Expression of Ig genes

Regulation of transcription and production of human antibodies

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Abbreviations

Ab	antibody
APC	antigen presenting cell
BSAP	B cell specific activator protein
C	constant
CSR	class switch recombination
D	diversity
FR	framework
H	heavy
HAMA	human anti-mouse antibodies
HLH	helix loop helix
HS	hypersensitive site
Ig	immunoglobulin
J	joining
L	light
LCR	locus control region
LPS	lipopolysaccharide
MAR	matrix attachment region
μE	IgH intron enhancer
PCR	polymerase chain reaction
sIg	surface immunoglobulin
3'E	3' enhancer
V	variable

Original papers

The present thesis is based on the following papers which will be referred to in the text by their Roman numerals.

I. Arulampalam, V., Furebring, C., Samuelsson, A., Lendahl, U., Borrebaeck, C.A.K., Lundkvist, I. and Pettersson, S. (1996) Elevated expression levels of an immunoglobulin transgene links the IgH 3' enhancer to the regulation of IgH expression. *Int. Immunol.* in press.

II. Furebring, C., Borrebaeck, C.A.K. and Pettersson, S. (1996) Evidence that the IgH 3' enhancer can act directly on a natural IgH promoter in vivo. *Manuscript.*

III. Danielsson, L., Furebring, C., Ohlin, M., Hultman, L., Abrahamson, M., Carlsson, R. and Borrebaeck, C.A.K. (1991) Human monoclonal antibodies with different fine specificity for digoxin derivates: cloning of heavy and light chain variable region sequences. *Immunology* **74**, 50-54.

IV. Simonsson Lagerkvist, A.C., Furebring, C. and Borrebaeck, C.A.K. (1995) Single antigen-specific B cells used to generate Fab fragments using CD40-mediated amplification or direct PCR cloning. *BioTechniques* **5**, 862-869.

V. Furebring, C., Ohlin, M., Pettersson, S. and Borrebaeck, C.A.K. (1996) Evaluation of novel control elements by construction of eukaryotic expression vectors. *Submitted.*

INTRODUCTION

The immune system protects us from being harmed by a variety of infectious microbial agents in our environment. One key component in the immune response against foreign particles is the antibody (Ab) molecule. Two functions are characteristic of every Ab molecule; (i) specific binding to an antigenic determinant, and (ii) mediator of effector functions. The latter involves binding and activation of complement, stimulation of phagocytosis by macrophages and triggering of granule release by mast cells. Abs are expressed exclusively by cells of the developmental line of B lymphocytes. Terminally differentiated B cells, so-called plasma cells, produce enormous amounts of Abs, which requires the corresponding genes to be very active in these cells. The expression of genes encoding the light and heavy-chain immunoglobulin (Ig) polypeptides is stringently regulated by a variety of different regulatory proteins. One of the best studied transcriptional regulatory mechanism is the Ig heavy chain gene. In the first part of this thesis, after a short introduction to the Ig molecule and its host the B cell, I will describe how the Ig gene expression is thought to be regulated.

The properties of an Ab makes it a suitable agent for therapeutic applications. The advent of hybridoma technology in 1975 made it possible to obtain Abs of defined specificity in large quantities (Köhler and Milstein, 1975). The murine Abs, however, suffer from several disadvantages. Therefore much effort has been focused on the development of human monoclonal Abs. In the second part of this thesis I will discuss different approaches in how to produce human Abs. The last part of the thesis is a presentation of my own projects, including the original papers whereupon this thesis is based.

Immunoglobulin structure

An antibody molecule consists of two identical light (L) polypeptide chains and two identical heavy (H) chains held together by a combination of noncovalent bonds and covalent disulfide bonds (Figure 1). Each polypeptide

chain can be divided into one variable (V) and one to four constant (C) domains. The antigen binding site is combined by the VL and VH domains. The variable region can be further divided into more conserved regions, the framework regions (FR), and hypervariable regions, often called the complementarity determining regions (CDR). The effector functions are determined by the structure of the constant region of the heavy chain. Abs are glycoproteins and the presence of carbohydrates on the Ab molecule is essential for some of the effector functions. Cleavage with the enzyme papain splits the Ab molecule into a Fab region, the antigen binding site and an Fc region, mediating effector functions.

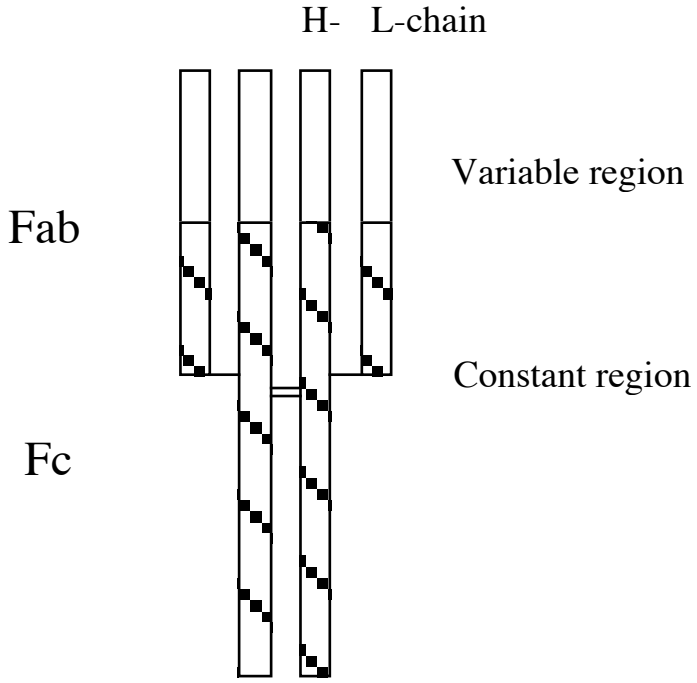

Figure 1. The immunoglobulin molecule

In humans, there are five classes of antibodies, IgM, IgD, IgG, IgA and IgE, each with its own isotype of H chain- μ, δ, γ, α and ε,respectively. The two isotypes of light chain, κ and λ are shared between the different classes.

IgM is the first class of antibody secreted in a primary immune response and it is preferentially produced as a pentamer. The low affinity of the molecule is compensated for by the high avidity mediated by its pentameric form.

IgD is expressed on the surface of naive mature B cells together with IgM. They are rarely secreted by an activated B cell and their function is unknown.

IgG is the major class of Ab in blood and is produced during secondary immune responses. There are four different subclasses IgG1-IgG4 which differ in their ability to activate effector functions. In general, protein antigens mainly induce IgG1 responses, and to a lesser degree IgG3 and IgG4, while polysaccharides induce IgG2 response.

IgA can be expressed either as a monomer or as a dimer. The dimeric form is the dominant class in secretions (milk, respiratory and intestinal secretions). IgA constitutes the first line of defense against pathogen invading the organism via the mucosa.

IgE is involved in some types of allergic responses through its high affinity binding to receptors on mast cells and basophils.

The generation of antibody diversity

The immune system is capable of producing a tremendous diversity of antibody specificities. Immunoglobulin genes are assembled during B cell differentiation from different gene segments that are combined through site-specific recombination. The variable regions are encoded by variable (V), diversity (D) and joining (J) segments. The human immunoglobulin heavy chain (IgH) locus contains approximately 50 VH, 20 DH and 6 JH gene segments. The Igκ locus contains 100 Vκ and 5 Jκ, whereas the Igλ contains 30 Vλ and 8 Jλ. These different segments, in the H chain locus, are joined by recombination as outlined in figure 2. The L chain locus undergoes a similar recombination process, with the exception that it lacks the D region genes.

