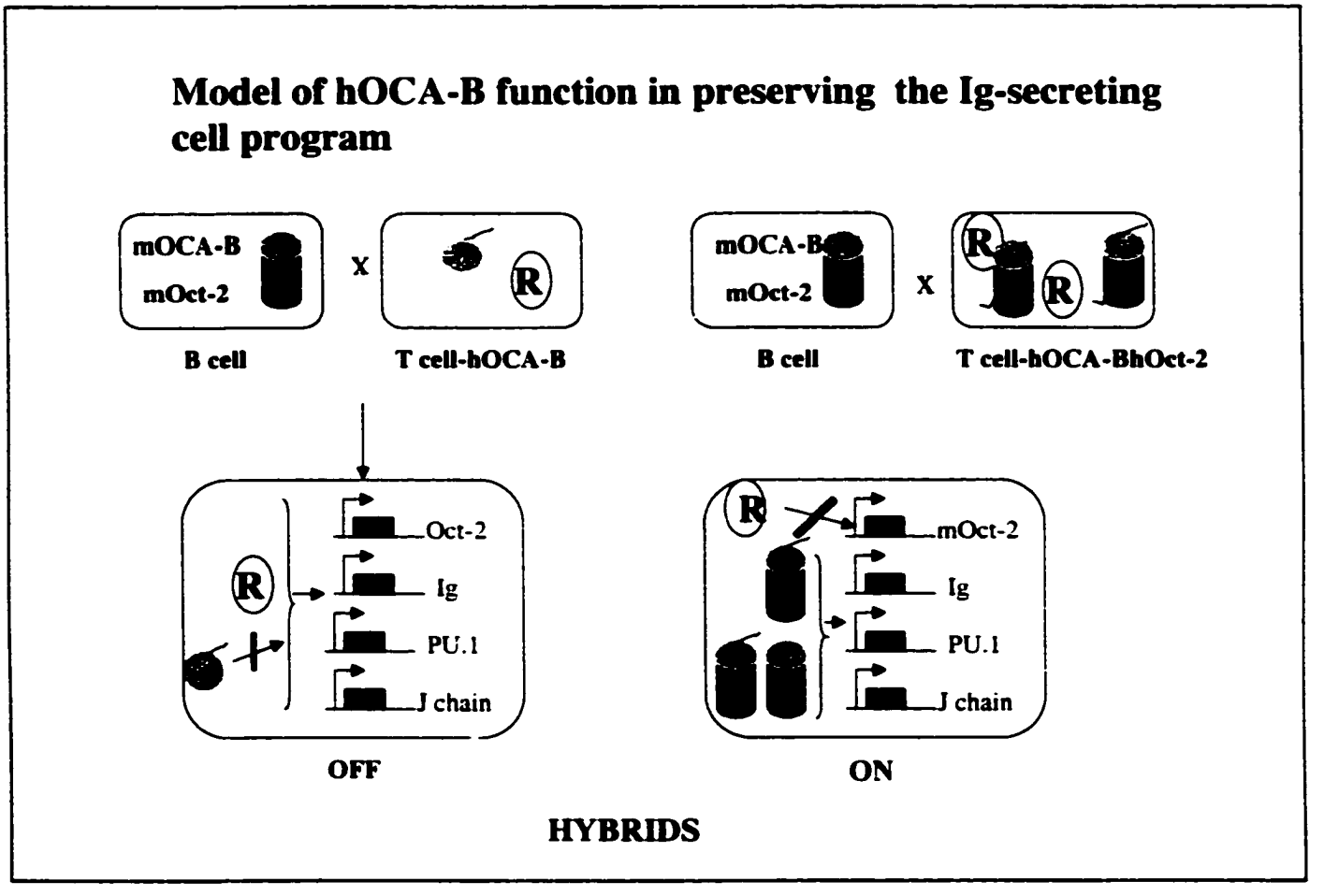
very informative since we are unable to obtain progenitors in PU.1$^{-/-}$ mice. We could also express the OCA-B vector and expect a phenotype similar to that in the Oct-2 experiments (that is, no effect on Ig gene expression upon overexpression of OCA-B at the pre-B cell stage). We might expect to see, therefore, a change in the hierarchy of B cell factors regulating B cell gene expression when using a pre-B cell as compared to an Ig-secreting cell.

