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THE UNIVERSITY OF ALBERTA

TRANSCRIPTIONAL REGULATION OF THE T CELL RECEPTOR β CHAIN GENE

by

HELEN MARIA VERHESEN MESSIER

A thesis submitted to the faculty of graduate studies and research in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MEDICAL SCIENCES (IMMUNOLOGY)

DEPARTMENT OF IMMUNOLOGY

EDMONTON, ALBERTA

SPRING 1994

ISBN 0-315-95236-9

UNIVERSITY OF ALBERTA

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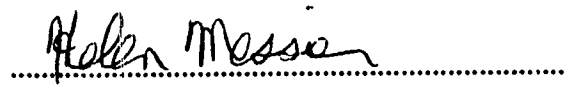
NAME OF AUTHOR:	HELEN MARIA VERHESEN MESSIER
TITLE OF THESIS:	TRANSCRIPTIONAL REGULATION OF THE T CELL RECEPTOR β CHAIN GENE
DEGREE:	DOCTOR OF PHILOSOPHY
YEAR DEGREE GRANTED:	SPRING 1994



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April 22, 1994

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FACULTY OF GRADUATE STUDIES AND RESEARCH

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TRANSCRIPTIONAL REGULATION OF THE T CELL RECEPTOR β CHAIN GENE

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In partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in Medical Sciences (Immunology)

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Date: November 15, 1993

DEDICATION

To

Tom

Mom and Dad

Thank you!

ABSTRACT

Transcription of the TCR β gene is regulated by multiple cis-acting elements. An AP-1 site and an adjacent inverted repeat (IR) in the Vβ2 promoter region are required for optimal TPA inducibility. I have demonstrated that jun/fos heterodimers bound to the Vβ2 AP-1 motif with a lower affinity as compared to the collagenase AP-1 motif. The 3' flanking IR bound the ets transactivator but not jun/fos heterodimers. Therefore, the individual contributions of jun/fos and ets transactivators explains the demonstrated cooperativity between the AP-1 and the 3' flanking sequence to confer optimal TPA inducibility to the Vβ2 promoter.

I have identified the inhibitory region as a silencer that contained a nuclear protein binding site, was orientation independent, and transferable to a heterologous promoter. There was a hierarchical interaction between the three cis-acting elements in the TCR β gene. The AP-1/IR element was capable of conferring TPA inducibility to the Vβ2 promoter. The region upstream from -739 to -343 abrogated basal as well as TPA inducible transcription. The presence of the β enhancer in cis overrode the effect of the inhibitory region, and was the only tissue specific cis-element.

The E3 motif within the core TCR β enhancer was found to be essential for enhancer activity. The p70 lupus autoantigen bound to the E3 motif in a sequence-specific manner. The p70 protein was unable to bind to an E3 mutant having reduced enhancer activity.

TCFβ1, a novel POU domain protein was identified by its binding to the critical E4 motif in the β enhancer. TCFβ1 represents a new class of POU

domain proteins. The expression of TCFβ1 in T cells and its ability to bind to multiple motifs in the TCR β enhancer support its role in regulating TCR β gene expression. The ability of recombinant TCFβ1 to bind octamer and octamer-related motifs, the expression of TCFβ1 in B cells, and the ability of TCFβ1 to transactivate mRNA promoters in a sequence-specific manner suggest that TCFβ1 has additional roles in regulating lymphoid gene expression.

Preface

Part of the work described in this thesis has been previously published in *The Journal of Immunology* (149:1980-1986, 1992), *Proc. Natl. Acad. Sci. USA* (90:2685-2689, 1993), and *Molecular and Cellular Biology* (13:5450-5460, 1993).

ACKNOWLEDGEMENTS

There are many people I am grateful to for their help, support and friendship.

Arun, we've come a long way together, thanks for sometimes pulling, and sometimes pushing me along. Thank you to my supervisory committee, Larry Guilbert, John Elliot, Tim Mosmann, Linda Pilarski, Hanne Ostergaard, and Charlotte Spencer for your guidance and concern. I appreciate the extra effort that was needed to facilitate a long distance degree. A special thanks to Richard Maki for your time and encouragement.

To everybody I worked with during the "daily grind" both in Edmonton and La Jolla, thank you for your advice, technical help, support and friendship, especially Sunil Mangal, Sak Igarashi, Anna Fu, Pat Kilgannon, Jitra Ratanavongsiri, Therese Fuller, Howie Brickner, Jody Flatt, Pat Fitzgerald, Joel Gaikwad, and Shailaja Kasibhatla.

Thanks so much to my parents, Peter and Barbara Verhesen, you have always been my source of stablility and love.

Tom, words cannot thank you enough for everything you've done. You've provided me with unending help, support, patience, advice, inspiration, encouragement, and love. This one's for you.

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LIST OF ABBREVIATIONS

AP-1	activator protein-1
ATF	activating transcriptional factor
B cell	bone-marrow derived cell
bp	base pair
BSA	bovine serum albumin
C	constant region
CAT	chloramphenicol acetyl transferase
CBF	core enhancer binding protein
CRE	cyclic AMP responsive element
D	diversity region
DMSO	dimethylsulfoxide
DN	double negative
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DNase I	deoxyribonuclease I
DP	double positive
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HLH	helix-loop-helix
Ig	immunoglobulin
IL-2	interleukin 2
IR	inverted repeat
J	joining region
kb	kilobase

kDa	kilodalton
MHC	major histocompatibility complex
μg	microgram
μl	microlitre
mM	millimolar
M	molar
mRNA	messenger RNA
NF	nuclear factor
nt	nucleotide
PEG	polyethylene glycol
Pu	purine
Py	pyrimidine
RNA	ribonucleic acid
SCID	severe combined immunodeficiency syndrome
SDS	sodium dodecyl sulfate
SLE	systemic lupus erythrematosus
SV40	simian virus 40
T cell	thymus derived cell
TCFβ1	T cell factor β1
TCR	T cell antigen receptor
TK	thymidine kinase
TLC	thin layer chromatography
TPA	12-o-tetradecanoyl-phorbol-13-acetate
TRE	TPA responsive element
V	Variable region

Chapter 1

INTRODUCTION

The mammalian immune system has evolved to protect the body from infectious pathogens. Although many different cells are involved in an immune response, lymphocytes are unique because they recognize antigen and this ensures the specificity of an immune response. Lymphocytes are composed of two classes; 1) B cells, which function as effector cells in the humoral immune response by producing antibodies; and 2) thymus dependent lymphocytes or T cells, which are involved in the cell-mediated portion of the immune response, such as delayed-type hypersensitivity, graft-vs-host disease, elimination of virally infected cells, and transplantation reactions. In addition, T cells are the dominant regulators of immune responses.

T cells recognize antigen only in association with self-major histocompatability complex (MHC) molecules via the heterodimeric T cell receptor (TCR) on the T cell surface (1). B cells, on the other hand, recognize native soluble antigen via their cell-surface immunoglobulin molecules, and respond by secreting antibody molecules with the same recognition specificity. The major histocompatibility co-recognition process ensures that T cells respond to foreign antigen only when it is present on an appropriate target cell.

There are two main classes of MHC molecules. The ubiquitously expressed class I MHC presents antigen from intracellular sources like viral proteins. Class II MHC, which is expressed by a subset of antigen presenting cells (APC's), presents fragments of extracellular foreign antigens. The T cell

accessory molecules CD8 and CD4 interact with MHC class I and class II respectively, correlating with T cell restriction subsets (2).

T Cell Development T cells are derived from hematopoietic precursor cells in the bone marrow that migrate and colonize the thymus as thymocytes (3). In the thymus, thymocytes develop into mature T cells. Thymocyte maturation consists of an ordered sequence of developmental stages that can be characterized by their expression of cell surface molecules. Based on their expression of CD4 and CD8, thymocytes can be divided into subsets. Upon entering the thymus, the cells proliferate extensively. On day 14 of murine gestation, most thymocytes are $CD4^-CD8^-$ double negative (DN) blast cells. By day 16 the cell-surface expression of CD4 and CD8 molecules can be seen.

Cells that express only low levels of CD8 but lack CD4 ($CD4^-CD8^{lo}$) on their surface, appear first, followed by cells which express high levels of both CD4 and CD8 molecules ($CD4^+CD8^+$, double positive) cells (4). The double positive cells arise from the $CD4^-CD8^{lo}$ cell population as suggested by the fact that $CD4^-CD8^{lo}$ cells mature into double positive cells spontaneously in vitro. The administration of anti-T cell receptor antibodies in vivo, however, will prevent this differentiation indicating a role for the T cell receptor in T cell development (5). Although at this stage T cell receptor expression is very low, the TCR plays an important role in regulating early T cell development. This will be discussed further under the ontogenic expression of TCR genes.

The expression of $CD4^+CD8^+$ double positive cells is followed by the appearance of $CD4^+$ and $CD8^+$ single positive cells within the next few days. The majority of functional mature T cells are of the $CD4^+$ or $CD8^+$ single positive phenotype. The change from double positive to single positive cells is accompanied by an increased expression of T cell receptor molecules on the

cell surface and a decrease in size from large blast cells to small lymphocytes. The change from a low to a high level cell surface expression of T cell receptor correlates with thymic selection events as discussed below (6).

The capability of a single DN stem cell to generate all four subsets of thymocytes indicates that double negative cells are the precursors of mature T cells (7). The next developmental step is less clear. It appears that double positive rather than double negative cells give rise to the mature single positive cell. Treatment with anti-CD8 monoclonal antibodies in vivo, blocks the development of $CD4^+$ cells indicating that single positive cells develop from $CD8^+$ precursors, most likely the double positive cells (8). Additionally, mice transgenic for a self reactive T cell receptor gene will arrest thymocyte development at the double positive stage indicating that the $CD4^+CD8^+$ stage is part of the developmental pathway (9). After the double positive cells are generated, T cell receptor specificities are selected in the thymus.

Thymic Selection Events The vast majority of double positive thymocytes die in the thymus rather than exiting to the periphery (3). This cell death is attributed to the selection processes occuring in the thymus. A negative selection event assures tolerance for self-antigen by eliminating all T cells with TCR's having a high affinity for self-antigen/MHC (10). A positive selection event targets only those T cells capable of recognizing antigen in association with self-MHC (11). Therefore, only T cells expressing a receptor that is self-MHC restricted as well as tolerant to self antigens will mature and exit the thymus as single positive cells. The expression and specificity of the T cell receptor, therefore, controls T cell maturation.

The focus of this thesis is on the regulation of T cell receptor β gene transcription. This review will address three areas that are relevant to an

understanding of T cell specific gene expression. The first part deals with our current understanding of the T cell receptor. The second section is an overview of general gene expression emphasizing transcriptional regulation. The final section is a discussion of the general rules of lymphoid specific gene expression.

THE T CELL RECEPTOR FOR ANTIGEN/MHC

The ability to grow antigen specific T cells clonally in culture provided the means to identify the antigen specific T cell receptor (12). Monoclonal antibodies were produced that recognized the clonotypic antigen-specific T cell receptor. The antibodies were assayed by their ability to block antigen specific activation of the T cell clone immunogen while not affecting other T cells (13). These antibodies were used to immunoprecipitate the T cell receptor molecules, and were found to be composed of acidic α chain and basic β chain glycoproteins with a core size of approximately 30,000 kDa (14).

The genes encoding the β chain of the T cell receptor were cloned by subtractive hybridization with B cell RNA (15). The cloning method was based on the assumptions that TCR genes are expressed exclusively in T cells, they undergo rearrangement solely in the same cells, and because they are cell surface molecules, they are part of the membrane-bound polysomal fraction.

The TCR α chain genes were cloned subsequently. T cell receptors are composed of homology units that in evolutionary terms relate them to B cell immunoglobulins as part of the immunoglobulin (Ig) supergene family. Each homology unit is ~110 amino acids long, has a disulphide bridge and a characteristic Ig fold consisting of two sheets of anti-parallel β strands. Pairs of homology units fold to create discrete polypeptide domains (16).

The confirmation that the genuine T cell receptor genes had been cloned came when the α and β genes from a particular T cell clone were transfected into T cells of a different antigen/MHC specificity. Only those transfectants that express both the introduced α and β genes contributed by the original T cell clone respond specifically to the appropriate antigen/MHC pair. Therefore, T cell specificity is defined as the recognition of both antigen

and MHC by a single T cell receptor composed of the α and β TCR chains (17, 18, 19).

Five other polypeptide chains, collectively referred to as the CD3 complex are structurally associated with the α/β TCR. The CD3 polypeptide chains co-immunoprecipitate with anti-TCR antibodies (20). The CD3 complex is required for the transduction of a signal from the T cell receptor leading to T cell activation (21, 22).

Although a majority of T cells express the α/β TCR, approximately 5% of mature T cells express the γ/δ TCR. The γ/δ receptors are expressed on the surface of subpopulations of T cells in both the thymus and the periphery. Their function, however, remains largely speculative. Like the α/β heterodimer, the γ/δ polypeptide chains are intimately associated with CD3 (23, 24).

The γ/δ and α/β T cells comprise separate lineages and follow different developmental pathways. This was demonstrated by mice with a targeted disruption of the TCR β chain gene. These mice lack a functional TCR β chain gene. α/β^+ T cells are not present in these mice, however, the γ/δ^+ T-cells are normal. Conversely, a knockout of the γ chain gene in mice has no effect on the development of α/β T cells. These data suggest that the generation of the α/β and the γ/δ lineages are mutually independent (25). Possible molecular mechanisms controlling α/β or γ/δ T cell lineage specific separation are discussed later.

Genomic Organization of T Cell Receptor Genes

Each TCR β polypeptide chain is composed of variable (V), diversity (D), joining (J), and constant (C) regions; the α chain has a similar composition but lacks the D region. The fine specificity of the α/β

heterodimer is determined by variable sequences at the amino-terminus. The V, D, and J gene segments are separated on the germ line chromosome and must undergo developmentally controlled genetic rearrangement events in the thymus before a functional transcript is produced. Subsequent to transcription, RNA splicing occurs to generate a mature mRNA transcript (26, 27).

β Chain The TCR β chain genes are found on chromosome 6 in humans and 7 in mice. There are two Cβ genes, each with 4 coding exons. The first two exons encode the external constant region domain, the third encodes the transmembrane domain, and the last exon encodes the cytoplasmic domain and the 3' untranslated region. The nucleotide and predicted amino acid sequences of the two Cβ genes are very similar to each other, and no functional differences between the two Cβ genes have been identified. Upstream of each C region is a cluster of seven Jβ regions and one Dβ region (28, 29). Each cluster contains one pseudo-Jβ gene.

Further upstream there are approxin ately 30 Vβ gene elements. The 5' most Vβ gene segment is Vβ2 which is approximately 800 kb 5' of the Cβ gene segment (30). The Vβ14 gene segment is located downstream of Cβ2, and requires an inversion event in order to be rearranged (31). Each Vβ gene segment is composed of two exons. The first exon encodes the leader sequence, while the second exon encodes the final part of the leader sequence and the rest of the variable domain. Most murine Vβ genes show little relation to each other and exist as single member gene families. Two three-member Vβ gene families comprise the exception (32). The human β-chain complex contains approximately 57 Vβ gene segments contained in 24 families (33). Vβ8 is the largest of these families consisting of 5 members (34).

α Chain The α-chain gene complex is located on chromosome 14 in both mice and humans. The α chain locus contains a single C region gene segment composed of four exons. Each exon encodes a separate domain. The four domains are 1) the constant region domain, 2) the region that forms the intrachain disulfide bond (characteristic of the Ig supergene family), 3) the transmembrane and intracytoplasmic domain, and 4) the 3' untranslated region (26). Located upstream of the C region gene segment are approximately 50 Jα region gene segments spread over 70 kb of DNA (35). The Vα gene elements are even further upstream and consist of approximately 100 segments comprised of up to 15 cross-hybridizing gene families. Like the Vβ elements, each Vα element consists of two exons.

γ and δ Chains The TCR γ chain gene is on chromosome 13 in mice and chromosome 7 in humans. It consists of Vγ, Jγ, and Cγ gene segments. The δ chain genes are unique because they are located within the α chain locus between the Vα and Jα elements. Rearrangements between Vδ, Dδ, and Jδ gene segments generate a functional TCR δ chain transcript (36).

Rearrangement

The T cell receptor genes rearrange in a manner reminiscent of immunoglobulin genes. The TCR β chain gene rearrangement precedes α chain gene rearrangement which is similar to the staggered rearrangement of heavy and light chain Ig genes. One major difference is that functional TCR gene rearrangement is restricted solely to T cells. All subsequent steps in the process of T cell development are contingent upon the successful rearrangement of the T cell receptor β, then α chain genes. Partial TCR β

rearrangement is seen in the earliest double negative thymocyte population (37), suggestive of the importance of the β chain in controlling further developmental events. Evidence from mice in which the TCR β chain has been disrupted indicate that in the absence of TCR β rearrangement the thymocyte developmental transition from double negative to double positive cells does not occur (25). Rearrangement of the TCR α locus is needed subsequently for the double positive to single positive transition (25).

Recombination Sequences Adjacent to each V, D, or J region is a conserved heptamer and nonamer sequence, separated by 12 or 23 base pairs (bp). Mostly based on work on Immunoglobulin genes the 12/23 rule of gene rearrangement has been formulated. A heptamer-nonamer motif spaced by a 12 bp nonconserved sequence rearranges to a 23 bp heptamer-nonamer motif. TCR genes also obey this 12/23 rule (39). In α and γ genes, these sequences regulate V/J rearrangement, whereas in β and δ genes an initial D/J rearrangement event is followed by V/DJ rearrangement. In the β chain locus the adjacent sequences make it possible for a Vβ to rearrange directly to a Jβ region or for two Dβ segments to join (40, 41, 42). The joining of two Dδ regions is also possible in the δ chain (43). Most of these rearrangements follow the looping-excision model in which the intervening sequences of DNA are deleted and can be detected as pieces of extra-chromosomal circular DNA (44, 45).

The TCR heptamer-nonamer sequences act as binding sites for site-specific recombinases and are interchangeable with the immunoglobulin heptamer-nonamer sequences. Possible candidates for the site-specific recombinases are recombination activation genes, RAG1 and RAG2. A targeted disruption of either RAG1 or RAG2 genes in the mouse germline

results in no V(D)J recombination and thus no mature B or T lymphocytes are generated is such animals (46, 47).

Rearrangement is Tissue Specific Since the recombination proteins, RAG1 and RAG2, and the sequences they recognize, the heptamer, and nonamer, are shared by both T and B cells, additional control elements must be present in T or B cells to ensure tissue specific rearrangement (48). According to the following observations, the controlling elements must reside in the respective TCR or Ig loci. 1) In mice transgenic for a TCR β mini-locus in germline configuration, the presence of the TCR Vβ, Dβ, Jβ and Cβ gene sequences in conjunction with the β enhancer were sufficient for the mini-locus gene segments to rearrange in a T cell-specific manner (49). 2) In mice transgenic for the Igκ gene, the presence of a cis-DNA element encompassing the 3' enhancer is required to restrict rearrangement of the transgene to the appropriate stage of of B cell development (50).

Order of α/β Rearrangement Although TCR β rearrangement normally precedes that of α (51), the initiation of β chain and α chain rearrangement is under separate control. This separate control of rearrangement can be seen in α and β knockout mice in which the rearrangement and expression of the β chain is not necessary for α chain rearrangement and transcription. Conversely, the α chain negative mice still rearrange and express the β chain gene (25).

The productive rearrangement and expression of the TCR β chain gene at one chromosomal locus is associated with the termination of rearrangement of the β chain on the second chromosome. This process of allelic exclusion was demonstrated in mice transgenic for a rearranged TCR β

gene. In these mice the presence of the β transgene inhibits rearrangement of the endogenous TCR β genes (52). Allelic exclusion ensures that each T cell expresses only one TCR specificity. Further rearrangement of the α chain gene is halted by the engagement of the α/β TCR on the double positive thymocytes during positive selection. The cessation of rearrangement is accomplished by the termination of RAG1 and RAG2 expression (53). The treatment of TCR+ human thymocytes with T cell activating agents such as antibodies that cross-link the TCR/CD3 complex or PMA plus a calcium ionophore results in greatly reduced levels of RAG transcripts (54). In addition, there is a dramatic decrease in thymic RAG1 expression in transgenic mice possessing a positive selecting MHC, but in mice with non-selecting thymi, high levels of RAG1 mRNA are still expressed (55).

Rearrangement and Transcription The stage, tissue, and allele specificity of rearrangement and the subsequent surface expression of the TCR chains are under transcriptional control. A number of experimental observations lend support to this. Transcription of unrearranged Ig and TCR genes always correlates with rearrangement, such that immediately prior to rearrangement sterile transcripts of unrearranged V and C gene segments are detected (56). These transcripts are not translated and are thus referred to as sterile. The presence of a short 1.0 kb β gene transcript during development demonstrates that an incompletely rearranged locus is being transcribed (57). It was demonstrated in mice transgenic with germline T cell receptor VDJ genes linked to the Ig Cμ enhancer (Eμ), that transcriptional activation via the Eμ is precisely correlated with recombination (58).

Alt and colleagues put forward the accessibility model to explain the dependence of recombination on transcription (58). This model suggests that

the act of transcription physically opens up the TCR or Ig chromosomal locus providing access to the recombination machinery. Cis-acting transcriptional regulatory elements are predicted to modulate this accessibility.

Generation of Diversity

There are three basic mechanisms used in generating diversity so that a limited number of gene segments can produce an essentially unlimited number of T cell receptor specificities. The mechanisms include recombinatorial diversity, combinatorial associations, and junctional diversity (26). Recombinatorial diversity is achieved by the random joining of the V-J or the V-D-J gene elements to produce a large number of different α and β chain genes. Combinatorial association is the random pairing of an α chain and a β chain to form heterodimers with a great variety of receptor-combining sites. Taken together, these two diversity generating mechanisms can potentially form 10^7 different TCR combinations (59). This huge potential for diversity is further increased by junctional diversity. Junctional diversity includes the imprecise joining of the gene segments, N-region diversity, and P-element diversity. N-region diversity is the random addition of nucleotides at the junctions of the germline encoded elements during rearrangement. This is probably mediated by terminal deoxynucleotidyl transferase (TdT) (60). P-element diversity is the template coded addition of nucleotides at the recombination junctions. P regions are usually 3 to 4 nucleotides in length (61).

The differences in the generation of TCR diversity compared to that of immunoglobulins is two-fold. The first difference is the absence of somatic hypermutation in T cell receptor genes. The second difference is that the TCR

Dβ gene segments can be joined to the Vβ's in all three translational reading frames, while in Ig genes each D_H is joined in only one reading frame (16).

Ontogenic Expression of TCR Genes

TCR γ/δ Expression The transcription, rearrangement and expression of the T cell receptor genes is developmentally controlled during thymic maturation. The first T cells detected during ontogeny are those expressing γ/δ T cell receptors on or before day 14 in the murine fetal thymus. At this time 6 - 7% of fetal thymocytes bear γ/δ receptors. The rearrangement and expression of γ/δ genes in T cells occurs in waves which subsequently exit the thymus and populate specific areas of the periphery (43).

The Importance of TCR β Expression On day 14 of murine gestation, the presence of a 1.0 kb transcript consisting of a rearranged DβJβ signifies the start of TCR β rearrangement (62). On day 15 Vβ to DβJβ rearrangement takes place, associated with the presence of 1.3 kb β chain transcripts (63, 64). By day 16, β chain expression is much higher and β/β homodimers are expressed on the cell surface in association with CD3 (65). The TCR β/β homodimer controls a number of developmental events. These events include the expansion of immature thymocytes, the initiation of CD4, CD8 gene expression and TCR α transcription, and the suppression of further TCR β rearrangement (66).

The requirement of the TCR β chain gene in thymocyte development was demonstrated by a number of experiments. In TCR α and β knockout mice, the presence of a functionally rearranged β chain only is necessary and sufficient to drive the double negative to double positive thymocyte transition and to expand the pool of thymocytes (25). T cell development in

rearrangement deficient SCID mice is arrested at the double negative stage. When these mice are crossed with mice expressing only the TCR β chain transgene, rescue of the DN cells to generate DP cells occurs (67, 68). Thymocytes in RAG1 or RAG2 deficient mice, like in SCID mice, are blocked at the double negative stage. The introduction of a β transgene into these mice results in the expression of the β gene on the cell surface and the complete restoration of both thymocyte number and double positive cells (25, 69).

Subsequent to β chain expression, TCR α gene transcription is detectable, and on day 17 there is an increase in α/β $CD4^+CD8^+$ T cells along with a proportional decrease in γ/δ cells. At this time the levels and specificity of α/β TCR's and the expression of accessory molecules involved in the process of selection, most notably CD4 and CD8, become important. The expression of the α/β TCR is associated with the double positive to single positive cell transition. Mice in which the α chain is "knocked out", display double positive arrest (25), and the introduction of both α and β transgenes into RAG deficient mice developmentally rescues the single positive cells (69).

THE REGULATION OF GENE EXPRESSION

The process by which a multicellular organism develops from a single cell involves a complex series of events. Cells must be capable of responding to developmental and environmental stimuli as well as generating and maintaining a differentiated state. These events are regulated by fluxes in the pattern of gene expression. Differential gene expression is regulated in multiple ways including transcriptional initiation, RNA processing, RNA transport from the nucleus to the cytoplasm, translation, the rate of mRNA degradation, and posttranslational modification of the resulting proteins. This portion of the review will focus on the regulation of transcription, which is a primary mechanism involved in controlling differential gene expression.

Two functionally distinct DNA sequences, the promoter and enhancer regions, are essential for regulating transcription by their interaction with sequence-specific DNA binding proteins (70).

Promoters

DNA fragments which are both necessary and sufficient to initiate basal transcription are called promoters (71). Promoters contain the start site for initiation of transcription and are defined as the regions of DNA involved in binding RNA polymerase II to signal where transcription should begin. Several kinds of promoter elements exist. The AT rich TATA box is required for basal transcription and ensures the correct initiation of transcripts. Additional elements located upstream of many promoters (upstream promoter elements) such as the CCAAT box tend to increase the rate of transcription (70). The activity and regulation of mRNA promoters are

mediated by general transcriptional initiation factors that interact with the minimal or core promoter elements of all mRNA genes, and by gene specific factors that recognize particular DNA sequences.

Mechanisms of Basal Transcription The proper initiation of transcription from promoters requires the participation of both RNA polymerase and additional accessory proteins known as general or basal transcription factors. The basal transcription reaction is centered on the TATA box and is generally considered to be common to the core promoters of all genes transcribed by RNA polymerase II (72). The first step in the transcriptional process is the sequence-specific binding of TFIID to the TATA box to form a template-committed pre-initiation complex. Commitment of the template ensures that the promoter remains active during nucleosome assembly (72, 73). TFIID is made up of the TATA binding protein (TBP) and a number of TBP associated factors (TAFs). The importance of TBP in transcription is suggested by the observations that TBP in association with various TAFs is required for transcription by all three RNA polymerases (74), and that 70% of the 180 C-terminal amino acids of TBP are identical in all eukaryotes (75). TBP is also important in the transcription of "TATA-less" RNA polymerase II promoters such as the "initiator" promoter of the terminal deoxynucleotidyl transferase gene (76). The next step in transcriptional initiation involves the sequential binding of a number of general transcription factors. TFIIB and RNA polymerase II both bind to the pre-initiation complex. RNA polymerase II is escorted to the complex by TFIIF. Pol II-TFIIF binding is followed by the addition of TFIIE, TFIIH, and TFIIJ to the complex (77). ATP is also required as an energy source during transcriptional initiation. RAP30/74 is TFIIF which is associated with an

ATP-dependent helicase activity whose likely role is to melt the DNA template to facilitate transcription (78). The initiation of transcription is characterized by the formation of the first phosphodiester bond of the RNA.

The carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II can be extensively phosphorylated. Interestingly, the nonphosphorylated form is part of the pre-initiation complex, while the phosphorylated form is part of the elongation phase. It was also observed that only the dephosphorylated form of the CTD interacts with TBP. Therefore, phosphorylation of the CTD may be needed to trigger the conversion of an initiation complex into an elongation complex by releasing RNA polymerase II from the preinitiation complex to start transcription (79, 80). A number of kinases capable of phosphorylating the CTD exist in the cell. These include TFIIH and a kinase associated with the Ku lupus autoantigen (79, 81).

Following transcriptional initiation, TFIID remains bound to the template and may be involved in the initiation of multiple rounds of transcription (82). The final step in transcription is elongation of the RNA transcript by RNA polymerase II. The elongation reaction may be modulated by the premature termination of transcription (83), or by an increase in the efficiency of elongation associated with transcription factors like TFIIS (84).

The rate of basal transcriptional initiation from the core promoter is regulated by sequences that act in a direction and orientation independent manner. These sequences can either upregulate (enhancers) or downregulate (silencers) basal transcription.