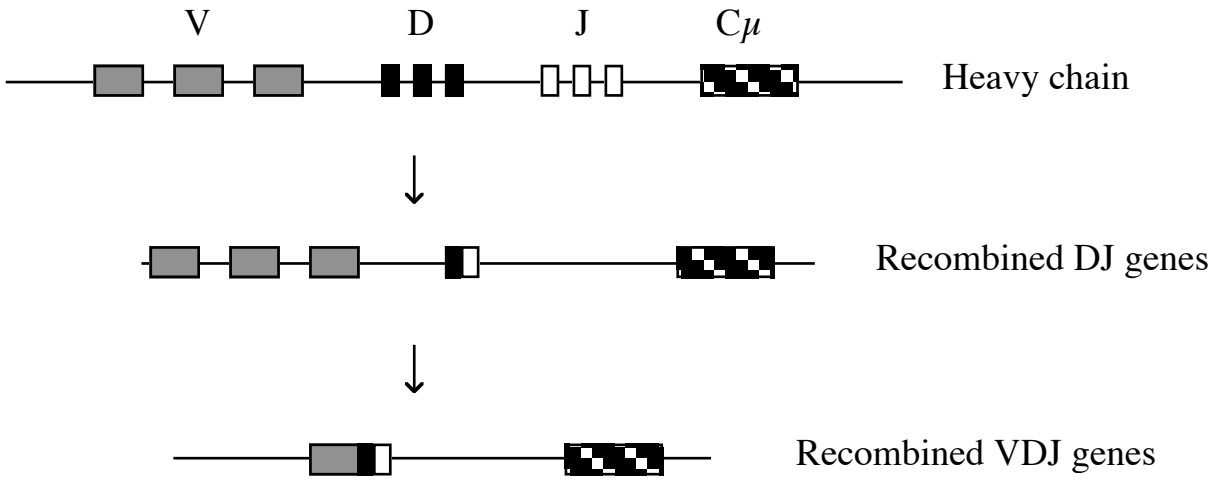

Figure 2. Recombination of antibody VDJ genes.

Further diversity is generated through junctional diversification, that is nucleotides can either be lost or added (P and N nucleotides) in the joining of V-(D)-J segments. Additional diversity can be derived from pairing of different VL/VH regions. Furthermore, after the B lymphocyte has been stimulated by antigen a somatic hypermutation mechanism may operate on the assembled variable regions.

B cell development

The cells of the immune system which are specialized to make antibodies are called B lymphocytes. They originate in the bone marrow and are derived from a multipotent stem cell. The different stages during differentiation of B lymphocytes, from precursor cells to antibody secreting cells, are outlined in figure 3. The different steps are defined by changes in the specific gene expression pattern.

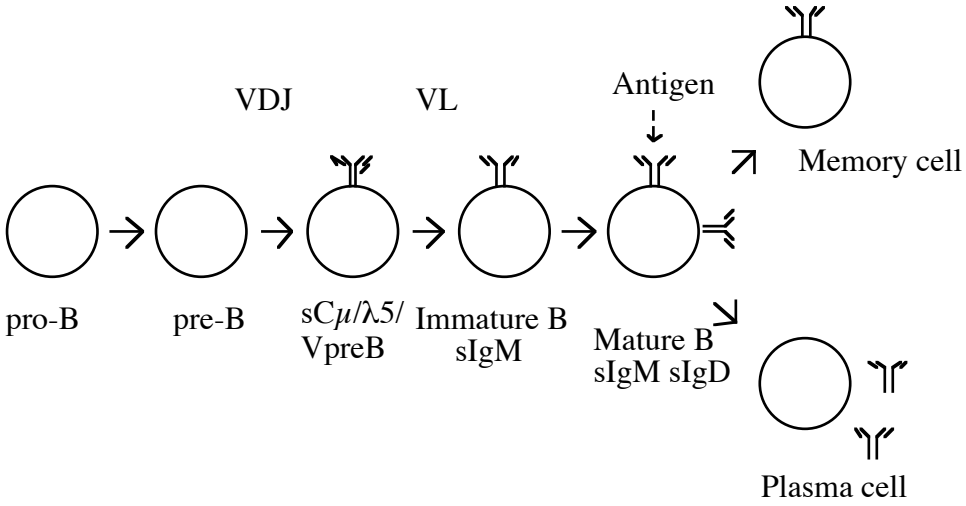

Figure 3. B cell differentiation.

At the pro-B cell stage, the Ig genes are in germline configuration but a sterile transcript is produced (Lennon and Perry, 1990). In pre-B cells, VDJ recombination of the IgH variable chain generates μ-chains which can associate with either the surrogate λ5 and VpreB light chains (Kerr et al., 1989; Kitamura et al., 1992) or the κ protein encoded by a germline JCκ transcript (Francés et al., 1994). This immature B cell receptor is expressed on the surface of the pre-B cell and can function as a signal-transducing surface receptor (Misener et al., 1991). Following the rearrangement of genes encoding Ig κ or Ig λ light chain, the IgM receptor is displayed on the surface of immature B cells. The mature B cell is antigen reactive and expresses IgM and IgD receptors. After encountering the correct antigen the mature B cell is activated and can differentiate into either memory or plasma cells. Differentiation to plasma cells include switch from surface Ig (sIg) expression to secretion of Abs and may include somatic mutation of Ig varible regions.

SIg on immature and mature B cells are noncovalently associated with the mb-1 gene product Igα and the B29 gene product Igβ (Sakaguchi et al., 1988; Hermanson et al., 1988). These molecules together form the B cell antigen receptor (BCR) complex. Igα and Igβ facilitates transport of the Ig molecule from the endoplasmatic reticulum to the cell surface and also have an important role in the signal transduction event (Matsuuchi et al., 1992; Nakamura et al.,

1993). Igα and Igβ are expressed at all stages prior to terminal differentiation into plasma cells.

B cell activation

B cell activation initially involves antigen binding to sIg. There are two kinds of antigen, T cell-dependent and T cell-independent. The T cell-independent pathway is triggered by two kinds of antigen, polysaccharides or potent polyclonal activators like lipopolysaccharide, (LPS). These antigen crosslink the sIg and thereby activate the B cells. However, the activation still requires T cell derived cytokines to stimulate Ig secretion (Pike et al., 1987). The T cell-dependent pathway is triggered by soluble proteins and relies on the possibility of the B cell to act as an antigen-specific presenting cell (APC). The B cell binds the antigen with its sIg. The antigen is internalized, processed into peptides and therafter presented on the cell surface bound to the MHC class II molecule. Finally the T cell recognizes the processed antigen on the B cell surface and the activation of the B cell starts. This presentation leads to cognate interactions, involving the release of T cell derived cytokines and increased expression of accessory molecules, which results in both T and B cell activation. A key feature of this cognate interaction is the induction on the T cell membrane of a ligand for CD40 (CD40L), which delivers an activational signal to the B cell (Noelle et al., 1992). Other important accessory molecules are the CD80, CD86 on the B cell and CD28 and CTLA-4 on the T cell (figure 4). Crosslinking CD40 promotes B cell proliferation and immunoglobulin class switch (Clark and Ledbetter, 1986; Jabara et al., 1990). If the CD40-CD40L interaction is blocked in vitro with soluble CD40 or CD40L specific Ab, the B cell can not proliferate indicating that this interaction is required for signaling (reviewed by Noelle et al., 1992). The interaction of CD28-CD80/CD86 prefentially activates the T cells to produce cytokines and to proliferate (Linsley et al., 1991). CTLA-4 deficient mice show massive expansion of activated T cells and an increase in serum Ig levels (Waterhouse et al., 1995). This data suggest that CTLA-4 may act as a negative regulator of T cell activation, and indirectly, B cell stimulation.

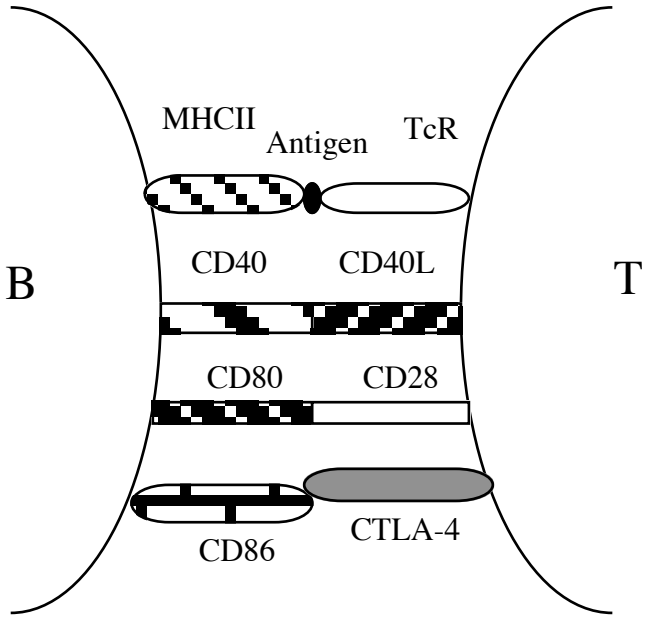

Figure 4. Interaction between B and T cell.

