NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

UNIVERSITY OF ALBERTA

STUDY OF *CIS*-ACTING ELEMENTS IN T CELL RECEPTOR β CHAIN GENES

BY

© JITRA RATANAVONGSIRI

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

FALL 1990

ISBN 0-315-64857-0

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: JITRA RATANAVONGSIRI

TITLE OF THESIS: STUDY OF *CIS*-ACTING ELEMENTS IN T CELL RECEPTOR β CHAIN GENES

DEGREE: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: FALL 1990

Jitra Ratanavongsiri

8824-27 Avenue

Edmonton, Alberta

T6K 2X4

Date: May 1 1990

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

STUDY OF *CIS*-ACTING ELEMENTS IN T CELL RECEPTOR β CHAIN GENE.

submitted by Jitra Ratanavongsiri
in partail fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences (Immunology).

Dr Arun Fotedar .....
Supervisor

Dr Thomas G Wegmann .....

Dr Bhagirath Singh .....

Dr David L Tyrrell .....

Dr Rich Barth .....
University of Rochester External Examiner

Date: May 1, 1990

ABSTRACT

The TCR αβ heterodimer recognizes antigen in association with self MHC. The functional α and β chain genes are generated by developmentally regulated rearrangement events between the germline V, D, and J gene segments. This work focuses on the analysis of *cis*-acting elements that control expression of the β chain gene.

In this study, multiple cis-acting elements have been identified in the mouse β chain gene. The minimum promoter region is located within 85 bp upstream of the Vβ2 transcription start site. Deletion of an additional 43 bp from the 5' end of this fragment abolished promoter activity. The presence of the conserved TCR β chain decanucleotide motif, an AP-1 consensus sequence and an inverted repeat in the deleted region suggests their role as targets for *trans*-acting factors regulating transcription of the β chain gene. Sequences between -343 and -85 increase transcription from the -85 fragment in T cells. Sequences further upstream of the Vβ2 promoter down regulate the Vβ promoter activity. The β chain enhancer located 5 kb downstream of Cβ2 enhances TCR gene transcription and can overcome the suppressive effect of the upstream negative regulatory elements.

The role of promoter and enhancer in the control of tissue specificity has also been analysed. The results indicate that expression of the Vβ2 promoter is lymphoid specific, i.e., only active in T cells but not in B cells. In addition, the β chain enhancer can enhance gene transcription of its cognate promoter in both T cells and fibroblasts.

Experiments in this study indicate that the Vβ2 promoter is TPA inducible. The TPA regulatory element in the Vβ2 promoter maps to the -85 to -62 region. This region includes an AP-1 consensus sequence and an inverted repeat motif. The TPA induction of TCR genes may play a role in the regulation of TCR gene expression.

Preface

Parts of the work described in this thesis have previously been published in the *Journal of Immunology* (144, 1111-1119, 1990). Results reporting on the identification of TPA responsive element in Vβ2 TCR promoter are submitted for publication.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr.Arun Fotedar for his support and enthusiasm during the course of this work. I am grateful to the members of my supervisory committee, Drs.Bhagirath Singh, Thomas G. Wegmann, D.L.J. Tyrrell, and Richard Barth for their helpful suggestions and to others for technical assistance and final preparation of the manuscript: Sack Igarashi, Sunil Mangal, Anna Fu, Helen Messier, Therese W. Fuller, Patrick Kilgannon, You Jun Shen, Dr. William E. Lee, Wilai Anomasiri, Nophanet Dhamborvorn, Wanjaree Mungsing and Kriengsak Srisuk.

A special thanks to my parents and Dom Kriangkum without whose support this work would not have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ag	antigen
AP-1	activator protein-1
AP-2	activator protein-2
AP-3	activator protein-3
AP-4	activator protein-4
AP-5	activator protein-5
ATP	adenosine triphosphate
B cell	bone marrow-derived cell
BAP	bovine alkaline phosphatase
bp	base pair
BSA	bovine serum albumin
C gene	constant gene
CAT	chloramphenicol acetyl transferase
cDNA	complementory DNA
CTL	cytotoxic T lymphocyte
D gene	diversity gene
DP	double positive
DN	double negative
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymine triphosphate
DMSO	dimethylsulfoxide

DNA	deoxyribonucleic acid
DNaseI	deoxyribonuclease I
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
HD	homeodomain
HLH	helix-loop-helix
hsp	heat shock protein
HSV	herpes simplex virus
IFN	interferon
Ig	immunoglobulin
IL-2	interleukin 2
IR	inverted repeat
J gene	joining gene
kb	kilobase
kd	kilodalton
MHC	major histocompatibility complex
mRNA	messenger RNA
MT	metallotheonein
NF	nuclear factor
nt	nucleotide
PEG	polyethylene glycol
PPD	purified protein derivative
Py	polyoma virus
RNA	ribonucleic acid
SCID	severe combined immunodeficiency syndrome

SDS	sodium dodecyl sulfate
SV40	simian virus 40
T cell	thymus derived cell
TBS	Tris-buffered saline
TCR	T cell antigen receptor
TF	transcription factor
TK	thymidine kinase
TLC	thin layer chromatography
TPA	12-*o*-tetradecanoyl-phorbol-13-acetate
TRE	TPA responsive element
V gene	variable gene
YAP-1	yeast AP-1

I. Literature Review

A. Introduction

The importance of thymus-dependent lymphocytes in the generation of an effective immune response was first described in the early nineteen sixties. T lymphocytes play a central role in cell mediated immune responses and in regulating both cellular and humoral immune responses. Unlike B cells that recognize soluble antigen, T cells recognize antigen only in association with self major histocompatibility complex (MHC) molecules (1).

This review is divided into three sections. The first deals with the structure and function of the antigen specific T cell receptor (TCR) and the genes that encode it. A discussion of the mechanistic regulation of eukaryotic gene expression is outlined in the second section. In particular, the roles of *cis*- and *trans*-acting elements in eukaryotic gene expression are discussed. Finally, in the last part, the recent literature on TCR gene regulation is reviewed.

B. Structure, Function and Molecular Biology of T-Cell Receptor

The TCR is a Single Receptor that Recognizes Antigen and Self MHC.

The ability of T lymphocytes to recognize antigen (Ag) only in association with self MHC is called MHC restriction. Cytotoxic T lymphocytes (CTL) kill cells that display foreign antigen and usually recognize the antigens in the context of class I MHC molecules whereas helper T cells augment T and B cell responses and tend to recognize antigen in the context of class II MHC molecules (2-5). This requirement of T cells for MHC restricted antigen recognition led to the postulation of dual receptor and single receptor models. The dual receptor model postulated that T cells have 2 receptors, one for antigen and the other for products of self MHC. The single receptor model on the other hand, proposed that the interaction of Ag and MHC molecules form a complex ligand on the cell surface of antigen presenting cells and is recognized by a single receptor on T lymphocytes. The latter model is now widely accepted, based on somatic cell genetics (6) and transfection experiments of TCR genes from one T cell to another (7-9). In addition, soluble Ia-antigen complexes

can stimulate the specific T cell response (10), suggesting that T cell antigen receptor is a single receptor that recognizes antigen complexed with self MHC molecules.

Structure of TCR

A major breakthrough in the study of TCR came from the ability to generate T cell lines and hybridomas coupled with the generation of clone specific antibodies against these cloned T cells. The clonotypic antibodies (11-17) bind specifically to the immunizing T cells in a clone specific manner. These clonotypic reagents block Ag/MHC dependent proliferation or IL-2 release by T cells in a clone specific manner (15,18). When coupled to sepharose beads, they activate in a clone specific manner in the absence of Ag/MHC (12,19). This suggests that the clonotypic antibodies bind to the T cell receptor. Biochemical analysis of the molecules recognized by these antibodies have been carried out by many investigators (11,12,15,20). The results suggest that in both mouse and human T cells, the antigen T cell receptor is composed of heterodimeric glycoproteins linked by disulfide bonds. The molecular weight ranges from 75,000 to 90,000 daltons. Upon reduction, these proteins resolve into two subunits varying from 38,000 to 44,000 daltons in the mouse. In human, one

subunit is larger with an apparent molecular weight of 40,000-49,000 daltons (21).

The two chains of the TCR differ in their isoelectric points; the alpha chain is more acidic than the beta chain (19,21). Similar to immunoglobulins (Ig), α and β chains are composed of variable and constant domains (19), each of which can be subdivided into different regions: a hydrophobic leader region, a variable region, a constant region, a transmembrane region and a short cytoplasmic tail. The deduced protein sequence of the β chain has 5 and 2 potential sites for N-glycosylation in mouse and human respectively (22-24). The α chain has 4 and 6 potential sites for N-glycosylation in mouse and human respectively (23,25,26). Based on the structural similarities between TCR and Ig, TCR is considered to be a member of the immunoglobulin gene superfamily.

Two Types of TCR

In addition to the $\alpha\beta$ heterodimeric TCR found in most of the T cells, a second type of TCR composed of γ and δ chains has also been described. $\gamma\delta^+$ T cells appear to bear the $CD3^+4^-8^-$ (double negative) phenotype, and are found to comprise only 0.5-1% of human peripheral blood T cells (27-

29) and about 3% of T cells in murine spleen and lymph nodes (30-32). γδ-bearing cells are also found in the fetal thymus (33), adult double negative thymocytes (34), nude spleen cells (35), dendritic epidermal T cells (36) and cells responding in mixed lymphocyte responses (31). In murine intestinal epithelium, $\gamma\delta^+$ cells are the predominant T-cell type.

The role of $\gamma\delta^+$ T cells in the immune system is not clearly understood at the present time. Some of the human and mouse $\gamma\delta^+$ cell lines exhibit cytolytic activity (37-39), whereas other $\gamma\delta^+$ cell lines, upon appropriate stimulation, can produce lymphokines like IL-2, IL-4 (40-42), GM-CSF and/or γ-interferon (42). The study of γδ+ hybridomas indicates that these cells can respond to PPD (purified protein derivative from *Mycobacterium tuberculosis*) as measured by IL-2 production (43). The present information, therefore, does not allow categorization of $\gamma\delta^+$ cells into cytotoxic or helper T cell subsets.

Earlier work on $\gamma\delta^+$ cells has focused on the possibility that these cells might represent an immature stage of T cell differentiation since rearrangement of γ gene precedes that of β chain. Several lines of evidence suggest that γδ and αβ receptor bearing T cells are two

distinct subpopulations that independently develop during thymic maturation. It is found that many $\alpha\beta^+$ T cell clones lack functional γ gene rearrangement(44,45). Recent study also indicates that the majority of α producing T cells have never rearranged their δ chain locus (46). Taken together, the evidence suggests that $\alpha\beta$ and $\gamma\delta$ T cells are distinct lineages. Nevertheless, since γ gene rearrangements are detected in most of the $\alpha\beta^+$ T cells, the possibility that both lineages are derived from common precursors is still open (47).

Cloning of $\alpha\beta$ TCR Genes

The mouse β chain gene was cloned by using substracted cDNA libraries (48). cDNA from a T cell hybridoma was hybridized with mRNA of a B cell hybridoma. The resulting substracted T cell specific cDNA was used to construct the library. It was then screened with a subtracted, T cell specific probe that was made from mRNAs encoding membrane bound proteins. The resulting clones that screened positive were themselves used as probes in Southern blots to determine if any of the cDNAs were transcribed from genes rearranged in T cells. By using such a procedure, the mouse TCR β chain was identified and isolated.

Human β chain gene was simultaneously and independently cloned by differential, replicate screening of a T cell lymphoma cDNA library with labeled cDNA from the same T cell lymphoma and cDNA from a B cell hybridoma (23). Of the 200 colonies that were identified with the T cell probe but not the B cell probe, one was identified as the beta chain of human TCR.

The murine α chain gene was subsequently isolated using T cell-B cell or T cell-T cell substracted cDNA libraries (25). The human α gene was identified using oligonucleotide probes based on amino acid sequence (26).

Genomic Organization of αβ TCR Genes

In general, the genomic organization of every TCR locus is similar to those of immunoglobulin genes. The V, D, J, and C gene segments are separated in the germ line (2). During maturation in the thymus to generate a mature TCR gene, two rearrangement events occur in the β chain gene, the first between D and J segments and the second between V and D-J segments. In the α chain, rearrangement occurs between V and J segments.

β Chain

The TCR β chain is located on chromosome 7 in the human and 6 in the mouse (50). There are ~30 Vβ gene segments in mouse (51,52) and over 100 in human (53,54). Unlike the Ig genes, Vβ gene segment families are small and there is little cross hybridization between members of Vβ gene segment families (51,53). In the mouse, with 3 exceptions, they are single member families. Each Vβ gene segment is composed of 2 exons; the first shorter exon encodes most of the leader sequence and the larger exon encodes the last 5 amino acids of leader sequences and the majority of the variable domain (55). Most of Vβ gene segments are located upstream of D, J and C genes except Vβ14 which is located 10 kb downstream of Cβ2 in an inverted transcriptional orientation (56).

Downstream of Vβ gene segments, there are two gene clusters. Each is composed of a single Dβ segment approximately 600 nucleotides 5' to a cluster of Jβ segments and 2-5 kb 5' of the first Cβ exon. In both human and mouse, the Jβ1 cluster contains 6 functional J gene segments. For Jβ2 cluster however, there are 7 and 6 functional J segments in human and mouse respectively (57-59). The Cβ1 and Cβ2 gene segments are highly conserved.

The Cβ genes of both human and mouse are divided into 4 exons; the first two encode most of the extracellular C domain, the third encodes a major part of the transmembrane region of the protein and the last encodes the cytoplasmic domain and the 3' untranslated region. The recombination signals similar to those of immunoglobulins are found downstream of Vβ gene segments, upstream and downstream of Dβ gene segments and upstream of the Jβ gene segments.

α Chain

The TCR α chain gene is located on chromosome 14 both in human (60) and mouse (61). The organization of the α chain is somewhat different from the β chain. First, there are a larger number (40-50) of Vα gene segments in both human (62) and mouse (63). Unlike the Vβ gene segments that rarely cross hybridize to each other, Vα gene segments are divided into closely related cross-hybridizing families, with an average size of 4-7 members (62). The Vα gene segments are also divided into 2 exons as described above in Vβ. The coding sequences of Jα are longer than those of immunoglobulins and β chain (63) and are also more numerous (~100) and spread over a long distance, i.e., over 70 kb in mouse and over 50 kb in human (64,65). There is only one C region in α chain gene and there is no Dα. The

recombination signals similar to those in the β chain gene are also used in the α chain gene.

An interesting feature of TCR genomic organization is that the gene encoding the δ chain of TCR is located between the clusters of Vα and Jα both in human and mouse. Current evidence suggests that there is some overlap in V gene usage between the α and δ chains (66). Nevertheless, the repertoire of Vδ genes appears to be limited. There are two Dδ and two Jδ with a single Cδ in mouse and two Dδ and three Jδ with a single Cδ in human.

The genomic organization of murine γ genes is different from other receptor gene loci including human γ genes. The interspersal of V and J-C gene segments of murine γ genes is analogous to the λ light chain. There are 7 Vγ, divided into 5 subfamilies and 4 Cγ, each of which is preceded by a single Jγ. Human γ genes on the other hand, are organized in a similar fashion to that of the β chain gene (67). So far there is no D element identified in the γ genes of TCR.

Rearrangement of αβ TCR Genes

The rearrangement of TCR genes follow the 12/23 rule of immunoglobulin gene rearrangement (68). The recombination signal located immediately proximal to each of the coding V, D and J segments is a highly conserved heptamer, separated from a conserved nonamer motif by a nonconserved spacer. The spacer can be either one (12 nucleotides) or two (23 nucleotides) turns of the DNA helix. A 12-nucleotide recognition sequence always recombines with a 23-nucleotide sequence.

In the β chain, the arrangement of these recombination signals allows different types of joining, i.e., Vβ can either join to the rearranged Dβ/Jβ segment or can directly join to the Jβ segment. Examples of both types of rearrangement have been found (69-71). Alternatively, Dβ–Dβ rearrangements are also possible and help generate more diversity. In the α chain, rearrangement between Vα and Jα gene segments obey the 12/23 rule (2).

The recombination signal sequences flanking δ gene segments allow various types of δ chain rearrangements, i.e., Vδ-Jδ, Vδ-Dδ-Jδ or Vδ-Dδ1-Dδ2-Jδ joinings. In murine γ genes, Vγ-Jγ rearrangements are preferred between the close

proximal genes within the same transcriptional orientation (67).

Most of the rearrangement of TCR genes follows the looping/excision model (72) of gene rearrangement. The existence of excision products in the form of extrachromosomal closed circular DNA in thymocytes provides direct evidence for this type of gene rearrangement (73,74). Analysis of the β gene sequences in the circular DNA indicates a reciprocal recombinant structure of V-D-J joining. Two pairs of recombination signal sequences are joined in a head-to-head fashion at the reciprocal joint (75). A reciprocal structure has also been reported in TCR α and δ chain genes (74). In addition to the looping model, unequal sister chromatid exchange has also been described (76). In the case of Vβ14 located 3' of Cβ2 in the opposite transcriptional orientation, gene inversion has been shown to be the mechanism used for gene rearrangement (56).