Enhancers

Enhancers are operationally defined as cis-acting DNA elements that increase the rate of transcription initiation from the start site of a linked

promoter in a distance or orientation independent manner (85). Upstream promoter elements, discussed previously also increase the rate of transcription but are dependent on their orientation and distance from the promoter. Both enhancer and upstream promoter elements bind sequence-specific DNA binding proteins that mediate their effects.

SV40 Enhancer The first transcriptional enhancer identified was an element ~110 bp upstream of the early simian virus 40 (SV40) promoter. The element consists of two identical sequences of 72 bp each, repeated in tandem (86, 87). Deletional mapping shows that the presence of either repeat by itself is adequate for increasing the rate of transcription, but the removal of both repeats results in a drastic decrease in gene expression. The SV40 enhancer also stimulates transcription when linked to a heterologous β-globin promoter. Subsequently, additional enhancers were identified in many other viral and cellular genes. They are located 1) upstream of the promoter as in SV40, 2) in the middle of an intron as in the immunoglobulin heavy chain gene (88), or 3) downstream of the gene as in the β-globin gene.

Detailed analysis of the SV40 enhancer elements reveals a structure that is common to other enhancers. The single 72 bp element has a modular structure, composed of three functional units of 15-20 bp each. In a reversion analysis of the SV40 enhancer, specific units or modules were mutated to reduce overall enhancer activity. Genetic revertants displaying increased levels of transcription were selected. Enhancer function is restored by the spontaneous duplication of the unmutated modules (89). A minimum of two enhancer modules is necessary for enhancement and duplication of any one module fully restores enhancer activity. In other words, each module cooperates with another module to enhance transcription, or acts

autonomously when present in multiple tandem copies (90, 91). The modular structure of cellular enhancers can be very complex, allowing transcription to be regulated in response to diverse signals, and also facilitating diverse expression patterns.

Enhansons Further analysis of the 15-20 bp enhancer modules reveals that they are composed of short, discrete sequences of DNA. These short DNA sequences are termed enhansons and appear to be the basic units of enhancer structure (92). Various types of enhansons function differently. One type of enhanson constitutes a functional enhancer module when present as a single copy. A second type of enhanson forms a functional enhancer module only when present as a tandem repeat. This type of enhanson displays strict spacing requirements between each element. A third type of enhanson forms a functional module only in combination with a different non-identical enhanson (93)

Each enhanson probably acts as an individual binding site for sequence-specific transcriptional activator proteins (94). The gradation of weak to strong enhancer activity is therefore regulated by the number and affinity of protein binding sites and the type of transcriptional activator that binds. The unique cell-specific enhancer activity of each of the SV40 enhancer elements results from the cell-specific activities of the proteins that bind to them (95). The combination, abundance, and interaction of all the proteins bound to a specific enhancer determines its range of expression.

Many enhancer modules appear to act synergistically with other modules or enhansons. Synergism is a cooperation between two enhancer elements that results in a greater enhancement of transcription than the sum of the individual contributions of each element (96). Synergism may be

achieved by the cooperative binding of adjacent proteins via a direct protein-protein interaction that increases their DNA binding affinities. For example, the synergistic effects of two palindromic steroid receptor binding sites results from the cooperative binding of the corresponding steroid hormone receptors (97). Synergism may also be functional, in which the synergistic elements are too distantly separated on the DNA to allow for direct contact between the bound proteins. An example can be found in the human beta interferon promoter in which the binding of two transactivators synergistically activates the promoter better than either transactivator alone (98).

Negative Regulation Negative regulatory sequences may involve a position-dependent interference with positive regulatory factors, or may be analogous to enhancers and repress transcription in a distance-independent manner. Cis-acting DNA sequences that mediate a position and orientation-independent repression of basal transcription are termed silencers (99). The similarity of silencers to enhancers has been shown in the chicken lysozyme silencer. This silencer is composed of modules that can either independently repress transcription as multimers or act synergistically with other modules. Each silencer module acts like a binding site for regulatory factors (100). Conceivably, silencers might also act by interfering with enhancer activity. For example, repressor proteins binding to the silencer sequence might prevent positive transcriptional proteins from binding DNA, or might inhibit their ability to transactivate once bound to DNA. Repressor proteins bound to silencers might also interfere directly with the formation or activity of the basal transcription complex (101).

Certain DNA elements can have either a positive or negative effect on transcription depending upon the regulatory proteins or signals present in a

particular cell type or at a certain stage of development. An example is the thyroid hormone receptor binding site. In the absence of ligand, the thyroid hormone receptor binds to its cognate binding site resulting in suppression of transcriptional activity from a thyroid hormone responsive promoter. The addition of thyroid hormone, on the other hand, results in stimulation of expression via the same thyroid receptor binding site (100, 102).

Transcriptional Activators

Eukaryotic transcriptional activators have a modular domain structure consisting of DNA binding and transactivation regions that are interchangeable among various transactivators. This modular structure was first demonstrated in domain swap experiments with Lex A, a bacterial repressor and GAL4, a yeast transcriptional activator. When the DNA binding domain of Lex A is fused to the activation domain of GAL4, the resulting fusion protein is able to activate transcription via the LexA binding site (103).

DNA Binding Domains The DNA-binding domains of sequence-specific transactivators include four types of motifs. These motifs are helix-turn-helix, zinc finger, leucine zipper, and helix-loop-helix. Each type of DNA binding domain has distinct functional properties and many are involved in the formation of selective protein dimers.

Eukaryotic homeodomains, such as those found in proteins encoded by the antennapedia and engrailed genes of *Drosophila*, and the prokaryotic transcriptional regulatory protein the lambda repressor, contain a helix-turn-helix DNA recognition domain. This domain consists of two to three α helices separated by a sharp β turn. The second α helix, which interacts with

the major groove of the DNA helix is termed the recognition helix and is critical for specificity (104, 105). The POU domain proteins such as the octamer binding proteins also use this type of motif to bind to specific DNA sequences. In addition to the homeodomain, the bipartite POU specific domain is also required for DNA binding activity (106). Homeodomain specificity may be enhanced by additional proteins which help to position the homeodomain on the DNA helix. However, eukaryotic homeodomain proteins interact with the DNA as monomers, whereas in prokaryotes, helix-turn-helix proteins often exist as dimers.

Three different structural classes of zinc finger DNA-binding domains have been identified. All require the presence of zinc for DNA binding and consist of a Zn^{2+} ion coordinated in a series of histidine/cysteine residues resulting in a finger-like structure formed around each Zn^{2+} ion. The transcription factor Sp1 is an example of the first class (107). In this class, the zinc finger is a 30 residue domain consisting of a two stranded β sheet and an α helix with one zinc ion bound to two cysteine and two histidine side chains (108). The α helix side chains provide specific nucleotide contacts when binding to DNA. Steroid hormone receptors fall into the second class, which consists of two α helices that are stabilized by the binding of two zinc ions, each bound by 4 cysteines to form side chains (109). These proteins usually exist as dimers where one α helix from each protein interacts with the major groove of DNA. Yeast activators such as GAL4 constitute the third class of Zn domains. This Zn domain has two closely spaced zinc ions sharing 6 cysteine residues (110). These proteins bind to DNA as dimers (111) and recognize a DNA sequence that has two-fold symmetry (112). A fourth class of zinc domain may be found in the GATA-binding proteins (113).

Recently it has been shown that two developmentally regulated isoforms of a *Drosphila* transcription factor differ in the DNA binding domain by one zinc finger. The two alternatively spliced forms have different DNA binding specificities, and regulate various sets of genes in different tissue and stage specific manners (114). These data suggest the modular structure of zinc finger domains and that a single factor can vary the type and number of its domains to control the transcription of a large number of genes.

The leucine zipper DNA-binding proteins contain a two stranded parallel α-helical coiled-coil dimerization motif or leucine zipper, required for dimerization and DNA binding. An adjoining basic region is also necessary for mediating DNA binding. The basic region is unstructured in the absence of DNA, but upon binding to its specific sequence, it acquires a high degree of α-helical structure (115). The DNA motif recognized by these proteins is 9-10 bp long and contains two-fold symmetry (116, 117). C/EBP, GCN4, fos, and jun belong to this category. Certain members of the leucine zipper family can form both homodimers and/or heterodimers with each other. The ability of these proteins to dimerize with various proteins increases their versatility in regulating transcription.

The helix-loop-helix motif is found in proteins like MyoD, cMyc, E12, and E47. This motif consists of two amphipathic α helices, separated by a loop. An adjacent basic motif is necessary for DNA binding, while the helix-loop-helix region mediates dimerization (118, 119). Other DNA binding domains in addition to the ones described exist. For example, AP-2, NFκB, TBP, and SRF (serum response factor) proteins do not contain any of the domains described above, nor do they contain domains that are similar to each other (120).

Activation Domains The activation domains of transcriptional regulating proteins are much less structured than their DNA binding domains (121), suggesting that the interaction of activators with their targets does not depend on precise structural complementarity. There is also no obvious sequence homology among the activating regions of various proteins (122), however, activation domains have distinctive amino acid compositions. Activation regions are often acidic, consisting of a high density of negative charges as found in the yeast transcriptional activator GAL4 (96), while proteins such as the CCAAT box binding family, CTF/NF-1, have activation domains rich in proline (123). The mammalian transcription factor Sp1 activates transcription through a domain characterized by a high glutamine content (107).

The mechanisms by which transcriptional activators increase the rate of transcription are not clearly understood. Based on experimental observations, a number of models have been proposed. In one model, transcriptional activators facilitate transcriptional initiation by interacting directly with a component of the general transcription machinery. For example, the ATF transcription factor when bound to DNA changes the conformation of TFIID bound at the TATA site, extending the region of DNA protected by the protein (124). Also, affinity chromatography has shown that the activation domain of the herpes simplex virion protein, VP16, can selectively interact with TBP and TFIIB (125, 126). VP16 activation mutants exist that reduce binding to TBP (127) and TFIIB (128), and conversely there are TFIIB mutations that do not bind to VP16 and fail to respond to activation, although these TFIIB mutants can still participate in basal transcription (129). Several negative factors are known to bind to TBP to

repress transcription like Dr1 (130, 131). Thus activators might increase transcription by preventing negative factor binding through direct competition for binding sites. Alternativley, general transcription factors might normally assemble non-productively at the promoter, unable to initiate RNA synthesis (132). The interaction of an activator protein with the general machinery might facilitate the assembly of productive initiation complexes. The fact that activator, TFIID, and TFIIB are all required during assembly of the pre-initiation complex to achieve high levels of transcription supports this model (126). Transcriptional activators might also function by overcoming repression by chromatin-associated proteins, such as Histone H1 (133).

The protein-protein interaction that connects the activator proteins to the basal transcription apparatus may involve intermediary proteins, termed adaptors or co-activators. In an in vitro transcription system, cloned TFIID proteins and basal factors required an additional crude protein preparation to initiate transcriptional activation with added Sp1, that was dispensable for basal transcription (134). This raises the possibility that the association of DNA-binding transcriptional activator proteins with different co-activators might be a major control point in transcription initiation. These adaptors might be involved in counter-acting nucleosome repression (135) or act as a bridge between the activator and the transcriptional machinery.

Recently, Reinberg and colleagues have demonstrated that in an in vitro transcription system, the response to an acidic activator, GAL4, requires ACF and Dr2 protein fractions in addition to TFIID, RNA polymerase II, and the other general transcription factors. ACF probably includes previously identified adaptors and Dr2 is DNA topoisomerase I. In the absence of activator, topoisomerase I represses basal transcription, while in the presence

of activator it stimulates transcription. They suggest that transcriptional activation initially requires the removal of molecules that maintain genes in a silent state, thereby making the genes responsive to subsequent activator proteins (136).

Control of Transcriptional Activator Activity The activity of transcription factor proteins can be regulated in a number of ways. Transcription factors can be sequestered in the cytoplasm by their interaction with a cytoplasmic protein or by a masking of their nuclear transport signal (137). They are thus unable to interact with their target DNA sequence in the nucleus. Activation results in the translocation of the factor into the nucleus. Control of NFκB activity is an example of a protein isolated in the cytoplasm, away from its target gene via its interaction with IκB until activated (Discussed further under Ig gene expression). Phosphorylation or dephosphorylation of transcription factors can either positively or negatively affect the ability of the DNA binding domain to bind its cognate sequence. For example phosphorylation of c-jun at certain sites inhibits its binding (138). The interaction of the transactivation domains with the transcriptional machinery c also be altered by phosphorylation/dephosphorylation. Phosphorylation of certain sites on CREB and c-jun will enhance their ability to stimulate transcription (139, 140). Glycosylation of protein factors might play a role. In vitro treatment of Sp1 with wheat germ agglutinin, a lectin which binds to certain sugar residues, specifically inhibits its transcriptional activity. The amount of a transcriptional activator present in a cell can be controlled by the levels of transcription of the protein. Interaction with other factors can either mask or unmask the activation domain. For example, in the absence of galactose, GAL80 will bind to GAL4 and cover its activating

region (96). In addition, dimerization with various other proteins can alter the DNA binding or the activation potential of different proteins. For example, jun homodimers bind DNA at a lower affinity than jun/fos heterodimers (141).

Enhancer Action at a Distance The mechanism of how enhancers function at a distance remains unclear, however a number of models have been proposed. In the first model, the DNA might loop, allowing the proteins bound to a distant enhancer to interact with conserved portions of the general transcription machinery, or with other proteins that bind near the promoter. There is a growing body of evidence in favor of this model, discussed in the previous section. In another model of how enhancers function at a distance, the proteins bound to an enhancer might activate a topoisomerase that would induce superhelical tension along the DNA or open up the chromatin until the altered DNA topology reached the promoter, which in turn might affect the binding or activity of proteins situated at the promoter. A different model suggests the enhancer could act as an entry point for DNA binding proteins that subsequently "slide" along the DNA to the promoter. Enhancers might also act as the initial binding site for proteins that subsequently help the binding of proteins to adjacent sequences, which in turn helps another protein to bind next to it and so on. Enhancers might also be involved in disruption of the chromatin, resulting in a release of nucleosome repression (142). In a final model, an enhancer might bind proteins that cause nearby genes to locate to a region of the nucleus where transcription factors are sequestered.

LYMPHOID SPECIFIC GENE EXPRESSION

The transcriptional expression of the TCR β chain gene is regulated at precise developmental time points, and is a prerequisite for further T cell development. Therefore by elucidating the molecular mechanisms controlling TCR β chain transcription, we can begin to understand how lineage specific patterns of rearrangement and expression are controlled during T cell differentiation, development, and activation. This part of the review summarizes the current knowledge of the T cell receptor β chain regulation and its similarities with the transcriptional regulation of other lymphoid specific genes, including the TCRα, TCRγ, TCRδ, and immunoglobulin genes.

Transcriptional Regulation of the TCR β Chain Gene

T cell receptor β gene expression is controlled at the transcriptional level by two cis-acting DNA elements. Each variable gene segment is associated with an upstream promoter region which interacts with a T cell specific enhancer located downstream of the Cβ2 gene segment.

Vβ Promoters Deletional analysis has been used to characterize the Vβ promoter regions. The minimal human Vβ8.1 promoter mapped 45 bp 5' of the transcriptional start site (143). The minimal promoter for the murine Vβ2 gene, the 5' most Vβ gene segment, mapped 85 bp 5' of the transcriptional start site (144). The murine Vβ8.3 promoter has also been characterized. Sequences upstream of the Vβ2 promoter increase its transcriptional activity as assayed by a CAT reporter gene. The sequences of 14

other murine Vβ promoter regions and the human Vβ8 family have been used as comparisons to identify common promoter motifs (145, 146).

The CAAT box, a common upstream promoter element in a variety of eukaryotic genes, is found upstream of the human Vβ8 gene family (146), and in 8 of the 14 murine Vβ's (147) including Vβ2 (144).

A conserved decanucleotide sequence upstream of the transcriptional start site is present in 13 out of 14 Vβ promoters studied. In the human Vβ8.1 promoter, no difference is seen in the transcriptional activity with or without the decanucleotide sequence, however, the transcriptional activity from the murine Vβ8.3 promoter is reduced 10-fold with the mutation or elimination of this sequence. The presence of this motif in the active Vβ2 promoter and its absence in the non-active promoter confirms the potential importance of the decanucleotide sequence (144). This also suggests that different Vβ genes may be controlled by different elements.

The decamer region is protected by an in vitro footprint in the Vβ8 genes, and is bound by nuclear proteins, specifically the CREB/ATF family of leucine zipper proteins (148). This binding is consistent with evidence that the decamer contains a consensus CRE (cAMP) and AP-1 binding sequence. However, a consensus CRE element but not a consensus AP-1 element alone can substitute for the decamer in vivo (145). Further mobility shift assays with the Vβ decamer motif identified two thymic specific nuclear protein complexes. One complex is first detected on day 16 in the fetal thymus, which parallels Vβ expression. No complexes are found in thymic extracts from SCID mice or from mature T cell lines, suggesting that the complexes represent transiently expressed Vβ transcriptional initiation factors (149).

An AP-1-like sequence is found in all members of the human Vβ8 family, and in 11 of 14 murine Vβ sequences examined by Anderson et al

(147). These AP-1 like sequences are also protected by T cell nuclear proteins as detected by in vitro footprints in the human Vβ8.1 and the murine Vβ2 promoter regions. An inverted repeat is present in the human Vβ8.1, the murine Vβ2, and in 12 of the 14 Vβ promoter sequences examined. The Vβ8.1 inverted repeat is also protected in vitro. These data suggest that the putative AP-1 binding sequence and the inverted repeat are both involved in Vβ regulation. As will be discussed later, the individual contributions of fos/jun heterodimers binding to the AP-1 site, and the ets transactivator binding to the inverted repeat are responsible for conferring TPA inducibility to the Vβ2 promoter.

AP-1 is a family of proteins encoded by multiple genes that can bind to AP-1 sites in the DNA. In mammals, members of this family include c-jun, junB, junD, fos and fra-1. AP-1 proteins undergo homo- and hetero-dimerization via their leucine zipper regions. This creates a variety of transcription factors with different functional properties. Synthesis of most of the AP-1 proteins can be induced by TPA (120).

Other potential regulatory promoter elements include four DNase I footprints, identified upstream of the Vβ TATA box from an α/β expressing T cell. One footprint is T cell-specific, and two of these protected regions have homology to octamer motifs found in the Ig enhancer (150).

The Vβ promoters are not T cell specific. Gottschalk and Leiden found that when activated by a heterologous enhancer, the Vβ promoter is active in T, B and other non-lymphoid cells (148). In studies with the Vβ2 promoter, an additional 300 bp upstream of the minimal promoter increases transcription in T cells and fibroblasts, but not B cells (144). Clearly there are elements other than the Vβ promoter that confer a T cell-specific phenotype on Vβ expression.

Negative Regulation Evidence indicates that negative trans-acting factors play a role in TCR β expression. In somatic cell hybrids of murine T lymphomas, the TCR β chain is rearranged in both parent cells, but expressed at low levels in one of the parents. Upon fusion, expression of TCRβ mRNA is repressed. This negative effect can be partially reversed by treatment with cycloheximide (151, 152).

A sequence which acts to inhibit transcription was identified approximately 700 bp upstream of the murine Vβ2 transcriptional start site. When present this negative element inhibits transcription from the Vβ2 promoter in fibroblast and B cells, in addition to T cells (144). As will be discussed later, the Vβ2 upstream region has been identified as a silencer. It is active in both orientations in T cells and in fibroblasts. This region specifically binds at least one nuclear protein.

Additional cis-acting negative elements are involved in controlling lymphoid lineage-specific gene expression. A silencer sequence in the γ gene was discovered using transgenic mice. Transgenics made with a 15 kb γ locus construct that lacked the 5' and 3' surrounding sequences have a reduced number of αβ T cells and high levels of γ mRNA are expressed in the remaining αβ+ cells. The transgenic mouse made with a construct containing the surrrounding DNA (40 kb construct) has normal levels of αβ T cells, that do not express γ RNA (153), indicating the presence of a γ gene silencer sequence that is active in αβ T cells. Likewise, a silencer sequence exists between the murine Cα gene segment and the α enhancer. The silencing sequence inhibits α transcription in γδ and all non-T cells, but not in αβ T cells. These silencers function in an orientation and distance

independent manner, and can even limit the activity of normally ubiquitous enhancers, such as the Moloney virus enhancer to α/β T cells (154).

TCR β Enhancer An enhancer element that is essential for the expression of a β transgene was mapped to approximately 5 kb 3' of the Cβ gene segment (155, 156). Transient transfection assays narrowed the region to a 550 bp region that conferred strong enhancer activity (156). The enhancer's position 3' of the Cβ structural gene guarantees that it will not be deleted during TCR β rearrangement.

The relative T cell specificity of the β enhancer has been demonstrated by a number of laboratories. In some instances TCR β enhancer activation was seen in B cells and fibroblasts in addition to T cells (144, 148, 155-157). Loh and colleagues suggested that the core β enhancer element is active in both B and T cells with additional flanking sequences acting as B cell silencers (157). Data also indicate that when the Vβ promoter is used in conjunction with the β enhancer, there is more activity in T cells, in contrast to when the β enhancer is used in conjunction with a heterologous promoter (148). The Vβ promoter and β enhancer are active in γ/δ T cells as well as α/β T cells (148). This observation correlates with the expression of a rearranged β transgene which is also not lineage-specific (158).

Through a combination of DNA footprinting and gel mobility shift assays, four different laboratories have mapped between four and seven nuclear protein binding sites within the core TCR β enhancer. Tβ1 to Tβ5 in the human (148), E1 to E4 (159), and βE1 to βE7 (157) in the mouse. The relationship between each of these motifs can be seen in Figure 1. The extensive overlap in the motifs mapped by each of the labs, and the high sequence conservation of these binding motifs in both mouse and human β

enhancers, suggest the importance of the identified regions as regulatory protein binding sites.

In one study, transient transfection and deletional analysis of the human β enhancer revealed that the central Tβ2, Tβ3 and Tβ4 motifs are each required for full enhancer activity. Deletion of the flanking Tβ1 and Tβ5 motifs has no effect on enhancer activity in mature T cells. Both α/β and γ/δ T cells express proteins which bind to all three Tβ2 to Tβ4 motifs. Non-T cells, however, lack at least one of these proteins (148).

Within the past year, a number of the proteins that bind to each of the enhancer motifs have been identified. As will be discussed later, the p70 lupus autoantigen and a novel POU domain protein, TCFβ1, bind to and likely regulate β enhancer activity. At the same time that the above proteins were cloned, additional β enhancer binding proteins were also identified. These additional β enhancer binding proteins are discussed below. It is very interesting to note that many of these proteins also have binding sites in the control regions of other lymphoid specific genes, including the Vβ promoter, suggestive of a coordinated control of lymphoid gene expression. The proteins that bind to the β enhancer are summarized in Figure 2. These proteins include p70, TCFβ1, CBF, CRE, GATA 3, Ets, TCF1α, TCF1, and E box proteins.

TCR β Enhancer Binding Proteins

CBF PEBP2a, also known as PEA2, is the polyomavirus core enhancer binding protein 2 (CBF). It binds to the conserved "core" motif (CTGTGGTAA) first identified in the SV40 enhancer and also present in the enhancer of several murine leukemia viruses. The "core" motif, when mutated in the Moloney virus enhancer, not only reduces transcription, but

also changes the lineage specificity of the virus from T cells to the erythroid lineage (160). In addition to its presence in the β enhancer, the core motif is also found in the TCR γ and δ enhancers as well as the Vβ2 promoter. PEBP2 is a core binding factor (CBF) and has homology to the *Drosophila* segmentation runt gene and the human AML1 gene involved in acute myeloid leukemia. PEBP2 mRNA is expressed preferentially in T cell lines, and has two potential binding sites within the β enhancer (Tβ3 and Tβ4). Transfection analysis reveals that enhancer activity of Tβ3 and Tβ4 is ~12 fold higher in T cells that express PEBP2a than in B cells that do not express this protein. Exogenous expression of PEBP2a in the B cell line, result in Tβ3/Tβ4 dependent transcriptional activity. Therefore, PEBP2a represents a family of transcription factors involved in the regulation of T cell specific gene expression (161).

CREB/ATF One of the best studied DNA transcriptional regulatory regions is the cAMP Response Element or CRE. The CRE is a palindromic octanucleotide motif (TGACGTCA) which is present in the transcriptional regulatory region of a large number of eukaryotic genes (162). Various studies have shown that 1) the presence of the CRE confers responsiveness to cAMP and calcium (163, 164), 2) the CRE binds to the cellular factor (ATF) and therefore, confers E1a responsiveness to several adenoviral genes (162), and 3) the CRE controls basal transcriptional activity of several enhancers including the T cell leukemia virus I (165) and the c-fos proto-oncogene enhancers (166). Several CRE binding proteins belonging to the ATF/CREB family of transcription factors have been cloned. The carboxy-terminal region of each of these proteins contains a dimerizing leucine zipper motif and an adjacent DNA binding basic region. The ATF/CREB proteins bind as both

homodimers and heterodimers (167). Of the proteins that have been cloned, CREB is a 43 kDa nuclear protein that responds to cAMP to induce transcription in a number of genes (168). Phosphorylation of CREB by PKA is critical for cAMP-inducible transcription (169). The second protein cloned was CRE-BP1 (ATF-2), which binds to the CRE as a heterodimer with Jun (167) and interacts with E1a to activate transcription from adenoviral promoters (170). The third protein cloned, CREB-2 (ATF-4), is ubiquitous in human tumor lines and mouse organs and regulates CRE responsiveness in a wide variety of cell types. Interestingly, overexpression of CREB-2 represses CRE dependent transcription in the CV-1 cell line (171).

In addition to the TCR β enhancer, CRE sites are found in the TCR α enhancer, and in all of the human and murine Vβ promoters (148, 172). All three of the CRE binding proteins and at least two more unidentified CRE binding proteins can bind to the TCR α enhancer (148). None of these proteins is specific to T cells but a thymocyte specific nuclear protein binds the CRE in the Vβ promoters. The expression of the Vβ promoter CRE binding protein correlates with TCR β gene rearrangement (149). The precise role of the ATF/CREB family of transcription factors in T cell receptor β chain regulation remains to be established. However, the fact that they can heterodimerize with the AP-1 family of transcription factors (Jun), their observed positive and negative roles in transcriptional regulation, and their response to calcium and cAMP are suggestive of their involvement in both T cell development and activation.

Gata 3 GATA-3 belongs to the four member family of GATA binding proteins. The erythroid transcription factor GATA-1 was the first member identified. The GATA family members each recognize the same consensus

DNA sequence and have a highly homologous zinc finger DNA binding domain (173). They also have a distinct, but overlapping pattern of tissue distribution and DNA recognition motifs. The ability of each member of the GATA family to transactivate correlates with DNA binding (174). Homologous recombination experiments have shown that GATA-1 is required for the normal development of the erythroid lineage, and is thus a lineage-specific determination gene (175).

GATA-3 is most abundantly expressed in T cells. Expression of GATA-3 starts at a very early stage of thymocyte development and correlates with the start of TCR β rearrangement (176). A larger GATA-3 transcript has been identified in kidney and in embryonic brain cells (177, 178).

Overexpression of GATA-3 can transactivate a reporter construct containing only the GATA-3 (Tα3) binding sequence, without the need for additional sequences (177). The GATA-3 binding sequence is found in the enhancers of all four TCR genes, and specific binding of in vitro synthesized GATA-3 is seen with the GATA motifs in the β, α and δ enhancers. The in vitro binding specificity of GATA-3 correlates with the activity of the TCR β enhancer in transient transfection experiments, suggesting a role for GATA-3 in regulating β gene expression (179).

Ets Proteins encoded by the Ets family of proto-oncogenes bind to purine rich DNA sequence motifs found in the regulatory region of a large number of genes (180 - 182). Among others, ets binding sites have been identified in the TCR α and β enhancers, the IL2 enhancer, and in several viral enhancers (183, 184). Multiple ets proteins are expressed in T cells including Ets-1, Ets-2, GABPa, Fli-1 and Elf-1 (185).

Expression of Ets-1 is developmentally regulated during thymic ontogeny, first appearing on days 17 and 18 in the mouse fetal thymus, a time course that parallels expression of the TCR α chain genes (186). The DNA binding domain is conserved in all ets members, and consists of a putative α helical region and an adjacent basic region.

Both Ets-1 and Elf-1 have distinct DNA binding specificities that recognize the same purine rich central core motif, but differ in their recognition of the 3' nucleotides flanking the core (185). Elf-1 binds to several T cell regulatory regions including the IL-2 enhancer and HIV-2 enhancer, but not to ets sites in the TCR α or β enhancers. Ets-1, on the other hand, binds preferentially to purine rich sites in the TCR α and β enhancers, but not to the IL2 enhancer (185). This may explain how the simultaneous presence of different ets proteins can regulate the transcription of various T cell-specific genes. Mutations in the Ets-1 binding site in the TCR α enhancer abolishes enhancer activity (184). However, overexpression of Ets alone does not transactivate the α TCR enhancer, suggesting the requirement of additional transcriptional factors for ets mediated enhancement. This observation is consistent with the fact that ets proteins usually require additional factors to interact with the basal transcription machinery (184).