REGULATION OF TRANSCRIPTION

DNA in the nucleus of eukaryotic cells is wrapped around histone cores, bound by nonhistone proteins and compacted into highly ordered chromatin structures. Gene activation correlates to an altered chromatin environment. The open chromatin has two special characteristics. The first is an increased sensitivity to digestion with DNaseI and the second is hypomethylation. DNA methylation is believed to be one way of regulating gene activity, where demethylation reflects an active gene. Gene transcription can be regulated by cis influences, dependent on the DNA sequences of genetic elements attached to a gene, and trans influences, dependent on the environment of the gene. Cis-acting control elements mediate their function by binding of nuclear proteins which assemble the DNA into a three dimensional configuration that is optimal for the transcription and expression of genes. Binding of trans-acting nuclear factors to the control elements may give the signal for opening of the chromatin structure. Promoters and enhancers represent two classes of DNA elements that appear to cooperate in the control of efficient transcription. The promoter region contains sequences upstream of the transcription initiation site that are involved in the tissue-specific activation or repression of transcription. Enhancer elements can potentiate transcription from an appropriate promoter region in a tissue-specific manner, somewhat independent of their orientation or distance from the promoter. The proteins important for gene transcription include basal transcription factors, positive and negative nuclear factors and co-factors which can modulate the function of specific nuclear factors. Control of the transcriptional activity of individual Ig gene loci is central to the regulation of B cell differentiation. The IgH locus appears to be activated at a very early stage of B cell development whereas the IgL locus is activated in late pre-B cells. In figure 5 the different promoters and enhancers important for IgH gene expression are outlined. Below the characteristics of these elements will be discussed.

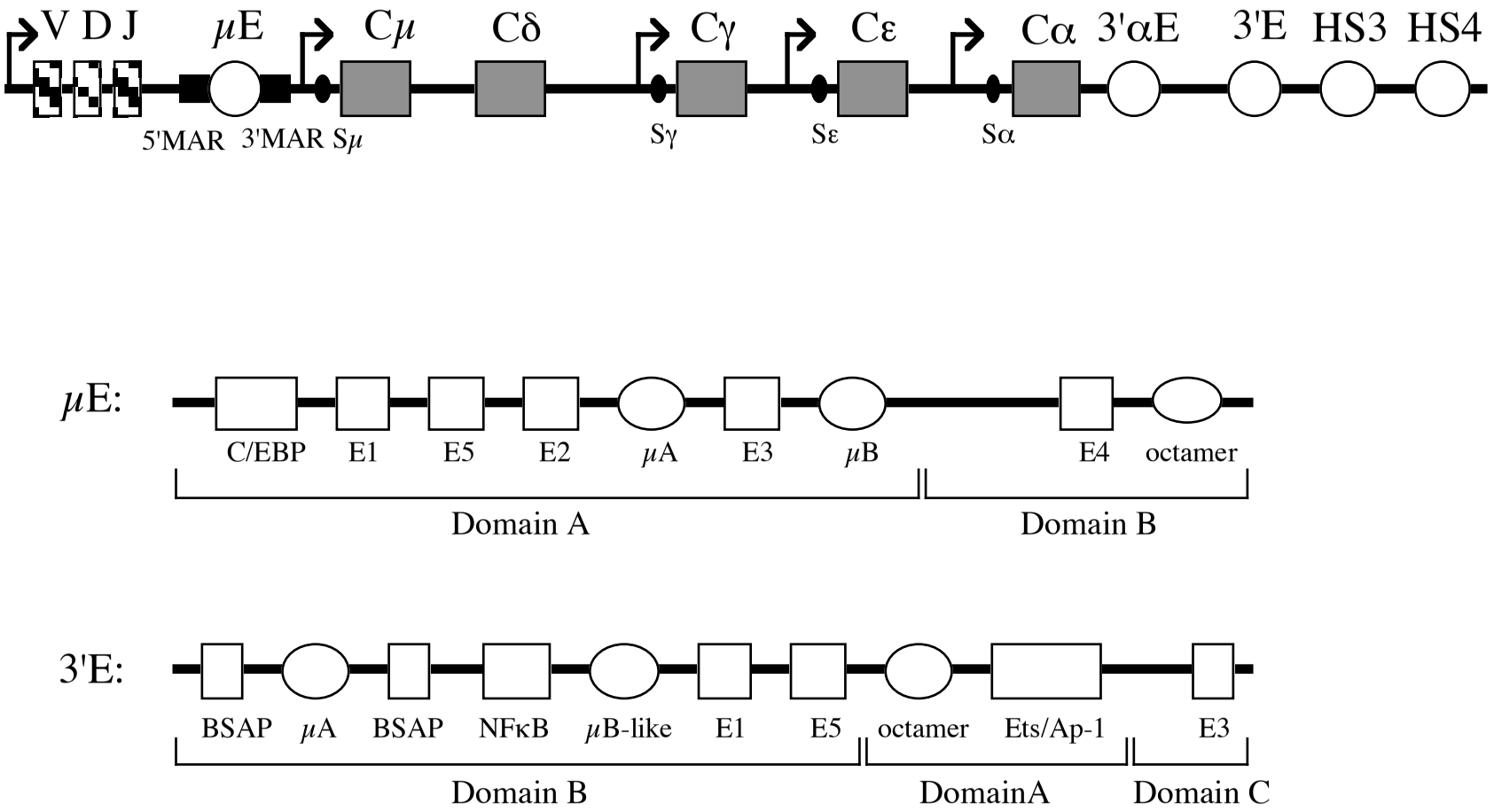

Figure 5. Regulatory elements of the mouse IgH locus

VH promoter

The VH promoters are located at the 5' end of each variable gene segment, between 150-200 bp upstream of the initiation site (Grosschedl and Baltimore, 1985) (figure 6). Two sequence motifs are shared by all Ig promoters, an octamer motif and a TATA box. The TATA box consensus sequence is the core promoter which interacts with the basal transcription machinery (Parvin et al., 1992). This motif is not cell-restricted. The octamer motif (ATTTGCAT or its inversion) is highly conserved among the VL and VH promoters (Falkner and Zachau, 1984). Mutations in the octamer motif reduces expression of the IgH gene in both transfection assays (Mason et al., 1985) and in transgenic animals (Jenuwein and Grosschedl, 1991). It has been shown that a single octamer motif is sufficient for conferring B cell specificity to a minimal promoter element consisting a TATA box (Dreyfus et al., 1987). A family of nuclear proteins bind to the octamer motif, two of them are present in B cells, Oct-1 and Oct-2 (Staudt and Lenardo, 1991). Oct-1 is expressed ubiquitously whereas the Oct-2 is largely B cell restricted (Staudt and Lenardo, 1991). Therefore it was proposed that the Oct-2 protein has a specialized function in B cells with respect to Ig transcription. However in Oct-2-deficient mice generated by gene targeting, the Ig genes are rearranged and transcribed normally (Corcoran et al., 1993). It was then suggested that other B cell specific components are responsible for lymphoid restricted transcription of Ig promoters. Recently an adaptor protein has been identified which is tissue specific and it stimulates transcription in conjunction with either Oct-1 or Oct-2 (Luo et al., 1992). An adaptor protein does not bind to DNA itself but can interact with DNA-binding proteins. This protein-protein interaction can then modulate the affinity of the DNA-binding protein. The adaptor molecule that binds the Oct-proteins have been cloned, by three different groups, as Bob-1, OBF-1 and OCA-B (Gstaiger et al., 1995; Strubin et al.,1995; Luo and Roeder, 1995). Several other sequence motifs are conserved in Ig promoters, including the heptamer, a pyrimidine-rich element and two E-box motifs. The heptamer is located close to the octamer and the two motifs can be bound cooperatively by Oct-binding proteins (Poellinger and Roeder, 1989). The

pyrimidine-rich sequences are important for full activity of the promoter (Eaton and Calame, 1987) and the PU-1 binds to this site (Schwarzenbach et al., 1995). The PU-1 is a member of the Ets family of proteins. The Ets proteins can bind DNA as a monomer or they can form multisubunit complexes which are stable only in the presence of DNA (Wasylyk et al., 1993). E-box motifs (CANNTG) bind proteins of the helix-loop-helix (HLH) family. The HLH proteins consist of two amphipathic helices separated by an intervening loop (reviewed by Kadesch, 1992). The HLH domain is preceded by a stretch of basic residues, which determine the DNA binding specificity. The role of these sites in the promoter is not established yet.