Generation of Diversity

Three basic mechanisms for generation of immunoglobulin gene diversity (germline diversity, combinatorial diversity and junctional diversity) are also

used by the TCR genes (2). Germline diversity is the utilization of a large number of different gene segments (V,D and J) for the formation V region. Although the number of V segments in TCR genes is relatively small compared to those of Ig, this is compensated by the large number of J segments. Combinatorial diversity is the random rearrangement of the different germ line gene segments. By calculating the number of V, D and J genes in both α and β, it is estimated that approximately 10^7 different TCR can be generated assuming that gene rearrangement occurs randomly (77). Junctional diversity in TCR includes the imprecise joining between segments and N-region diversification, the addition of random nucleotides into the junction between the joined gene segments during gene rearrangement. In contrast to the immunoglobulin genes, no evidence for somatic hypermutation has been demonstrated in the generation of TCR gene diversity (51,52,71,78,215).

TCR Expression during T Cell Ontogeny.

$\gamma\delta$ bearing T cells appear first in ontogeny and can be detected by day 14 of gestation in the mouse fetal thymus (33,79). On day 15, the β transcripts can be detected. On day 16, the expression of the β transcript increases and α

chain transcript is detectable. After day 16, the proportion of γδ bearing cells decreases in parallel with the increase of $CD4^+$ and/or $CD8^+$ cells and expression of αβ bearing T cells (80-82). The level of TCR expression in thymocytes varies from low to high depending on the stage of maturation. Recent studies suggest that the level of TCR expression may play an important role during thymic selection (83-85).

Selection of MHC-Restricted T Cells

A considerable amount of evidence from the past decade suggests that self MHC recognition by T cells is "learned" during T cell development in the thymus (86). Through multiple stages of T cell differentiation, $CD4^-CD8^-$ (double negative; DN), TCR^- T cell precursors acquire $CD4^+CD8^+$ (double positive; DP) phenotype and subsequently mature to $CD4^+,TCR^+$ or $CD8^+,TCR^+$ (or single positive) T cell subsets. Thymic education involves both positive and negative selection, i.e., thymocytes that recognize exogenous antigens in association with self MHC are positively selected by the MHC in the absence of antigen whereas the autoreactive T cells specific to autoantigen and self MHC are eliminated.

Earlier evidence that supported positive selection has come from the studies of radiation-induced bone marrow chimera and *in-vivo* antibody blocking experiments (87). Recent studies using TCR transgenic mice have indicated that positive selection occurs around the time the DP cells differentiate into single positive thymocytes. Class II MHC expressing epithelial cells of the thymic cortex are responsible for such selection (84). A study of the H-Y/H-$2D^b$ specific transgene receptor in SCID mouse showed that in a non-relevant MHC environment, thymocytes expressing the transgene receptor accumulated at the immature DP stage. This suggests that cells that have no affinity for MHC cannot be positively selected.

Direct support for negative selection has come from the observation that peripheral $V\beta17a^+$ T cells, which react with I-E molecules, were deleted in $I\text{-}E^+$ mice (89,90). A similar observation has been made in the deletion of $V\beta8.1$ and $V\beta6$ in $Mls\text{-}1^a$ mice (91,92). This clonal deletion was mediated by the radio-sensitive bone-marrow derived dendritic cells inducing thymocytes at or around the DP stage to undergo apoptosis (87).

A recent study (83) on the density of TCR on thymocytes indicated that cortical thymocytes in the early

stage of differentiation have low density of αβ TCR as compared to the more mature medullary thymocytes or peripheral T cells. The TCR^{low} DP cells are divided into two distinct subsets; one with an αβ receptor uncoupled to CD3 and resistant to the elimination by engagement of the TCR, and one with a coupled receptor that is sensitive to deletion. This indicates that the αβ TCR can mediate both positive and negative selection in TCR^{low} cells, as well as activation of mature TCR^{hi} cells. The difference is probably due to unique coupled or uncoupled signal transduction pathways in each cell type.

C. Eukaryotic Gene Expression

The control of gene expression in eukaryotic cells is much more complex than in prokaryotic species. In bacteria, transcription and translation are coupled. Thus the major control is at the level of initiation of transcription. In higher eukaryotes, DNA is packaged into chromosomes in which the basic repeating unit is the nucleosome consisting of 150-200 base pairs of DNA wrapped around the histone octamer. In eukaryotic cells, transcription occurs in the nucleus, whereas the site of translation is in the cytoplasm. The transportation of mRNA from nucleus to

cytoplasm is intimately associated with mRNA processing (splicing etc). After mRNA is translated into protein, post translational modification such as glycosylation and phosphorylation takes place in the pathway to form the mature functional protein.

Problems Related to Eukaryotic Gene Regulation

There are two important problems that we have to address to comprehend the complexity of eukaryotic gene regulation. Firstly, although all somatic cells inherit the same DNA (almost), each differentiated cell type has an "epigenetic memory" to distinguish active transcription units from transcriptionally silenced genes. This memory has to be maintained throughout multiple cycles of cell division of the differentiated cells. Secondly, each specific cell type responds to a variety of extracellular signals by inducing gene expression of only the appropriate gene. Failure to do this in a tightly regulated manner would be fatal. Thus, there is no doubt that complex hierarchial mechanisms must exist to ensure this.

Multiple Mechanisms Control Eukaryotic Gene Expression

To date, it has been shown that the control of gene expression can be at different levels, such as at the level of chromatin structure, state of DNA methylation, control of transcription initiation, RNA splicing, mRNA stability, translation and post-translational modification. Only the pretranscriptional and transcriptional controls are further described.

Pretranscriptional and transcriptional control are the major modes of gene regulation. Several lines of evidence lead to this conclusion. Firstly, RNA-DNA hybridization experiments indicate that different populations of nuclear RNA molecules are present in different developmental stages or different tissues in the same organism (93). Secondly, *in vitro* transcription assays of chromatin prepared from different stages or tissues of the same organism yielded different types of gene products (94). Thirdly, there is a strong correlation between changes in the chromatin structure and gene activity (95). Taken together, these strongly suggest the existence of the pretranscriptional and transcriptional control in eukaryotic gene expression.

Altered chromatin structure during gene activation is indicated by the increased sensitivity to nuclease digestion (DNaseI, micrococcal nuclease, Staphylococcal

nuclease, etc.) suggesting an unfolding or disorganization of the chromatin. In the transcriptionally silent genes, the nucleosome structure is in a condensed conformation. In the highly transcribable genes, this varies among different genes studied. A number of genes such as large rRNA lack nucleosome organization during transcription (96). Isolated oligonucleosomes, on the other hand, are efficiently transcribed without any loss of histones (97). Study on *in vivo* chromatin structure of heat shock protein 70 (hsp70) genes of *Drosophila melanogaster* suggests that histone H4 remains bound to actively transcribed DNA (98). Recent study indicates that during transcription of hsp70, histones are retained on the coding region but not on the promoter region. The pattern of histone-DNA interaction, however, is altered (99), i.e., the more flexible N- and C-terminal region of histones remain associated with DNA while the central globular region of histones are partially displaced. The nuclease hypersensitive sites in or around the regulatory regions are detected not only when a gene is being transcribed, but also in a gene that is potentially activatable. It appears to be a necessary but not sufficient condition for gene expression.

DNA methylation occurs at the C residue in the CpG sequence. Undermethylation normally correlates well with

gene activity. Methylation is thought to regulate gene expression by either crowding the major groove of DNA by the 5' methyl group or activating the binding of the repressor protein. The former idea is supported by the finding that some of the transcription factors can only bind to the unmethylated but not the methylated DNA (100). Some proteins, however, can bind equally well to both unmethylated and methylated DNA (101,102). The latter idea is presently supported by the finding of a nuclear protein that binds specifically to DNA containing methylated CpGs regardless of the sequence (103,104). Methylation and transcription, however, are not always coupled.

Control of Gene Transcription

In recent years, rapid advances in our understanding of eukaryotic gene transcription have been made. Here, the role of *cis* and *trans*-acting elements in the control of gene transcription by RNA polymerase II is discussed.

Promoter

Gene transcription of eukaryotic cells is regulated by multiple *cis*-acting elements scattered within a transcription unit (105). The control region in the

immediate vicinity of a transcription start site is called a promoter (106,107). Within a promoter, a highly conserved TATA box similar to Pribnow's box in bacteria is located at around 30 bp upstream of the transcription start site and has been shown to be the binding site of the general transcription factors. The function of the TATA box is to ensure the accurate transcription initiation. However, some promoters such as adenovirus 2 EIIa, IVa2 lack a discernable TATA motif. It is proposed that these promoters may bind to the same general transcription factors as TATA-box containing promoters or different transcription factors may be involved in the DNA-protein interaction (106).

Besides the TATA box, a promoter also contains other *cis*-acting elements designated upstream promoter elements. Mutagenesis studies suggest that the strength of promoters is determined by the number and type of the upstream promoter elements. The commonly found upstream promoter elements are GC and CAAT boxes. A large number of less common elements are found in special types of signal-dependent transcription regulation, such as in response to hormones or growth factors. Gel retardation (108) and DNaseI footprinting experiments (109) show that these elements can bind to *trans*-acting nuclear proteins and can function regardless of their orientation with respect to

the TATA box. However, the position of these upstream elements on the helical DNA is important, i.e., they have to be on the same side of the α-helix. Insertion of nucleotides between the upstream elements and the TATA box to change their position in the helical structure can decrease the level of transcription (110), suggesting that the interaction among the proteins that bind to upstream elements and the TATA box requires the appropriate stereospecific alignment of the proteins on the DNA helix.

Formation of the Transcription Initiation Complex

In mammalian cells, there are at least five general transcription factors in addition to RNA polymerase II that are required for the formation of a transcription preinitiation complex (241,111). These are TFIIA, TFIIB, TFIID, TFIIE and TFIIF.

TFIID and RAP30 (RNA polymeraseII associating protein 30), a subunit of TFIIF, display a sequence similarity to the bacterial sigma factor (112,117). RAP30/74, known as TFIIF, contains an ATP-dependent DNA helicase whose function is to melt the DNA at the transcription start site (112). In yeast, TFIID and TFIIA homologues have been

identified and yeast TFIID has already been cloned (113-117).

RNA polymerase II is composed of 8-10 subunits ranging in molecular weight from 240 kd to 10 kd (241). The largest subunit exists in three forms distinguished by the modification at the C-terminal domain of the molecule. DNA sequence analysis and the deduced protein sequences indicate that RNA polymerase II is highly conserved. In addition, the genomic organization in mouse and human also appears to be conserved. The amino end of this largest subunit contains a zinc binding domain, suggesting a role in DNA binding (118). Interestingly, in eukaryotic cells, the carboxy end of the largest subunit contains a repeated heptapeptide region, Tyr-Ser-Pro-Tyr-Ser-Pro-Ser; 52 times in the mouse (118), 26 times in yeast (119). It has been shown in functional studies that a certain number of repeats is required for the function of RNA polymerase II *in vivo* (120-122). Although the molecular interaction of the RNA polymerase II subunits is not yet known due to limited information on the smaller subunits, RNA-protein cross linking studies showed that during transcription, the mRNA is in close proximity to the largest and the second largest RNA polymerase II subunits (123).

Kinetic studies of transcription initiation complex formation indicate that TFIIA, although it has no known DNA binding properties, is the first factor that forms the complex, followed by or coincidental with the binding of TFIID and RNA polymerase II. The rapid addition of other TFs and protein phosphorylation by a protein kinase results in the formation of an active transcription initiation complex. The whole process, however, is driven by some specific *trans*-activators (discussed below).

How does a Promoter Function?

The function of the promoter is to control the initiation of transcription. Although factors involved in the preinitiation complex formation are present in all cells, specific *trans*-activators which bind to the upstream promoter elements determine the activation of gene transcription.

Two possible mechanisms for transcription stimulation by specific *trans*-activators have been proposed (124). In the first, the specific activators initiate the transcription by recruiting one or more of the general transcription factors to facilitate assembly of a preinitiation complex. Experimental evidence that supports

this idea has come from the study of adenovirus E4 promoter and a cellular factor called ATF. By using DNaseI footprint analysis (125) and transcription assays (126), it was shown that initially ATF bound to TFIID and then to the promoter. These interactions in turn facilitated promoter recognition by RNA polymerase II and other general initiation factors. In the second model, the activators enhance some steps following assembly of the preinitiation complex. In both cases, the strength of the promoter is determined by the number and interaction of the upstream promoter elements, the presence of negative regulatory elements, the type and amount of *trans*-acting factors that are present in the cells, and the change of *trans*-acting factors upon a specific induction.

Enhancer

The *cis*-acting element that enhances the transcription from a promoter in a distance and orientation independent manner is called an enhancer (129-131). Enhancers were first described in the SV40 early promoter (127,128). It is located in the 72 bp tandem repeat of SV40 DNA and its deletion reduces the early gene expression by two orders of magnitude. SV40 enhancer can also enhance transcription of heterologous promoters such as the herpes simplex thymidine

kinase (HSV-tk), rabbit β-globin, conalbumin, chick lysozyme and mouse metallothionein. Similar enhancer elements were subsequently found in a number of eukaryotic viruses as well as in the mammalian genes, such as α-interferon, histones, chymotrypsin, β-globin and Ig genes (130). An example of a tissue specific enhancer is the Ig enhancer located within J-C intron of Ig genes. It is functional only in B cells but has no transcriptional stimulatory effect in other somatic cells (e.g., fibroblasts).

Enhancers are often composed of multiple short (8-10 bp) sequence motifs. These motifs were frequently identified by sequence comparisons among different enhancers and were subsequently proved functional by saturation mutation analysis. The enhancer elements function as *trans*-acting nuclear protein binding sites. Therefore, the enhancer appears to interact with a variety of factors. Studies of the organization of the SV40 enhancer (132), the best known enhancer, indicates that it is composed of various enhancer elements separated from each other by roughly 100 bp. Each element is capable of enhancing gene transcription and is composed of enhansons, short DNA sequences that form the basic units of enhancer

structure. Whether the enhanson structure is obeyed in other enhancers remains to be proved.

Inducible Enhancer

Upon induction by a specific signal, this type of enhancer can increase the rate of initiation of transcription. The inducible enhancers are found in a variety of eukaryotic genes such as genes for heat shock, metallothionein, β-interferon, and c-fos etc. The inducible enhancer elements are sometimes closely associated with their promoters or may be located at a distance. They can function on heterologous promoters and the elements are normally short DNA sequences of 8-15 base pairs (105).

TPA Inducible Enhancer

One example of an inducible enhancer that will be further discussed here is the TPA-responsive element AP-1.

TPA (12-*o*-tetradecanoyl-phorbol-13-acetate) is a tumor promoter that can enhance the effect of the subcarcinogenic dose of an initiating carcinogen (133-135). Subsequent studies demonstrated that the transcription of many cellular proto-oncogenes like *c-fos* (136), *c-myc* (137), *c-*

sis (138) and *pro-1* (139) can be enhanced by TPA. In addition, transcription from other eukaryotic genes, such as human collagenase (140), rat stromelysin (140), human MTIIA (141) as well as SV40 and polyoma viral gene expression is also induced by TPA. There are many TPA responsive elements (TRE) identified from such genes in mammalian and yeast cells such as AP-1, AP-2, AP-3, AP-4, AP-5, NF-κB and YAP-1 (yeast AP-1)(142-145).

AP-1, the prototype of TPA inducible elements has been shown by transfection experiments in Hela cells to have the characteristic of an enhancer. In the presence of TPA, AP-1 further enhances the transcription by 5-10 fold (145). The AP-1 binding proteins are biochemically defined by their ability to recognize the TGACTACA motif (151).

There may be more than a single mechanism used by TPA to enhance the transcription via the AP-1 binding site. Evidence shows that TPA can stimulate protein kinase C which may in turn activate the AP-1 proteins by post-translational phosphorylation events (142,145,151). Alternatively, TPA increases transcription of the *trans*-acting factors such as JUN and FOS which bind the AP-1 segment (146-148). Serum factors also have this similar effect (149). The possibility that TPA may act directly on

the *cis*-element, however, has not been ruled out. Since AP-1 is a family of proteins that share the same DNA binding specificity, multiple factors within the family are shown to interact to the AP-1 responsive element (146,147,150). Tissue distribution of these factors will also determine the degree of TPA responsiveness.