TCF-1α/LEF-1/TCF1 Human TCF-1α, its mouse equivalent, LEF-1 (lymphoid enhancer binding factor-1), and TCF1 are members of a family of proteins that have an 85 amino acid homology with the high mobility group (HMG) of non-histone chromosomal DNA binding proteins. The LEF -1 and TCF1 proteins were identified as TCR α enhancer binding proteins that recognize the pyrimidine rich CCTTTGAA sequence (Tα2). Additional evidence indicates that TCF-1α binds to Py rich elements in the IgH, TCR β,

and δ enhancers, and promoters of lck, CD3γ, and δ, CD4 and HIV (187, 188). LEF-1 is expressed at all stages of development in α/β and γ/δ T cells and in mouse pre-B cells. The expression of TCF-1α mRNA is increased by agents which activate immature T cell lines such as phorbol esters (188, 189).

TCF1 is encoded by a separate gene from that of TCF-1α and LEF-1, and its expression is limited to thymocytes and mature T cells (190). Multiple molecular weight proteins, presumably alternatively spliced forms of TCF-1α/LEF-1 and TCF-1 have been identified (188, 191). The splice variants differ by the presence or absence of proline and serine/threonine rich amino- and carboxy-termini. These regions are suggestive of activation domains, and thus the splice variants may have different activation potentials.

Overexpression of LEF-1 and TCF1 in more mature B cells (cells lacking endogenous LEF-1), can transactivate reporter constructs containing their cognate binding sites (189). The LEF-1 binding site is required for full TCR α enhancer function. However, multimerized LEF-1 sites alone are unable to augment basal promoter activity, indicating that the function of LEF-1 may depend on other transcriptional regulators (187, 189). The LEF-1/TCF1α and TCF1 proteins have a DNA binding domain that interacts with the minor groove of the DNA helix, and this interaction with DNA produces a sharp bend in the DNA helix. Therefore, LEF-1 may act as an architectural protein by facilitating protein/protein contacts through the alignment of distant protein binding sites by bending the DNA helix (192). By facilitating the assembly of a higher order complex of proteins, LEF-1/TCF-1α and TCF1 may play a central role in regulating the interaction of the distinct proteins that bind to the TCR enhancer regions.

E box Proteins Originally isolated from plasmacytoma cells, the μEBP-E protein binds to E sites within the IgH enhancer, the TCR β enhancer, and the V_H promoter (193). The Ig/EBP-1 protein binds to E sites in a manner indistinguishable from that of μEBP-E. Both proteins are members of the C/EBP family of basic zipper proteins (bZIP). There are five identified members of the bZIP activator C/EBP family, C/EBP-1, Ig/EBP-1, NF-IL6, CRP1 and CRP3. All members have a high degree of homology in the basic zipper region and are capable of forming heterodimers. All of the C/EBP member proteins recognize identical DNA motifs (193 - 195). The expression of Ig/EBP-1 and NFIL-6 is not restricted to B cells, however, they are the only members of the family that show a high level of expression in B cells. This observation has led to the idea that μEBP-E is a mixture of Ig/EBP-1 and NF-IL6 binding. Expression of NF-IL6 is inducible by LPS, IL1 or IL6, suggesting its importance in regulating gene expression in B cells (196).

Additional TCR β Control Regions

The presence of DNase I hypersensitive sites are the result of a change in chromatin structure and often correlate with tissue specific gene expression, indicative of an increased accessibility of the DNA to transcriptional proteins (197). For example, a T cell specific DNase I hypersensitive site is present in the β enhancer region. Additionally, a DNase I hypersensitive site is found just upstream of the enhancer. This DNase I hypersensitive site is only present in cells expressing the TCR β chain (198).

Hypersensitive sites in a region of the J-C intron are homologous to the κB site in the Ig κ chain enhancer, and are preferentially active in phorbol ester stimulated T cells (199). Initial attempts to identify an enhancer element within the Jβ-Cβ intron (143), as is found in Ig genes, proved unsuccessful.

Analysis of the region within the Jβ2-Cβ2 intron using DNase I protection and gel-shift assays, reveals seven protein binding sites (200). The sequence of one of these regions is homologous to the Y box binding site that is found in the promoters of several MHC class II genes, and also within the 3' Cβ enhancer (201). Therefore, this region may play a transcriptional regulatory role.

Another possible β chain enhancer element is upstream of the human Vβ8.1 promoter. A 230 bp fragment located 570 bp from the transcriptional start site, confers a T cell specific expression phenotype to a heterologous promoter. This fragment acts in an orientation-independent manner, suggestive of an enhancer element (143). Further analysis is needed to determine what role, if any, this region plays in controlling TCR β gene expression.

TCR α Chain Gene

Like the TCR β chain, expression of the α chain of the T cell receptor is tightly regulated during ontogeny and is expressed solely in α/β T-cells. Cell-surface expression of the α/β heterodimer is dependent on the expression of the α chain gene. The similarities between the TCR β and α chains extends to their transcriptional control mechanisms. The Vα promoters alone, like Vβ promoters, are not tissue-specific and show very weak transcriptional activity. An analysis of the Vα11 minimal promoter, reveals an essential transcriptional element called the GT box. The GT sequence is present in 3 out of four Vα promoters and is also found in the α enhancer. The ubiquitous proteins that bind to this sequence are novel members of the Sp1 family of Zn finger proteins (202). Based on the TCR β genes, the finding of an enhancer located 4.5 kb 3' of TCR α constant region was not unexpected

(203). The whole TCR α enhancer is a strong transcriptional activator in α/β+ T cell lines, with both the Vα and heterologous promoters, and it is inactive in the non-T cell lines tested. There is a high degree of sequence conservation between the mouse and human TCR α enhancers (203).

The murine minimal enhancer has two critical nuclear protein binding sites, Tα1 and Tα2 detected by DNase I footprinting. Both sites are required for activity of the minimal enhancer. In addition, the 20 bp spacing between Tα1 and Tα2 is critical. Tα2 can act as an activator in a TCR α context, but is repressive in several heterologous promoter/enhancer contexts, such as SV40 (184, 204). Two additional protein binding elements are present in the human α enhancer, Tα3 and Tα4. These sites are not critical for enhancer function but compensate for mutations in Tα1 and Tα2. The four protected sites in the TCR α enhancer are bound by at least seven T cell nuclear protein complexes (191). Therefore, TCR α expression is controlled by interactions between the proteins binding to the four partially redundant sequence motifs in the TCR α enhancer.

The molecular similarities between TCR β and α chain expression become clearer upon examining the identity of the enhancer binding proteins. Tα1 has a cAMP consensus sequence (CRE) and binds a set of ubiquitous CRE-binding nuclear proteins (CREB) (148). Tα2 binds at least four nuclear protein complexes, one of which is T cell specific (205). TCF1 and TCF-1α both bind to a pyrimidine-rich region in Tα2. A mutation in the TCR α enhancer which abrogates TCF1α binding, drastically decreases enhancer activity (188). Ets-1 binds the 3' end of Tα2, and as mentioned previously Ets-1 expression correlates with TCR α expression. Mutation of the ets binding site abolishes TCR α enhancer activity, but ets-1 alone cannot transactivate. Tα3 contains overlapping GATA, E box (κE2), and AP-2 like motifs. Tα4

binds three ubiquitously expressed nuclear proteins complexes. The factors that bind to the TCR β enhancer must also be involved in the coordinate regulation of the TCR α enhancer.

TCR γ Chain Gene

A murine transcriptional enhancer region is found approximately 3 kb downstream of the Cγ1 gene. Multiple copies of this enhancer, like the TCR β enhancer, are active in both α/β and γ/δ T cell lines, but not in other cell types. The importance of the γ enhancer is confirmed in transgenic mice. The Cγ1 transgenic gene construct is expressed at high levels in T cells, only when it contains the γ enhancer sequence. The minimal enhancer is contained in a 200 bp fragment. DNase I footprinting identifies six nuclear protein binding sites within the 200 bp fragment. An analysis of the enhancer sequence reveals similarities to a number of previously identified sequences, including an ets binding motif, an AP-2 binding site, a κE2 site, and sequences similar to Tα2 and CBF (206, 207).

TCR δ Chain Gene

As the TCR δ genes are found within the locus of the TCR α genes, this led to the original hypothesis that these two genes would share regulatory elements. A T cell-specific δ enhancer, however, was identified in the Jδ3-Cδ intron of the human TCR δ locus. This enhancer increases transcription from heterologous as well as Vδ promoters in both α/β and γ/δ T cells (208). A δ enhancer in the same chromosomal location was also confirmed in the murine δ locus (209). The minimal enhancer is a 250 bp region, in which footprinting analysis identified 7 distinct nuclear factor binding sites δE1 to δE7.

A 60 bp fragment containing the δE3 and δE4 motifs displays good enhancer activity. Further analysis indicates that the δE3 site, when multimerized, acts alone as an enhancer. The δE3 site binds two distinct proteins. The NF-δE3C protein is ubiquitous and identical to the μE3 binding factor in the immunoglobulin enhancer. The NF-δE3A protein is T cell specific and may play a dominant transcriptional role through activation of the δE3 site. Sequence analysis reveals a number of common motifs. GATA binding sites are found in δE1 and δE4. A κE3 like sequence is found in δE3, an Ets-1 binding site exists in δE5, and a TCF-1/LEF-1 binding site in δE7 (208, 210). The precise roles that each of the binding factors have on δ transcription before and after rearrangement remains to be established.

Immunoglobulin Gene Expression

Immunoglobulin gene expression during B cell development is similar to that of T cell receptor gene expression during T cell development. Ig gene expression is restricted to B cells and is critical for Ig gene rearrangement and B cell development. The transcriptional control of Ig genes is dependent on the coordinate interaction of members of various protein families and their recognition of a variety of conserved DNA motifs. Both enhancer and promoter sequences are involved in the restriction of immunoglobulin expression to B cells (211). It is not surprising, therefore, that TCR and Ig transcriptional control regions have certain DNA motifs and binding proteins in common. Given the similarities between Ig and TCR gene expression, an understanding of the well-studied immunoglobulin genes, which have long been used as a model for tissue-specific gene expression, will invariably shed light on the processes that control TCR gene expression.

Immunoglobulin Promoter Motifs Immunoglobulin promoters are associated with each variable region gene. Upstream of the TATA box, is a conserved eight nucleotide sequence known as the octamer motif, which is present in all Ig promoters. This motif is critical for conferring B cell-specific activity to the promoter (212), and deletion or mutation of this motif drastically reduces B cell-specific transcription. The presence of the octamer motif alone is sufficient to confer lymphoid restricted promoter activity (213). The octamer motif is also required for optimal transcription in vitro when B cell extracts are used, but has no effect on transcription when HeLa extracts are used (214). In conjunction with the Ig enhancer, the V_H octamer sequence is necessary for high level expression of μ transgenes in B cells (215). Octamer motifs are also present in the enhancer regions, and 5' of the J_H chain gene (216). The octamer sequence however, is not confined to immunoglobulin genes, it is also found in and required for expression of the promoters of the thymidine kinase, IL2, histone H2B, and small nuclear RNA genes (217 - 220).

Additional sequences are important for full transcriptional activity from the Ig V_H promoters. Upstream of the octamer sequence is a conserved heptamer sequence, and further 5' a polypyrimidine tract is present (221). In the presence of the octamer element, mutation of the heptamer sequence reduces promoter activity by 30 - 80%. The heptamer sequence is a low affinity octamer binding site and shares cooperative binding characteristics with an adjacent octamer motif (222). An E site and μE3 motif identified in the Ig enhancer are also found in a number of V_H promoter regions (223). In the murine V_H1 promoter, an E site that is bound by protein is required for full promoter activity. A conserved site that is responsive to IL5 plus antigen is also present in the promoter region, and is termed VDSE (224).

Octamer Binding Proteins Two proteins were originally identified which bind to the octamer sequence. The Oct-1 (OTF-1) protein (225), is ubiquitously expressed, and the Oct-2 (OTF-2) protein (226), is found abundantly in B cells, several T cell lines, certain myeloid and erythroid cells and in non-lymphoid brain and kidney cells (220, 227, 228). Both proteins are members of the POU domain family of transcriptional activators. The POU proteins are related to homeodomain proteins, and in addition to the homeodomain contain a POU specific domain (106). The Oct-1 and Oct-2 proteins selectively stimulate in vitro transcription from histone H2B and Vκ promoters, respectively. Oct-1 is implicated in the activation of snRNA promoters via a selective activation domain (229), and implicated in Ig expression in association with a coactivator (230), while Oct-2 selectively activates mRNA promoters in an octamer-dependent manner (229).

The Oct-2 protein was thought to be the factor responsible for B cell specific expression of immunoglobulin genes via its interaction with the octamer motif (231, 232). Many observations support this conclusion. Oct-2 is present in B cells, it binds to the octamer motif which is essential for Ig expression, and it preferentially activates mRNA promoters (233). In somatic cell hybrids between B cells and fibroblasts, both Ig and Oct-2 expression are inhibited simultaneously. In the same hybrid system, Ig promoter constructs can be activated by the transfection of Oct-2 expression vectors (234).

The essential role of the Oct-2 protein in immunoglobulin gene expression has recently become questionable. In an Ig expressing cell line in which both alleles of Oct-2 are "knocked out" by homologous recombination, Ig gene transcript levels are unperturbed (235). In addition, the generation of Oct-2 negative mice indicates that Oct-2 is not required for the development of immunoglobulin-bearing B cells, Oct-2 expression is, however, required

later for B cell maturation (236). In B X T cell hybrids, Ig genes are extinguished. The octamer motif is sufficient to confer this extinction, however, Oct-1 and Oct-2 are both still expressed and capable of binding octamer motifs in these hybrids (237). Therefore, it is likely that another octamer binding protein is involved in controlling Ig expression.

Immunoglobulin Enhancers

The μ heavy chain gene is expressed at the earliest pre-B cell stage of differentiation. The μ enhancer is a critical component of this specific gene activity. The immunoglobulin heavy chain intronic enhancer in both the human and mouse is located approximately 500 bp downstream of the 3' most J_H region in the J_H-C_H intron (238). This location allows the enhancer to influence the promoter region of the V_H gene that is brought into close proximity by the rearrangement event. The unrearranged V_H genes upstream fall outside of the enhancer's primary sphere of influence, and are thus transcribed at a much lower level (56). The promoter/enhancer proximation effect was observed in the HAFTL-1 cell line, in which the joining of distal D segments is accompanied by an increase in the steady state level of transcripts initiating 5' of the D region (239). The enhancer is also 5' of the μ switch region, and is therefore still present after isotype switching.

The μ enhancer region activates transcription of cis-linked genes in a B cell-specific manner. The μ enhancer alone is sufficient to confer the correct expression of a heterologous gene to B lymphocytes (240). Also, when the Ig heavy chain gene is transfected into fibroblasts, enhancer dependent transcription occurrs only with the injection of B cell nuclear extracts (241). A central core of the μ enhancer alone enhances non-lymphoid specific transcription. However, the entire enhancer is B cell-specific, suggesting the

presence of negative elements within the enhancer that are active in non-B cells (242). The NF-μNR protein may act as a tissue specific negative regulator by binding to four sites near the enhancer. This binding represses μ enhancer activity in some T cells lines, and pre-B cells (243).

μ Enhancer Motifs In vivo DMS footprinting revealed up to 8 regions within the enhancer having a B cell specific pattern. These included 5-eight nucleotide consensus sequences named μE1, μE2, μE3, μE4, and μE5 (244). This E motif sequence is also present thrice within the mouse κ light chain enhancer (245). In addition, the octamer element that is important in regulating Ig promoters is also present in the m enhancer, and μB, E box and π sites are also present (246).

In transfection experiments, mutation of the enhancer octamer motif leads to a 10%-40% reduction in activity from the 700 bp enhancer, and a 90% reduction in enhancer activity in the 300 bp version of the enhancer. These octamer mutations exert their effect in a B cell-specific manner (233, 247). Destruction of the μB site decreases enhancer activity in B cells but has no effect in fibroblast cells. Deletion experiments suggest that both the octamer and μB motifs are involved in conferring B cell specificity in pre-B cells, but either one alone will suffice at later stages of development (248).

The π motif confers strong enhancer activity in pre-B cells but not in mature B cells. The E box site can be bound by μEPB-E and Ig/EBP-1. These are members of the C/EBP family of E-box binding proteins which also includes NF-IL6, CRP1 and CRP3. Ig/EBP-1 is present in early B cell lines, while both Ig/EBP-1 and NF-IL6 are present at later B cell stages (249) NF-IL6 is inducible by LPS (196).

The μE motifs can compensate for each other. For example, enhancer activity decreases only when the μE1, μE2 and μE4 sites are mutated simultaneously (247). Mutagenization of μE3, however, results in a decrease in enhancer activity to 40% of the wild type enhancer (246). Despite their functional and sequence similarities, the μE sites are capable of binding distinct nuclear proteins found in non-lymphoid as well as B cells (250). The μE5 site is unique in that mutation of this site results in the abrogation of enhancer repression in fibroblast cells, discussed further in the next section (251).

E Motif Binding Proteins E motif binding proteins include a family of helix-loop-helix (HLH) DNA binding proteins. The HLH motif has been found in MyoD, c-myc, max, the protein encoded by the *Drosophila daughterless* gene and other developmentally important proteins (119). These proteins have a basic region adjacent to the helices (bHLH), which in addition to dimerization, is required for binding to DNA (252). There are three classes of HLH proteins divided according to their ability to dimerize with themselves and with other HLH proteins (119). The formation of tissue specific heterodimers of E motif binding bHLH proteins is a versatile method in which these proteins can control transcription.

The E2A gene encodes members of the ubiquitous HLH proteins. The E2A proteins each arise by differential splicing. These include E12, E47 and E2-5, all of which bind to the μE5 motif in the heavy chain enhancer in addition to the κE2 motif in the light chain enhancer (253). The E2-2 protein is structurally similar to E2A and also has the same binding specificity.

The μE3 motif in the heavy and light chain enhancers are bound by three HLH proteins, TFE3, TFEB and USF (254, 255). When bound to their

cognate motifs, E2-5 and TFE3 can directly activate enhancer dependent transcription. The TFEC protein is the newest bHLH family member and is capable of forming heterodimers with TFE3 to bind the μE3 motif. TFEC cannot transactivate on its own, and cotransfection with TFEC strongly inhibits TFE3 transactivation (256). Transfection experiments in fibroblast cells indicate the presence of the μE5 site inhibits approximately 100 fold the ability of exogenously overexpressed TFE3 to activate transcription through the μE3 site. However, when E2-5 is overexpressed in fibroblasts, it can activate transcription through the μE5 site so that the μE3 site is no longer repressed. It appears that the E2-5 protein displaces a fibroblast repressor at the μE5 site (257). Another HLH protein, Id inhibits IgH(~2 fold) and Igκ(~5 fold) enhancer activity in transient transfections in B cells (258). Id proteins lack the DNA binding domain, therefore heterodimers with Id lack the ability to bind DNA. This is a method in which bHLH proteins can form active versus inactive heterodimers (259). Further versatility in transcriptional control by bHLH proteins is their differential binding affinity. The binding affinity of the E2A bHLH protein is higher in B cells than in fibroblast cells, although the same level of E2A mRNA is expressed in both cell types (260). This cell-type specificity of DNA binding activity may be controlled by post-translational modifications of these proteins.

3' IgH Enhancer A second B cell specific enhancer is located 25 kb downstream of the C region gene. It contains both an octamer and a μE5 motif (261). This enhancer may be active after switch recombination, or may regulate the sterile transcripts originating from the IgH C regions.

Igκ Enhancer An Igκ enhancer element was identified within the Jκ-Cκ intron by deletional and transfectional analysis, approximately 700 bp 5' of the Cκ gene, (262). The Igκ enhancer is active only in mature B cells and plasmacytomas. Four nuclear factor binding sites are present within the κ enhancer. Three E sequences, κE1 to κE3, similar to the μE sequences found in the Ig heavy chain enhancer are present, as is a κB motif. Mutations in κE2 and κE1 reduce enhancer activity, but mutations in κE3 have much less effect on activity of the κ enhancer. The κB motif alone has B cell-specific enhancing activity, and is responsible for B cell stage-restricted expression. Mutation of the κB motif completely abolishes κ enhancer activity (244, 263).

The κB motif acts as an inducible enhancer under the same conditions that lead to NF-κB binding (263). The NF-κB protein binds to the κB motif and its nuclear activity correlates with κ gene expression. The NF-κB protein is only found in the nucleus of B cells that transcribe the κ light chain gene. It can be induced in pre-B cells by LPS and in non-B cells by TPA. Induction occurs by a post-translational mechanism, since protein synthesis is not required for NF-κB activation (264). In its inactive form, NF-κB is complexed with IκB, a specific inhibitor, and is confined to the cytoplasm. Upon activation, during the pre-B to B cell transition, IκB dissociates and the active NF-κB translocates to the nucleus to stimulate its target enhancer (265)

The NFκB protein can induce many genes that are important for immune function, including IL-2, IL-2Rα, IL-6, TNF-α, GM-CSF, TNF-β, MHC class I and II, and β-interferon. It is also involved in viral gene activation of CMV and HIV. Additionally, many diverse signaling pathways can induce NFκB (266).

Ig κ 3' Enhancer Downstream of the Cκ region, is a strong tissue-specific enhancer (267). It is weakly active in pre-B cells, but is much stronger than the intronic enhancer in mature B cells. The sequence contains homology to two NF-κB sites, and a μB site. However, the 3' enhancer has strong activity in the absence of NF-κB. Experiments with transgenic mice indicate that deletions of the 3' enhancer, reduce κ gene expression and deregulate allelic exclusion (224). As discussed previously, the area encompassing the 3' enhancer also acts as a negative cis-element to inhibit κ gene rearrangement in T cells and pro-B cells (50).

Shared Nuclear Proteins and Binding Sites

The TCR promoters and enhancers and the IL2, Ig and other lymphoid specific genes, all share common nuclear protein binding sites and transcriptional factors with that of the TCR β chain genes. These include, octamer and octamer-related proteins, CREB, GATA-3, E-box proteins, Ets, CBF, TCF-1α and TCF-1. This sharing of factors allows coordination of tissue specific gene expression during lymphocyte development and activation. However, each lymphoid specific gene displays a unique, precise lineage and temporal expression, and so far no lineage specific protein complexes have been identified.

How are T cell specific genes containing the same or similar cis-acting sequences, uniquely regulated?

1. Combinatorial diversity may allow interaction between different transcription factors. Different combinations of transcriptional factors binding to various DNA elements may result in different transcriptional effects.

2. Different members of a protein family may either exhibit different DNA-binding specificities, or need adjacent proteins for optimal binding.

3. Negative elements or repressor sequences may play a role in restricting expression to certain tissues.

4. The DNA-chromatin context of the motif may or may not allow various proteins to bind to their cognate sequence.

5. T cell specific post-translational modifications, or T cell-specific factors that interact with ubiquitous DNA binding proteins may help to control tissue specificity.

6. Transactivator proteins may preferentially interact with specific promoters.

7. Important T cell specific binding proteins may not yet be identified.

TCR β gene expression is controlled by multiple T cell nuclear proteins. All of the above methods have been demonstrated, or are likely to play a role in controlling TCR β and lymphoid specific expression.

PROJECT AND RATIONALE

In order to understand immune function, it is crucial to comprehend the mechanisms which determine immune cell phenotype and which regulate lymphoid maturation. The rearrangement and expression of the T cell receptor β chain gene is an essential component in defining the T cell lineage. The T cell specific transcription of the TCR β chain gene is a prerequisite for the recombination and expression of the TCR during T cell development. By understanding how transcription of the TCR β chain gene is controlled an understanding of a critical component regulating T cell development is gained. Therefore, my project involved studying the transcriptional regulation of the TCR β chain gene, in order to shed light on the process of T cell development and thus immune function.

When I started work on this thesis, multiple cis-elements controlling TCR β gene transcription had been identified. These included the Vβ promoter elements and the downstream β enhancer. There was, however, only speculative knowledge of the transcriptional proteins that bind to these cis-regions. Since the regulation of gene expression is controlled by DNA binding proteins, I started to characterize the proteins which bind to the TCR β chain gene cis-elements. Identifying the proteins that bind to the cis-elements would help to dissect the mechanisms controlling TCR β chain transcription and lead to a better understanding of β gene expression. Additionally, identifying the DNA-binding proteins would generate novel tools with which to further dissect the process of T cell development.

I succeeded in identifying a number of proteins that bind to the cis-elements to control Vβ2 gene transcription. Vβ2 is the 5' most Vβ gene thus the elements that control Vβ2 gene expression may also play a role in

controlling the expression of the entire Vβ locus. I have shown that the combination of fos/jun heterodimers, in association with the ets protein, bind to the TPA inducible region of the Vβ2 promoter and together confer optimal inducibility. This work is discussed in section I and was published in *The Journal of Immunology* (149:1980-1986, 1992).

I have also demonstrated the dominance of the β enhancer in the ternary interaction between an upstream inhibitory element, and the TPA inducible element to control β gene transcription. This is discussed in section II. My evidence indicates that the β enhancer is the only cis-element responsible for conferring T cell-specific expression to the β chain gene. Therefore, the identification of proteins binding to the enhancer is essential for further understanding the process of β gene transcription. Towards this goal, I have identified the p70 lupus autoantigen as a protein that binds in a sequence-specific manner to a transcriptionally important motif in the β enhancer. I have implicated a role for the p70 protein in controlling β gene transcription. This work is discussed in section IIIA and was published in *Proc. Natl. Acad. Sci. USA* (90:2685-2689, 1993). Finally, I have identified and characterized a novel POU domain protein, TCFβ1 which binds to the β enhancer. POU proteins are important in the generation and maintenance of specific lineages. I have shown that TCFβ1 binds to multiple motifs within the β enhancer. In addition, it binds to the transcriptionally important immunoglobulin octamer motifs. I characterized the lineage-specific tissue distribution of TCFβ1, and have also demonstrated that TCFβ1 is capable of transactivating in a sequence specific manner. TCFβ1 is therefore an important candidate in controlling lymphoid-specific gene expression. This work is discussed in section IIIB and was published in *Molecular and Cellular Biology* (13:5450-5460, 1993). Within the past year, other β enhancer binding

proteins were identified by various labs. Together with my data, the identification of the TCR β enhancer binding proteins provide a clearer picture of the mechanisms of β gene regulation.

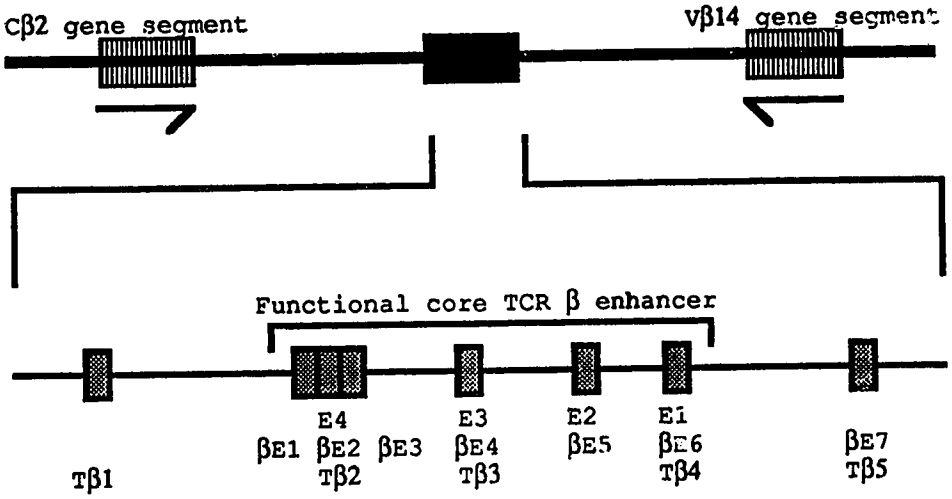

Figure 1. Map of the TCR β enhancer. The footprints identified in three studies are indicated.