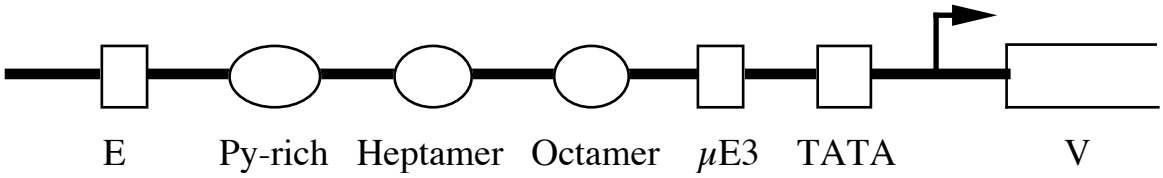

Figure 6. The VH promoter.

Conserved promoters, I promoters, are located 5' of each constant region. These promoters lack the TATA-box but contain multiple initiation sites (Rothman et al., 1991). The activity ot these promoters is reflected by the appearance of sterile transcripts from constant genes prior to class switch recombination (CSR). The sterile transcription may regulate the corresponding gene rearrangement through altering the accessibility of the DNA. The I promoter may contain a unique combination of motifs mediating the action of various cytokines and other T cell influences that are known to regulate selection of that isotype.

IgH intron enhancer (*μ*E)

The enhancer located between the JH and C*μ* was one of the first cellular (nonviral) enhancers recognized (Banerji etal., 1983; Gillies et al., 1983; Neuberger, 1983). In both the human and murine loci this enhancer lies 3' of the most downstream JH region and contains a 220 bp core element, the *μ*E. It is

located 5' of the switch region and where it is retained after switch recombination. In transfection assays this enhancer appears to be active at all B cell stages (Grosschedl and Baltimore, 1985). The generation of mice lacking the μE has demonstrated its importance for efficient DNA demethylation, germline Ig transcription and VDJ recombination during B cell development (Serwe and Sablitzky, 1993; Chen et al., 1993). Deletion of μE also strongly suppresses switch recombination at the Cμ switch region, whereas the switch region of the γ1 gene is efficiently rearranged (Gu et al., 1993).

μE is flanked by two Matrix attachment regions (MARs) which mediate an association with the nuclear matrix (Cockerill et al., 1987). These MARs have been shown to be essential for transcription of a rearranged μ gene in transgenic B lymphocytes (Forrester et al., 1994). It has been suggested that the μE and its flanking MARs are largely responsible for inducing chromatin alterations (Cockerill et al., 1987). The MARs contain binding site for two nuclear factors, NFμNR and SATB-1, which may participate in the repression of enhancer activity in non-B lymphoid cells (Scheuermann and Chen, 1989; Dickinson et al., 1992).

Functional and biochemical analyses have identified a set of nuclear protein binding sites in the μE, like the E-boxes, octamer binding and Ets binding motifs (Staudt and Lenardo 1991; Ephrussi et al., 1985) (Figure 5). There are five E-boxes (Eμ1-Eμ5) which bind a variety of homo or heterodimerizing trans-acting factors belonging to the HLH family. Mutational studies have suggested that the E-box elements contribute to the transcriptional regulation of the IgH gene but do not determine lymphoid specificity (Lenardo et al., 1987; Perez-Mutal et al.,1988). The octamer element, also present in Ig promoters, bind the Oct-1 and Oct-2 proteins. Mutation of these sequences in the context of entire IgH enhancer has only very little effect on its activity in lymphoid cells (Perez-Mutal et al., 1988; Jenuwein et al., 1991), suggesting the presence of other motifs that confer the lymphoid specific activity of the μE. The Ets-binding sites, μA and μB, are required for optimal μE activity in B cells (Liebermann et al., 1990; Nelson et al.,

1993). The μA motif binds at least five different Ets family members, presumably with varying affinites, whereas the μB motif binds the PU-1 (Nelson et al., 1993).

3' enhancer (3'E)

The observation that certain cell lines, in which the μE was deleted, still transcribed the IgH gene (Eckhardt and Birshtein, 1985; Wabl and Borrows, 1984) led to the notion that the μE might serve an important function in early B cell stages, while other control elements might be more important in late stages. Transgenic studies have also shown that the IgH transgene was not expressed at the same high level as the endogenous IgH gene (Sturb, 1987). Subsequently, a second B cell specific enhancer 3' of the Cα coding sequence was identified, both in mice (16kb downstream) and in rat (27kb downstream) (Pettersson et al., 1990; Dariavach et al., 1991; Lieberson et al., 1991). It has not yet been identified in the human genome. In transfection assays the 3'E activity was only detected in cell lines representing terminally differentiated B cells (Lieberson et al., 1991; Darivach et al., 1991). Transgenic studies confirmed this late B cell activation profile (Arulampalam et al., 1994). In these animals, enhancer activity was observed in both in vivo activated splenocytes and in B cells of the peritoneal cavity. The 3'E activity late in B cell development also correlates with the demethylation and hypersensitive site formation over this region (Giannini et al., 1993). Potential function of the 3'E includes CSR, somatic hypermutation or increased Ig gene expression. The targeted deletion of the 3'E in mice revealed its role in control of CSR (Cogné et al., 1994). These mice were deficient in serum IgG2a and IgG3, but had normal levels og IgM and IgG1. In transgenic studies it has been shown that the Ig gene expression is higher if the transgene is controlled by the μE+3'E, as compared to the transgene controlled by the μE alone (paper I). This augmentation was seen at both the mRNA and protein levels. In cell lines, loss of both the μE and the 3'E completely abolished IgH gene expression, demonstrating the importance of 3'E for gene transcription (Lieberson et al., 1995).

The 3'E contains a number of nuclear protein binding sites (figure 5), some of which are identical to those found in the μE. The 3'E can be divided into three functional domains (Grant et al., 1992). Domain A and B confer lymphoid specific reporter gene activity whereas domain C appears to be active in all cell lines used.

Domain A, the strongest transcriptional domain, contains an octamer motif and an Ets-1/AP site. Mutations in the octamer site reduces the activity, but the cell specific activity is likely to rest with the Ets/AP-1 site. A DNA binding complex, nuclear factor of activated B cells (NFAB), binds to the Ets/AP-1 site. This complex contains the tissue restricted Ets protein, Elf-1 and the AP-1 protein (dimer of Jun-B and c-Fos) (Grant et al., 1995). This binding is only observed in activated B cells.

Domain B contains a range of nuclear factor binding sites. The E-box motifs E1 and E5 have been shown to be important for enhancer activity (Grant et al., 1992; Meyer et al., 1995). The E2A gene products E12 and E47 can bind to the E5 site and the zink finger protein YYI binds to the E1 site. The μA- and μB-like motifs also bind Ets proteins. The activity of the μB has not been characterised, whereas the μA contribute to the transcriptional actvity of domain B (Grant et al., 1992). There are also two binding sites for B cell specific activator protein (BSAP) in domain B. BSAP, a recently identified member of the Pax-gene family of transcription factors, is expressed in the B lineage from the pro-B to the mature B cell stage, however, it is not found in plasma cells (Singh and Birshtein, 1993, Neurath et al., 1994). When the BSAP binds to the motif in the 3'E it exerts a negative effect on the transcriptional activity. This might explain why the 3'E is active in plasma cells only.

Domain C has only one protein binding motif, E3, which can bind the DNA-binding protein, USF, in splenic nuclear extracts (Arulampalam et al., 1995). USF is a member of a distinct class, denoted basic-HLH-zip proteins. These proteins are distinguished by the presence of leucine zippers adjacent to their HLH motifs (Kadesch, 1992).

Additional control elements

An enhancer has been identified close to the Cα gene (Matthias and Baltimore, 1992). This enhancer has quite weak lymphoid specific activity. The location of this enhancer close to the Cα suggested that it might be involved in controlling switch recombination to Cα, although this has not yet been confirmed.

Analysis of the 3' end of the IgH locus revealed four DNaseI hypersensitive sites (HS), two of which (HS1 and HS2) mapped to the 3'E (Madisen and Groudine, 1994, Michaelson et al., 1995). The enhancer activity of HS3 and HS4 is restricted to B cells, HS3 is only active in plasma cells whereas HS4 is active in pre-B as well as plasma cells. HS3 and HS4 contain multiple binding sites for transcription activators commonly associated with Ig enhancers, the significance of these different motifs have not been confirmed.

Locus control region

The most well-characterised locus control region (LCR) is at the 5' end of the human β-globin locus. The LCR consists of five different regulatory elements which are involved in the regulation of replication, chromatin structure and transcription of the entire locus (Crossley and Orkin, 1993). Transgene constructs containing the LCR show copy number dependent expression of linked globin genes independent of the site of integration of the transgene in the host genome (Grosveld et al., 1987; Blom van Assendelft et al., 1989). The LCR regulates transcription of the different β-globin genes through competion of promoters with the LCR elements (Wijgerde et al., 1995).