AP-1: Family of Enhancer Binding Proteins

AP-1 was initially isolated from the nuclear extract of Hela cells by DNA affinity chromatography (151). The biochemical study of AP-1 indicated that it is composed of various different proteins that share sequence specific DNA binding properties. These include the gene products of *c-jun, c-fos, junB, junD* and *fra*1 (146, 147, 242-244).

c-jun is the homologue of a viral oncogene, *jun* (152,153). The discovery of Jun/AP-1 came from the observation that v-Jun has sequence homology with the DNA binding domain of GCN4, a yeast transcription factor (154). Since the GCN4 binding sequence is almost identical to the AP-1 motif, it was suggested that Jun might be related to the AP-1 proteins. In addition, the antibody to Jun cross reacted with AP-1 (155). Subsequent isolation of *c-jun* from human genomic library revealed that c-Jun can bind to the

AP-1 motif and that the sequences of several AP-1 tryptic peptides were identical to the predicted amino acid sequence of Jun. Hence, Jun is one of the AP-1 peptides (156,157).

c-fos is a proto-oncogene whose product is involved in gene regulation (148). Fos forms a complex with the protein, p39 and binds to the AP-1 motif. Antibody against Jun cross reacts with p39. Additional studies on protein structure indicate that p39 is indeed Jun (146,158,159).

A number of jun-related and fos-related genes that encode for AP-1 proteins have now been identified. How these gene products react with each other in the binding to AP-1 is not clearly understood. Recent studies of Fos and Jun indicated that both proteins can form a heterodimeric complex via the leucine zipper interaction (160-164)(see below, 'DNA binding motif'). Jun, but not Fos, can form a homodimeric protein that can also bind to the AP-1 motif but with a relatively low affinity (165). Nevertheless, a Fos homodimer can be generated if the leucine zipper structure in one of the Fos molecule is replaced by that of Jun. This homodimeric protein can also bind to the TRE (165). This fact supports the idea that Fos and Jun form a

nearly symmetrical DNA binding site that interacts with the palindromic TRE (5'TGACTCA3').

How do Regulatory Elements Work at a Distance?

There are four models that may explain the signal transmission between distant promoters and enhancers. These are looping, twisting, sliding and oozing models (167,168).

In the looping model, proteins bind to the DNA at separate sites and the looping or bending of the intervening sequences allows the interaction between these proteins. The twisting model proposes that the binding of the regulatory protein to DNA changes the DNA conformation. This altered form of DNA allows the binding of other proteins for the initiation of transcription. In the sliding model, a protein recognizes a specific site on the DNA and then moves along to another specific sequence where it initiates transcription. Finally, for the oozing model, the binding of a regulatory protein at one specific site provides help for the binding of another protein to the adjacent sequences, which in turn helps another to bind next to it, and so on, until the site of transcription initiation is reached.

The twisting model has now been ruled out by the experiment of Plon and Wang (169) who demonstrated that the enhancer can function cooperatively with the promoter even when they are topologically separated. This means that the twisting of the enhancer cannot be responsible for gene activation at the promoter site. So far, there is no evidence that strongly argues for or against the sliding or oozing model. At the present time, the looping model seems to be favored.

Interactions between and within Promoter and Enhancer Elements

Recently, two models that explain signal integration within a promoter or enhancer have been proposed (166). The first model deals with the interaction of several transcriptional activator proteins that are located relatively far apart. In this model, the proteins are brought into direct contact by bending of the DNA. The second model deals with the interaction of several transcriptional activator proteins that bind to the adjacent sites in DNA. This model proposes that the activator proteins may interact with each other indirectly through binding with another protein molecule which helps

integrate the signal from transcription factors. Whether these models are used in the cells remains to be answered.

DNA Binding Motifs

The DNA binding domains of sequence specific DNA binding proteins bear four types of motifs; helix-turn-helix, zinc finger, leucine zipper and helix-loop-helix (170-172).

Helix-turn-helix motif was initially described in lambda cro and C1 proteins and in the catabolite activator protein (CAP) of *E.coli*. These proteins share a similar secondary structure of two alpha helices separated by a relatively sharp beta turn (173) and bind to the major groove of the DNA helix. In eukaryotic cells, the helix-turn-helix motif is identified in the homeodomain (HD) of *Drosophila* homeotic gene products, *antennapedia, fushi tarazu, ultra bithorax,* the octamer binding factors, i.e., Oct-1 , Oct-2, the pituitary specific factor, Pit-1 and a developmental regulatory protein of the nematode *Caenorhabditis elegans,* Unc-86 etc. A subclass of the HD-containing protein family known as POU domain is identified in Pit-1, Oct-1, Oct-2 and Unc-86 (174). These factors share a large conserved region (POU-specific subdomain) in

addition to the homeodomain. In contrast to other HD-containing proteins that can bind and directly activate transcription of target genes, POU proteins require both the POU specific subdomain and a homeodomain in order to function, as deletion of the POU specific region debilitates DNA binding activity in a gel retardation assay (175).

The zinc finger motif was first identified in the RNA polymerase III transcription factor, TFIIIA (176) and subsequently in a variety of DNA binding proteins such as Sp1, GAL4, steroid hormone receptor etc (177). They are divided into 2 classes according to the number and position of the cysteine and histidine residues available for zinc coordination (178). In the C_2H_2 class, e.g., TFIIIA and Sp1, the basic structural unit is composed of pairs of cysteins and histidines separated by a loop of 12 amino acids. The finger motif is tandemly repeated a minimum of 2 times with a linker of 7 to 8 amino acids separating the units. The Cx class, such as GAL4 and the steroid receptors, has a variable number of conserved cysteines for metal chelation. In TFIIIA, it was indicated that the finger binds to the major groove of the helix (179). A zinc molecule was shown in Sp1 and GAL4 to be necessary for the binding of protein to the DNA (180).

The leucine zipper is found in C/EBP, a heat stable, rat liver nuclear protein (181), in FOS, MYC, JUN and GCN4, etc. The protein has a helical structure with hydrophobic amino acids, namely leucine, on one side and hydrophilic amino acids on the other side. The leucine residues are located at every seventh residue in the helix structure (7 amino acids/turn). Dimerization of two homo or hetero proteins results in the formation of hydrophobic interaction between two helices by the interlocking of the leucine side chains, the so called leucine zipper(182). This type of interaction is believed to be involved not only in creating protein-DNA interactions but also in the protein-protein interactions that are not directly involved in DNA recognition.

Helix-loop-helix motif (HLH) has been recently identified in various proteins such as mammalian MYOD, CMYC, LMYC, NMYC proteins and two immunoglobulin kappa chain enhancer binding proteins (E12 and E47) (172). It consists of 2 amphipatic helices separated by a loop region of variable length. Studies with E12 and E47 suggest that the HLH region is sufficient to allow these proteins both to dimerize and to bind to specific DNA sequences (183). In

addition, various members of the HLH family can form homo- and heterodimers similar to the leucine zipper motif.

D. Control of TCR Gene Transcription

The analysis of the regulation of TCR gene transcription is still in its infancy. There are many interesting questions regarding TCR expression. For example, how do T cells control the TCR gene rearrangement?, What is the mechanism that controls tissue specific expression of TCR?, What is the role of TCR in T cell development?, Are αβ and γδ T cells derived from the same precursor cells? Most of these questions still remain to be answered. Here, the current information of TCR gene transcription is reviewed.

TCR Enhancer and Tissue Specific Expression

The Ig enhancer located within the J-C intron is responsible for tissue specific expression of Ig genes. Initial attempts to isolate the TCR enhancer from the J-C intron of TCR genes, however, were not successful (184,185). The mouse TCR β chain enhancer was first successfully identified 5 kb downstream of the Cβ2 structural gene segment (184, 185). By using transient

expression assays, Krimpenfort, *et al* (184) demonstrated that the TCR β enhancer is lymphoid specific, i.e., it can function in both T and B cells but not in non-lymphoid cells. In addition, McDougall, *et al* (185) found the TCR β enhancer to be weaker than the SV40 enhancer.

The α chain enhancer was initially reported by Luria, *et al* (186) to be located within the JαCα intron. This result, nevertheless, was not confirmed by other investigators. Recently, Winoto and Baltimore (187) and Ho, *et al* (188) mapped the murine and human α chain enhancer 3 kb downstream of the Cα structural gene segment. They also showed that the Vα promoter is transcriptionally inactive in the absence of the enhancer. The α enhancer enhances the transcription of a heterologous promoter only in T cells, but not in B cells or fibroblasts. Nuclear extracts from T cells can bind specifically to this enhancer, thus supporting the functional data that suggests the TCR α enhancer is T cell specific.

Functional Elements Identified in the TCR vβ Promoters

Anderson, *et al* (189) and Lee, *et al* (190) observed a unique conserved decanucleotide sequence (5'AGTGACATCA3')

present not too far upstream from the transcription start site in 13 out of 14 of Vβ promoters studied. The core sequence is similar to the AP-1 binding sequence and to the c-AMP responsive element (CRE). A 5' deletional analysis of the human Vβ8.1 promoter showed no difference in transcriptional activity between promoter fragments with or without the decanucleotide motif (191). Anderson, *et al* on the other hand, while studying the murine Vβ8.3 promoter demonstrated that elimination of the decamer motif by either deletion or mutation, can reduce the transcription activity of the Vβ8.3 promoter by 10 fold. Footprinting analysis indicates the binding of EL-4 nuclear protein to the decamer sequence (192), thus supporting the conclusion that this decanucleotide sequence is important for TCR promoter activity. A consensus cAMP responsive element (CRE) but not a consensus AP-1 site, can functionally substitute for the decamer in transient expression assays.

Diamond, *et al* (191) identified promoter activity in a human Vβ8.1 promoter fragment 45 bp upstream of the transcription start site. The Vβ promoter is T cell specific, i.e., does not function in B cells or fibroblasts. They also found a T cell specific enhancer element (-800 to -570) that acts in an orientation and distance independent manner.

Chromatin Structure of TCR Genes

Changes in chromatin structure often reflect the developmental regulation of genes expressed in a tissue specific manner. The presence of DNaseI hypersensitive sites in the chromatin structure, in many examples, correlates with active or potentially active gene expression. The hypersensitive regions are frequently shown to be within or close to the regulatory regions. Studies of Ig genes indicate the presence of DNaseI hypersensitive sites in the enhancer region of the J-C intron (193-196). In the TCR β gene, the DNaseI hypersensitivity has been detected in various regions. Bier (198) has shown that the region in the Jβ2-Cβ2 intron of TCR β gene contains the tissue specific DNaseI hypersensitive site, leading to the initial speculation that TCR enhancer may be located within the J-C intron. It was subsequently shown that this region does not have enhancer activity but contains a TPA inducible and NF-KB like element (236). A more recent study by Hashimoto (199) indicates two DNaseI hypersensitive sites downstream of Cβ2 region. One of these is located in the enhancer region found in all T cell lines but not in other tissues. Another hypersensitive site was mapped between Cβ2 and the putative enhancer region found only in

β chain transcriptionally active T cell lines. The second hypersensitive region contains some DNA sequences homologous to the typical regulatory elements such as AP-1, core sequences of SV40 and polyoma enhancers, Ig octamer, and sequences similar to the protein binding site detected by chemical modification using DMS in the IgH enhancer. Hashimoto has concluded that while the first hypersensitive site maps a region involved in tissue specificity, the second hypersensitive site may be involved in both stage and tissue specificity, thus suggesting that changes in chromatin structure reflect aspects of TCR tissue specific gene expression.

State of Methylation in TCR β Chain Genes

The state of DNA methylation is often associated with the transcriptional activity of eukaryotic genes. Evidence has been accumulating that methylation of DNA plays an important role in controlling gene expression in a cell or tissue specific manner (200,201). Sakamoto,*et al* (202), while attempting to use the methylation state of TCR β gene as a marker for different leukocyte populations (T cells, B cells, monocytes and large granular lymphocytes) have found that in T cells that express a complete 1.3 kb β chain transcript, most of the CCGG sequences in the β chain gene

are not methylated. In contrast, B cells that produce low level of the 1.0 kb incomplete β chain transcript and monocytes contain highly methylated CCGG sequences. The Jβ2 but not Jβ1 region of TCR β gene, however, is hypomethylated in both human B cells and monocytes (203). The fact that B cells and monocytes have an identical methylation pattern but are different in their ability for TCR gene transcription suggests the involvement of another regulatory control in TCR gene expression. Therefore, although these studies indicate a good correlation between hypomethylation and TCR gene expression in T cells, the state of DNA methylation alone may not be enough to explain the tissue specificity of antigen T cell receptor.

E. Project and Rationale

Understanding the T cell receptor for antigen plus self MHC has been the main goal in immunological research for the past decade. Only recently, the polypeptide chains comprising the TCR and the genes that encode them have been discovered. The puzzle of how T cells control TCR gene expression in a cell type and stage specific manner, however, has not yet been resolved. In the Ig gene, the promoter and enhancer has been shown to play a role in the control of tissue specific expression in B cells. Similar

mechanisms have been postulated to be used by TCR genes as they are similar in structure, function and genomic organization.

The objective of the work presented in this thesis is to investigate the *cis*-acting elements that control the expression of TCR β chain gene. When this project started, the TCR β promoter and enhancer had not been identified. Therefore, our first goal was to identify the TCR β promoter (Section A). This involved the generation of a murine T cell genomic library, isolation and characterization of the TCR beta chain gene. Finally, the TCR β promoter was analysed by deletional analysis using transient expression assays.

The next step of the study was to analyse the role of TCR β chain promoter in the control of tissue specific expression. While this was being analysed, the first murine TCR β enhancer was identified downstream of the Cβ2 gene segment. Therefore, the interaction of the TCR β promoter and enhancer both in terms of their cooperativity and their role in tissue specific expression was also investigated. The results are presented in part II.

As a result of the TCR β promoter analysis, some interesting sequences were identified in the minimum promoter fragment. One of these sequences is an AP-1 binding site which has been previously shown by others to enhance gene transcription by the phorbol ester, TPA. The last part of this work, as discussed in part III focuses on the role of TPA in TCR β gene transcription. As shown here, the TCR β promoter is inducible by TPA and the element responsible for this induction in TCR β promoter was characterized.

Overall, this work indicates that there are multiple regulatory elements that control TCR gene transcription. The TCR β promoter is inducible by TPA and thus may serve as another control mechanism for the regulation of TCR β chain gene.

II. Materials and Methods

Cells

B.1.1 is a T cell hybridoma which releases IL-2 when stimulated with poly 18 (EYK(EYA)5) and IA^d expressing antigen presenting cells (204). It was generated by fusing Balb/c poly 18 specific T cell blasts with BW5147. EL-4 (T cell), MOPC-21 (B cell), NIH3T3 and Balb/c3T3 (fibroblasts) and F9 (teratocarcinoma) were obtained from ATCC. PA317 cells (205) are 3T3 cells transfected with a defective Moloney Murine Leukemia virus and were a kind gift from Dr. A. Dusty Miller (Seattle).

Generation of B.1.1 Genomic Library

B.1.1 Genomic DNA Preparation

Genomic DNA was prepared from B.1.1 cells by the method of Blin and Stafford (206). In brief, B.1.1 cells grown in RPMI 1640 and 10% fetal calf serum were washed twice in phosphate buffered saline and the cell pellet was resuspended in ice-cold TE (10 mM Tris (pH 8.0), 1 mM EDTA) at a concentration of 10^8 cells/ml. Ten volumes of 0.5M EDTA (pH 8.0), 100 µg/ml proteinase K and 0.5% sodium sarkosyl were added to the tube and the mixture was

incubated at 50°C for 3 hours. The sample was then phenol extracted three times and extensively dialysed against a solution of 50 mM Tris (pH 8.0), 10 mM EDTA and 10 mM NaCl. After dialysis, the sample was treated with 100 µg/ml RNaseA at 37°C for 3 hours, phenol/chloroform extracted twice and dialysed extensively against TE. The DNA was stored at 4°C.

Library

Genomic DNA from B.1.1 cells was partially digested with BamHI under conditions that gave the highest yield of DNA fragments in the 15-20 kb range. The BamHI cut DNA was size selected by sucrose gradient centrifugation. In short, 38 ml of 10-40% sucrose density gradient was prepared in a Beckman SW27 polyallomer tube. The sucrose solutions were made in a buffer containing 1M NaCl, 20 mM Tris (pH 8.0) and 5 mM EDTA. Five hundred micrograms of partially digested B.1.1 DNA resuspended in 500 µl TE was heated at 68°C for 10 minutes, cooled to 20°C and overlayed onto the gradient. The sample was centrifuged at 26,000 rpm for 24 hours at 20°C. After centrifugation, fractions of 500 µl were collected from the bottom of the centrifuge tube. Aliquots of DNA from every third tube were run on 3% agarose gels to determine the size of DNA. The fractions containing DNA fragments in the 15-20 kb size range were

pooled, dialysed against TE and ethanol precipitated. The DNA pellet was dissolved in TE to yield a concentration of 500 µg/ml. The DNA solution yield was reanalysed by running 0.5 µg DNA on 0.3% agarose gels to confirm the accuracy of the size distribution of the partially restricted genomic DNA.