The Ig-secreting cell is the effector cell in an immune response. Therefore, it is crucial to understand the role of B cell factors that are present at this mature state. Our studies have shown that OCA-B and Oct-2 are the necessary factors for expression of the Ig gene at the plasma cell stage. One would expect that our studies could provide support to understand the aetiology of a particular disease. Consistent with this point, recent clinical studies on classical Hodgkin's disease (cHD) have correlated the lack of immunoglobulin expression to the lack of OCA-B and Oct-2 (134, 135). Hodgkin's disease is a lymphoproliferative disease of predominantly B-cell origin where the B cells are not able to transcribe immunoglobulin despite the presence of rearranged immunoglobulin genes. The investigators are sure that the lack of expression of immunoglobulin is not due to the presence of crippled Ig genes since the immunoglobulin gene coding capacity is intact.

These somatic cell fusion studies have allowed us to set up a model by which OCA-B functions in preserving the phenotype of the Ig-secreting cell (Figure 5.5). In a B cell, endogenous murine OCA-B and Oct-2 are turned off upon fusion to a T cell that

expresses exogenous OCA-B. The presence of a repressor is shown in the T cell. Upon fusion, only exogenous OCA-B is expressed. It is not sufficient, however, to rescue all B cell genes. When similar fusions are done but now OCA-B and Oct-2 are expressed in the T cell before fusion, then all B-cell tissue-specific genes are turned on. The conclusion from these experiments are that OCA-B and Oct-2 are sufficient to maintain the phenotype of the Ig-secreting B cell.

Figure 5.5: Model of hOCA-B function in preserving the Ig-secreting cell program. Upon fusion of a T cell overexpressing the OCA-B gene with a B cell, all tissue-specific genes are turned off. The repressor takes over the transcriptional control of the genes and silenced them all. However when both OCA-B and Oct-2 are maintained, then all tissue-specific genes are turned on. Moreover endogenous OCA-B and Oct-2 are not turned off and there is more functional complex for rescue. The repressor in this case does not have control of these genes. In conclusion, OCA-B and Oct-2 are sufficient to maintain the genetic program of an Ig-secreting cell and they prevent the transcriptional silencing that is typical between cell fusions.