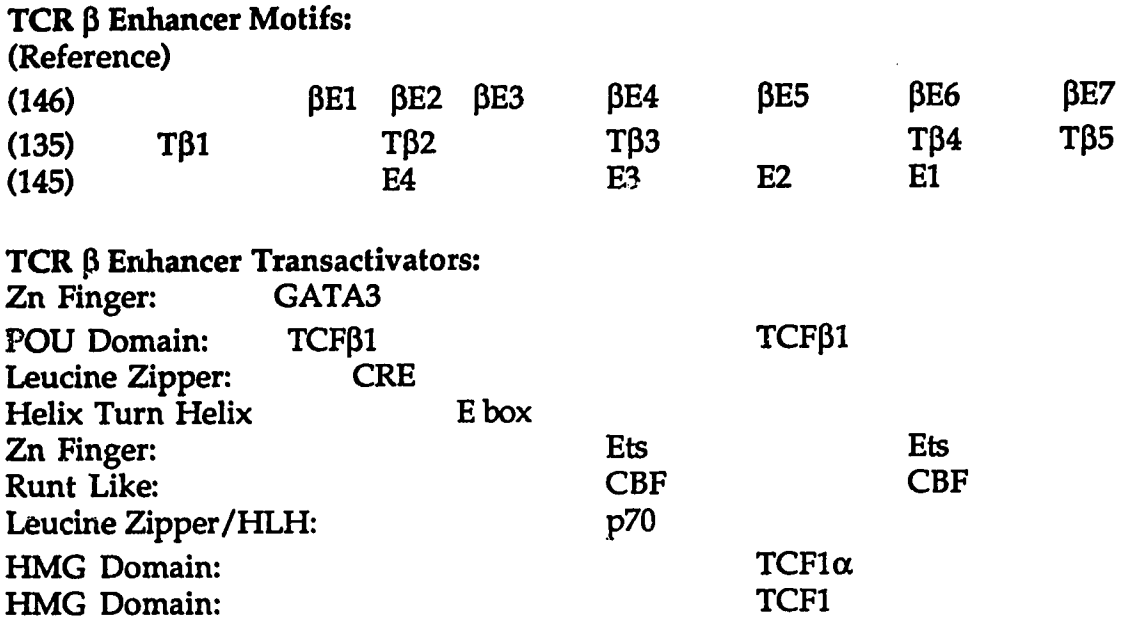

Figure 2. T cell receptor β enhancer binding proteins. The proteins, and the motifs within the enhancer to which they bind are identified. The type of DNA binding motif present in each protein is also indicated.

Chapter 2

MATERIALS AND METHODS

In Vitro Transcription and Translation

Preparation of an RNase-free vector The DNA insert encoding the appropriate protein was subcloned into an ATG Bluescript vector. The ATG codon was introduced into the Bluescript vector by using adapter double-stranded oligonucleotides (5' AGCTTCAACCAGCCTCCCGCGACGATGG 3'). The template was linearized with an enzyme 3' of the coding sequence in order to produce run-off transcripts of a discrete size. The linearized plasmid was then treated with 10 μg Proteinase K at 37° C for 30 minutes. It was subsequently extracted once with phenol:chloroform, and once with chloroform, then ethanol precipitated. The plasmid was resuspended in DEPC treated (RNase free) water to a concentration of 1μg/μl.

Transcription Reaction Capped RNA was synthesized in a reaction containing 1 μg linearized plasmid, 1X transcription buffer (40 mM Tris-Cl (pH 7.5), 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine), 0.4 mM UTP, 0.4 mM CTP, 0.4 mM ATP, 0.12 mM GTP, 5'7meGppp5'G CAP analogue, 30 mM DTT, RNase Inhibitor, and 10 Units (U) of the appropriate RNA Polymerase (either T7 or T3) that would generate the sense RNA strand. The addition of the transcription components was carried out at room temperature, and subsequent incubation was done at 37°C for one hour. The DNA template was removed following the synthesis of capped RNA transcripts by incubation at 37°C for 10 minutes with 10 Units of RNase-free DNase I. The RNA was then extracted with phenol:chloroform, ethanol precipitated in the presence of sodium acetate, and resuspended in RNase-free TE. The RNA

could be visualized on a denaturing agarose gel, with ethidium bromide staining.

Translation Reaction The capped RNA transcripts were translated using rabbit reticuloycyte lysates (Promega, Madison, WI). The reaction included 2 µl RNA substrate, 40 Units of RNase Inhibitor, 20 µM amino acid mixture (minus methionine), 40 µCi ^{35}S-methionine (1200 Ci/mmole), and 35 µl nuclease treated lysate, to a final volume of 50 µl. The reaction was incubated at 30°C for 30 min and the translation products were visualized by SDS-polyacrylamide gel electrophoresis

Reverse Gel Shift Assay

The ^{35}S-labelled protein was generated by in vitro transcription and translation. Linearized plasmid DNA containing the motif of interest or plasmid alone as control was incubated with 1 µl of a 50 µl reticulocyte lysate reaction in the presence of 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM dithiothreitol (DTT), 1mM EDTA, 5% glycerol and 50 ng poly dI:dC as nonspecific competitor. After 15 minutes at room temperature, the reactions were run on 4% nondenaturing polyacrylamide gels in 0.25X TBE buffer (1X TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA) with 4% glycerol. Gels were run at 4°C to prevent overheating and the subsequent dissociation of the protein-DNA complex.

Gel-Shift Assay

The following conditions were used to perform the gel retardation assays. The probes used were either oligonucleotides or DNA plasmid inserts. The oligonucleotide probes were generated on a Gene Assembler (Pharmacia, Cambridge, England) and were end-labelled with Klenow and ^{32}P-dCTP. The

labelled sense and antisense strands were then annealed by slow cooling to form a double stranded DNA probe. The isolated inserts were labelled with ^{32}P-dCTP using a random primed method (268). The subsequent binding conditions varied according to the particular protein used. Nuclear extract (5 to 10 μg) was preincubated in the presence of 10 mM Tris-Cl (pH 7.5), 50 mM NaCl, 1 mM dithiothrietol, 1 mM EDTA, 5% glycerol, and 4 μg poly dI:dC before the addition of 25,000 cpm of probe. Binding reactions for in vitro translated proteins and bacterially expressed recombinant proteins were done with 1 μl of the reticulocyte lysate reaction or 1 μl of the purified protein preincubated with 10 mM HEPES (pH 7.8), 4 mM $MgCl_2$, 0.1 mM EDTA, 4 mM spermidine, 100 mg/ml BSA, 2 mM dithiothreitol, 15% glycerol, and 2 μg poly dI:dC, before addtion of 10,000 cpm of probe. For competitive gel-shift assays, the cold competitor motif was added to the reaction immediately before the probe. The reactions were incubated at room temperature for 15 minutes before running them on 4% nondenaturing polyacrylamide gels in 0.25X or 0.5X TBE.

Bacterial Overexpression of Recombinant Protein

The cDNA encoding the C-terminal portion of the p70 lupus autoantigen or the TCFβ1 cDNA insert were cloned inframe into the Eco RI site of the pRSET B vector (Invitrogen, San Diego, CA). For protein expression the plasmids were transformed into *Escherichia coli* BL21(DE21) containing the pLys E (p70) or pLys S (TCFβ1) plasmid (Novagen). In the pLys E vector-host system, very little expression of the protein occurs before induction. This tight regulation of expression was essential because a leaky promoter, which expresses the C-terminal portion of p70 prematurely kills the bacteria. The bacterial culture was grown in M9ZB (1 mg/ml NH_4Cl, 3

mg/ml KH_2PO_4, 6 mg/ml Na_2HPO_4, 1mmol $MgSO_4$, 4mg/ml glucose, 50 mg/ml Bactotryptone, and 25 mg/ml NaCl) at 37°C to an OD_{600} of 1.0. Protein expression was induced by adding 1 mM IPTG followed by 4 hours incubation at 37°C. The bacterial pellet was resuspended in 20 mM Tris-Cl (pH 7.4), 500 mM NaCl, 10% glycerol, 1 mM EDTA, 0.1% NP40, 1 mM PMSF, 5 mg/ml leupeptin, and 1% (v/v) aprotinin. After sonication and multiple freeze-thaw cycles to lyse the bacteria, the desired protein was preferentially precipitated with a final concentration of 33% ammonium sulfate. This protein preparation was found to be more than 95% pure as assayed by densitometric analysis of the recombinant proteins on a Coomassie Blue stained polyacrylamide gel.

Transfections

Cells growing in suspension such as EL-4 cells were transfected by the DEAE-dextran procedure (269). In short, the cells were grown in Dulbelco's Modified Eagle Medium (DMEM) supplemented with 7% fetal calf serum. While in log phase growth, the cells were washed three times in DMEM and once in Tris Buffered Saline (TBS: 25mM Tris (pH 7.5), 137 mM NaCl, 5mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4). A total of 2×10^7 cells were mixed with 15-20 μg of DNA and 500μg/ml DEAE-dextran and incubated at 37°C for 30 to 120 minutes. The cells were subsequently washed one time with TBS and 2 times with DMEM then plated in DMEM with 7% fetal calf serum and grown at 37°C with 7% CO_2. The cells were harvested after 48 hours.

COS-7 cells were also transfected with DEAE-dextran with the following modifications. Cells were added to 100 mm dishes at 5×10^5 cells/plate the day before transfection. The next day the cells were washed

once with phosphate buffered saline (PBS), and incubated with 2 μg/ml DNA and 200 μg/ml DEAE-dextran in 4 ml DMEM with 7% fetal calf serum at 37°C for 4 hours. This was followed by three washes with PBS and one wash with DMEM. Complete media was added and the cells remained in culture 36 to 48 hours before harvesting.

The calcium phosphate method (270) was used to transfect adherent cells such as NIH3T3 and HeLa. Briefly, 5×10^5 cells were plated on 100mm dishes approximately 24 hours prior to transfection. Two to four hours before transfection, the cells were fed with 9 ml of fresh DMEM with 7% fetal calf serum. The DNA precipitate was prepared by adding the DNA (20 μg) in 500 μl of 25 mM $CaCl_2$ dropwise to an equal volume of 2X HEPES Buffered Saline (2X HEBS: 280mM NaCl, 50 mM Hepes, 1.5 mM Na_2HPO_4 (pH 7.02)), and was allowed to sit at room temperature for 20 minutes. The DNA precipitate was then added dropwise onto the cells. After 4 hours of incubation at 37°C for NIH3T3 and 16 hours at 37°C for HeLa, the cells were washed twice with phosphate buffered saline and fed with DMEM containing 7% fetal calf serum. The cells were harvested 48 to 60 hours post-transfection.

TPA Induction

Twenty-four hours after transfection TPA (dissolved in DMSO) was added to the cells at a final concentration of 60 ng/ml of media. The uninduced control received DMSO alone. The cells were then incubated at 37°C until they were harvested.

CAT Assay

After harvesting, the cell pellet was washed once in TBS and resuspended in 100 μl of 250 mM Tris-Cl (pH 7.5) and 1mM EDTA. A cell

extract was made by freezing and thawing thrice, followed by a spin at 10,000 rpm for 10 minutes to remove the cell debris. The protein concentration of the extract was determined using either the Lowry method (271) or the Bradford assay (Bio-Rad). Equal amounts of protein were used for each sample in the subsequent CAT assay. The samples were brought up to 124 µl in extract buffer and heated at 60°C for 10 minutes in order to destroy any deacetylase activity. After the extract had cooled, 24 µl of Acetyl Coenzyme A (3.0 mg/ml) and 0.1 µCi of ^{14}C-chloramphenicol was added. After a 5 to 18 hour incubation at 37°C, the sample was extracted with 1 ml ethyl acetate, lyophilized and redissolved in 20 µl of ethyl acetate. The sample was spotted on silica gel thin layer chromatography plates and run in a mixture of chloroform:methanol (95:5). The TLC plate was exposed to X-ray film at room temperature. The percent conversion was determined by counting the TLC plate in a scintillation counter, and expressed as percentage of ^{14}C chloramphenicol to 1 and 3 acetylated chloramphenicol. Cotransfection with pCH110, a β-galactosidase expression plasmid, followed by analysis of β-galactosidase activity was used to standardize all CAT assays (272).

RNase Protection Assay

The antisense RNA probe (100 to 500 nucleotides long) corresponding to a portion of the transcript of interest was generated by in vitro transcription as described earlier with the following exceptions. GTP was used alone without the 5' Capping analogue, and ^{32}P-UTP (800 Ci/mmol) was used in the place of "cold" UTP. The plasmid template could be linearized at specific sites in order to generate a probe of a distinct size. For each RNA sample, 8 x 10^4 cpm of ^{32}P-labelled probe was mixed with 1 to 10 µg of total or poly A$^+$ RNA (in the case of human thymus RNA) along with 20 µl of hybridization

buffer (80% deionized formamide, 100 mM sodium citrate (pH 6.4), 300 mM sodium acetate (pH 6.4), and 1 mM EDTA). This hybridization reaction was denatured at 90°C for 5 min and incubated at 42°C overnight. Digestion of the hybridized RNA's was done at 37°C for 30 min using a mixture of 250 Units/ml RNase A plus 10,000 Units/ml RNase T1. This mixture digests all single stranded RNA, leaving the hybridized double-stranded RNA intact. The RNase digestion was stopped using an RNA Inactivation/Precipitation mixture (Ambion, Austin, TX) which contains guanidium isothiocyanate. The precipitated RNA was pelleted by centifuging at 4°C and was resuspended in 8 µl of loading buffer (0.5 M NH_4OAc, mM EDTA, 0.2% SDS). The resuspended RNA was subsequently run on an 8% denaturing polyacrylamide gel, and the protected bands were visualized by exposing the gel to X-ray film.

Large Scale Plasmid Preps

Bacteria containing the plasmid of interest were grown overnight in 500 ml of TB (0.231% KH_2PO_4, 1.25% K_2HPO_4, 1.2% Tryptone, 0.4% glycerol) plus the appropriate antibiotic. The bacteria were pelleted by centrifuging at 5,000 rpm for 10 minutes. The pellet was resuspended in 18 ml of Solution I (50mM glucose, 25mM Tris-Cl(pH 8.0), 10mM EDTA(pH 8.0)). 2 ml of lysozyme (10 mg/ml) was added and the solution was allowed to sit on ice for 5 minutes. 40 ml of freshly prepared Solution II (0.2N NaOH, 1% SDS) was subsequently added, and the contents were mixed thoroughly. After 10 minutes at room temperature 15 ml of Solution III (60 ml 5M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O) was added. The contents were mixed by shaking and sat on ice for 10 minutes. The bacterial lysate was subsequently centrifuged at 4000 rpm for 15 min at 4°C. The supernatant was

then filtered through four layers of cheesecloth and 0.6 volumes of isopropanol was added. After 10 minutes at room temperature, centrifugation was again performed at 5000 rpm for 15 minutes. The resulting pellet was washed with 70% ethanol and resuspended in 3 ml of TE (pH 8.0). This nucleic acid solution was then mixed with 3 ml of ice cold 5M LiCl, mixed and centrifuged at 10,000 rpm for 10 minutes. An equal volume of isopropanol was added to the supernatant and the nucleic acids were precipitated by centrifuging at 10,000 rpm for 10 minutes. The subsequent pellet was dissolved in 500 µl TE (pH 8.0) containing 20 µg/ml of RNase A and was incubated at 37°C for a minimum of 30 minutes. An equal volume of 13% PEG in 1.6 M NaCl was then added to precipitate the plasmid DNA and the DNA pellet was redissolved in 100 ul TE, extracted in succession with phenol, phenol/chloroform, and chloroform and finally ethanol precipitated in the presence of ammonium acetate. The plasmid pellet was resuspended in sterile water and stored at 4°C. Alternatively, the plasmid was purified by a CsCl/EtBr equilibrium gradient centrifugation (268), which was done instead of the PEG precipitation.

Double-stranded sequencing reactions were performed with the USB (Cleveland, Ohio) Sequenase Kit as per manufacturers instructions.

DNase I In Vitro Footprinting

A singly ^{32}P end-labeled DNA probe was prepared by digesting the plasmid containing the appropriate insert with an enzme which generates a 3' overhang. This was "filled in" with ^{32}P-labeled nucleotides using Klenow (260). The plasmid was then digested with a second enzyme and subsequently gel purified to generate a probe that is labeled only at one end. For each reaction 50,000 cpm of probe was mixed with 10 µl of 10% polyvinyl alcohol,

and 4 μg poly dI:dC in a volume of 25 μl. This was incubated with 25 μl of nuclear extract in 50 mM Tris-Cl (pH 7.9), 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20% (v/v) glycerol, and 0.1 M KCl. Binding took place on ice for 15 minutes, followed by the addition of 50 μl of 10 mM $MgCl_2$ and 5 mM $CaCl_2$, both are needed for optimal DNase I activity. Two microlitres of 100-fold dilutions of DNase I were quickly mixed into the different reaction tubes and incubated at room temperature for 60 seconds. The digestions were terminated by adding 90 μl of STOP solution (20 mM EDTA, 1% (w/v) SDS, 0.2 M NaCl, and 325 μg/ml yeast RNA). The samples were extracted with phenol/chloroform, ethanol precipitated, and run on 6% denaturing polyacrylamide gels. In order to accurately map the footprint, the G and G + A Maxam-Gilbert ladders (273) were run in parallel lanes.

Preparation of Nuclear Extracts

Nuclear extracts were prepared by a modified Dignam's protocol (274). Briefly, cells grown in culture were harvested and washed in PBS supplemented with $MgCl_2$. The cell pellet was resuspended in Buffer H (10 mM Tris-Cl (pH 7.9), 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT). The cells were allowed to swell for 15 minutes on ice and were lysed by douncing 20 times with a type B pestle. The nuclei were collected by centrifugation, and resuspended in Buffer D (50 mM Tris-Cl (pH 7.5), 10% sucrose, 0.42 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 20% glycerol, 2 mM DTT, and 0.1 mM of PMSF, 2 μg/ml each of aprotinin, leupeptin, trypsin inhibitor, pepstatin A, and benzamidine). The nuclei were dounced approximately 30 times and stirred on ice for 30 minutes. Following a high speed centrifugation, the supernatant was ammonium sulfate precipitated. The protein pellet was resuspended in TM

Buffer (TM: 50 mM Tris-Cl (pH 7.9), 12.5 mM $MgCl_2$, 1mM EDTA, 0.1M KCl, 20% glycerol, and 2mM DTT), and the nuclear protein fractions were collected after running the extract through a P-10 column at 4°C..

Isolation of Total RNA and Poly A$^+$ RNA

Total RNA was isolated from tissue culture cells, as well as from mouse tissues by using the RNAzol B method (Cinna/Biotecx Laboratories International Inc). In short, the samples were homogenized in RNAzol B and extracted by the addition of chloroform. The aqueous phase was collected and the RNA was precipitated with isopropanol. The ethanol washed RNA was resuspended in DEPC treated water. and the concentration was determined by spectrophotometry.

Poly A$^+$ RNA was subsequently isolated from the total RNA using the PolyATtract mRNA Isolation System (Promega, Madison, WI). The manufacturers protocol was followed as outlined in the technical manual. Briefly, the total RNA is denatured and annealed to a biotinylated-oligo(dT) probe. The annealed RNA is then captured using streptavidin paramagnetic particles, which are collected by placing the tube containing them in a magnetic rack. The particles and their associated poly A$^+$ RNA remain on the side of the tube, while the remaining RNA and contaminants can be washed off.

Northern Analysis

Northern blotting was carried out using the protocol of Fourney et al. (275). Essentially, the RNA was run on a denaturing agarose/formaldehyde gel in a 1X MOPS/EDTA buffer, and the RNA was visualized under UV light by the addition of EtBr to the sample buffer. After a mild NaOH treatment,

the RNA was transferred to Immobilon N (Millipore, Marlborough, MA), a positively charged, hydrophobic nylon membrane by capillary transfer in 10X SSC. The use of a sponge for a wick greatly improves the efficiency of transfer. The membrane was then probed with either ^{32}P-labeled cDNA or RNA probes using standard methods as described in Maniatis et al (260). The prehybridization/hybridization solution consisted of 5X SSPE, 10X Denhardt's, 50% formamide, 2% SDS, and 100 µg/ml denatured and sheared salmon sperm DNA. Prehybridization was done at 42°C for >2 hours, followed by the addition of the denatured probe. Hybridization was done for 18 to 24 hours also at 42°C. The blot was washed several times in 2X SSC and 0.5% SDS at room temperature, followed by a wash at 50°C in 0.1X SSC and 0.1% SDS. The blot was then exposed to X-ray film with two intensifying screens at -70°C.

Chapter 3

RESULTS

I. IDENTIFICATION OF PROTEINS BINDING THE TPA INDUCIBLE ELEMENT OF THE Vβ2 PROMOTER

Changes in the level of expression of the T cell receptor genes occur at critical stages during T cell development. For example, double positive cells express low to intermediate levels of surface TCR, while single positive cells express high levels (276). Phorbol esters like TPA mimic signals from cell surface receptors by activating critical signal transduction molecules like protein kinase C (PKC), which in turn activate gene transcription.(277, 278). Transcript levels of α and β TCR genes are increased when protein kinase C is activated by TPA in both thymocytes and pre-T cell lines. TPA induction increases α and β gene expression but decreases γ and δ transcripts, whereas calcium ionophores elevate γ and δ transcripts but decrease α and β transcript levels (279 - 281). Since expression of α/β and γ/δ heterodimers on T cells are mutually exculsive (38, 282, 283, 153), this coordinated regulation suggests that TPA may mimic a relevant physiologic stimulus in T cell ontongeny.

An element that responds to TPA induction is present in the Vβ2 promoter (144). Transient transfection assays map this element between -85 and -42 upstream of the transcriptional start site. This region contains the decamer motif, an AP-1 motif and an inverted repeat (284, 147, 285) (Figure 3). More specifically, optimal inducibility is conferred by the AP-1 and inverted repeat motifs when present together. Individually these motifs have little effect on inducibility. This is surprising because the prototypical AP-1 motif

from the human collagenase gene is able to confer TPA inducibility on its own. The only difference in the Vβ2 and the collagenase AP-1 motifs, is a single G to T base substitution (Table 1). The AP-1 consensus motif, otherwise known as the TPA responsive element (TRE) has previously been identified as the only sequence required for conferrring TPA inducibility to heterologous promoters (285, 286). AP-1 (activator protein 1) is a family of proteins encoded by multiple genes that can bind to AP-1 sites in the DNA. In mammals members of this family include c-jun, junD, c-fos, fosB and fra-1. The c-jun transactivator forms a heterodimer with c-fos via their respective leucine zipper motifs. Jun/fos heterodimers bind to the DNA AP-1 sequence with a log fold higher affinity than c-jun homodimers (287 - 292). Fos/jun heterodimers are inducible by TPA. Treatment by TPA causes rapid transcription of c-fos and c-jun, as well as inducing post-translational modifications. For example, activation of PKC by TPA causes dephosphorylation of certain sites on c-jun that correlates with increased AP-1 binding activity (293). In addition, phosphorylation of different sites on c-jun following TPA treatment is responsible for increasing the transactivation potential of c-jun (294, 295).

Interaction of Vβ2 AP-1 Motif with AP-1 DNA Binding Proteins

I wanted to determine if the G/T base substitution in the Vβ2 AP-1 motif weakens the functional DNA-protein interaction as compared to the prototypical collagenase AP-1 motif. To test this jun B/c-fos* heterodimers were generated by in vitro transcription and translation. A gel mobility shift assay was used to monitor binding of jun B/c-fos heterodimers to the

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* The junB and junD cDNA's were a gift from Dr. D. Nathans, Johns Hopkins. The c-fos cDNA was kindly provided by Dr. I. Verma, Salk Institute.

collagenase AP-1 motif probe. The collagenase AP-1 motif alone is sufficient for mediating TPA-stimulated transcriptional activity (296). As a competitor in this assay, the collagenase AP-1 motif competed at least 16 fold better than the Vβ2 AP-1 motif determined by the amount of competitor oligonucleotide required to abolish binding (Figure 4). Jun D in combination with c-fos, jun B, or c-fos alone did not bind to the AP-1 motif (Data not shown). These results indicate the c-fos/jun B heterodimer bound to the collagenase AP-1 motif with an affinity at least 16 times higher than to the Vβ2 AP-1 motif. This difference in affinity helps explain why the Vβ2 AP-1 motif was insufficient to confer optimal TPA inducibility by itself.

Since the 3' flanking inverted repeat region was required in addition to the Vβ2 AP-1 motif to confer optimal TPA inducibility, this inverted repeat region might help to increase the affinity of jun B/c-fos heterodimers for the Vβ2 AP-1 site. To test this, the ability of the -85 to -73 (IR), -84 to -62 (AP-1/IR), and the -75 to -62 (AP-1) oligonucleotides to compete for binding to the collagenase AP-1 motif probe in a gel-shift assay was determined. The -73 to -62 oligonucleotide containing only the inverted repeat region did not compete for binding. The -84 to -62 oligo, which contains both the Vβ2 AP-1 motif and the 3' flanking inverted repeat region competed as efficiently as the -85 to -73 oligonucleotide which contains only the Vβ2 AP-1 motif (Figure 4). This argues for the absence of any binding sites for jun B/c-fos heterodimers in the inverted repeat region and also for the inability of the inverted repeat region to increase the affinity of jun B/c-fos heterodimers for the Vβ2 AP-1 sequence.

An alternative explanation to the dual requirement of the 3' inverted repeat region and the AP-1 region, is that TPA inducibility is conferred by the binding of a distinct transactivator to the inverted repeat region. The

transactivator would act in association with jun B/c-fos heterodimers. To test whether another protein binds to the inverted repeat motif, TPA induced EL-4 extracts were used in competitive gel-shift assays (Figure 5). When the -75 to -62 (IR) oligo was used as a probe, the -75 to -62 oligo competed, whereas the -85 to -73 oligo (AP-1 motif) did not. These data suggest that sequence-specific proteins that bind to the 3' flanking region do not bind to the Vβ2 AP-1 motif. In the converse experiment, using the -85 to -73 oligo (AP-1 motif) as a probe, the -85 to -73 oligonucleotide competed while the -75 to -62 oligo (inverted repeat motif) did not compete. These data confirm that proteins which bind to the Vβ2 AP-1 motif cannot be competed by the 3' flanking sequence. This demonstrates that proteins in addition to jun B and c-fos bind to the -84 to -62 region of the Vβ2 promoter.

Identification of the Second Transactivator

The presence of an ets-like motif GAGGAAGT in the 3' flanking inverted repeat region provided a clue in determining the identity of the additional transactivator. The probable role of ets transactivator involvement was directly tested by assaying the ability of the polyoma canonical ets motif and the Vβ2 inverted repeat motif to cross-compete in gel-shift assays (Figure 5). In competition experiments using the inverted repeat probe, both the inverted repeat and the polyoma ets motif competed, while the Vβ2 AP-1 motif did not compete. Identical results were obtained using the polyoma ets motif as a probe, in which both the ets motif and inverted repeat oligos competed, whereas the Vβ2 AP-1 motif did not compete. This implies that the ets protein binds the inverted repeat motif in the Vβ2 promoter.

The conclusion that ets binds to the inverted repeat region was then directly tested by generating an in vitro transcribed and translated ets-2 protein*. The recombinant ets protein bound to the Vβ2 inverted repeat motif (Figure 6). The IR-ets DNA-protein complex was successfully competed with the Vβ2 inverted repeat motif but not with the Vβ2 AP-1 motif. This is consistent with the binding specificity of the EL-4 nuclear extract (Figure 5). It can thus be concluded that ets-2 binds the Vβ2 inverted repeat in a sequence-specific manner.

The affininity of jun/fos heterodimers for the Vβ2 AP-1 motif is not increased by the presence in cis of the inverted repeat motif. The 3' flanking inverted repeat binds the ets transactivator but not jun/fos heterodimers. The demonstrated cooperativity between the AP-1 and the 3' flanking sequence to confer TPA inducibility to the TCR Vβ2 promoter can best be explained by the cumulative individual contributions of the jun B/c-fos heterodimer binding to AP-1 and the ets transactivator binding to the inverted repeat.

Discussion

An analysis of the TCR Vβ2 promoter indicates that the it is TPA-inducible. The TPA inducible element maps between -85 and -62, a region which contains the conserved AP-1 and inverted repeat motifs. Both motifs are required for conferring optimal TPA inducibility. The -84 to -62 Vβ2 motif and the prototypic collagenase TRE element are equally inducible. While both the TCR decamer (-64 to -53) (145) and the -84 to -62 motifs increase basal transcription, only the -84 to -62 motif confers TPA inducibility (297).

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*The ets-2 cDNA was kindly made available by Dr. R. Maki, La Jolla Cancer Research Foundation.

The conserved AP-1 or TRE motif has previously been identified as the DNA sequence that confers TPA inducibility to heterologous promoters (285, 286). The Fos and Jun family of leucine zipper transcription factors bind to the AP-1 site to mediate this inducibility (289 - 292). The TCR Vβ2 promoter, on the other hand, requires the 3' flanking inverted repeat motif (-61 to -73) in addition to the AP-1 motif (-75 to -82) to confer inducibility by TPA. The decreased affinity of the core Vβ2 AP-1 motif for jun/fos heterodimers explains its inablity to confer optimal TPA induciblity. The decreased binding affinity results from a single G to T base substitution. The conclusion that the ets protein binds the Vβ2 inverted repeat motif is based on the ability of the canonical polyoma ets and Vβ2 inverted repeat motifs to cross-compete, and on the ability of recombinant ets-2 protein to bind the inverted repeat motif.