The B.1.1 DNA fragments were cloned into EMBL 3 by ligating 0.4 µg B.1.1 DNA with 1 µg EMBL 3 arms (molar ratio=1:1) in 50 mM Tris (pH 8.0), 7 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ATP (pH 7.5) and 0.7 unit T4 DNA ligase in a total volume of 5 µl. The ligation mixture was incubated at 4°C overnight and then *in vitro* packaged (Gigapack Plus, Vector cloning system, San Diego). The recombinant lambda phage was plated on P2392 strain of *E.coli*. The number of recombinant clones obtained ranged from $3x10^5$ to $6x10^5$ pfu/µg lambda DNA.

Screening of Genomic Library

The B.1.1 genomic library was plated at a density of 30,000 pfu/plate. The plaques were transferred to nylon membranes (Colony/plaque screen disc, NEN Research Products) by overlaying for 2-3 minutes. The membranes were then denatured in 0.5 N NaOH, neutralized in 1.0 M Tris (pH

7.5) and air dried at room temperature. The TCR β probe was radiolabelled (207) by incubating 0.1 µg of heat denatured probe with 50 µCi ^{32}P-α-dATP, 0.1 µg oligodeoxy-ribonucleotides (Pharmacia, cat.#27-2166), 20 µM dNTPs (dATP, dCTP, dGTP, dTTP), 1 unit of Klenow fragment in 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 200 mM HEPES and 1 µg BSA in a total volume of 25 µl at room temperature for 3 hours. The labelled probe was separated from unincorporated radioactive nucleotides by running it through a sephadex G-50 column with 10 mM Tris pH 8.0 and 1 mM EDTA. The membranes were prehybridized in 1% SDS, 1 M NaCl and 10% dextran sulfate at 65°C for at least 6 hours. Hybridization was done by adding > 100 µg/ml denatured salmon sperm DNA and < 10 ng/ml radioactive probe into the prehybridization mix, and incubated at 65°C for an additional 16-24 hours. The washing steps were as follows: twice with 2xSSC (1xSSC: 0.15 M NaCl and 0.015 M sodium citrate ,pH 7.0) at room temperature for 5 minutes, twice with 2xSSC and 1%SDS at 65°C for 30 minutes, and twice with 0.1xSSC at room temperature for 30 minutes. The autoradiographs were developed after exposing Kodax X-ray film with an intensifying screen at -70°C overnight.

Lambda DNA Preparation for Genomic Clone Analysis

Lambda DNA was prepared as described by Yamamoto (208). The lambda phages were incubated until confluence. The plate lysate stocks were prepared by adding 5 ml of SM (100 mM NaCl, 50 mM Tris.Cl pH 7.5, 8 mM $MgSO_4$ and 0.01% gelatin) to the plate and incubating at 4°C for several hours with intermittent shaking. The supernatants were removed and incubated with RNaseA 10 µg/ml and DNaseI 10 µg/ml at room temperature for 1 hour. Solid sodium chloride was added to a final concentration of 1 M. The lysates were centrifuged at 11,000xg for 10 minutes at 4°C to remove debris. The phages were precipitated by adding PEG6000 to a final concentration of 10% w/v, incubated on ice for 1 hour and centrifuged at 11,000xg for 10 minutes at 4°C. The pellet was gently resuspended in SM (8 ml for each 500 ml of supernatant) and PEG removed from the lambda particles by chloroform extraction. Cesium chloride was added to the phage suspension (in the aqueous phase) at a concentration of 0.75 gm/ml and centrifuged in a Beckmann SW50.1 rotor at 35,000 rpm for 24 hours at 25°C. After centrifugation, a bluish band of phage particles was collected, and the cesium chloride dialysed away at room temparature for 1 hour against a 1000-fold volume of 10 mM NaCl, 50 mM Tris.Cl (pH 8.0) and 10 mM $MgCl_2$ with one

buffer change. The lambda DNA was extracted from the particles by vigorously shaking with phenol. The DNA was then extracted with phenol/chloroform and dialysed extensively at 4°C in TE (1,000-fold volumes with 3-4 changes of buffer in 48 hours).

Primer Extension Assay

Total RNA preparation

Total RNA was prepared by the guanidium/cesium chloride method (209). B.1.1 cells grown in RPMI 1640 supplemented with 10% fetal calf serum were washed twice with phosphate buffered saline pH 7.4 and the cell pellet resuspended in 5 volumes of G-solution (4 M gaunidium isothiocyanate, 5 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol and 0.5% sodium sarkosyl). Cesium chloride was then added to a concentration of 1 gm/2.5 ml. The mixture was then overlayed onto 1.2 ml cushion of 5.7 M CsCl in 0.1 M EDTA (pH 7.5) in a Beckmann SW50.1 polyallomer tube and centrifuged at 35,000 rpm for 16 hours at 25°C. After centrifugation, the upper layer solution was removed and replaced by G solution. The G solution was again removed and refilled 3-4 times. Finally, all the supernatant was removed. The RNA pellet was dried and resuspended in 10 mM Tris (pH 7.4), 5 mM EDTA, 1% SDS. The

RNA solution was extracted twice with phenol/chloroform, once with chloroform and precipitated with ethanol. The RNA was stored as a precipitate in ethanol at -70°C until used.

Assay

The 24-mer synthetic oligonucleotides complementary to nucleotides +67 to +90 of the Vβ2 leader sequences (5' GCACAGAATGCAAAACTGCCACAT 3') was synthesized at the Regional DNA Synthesis Laboratory (Calgary) and was used in a primer extension assay for mapping the Vβ2 transcription start site in B.1.1 cells. The oligonucleotide primer was end labelled with ^{32}P by incubating 0.1 µg of primer with 100 µCi ^{32}P-γ-ATP and 1 unit of T4 polynucleotide kinase in 10 mM $MgCl_2$, 20 mM Tris (pH 7.6) and 4 mM 2-mercaptoethanol at 37°C for 3 hours The radiolabelled oligonucleotide was purified through a sephadex G-50 column equilibrated with 10 mM Tris pH8.0 and 1 mM EDTA and ethanol precipitated. Twenty micrograms of total RNA from B.1.1 cells were annealed to $2x10^5$ cpm of end labelled primer in 250 mM KCl, 10 mM Tris (pH 7.5), and 1 mM EDTA in a total volume of 5 µl at 65°C for 60 minutes and allowed to cool to room temperature. The primer-RNA mixture was then adjusted to 60 µl in the presence of 60 mM NaCl, 10 mM Tris (pH 8.3 at 42°C), 10 mM DTT, 1 mM dNTPs, 50 µg/ml actinomycin D, 8 mM $MgCl_2$ and 1 unit reverse trnscriptase. The mixture was

incubated at 42°C for 1 hour. The primer extension product was heat denatured for 2 min and run on an 8% sequencing gel along with known end labelled molecular weight markers.

S1 Nuclease Assay

A 60-mer synthetic oligonucleotide probe extended from the Vβ2 leader sequence to -5 was end labelled by PNK reaction as described in the primer extension assay and purified on 8% polyacrylamide gel electrophoresis. Twenty micrograms of total RNA was mixed to 10^5 cpm probe in 167 µM Hepes pH 7.5, 1 M NaCl and 0.3 mM EDTA in the total volume of 30 µl. The mixture was incubated at 70°C for overnight and then at 37°C for 5-10 minutes. The S1 nuclease reaction was done by adding 270 µl S1 digestion buffer (0.28 M NaCl, 0.05 M sodium acetate pH4.5, 4.5 mM zinc acetate and 20 µg/ml salmon sperm DNA), 500 units/ml S1 nuclease (Sigma) and incubated at 30°C for 1 hour. The reaction was stopped by the addition of 3 µl 0.5 M EDTA and ethanol precipitated. The S1 digested product was then resuspended in 3 µl 0.1 M NaOH and mixed with 3 µl sequencing dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF), heat denatured for 2 minutes and run on an 8% sequencing gel along with the end labelled probe and known molecular weight markers.

Sequencing

M13 Single Stranded DNA Preparation

M13 culture was grown by inoculating 100 µl of an overnight JM101 culture with 10^5 pfu M13 in 5 ml of YT broth (1L: 8 gm Tryptone, 5 gm Yeast Extract, 5 gm NaCl) and shaken at 250 rpm, at 37°C for 6 hours. The culture was centrifuged in a microfuge for 5 minutes and the supernatant containing M13 virus particles was collected. The M13 virus particles were precipitated by adding 1/4 volume of 20% PEG in 2.5 M NaCl and allowing in the mixture to sit at room temperature for 10 minutes. The pellet was dried and resuspended in 100 µl TES (10 mM Tris pH 7.4, 5 mM EDTA and 1% SDS), extracted in succession with phenol and chloroform and finally ethanol precipitated. The M13 ssDNA was store at 4°C (not frozen).

Rapid Double Stranded Plasmid DNA Preparation for Sequencing

Ten millilitres of an overnight culture grown in TB (1L: 2.31 gm KH_2PO_4, 12.5 gm K_2HPO_4, 12 gm Tryptone, 4 ml glycerol) was centrifuged at 4,000 rpm for 5 minutes. The pellet was resuspended in 250 µl of 250 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA by vortexing and 500 µl of 0.2 M

NaOH and 1% SDS was added. The contents of the tube were mixed by inversion for 3 minutes and 250 µl of 5 M potassium acetate (equal volume of 3 M potassium acetate and 2 M acetic acid) was added. The sample was centrifuged at 4,000 rpm for 5 minutes and the supernatant transferred to a new eppendorf tube. The pDNA was precipitated by adding 0.5 ml isopropanol and centrifuged for 5 minutes. The DNA pellet was dissolved in 20 µl TE (10 mM Tris pH 8.0 and 1 mM EDTA) and treated with RNaseA (40 µg/ml) at 37°C for 30 minutes. An equal volume of 13% PEG in 1.6 M NaCl was then added to precipitate DNA and the DNA pellet was redissolved in 100 µl TE, phenol/chloroform (1:1) extracted once, followed by chloroform extraction and ethanol precipitated in the presence of 0.5 M ammonium acetate. The pellet was dried, resuspended in 10 µl TE and stored at 4°C.

Prior to the sequencing reaction, the pDNA (2-4 µg) was treated with 0.4 M NaOH at room temperature for 5 minutes and ethanol precipitated. The DNA pellet was dried and dissolved in 8 µl distilled water.

Sequencing Reaction

Sequencing reaction was done by standard dideoxy procedure using sequenase™ (US Biochemicals) enzyme

instead of Klenow. In brief, the mixture of M13 DNA or alkali denatured pDNA (7 µl), 1 µl primer (5 ng/µl) and 2 µl 5X sequencing buffer (200 mM Tris pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) was incubated at 65°C for 2 minutes and allowed to cool slowly to room temperature over a period of about 30 minutes. It was then mixed with 2 µl diluted labeling-mix (1:5; labelling-mix: 7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP), 1 µl 0.1M DTT, 0.5 µl ^{35}S-α-dATP (1,000 Ci/mmole), 2 µl of diluted sequenase (1:8) and incubated at room temperature for 5 minutes. After incubation, an aliquot of 3.5 µl of the reaction mixture was transferred to each of 4 tubes, containing 2.5 µl of ddATP or ddTTP or ddCTP or ddGTP termination mix. The tubes were further incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 4 µl sequencing dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF), heat denatured and run onto 8% denaturing polyacrylamide gel. After electrophoresis, the gel was dried and exposed to x-ray film with an intensifying screen at -70°C for overnight.

Cloning of pDNA Contructs

Conversion of DNA Overhangs to Blunt Ends

The DNA overhangs generated by restriction enzyme were blunted by T4 DNA polymerase treatment. DNA (1 µg) was

incubated with 2.5 units of T4 polymerase and 200 mM dNTPs in 33 mM Tris-acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol and 100 µg/ml BSA at 37°C for 15 minutes. The reaction was stopped by the addition of 1 µl 0.5 M EDTA, phenol/chloroform (1:1) extracted and ethanol precipitated.

Dephosphorylation of DNA

Vector DNAs, to help prevent self ligation, were dephosphorylated by bovine alkaline phosphatase (BAP) prior to ligation reaction. Briefly, DNA (1µg) was incubated with 1 unit BAP in the presence of 50 mM Tris (pH 8.0) at 65°C for 30 minutes. The reaction was stopped by adding 1 µl of 0.5 M EDTA, phenol/chloroform (1:1) extracted three times and ethanol precipitated.

Gel Purification of DNA

For ligation purpose, DNA fragments were purified by agarose gel electrophoresis and recovered by trapping with DE 81 paper (Whatman). This was done by inserting a piece of DE81 paper in front of the migrating DNA band in the agarose gel. Electrophoresis was continued until the DNA band was transferred to the paper. The DE81 (with the trapped DNA) was washed 3 times in 10 mM Tris pH 7.4, 0.1

mM EDTA, 0.1 M NaCl. The DNA was eluted with 10 mM Tris pH 7.4, 0.1 m EDTA, 1 M NaCl. The eluted DNA was mixed with an equal volume of distilled water and precipitated with 4 volumes of ethanol.

Ligation Reaction

The blunt end and sticky end ligations were done by mixing vector and insert DNA (gel purified) at 1:1 molar ratio (ranging from the total DNA of 200 ng to 1000 ng) in 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 5% PEG8000, 1 mM ATP, 1 mM DTT and 1 unit T4 ligase in a total volume of 10 to 25 µl and incubated at room temperature for at least 4 hours. The reaction was stopped by the addition of 1 µl of 0.5 M EDTA. The ligation mix could be kept at 4°C before transforming the competent bacteria.

Bacterial Transformation

Preparation of Competent Host Cells

The bacterial culture was prepared by inoculating 5 ml overnight culture into 500 ml LB (1L: 10 gm Tryptone, 5 gm Yeast Extract, 10 gm NaCl) and vigorously shaking at 37°C until OD_{610} was between 0.4 and 0.6. The culture was then centrifuged at 4,000 rpm for 10 minutes. The pellet was gently resuspended in 250 ml ice cold, sterile 50 mM $CaCl_2$, left on ice for 30 minutes and spun down at 4,000 rpm for

10 minutes. The bacteria were resuspended in 42.5 ml cold 50 mM $CaCl_2$ and 7.5 ml sterile glycerol and frozen in small aliquots at -70°C. In my hands, the transformation efficiency was in the order of 10^6 colonies/μg supercoiled DNA.

Transformation of Competent Host Cells

An aliquot of ligation mix (10 to 100 ng of DNA) was added to 50 μl of competent host cells and incubated on ice for 1 hour, then heat shocked at 42°C for 2-3 minutes and plated on agar plates containing the appropriate antibiotic. The plates were incubated overnight at 37°C.

Plasmid DNA

pSV2cat was a gift from Dr. J. Elliot (Stanford). The 4.5 kb BamHI-KpnI fragment containing the Cβ2 downstream enhancer was kindly provided by Drs. R. Barth and Leroy Hood (Caltech). The Col-TREx3/TKcat (145), Col-TREx1/TKcat (145), TREΔ-72/TKcat (145) and c-jun expression vector (240) have been described previously and made available to us by Dr. M. Karin.

For the sake of clarity in describing them, the pDNA constructs used in this study were divided into groups

depending on the way the constructs were generated (Figure 1). Standard recombinant DNA procedures were used (210).