It has been proposed that the IgH locus contains two LCRs. The μE and its flanking MARs form a potential LCR upstream of the Cμ. This LCR is required for VDJ recombination at early stages of B cell development (Serwe et al., 1993, Gu et al., 1993). The other potential LCR is located at the 3' end of the IgH locus and would include the 3'E, HS3 and HS4. Madisen and Groudine (1994) have demonstrated that these three elements confer copy number-dependent expression of a linked myc-gene in vitro. We have shown that the 3'E can confer

position independent expression of a linked transgene (paper I) but no copy number-dependent expression was achieved. The missing elements to achieve copy number-dependent expression might be the HS3 and HS4. The potential 3' end LCR might also regulate transcription of different constant isotypes through competion with the different promoters. At least the 3'E can direct transcriptional activity of specific promoters (Cogné et al., 1994). Mice with targeted deletion of this enhancer did not produce γ3 and γ2a germline transcript.

PRODUCTION OF HUMAN ANTIBODIES

Monoclonal Ab became available when Köhler and Milstein demonstrated that cultured mouse myeloma cells could be fused to spleen cells from immunized mice (Köhler and Milstein, 1975). The hybrid cells (hybridoma) grew continously in culture (a property aquired from the myeloma cells) and continued to produce large quantities of Ab (a property obtained from the spleen cells). The mAbs have an enormous potential as therapeutic and diagnostic agents because of their remarkable specificity and affinity for their target. Rodent mAb unfortunately has several disadvantages, such as a short half-life in serum, only some of the different classes can trigger human effector functions and the Abs elicit an unwanted immune response in patients (human anti-mouse antibodies or HAMA). HAMA can result in enhanced clearance of the Ab from the serum, blocking of its therapeutic effect. Only a fraction of the anti-mouse immune response is directed to the variable region of the rodent Abs. To reduce the HAMA response it is therefore necessary to express potentially therapeutic Ab either as chimeric, humanized (engraftments of mouse CDR into the human framework region) or human monoclonal Abs. Recent development in both monoclonal Ab strategy and the use of recombinant DNA technology have now made it feasable to produce human mAb with the desired specificity. Below, the different steps, outlined in figure 7, leading to antigen-specific human Ab will be discussed.

In vitro generation of antigen-specific Abs

Due to ethical problems associated with immunization of humans much interest has been focused on techniques to generate antigen-specific Ab, using in vitro systems. Human B cells may be obtained from spleen, tonsils, lymph nodes, bone marrow or peripheral blood, the latter being the most accessible source. An approach to generate antigen-specific Abs is to use in vitro immunization techniques. However, in vitro immunization of human lymphocytes was initially more difficult to achieve than that of the murine counterpart. The reason for this

might be that peripheral blood cells contain cytotoxic T cells, monocytes, granulocytes and NK cells, which abrogate the antigen-specific activation of B cells (Borrebaeck et al., 1988). These cells must therefore be removed from the peripheral blood cells by L-leucyl-L-leucine methyl ester (LeuLeuOMe) treatment before an in vitro immunization of the B cells can take place (Borrebaeck et al., 1988). Briefly, the cells are cultivated with antigen in medium containing supernatant from irradiated pokeweed mitogen (PWM) stimulated T cells and recombinant IL-2. Immunization of naive B cells using this protocol only generates primary immune responses.

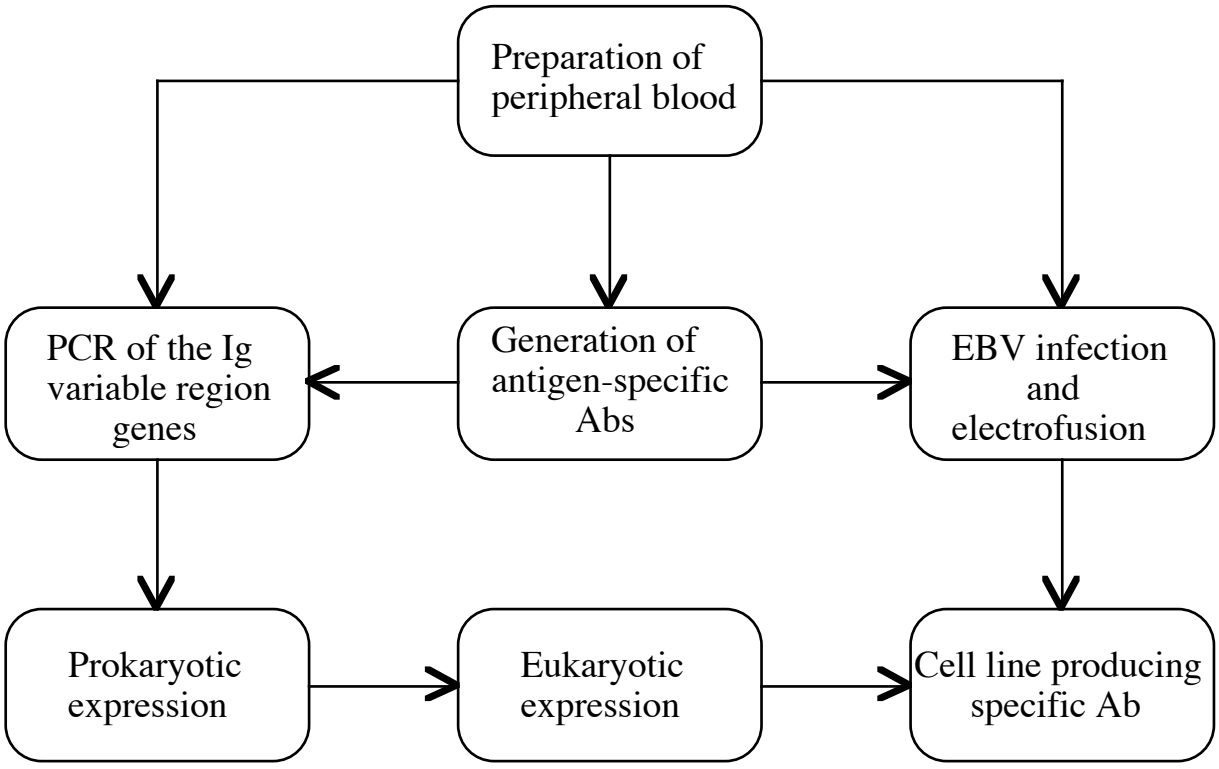

Figure 7. Establishment of human monoclonal Ab-producing cell lines.

B cells can be activated antigen-specifically and simultaneously be costimulated in vitro via their CD40 surface molecules. The stimulation can be delivered using either soluble recombinant CD40L or CD40 specific Ab in combination with cytokines (Nonoyama et al., 1993; Armitage et al., 1993). Using this CD40-system it is possible to maintain a long term B cell culture and polyclonal isotype switch has also been reported (Splawski et al., 1993).

Antigen-specific activation of B cells in vitro could be achieved after stimulating the B cells with anti-sIg Abs in the presence of T helper cells and Staphylococcal Enterotoxin A (SEA) (Ingvarsson et al., 1995). Increased crosslinking of the sIg enhanced Ig production. In this manner specific Ab was obtained against both primary and secondary antigens.

Chin et al. (1995) have been able to activate B cells antigen-specifically and induce isotype switch to achieve antigen-specific IgG production, using their primary and secondary antigen driven cultures. The secondary immunization is supported by antigen activated T helper cells and stimulation via the CD40 pathway.

Immortalization

One of the most important technologies in the production of monoclonal Ab is the establishment of immortal cell lines. The most frequently applied technique uses Epstein-Barr virus (EBV) to infect and activate human B cells (Steinitz et al., 1979). To rescue the transformed B cells they can be further fused to myeloma cells which stabilizes the Ab production (Kozbor et al., 1982).

Another alternative to immortalizing monoclonal Abs is to use DNA recombinant technology, which will be discussed below.

Polymerase chain reaction

Since the first report of specific DNA amplification using the polymerase chain rection (PCR) in 1985, the number of different applications has grown steadily, as have modifications of the basic method. An alternative way of immortalizing human Ab is to use this powerful PCR technique for amplification of the VL and VH chains. The concept is simple: a mixture of oligomer primers in the 5' leader sequences or framework 1 region with 3' constant or framework 4 region permits the amplification of any Ig variable region (Larrick et al., 1989a; Coloma et al., 1991; Marks et al., 1991a). The method has been used to obtain variable regions of both heavy and light chains from single human B cells (paper

IV; Küppers et al., 1993). These fragments can thereafter be cloned into either prokaryotic or eukaryotic expression vectors.