The jun/fos heterodimer binds the Vβ2 AP-1 motif but not the Vβ2 inverted repeat motif; the ets transactivator, conversely, binds the Vβ2 inverted repeat motif but not the Vβ2 AP-1 motif. Since both motifs are required for optimal inducibility by TPA, the contributions of the jun/fos heterodimer in association with the ets transactivator likely confers this inducibility to the Vβ2 promoter. The cooperativity between fos/jun and ets demonstrated in polyoma gene expression (298, 299) is consistent with this conclusion. The Vβ2 data extend the polyoma observations by distinguishing two functional types of AP-1 sites. The AP-1 site in the collagenase gene confers optimal TPA inducibility by itself while the AP-1 site in the TCR Vβ2 promoter requires a flanking ets-2 site to confer optimal TPA inducibility. In addition, these results characterize an interaction between fos/jun heterodimers and ets in a TCR Vβ promoter. The binding of fos/jun proteins to the AP-1 motif, and ets-2 to the inverted repeat, concurs with footprinting

experiments done with the human Vβ8.1 promoter region in which AP-1 like sequences and the IR motif were both protected (145).

Ets belongs to a family of protooncogenes that are sequence-specific transactivators. The ets binding site is found in the regulatory region of a number of eukaryotic genes. T cells express many different members of the ets family of proteins including ets-1 and ets-2. Like expression of fos and jun, TPA treatment also increases ets expression.

Phorbol esters, like TPA, activate protein kinase C within the cell. The activation of PKC is an important component in T cell activation. The characterization of a TPA element in the Vβ2 promoter agrees with previous reports of phorbol ester enhancement of TCR transcription. Transcript levels of α and β TCR genes are increased by PKC activation in Jurkat (279), a human T cell tumor, human thymocytes (280), and EL4, a murine T cell tumor. An NF-κB TPA inducible element is present in the Jβ-Cβ intron (199). This raises the possibility that the Vβ2 TPA element, which is 800 kb from Cβ2, and the TPA element in the Jβ-Cβ intron, could both be involved in inducing transcription at the time of rearrangement, thus opening up the entire β locus to the recombination machinery (56). The presence of the inducible 1.3 kb (VβDβJβCβ) and 1 kb (DβJβCβ) transcripts at the time of β gene rearrangement (279) argues in favor of this hypothesis. Alternatively, the TPA elements may be involved in induction of β transcripts after TCR β gene rearrangement. The fact that an AP-1 like element is found in all members of the human Vβ8 family and in 11 out of 14 murine Vβ's analyzed, and an inverted repeat motif is equally abundant (147), argues in favor of the TPA signal controlling each Vβ separately. Separate control of each Vβ would be expected if the TPA element exerted its effect after rearrangement.

Fluxes in TCR gene transcription are the driving force behind T cell maturation. Intriguingly, TPA increases α and β transcripts but decreases γ and δ transcripts, while calcium ionophores elevate γ and δ transcripts but decrease α and β transcript levels (281). The fact that α/β T cells express little or no γ/δ RNA and that γ/δ T cells express little or no α/β RNA suggests that the coordinated TCR expression observed with calcium ionophores and TPA is physiologically releveant and that TPA may mimic a physiological stimulus in T cell ontogeny.

The presence of TPA induces both the fos/jun heterodimers and the ets transactivator which are found in high levels in T cells but are not T cell specific. Consistent with this expression pattern, the Vβ2 TPA element is active in T cells and fibroblasts (297). However, the TCR β genes are rearranged and expressed in T cells only. The physiological effects of such an element that is not T cell-specific could be restricted to T cells if it interacted with other cis transcriptional elements or with the TCR β enhancer to render it specific to T cells.

Ets binding sites identified in the TCR β, α and δ gene enhancers are essential for enhancer activity (184). The finding that the ets transactivator binds to both the promoter and enhancer regions of the Vβ2 gene to control gene expression is consistent with a number of other genes, in which the same protein binding site is present in both the promoter and enhancer regions. The immunoglobulin gene which contains an octamer site in both the promoter and enhancer is an example. The binding of the same transactivator to two different regulatory DNA elements of the same gene may be an important strategy in transcriptional regulation.

The identification of a novel AP-1 site that requires the adjacent ets binding site for optimal TPA inducibility describes a new mechanism by

which AP-1 binding proteins can control transcription. The single base substitution in the Vβ2 promoter AP-1 binding site decreases the binding and thus transactivation potential of fos/jun heterodimers. Combining the AP-1 site with the adjacent ets site allows the cell to regulate three transactivators rather than only two in order to control TPA inducible transcription of the Vb2 promoter. The availability and activity of various combinations of the three transactivators may dicatate differing levels of transcription. Fos and jun proteins alone are able to induce the Vβ2 promoter, however, the addition of ets increases this ability. Evidence indicates that ets and fos/jun proteins can cooperate with each other in transcriptional activation (299). Our data suggest that the binding affinity of fos/jun heterodimers can control an ets-fos/jun interaction in transcriptional activation. Decreasing the affinity of fos/jun heterodimers for the TPA site in the Vβ2 promoter lowers the ability of fos/jun heterodimers to transactivate via the AP-1 site, and increases the requirement for another transactivator to confer optimal TPA inducibility. It remains to be determined if this novel mechanism of controlling the binding and thus activation potential of transactivators as demonstrated in the TCR Vβ2 gene promoter will have a more general significance in the transcriptional regulation of other genes.

TABLE I*

AP-1 CONSERVED SEQUENCES IN DIFFERENT TPA INDUCIBLE GENES

Human Metallothionen gene	CAAGTGACT**CAG**CGCGGGGCGTGTGCA
Human Collagenase gene	AGCATGAGT**CAG**ACACCTCTGGCTTTC
Rat Streptolysin gene	ATTATGAGT**CAG**TTTGCGGGTGACTCT
SV40 Element 1	TCAATTAGT**CAG**CAACCATAGTCCCGC
SV40 Element 2	AAAATTAGT**CAG**CCATGGGGCGGAGAA
Mouse IL-2 gene	CCATTCAGT**CAG**TATATGGGGTTTAAA
TCR $V_{\beta 2}$ gene	AAGCTTAGT**CAG**TTCCTGAGGAAGTC

*Sequences are described in 300 and 301.

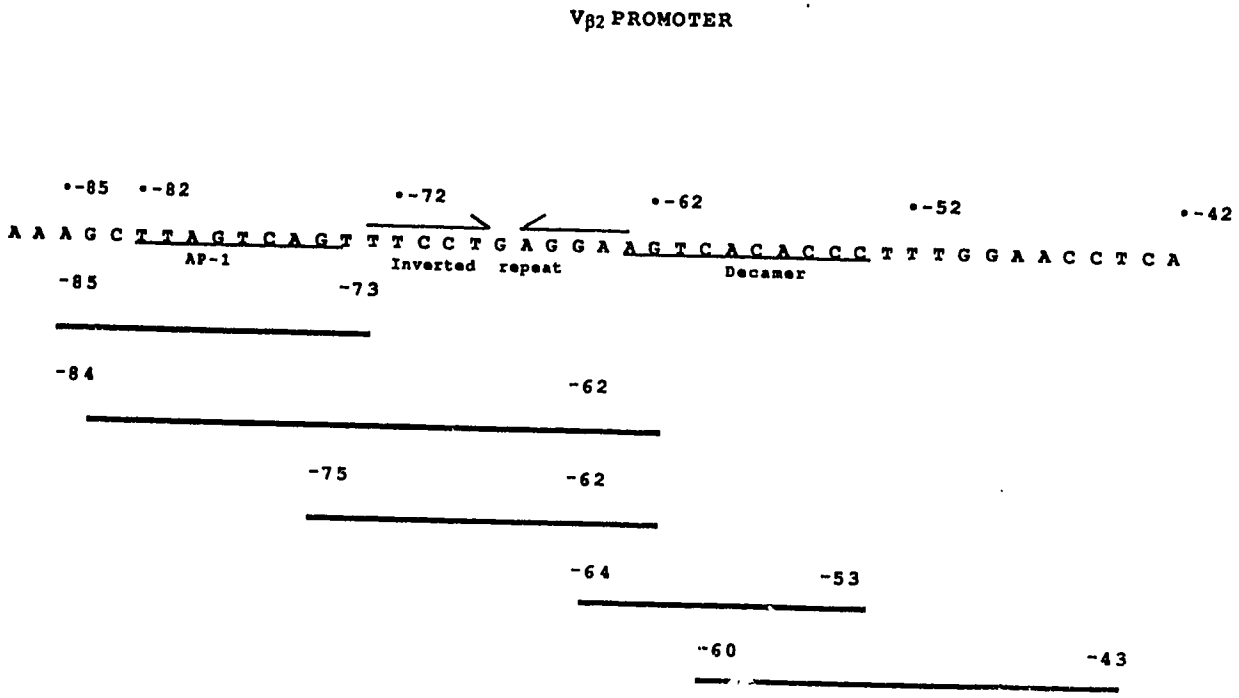

Figure 3. Map of the transcriptional elements within the Vβ2 promoter region (144).

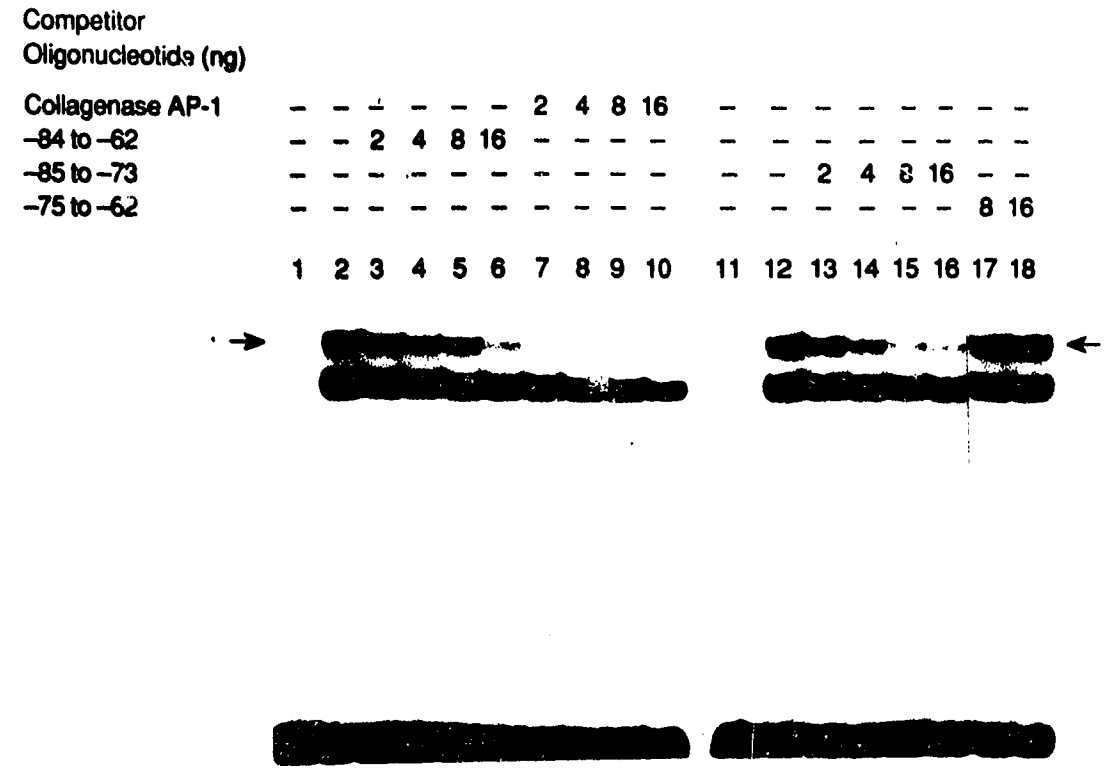

Figure 4. Gel shift assays of in vitro translated jun B/c-fos heterodimers using a collagenase AP-1 probe. Jun B or C-fos homodimers did not bind. Competition experiments were performed with excess cold double-stranded oligonucleotides. Arrow indicates the position of the specific DNA-protein complex. *Lanes 1* and *11*: Probe alone; *Lanes 2 and 12:* Probe + jun/fos + dI:dC (500ng); the shifted band was competed out with excess cold -84 to -62 (AP-1/IR) oligo in *lanes 3 to 6:* collagenase AP-1 oligo in *lanes 7 to 10:* -85 to -73 (AP-1) oligo in *lanes 13 to 16:* or the -75 to -62 (IR) oligo in *lanes 17 and 18.*

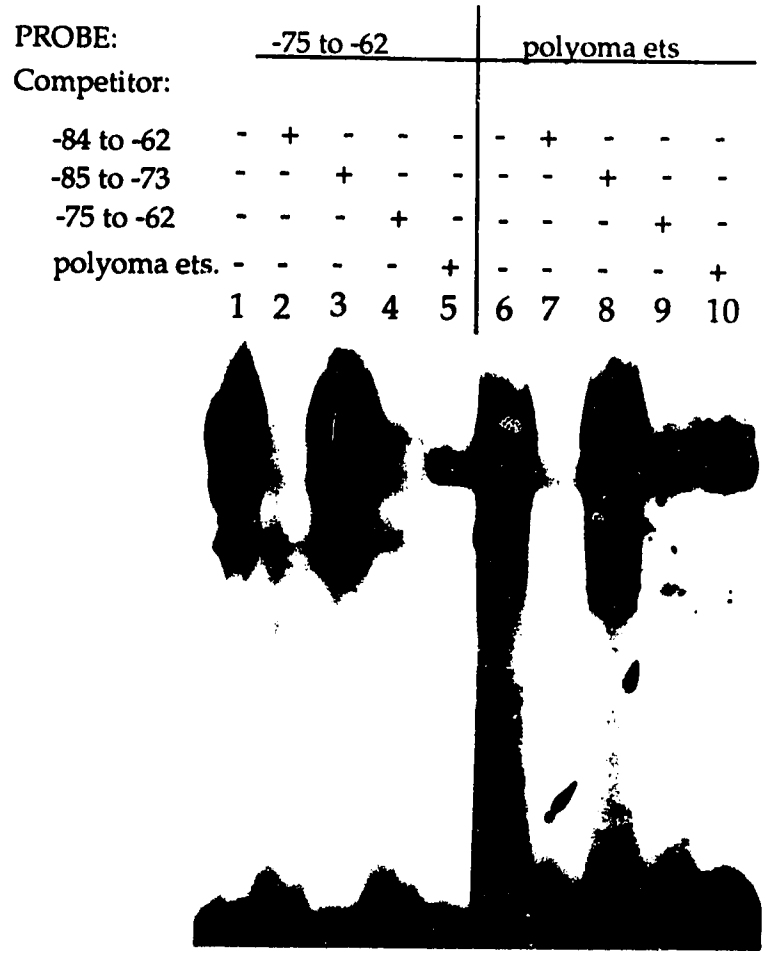

Figure 5. Binding of ets transactivator to the inverted repeat motif (-75 to -62) in the TCR Vβ2 promoter. Cross-competition experiments in gel shift assays of the inverted repeat (-75 to -62) probe in *lanes 1 to 5;* and the polyoma canonical ets probe in *lanes 6 to 10* with TPA induced EL4 extracts. The comparative ability of 100 ng of the -75 to -62 *(lanes 4 and 9),* the -85 to -62 *(lanes 2 and 7),* the -85 to -73 *(lanes 3 and 8)* and the polyoma ets *(lanes 5 and 10)* double stranded oligos to compete in gel shift assays was determined.

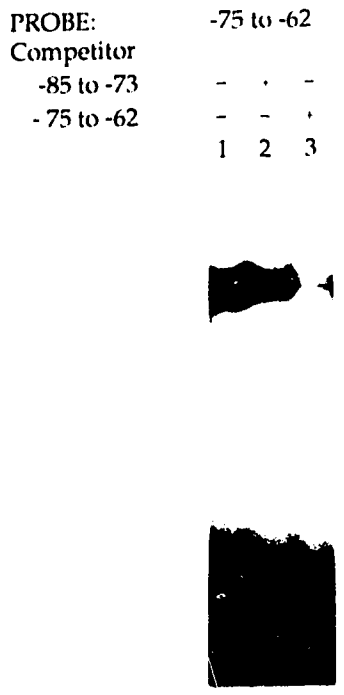

Figure 6. In vitro transcribed and translated ets-2 protein binds the inverted repeat (-75 to -62) motif. The ability of ets-2 protein to bind the -75 to -62 probe in a sequence specific manner was determined by comparing the ability of the -85 to -73 *(lane 2)* and the -75 to -62 *(lane 3)* double stranded oligonucleotides to compete (50 ng of each competitor) in this gel-shift assay.

II. THE HIERARCHICAL INTERACTION BETWEEN DIFFERENT ELEMENTS IN THE β LOCUS

A deletional analysis of the Vβ2 promoter upstream region suggested the presence of a transcriptional inhibitory element. The -42 to +31 promoter fragment was not TPA inducible, whereas the -85 to +31 and -243 to +31 Vβ2 promoter fragments were TPA inducibile, indicating the presence of a TPA inducible element between -42 and -85. Surprisingly, the -700 to +31 and -2000 to +31 Vβ2 promoter fragments were not inducible by TPA (Figure 7). This suggests sequences upstream of -343 in the Vβ2 promoter inhibit TPA inducibility. This is consistent with data indicating that this region can inhibit basal expression from the Vβ2 promoter in T cells (144).

Effect of the Vβ2 Inhibitory Region on Basal Transcription from a Heterologous Promoter

To ascertain if the Vβ2 upstream inhibitory sequence is a silencer element, the fragment from -343 to -739 (Bgl II to Eco RI) was cloned upstream of the heterologous thymidine kinase promoter. The presence of this fragment reproducibly inhibited TKCAT activity (Figure 8). The ability to transfer inhibition to a heterologous promoter is indicative of a silencer element. The Vβ2 upstream region was also able to inhibit transcription when present in either orientation, also indicative of a silencer element. This data suggests that the region upstream of -343 in the Vβ2 gene is a silencer.

Effect of the Vβ2 Inhibitory Region on Inducible Transcription From a Heterologous Promoter

The silencer region was identified by its inhibition of TPA induciblity in the Vβ2 promoter. It was shown that this region could inhibit basal expression from a heterologous promoter. If the silencer interaction with the TPA region is genuine, such an interaction should be transferrable to a heterologous promoter. This was tested by generating two contructs in which the Vβ2 promoter fragments were cloned upstream of the heterologous TK promoter in the pBLCAT2 vector. One fragment, from -739 to +31, contained both the inhibitory and TPA inducible elements, while the second fragment from -343 to +31 contained only the TPA inducible element. Cloning fragments into the vector in the antisense orientation prevented transcripts initiated from the Vβ2 promoter from causing CAT expression. The transcriptionally active elements, the silencer, and TPA inducible element, on the other hand, would still be able to affect the TK promoter and thus CAT activity. The construct containing only the TPA inducible element conferred TPA inducibility to the TK promoter. In contrast, TPA inducibility was completely absent from the construct that contained both the TPA element and the inhibitory region (Figure 9). These results indicate that the 5' inhibitory element abrogates TPA inducibility and that this interaction can be transferred from the Vβ2 promoter to a heterologous promoter. These data also indicate that both the TPA element and the silencer element are orientation independent.

The Active Fragment of the Inhibitory Region Binds Protein

If the inhibitory region is a genuine silencer element, it should act as a binding site for repressor proteins. To test this a number of restriction fragments of the -343 to -739 inhibitory element were cloned into the puc 18 vector and used as probes in gel shift assays. Using nuclear extracts from EL4 T

cells, nuclear proteins bound at least one area within the 400 bp inhibitory region (Figure 10). This protein-DNA complex could be abolished by competition with the non-radioactive specific competitor used as the probe, but not with irrelevant DNA fragments.

To further map the exact protein binding motifs of the inhibitory element, DNase I footprinting was done using the same Dde I fragment that was used in the gel-shift assays. A protein present in EL4 nuclear extracts protects a portion the Dde I fragment, forming an in vitro footprint (Figure 11). The specific binding of at least one nuclear protein to the 5' Vβ2 promoter region is consistent with the definition of a silencer, and suggests that the Vβ2 upstream region may act mechanistically like a silencer element.

TCR β Enhancer is a Dominant Element Which Regulates Transcription of the β Locus

Data presented earlier indicate that the sequence between -85 and -62 within the Vβ2 promoter acts as a TPA inducible element, that sequences 5' to this are inhibitory, and that the presence of the inhibitory element with the TPA element actually abrogates TPA inducibility. These interactions between the inhibitory and TPA inducible elements can be transferred to a heterologous promoter. To determine the role of the TCR β enhancer on the interaction between the TPA and inhibitory promoter elements, transient transfection experiments were undertaken in EL4 cells. The presence of the TCR β enhancer in cis overrides the effect of the inhibitory sequence on TPA inducibility (Figure 7).

In T cells the TCR Vβ2 minimal promoter (-42) is not TPA inducible since it lacks the TPA element (AP1/IR), the -343 construct is TPA inducible since it contains this element. The inhibitory sequence 5' of -343 abrogates

TPA inducibility, but the presence of the β enhancer in cis overrides the effect of the inhibitory region on TPA inducible expression. These data suggest a dominant role of the β enhancer in regulating β gene expression.

The β Enhancer Controls Tissue Specific Gene Expression

The importance of the β enhancer in controlling tissue specificity was tested by transfecting the Vβ2/CAT constructs shown in Figure 7 in NIH3T3 fibroblast cells. In this non-T cell line, the TPA inducible element was active (-343 construct), and the inhibitory region was still able to abrogate TPA inducibility (-1635 construct). However, the β enhancer was unable to overcome the effect of the inhibitory element (Figure 12). These results indicate that the β enhancer is the only cis-acting element in the TCR β chain gene identified so far that confers tissue specificity.

The tissue specificity of the β enhancer was also seen in constructs in which the β enhancer was cloned upstream of the heterologous Thymidine Kinase promoter. In these constructs the enhancer was active in EL4 T cells, but had no enhancing activity in NIH3T3 fibroblast cells, indicating the T cell-specific activity of the β enhancer.

Discussion

The interaction between three elements active in the TCR Vβ2 promoter has been analyzed and a number of conclusions can be drawn. 1) The inhibitory region acts like a silencer motif, it is transferrable to a heterologous promoter and acts in an orientation independent manner; 2) The inhibitory region is capable of inhibiting both basal and TPA inducible gene expression and is active in T cells and fibroblasts; 3) The inhibitory sequence binds to at least one nuclear protein; 4) The TCR β enhancer is the

dominant tissue-specific element; and 5) The enhancer is able to override the effect of the inhibitory sequence on both basal and inducible gene expression in a tissue specific manner.

Evidence that the Vβ2 inhibitory region is a silencer, like the silencer elements originally described in yeast (302), is consistent with the presence of multiple regulatory elements between -739 to -343 and -2570 to -1902 (144) that have homology to gene silencers found in the chicken lysozyme gene (303), the mouse immunoglobulin heavy chain gene (304, 305) and many other eukaryotic silencers (306 - 308). The Vβ2 promoter region that demonstrates functional inhibition binds proteins and contains conserved motifs also found in other silencers.

The prototypical silencer element containing silencer homology motifs is upstream of the chicken lysozyme gene. This element has a modular structure similar to enhancers and binds to proteins that can activate as well as repress gene transcription through interacting with the promoter. Therefore, since the TCR Vβ2 upstream silencer contains similar homology motifs, it may be capable of mediating complex transcriptional control.

Silencers might exert their inhibitory effects on transcription in a number of different ways. They might inhibit the binding of positively acting transcriptional factors, or they might inhibit either the basal or activated transcriptional process by direct protein-protein interactions. An inhibition of DNA looping thus preventing positive factors from interacting with the basal transcriptional machinery, or a sequestration of positive factors making them unavailable for transcription are also possible silencer mechanisms.

The 500 bp TCR β chain enhancer identified by Krimpenfort et al (156) dominates the interaction between the three TCR β transcriptional elements. Its presence abrogates the ability of the inhibitory region to repress basal and

inducible transcription. The β enhancer is so far the only element that confers T cell-specific expression to the β chain gene. This agrees with data from McDougall et al (155) and Krimpenfort et al (156) in which TCR β enhancer activity was absent in fibroblasts.

A silencer, a TPA element, and an enhancer have been described in the control of Vβ2 transcription. The question then arises as to the role these elements play in regulating TCR β gene expression during development. The inhibitory region may be involved in helping to restrict β gene expression to T cells. This role of the silencer is supported by the inability of the enhancer to overcome repression of TPA inducibility, mediated by the inhibitory region in fibroblast cells. The interaction between the inhibitory, TPA inducible and enhancer elements might form the basis of transcription of unrearranged Vβ genes prior to rearrangement. This could be accomplished by the transient neutralization of silencer activity at the appropriate stage of T cell maturation. Alternatively, the elements may be responsible for fluxes in TCR β transcription after rearrangement.

The coordinated interaction of the three elements provides a model system which indicates the importance of the β enhancer. It is the dominant element in this interaction and is the only one of these elements that confers tissue specificity. Therefore, in order to understand β gene expression, it is essential to understand the proteins that bind to the TCR β chain enhancer and regulate enhancer activity.

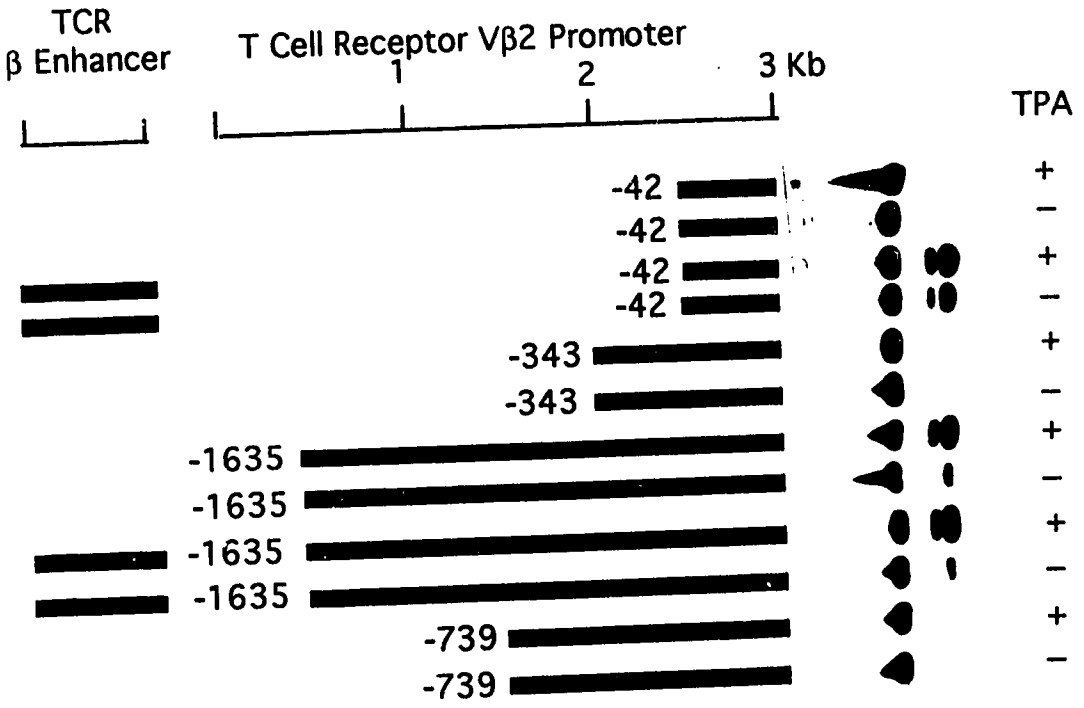

Figure 7. Demonstration of the functional interactions between the TPA inducible, inhibitory, and enhancer elements in the TCR Vβ2 gene in T cells. Transient transfection experiments of constructs in which 5' nested deletions of the TCR Vβ2 promoter region drive CAT expression in EL4 cells. The constructs used from -1635 to +31 are shown. In certain constructs the TCRβ enhancer was added 5' of the promoter region. Transfected cells were induced with TPA in DMSO or DMSO alone 12 hours before harvesting. The amount of extract used in each CAT assay was standardized to β-galactosidase expression from co-transfection of the pCH110 plasmid (SV40 promoter). A representative experiment of n>9 is shown.

EL-4

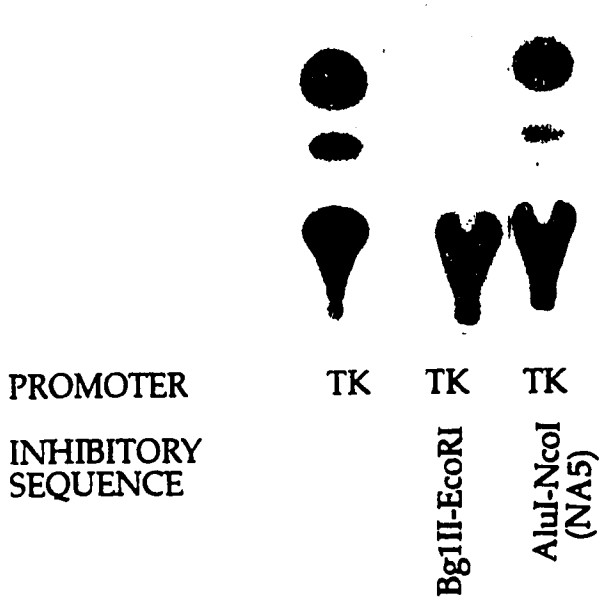

Figure 8. Effect of the inhibitory sequence on heterologous Thymidine Kinase promoter activity. The Bgl II-Eco R1 (-343 to -739) inhibitory region fragment was cloned upstream of the TK promoter in TKCAT. Alu I-Nco I (NA5) is the the core β enhancer, also cloned upstream of the TK promoter. Transient transfections were done in EL4 cells. The CAT assay was standardized by cotransfection of a β-galactosidase expressing plasmid (pCH110).