Group 1 pTCRcat and pTKcat

pTCRcat was a series of pDNA in which various fragments of the TCR Vβ2 putative promoter drive chloramphenicol acetyl transferase (CAT) gene expression. pTCRcat and pTKcat were derived from pSV2cat by replacing the SV40 promoter and enhancer region with the TCR putative promoter and TK promoter respectively.

pSScat, pEScat and pTKcat SS is a fragment of TCR Vβ2 5' upstream region that extends from SalI to SphI (-2,570 to +31). ES is a EcoRI-SphI fragment of TCR Vβ2 promoter (-343 to +31). TK promoter was a -198 to +51 PvuII-BglII fragment from Herpes simplex thymidine kinase gene. The SV40 promoter and enhancer were removed from pSV2cat by digesting with AccI and HindIII. pSScat, pEScat, and pTKcat were generated by inserting SS, ES or TK fragment into the 4.5 kb fragment of the promoterless and enhancerless CAT vector by blunt end ligation. The DNA overhangs were blunted by T4 DNA polymerase, gel purified and ligated at room temperature for 4 hour prior to transformation (see

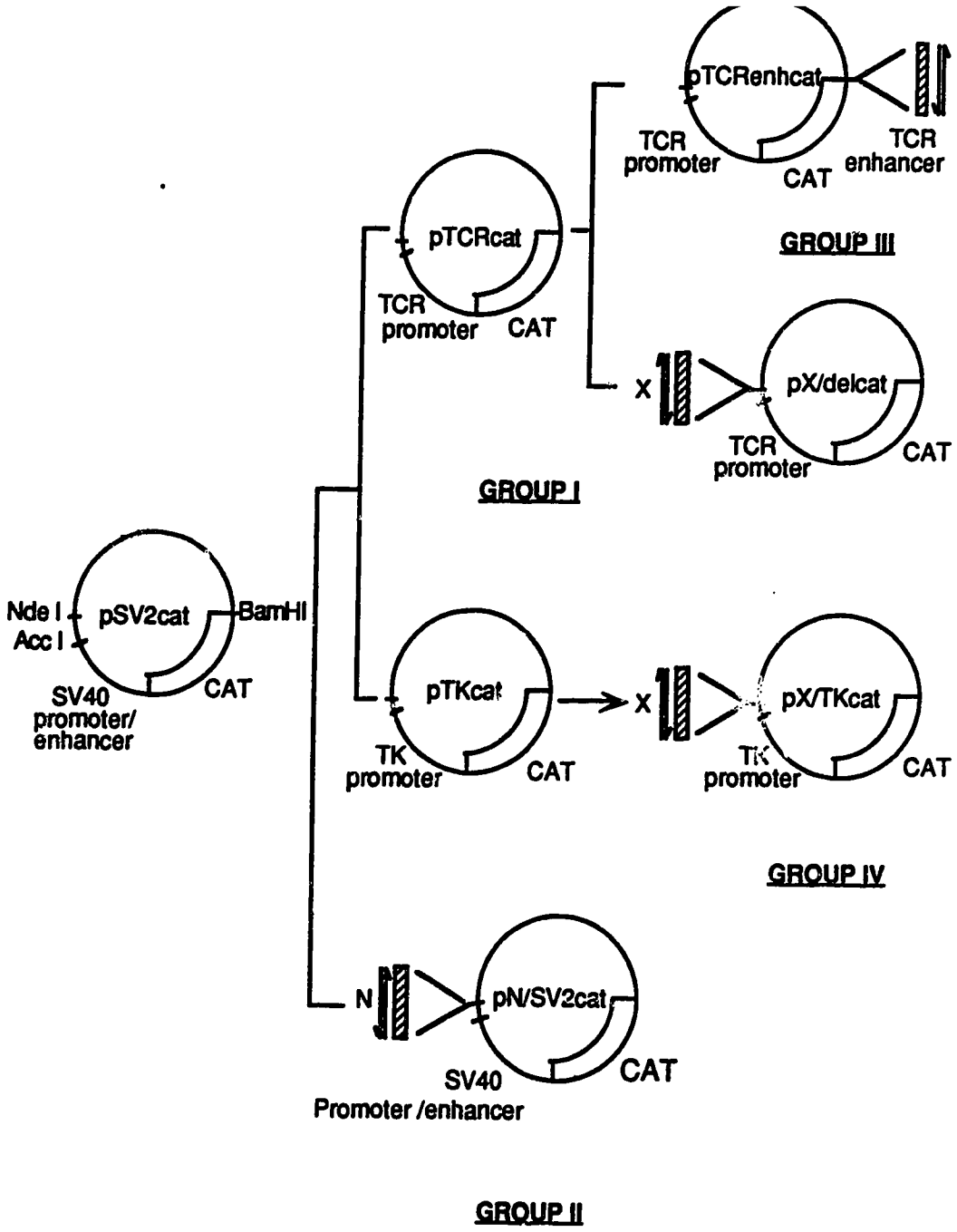


Figure 1 Four groups of plasmid DNA constructs

above "Cloning of pDNA Constructs"). The constructs were analysed by restriction enzyme digestion, Southern and sequence analysis.

pXScat, pAScat, pBScat, pHScat These constructs were made by digesting pSScat with NdeI and the respective enzymes (XbaI for XS, AccI for AS, BglII for BS and HindIII for HS). The fragments were blunt ended, gel purified and self ligated as described above.

pdel1cat and pdel2cat These constructs were made by digesting pEScat (-343 to +31 TCR promoter) with HindIII which removed a fragment between nucleotide -287 to -85 from ES promoter. The DNA was further deleted at both ends by treating with Exonuclease III at various incubation times, followed by mung bean nuclease digestion (ExoIII/Mung reagent kit, Stratagenes) and self ligation. The constructs made by this preparation were sequenced and two of them, pdel1cat and pdel2cat, were selected. pdel1cat contained the sequence between -42 and +31 of TCR promoter without any other TCR upstream sequences. Similarly, pdel2cat contained the sequences only from -18 to +31 of TCR gene.

Group 2 pN/SV2cat

pN/SV2cat was a series of pDNA constructs derived from pSV2cat by inserting various DNA fragments of TCR 5' flanking region upstream of the SV40 promoter and enhancer regions. pSV2cat was cut with AccI and the fragment ligated into pSV2cat by blunt end ligation as described. The constructs were analysed by restriction enzyme digestion. The name of the constructs followed the name of the fragment inserted and /SV2cat.

Group 3 pTCRenhcat

The pTCRenhcat constructs were derived from the pTCRcat vector. They contained various (blunt ended) fragments of TCR enhancer cloned into pTCRcat vectors at the BamHI site, downstream of CAT structural gene. In some cases, the enhancer was cloned at the NdeI site just upstream of the TCR Vβ2 promoter. All the cloning was done by blunt end ligation of the enhancer fragment into the respective pTCRcat vector. They were analysed by restriction enzyme digestion, Southern and sequence analysis. The nomenclature of these vectors is illustrated by the following example. The pESIcat construct was generated by cloning ES fragment of TCR promoter and

fragment I of TCR Vβ2 enhancer downstream of the CAT genes into the promoterless and enhancerless SV0cat vector.

Group 4 pX/delcat and pX/TKcat

These are two series of pDNA constructs derived from pdel1cat and pTKcat by inserting double stranded oligonucleotides (X) upstream of the del1 or TK promoter. There are 4 different double stranded oligonucleotides used in the generation of these pDNA constructs. The sequences and nomenclature are as follow:

OLIGONUCLEOTIDES	**REGION**
5' TATGAGCTTAGTCAGTTCATA 3' 3' ATACTCGAATCAGTCAAGTAT 5'	-85 to -73
5' TATGCTTAGTCAGTTTCCTGAGGAAGCATA 3' 3' ATACGAATCAGTCAAAGGACTCCTTCGTAT 5'	-84 to -62
5' TATGTTTCCTGAGGAAGCATA 3' 3' ATACAAAGGACTCCTTCGTAT 5'	-75 to -62
5' TATGAGTGATATCACTTCATA 3' 3' ATACTCACTATAGTGAAGTAT 5'	TCR decamer

Preparation of Double Stranded Oligonucleotides

The complementary strands of oligonucleotides were mixed together at 1:1 molar ratio in 0.1 M NaCl, 10 mM Tris pH 7.8, 1.0 mM EDTA, incubated at 65°C for 5 minutes and

allowed to anneal by incubating at 57°C for 1-2 hours. The mixture could be stored at -20°C.

p1x(-85 to -73)/delcat, p1x(-84 to -62)/delcat, p1x(-75 to -62)/delcat, p1x(-62 to -53)/delcat The corresponding double stranded oligonucleotides (0.4 ng) were mixed with NdeI digested pdelcat (25 ng) in 50 mM Tris pH 7.6, 10 mM $MgCl_2$, 5% PEG8000, 1 mM ATP, 1 mM DTT and 1 unit T4 ligase in a total volume of 10 µl. The mixture was incubated at 45°C for 5 minutes and quick chilled to 4°C. The ligation reaction was carried on at 4°C overnight before transforming the bacteria. The constructs were analysed by double stranded sequencing.

p2x(-85 to -73)/delcat and p3x(-84 to -62)/delcat The corresponding double stranded oligonucleotides (0.4 ng) were ligated into NdeI digested pdelcat in 50 mM Tris pH 7.6, 10 mM MgCl2, 5% PEG8000, 1 mM ATP, 1 mM DTT and 1 unit T4 ligase in a total volume of 10 µl at room temperature overnight followed by transformation. The constructs were analysed by double stranded sequencing.

p5x(-85 to -73)/TKcat and p3x(-84 to -62)/TKcat The corresponding double stranded oligonucleotides were cloned into pUC18 to yield a multimer of oligonucleotides. The

5x(-85 to -73) and 3x(-84 to -62) were cut out of pUC18 and inserted into pTKcat at NdeI site by blunt end ligation (see 'Cloning of pDNA Constructs'). After the bacterial transformation, the constructs were analysed by double stranded sequencing.

Transfection

EL-4 and MOPC-21 were transfected by the DEAE-dextran procedure (211). Briefly, the cells grown in DMEM and 10% fetal calf serum were washed thrice with DMEM and once with Tris buffered saline (TBS: 25 mM Tris (pH 7.4), 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM Na_2HPO_4). A total of 2 x 10^7 cells were mixed with 20 µg DNA and 500 µg/ml DEAE-dextran in TBS and incubated at room temperature for 30 minutes. After this step, EL-4 cells were washed once with TBS, twice with DMEM and plated in complete medium (DMEM supplemented with 10% fetal calf serum). For MOPC-21, the cells were washed once in TBS and further incubated with 200 µg/ml chloroquine in DMEM at 37°C for 3 hours, then washed 3 more times and plated in complete medium. The cells were harvested after 48 hours in culture.

Transfection of NIH3T3, Balb/c3T3, PA317 and F9 was performed by the calcium phosphate method. The cells were grown in a 100 mm culture dish until approximately 30% confluent. Four hours prior to transfection, cells were fed with 9 ml of fresh medium and the DNA precipitate was prepared by a standard procedure (212). Briefly, DNA (20 µg) in 500 µl of 250 mM $CaCl_2$ was added dropwise to an equal volume of 2x HEPES buffered solution (280 mM NaCl, 50 mM HEPES, and 1.5 mM Na_2HPO_4, pH 7.1) and was allowed to sit at room temperature for 20 minutes. The DNA precipitate was then added dropwise over a 100 mm dish of cells. After 4 hours of incubation at 37°C (16 hours at 37°C for F9), the cells were washed twice with phosphate buffered saline and fed with complete medium. The cells were harvested after 48 hours in culture.

Transfection efficiencies were monitored by cotransfecting 5 µg of pCH110 with 15 µg of the test plasmid. pCH110 is a plasmid DNA contruct in which the SV40 promoter and enhancer drives the expression of the β-galactosidase gene. Forty-eight hours after transfection, the cells were divided into 2 fractions; 1/3 for the β-galactosidase assay and 2/3 for the CAT assay.

TPA Induction

Twenty-four hours after transfection, TPA (dissolved in DMSO at a concentration of 10^{-2} M) was added to the culture at a final concentration of 60-100 ng/ml. The uninduced control received DMSO alone. The cells were harvested 12 hours after TPA induction.

CAT Assay

Cells were washed twice in TBS and the pellets were resuspended in 100 µl of 250 mM Tris (pH 7.5). Cell extracts were prepared by three cycles of freezing and thawing and the cell debris was removed by spinning in a microcentrifuge for 10 minutes at 4°C. The protein concentration of the samples were determined by Lowry's procedure and CAT assay from each experiment was done with an equal amount of protein. Cell extracts were pretreated with 1 mM EDTA pH 8.0 at 60°C for 10 min. CAT assay was done by incubating the extract with 0.1 µCi ^{14}C chloramphenicol, 0.5 mM acetyl CoA and 250 mM Tris (pH 7.5) in the total volume of 150 µl at 37°C for 5 hours (unless otherwise indicated). Chloramphenicol and its derivative were extracted from the reaction mixture into 1 ml of ethyl acetate, lyophilized and redissolved in 15 µl of ethyl

acetate. The sample was spotted on a silica gel plate and thin layer chromatograms were run in chloroform:methanol (95:5). The TLC plate was exposed to x-ray film at room temperature overnight. CAT enzyme activity was measured by counting the TLC plate in a scintillation counter and expressed as percentage conversion of ^{14}C chloramphenicol to 1- and 3-acetylated chloramphenicol.

β-Galactosidase Assay

β-galactosidase activity was determined as described by Miller (213). Cells were washed with TBS and the extracts were prepared in 100 µl freeze-thaw buffer (0.25 M sucrose, 10 mM Tris pH 7.4, 10 mM EDTA) as described in CAT assay. Protein concentration was determined by Lowry's method. The β-galactosidase reaction was done by mixing 25 µl of cell extract with 175 µl of Z buffer (60 mM Na_2HPO_4, 40 mM $NaHPO_4$, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol adjusted to pH 7.0) and 40 µl of *o*-nitrophenyl-β-D-galactopyranoside (ONPG, 4 mg/ml in Z buffer). Reaction was incubated at 30°C in the dark until a yellow color developed. To stop the reaction, 100 µl of 1M Na_2CO_3 was added and the OD420 was measured. The β-galactosidase specific activity was calculated as nanomoles of ONPG cleaved (A420/0.0045) per minute per milligram of protein.

III Results

A. Identification of a TCR β Chain Promoter

Isolation of a TCR β Chain Gene

B.1.1 is a poly 18 (EYK(EYA)$_5$) specific and IA^d restricted T cell hybridoma. The analysis of B.1.1 cDNA clones (Kilgannon and Fotedar, manuscript in preparation) indicated that B.1.1 cells utilized Vβ2, Dβ1.1 and Jβ1.3 gene segments in its TCR β chain. An EMBL-3 library was made from genomic DNA from the B.1.1 hybridoma. The B.1.1 TCR β gene was identified by screening the B.1.1 genomic library with Vβ2 and Cβ specific probes. Three clones from ~10^6 recombinants hybridized to both Vβ2 and Cβ specific probes. The restriction maps of the three inserts were determined by standard methods. The results of these analysis showed that all three recombinant clones contained an identical fragment of the B.1.1 TCR β chain gene. The restriction map and genomic organization of cloned B.1.1 TCR gene are shown in Figure 2. In addition to the complete rearranged β chain gene, the genomic clone extended 2.6 kb of 5' of the Vβ2 coding segment to 4.5 kb downstream of Cβ1. For technical convenience, a fragment from SalI to KpnI (3 kb) carrying part of the Vβ2 coding sequences and

the 2.6 kb 5' flanking region was subcloned into pUC18. This 2.6 kb upstream region was subsequently used for analysis of the TCR Vβ2 promoter.

Sequence Analysis of the 5' Flanking Region of Vβ2 TCR Gene

The 5' flanking region of TCR gene from SalI to KpnI was sequenced. This was done by sequencing the various overlapping DNA fragments cloned into m13mp18 and/or m13mp19. The sequencing strategy is described in Figure 3. The sequence shown in Figure 4 includes 2.6 kb upstream of the Vβ2 leader sequence. Some of the conserved sequences identified in other TCR or other eukaryotic genes are also identified. The motifs that merit discussion include the A-T rich region at -31 to -28 (+1 as the transcription start site: see below). This is the probable TATA box of the Vβ2 TCR promoter, although it is different from the canonical TATA motif. A decanucleotide motif is conserved in 13 of 14 Vβ promoters (5'AGTGAT/CG/ATCA3') and was first analysed by Anderson, *et al* (189) and Lee, *et al* (190). This decamer motif was seen twice in the TCR Vβ2 gene; one at the position -63 to -54 (5'AGTCACACCC3', 70% homology) and the other at -257 to -246 (5'ACACATATCA3', 70% homology) An AP-1 binding motif (5'TGAGTCA3') (145) was found at -82 to

-75 (5'TTAGTCAG3') (145). CAAT box (CP-1) was located at -294 to -287 (5'ATTGGCC3') in the reversed orientation. Two different inverted repeat sequences were found at -149 to -139 (5'GAGCATTGCTC3') and at -73 to -63 (5'TTCCTGAGGAA3'). A direct repeat was seen at -170 to -151 (5'TGTGCATGAGTGTGGATGAG3'). Another repeat appeared at -1,332 to -1,282 (5'AAA(TAAC)5TAAA(CAA7)5AAA3'). The functional significance of these sequences will be discussed later.

Mapping the Transcription Start Site of Vβ2 TCR Gene

To help identify the TCR promoter, the transcription start site of beta chain gene had to be located. To do this, a primer extension assay was performed by annealling B.1.1 total RNA to a radiolabelled synthetic oligonucleotides complementary to nucleotide position +67 to +90 of the leader sequences (Figure 4). The oligonucleotide primer was extended by Reverse Transcriptase and the product was run on a sequencing gel (see materials and methods). Figure 5 showed the primer extension product run along with a known sequencing reaction. The transcription start site of Vβ2 TCR gene was located 66 nucleotides upstream of the leader sequences (+1 in Figure 4).

To confirm this and rule out the presence of an intron in the 5' untranslated region, an S1 protection assay was performed. The probe used was a 60-mer synthetic oligonucleotides extending from the Vβ2 leader sequence to -6. The results from the S1 protection analysis (Figure 6) were in agreement with the primer extension assay with only 1 nucleotide difference. The small difference between the two methods is often observed in the analysis of the transcription start site of many eukaryotic genes.