Prokaryotic expression

Using bacterial expression technology, different types of Ab fragments can be made in fully functional form in E. coli. This technology can be extended further to cloning and expressing libraries of VL/VH chains from the immune system. The construction of libraries of single chain Fv or Fab fragments of antibody molecules that are expressed on the surface of filamentous phages and the selection of specific recombinant Ab offers a new and powerful mean for generating monoclonal Ab (reviewed by Hoogenboom et al., 1992a; Marks et al., 1992a). A major goal of recombinant Ab technology is to develop Ab libraries of large size and diversity to facilitate the isolation of Abs of every conceivable specificity, among them Abs with high affinity. The rearranged V-genes can be derived from immunized (Barbas et al., 1991; Clacksons et al., 1991) or unimmunized B cells (Marks et al., 1991b). The naive libraries are easier to obtain but mostly low affinity binders have been isolated (Marks et al., 1991b). An alternative approach to creating diverse libraries is to use a collection of germline VH segments fused to synthetic CDR3 regions in vitro (varying in length between 6 and 15 amino acids) and combined with one or multiple light chains (Hoogenboom and Winter, 1992b; de Kruif et al., 1995a). Fully synthetic libraries have also been described (Barbas et al., 1992; Söderlind et al., 1995).

Several methods for antigen-specific selection of high affinity Abs have been described. These include selection of phage Abs binding to antigen-coated tubes (Marks et al., 1991b) or binding to antigen on a column matrix (Clackson et al., 1991). One alternative is to use flowcytometry based selection for finding Ab against cell surface antigens (de Kruif et al., 1995b). To mimick in vivo antigen selection, Duenas and Borrebaeck (1994) used a fusion protein consisting of antigen fused to the pIII protein. Using an engineered helper-phage with deleted functional gIII made it possible to select high affinity Abs.

Since most libraries are obtained from naive repertoires, methods for affinity maturation in vitro have been developed. These include in vitro maturation of Ab using codon based mutagenesis (Glaser et al., 1992), error prone PCR (Casson and Menser, 1995) or spiked oligonucleotide based mutagenesis (Hermes et al., 1989). One alternative is to use the chain-suffling technique (Marks et al., 1992b).

After selecting a high affinity Ab to a desired antigen, it could be either expressed in E.coli and purified, or cloned in an eukaryotic expression vector to produce the entire Ab molecule.

The use of Ab fragments may be advantageous in some therapeutical applications, as they penetrate tissue more readily and are cleared more rapidly from serum. This may help in neutralizing and clearing drugs from the serum. Engineering of recombinant Ab has also resulted in the construction of a new generation of molecules. Monovalent sFv Abs have been converted into bivalent monospecific or bispecific reagents using e.g. dimerization domains (Pack et al., 1993) or direct coupling (Holliger et al., 1993). In addition, Fv can be fused or coupled to a variety of cytokines, enzymes or radioactive entities.

Eukaryotic expression

Gene transfection provides a method for making novel Ig molecules. The tranfected cells (transfectomas) grow continously in culture and produce the Ig specified by the transfected gene. To create a transfectoma cell line synthesizing a novel Ab, both Ig heavy and light chains must be transfected into the same recipient cell line. Although both chains can be contained in a single vector, it is usually more convenient to construct separate light and heavy chain vectors, which are simultaneously transferred and expressed. A vector should contain regulatory elements, VL/VH region genes, a genomic constant region including polyA signal and splice signals and a selectable marker, as outlined in figure 8.

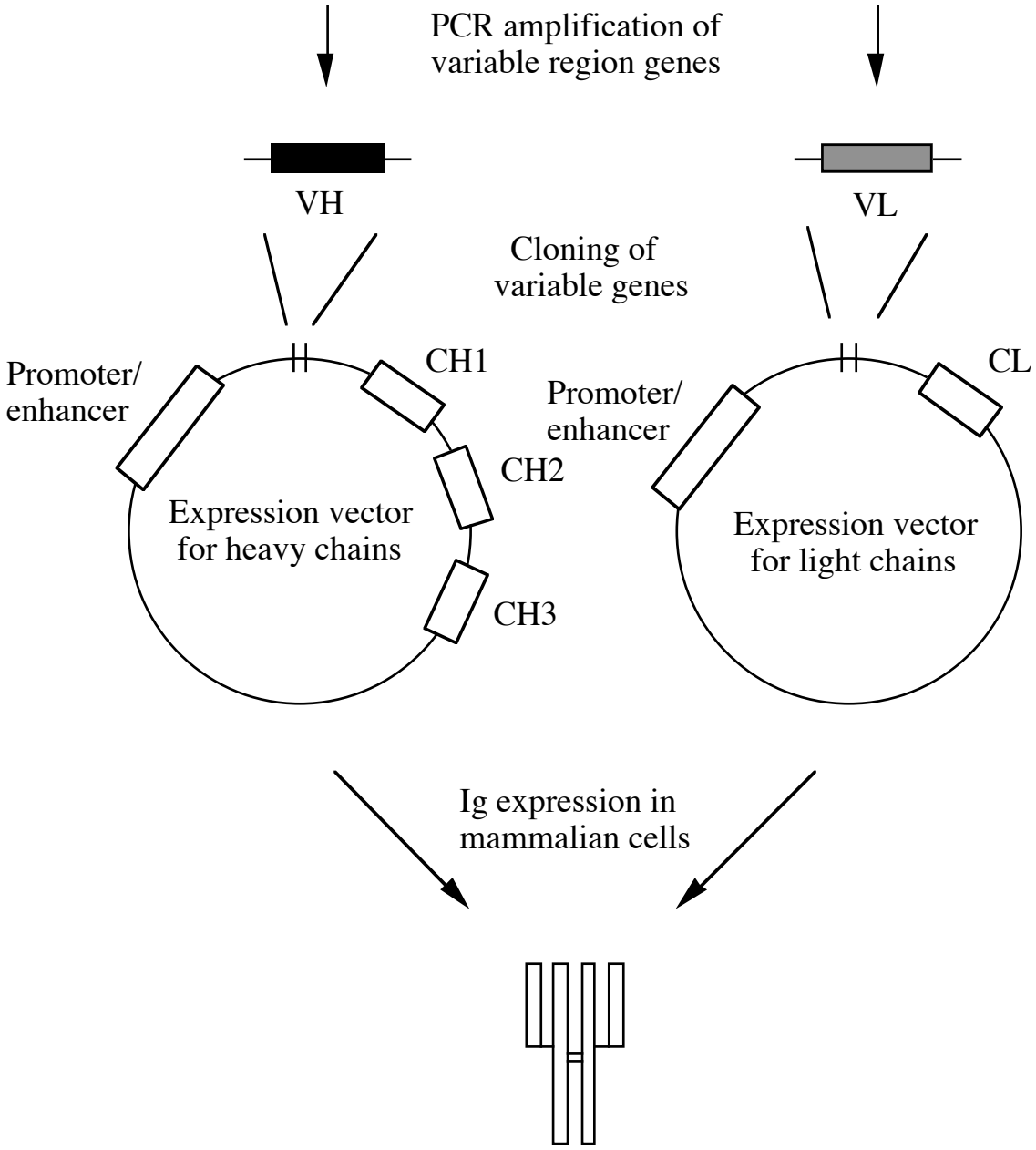

Figure 8. Eukaryotic expression of Ig genes.

The variable region genes can be obtained either as genomic DNA from a bacteriophage lambda library or as cDNA from any B cell, hybridoma or phage-display-selected Ab fragment. There are several methods for stably introducing DNA into eukaryotic recipient cells, such as protoplast fusion (Sandri-Goldin et al., 1981) or electroporation (Potter et al., 1984). Stable transfectomas are produced with frequencies of 10^{-3} to 10^{-7} depending on the recipient cell line. The vector must therefore contain a biochemically selectable marker, which permits the selection of the rare, stably transfected cell line. Two frequently used

selectable markers are the gpt-gene (bacterial xanthine-guanine phosphoribosyltransferase gene) or the neo-gene (aminoglycoside phosphotransferase gene). Both enzymes, when expressed, endow a dominant biochemically selectable phenotype to mammalian cells so that they can be used for selection in non drug-marked recipient cells.