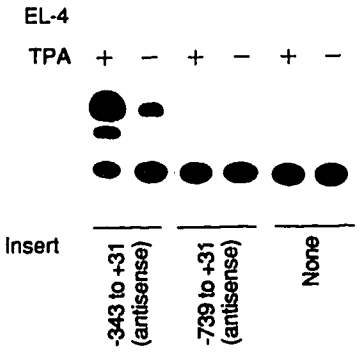

Figure 9. The abrogating ability of the inhibitory sequence on the TPA inducible element in the Vβ2 promoter can be conferred to a heterologous Thymidine Kinase promoter. Two fragments -743 to +31 (contains both the inhibitory and TPA inducible elements) and -343 to +31 (contains only the TPA inducible element) were cloned upstream of the TK promoter in the pBLCAT2 vector in the antisense orientation. In this orientation transcripts initiated from the Vβ2 promoter would not cause CAT expression, but the transcriptionally active elements would be able to affect TK promoter and thus CAT activity. Transient transfection experiments were done in EL4 cells, and CAT assays were standardized by co-transfection of the pCH110 plasmid.

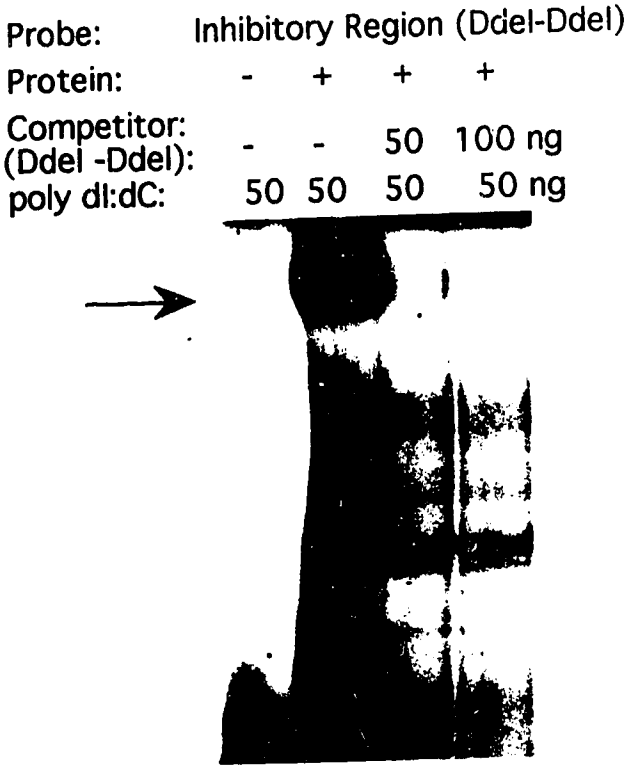

Figure 10. Gel shift assays using the inhibitory region probe and EL4 nuclear extracts. The probe is a 400 bp Dde I-Dde I subfragment of the Bgl II-Eco RI Vβ2 inhibitory region. *Lane 1:* Probe alone, *Lane 2*: Probe+Extract+dIdC, *Lanes 3 and 4:* Probe + Extract + dIdC + 50 and 100 ng of the specific competitor (Dde I-Dde I). The arrow indicates the position of the specifice DNA-protein complex. Irrelevant DNA fragments did not compete.

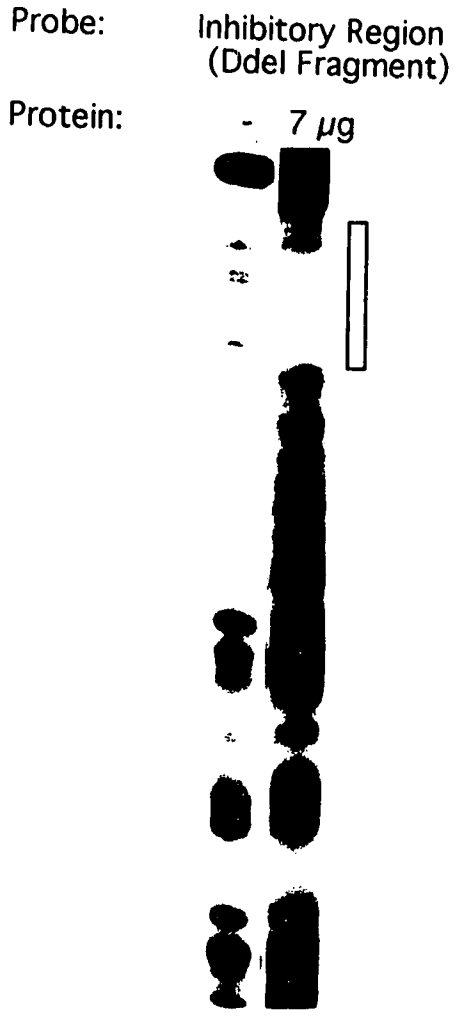

Figure 11. DNase I footprinting of a Vβ2 inhibitory region probe (Dde I-Dde I). *Lane 1:* Free Probe, *Lanes 2* Bound probe with 7 μg EL4 nuclear extract. The protected region is indicated by a rectangle, as seen by the disappearance of three bands upon the addition of extract. The darker bands on either side of the protected region are likely due to DNase I hypersensitivity caused by an opening up of the DNA surrounding the bound protein.

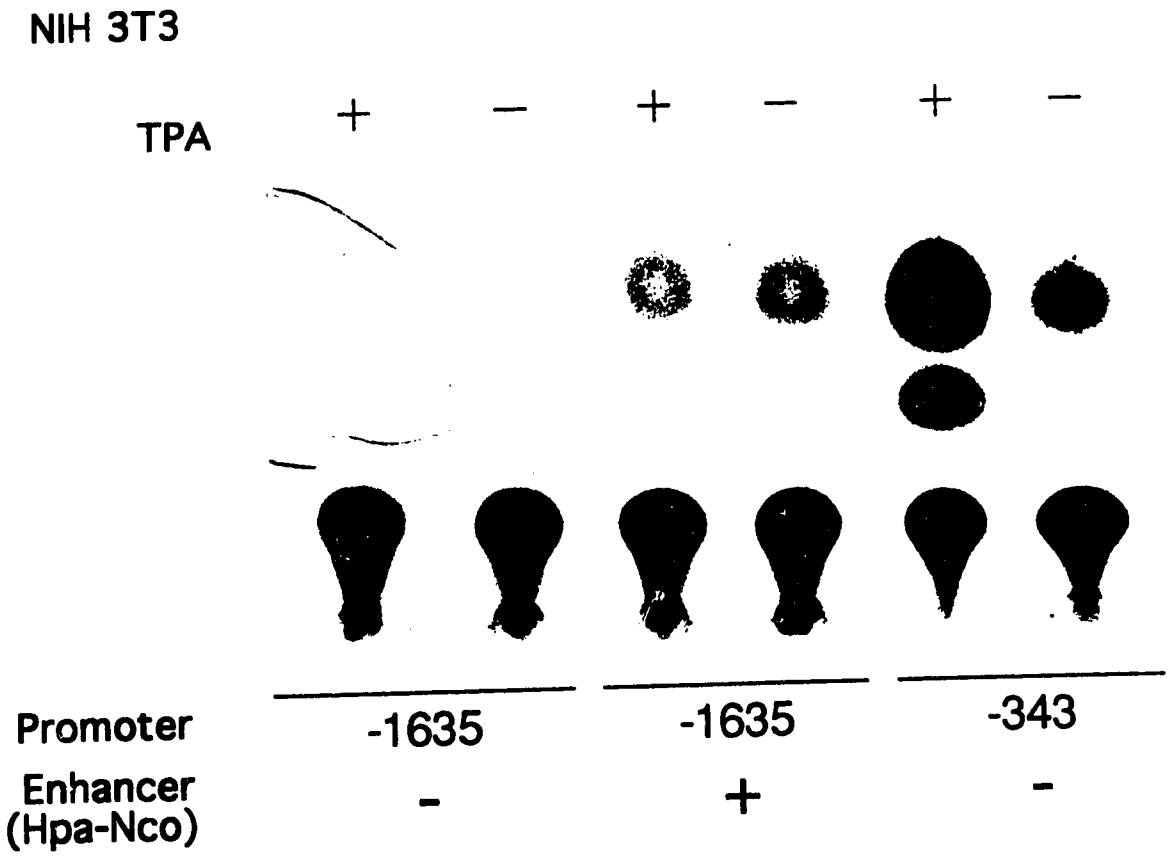

Figure 12. Tissue specificity of the interaction between the transcriptional elements in the TCR β chain gene. Interaction of TPA inducible (within -343 fragment), inhibitory (-343 to -1635) and β enhancer elements in NIH3T3 cells. The Vβ2 promoter region drives CAT expression. The TCR β enhancer was cloned upstream of the 5' inhibitory region. Transient transfections were done in NIH3T3 cells with the addition of TPA/DMSO (+) or DMSO only (-). CAT assays were standardized by cotransfection of a β-galactosidase expression plasmid (pCH110). The abrogating influence of the inhibitory region on the TPA inducible element was not overcome by the presence of the β enhancer.

III. IDENTIFICATION OF β ENHANCER BINDING PROTEINS

The β enhancer, located 3' of the Cβ2 gene is the dominant element in controlling β gene expression during ontogeny and ensuring that expression occurs exclusively in T cells. DNase I footprinting experiments done in our lab (159) identified 4 motifs within the core TCR β enhancer that are bound by proteins, suggesting the importance of these motifs in controlling β enhancer activity. In order to understand how the enhancer mediates its activity, it is essential to identify and understand the proteins that bind to these critical cis-motifs.

III.A. p70 BINDS THE β ENHANCER E3 MOTIF

The E3 Motif is Critical for TCR β Enhancer Activity

In vitro footprints were mapped to the E3 motif in both mouse and human DNA (148, 157), and the E3 motif is 100% conserved between mouse and human (148). In addition, an in vivo interaction of DNA-protein in the T cell chromatin was identified at this site (S. Mangal in 309). All of these observations strongly suggest that the E3 motif is important for TCR β enhancer activity. See Figure 1 for identification of the E3 motif. To prove the critical role of the E3 motif in the regulation of β enhancer activity substitutional mutagenesis of the E3 motif in the TCR β enhancer fragment (positions 440 - 780) was conducted (Table III). This mutated enhancer was put in the pBLCAT2 vector in which the Thymidine Kinase (TK) promoter drives CAT reporter gene activity. These mutations of the E3 motif decreased β enhancer activity by >80% in transient transfections into EL4 cells (Figure

13). Therefore the conclusion that the E3 motif is critical in regulationg TCR β enhancer activity is correct.

Binding Specificity of the p70 Lupus Autoantigen

To identify the proteins that bind to the E3 motif, a southwestern screening of a λgt11 Jurkat T cell library, using the E3 motif as a probe was undertaken. The screening identified two clones encoding proteins that bound to the E3 motif but not to the AP-1 or to the Ets motifs (H. Brickner in 309). The sequencing of the genes encoding the proteins revealed that these E3-binding proteins were encoded by the C-terminal portion of the p70 lupus autoantigen gene. If the p70 protein encoded by the cloned gene regulates β enhancer activity via its binding to the E3 motif, it must bind to this motif in a sequence-specific manner. It was therefore critical to determine if a recombinant p70 protein could specifically bind to the E3 motif in vitro. To test this the C-terminal portion of p70 including amino acids 111 - 222, obtained from the Jurkat library, was cloned into the pRSET B vector and expressed in *E. coli* . The resulting recombinant protein was tested in gel mobility shift assays. The p70 protein bound the E3A probe (Figure 14). Formation of the E3A-p70 DNA-protein complex was blocked by competition with nonradioactive E3A or βE1 (which completely overlaps the the E4A motif), but not by Vβ2 AP-1 (-85 to -73) or Vβ2 Ets (-75 to -62) motifs (Table 2). The wildtype E3A motif competed for binding to the p70 protein, whereas, the mutant E3A motif (Table 3) failed to compete (Figure 14). The substitution of this nonbinding mutant E3A motif into the wild-type β enhancer reduced enhancer activity by 70% (148). This result supports the conclusion that p70 lupus autoantigen binds DNA in a sequence-specific manner and likely acts as a positive factor in regulating TCR β enhancer activity.

Further support of the binding specificity of the p70 protein was shown in supershift assays, in which anti-Ku (p70/p80) antisera could specifically supershift the E3 DNA-protein complexes but not irrelevant DNA-protein complexes in a gel-shift assay with Jurkat nuclear extracts (J. Gaikwad in 309). These data demonstrate that a majority of the E3-binding proteins in T cells bear Ku (p70/p80) autoantigenic determinants. A UV cross-linking assay identified a 70 kDa and an 80 kDa protein from T cell nuclear extracts that bound to the E3 motif in a sequence-specific manner (T. Fuller in 309). The UV cross-linking assay utilized a ^{32}P-labeled, bromodeoxyuridine substituted DNA probe for the E3 region. The probe was bound by EL4 nuclear extracts and subsequent exposure to ultraviolet light permanently cross-linked the bound proteins to the DNA. The molecular weight of the E3-binding proteins could then be visualized by SDS-PAGE. The fact that the p70 lupus autoantigen binds the E3 motif agrees with the molecular weight of the 70 kDa E3-binding protein identified by UV cross-linking.

Discussion

The generation of autoantibodies against cellular constituents such as DNA, RNA and proteins is common in patients with systemic lupus erythematosis (SLE). The p70 lupus autoantigen is a target of autoantibodies, found in approximately 10% of patients with SLE and related autoimmune disorders (310 - 315). p70 comprises part of the Ku lupus autoantigen. The Ku antigen is a heterodimer consisting of 70 and 80 kDa subunits (p70 and p80), and the production of anti-Ku autoantibodies correlates strongly with particular subsets of autoimmune disease (310, 311).

The physiological function of the Ku lupus autoantigen is still unclear. It has been suggested that the p70/p80 heterodimer interacts with the free

ends of linear double-stranded DNA (316, 317). Other studies indicate that purified proteins protecting specific DNA motifs bear Ku serological determinants (318 - 320). This suggests that Ku-like proteins might be sequence-specific DNA binding proteins. It is not resolved, however, whether Ku is actually a sequence-specific DNA binding protein or whether it simply co-purifies with other DNA binding proteins.

The demonstration that the p70 protein binds specifically to the E3 motif in the TCR β enhancer is the first to show that p70 can bind DNA in a sequence-specific manner. The evidence is based 1) on the ability of recombinant p70 lupus autoantigen expressed in bacteria to bind the E3 motif, and 2) on a mutant E3 motif being a less effective competitor than the wild-type E3 motif. The fact that the nonbinding mutant has markedly reduced enhancer activity, suggests a probable role for p70 in TCR β expression. The ability of Ku monospecific antiserum to specifically supershift the E3 DNA-protein complex in gel-shift assays with Jurkat nuclear extracts provides further evidence that p70 specifically binds to the E3 motif (309).

The ability of the E3 motif to bind a 70 kDa and an 85 kdDa protein, as identified in UV cross-linking experiments (309), suggest that the p80 component of the Ku antigen complex might also bind to the E3 motif. This is consistent with the ability of lupus Ku-monospecific antiserum to bind most of the E3 DNA-protein complexes. The majority of the 70 and 85 kDa E3-bound proteins form distinct DNA-protein complexes in gel shift assays. The above implies that a simple heterodimer with a 1:1 stoichiometry between the p70 and p80 polypeptides does not explain its ability to bind DNA in a sequence-specific manner. It has previously been suggested that p70 and p80 form a 1:1 stoichiometrical heterodimer which binds nonspecifically to DNA ends (316, 317). However, p70 can strongly bind to the E3 motif on its

own, suggesting that the p70 lupus autoantigen is able to bind DNA as either a monomer or homodimer. In addition to the sequence-specific interaction of the p70 monomer or homodimer, heterodimers of p70 and p80 may have a high nonspecific DNA-binding ability. Furthermore, p70/p80 heterodimers may also bind DNA in a sequence-specific manner.

The conclusion that the p70 lupus autoantigen binds DNA in a sequence-specific manner and regulates transcription is consistent with the observation that purified proteins expresssing Ku determinants protect transcriptionally relevant motifs (318, 320). Transcription of the human U1 promoter is abrogated by depletion of molecules bearing Ku determinants and restored by addition of proteins bearing these determinants (320).

A high number of people with autoimmune disease might have antibodies to DNA-binding proteins like p70, if these antibodies are capable of inducing antiidiotypic antibodies having specificity for DNA. This possibility is consistent with the fact the presence of antibodies to p70/p80 in autoimmune sera correlates with large amounts of anti-DNA antibodies (312).

The p70 lupus autoantigen is expressed in human liver, thymus, thyroid, and spleen (313, 321, 322). Yaneva et al (322) did not detect transcripts in human brain, muscle, or liver. The tissue distribution of p70 is not sufficient to explain the lymphoid specificity of the TCR β enhancer. The p70 lupus autoantigen must therefore interact with other transactivators in regulating β enhancer activity. This is consistent with evidence that no TCR β enhancer binding proteins previously identified are α/β T cell restricted (Chapter 1).

Both the βE1 and E3A motifs (Table II) bind to the p70 lupus autoantigen as evidenced by their ability to compete in gel-shift assays. The

sequence differences in the two motifs suggest that the p70 lupus autoantigen can recognize a degenerate motif in a sequence-specific manner. Other proteins with high nonspecific DNA binding activity in addition to a sequence-specific binding activity have been described (323). The ability of the same protein to be involved in two types of interactions with DNA suggests that p70 plays a more general role in transcription. Indeed, the Ku autoantigen has been identified as a DNA associated component of a RNA polymerase II CTD kinase. The CTD kinase phosphorylates RNA Polymerase II and is associated with the initiation of transcriptional elongation (324). The connected kinase activity of the Ku autoantigen is associated with its affinity for binding to the ends of DNA (325, 326). This makes sense for the T cell receptor genes if during rearrangement the exposed DNA ends attract the Ku autoantigen, allowing it to enter the DNA locus. It can then move along the DNA to find its specific sequence, and upon binding to the E3 motif it may influence transcription perhaps by phosphorylating RNA polymerase. This and other studies (320, 327, 328) suggest a functional mechanism in support of the conclusion that p70 regulates TCR β gene transcription.

TABLE II

TCR β Enhancer Motifs

Name of the motif	Sequence
	E4 MOTIF
	GCATCTCACCCCAGGTCTGGCTGTTTATCTGTAAGTAACATCAGCACCAAGTAAGAATGG
βE1:	5' TCTCACCCCAGGTCTGGCTGTTTATCTGTAAGTAACA 3'
E4A:	5' TCTGGGTGTTTATCTGTAAGTA 3'
βE2:	5' TATCTGTAAGTAACATCAGCACCAAGTAAGAATGG 3'
E4B:	5' GTAAGTAACATCAGCACCAAG 3'
βE3:	5' GTAAGAATGG
	E3 MOTIF
	CCACCTGCCATAGCTCCATCTCCAGGAGTCACAACAGGATGTGGTTTGACATTTACCAGGT
βE3:	CCACCTGCCATAGCTCCATCTCCAGGAGTC 3'
E3A:	5' CCAGGAGTCACAACAGGATGTGGTTTG 3'
E3B:	5' CAACAGGATGTGGTTTGACATTTAC 3'
	E2 MOTIF
	CCTACATCTGGGGTGCCTGTGAATGCTCCCCCACTCACTCACATTCTGAGCATTTTGGGAA
βE5:	5' CTGTGAATGCTCCCCCACTCACTCACATTCTGAGCATTTT 3'
	E1 MOTIF
	CCACACTTGCCACATCCTGTCTTCAAACCCTTCTCATGCAGCCCTTTCTACCTCAGCCTCT

TABLE III

Wild Type and Mutant Motifs Used In DNA Binding Assays.

TCR βEnhancer Motifs.

βE1 wild type:	5' TCTCACCCCAGGTCTGGCTGTTTATCTGTAAGTAACA 3'
βE1 mutant#1:	5' TCTCACCCCAGGGAGTTTTGTTTATCTGTAAGTAACA 3'
βE1 mutant#2:	5' TCTCACCCCAGGTCTGGCGTGGGCTCTGTAAGTAACA 3'
βE1 mutant#3:	5' TCTCACCCCAGGTCTGGCTGTTGCGATGTAAGTAACA 3'
βE1 mutant#4:	5' TCTCACCCCAGGTCTGGCTGTTTATCTTGCCTGCACA 3'
βE5 wild type:	5' CTGTGAATGCTCCCCCACTCACTCACATTCTGAGCATTTT 3'
βE5 mutant:	5' CTGTGAATGCTCCCCCACGACAGAACATTCTGAGCATTTT 3'
Wild type E3A:	5' CCAGGAGTCACAACAGGATGTGGTTTG 3'
Mutant E3A:	5' CCAGGAGTCACACCAAGATCTTGGTTC 3'

TCR Vβ2 Promoter Motifs.

Vβ2 AP-1:	5' TATGAGCTTAGTCAGTTCA 3'
Vβ2 ets:	5' TATGTTTCCTGAGGAAGCA 3'

POU Protein Binding Motifs.

Octamer motif:	5' ATGAATATGCAAATCAGGTGA 3'
Mutant octamer motif:	5' ATGAATATGCCCCTCAGGTGA 3'
Pit motif:	5' CCTGATTATATATATATTCATGAAGGTG 3'
Mutant Pit motif:	5' CCTGATTATATATAGCGGACTGAAGGTG 3'

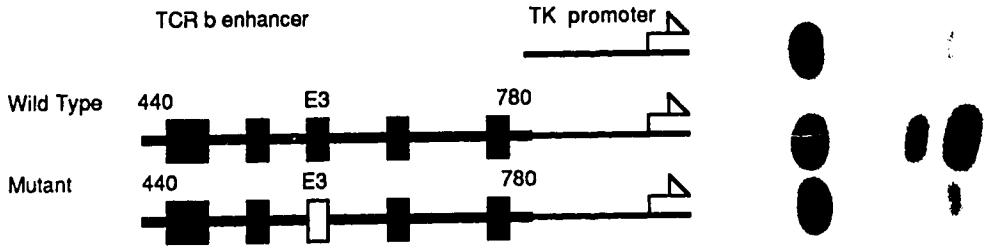

Figure 13. The E3 motif is critical for TCR β enhancer activity. The enhancing activity of the wild-type and mutated TCR β enhancer fragment (positions 440-780) was read from the Thymidine Kinase promoter in the pBLCAT2 vector. Transient transfections were done in EL4 cells. CAT activity was standardized by co-transfection of the pCH110 β-galactosidase expression plasmid. Similar results were seen in mutiple experiments.

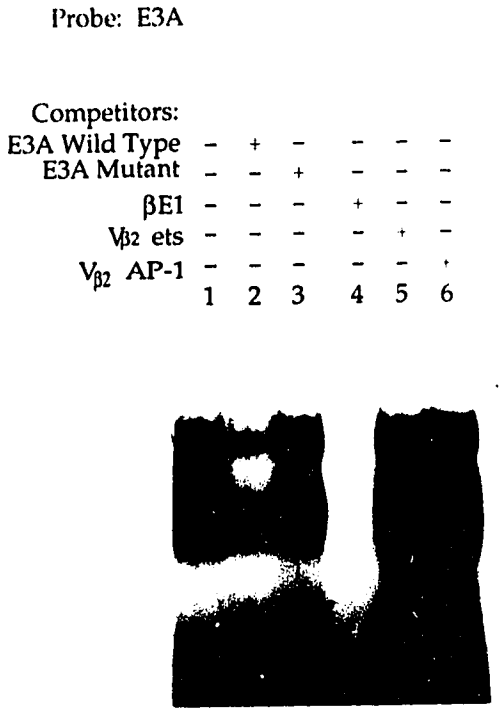

Figure 14. The p70 lupus autoantigen binds the E3A motif in a sequence specific manner. The bacterially expressed recombinant C-terminal portion (aa 111-222) of the p70 lupus autoantigen was analyzed in gel-shift assays with the E3A probe. A panel of nonradioactive excess (400 ng) oligonucleotides were tested for the ability to compete with the E3A probe. The competitors included, *lane 2* - wild-type E3A, *lane 3* - mutant E3A, lane 4 - βE1, *lane 5* - Vβ2 Ets, and *lane 6* - AP-1 motifs.

III.B. TCFβ1 IS A NOVEL PROTEIN THAT BINDS THE β ENHANCER

The Role of the E4 Motif in β Enhancer Activity

Substantial evidence supports the importance of the E4 motif in β enhancer activity. The E4 region is approximately 85% conserved between mouse and human genes, and in vitro footprints mapped to the E4 motif in both mouse and human DNA (148, 157). Substitutional mutagenesis of the E4 motif results in the reduction of enhancer activity (148).

To further prove the importance of the E4 motif in the regulation of β enhancer activity, a deletional analysis was done. Specific motifs were deleted from the functional core of the TCR β enhancer, and these fragments were cloned upstream of the Thymidine Kinase promoter in the pBLCAT2 vector. Deletion of the E1 region decreased CAT activity to 21%, whereas deletion of the E4 region dramatically decreased CAT activity to only 0.5% of wild-type enhancer activity (Figure 15). These results indicate the E4 motif is crucial for the ability of the β enhancer to regulate gene expression.

A TCR β Enhancer Binding Protein

To identify genes which encode E4-binding proteins, a Jurkat T-cell λgt-11 library was screened using the E4A motif as a probe (H. Brickner in 329). This screening identified a number of clones encoding E4A-binding proteins. The isolated inserts were cloned into an ATG Bluescript vector so that the binding specificity of the clone could be confirmed using an in vitro generated protein. Using the cloned gene as a template, an ^{35}S-labeled protein was transcribed and translated in vitro and visualized on denaturing protein gels by autoradiography. An approximately 50 kDa protein could be visualized in sense RNA programmed lysates but not in either unprogrammed lysates or

lysates programmed with inappropriate RNA (Figure 16). The labeled protein was then tested in a reverse gel shift assay and found to form a DNA-protein complex specifically with the plasmid containing the E4A motif; but the labelled protein did not bind to plasmid containing the E3A motif, nor to puc 18 plasmid alone. This suggests the gene which encodes an E4 motif binding protein had been cloned, and that binding to E4 occurs in a sequence-specific manner (Figure 16).

TCFβ1 Is a Novel POU Domain Protein

Sequence analysis of the cloned gene followed by a computer data base search revealed a novel gene which we named TCFβ1. A conceptual translation of the cDNA identified a region homologous to the POU domain in the carboxyl end of the protein (Figure 17). This similarity extended through the POU-specific domains A and B, and the POU homeodomain. The WFC motif which is found within the POU homeodomain, and is conserved in all POU domain proteins, is also present in the TCFβ1 protein.

Although TCFβ1 is clearly a POU domain protein it has significantly more divergence (~26%) at the consensus POU residues than does any other POU protein (<85%). Since the POU domains of all known POU proteins are more closely related to each other than TCFβ1 is to any one of them, we suggest TCFβ1 is a novel POU protein which belongs to an as yet undescribed POU subfamily. In accordance with the nomenclature (330), we have tentatively classified TCFβ1 as the only known member of POU domain subfamily VI (Figure 18). The divergence in amino acid identity of TCFβ1 from other known POU proteins is most significant in the POU-specific domain A region. The TCFβ1 protein has approximately 48% identity with

other POU members, whereas the other POU proteins are approximately 70% identical when compared with each other in this N-terminal region.

The sequence of TCFβ1 differs dramatically outside the POU domain. Three direct repeats (underlined in Figure 17) can be identified in the proline-rich N-terminal portion of the TCFβ1 protein. Direct repeats have also been identified in the proline-rich activation domains of some transactivators such as AP-2 (331) and CTF-1 (332). Another proline-rich activation domain was identified in the Oct-2 protein (231). The N-terminal region of TCFβ1 also has a high β-pleated sheet content, as suggested by Chou-Fasman and Robson-Garnier algorithm analysis of the TCFβ1 amino acid sequence. Two other POU domain transactivators, Pit-1 and Oct 3/4 have a similarly high β-pleated sheet content in this region. Because of its similarity with other transcriptional regulatory proteins, these structural characteristics together suggest that TCFβ1 is a novel DNA-binding protein which regulates transcription.