Identification of the Vβ2 Promoter

Transient expression assays were used to identify the TCR promoter. The test plasmid DNA constructs used in this study were pSV2cat derivatives. pSV2cat was a transient expression vector in which the SV40 promoter and enhancer drives chloramphenicol acetyl transferase (CAT) gene expression. The CAT enzyme is not present in eukaryotic cells, thus the possibility of interference due to the endogenous production of the indicator gene was eliminated. In addition, CAT assay is very sensitive allowing the detection of enzyme at the picogram level.

To help delineate the minimal sequence required for promoter activity, a series of 5' nested deletion fragments

of the Vβ2 promoter were cloned into pSV0cat (see materials and methods and Figures 1 and 2). pSV0cat was a derivative of the pSV2cat vector from which the SV40 promoter and enhancer had been cut out. These constructs included the Vβ2 upstream region from SalI to SphI (-2,570 to +31; pSScat), XbaI to SphI (-1,902 to +31; pXScat), AccI to SphI (-1,635 to +31; pAScat), BglII to SphI(-739 to +31; pBScat), EcoRI to SphI (-343 to +31; pEScat), HindIII to SphI (-85 to +31; pHScat), -42 to +31 (pdel1cat) and -18 to +31 (pdel2cat). The promoter activity was tested by transfecting the pTCRcat constructs into EL-4 T cells (see Materials and Methods) and CAT enzyme was assayed as an indicator of promoter activity. Transfection efficiency was monitored by cotransfection with pCH110. pCH110 is a plasmid DNA containing SV40 promoter and enhancer driving β-galactosidase gene expression. Transfection efficiency as monitored by the level of β-galactosidase activity was usually very reproducible. In addition, each transfection experiment had been repeated 4 to 10 times. Thus, the different levels of CAT expression seen in these experiments reflected real differences in transcriptional activity of each construct and was not due to experimental fluctuation in transfection efficiencies.

The results in Figure 7 and Table 1 clearly demonstrated that the HindIII-SphI (HS; -85 to +31) fragment could drive transcription of CAT, in EL-4 cells. Thus suggesting that 85 bp upstream of the transcriptional start site in the Vβ2 gene possessed promoter activity. To help delineate more accurately the active sequences in the HS fragment, two more deletional constructs, del1 (-42 to +31 with a TATA box) and del2 (-18 to +31 no TATA box) were also tested. Both of these constructs were transcriptionally inactive, thus suggesting that sequences between -85 to -42 were the targets of positively regulating transacting factors. The potentially interesting and conserved sequences identified in this region of the HS promoter fragment include an AP-1 sequence 5'TTAGTCAG3' (-82 to -75), an inverted repeat 5'TTCCT G AGGAA3' (-73 to -63) and a sequence 5'AGTCACACCC3' (-63 to -54) homologous to a previously described conserved decanucleotide motif (189,190). In addition, the presence of a conserved TCR β chain nonamer sequence (190) within the functional promoter HS fragment is consistent with a probable functional role in TCR β gene expression.

The EcoRI-SphI fragment (ES; -343 to +31) was 2-16 fold more efficient in driving CAT expression as compared to the HS fragment. Therefore, although the -85 to +31 HS

fragment had promoter activity, sequences from -343 to -86 increased this transcription in T cells. A number of interesting motifs can be identified in this region. These include an inverted repeat 5'GAGCATTGCTC3' (-149 to -139), a direct repeat 5'TGTGCATGAGTGTGGATGAG3' (-170 TO -151), a motif homologous to the conserved decanucleotide sequence 5'ACACATATCA3' (-257 to -248) and a CAAT box sequence 3'GG[illegible] (-288 to -294).

Transfection of pDNA constructs that carried fragments of TCR promoter larger than pEScat, such as pSScat (-2,570 to +31), pXScat (-1902 to +31), pAScat (-1,635 to +31) and pBScat (-739 to +31) did not increase the promoter activity. In fact, the level of CAT expression was always below that of pEScat, suggesting the presence of some negative regulatory element(s) upstream of -343, capable of overcoming the enhancing effect of -343 to -86 fragment. This negative regulatory element was possibly located in the -739 to -343 (BglII to EcoRI) region since it was the common sequence shared by all of the constructs that had weak promoter activity. Alternatively, there could be multiple negative elements scattered over the -2,570 to -343 region.

Presence of Negative Regulatory Elements Upstream of the Vβ2 Promoter

Functional test To prove that negative regulatory elements were present in the upstream fragment of Vβ2 promoter (SalI to EcoRI; -2,570 to -343), various DNA fragments from the -2,570 to -344 region (Figure 8) were cloned upstream of SV40 promoter and enhancer in the pSV2cat vector at the AccI site (pN/SV2cat, see Materials and Methods, Figure 1 and 8). This included fragments from SalI-XbaI (-2,570 to -1902), SalI-AccI (-2,570 to -1,635), SalI-EcoRI (-2,570 to -343), BglII-BglII (-2,500 to -739), AccI-BglII (-1,635 to -343) and BglII-EcoRI (-739 to -343). The pN/SV2cat constructs and pSV2cat were transfected into EL-4 cells and CAT assays were done for 1 hour. Reduction of CAT expression of the test constructs relative to pSV2cat indicated the presence of negative regulatory elements.

Figure 9 and Table 2 clearly showed the presence of negative regulatory elements in TCR upstream promoter fragment. The suppression was orientation independent. In particular, the strong suppressive effect was observed in the constructs that carried BglII-EcoRI (BE; -739 to -343) or SalI-XbaI (SX; -2,570 to - 1902) fragment. This suppression was not due to the change in the size of pDNA

since insertion of an irrelevant DNA did not affect the level of CAT expression (Messier and Fotedar, unpublished result).

Sequence comparisons The sequences of SalI-XbaI and BglII-EcoRI fragments were compared to other eukaryotic negative regulatory elements. Figure 10 showed multiple homology regions between the TCR negative upstream elements and various silencers. The functional significance of these elements in TCR gene expression awaits further study.

Discussion

In this study, a mouse Vβ2 promoter of TCR gene isolated from B.1.1 genomic library has been identified. By using a transient expression system and 5' deletional analysis, it was shown that multiple regulatory elements were present within a 2.6 kb 5' flanking region of the Vβ2 structural gene segment. The minimum sequence conferring the Vβ2 promoter activity was mapped to the -85 to +31 fragment. Sequences between -343 and -85 significantly enhanced the transcriptional activity of the -85 to +31 Vβ2 promoter. In addition, a region upstream of -343 contained a negative regulatory element.

The finding that the -85 fragment had promoter activity whereas the -42 fragment was silent was comparable to the study of human Vβ8.1 (191) and mouse Vβ8.3 promoters (192). In the human Vβ8.1, the -45 fragment was shown to be essential but not as efficient as the larger -343 fragment for driving gene transcription. Similarly, in the mouse Vβ8.3, sequences between -84 and -33 were demonstrated to be critical for the promoter activity as the -33 fragment failed to initiate gene transcription while the -84 fragment had promoter activity. The significance of the sequence between -85 and -42 in the Vβ2 promoter was also supported by the finding that this region bound nuclear proteins (Fuller and Fotedar; unpublished data). In addition, the AP-1 binding sequence, an inverted repeat sequence and the TCR β promoter conserved decanucleotide motif are also located in this region.

A conserved decanucleotide motif, 5'AGGTGAT/CG/ATCA3', was identified in 13 out of 14 Vβ promoters by Anderson *et al* (189). In this Vβ2 promoter, it was identified at 2 locations; -63 to -54 (5'AGTcACAcCt3') and -257 to -246 (5'AcacATATCA3'). In both locations, this motif was protected in DNaseI footprinting experiments (Fuller and

Fotedar, unpublished data), suggesting its possible functional role in TCR gene transcription. The functional significance of this sequence has only so far demonstrated by Anderson *et al* (192). Deletion or mutation of the decamer sequence from the complete Vβ8.3 promoter abolished promoter activity in their study. Diamond *et al* (191), on the other hand, did not find a significant difference in human Vβ8.1 promoter activity when the decamer sequence was deleted in a 5' deletional analysis. Our analysis showed that the effect was weaker than other motifs present in this region. The role of the decamer motif in the Vβ2 promoter is discussed further in Section C.

The AP-1 motif extends from -82 to -75 (5'TTAGTCAG3') in the TCR Vβ2 promoter. The role of the AP-1 motif in regulating transcription can be argued for on a number of grounds. Firstly, the deletional experiment described here identified the -85 to -43 region as being critical for Vβ2 chain transcription. Secondly, AP-1 like sequences (75-87% homologous to the consensus AP-1 sequence) were protected in footprinting experiments done with the human Vβ8.1 promoter regions (192). In addition, a T cell specific hypersensitive site was present just downstream of this protected region. Similar footprinting experiment done in the Vβ2 promoter also indicated the protection of the AP-1

binding site (Fuller and Fotedar; unpublished data). Thirdly, AP-1 like sequences were found in all expressed members of the human Vβ8 family (215) and in 11 of 14 partial Vβ sequences described by Anderson (189). The direct role of the AP-1 binding sequence in TCR Vβ2 transcription has been directly analysed and described later in this thesis.

In addition to the presence of decamer motif and AP-1 sequence in the -85 to -43 region, an inverted repeat sequence is located at -73 to -63. Other repeats are also found in the 5' flanking region of Vβ2 gene. Inverted repeats are of interest because of their potential ability to form cruciform structures which allow for a more dynamic structure and a potential role in regulation. Interestingly, the inverted repeat found at -73 to -63 region of the Vβ2 promoter (Fuller and Fotedar; unpublished data) and human Vβ8.1 putative promoter region (216) are all protected in DNA binding experiments. Inverted repeats can also be seen in 12 out of 14 partial 5' upstream Vβ sequences (189). Nevertheless, their role in TCR gene transcription remains obscure.

A CAAT box sequence at -288 to -294 (5'ATTGGCC3') is in the reversed orientation and is identical to the CAAT

box in the mouse β globin gene where the functional relevance in transcriptional regulation is better understood from mutagenesis experiments (217,219). CAAT box sequences are conserved in all expressed members of the human Vβ8 family (215) and are protected in DNA binding studies only on the noncoding strand (216). CAAT box like sequences upstream of the cap site can be identified in 8 out of 14 Vβ partial sequences reported by Anderson (189).

The strong promoter activity of the pEScat (-343 to +30) construct as compared to the smaller promoter construct, pHScat (-85 to +30) (Figure 7 and Table 1) may partly be explained by the presence of the additional positive regulatory elements such as CAAT box and the core sequence of polyoma enhancer. Moreover, other positive *cis*-acting elements not yet identified may be present in the -285 to -86 region. By using gel retardation assay and competition with excess cold specific competitor as well as DNaseI footprinting analysis, Fuller and Fotedar (unpublished data) have found that the -285 HindIII to HindIII -86 fragment binds nuclear proteins distinct from those binding to the HS (-85 to +30) fragment, suggesting that the strong promoter activity of the ES fragment may be due to its ability to bind additional positive acting transcriptional factors. Whether the nature of this

positive regulatory element is similar or different from the distal enhancer element located at -800 and -570 of human Vβ8.1 promoter (191) awaits further study.

Sequences upstream of -343 of the Vβ2 promoter contain negative regulatory elements. This was shown by: 1) transfection of pSScat, pXScat, pAScat and pBScat resulted in decreased CAT activity as compared to pEScat 2) sequences upstream of -343 suppressed the expression of heterologous promoters. Insertion of the putative negative regulatory fragment upstream of SV40 promoter and enhancer in pSV2cat markedly decreased CAT expression in an orientation-independent fashion. Whether this negative regulatory element can function at a distance is under investigation.

Similar suppressive effects of this negative element have also been shown by Messier and Fotedar (Unpublished results) using the herpes simplex TK promoter. The transcriptional suppression on TK promoter was more pronounced than on the SV40 promoter/enhancer. Similar observations have been made in the study of chicken lysozyme silencers (220). This suggested that the negative regulatory elements may play an important role in the

control of both tissue specific and inducible gene expression.

The finding of multiple negative regulatory elements clustering in a short DNA region (BglII-EcoRI (-739 to -343) and SalI-XbaI (-2,570 to -1902)) in TCR gene was not unexpected as it has also been observed in other eukaryotic gene silencers (220-234). A DNA fragment that can partially downregulate transcription was also found in human Vβ8.1 upstream promoter region (191) whereas this was not observed in mouse Vβ8.3 promoter studies(192).

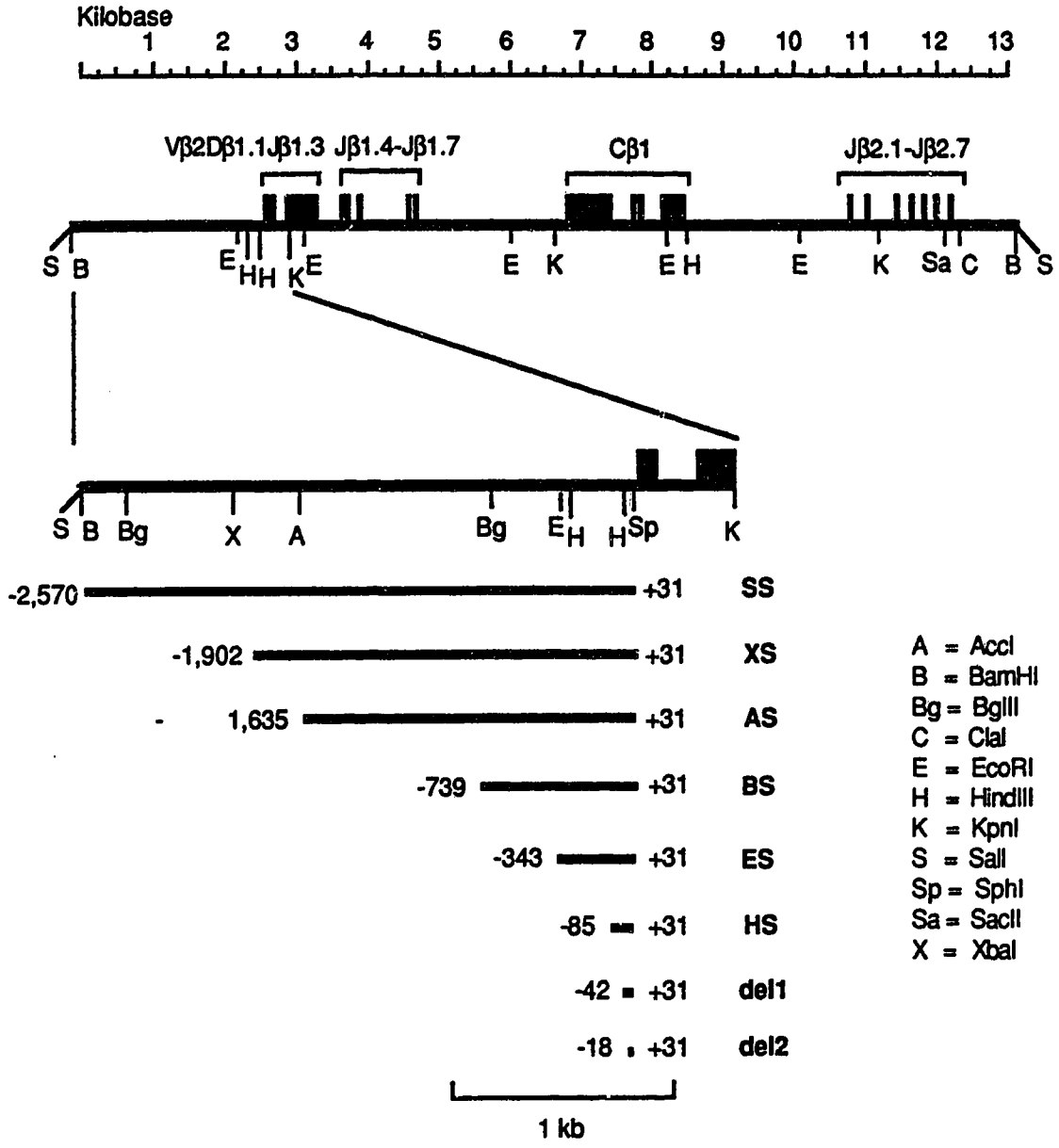


Figure 2 Restriction map of the B.1.1 functionally rearranged TCR β chain gene. The 5' nested deletions of the T cell receptor β chain promoter are shown. These include: SS=SalI-SphI (-2,570 to +31), XS=XbaI-SphI (-1,902 to +31), AS=AccI-SphI (-1,635 to +31), BS=BglII-SphI (-739 to +31), ES=EcoRI-SphI (-343 to +31), and HS=HindIII-SphI (-85 to +31) fragments. Two more deleted constructs generated by ExoIII deletion from the HindIII site in HS are del1 and del2.