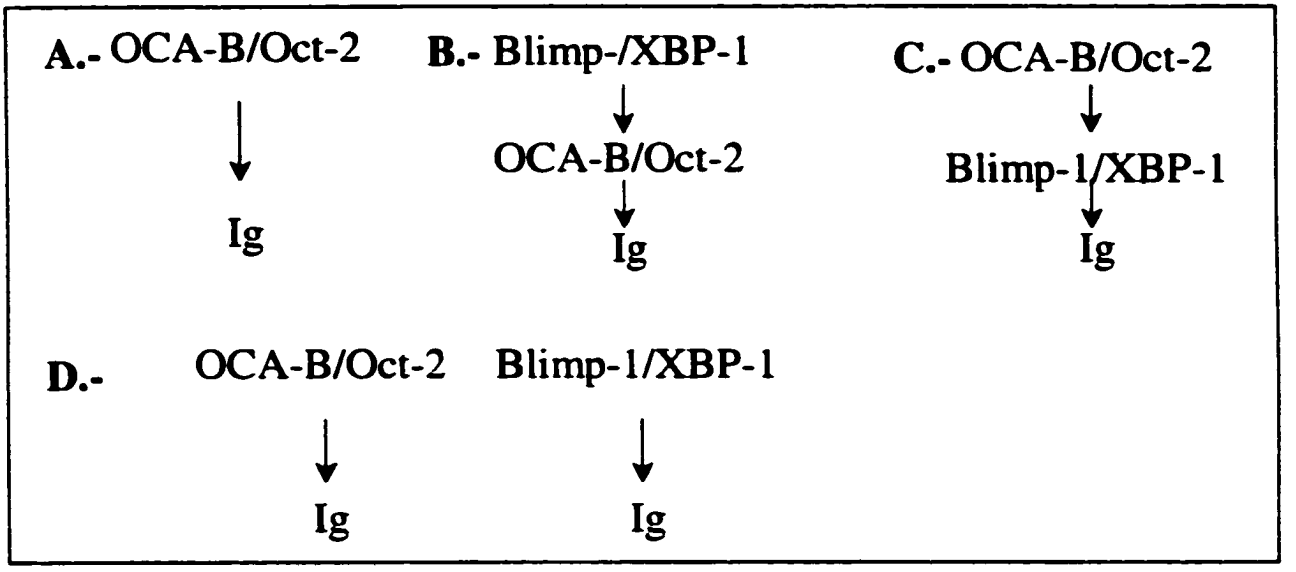


Our studies have provided new insights into the hierarchy of B cell factors working at the antibody-secreting plasma cell stage. Our working hypothesis is that Oct-2 with coactivator OCA-B are sufficient to maintain the genetic program of the Ig-secreting cell, whereas PU.1 plays a subordinate role to Oct-2 and OCA-B. There have been two other factors described which are as necessary for this particular stage of B cells: Blimp-1 (B lymphocyte-induced maturation protein) and XBP-1 (68, 69). Blimp-1 is specifically expressed in activated B and plasma cells, whereas XBP-1, although found ubiquitously, is upregulated by stimuli that induce plasma-cell differentiation. Both are expressed in plasmacytomas. These two factors can independently induce the B cell lymphoma line BCL1, which represents an activated B cell, to differentiate into a plasma cell, providing a good working model to test for master switch genes that can drive B cell differentiation. Some of the phenotypic changes associated with B cell differentiation to the plasma cell stage are induction of J chain message, immunoglobulin secretion, up-regulation of Syndecan-1, and increased cell size and granularity. Based on these findings, we need to integrate factors Blimp-1 or XBP-1 into this newly established hierarchy at the Ig-secreting cell stage.

Reasoning reciprocally, we would predict that an Ig-secreting cell line that lacks both OCA-B and Oct-2 should not be able to express Ig or other B cell-specific genes unless other factors can compensate for the double mutation. In order to knockout both genes consecutively at this particular stage, one first has to select an Ig-secreting cell line that has only one copy of each gene in its genome. One needs to use different selectable markers for each gene to reject those clones that have randomly integrated and keep those

where targeted deletion occurred. As we all know, just targeting a single gene is very tedious and can take a long time. Therefore, one is looking at a long but probably a feasible experiment. If we can obtain an Ig-secreting cell line that has knocked out both the OCA-B and Oct-2 genes, we could check expression levels of Blimp-1 or XBP-1 factors known to be master gene regulators at this stage of B cells. After knockout of both OCA-B and Oct-2 genes, we would predict that Ig is turned off (Figure 5.6 A). Whether these factors are acting directly or indirectly at the promoters is not known. If Ig is off and levels of Blimp-1 or XBP-1 are on in these knockout cell lines, we can say that Blimp-1 or XBP-1 are acting earlier in the hierarchy as OCA-B and Oct-2 to control B cell gene expression (see Figure 5.6 B). If Blimp-1 and XBP-1 levels are not on, then we can say these factors are acting late in the hierarchy (Figure 5.6 C). If upon knockout of OCA-B/Oct-2 we encounter that Ig is still on (refer to Figure 5.6 D), we have the possibility that two different pathways allow us to get to the plasma cell stage.

Figure 5.6. Possibilities upon knockout OCA-B/Oct-2 in an Ig-secreting cell line. Hierarchy with respect to Blimp-1/XBP-1 factors.



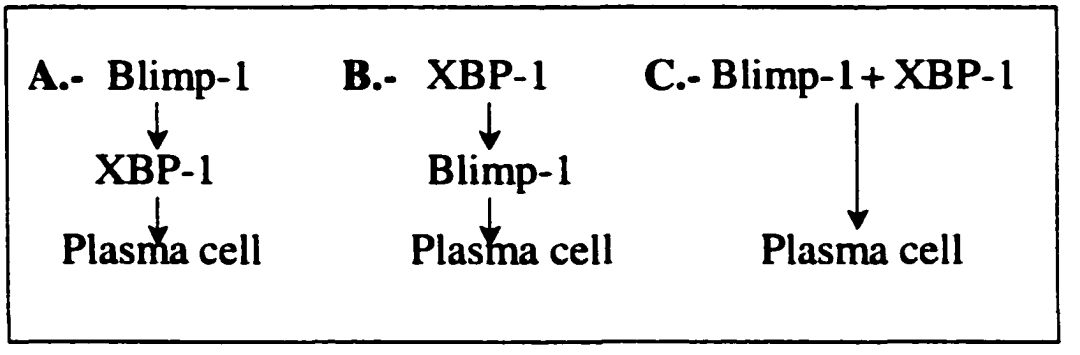
If we want to attempt deletion of both of these genes (OCA-B and Oct-2) in vivo, it is even more difficult and requires a technique that is still being worked out. Recall that these knockouts have been obtained; however, there was an arrest at the maturation of B cells previous to the Ig-secreting cell. The idea is to use conditional inactivation of one gene in a mouse that has incorporated lox P sites surrounding the gene (introduced by homologous recombination in embryonic stem cells). We can then conditionally inactivate the "floxed" gene by crossing these mice with another that expresses the Cre recombinase only in the B cell lineage. Successful conditional deletion of the ligand binding chain of tumor growth factor-β (TGF- receptor□ β, named TβR type II), (136) only in B cells was accomplished by intercrossing a mouse with lox P sites TβR (floxed) with a mouse expressing the Cre recombinase only in the B cell lineage through knock in