Tissue Distribution of TCFβ1

A panel of human T cell lines including Jurkat, HUT-74, MOLT3, and MOLT4 were tested for TCFβ1 expression in a Northern analysis using poly-A$^+$ RNA. All lines were positive for TCFβ1 expression (Figure 19A). This result confirms the expectation that if TCFβ1 is a regulator of β gene expression it should be expressed in T cells. As is also expected, TCFβ1 is expressed in human thymus. Human thymus poly-A$^+$ RNA was tested by both Northern analysis and by RNase protection assays (Figure 19B and 19C). In addition to being expressed in T lymphocytes, TCFβ1 is expressed in the human B-cell lines Raji and Daudi (Figure 19A). The expression of TCFβ1 in both B and T cells is consistent with the reported activity of the minimal TCR

β enhancer in some B-cell lines (156, 157). The fact that TCFβ1 is expressed in B cells is also very interesting because of the demonstrated role of octamer-binding POU domain proteins in regulating Ig gene expression(215, 221, 230-232). The major TCFβ1 transcript in lymphoid cells is approximately 2.8 kb long. However, HUT-74 T cells also express a smaller TCFβ1 transcript. One possibility is that this smaller transcript arises by alternative splicing, although this requires further verification.

The tissue distribution of the TCFβ1 gene transcript was determined by Northern analysis of poly-A^+ RNA from a panel of human tissues. The expression of TCFβ1 in human brain and skeletal muscle, but not in liver, kidney, heart, placenta, or pancreas indicates that TCFβ1 is expressed in a lineage-restricted fashion. In addition to the 2.8 kb TCFβ1 transcript, there is a 5 kb TCFβ1 transcript seen only in human brain and skeletal muscle (Figure 19D). A genomic Southern indicated that TCFβ1 is a single copy gene (Messier and Fitzgerald), and thus cross-hybridization with other members of a gene family would not account for the TCFβ1 RNA observed in the various tissues.

TCFβ1 Recognizes Multiple Motifs Within the β Enhancer

To analyze the DNA binding characteristics of TCFβ1, bacterially expressed recombinant TCFβ1 was generated. The purified TCFβ1 protein was visualized on SDS-polyacrylamide gels and found to be >95% pure as assayed by densitometric analysis of the recombinant proteins on a Coomassie Blue stained polyacrylamide gel (Figure 20). As expected, this purified TCFβ1 bound the βE1 oligonucleotide from the TCR β enhancer (Figure 21). The βE1 oligonucleotide completely overlaps the E4A motif (TableII), while the flanking sequences found in βE1 stabilized the DNA-protein complex in gel

shift assays. A panel of βE1 mutants (Table III) were then analyzed for their ability to abolish the βE1-TCFβ1 DNA-protein complex in gel shift assays. Mutants #2 and #3 were dramatically less competitive for binding than mutants #1 and #4. Mutants #1 and #4 competed as effectively as the wild-type βE1 motif, such that 100 ng of each completely abolished the βE1-TCFβ1 DNA-protein complex (Figure 21). The inactive βE1 mutants #2 and #3 are mutated in the region which is 100% conserved between human and mouse genes (148, 156, 157). The view that TCFβ1 regulates β enhancer activity is consistent with the reported inability of mutant #3 to drive transcription in T-cells (179).

Previous TCR β enhancer studies suggest that β enhancer-binding proteins bind to multiple motifs within the β enhancer (148, 157). To establish whether TCFβ1 can also bind to multiple motifs, a panel of TCR β enhancer motifs were tested in gel shift assays for their ability to compete with the βE1 motif for binding the TCFβ1 protein. The ability of TCR β enhancer motifs βE2 and βE5 to abolish the TCFβ1-βE1 DNA-protein complex indicated that TCFβ1 bound to multiple motifs in the β enhancer. This conclusion was supported by the identification of a mutant βE5 motif (Table 3) which competed less effectively than the wild-type βE5 motif (Figure 22).

TCFβ1 Binds To Octamer and Octamer-Related Motifs

The above mutational analysis suggests that mutating an AT-rich region to a GC-rich region abrogates the binding of TCFβ1 to its cognate motif in the TCR β enhancer. Since similar AT to GC mutations in the octamer motif abolished binding by other POU domain proteins, the ability of TCFβ1 to bind to the octamer motif was examined. The immunoglobulin octamer motif was used as a probe in gel shift assays. TCFβ1 bound in a sequence-

specific manner to the Ig octamer motif (Figure 23). This binding was successfully competed by a wild-type octamer motif, but not a mutant octamer motif (Table 3). Similar results were also obtained when the Pit motif from the prolactin promoter was used. The Pit motif is the DNA binding sequence of Pit-1, a pituitary POU domain protein. The ability of TCFβ1 to bind the octamer and octamer-related Pit motifs, both of which are not present in the TCR β enhancer, suggests that TCFβ1 may have additional functional roles in lymphoid cells.

TCFβ1 Is A Transactivator

A cotransfection assay was used to determine the transactivation characteristics of TCFβ1. The reporter plasmid contained a multimerized (6X) Pit motif cloned upstream of either a minimal β globin promoter or the snRNA U2 promoter*. By themselves, these promoters have no transcriptional activity (229). The ability of TCFβ1 to transactivate was determined by an RNase protection analysis of the appropriately inititated β globin or U2 transcript. The α globin plasmid was used as a control for transfection efficiency and its activity was also determined by RNase protection. The DNA-binding Pit domain replaced the DNA-binding domain of Oct1, Oct2 and TCFβ1 in the 1P1, 2P2, and TFT constructs, respectively. The TTT construct is TCFβ1 alone. As discussed previously TCFβ1 is capable of binding to the Pit motif. The cDNAs were overexpressed from a cytomegalovirus (CMV) promoter. When overexpressed in HeLa cells, the TCFβ1 cDNA transactivated the β-globin promoter in a Pit motif-dependent manner. The transactivation of TCFβ1 was even higher than that of Oct2

* The Oct1 and Oct2 expression plasmids and the β globin and U2 reporter plasmids were kindly supplied by Dr. W. Herr.

(Figure 24). When reporter plasmids that had either no motifs, or an inactive motif (dpm 8) were cotransfected with the TCFβ1 expression plasmid, there was no transactivation. Overexpression of TCFβ1 containing the Pit POU DNA-binding motif, had minimal effect on the Pit-dependent U2 promoter (Figure 25). The Oct1 plasmid (1P1) on the other hand, was very efficient at transactivating this construct. This result indicated that TCFβ1 may be a transactivator capable of regulating mRNA promoters. To test whether TCFβ1 could transactivate via the E4 motif, multiple copies of the E4 motif were cloned upstream of the thymidine kinase promoter in the pBLCAT2 vector. Both Oct2 and TCFβ1 were efficient at transactivating via the β enhancer E4 motif, indicating that E4 is a genuine POU binding motif (Figure 26).

Discussion

The TCR β enhancer is critical for β chain gene expression. TCFβ1 is a novel POU domain protein that binds to a crucial motif in the β enhancer. TCFβ1 represents a new class of POU proteins distantly related to other known POU domain proteins. The POU-specific plus the POU homeodomain are present in the C-terminal region of TCFβ1 and act as the DNA binding domain. Deleting POU-specific domain A from TCFβ1 reduced its affinity for the octamer motif (P. Muchowski, unpublished results). This lower affinity implies that like other POU domain proteins (330), TCFβ1 requires the POU-specific domains for high affinity DNA binding. The comparative inability of TCFβ1 to bind a transcriptionally inactive mutant motif (179), indicates a role for TCFβ1 in regulating TCR β enhancer activity. The regulatory role of TCFβ1 is futher supported by its ability to bind multiple motifs in the TCR β enhancer, as well as its lineage restricted pattern of

expression. The expression of TCFβ1 in B cells is consistent with transfection data in which the TCR β enhancer was active in certain B cell lines (144). The ability of TCFβ1 to transactivate in a pit motif-dependent manner suggests that it is an authentic transcriptional regulatory factor involved in lymphoid gene expression. The cotransfection studies using the multimerized E4 motif upstream of the Thymidine Kinase promoter indicate that TCFβ1 and Oct2 transactivate in an E4A-dependent manner. This finding advocates that E4A is a genuine POU-binding motif and suggests a role for POU proteins in regulating TCR β gene expression.

The E4 region of the β enhancer, in addition to TCFβ1, binds CRE (148, 157, 171), E-box (157), and GATA motif binding proteins. As discussed in chapter 1, each of these motifs have been identified in the enhancer regions of at least one other TCR gene. In contrast to the inactivity of the TCR β enhancer in HeLa cells, the expression of TCFβ1 in these same cells is not contradictory. None of the TCR β enhancer motifs bind to T cell specific nuclear proteins, as determined by gel-shift assays (148). This suggests that the lymphoid cell-specific activity of the TCR β enhancer results from the cumulative contributions of many transactivators each with a tissue distribution that is not strictly lymphoid cell-specific. To understand β gene regulation, the identification of the transactivators conferring a lymphoid cell-specific activity to the TCR β enhancer is crucial.

The presence of GATA-3, which is expressed in T cells but not in B cells or macrophages, implies that GATA-3 is important for the regulation β enhancer activity. Interestingly, GATA-3 and TCFβ1 bind to partially overlapping motifs within the β enhancer. The mutation of the GATA-3 motif which abrogates its binding, also diminishes binding of TCFβ1 to the E4 motif. It remains unresolved whether this is indicative of a cooperative or

antagonistic functional interaction between TCFβ1 and GATA-3, or whether it involves the differential interaction of the two transactivators at different stages of T cell development.

POU domain proteins have been shown to bind the octamer and related motifs. These octamer and octamer-related motifs regulate transcription of both ubiquitous and lymphoid specific genes. The octamer motif is essential for immunoglobulin gene expression. The addition of octamer motifs uptream of a minimal Ig promoter is sufficient for lymphoid specific activity (213). Also, in mice transgenic for the V_H gene, Ig gene expression is decreased approximately 30 fold by the introduction of a non-binding mutation of the octamer motif in the transgenic V_H promoter. The decrease was evident even in the presence of a complete Ig enhancer (215). Octamer motifs are also involved in the induction of interleukin 2 gene expression by signals from the T cell receptor (333).

POU proteins regulate lineage specific gene expression via their interaction with octamer-like motifs (102, 312). Mutations of POU genes affect this interaction, resulting in aberrations in the generation and maintenance of specific lineages (334 - 339). Of a number of previously described POU-domain proteins only Oct1 and Oct2 are expressed in lymphocytes (330, 340). An octamer motif upstream of a small nuclear RNA (snRNA) promoter regulates snRNA expression independent of cell type. This agrees with the ubiquitous expression of Oct1. The observation that octamer motifs placed upstream of mRNA promoters confers lymphoid specific activity correlates with the expression of Oct2. There are two possibilities that might explain how the octamer motif can display activity that is both ubiquitous and tissue restricted. The first possibility is quantitiative, and suggests that Oct1 is a weaker activator than Oct2 and requires the binding of additional factors. In

support of this model, evidence indicates that Oct1 is dependent on an unidentified lymphoid protein for optimal activity in Ig promoters (230, 341). The second possibility is qualitative, and suggests that Oct1 selectively activates snRNA promoters in a ubiquitous manner through a distinct activation domain. In support of this model evidence indicates that Oct1 preferentially activates snRNA promoters, while Oct2 preferentially activates mRNA promoters. A unique snRNA activation domain has been mapped to the C-terminal portion of Oct1 that is absent in Oct2 (229).

Despite the apparent importance of Oct2 in regulating lymphoid expression, the octamer motif can still regulate mRNA promoters in T cells lacking Oct2 (333). For instance, the octamer motif in the IL2 enhancer is the dominant regulatory motif, even in Jurkat cells which do not express Oct2 (333, 342). Also, disruption of both alleles of the Oct2 gene in a B cell line, as well as in mice, did not abrogate Ig gene expression. To account for these observations, it is necessary to postulate that Oct1 protein regulates mRNA promoters in addition to snRNA promoters (230, 341). The identification of a unique Oct1 activation domain which preferentially regulates small nuclear RNA promoters (229), however, limits such explanations. An alternative explanation is the involvement of novel octamer binding POU-domain proteins. Support for this is found in the B X T cell hybrids, where the extinguishing of Ig genes is dependent on the octamer motif, but Oct1 and Oct2 are still expressed and are capable of binding (223). The identification of a novel POU domain protein, TCFβ1, that is present in lymphoid cells, suggests that regulation of transcription by lymphoid POU domain proteins is more complex than previously thought. The fact that TCFβ1 is expressed in both T and B cells, is capable of transactivating mRNA promoters, and in addition to the TCR β enhancer, binds the Ig octamer and octamer-related pit motifs in a

sequence specific manner, suggests a role for TCFβ1 in regulating the expression of other lymphoid genes. Like other POU proteins, TCFβ1 may be an important lineage determining protein.

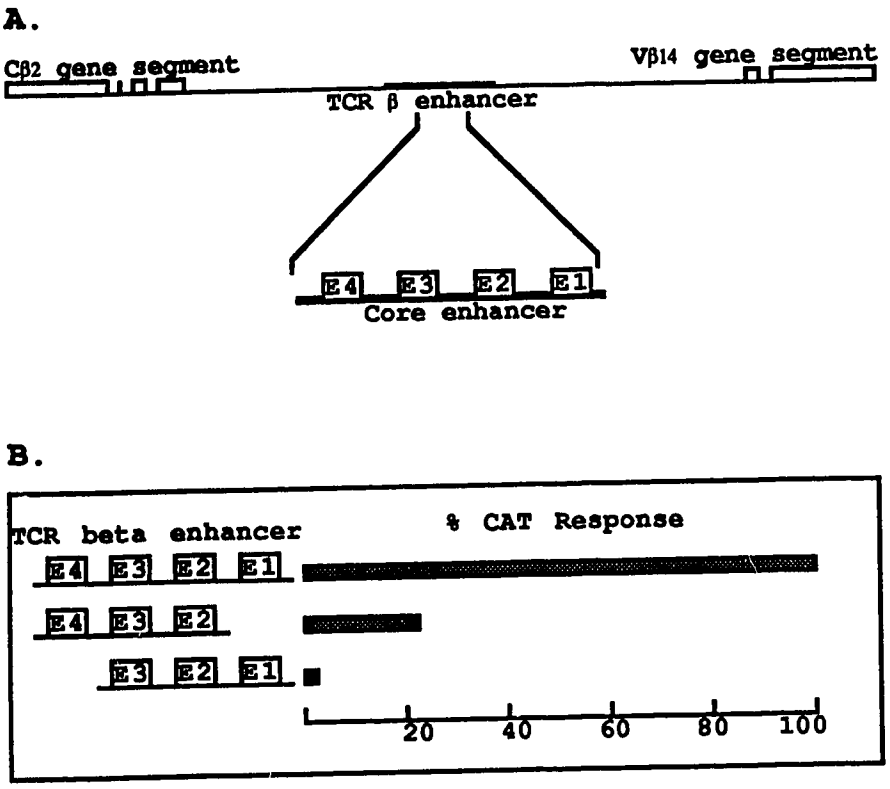

Figure 15. Role of the E4 motif in TCR β enhancer activity. (A) Schematic cartoon of the protein-binding sites in the core TCR β enhancer. (B) Deletional analysis of the functional core of the TCR β enhancer. The different fragments of the TCR β enhancer were cloned upstream of the TK promoter in the pBLCAT2 vector, and were transiently transfected into EL4 cells. Data represent percent activity of the β enhancer fragment (bp 521 to 780); the activity of the TK promoter (1.4%) was enhanced by this β enhancer fragment to 85%. Deletion of the E1 region decreased CAT activity to 21%, whereas deletion of the E4 region decreased CAT activity to 0.5%. The same 4 fold difference in the CAT activity of the 2 enhancer deletions was seen in multiple experiments (n>4).

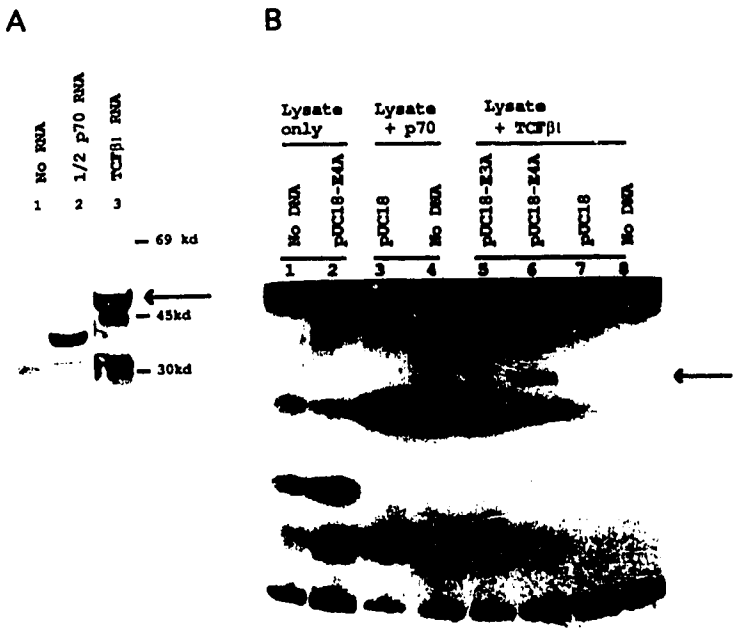

Figure 16A. Visualiation of the ^{35}S-methionine-labeled TCFβ1 protein generated by in vitro transcription and translation. The reticulocye lysates were programmed with either TCFβ1 RNA or a control RNA encoding the C-terminal half of the p70 lupus autoantigen. **16B.** TCFβ1 binds to the E4A motif in a sequence-specific manner in a reverse gel-shift assay. The ability of unprogrammed reticulocyte lysates or those programmed with an irrelevant RNA (p70) or TCFβ1 RNA to bind plasmid DNAs was determined in a reverse gel-shift assay. The plasmid DNAs included pUC18, the E4A motif cloned in pUC18, and the E3A motif cloned in pUC18. The arrow identifies the specific E4A-TCFβ1 DNA-protein complex.

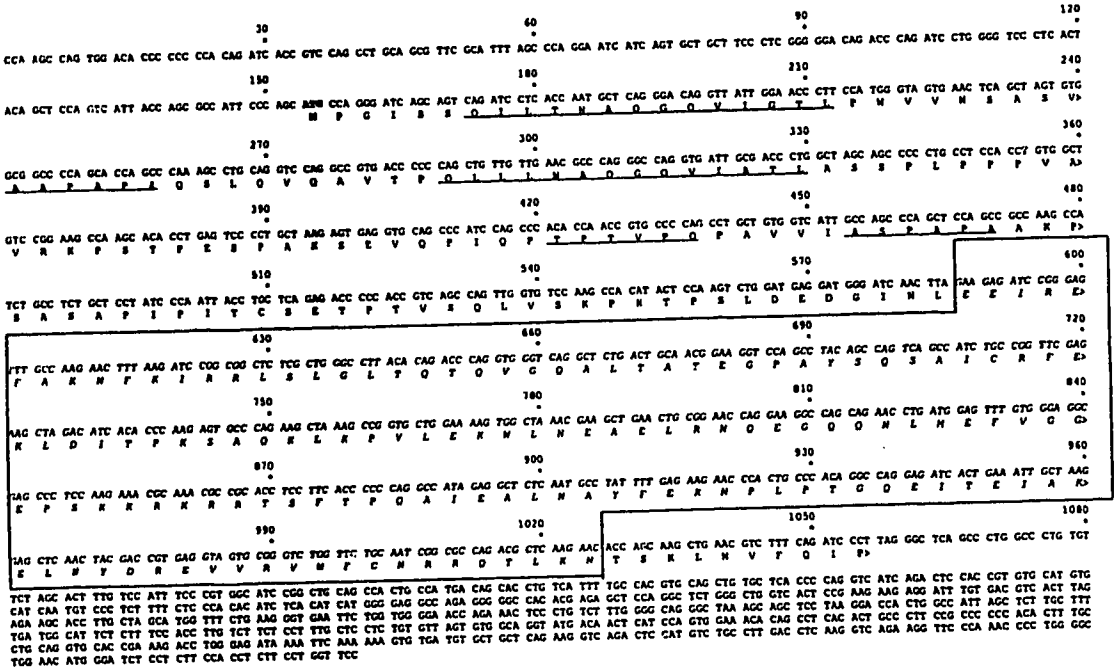

Figure 17. Nucleotide and predicted amino acid sequence of TCFβ1. The presumptive initial methionine is shown in boldface type. The conserved POU domain of the TCFβ1 protein is boxed. The direct repeats in the N-terminal region of the TCFβ1 protein are underlined.

COMPARISION OF THE POU DOMAIN SEQUENCES OF RELATED POU DOMAIN PROTEINS

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GENE   CLASS ---------- POU Specific Domain ----------        -------- Linker -----
                --POU Specific Domain A--        ---POU Specific Domain B---
Pit-1     (I)  RE LEQFANEFKVRRIKLGYTQTNVGEALAAV HGSEF     SQTTICRFENLQLSFKNACKLKAILSKWLEEAE  QVGALYNEKVGANE

Oct1      (II) EE LEQFAKTFKQRRIKLGFTQGDVGLAMGKL YGNDF     SQTTISRFEALNLSFKNMCKLKPLLEKWLNDAE  NLSSDSSLSSPSALNSPGIEGLS
Oct2      (II) EE LEQFARTFKQRRIKLGFTQGDVGLAMGKL YGNDF     SQTTISRFEALNLSFKNMCKLKPLLEKWLNDAE  TMSVDSSLPSPNQLSSPSLGFEPAG

cfla1   (III) DD LEAFAKQFKQRRIKLGFTQADVGLALGTL YGNVF     SQTTICRFEALQLSFKNMCKLKPLLQKWLEEAD  STTGSPTSIDKIAAQG
Brn-1   (III) DD LEQFAKQFKQRRIKLGFTQADVGLALGTL YGNVF     SQTTICRFEALQLSFKNMCKLKPLLNKWLEEAD  SSTGSPTSIDKIAAQG

unc-86   (IV) RQ LETFAEHFKQRRIKLGVTQADVGKALAHL KMPGV GSL SQSTICRFESLTLSHNNMVALKPILHSWLEKAE  EAMKQKDTIGDINGILPNT
Brn-3    (IV) RE LEAFAERFKQRRIKLGVTQADVGSKLANL KIPGV GSL SQSTICRFESLTLSHNNMIALKPILQAWLEEAE  GPQREKMNKPELFNGG
i-pou    (IV) RE LEAFAERFKQRRIKLGVTQADVGKALANL KLPGV GAV SQSTICRFESLTLSHNNMIALKPILQAWLEEAE  AQAKNKRRDPDAPSVLPAG

Oct3/4    (V) KE LEQFAKLLKQKRITLGYTQADVGLTLGVL FGKVF     SQTTICRFEALQLSLKNMCKLRPLLEKWVEEAD  NNENLQEICKSETLVQA

TCFβ1    (VI) EE IREFAKNFKIRRLSLGLTQTQVGQALTAT EGPAY     SQSAICRFEKLDITPKSAQKLKPVLEKWLNEAE  LRNQEGQQNLMEFVGGEPS

Consensus      LE FA  FK RRI LG TQ DVG ALA L     E     SQSTICRFE L LS KNM KLKPLL KWLEEAE
                                          MG      V       T           N A A   I  A  ND D
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GENE   CLASS ------------- POU HOMEO DOMAIN -------------------------
               -Basic--   ---Helix1--          --Helix2--        --Helix3 WFC---
Pit-1     (I)  RKRKRRTTI  SIAAKDALERHFG  EHSKP  SSQEIMRMAEEL  NLE  KEVVRVWFCNRRQREKR

Oct1      (II) RRRKKRTSI  ETNIRVALEKSFL  ENQKP  TSEEITMIADQL  NME  KEVIRVWFCNRRQKEKR
Oct2      (II) RRRKKRTSI  ETNVRFALEKSFL  ANQKP  TSEEILLIAEQL  HME  KEVIRVWFCNRRQKEKR

cfla1   (III) RKRKKRTSI  EVSVKGALEQHFH  KQPKP  SAQEITSLADSL  QLE  KEVVRVWFCNRRQKEKR
Brn-1   (III) RKRKKRTSI  EVSVKGALESHFL  KCPKP  SSQEITNLADSL  QLE  KEVVRVWFCNRRQKEKR

unc-86   (IV) DKKRKRTSI  AAPEKRELEQFFK  QQPRP  SGERIASIADRL  DLK  KNVVRVWFCNQRQKQKR
Brn-3    (IV) EKKRKRTSI  AAPEKRSLEAYFA  VQPRP  SSEKIAAIAEKL  DLK  KNVVRVWFCNQRQKQKR
i-pou    (IV) EKK  RTSI  AAPEKRSLEAYFA  VQPRP  SGEKIAAIAEKL  DLK  KNVVRVWFCNQRQKQKR

Oct3/4    (V)  RKRKRTSI  ENRVRWSLETMFL  KCPKP  SLQQITHIANQL  GLE  KDVVRVWFCNRRQKGKR

TCFβ1    (VI) KKRKRRTSF  TPQAIEALNAYFE  KNPLP  TGQEITEIAKEL  NYD  REVVRVWFCNRRQTLKN

Consensus      RKRKKRTSI      K  LE  F     PKP  S   IT  A  L   LE  KEVVRVWFCNRRQK KR
               KRKRR          R              QR  T    A          MK   N I       Q
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Figure 18. Sequence comparison of the POU domain of TCFβ1 with other POU domain proteins. The region compared extends from the POU-specific domains A and B to the POU homeodomain. The POU domain proteins are classified by Rosenfeld (330). There are additional class III and class V POU domain proteins which are not shown.

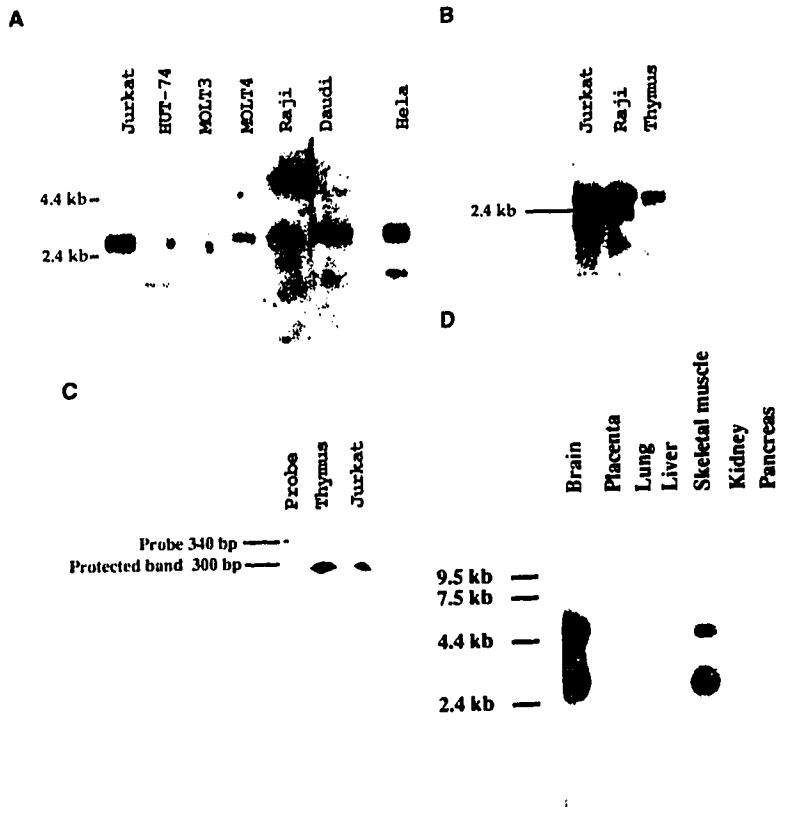

Figure 19A. Expression of TCFβ1 in human T and B cell lines. One microgram of poly A+ RNA was run in each lane. The probe was a full-length Jurkat TCFβ1 cDNA clone. The major transcript is ~2.8 kb. HuT-74 also reproducibly expresses a smaller transcript. **19B.** Expression of TCFβ1 in human thymus, determined in a Northern assay. One microgram of poly A+ RNA was run in each lane, and the Northern blots were probed with a TCFβ1 cDNA probe. The major TCFβ1 transcript in the thymus is ~2.8 kb long. **19C.** Expression of TCFβ1 in human thymus, determined in an RNase protection assay. The antisense RNA probe corresponded to nt 675 to 975 of the TCFβ1 cD **19D.** Expression of TCFβ1 in normal human tissues. A human multiple-tissue Northern blot of poly A+ RNAs was obtained from Clontech. The probe was a TCFβ1 antisense RNA probe (nt 675 to 975). Identical results were obtained with a labeled TCFβ1 cDNA probe. In addition to the 2.8 kb TCFβ1 transcript, a transcript of ~5 kb is easily detectable in brain and skeletal muscle.