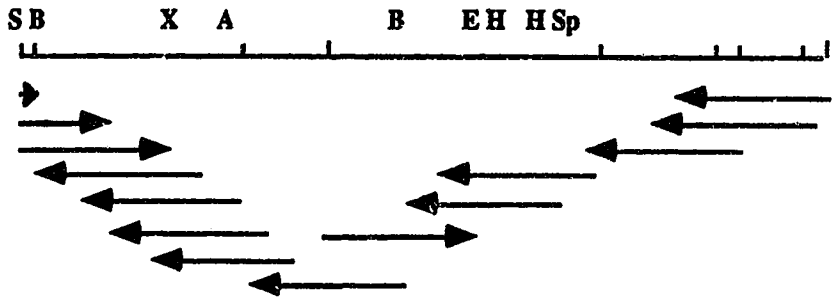
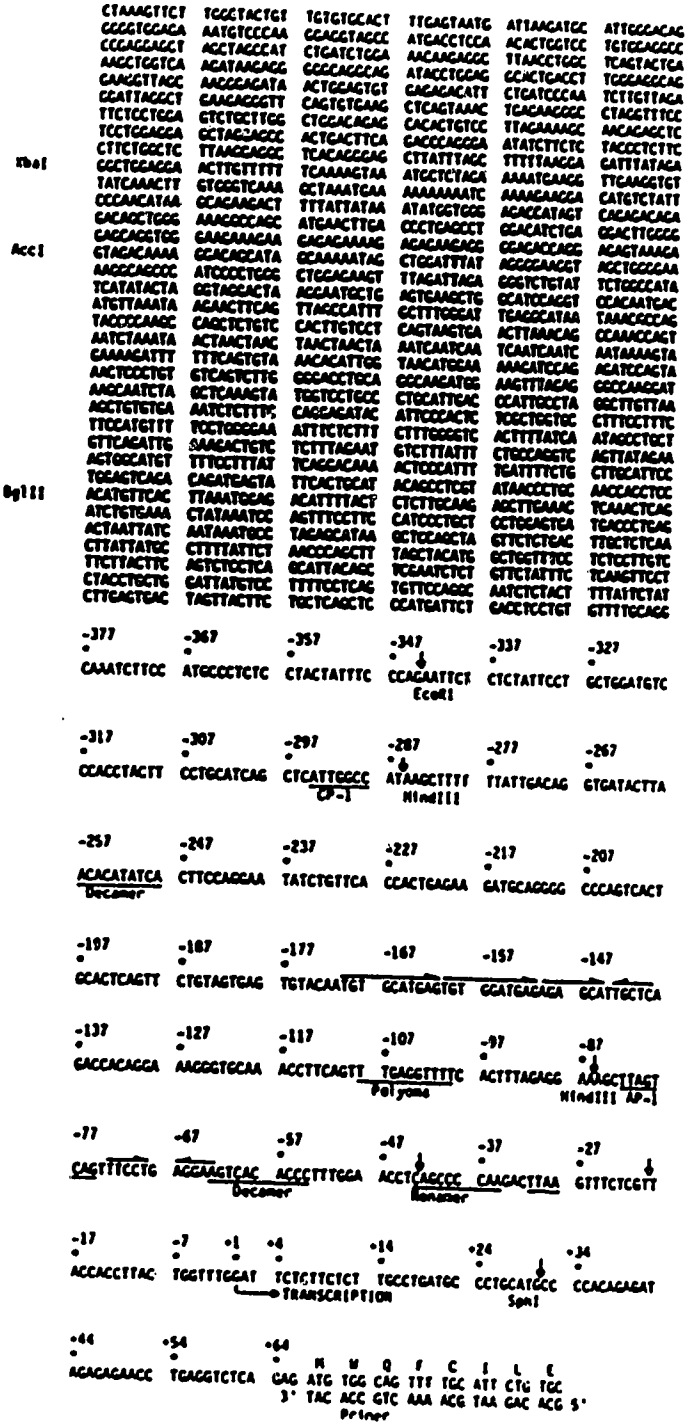


Figure 3 Sequencing strategy of the 5' flanking region of β chain gene. Arrows indicate the direction in which the fragments were sequenced.

Figure 4 The nucleotide sequence of the 5' region upstream of the Vβ2 coding sequence. The relevant conserved sequences in the functional Vβ2 promoter are underlined and the motifs identified. The conserved consensus sequences for CP-1 (218), a TCR β chain decamer sequence (189, 190), polyoma enhancer (184), AP-1 (145) and a TCR β chain nonamer sequence (190) have been described elsewhere. Direct and inverted repeats are identified by horizontal arrows. Vertical arrows identify the deletion constructs used to map the relevant sequences for the β promoter deletion analysis. The horizontal arrow identifies the start site of transcription. The antisense oligonucleotide used for primer extension experiments is identified as primer.


XbaI
AccI
BglII
-377
-367
-357
-347
-337
-327
CAAATCTTCC ATGCCCTCTC CTACTATTTC CCAGAATTCT CTCTATTCCT GCTGGATGTC
EcoRI
-317
-307
-297
-287
-277
-267
CCACCTACTT CCTGCATCAG CTCATTGCCC ATAAGCTTTT TTATTGACAG GTGATACTTA
CP-1
HindIII
-257
-247
-237
-227
-217
-207
ACACATATCA CTTCCAGCAA TATCTGTTCA CCACTGAGAA GATGCAGGGG CCCAGTCACT
Decamer
-197
-187
-177
-167
-157
-147
GCACTCAGTT CTGTAGTGAG TGTACAATGT GCATGAGTGT GCATGAGAGA GCATTGCTCA
-137
-127
-117
-107
-97
-87
GACCACAGGA AAGGGTGCAA ACCTTCAGTT TGAGGTTTTC ACTTTAGAGG AAAGCTTAGT
Polyoma
HindIII AP-1
-77
-67
-57
-47
-37
-27
CAGTTTCCTG AGGAAGTCAC ACCCTTTGGA ACCTCAGCCC CAAGACTTAA GTTTCTCGTT
Decamer
Hexamer
-17
-7
+1
+6
+14
+24
+34
ACCACCTTAC TGGTTTGGAT TCTGTTCTCT TGCCTGATGC CCTGCATCCC CCACAGAGAT
TRANSCRIPTION
SpeI
+44
+54
+64
AGAGAGAACC TGAGGTCTCA GAG ATG TGG CAG TTT TGC ATT CTG TGC
3' TAC ACC GTC AAA ACG TAA GAC ACG 5'
M M Q F C I L C
Primer

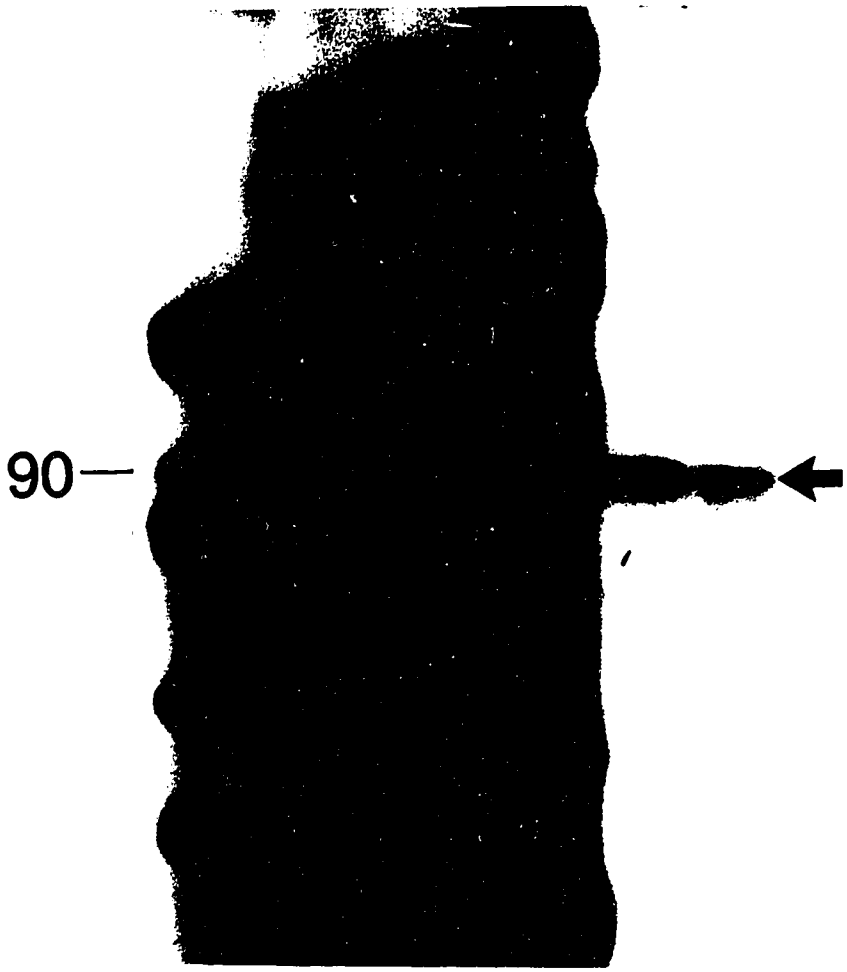


Figure 5 Mapping the transcription start site of the Vβ2 gene in B.1.1 by primer extension assay using an oligonucleotide extending from +90 to +67. The primer extension product is identified by a horizontal arrow.

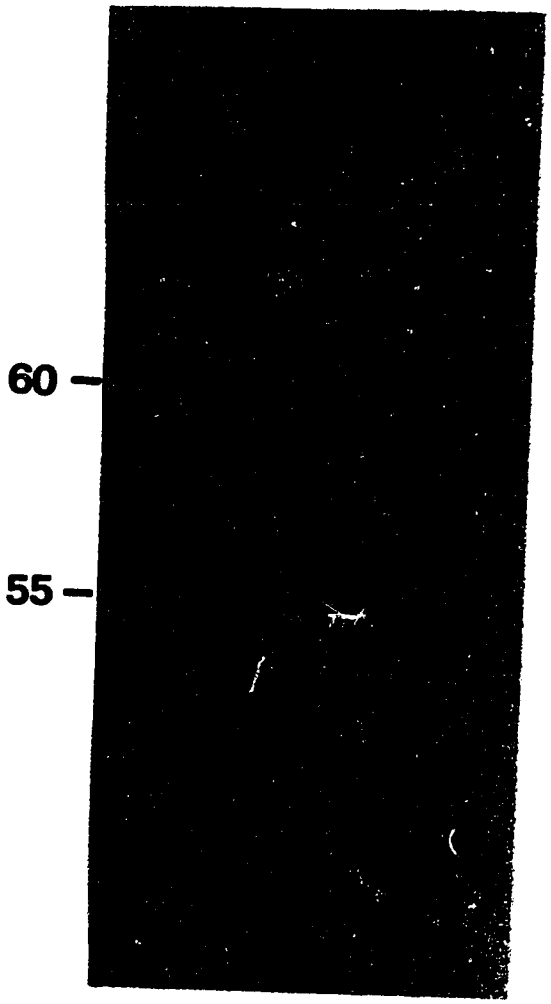


Figure 6 Mapping the transcription start site of the Vβ2 gene in B.1.1 by S1 nuclease protection assay. The probe was a 60-mer oligonucleotides extending from +90 to -6. The protected band was at 55 nucleotides in length.

Promoter	–	SV2	SV2	SS	BS	XS	AS	ES	HS
Enhancer	–	SV2	SV2	–	–	–	–	–	–

Figure 7 CAT expression of pTCRcat in EL-4 cells. The promoter fragments in these constructs are identified by their symbols in Figure 2. Two control plasmids, one in which the SV40 promoter/enhancer drives CAT expression (pSV2cat) and the other being a promoterless and enhancerless construct (pSV0cat), were also used in these transfection experiments.

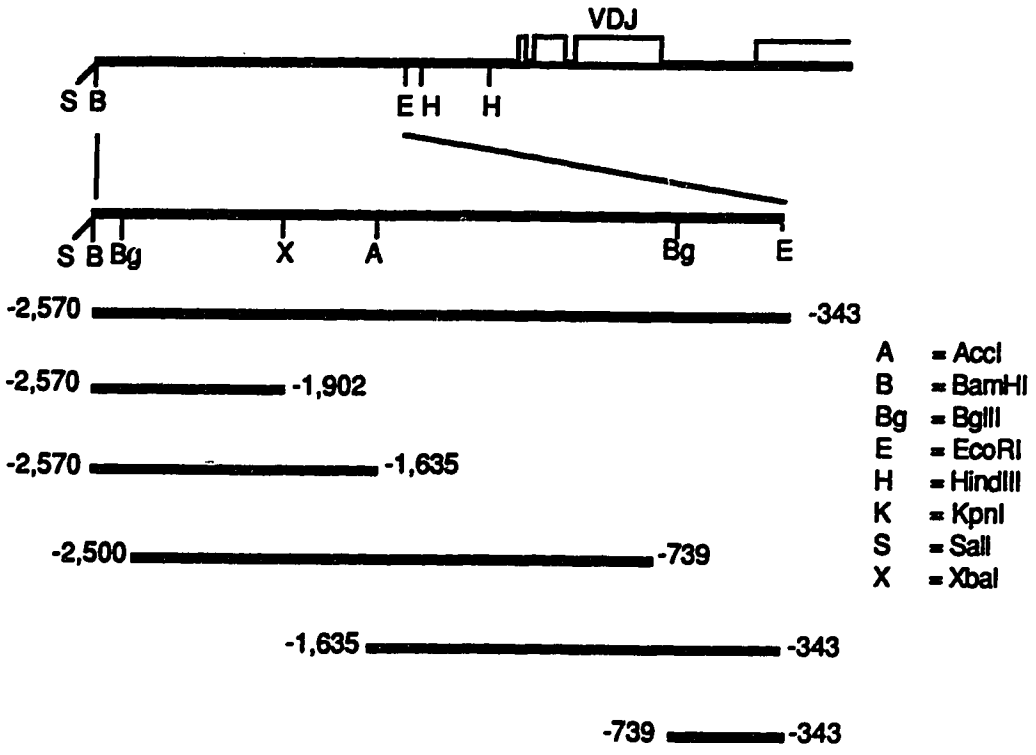


Figure 8 Restriction map of B.1.1 TCR β chain negative control region. Fragments tested for their inhibitory effect include SE=SalI-EcoRI (-2,570 to -343), SA=SalI-AccI (-2,570 to -1,635), BB=BglII-BglII (-2,500 to -739), AE=AccI-EcoRI (-1,635 to -343) and BE=BglII-EcoRI (-739 to -343).

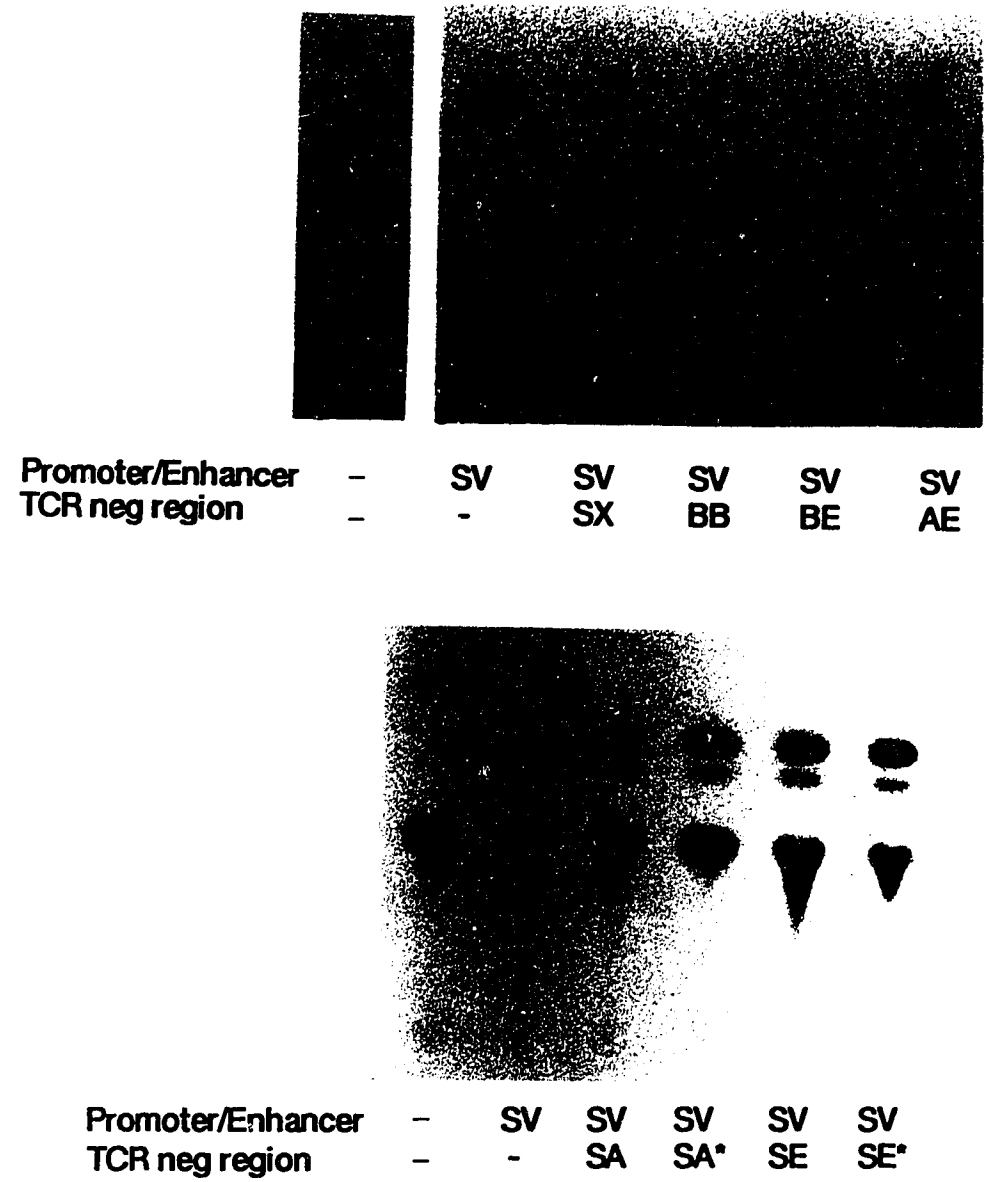


Figure 9 CAT expression of pN/SV2cat in EL-4 cells. The fragments of negative region were identified by their symbols in Figure 8. SA* (-1,635 to -2,570) and SE* (-343 to -2,570) are in the antisense orientation with respect to the promoter and enhancer.