of the Cre into the CD19 locus (137). For our purposes, we would need to use a knock-in mouse that activates the Cre recombinase late in the B cell development. For instance, place the Cre under the transcriptional regulation of the J chain gene that is known to be activated at this cell stage. In this way, we could accomplish cell-type and stage specific mutagenesis using the Cre/lox P system.

One can also make use of the published knockout experiments to learn about transcription factor hierarchies. We can ask whether mutation of one gene can influence the expression of another or if one gene is able to compensate for the mutation of another or if one gene acts at more than one place within the hierarchy. For instance, we can check the expression levels of the Blimp-1 factor in knockout experiments of the XBP-1 gene. Since XBP-$1^{-/-}$ mice died in utero, the RAG-2 complementation system was used to analyse XBP-1-deficient lymphocytes from adult chimaeric animals. These animals can make XBP-$1^{-/-}$ B cells which can be activated and express cellular markers characteristic of normal activated B cells. XBP-1-deficient lymphocytes can also form germinal centers; however, these cells cannot further differentiate into plasma cells. There is a block between full stage B cell activation and differentiation into a plasma cell. Levels of expression of Blimp-1, which has also been shown to drive B cell differentiation, was not affected in these $XBP^{-/-}$ deficient activated B cells. This demonstrates that Blimp-1 cannot compensate for XBP-1 in the formation of the Ig-secreting cell. Beyond this we can consider two possibilities: XBP-1 is acting later than Blimp-1 and a second that XBP-1 and Blimp-1 are required for the formation of plasmacyte. In order to distinguish between these, we need to investigate the reciprocal, that is check levels of expression of

XBP-1 in Blimp-1 knockout mice. If Blimp-1 is needed in plasmacyte formation, Blimp-1 deficient activated B cells will not be able to progress to the plasma cell stage (Figure 5.7 A or B). If XBP-1 is present in these Blimp-$1^{-/-}$ mice, then we can say that Blimp-1 is not required for the XBP-1 expression and most likely that XBP-1 is not acting later than Blimp-1 (B) but rather both factors are required for plasma cell formation (C).

Figure 5.7. Hierarchy of Blimp-1 with respect to XBP-1 in an Ig-secreting cell.



We can then use similar reasoning by checking the expression of OCA-B / Oct-2 in these XBP-1-deficient lymphocytes. We could find out if these genes are expressed. If they are, they are not sufficient to induce plasma cell formation. On the contrary, we could find out that these genes are not expressed in which case we would conclude that they are directly or indirectly regulated by XBP-1 and, therefore, lie downstream of this factor. We could then ask whether reconstituting expression of these factors in the XBP-$1^{-/-}$ mice would reconstitute plasma cell formation. To do the reciprocal, one has to have the results on the knockout strategy as I described above.

We can make use of the in vitro model cell line BCL1. Based on the cellular markers that are available, Syndecan-1 and J chain, one can establish which factors allow for progression to the plasma cell stage. I would also try overexpression of both OCA-B and Oct-2 to see if we get progression to the plasma cell stage.

These somatic cell fusion studies have succeeded in identifying the hierarchy of B cell factors needed for B cell-specific expression at the Ig-secreting cell stage. OCA-B and Oct-2 are at the apex of this hierarchy with PU.1 playing a subordinate role to OCA-B and Oct-2 at this cell stage.

Table IV: Summary of data in somatic cell fusion experiments

Ig-secreting	T cell	informative hybrids	Ig-expressing	%Ig rescue
MP	BW	7	0	0
MPPU.1#1	BW	9	0	0
MP	BWPU.1#1	11	0	0
MPOCA-B 8/6 A+B	BW	17	16	94
MP	BWOCA-B#10	7	1	14
MP	BWOCA-B- Oct-2#1	4	4	100
MP	BWOCA-B- Oct-2#4	5	5	100

CHAPTER 6

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