There are two possible ways of creating an eukaryotic expression vector, to use either viral or endogenous regulatory elements. Viral promoter/enhancer pairs from cytomegalovirus (CMV) (Bebbington et al., 1992) and SV40 (Morrison et al., 1995) have been used successfully to achieve high levels of Ab expression. Viral based vectors can be transfected into either myeloma cell lines or Chinese hamster ovary cells (CHO). The Ig gene expression was for a long time thought to be regulated by the V promoter and the intron enhancer. Therefore most vectors based on endogenous control elements use this promoter/enhancer combination. The rate of Ab secretion attainable using these vectors is generally considerably lower than those of comparable parent hybridomas (Nakatani et al., 1989; Walls et al., 1993). Recent observations indicate that other enhancer elements are important for Ig gene expression. Including the 3'E to the IgH vector increased the IgH expression five fold (Mocikat et al., 1993; Mocikat et al., 1995a; Paper V). Vectors based on endogenous control elements are also transfected in myeloma cells.

Gene amplification can be used to increase the level of Ig expression. Two alternative selectable markers have been described in Ig expression vectors, the glutamine synthetase (GS), and the dihydrofolate reductase (DHFR). Cointroduction of heavy and light chain constructs with subsequent amplification using the DHFR-marker resulted in as much as 25-fold increase in secretion of intact Ab relative to unamplified cells (Dorai and Moore, 1987). The GS-marker in combination with a vector, based on viral control elements, containing both heavy and light chain, also resulted in higher secretion levels of Ab (Bebbington et al., 1992).

Using eukaryotic Ig expression vectors it is also possible to introduce novel functions not normally found in the Ig molecule. To achieve optimal effector functions either the constant region can be changed into an other region

(reviewed by Shin et al., 1992; Brekke et al., 1993) or the IgG1 can be converted into a pentamer IgG1 (Smith and Morrison, 1994). It is also possible to produce fusion proteins with e. g. cytokines (Morrison et al., 1995).

One alternative way of producing chimeric Abs is to, through homologous recombination, introduce the desired human constant gene segment into the Ig loci of any hybridoma cell line of interest. The advantages of using this system is that all regulatory elements needed are already in the loci and the variable gene does not have to be isolated from the hybridoma and finally that one recombination vector can be used for all specificities. There are two kinds of recombination vectors available, replacement or integration vectors (Capecchi et al., 1989). The replacement vector contains a two-sided homology flank neighbouring the heterologous region, whereas the integration vector contains one homology flank within which the construct is linearized, thus giving rise to a duplication of the target sequence. Using these vectors, the Ig expression levels are comparable with the original hybridoma levels (Mocikat et al., 1995b; Baker et al., 1994). The disadvantage in using these vectors is a frequent occurance of clones that coexpress Ig from two species. So far this approach to produce Abs has only been done using murine hybridomas.

THE PRESENT INVESTIGATION

The aim of this work has been; (i) to increase the understanding of how the Ig gene expression is regulated and (ii) to generate and produce human monoclonal Abs.

(i) We have focused our interest on the regulatory elements in the 3' end of the IgH locus. We chose these elements because of their potential role in regulating high Ig gene expression. In paper I and II we have investigated, using transgenic mice, the 3' enhancers role in controlling Ig gene expression. In paper V we have used different regulatory elements for optimizing an eukaryotic expression vector.

(ii) Our goal has been to develop techniques for obtaining human monoclonal Abs. Different approaches have been used such as PCR immortalization of variable regions, prokaryotic and eukarotic expression. These approaches will be discussed in paper III-V.

Paper I: Elevation of immunoglobulin gene expression in transgenic animals.

In paper I we investigated if the 3'E in combination with the μE can induce higher Ig gene expression in transgenic animals. Mice harbouring rearranged IgH transgenes, potentiated by the VH promoter/μE pair (Vμ1) or by the VH promoter in combination with the μE/3'E (Vμ3) were generated. Analysis of these mice demonstrated that the specific IgH gene transcription was at least five fold higher after addition of the 3'E to the transgene. The enhancer activity is mainly found in the B cell compartment. Separation of splenic cell populations revealed that the transgene expression is restricted to in vivo activated B cells. The expression level in resting B cells are comparable between the animals, whereas the elevation in expression is significantly higher in the activated B cells in the Vμ3 mice. There is some specific IgH gene expression observed in non-lymphoid tissues as well. Non-lymphoid expression of other IgH transgenes has been observed earlier (Grosschedl et al., 1984; Arulampalam et al., 1994). The

detected expression in non-lymphoid tissues may be due to contaminating B cells. Alternatively the aberrant activity of the enhancers may reflect utilisation of trans-acting factors that are present in lymphoid as well as non-lymphoid tissues.

We have also shown that the transgene can be stimulated in a T cell dependent manner after immunization with an appropriate antigen. Both Vμ1 and Vμ3 had increased serum levels of NP-specific Abs.

The high level of transgene expression prevents rearrangement of endogenous genes, although incompletly (Weaver et al., 1985; Nussenzweig et al., 1987). The endougenous expression is higher in the Vμ1 mice as compared to the Vμ3 mice. The higher IgH gene expression in the Vμ3 mice may downregulate the endogenous expression more efficiently.

Transgene expression in a position independent but not copy-number dependent manner was observed in the Vμ3 mice. Therefore we conclude that the 3'E can be part of a potential LCR but can not function as such on its own.

In conclusion, the 3'E can upregulate Ig gene expression in transgenic mice. These findings are supported by the observations that adding the 3'E to an eukaryotic expression vector controlled by the VH promoter/μE pair increased the Ig gene expression five fold (Paper V; Mocikat 1993).

Paper II: The IgH 3' enhancer can act directly on a natural IgH promoter in vivo

In paper II we wanted to study the biological function of the 3'E. To investigate if the 3'E alone can function in conjunction with the VH promoter, mice harbouring rearranged IgH potentiated by the VH promoter/3'E pair were generated. Analysis of these mice revealed that the activity of the 3'E is restricted to lymphoid-specific tissues. There was no activity seen in non-lymphoid tissues. The serum levels of antigen-specific Abs were much higher in the transgenic

mice as compared to normal mice indicating that the transgene product is secreted into the serum.

One possible mechanism whereby the 3'E could potentiate VH promoter induced transcription over such long distances is represented by the looping model (figure 9).

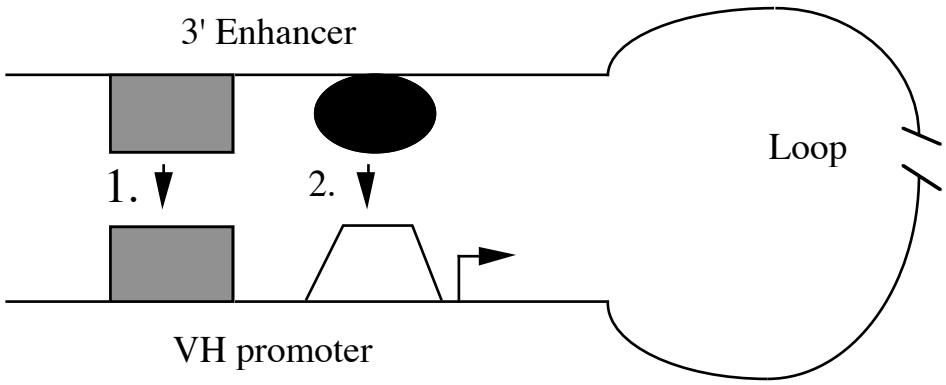

Figure 9. The looping model. Interaction of homologous (1) or heterologous (2) factors bound at the promoter and enhancer, with the looping out of intervening DNA.

This suggests that activators bound at a distance interact with proteins bound at the promoter, causing a looping out of intervening sequences. Members of the bHLH-zip family can bind to DNA as homo or heterodimers. These proteins can also form tetramers (Ferre-D'Amare et al., 1994). This finding suggests that bHLH-zip tetramers, by binding to DNA motifs in both the VH promoter and the 3'E, may provide a physical bridge between the two control regions.

Support for the possible VH promoter/3'E interaction is observations by Lieberson et al. (1995). They showed that a cell line, lacking the μE, loses the ability to express IgH gene upon the targeted deletion of the 3'E.

Paper III: Cloning of variable regions from three different human Abs that differ in their reactivity against digoxin derivates.