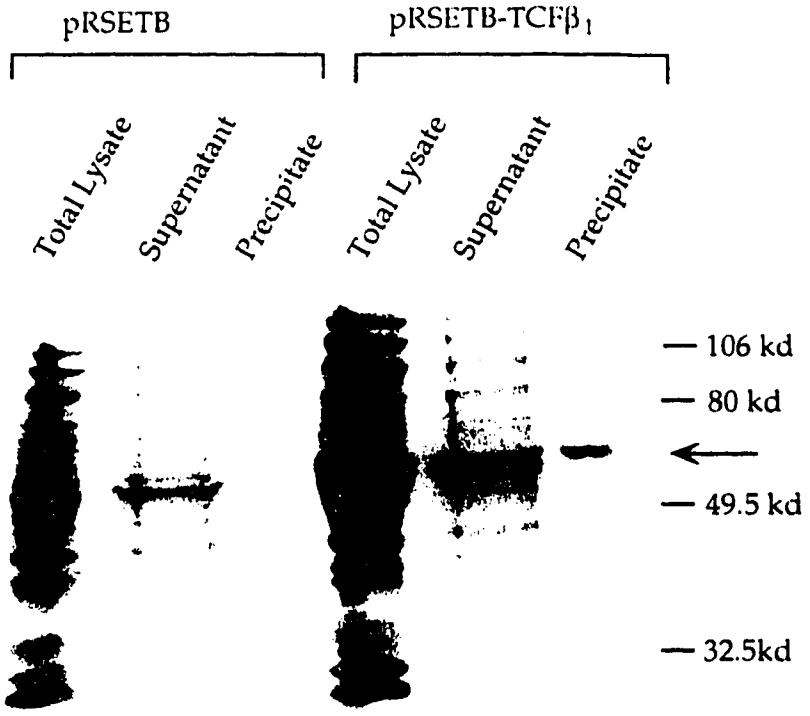

Figure 20. Generation of bacterially expressed recombinant TCFβ1. The pRSET B vector containing the TCFβ1 insert was used to transform *E. coli* BL21(DE3) containing plasmid pLys S. The TCFβ1 protein was expressed by induction with IPTG and was purified by ammonium sulfate precipitation. The total bacterial lysate, supernatant, and resuspended precipitated TCFβ1 protein were run on parallel lanes on a 10% denaturing polyacrylamide gel.

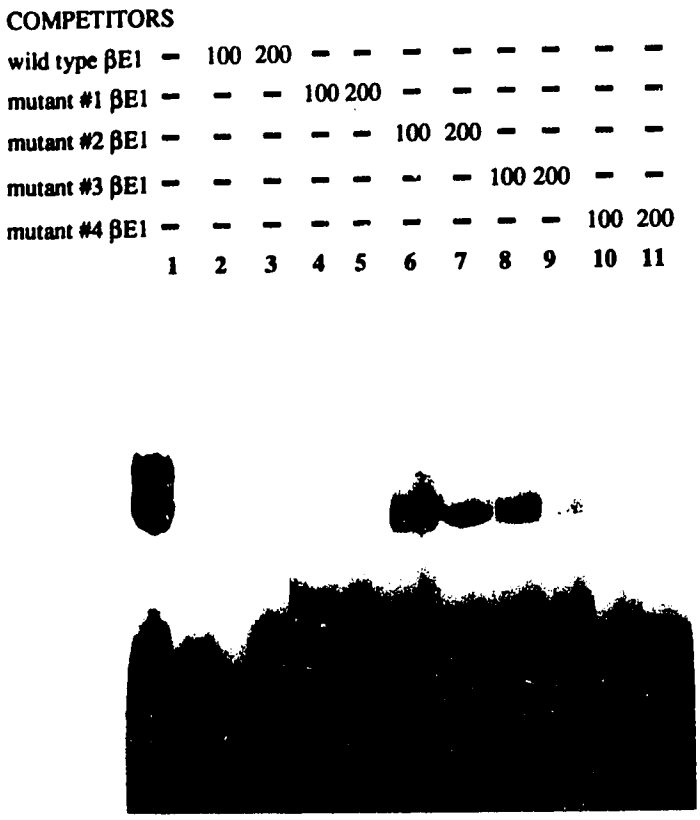

Figure 21. Recombinant TCFβ1 binds the βE1 motif in the TCRβ enhancer in a sequence-specific manner. The βE1 motif, which completely overlaps the E4A motif, was used as a probe and incubated with purified recombinant TCFβ1. Wild-type βE1 and a panel of mutant βE1 motifs were used as competitors (100 to 200 ng) in a gel-shift assay. Wild-type and βE1 mutants 1 and 4 were effective competitors, whereas mutants 2 and 3 were less effective.

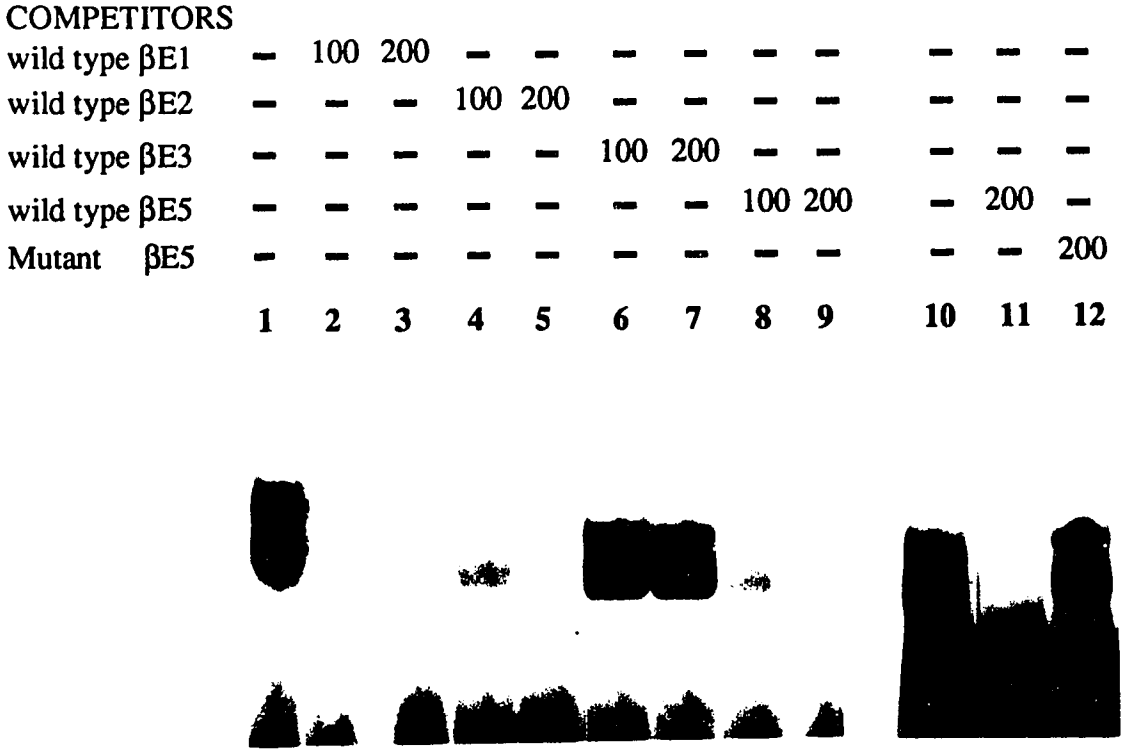

Figure 22. Recombinant TCFβ1 binds multiple motifs in the TCR β enhancer. The βE1 motif was used as a probe and incubated with purified recombinant TCFβ1. The ability of wild-type βE1 (100 to 200 ng) and other TCR β enhancer motifs (βE2, βE3, and βE5) to abolish the βE1-TCFβ1 DNA-protein complex was determined in a gel-shift assay. The mutant βE5 motif is a less effective competitor than the wild-type βE5 motif.

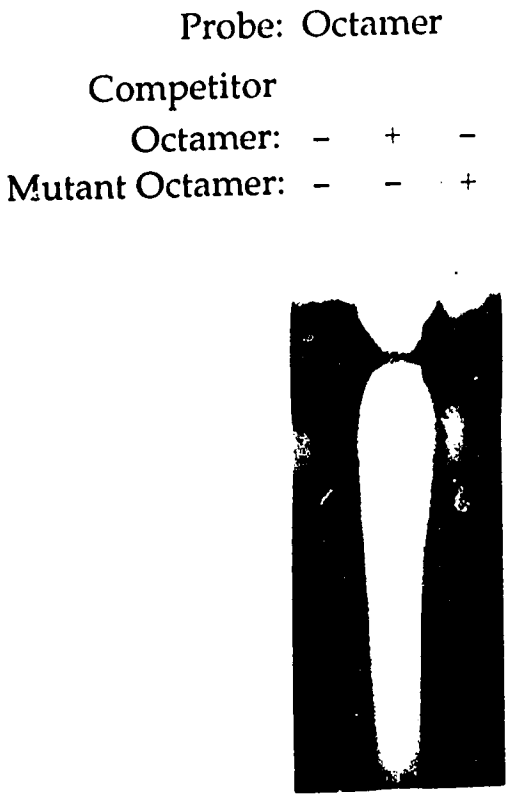

Figure 23. Bacterially expressed recombinant TCFβ1 binds the Ig octamer motif in a sequence-specific manner. The octamer motif was labeled, and the ability of octamer and mutant octamer motifs (shown in Table 3) to abolish the DNA-protein complex in a gel-shift assay was determined. The wild-type octamer motif competed, whereas the mutant octamer did not.

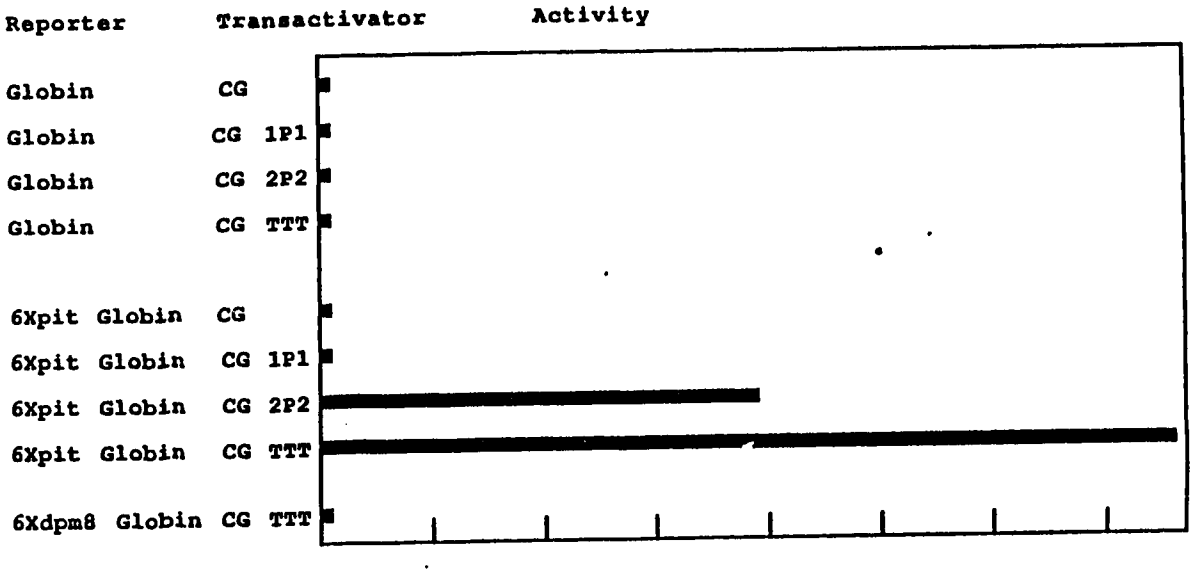

Figure 24. Transactivation characteristics of TCFβ1 with the β-globin promoter, containing multimerized Pit or inactive Pit (dpm 8) motifs. The activity of the cotransfected reporter construct in HeLa cells was determined by RNase protection of appropriately initiated transcripts from a β-globin promoter. The bar graph represents a densitometric analysis of the protected bands from an autoradiograph. Activities have been corrected for variations in transfection efficiencies by determining the activity of a cotransfected control plasmid, pα 4x(A+C). The cDNAs of the POU domain proteins are expressed under the control of the cytomegalovirus promoter in the CG vector. The DNA-binding POU domains of Oct1 and Oct2 in 1P1 and 2P2, respectively, are swapped with the Pit-1 POU domain (229). A representative experiment of n>4 is shown.

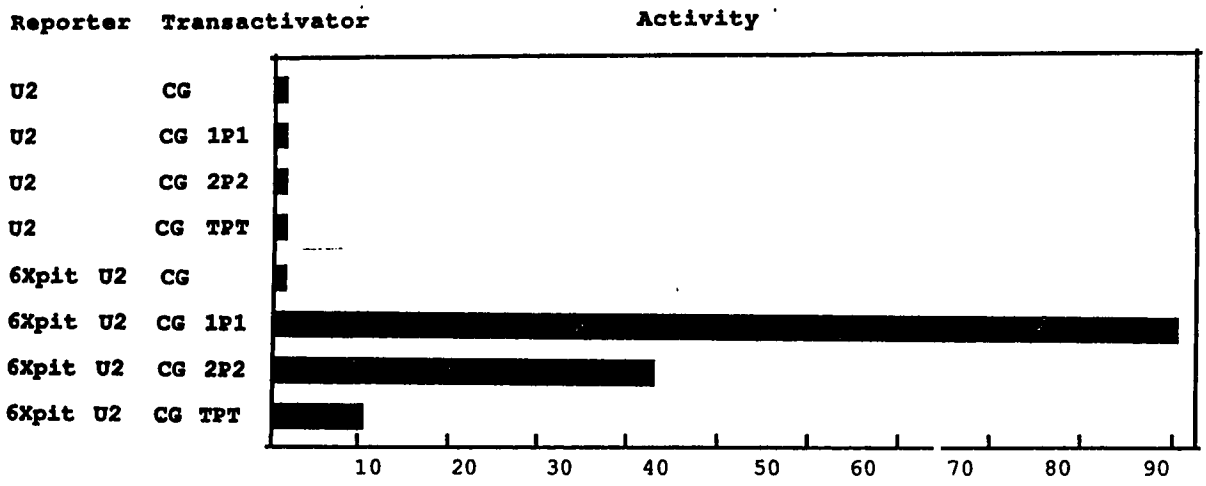

Figure 25. Transactivation characteristics of TCFβ1 with the U2 promoter, containing multimerized Pit motifs. The DNA-binding POU domains of Oct1, Oct2 and TCFβ1 in 1P1, 2P2, and TPT, respectively, are swapped with the Pit-1 POU DNA-binding domain. The cDNAs of the POU proteins are expressed under the control of the cytomegalovirus promoter in the CG vector. The activity of the cotransfected reporter contruct in HeLa cells was determined by RNase protection of appropriately initiated transcripts from a U2 promoter. The bar graph represents a densitometric analysis of the protected bands from an autoradiograph.

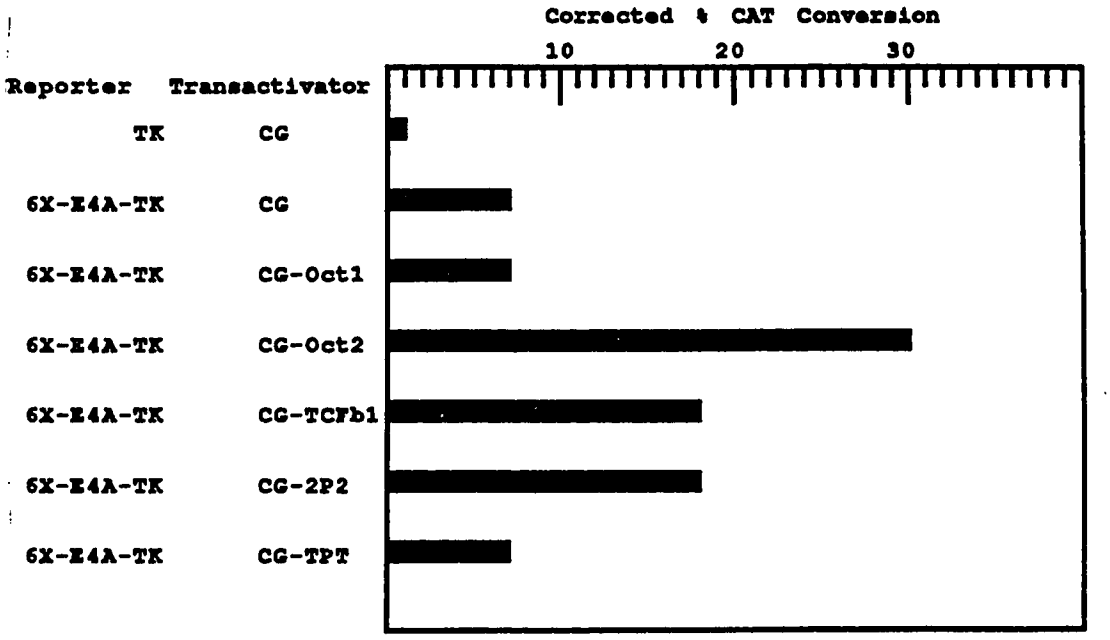

Figure 26. Transactivation from the E4A motif. The cDNAs of Oct1, Oct2 and TCFβ1 are under the control of the cytomegalovirus promoter in the CG vector. Multimerized E4A motifs were cloned upstream of the Thymidine Kinase promoter, in the pBLCAT2 vector. The POU cDNAs were cotransfected with the reporter construct in HeLa cells, and CAT reporter activity is shown (n=2).

Chapter 4

SUMMARY AND FUTURE DIRECTIONS

In order to explain T cell receptor β gene expression during T cell development, this thesis examined the mechanisms of β gene transcriptional regulation. The cis-acting sequences involved in regulating transcription were initially examined. The TPA inducible element was analyzed and results indicate the binding of fos/jun and ets proteins mediates its activity. The Vβ2 upstream inhibitory region was characterized, and the hierarchical dominance of the β enhancer was examined. The interaction between the TPA inducible element, the inhibitory region and the β enhancer, three cis-acting elements that control β gene expression was elucidated. Because the β enhancer was dominant in controlling this interaction it was subsequently examined in more detail. Two proteins which bind to the enhancer to influence transcription, the lupus autoantigen p70, and the novel POU protein TCFβ1, were identified. From these studies a number of conclusions emerged:

1. The TPA inducible element in the Vβ2 promoter region is dependent on both the AP-1 motif and the inverted repeat motif for optimal inducibility. The single G/T base substitution in the Vβ2 AP-1 site, compared to the prototypical collagenase AP-1 motif, decreases its affinity for binding the fos/jun heterodimer. The decrease in binding decreases the ability of the AP-1 site to confer TPA inducibility on its own. The ets transactivator binds to the inverted repeat motif and together with the fos/jun heterodimer confers optimal TPA inducibility to the Vβ2 promoter. This is the first demonstration of a functionally different AP-1 binding site, and the first

implication of the ets transactivator in binding to a Vβ promoter region to control gene expression.

2. The region upstream of the Vβ2 promoter from -739 to -343, can act like a silencer to inhibit basal and TPA inducible gene expression. It is orientation independent, transferable to a heterologous promoter, and is active in fibroblasts as well as T cells. The active fragment binds to at least one nuclear protein complex. This is the only characterized silencer-like element present in any Vβ regulatory region. Since Vβ2 is the 5' most Vβ gene segment, the inhibitory region may affect the entire locus.

3. There is a functional interaction between the TPA inducible element, the silencer element, and the β enhancer. The inhibitory region is capable of silencing both basal and TPA inducible expression from the Vβ2 promoter. A hierarchy exists within this interaction in which the β enhancer dominates. The presence of the β enhancer abrogates the silencer's inhibition on both basal and inducible expression. This is a novel hierarchical interaction, and likely plays a role in regulating β gene expression during T cell maturation.

4. The TCR β enhancer located downstream of the Cβ2 gene segment is critical in overcoming the effects of the inhibitory region in Vβ gene expression. The TCR β enhancer is the only element in the β chain gene that is capable of conferring T cell-specific expression.

5. The E3 motif within the β enhancer is critical for enhancer activity. The p70 lupus autoantigen binds to this motif in a sequence-specific manner. This is the first demonstration that p70 can bind to DNA in a sequence-specific manner, and that p70 plays a probable role in regulating TCR β gene transcription.

6. The E4 motif plays an important role in the regulation of β enhancer activity. TCFβ1 is a novel POU domain protein that binds to the E4 motif. TCFβ1 belongs to a new subfamily of POU domain proteins, and is expressed in a lineage restricted manner, including in T cells and thymus. Recombinant TCFβ1 purified to near homogeneity binds to multiple motifs within the β enhancer, as well as to octamer and octamer-related motifs. TCFβ1 is a transactivator capable of regulating mRNA promoters. These characteristics of TCFβ1 suggest that it plays an important role in lymphoid gene regulation.

The process of T cell development begins with lymphoid precursor cells in the bone marrow that do not express any T cell specific genes. Upon entering the thymus, however, the cells begin to express lineage-specific genes at precise developmental time points. The expression of the β TCR gene defines the αβ T cell lineage. Lineage-specific rearrangement of the TCR genes is followed by a further increase in TCR gene expression. The expression of other T cell-specific genes such as the CD3 chain genes, CD4, and CD8 genes is also under strict temporal control during development. Activation of mature T cells results in the expression of other T cell specific genes such as IL2.

The coordinate induction of sets of tissue- and lineage-specific genes defines development. This process can be accomplished in an efficient manner by a common set of transcriptional factors that regulate the transcription of a number of tissue-specific genes. Since the expression of the T cell receptor β chain gene is an essential component of T cell lineage determination, an understanding of how T cell receptor β gene expression is regulated during T cell differentiation sheds light on the process of T cell development and therefore immune function.

The cis-acting transcriptional regulatory elements such as the enhancer, TPA inducible, and inhibitory region have been identified and characterized. In addition, several of the transcription factors which bind to these elements have been identified and cloned, including fos/jun, ets, p70, and TCFβ1. The TCR β gene is regulated by a coordinated interaction of these proteins binding to the cis-controlling elements and their action at various stages of T cell development. By knowing the identity of the transcriptional factors such as fos/jun, ets, p70, and TCFβ1 we can begin to understand the role each plays. There are, however, many questions arising from this work that need to be addressed in order to gain a more complete understanding of β gene regulation, and therefore T cell development. The questions revolve around 1) how the expression of the lineage-specific β chain gene can be uniquely controlled by the same transcription factors that control other T cell genes, and 2) how non-αβ T cell specific transcription factors can control lineage-specific TCR β gene expression. The identification of the transcription factors now allows us to approach these questions. A number of possibilities can be envisioned, and subsequently tested.

The unique pattern of expression of the TCR β chain gene compared to other T cell specific genes could be accounted for by combinatorial diversity, in which the different combinations of nuclear protein binding sites leads to differential gene expression. The various transcription factors interact with each other to increase gene expression or to bind cooperatively. An example of a unique combination of transcriptional factor binding sites in the β gene was demonstrated in the Vβ2 promoter. The interaction of fos/jun and ets binding sites leads to optimal TPA inducibility. None of the T cell-specific cis regulatory regions have identical combinations of protein binding sites,

suggesting the importance of combinatorial diversity in uniquely regulating transcription.

It is important to distinguish between the involvement of each transcription factor at all stages of development versus involvement specific developmental stages. Knowing when a transactivator is active may explain the temporal expression of genes during development. It is essential to keep in mind the developmental stage of a cell line when interpreting transfection data, since various transactivators may or may not be active at that particular stage. This may also explain the redundancy of cis-elements like Tβ1 and Tβ5 in the human β enhancer. These elements may be important only at specific time points during development. An example is the cis-element interaction between the TPA-inducible, the inhibitory region, and the β enhancer. This interaction was determined by transfections in the EL4 cell line. EL4 is a mature T cell line, and it should be kept in mind that at other stages of T cell development the elements might interact differently. An example would be if the inhibitory region is transiently neutralized to allow rearrangement at the appropriate time.

Common sequences in the promoter and enhancer regions of the β chain gene might lead to a coordinated regulation. For example ets binds to both the Vβ2 promoter and the β enhancer. Ets mediated binding and transcription might open up the chromatin from both ends of the locus to facilitate rearrangement. Since the β chain is the first to rearrange in αβ T cells, an ets site in both the promoter and enhancer may ensure that β rearrangement occurs first, while in the TCR α chain gene a single ets site is present solely in the enhancer, perhaps resulting in delayed rearrangement. This is analogous to the octamer sequence being present in both the promoter and enhancer of IgH genes, but only a single octamer sequence being present

in the later rearranging Ig κ and λ chain genes. The interaction of enhancer binding proteins with specific promoters can also limit transcription from certain promoters. For example, TCFβ1 appears to activate transcription from mRNA promoters only. Therefore, although TCFβ1 may bind to the control regions of snRNA promoters, it probably does not play a transcriptional regulatory role.

No lineage specific DNA binding proteins have been identified. However, there may be lineage specific factors that do not bind DNA themselves but interact with the DNA binding proteins. For example TCFβ1 may interact with an αβ lineage-specific non-DNA binding protein. It would be interesting to do a protein-protein screening to identify such linker proteins. Linker proteins may also be needed to facilitate interaction of the DNA-binding proteins with the basal transcriptional machinery. Ets for example needs other factors to interact with the basal transcriptional machinery, p70 and TCFβ1 may or may not need additional factors.

Lineage specific rearrangement may lead to lineage specific β gene expression. Rearrangement of the β chain gene rather than β chain gene expression may signal commitment to the αβ T cell lineage. The specific recombination may potentiate further transcription. For example, evidence indicates that the Ku protein binds to DNA ends and can slide along the DNA (343). The process of rearrangement offers an ideal situation for p70 to enter the TCR β gene locus to control subsequent β gene expression.

Post-translational modification of the transcription factors may alter their DNA binding or regulatory activities in a lineage-specific manner. One way that this could be accomplished is by the nuclear location of factors at specific developmental stages. The cellular localization of TCFβ1 could be tested by con-focal microscopy of various thymocyte populations. Protein

splice variants may be lineage or stage specific. For example, a smaller TCFβ1 transcript is found in HUT-74 cells in addition to the size of transcript seen in Jurkat cells. Both cell lines are T cells but may represent different stages of T cell development or activation in which alternatively spliced forms of TCFβ1 are active. Alternative splicing may change the DNA binding specificity or the regulatory ability of a protein. A larger TCFβ1 transcript is found in skeletal muscle and brain lineages, and may also be due to alternatively spliced forms each having lineage-specific regulatory activities.

Post-translational phosphorylation is a common method of controlling the binding and regulatory activity of transcriptional proteins. We know that jun and ets can both exist in phosphorylated and non-phosphorylated forms. The regulatory and DNA binding activity differs according to the phosphorylation states of the protein. The potential of TCFβ1 to be phosphorylated still needs to be determined. Previous studies show that the Ku lupus autoantigen is associated with a p350 kinase activity. p70 may interact with the basal transcriptional machinery, through its associated kinase activity, to phosphorylate the CTD of RNA polymerase II or other transcriptional proteins. Since p70 and TCFβ1 bind to neighbouring sites within the β enhancer, the Ku-associated kinase might regulate TCFβ1. It remains to be determined if p70 binds to the E3 site in the β enhancer in association with the kinase, and if TCFβ1 is phosphorylated. It is of interest to note that TCFβ1 binds to the octamer sequence, that p70 has also been found associated with the octamer sequence, and that the Ku associated kinase activity can phosphorylate Oct1 and Oct2. This suggests an interaction between TCFβ1 and p70, perhaps during B cell development, Oct2 and TCFβ1 bind to the octamer sequence at different time points, and both might interact with p70.

Lineage specific expression can be regulated by negative sequences that restrict expression to the appropriate cell. The Vβ2 upstream region acts like a silencer and may be responsible for silencing the entire β locus in non-αβ T cells. However, the identity of the inhibitory region binding proteins remains to be established. Additionally, the time during development at which the interaction between the inhibitory, TPA inducible and enhancer elements occurs is not yet determined. The role this interaction plays in inducing transcription at the appropriate developmental stage, and in limiting transcription in the inappropriate tissue or stage needs to be determined. Since the murine and human β enhancer regions are so similar, it would be interesting to determine if a silencer is also present in the human Vβ locus. Negative transcriptional regulation can be mediated via negatively acting DNA binding proteins. The same factor may act as both an activator and a repressor of transcription at different developmental time points. For example, ets-1 can both inhibit and activate transcription via the β enhancer depending on external cellular signals (343).

The overall DNA chromatin context of the TCR β locus may render it open or closed to the activity of various transcriptional proteins. Therefore, locus control regions, although not yet identified, may play a role in lineage determination.

The proteins I have identified, can now be used to generate novel tools to further dissect β gene regulation. The technologies available for "knocking out" the genes encoding TCFβ1 and other transcription factors by homologous recombination, expression of the different forms of TCR β gene control elements in transgenic mice, and biochemical analysis of the transcription factors binding to the β control elements will allow the goal of thoroughly understanding β gene transcription to be reached.

Chapter 5

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