The relation between Ab specificity and amino acid sequences of CDR1-3, in both heavy and light chains of human monoclonal Abs, was investigated to determine if a particular VH/VL gene segment is utilized during the primary immune response against a hapten. Human monoclonal IgM Abs against digoxin were established after in vitro immunization of human peripheral blood lymphocytes. Three human monoclonal Abs were generated, which differ in their fine-specificity against digoxin and several digoxin analogues. The variable heavy and light chain regions were PCR amplified using leader sequence and constant region specific primers, and subsequently sequenced. Comparison of the DNA sequences with those of the GenBank/EMBL databases revealed that the different variable regions of the Abs belonged to different subfamilies. The complementarity determining regions of the three Abs showed considerable differences, both in amino acid sequnce and in length. Thus, despite very similar reactivity of the three Abs against digoxin there are significant variations in their amino acid sequences. We therefore conclude that binding specificity is not a consequence of an apparent restriction of particular gene segments in the primary human immune response against digoxin.

The PCR based protocol first used in this study can be a useful general tool for studies on the relationship between the structure of an Ab variable regions and its antigen binding function. The variable regions can be cloned into prokaryotic expression vectors for studies of mutagenic variants of the variable regions.

Paper IV: Generation of human Ab fragments from single B cells.

A method for generating human Ab fragments from single B cells is described in paper IV (figure 10). Two different approaches were examined

for the generation of human Abs; either (i) the direct isolation of antigen-specific B cells followed by PCR-amplification of the variable genes from this single cell, or (ii) cloning antigen-specific B cells by limiting dilution in an EL-4/CD40 based cell culture and then PCR amplification of the variable region genes. Both these approaches allow us to immortalize antigen-specific variable regions and to retain the original pairing.

The antigen-specific sIg^+ B cells were selected using antigen-coated magnetic beads. The magnetic bead rosetted B cells were isolated as single cells using a simple manual pipetting procedure. The immortalization procedure used is based on PCR technology. To minimize the number of steps involved in obtaining the desired VL/VH genes, the mRNA was isolated with oligo dT magnetic beads and thereafter the cDNA synthesis was performed directly on the beads. The sensitivity of the PCR procedure was increased using nested PCR, as compared to earlier protocols (Larrick 1989b). In the first step leader sequence and constant region specific primers were used followed by FR1 and FR4 specific primers in the second step.

For selection of specific B cells against recall antigen it is possible to use this single cell approach with direct PCR immortalization of the genes. In activated B cells the mRNA level is much higher as compared to the level in resting B cells. Therefore we developed a cellular amplification step for rare binders, e. g. primary response specificities. A combination of the EL-4 system described by Zubler et al. (1985) and the CD40 system described by Banchereau et al. (1991) was used for cellular amplification of antigen-selected B cells. This combination of cell cultures resulted in specific clones from approximately 10% of the magnetically selected B cells. The positive clones were immortalized using the PCR protocoll described above. The VL/VH from a single cell and single cell clone respectively were cloned into a procaryotic expression vector and expressed as soluble Fab fragments.

A further advantage in using the cellular amplification procedure is the opportunity to test for antigen-specificity in an ELISA. If cross-reactive antibodies are selected, the directly isolated B cells can not be analysed until the genes have been cloned into prokaryotic or eukaryotic expression vectors.

In conclusion, we have developed a method for generation of human Abs selected from single antigen-specific B cells. The approach offers the possibility of investigating the original variable region combinations and is very fast and efficient compared to conventional hybridoma technology.

Paper V: Design of an eukaryotic IgH expression vector

In paper V an eukaryotic IgH expression vector was developed using different regulatory elements. Conventional vectors rely on the VH promoter in combination with the μE. The Ig expression levels using these vectors are substantially lower than the levels achieved in the parental hybridomas. Recent findings suggested that other control elements might be important for Ig expression. Therefore we evaluated if these novel enhancer elements could contribute to higher Ig gene expression in a myeloma cell line. A backbone vector was constructed, which contains the VH gene (from a NP-specific Ab), the γ3 constant region, the VH promoter and the μE. Different enhancer elements were added to the backbone vector , such as the 3'E (1kb), 3'E (4kb) and finally the combination of the 3'E, HS3 and HS4. Inclusion of the 3'E in the backbone vector resulted in a five fold increase in Ig expression, whereas including the 3'E (4kb) or the 3'E/HS3/HS4 did not upregulate Ig expression any further. The absence of increased expression using the combination 3'E/HS3/HS4 elements is most probably related to the characteristics of the host cell used in these experiments. Observation by Michelson et al. (1995) indicated that the activity of the HS4 and 3'E are host cell dependent.

To analyse if there is a correlation between the number of enhancer elements and transcriptional enhancement, we cloned the 3'E on both sides of a reporter gene. However, no copy-number dependent increase in transcription of the gene was observed. We also assessed if the position of the 3'E in the vector was of importance, since Mocikat et al. (1995) claim that the 3'E activity is suppressed if it is located in close proximity to the μE. In our vector the position of the 3'E did not affect the expression levels.

Therefore we conclude that in our expression vector the 3'E upregulates the immunoglobulin expression in a position-independent way. This vector, designated pTIF-1 (figure 11), can be used for expression of any antibody of interest, using PCR immortalization of the variable heavy chain region. Furthermore, the constant region can be exchanged which facilitates studies of antibody effector functions.

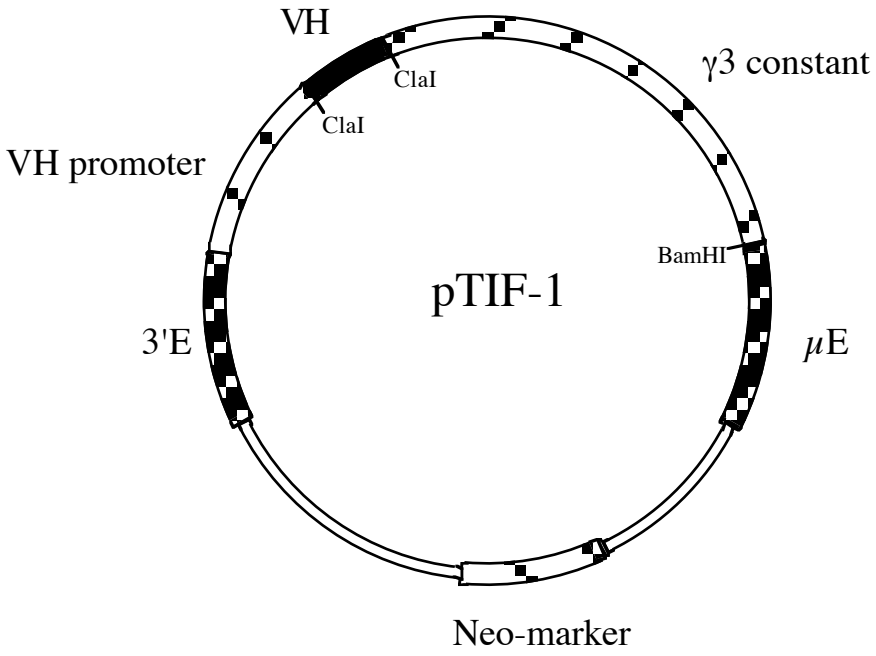

Figure 11. The eukaryotic IgH expression vector: pTIF-1.

Concluding remarks

This thesis describes studies for understanding the role of the 3' enhancer in regulation of Ig gene expression. Although progress has been made, several questions remain to be solved, such as is the 3'E, HS3 and HS4 combination a potential LCR? This can be answered after generating transgenic animals harbouring transgenes potentiated by these enhancer elements. Our eukaryotic expression result was less elucidating regarding these control elements. Preliminary data, however, indicate that the expression is cell line dependent, since we do see an upregulation of Ig gene transcription in the cell line S194, but not in J558L. Therefore I conclude that a careful investigation regarding the cell line used for transfection of eukaryotic expression vectors needs to be done. The optimal cell line would be a human cell line to achieve a proper glycosylation pattern. Unfortunately, the stable host cells available for transfection are of mouse x human origin at best.

The different approaches possible for achieving human Abs are expanding all the time. In this thesis I have described some of the approaches to generate and produce human Abs. It would be a challenge to combine some of these approaches to achieve a high affinity Ab against desired antigen. For example combining the methods described in paper IV and in paper V could be one approach. If the Ab of interest is derived from a primary response, an intermediate step involving affinity maturation of the genes will be necessary. The advantage in using the approach in paper IV is that the Ab retains the original VH/VL pairing which is nomally lost in combinatorial antibody libraries.

In conclusion, I do believe that in the near future a whole range of possible therapeutic agents based on the Ab molecule will be available.

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