Figure 10 Sequence comparisons between the Vβ2 negative upstream elements and various eukaryotic silencers. These are chicken lysozyme N-2.4kb, N-0.25kb and N-1.0kb (220), rat insulin 1 repetitive element (INS1;227), and unique element (INS2; 230), human β-interferon (β-IFN; 223), mouse immunoglobulin heavy chain (mIgH; 226, 234), rat growth hormone (rGH; 228), polyoma, SV40 and adenovirus 2 (Py, SV40, Ade2E1a; 221), MoMSV (224), HIV (231) and mouse H-2L^{d} (197).

HOMOLOGY BOX 1		HOMOLOGY BOX 2	
CACCCTCTCTG	-1.09N-1.0	GATTCTCCTCC	-0.97kb N-1.0
CAACCTCTCTG	-1.11N-1.0	GATTTTACTGC	-229kb N-0.25
TACCCTCTCTG	-271 INS2	GATCCTCTTCC	-2.47kb N-2.4
GCCCCTCTCTG	-112 INS2	GACACTCCTCC	86 INS1
GGTCCTCTCTC	-46 β-IFN	CCATCTCCTCA	-147bp $H2L^d$
AACCCTCTCAC	140 mIgH	CGGTCTCCTCT	560 MoMSV
AGACCTCTCTA	3 mIgH	GAATCTCCTCC	-142bp rGH
GAGCCTCTCTA	-366 bp rGH	CATTCTCGTCC	37 SV40
AAGCCTCTCTA	5225 PY	TTTTCTCCTCC	+40 Ade2E1a
ANCCTCTCC	**CONSENSUS**	ANTCTCCTCC	**CONSENSUS**

Vβ2 TCR		Vβ2 TCR	
GCCCTCTCC	-365 to -357	CCTCTCCTAC	-363 to -354
TTCCTCTCC	-571 to -563	AGTCTCCTCA	-547 to -538
AGCCTCTAT	-1942 to -1934	AGTTTCCTTC	-717 to -708
ATCTTCTCT	-2015 to -2007	AAGTTCCTCC	-1924 to -1933
ACCCTCTTC	-2006 to -1998	AATCTCCTTT	-1944 to -1953
ACCCTCTTG	-2127 to -2119	TAGCTCCTCC	-2006 to -1997
GTCCTCTCT	-2486 to -2494	ACCCTCTTCC	-2044 to -2053
		ATTCTGATCC	-2160 to -2151
		TTTCTCCACC	-2435 to -2426

HOMOLOGY BOX 3

ATGCAGTAAAA	-221bp N-0.25
ATGCAGGATCT	-36bp HIV
ATGCAGTTTTA	804 INS1
ATATAGTAAAA	1073 INS1
AGCAAGTAAAA	1181 INS1
ACATAGTAAAA	1622 INS1
TAAGAGTAAAA	85 mIgH

Vβ2 TCR

ATAGAATAAAA	-438 to -448
AGAGAGTAAAA	-764 to -774

TABLE 1

PROMOTER ACTIVITY OF 5' DELETION CONSTRUCTS OF THE TCR β CHAIN PROMOTER IN EL-4 CELLS

CONSTRUCT[1]			% CONVERSION[2]			
Name	Promoter	Enhancer	Exp.#1	Exp.#2	Exp.#3	Exp.#4
SV0cat	-	-	0.4	0.1	0.1	0.1
SV2cat	SV	SV	78	84	81	ND[3]
SScat	SS	-	3.5	0.49	ND	ND
XScat	XS	-	4.9	ND	1.5	ND
AScat	AS	-	2.2	1.0	2.8	ND
BScat	BS	-	2.1	ND	3.7	ND
EScat	ES	-	13.1	8.6	14.3	ND
HScat	HS	-	1.6	2.9	3.7	4.3
del1cat	del1	-	0.4	ND	ND	0.1
del2cat	del2	-	0.2	ND	ND	ND

1. pSV0cat was derived from pSV2cat. All the other vectors have TCR β chain promoter fragments driving CAT expression. The symbols are explained in Figure 2.

2. Transfection efficiencies were monitored by cotransfecting the test plasmids with pCH110, the β-galactosidase expression vector. The % CAT conversion values were normalized to the level of β-galactosidase level. These experiments have been repeated in different transfection experiments at different times (n=10) and gave similar results.

3. Not done.

TABLE 2

EFFECT OF THE TCR NEGATIVE CONTROL REGION ON pSV2cat

CONSTRUCT[1]		RELATIVE CAT ACTIVITY[2]			
Name	Negative control fragment	#1	#2	#3	#4
pSV2cat	-	1.00	1.00	1.00	1.00
pSX/SV2cat	SX	ND[3]	0.36	0.27	0.43
pSA/SV2cat	SA	0.69	ND[3]	0.46	ND
pSA*/SV2cat	SA*	0.52	ND	ND	ND
pSE/SV2cat	SE	0.58	0.78	0.57	ND
pSE*/SV2cat	SE*	0.71	ND	ND	ND
pBB/SV2cat	BB	ND	0.52	0.62	ND
pAE/SV2cat	AE	ND	0.52	ND	ND
pBE/SV2cat	BE	ND	0.29	ND	0.37

1. The constructs were derived from pSV2cat by inserting the negative control fragment upstream of SV40 promoter. The symbols were explained in Figure 8.

2. Transfection efficiencies were monitored by cotransfecting the test plasmids with pCH110, the β-galactosidase expression vector. The % CAT conversion values were normalized to the level of β-galactosidase level. The CAT activity of pSV2cat was set to 1, and the others were normalized to pSV2cat. These experiments have been repeated in different transfection experiments at different times (n=10) and gave similar results.

3. Not done

* The negative control fragment was in the reversed orientation with respect to the promoter.

B. Interaction of TCR Promoter and Enhancer and Their Role in Tissue Specific Expression

Interaction of TCR Promoter and Enhancer

The ability of sequences downstream of the Cβ2 gene to enhance transcription from the Vβ2 promoter was analysed. The TCR beta chain enhancer was located 5 kb downstream of the Cβ2 structural gene segment (184,185). The enhancer fragment used in this study was a kind gift from Drs. R Barth and L. Hood. The restriction map of the 5 kb BamHI-KpnI enhancer fragment is shown in Figure 11. Enhancer fragment #I is a 0.5 kb HpaI-NcoI fragment; #III is a 2.5 kb HindIII-NcoI fragment; #V is 1.5 kb BglII-BglII fragment and #VII is the 5 kb BamHI-KpnI fragment. These fragments of β chain enhancer were inserted into pTCRcat vector at the BamHI site, downstream of the CAT structural gene (pTCRenhcat, see Materials and Methods). The orientation of the enhancer fragment with respect to the Vβ2 promoter and the construct nomenclature are summarized in Figure 12. The 4.5 kb BamH1-KpnI fragment and its subfragments enhanced transcription from the Vβ2 promoter in T cells. The smallest fragment which enhanced transcription from the Vβ2 promoter was the 500 bp HpaI-NcoI fragment #I (Figure 13 and Table 3). This enhancement was seen in either

orientation whether it was present either downstream (BamHI site) or upstream (NdeI) of the cap site. We also investigated the ability of the enhancer sequences to enhance transcription from AS, ES and HS fragments of the Vβ2 promoter. The results suggested that irrespective of the length of the 5' promoter sequence used, enhancement could be demonstrated. The ability of enhancer sequences to dramatically increase transcription from the repressive sequence containing AS promoter fragment suggests that their repressive influence on the Vβ promoter can be overridden when the enhancer is present in *cis*. This enhancement could be seen with any fragment which contained the fragment #1 sequence (HpaI-NcoI 500 bp fragment). The overriding of this repression in the presence of the enhancer suggests a probable functional role during T cell development.

Mouse TCR β Chain Promoter *vs* Tissue Specific Expression

To examine whether the mouse TCR β chain promoter plays a role in tissue specificity, pDNA constructs that carried TCR promoter (pTCRcat, see Materials and Methods and Figure 2) were transfected into B cells (MOPC-21) and non lymphoid cells (NIH3T3, Balb/c 3T3 and PA317). The

transfection of MOPC21 was done by the DEAE-dextran method followed by an additional chloroquine treatment. All of the fibroblast lines were transfected by calcium phosphate procedure. CAT assays for these transfections were done for 5 hours and the results were shown in Figures 14, 15, Tables 4 and 5. It was clearly shown that mouse TCR Vβ2 promoter was not active in B cells since none of the pTCRcat constructs could be expressed in MOPC-21 cells. On the other hand, mouse TCR β chain gene promoter was active in all fibroblast lines used in this study. Once again, the transfection data in fibroblasts confirmed that the -85 fragment had promoter activity whereas fragments smaller than that (-42 or -18 bp fragments) were not sufficient to promote gene transcription. These results suggested that sequence between -85 and -42 of Vβ2 promoter played a role in the control of tissue specific expression in lymphoid cells. The expression of TCR promoter in non-lymphoid cell lines suggested that a different mechanism regulated TCR gene expression.

TCR Enhancer *vs* Tissue Specific Expression of TCR Genes

The role of TCR enhancer in the control of tissue specificity was also analysed. A series of pTCRenhcat

constructs (Figure 12) was transfected into NIH3T3, Balb/c3T3 and PA317. The CAT activity was assayed for 5 hours and results are shown in Figure 15 and Table 6. Surprisingly, CAT expression was also seen in all fibroblast lines studied. That is, the TCR β enhancer can cooperate with the TCR Vβ2 promoter to increase the level of gene expression in fibroblasts, suggesting that both TCR promoter and enhancer may not have a direct function in the control of tissue specific expression in non-lymphoid cells (also see Discussion).

Discussion

In this part of the study, the interaction of TCR promoter and enhancer and their role in tissue specific expression were analysed. The TCR enhancer located downstream of the Cβ2 gene can enhance gene transcription of TCR Vβ2 promoter in EL-4 T cells (Figure 13) as shown previously by Krimpenfort *et al* (184). A 500 bp HpaI-NcoI fragment #I is sufficient to confer the enhancer activity. The finding that TCR β enhancer can override the suppressive effect of the negative regulatory element in the Vβ2 promoter implicates its role in controlling the quiescent state of the promoter in unrearranged TCR genes. Thus, transcription of TCR genes can be activated only when

the Cβ2 downstream enhancer is brought into close proximity by TCR gene rearrangement.

The role of TCR promoter in tissue specific expression was also examined. The TCR Vβ2 promoter is active in T cells (EL-4) but not in B cells (MOPC-21), suggesting either the absence of positive regulatory *trans*-acting factor in B cells or the presence of a B cell specific negative *trans*-acting factor. The latter idea is now supported by the finding that multiple *trans*-acting factors can bind to the same DNA sequence. Thus the interaction between *cis* and *trans*- acting factor in one cell may result in gene activation whereas in the other cells, it may shut off gene transcription. In contrast to B cells, the Vβ2 TCR promoter is active in all fibroblast lines studied (NIH3T3, Balb/c3T3 and PA317). This is different from the human Vβ8.1 promoter (191) located 45 bp upstream of transcription start site, that can only express in T cells, not B cells or fibroblasts (Hela and COS cells). This discrepancy may be due to the difference in cell lines used. The finding that TCR Vβ2 promoter was active in fibroblasts suggested that different mechanisms are used by B cells and fibroblasts to prevent TCR gene expression. It also leads us to suggest that either the chromatin structure of the TCR genes in fibroblasts or the presence

of fibroblast repressor sequences help ensure the transcriptinal silence of TCR genes in fibroblasts.

The result in Figure 15 showing that the TCR β enhancer can markedly enhance gene transcription of Vβ2 promoter in fibroblasts are not predictable from the data from transgenic mice (184). When rearranged TCR β chain genes are used to make transgenic mice, primarily T cell specific transcription was seen, although a low level of aberrant expression in non T cells was also detected. The discrepancy of the results due to the difference in systems used in both studies is not so surprising as similar observation has been seen before in the study of other eukaryotic genes such as HLA-DRα (235). Again, it suggests that chromatin structure may play an important role in the control of TCR gene expression.

Among the studies in which transient expression assays were used to analyse the TCR β enhancer, McDougall *et al* (185) and Krimpenfort *et al* (184) have shown that the β enhancer is not functional in fibroblasts. This discrepancy is probably due to their use of SV40 and pim-1 promoter with TCR β enhancer to demonstrate tissue specificity. In fact, this was shown to be the case. Messier and Fotedar (unpublished data) have recently demonstrated that the β

enhancer can enhance transcription from the Vβ2 promoter but not the TK promoter in NIH3T3 cells. However, the β enhancer can still enhance the transcription of TK promoter in EL-4 cells. This implies that the interaction seen between an enhancer and a heterologous promoter may not be extrapolatable to the interaction of a promoter with its cognate enhancer.

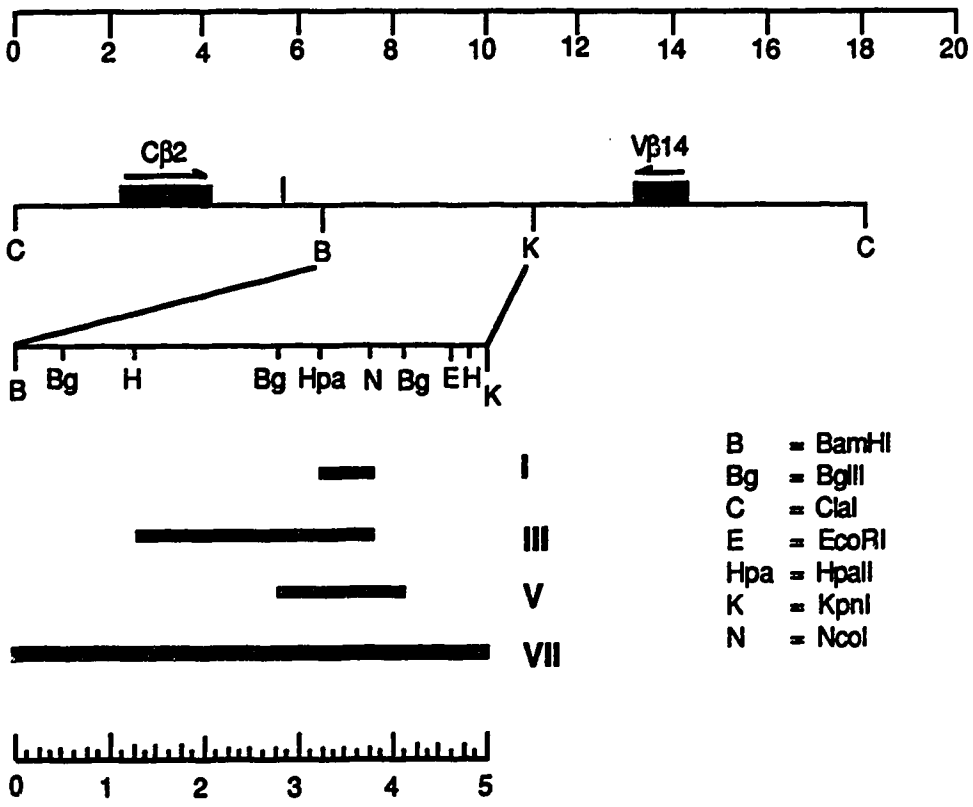


Figure 11 Restriction map of the TCR β enhancer region. The fragments tested for the enhancer activity are identified. Fragments I (HpaI-NcoI, III (HindIII-NcoI), V (BglII-BglII), and VII (BamHI-KpnI) were used for this analysis.

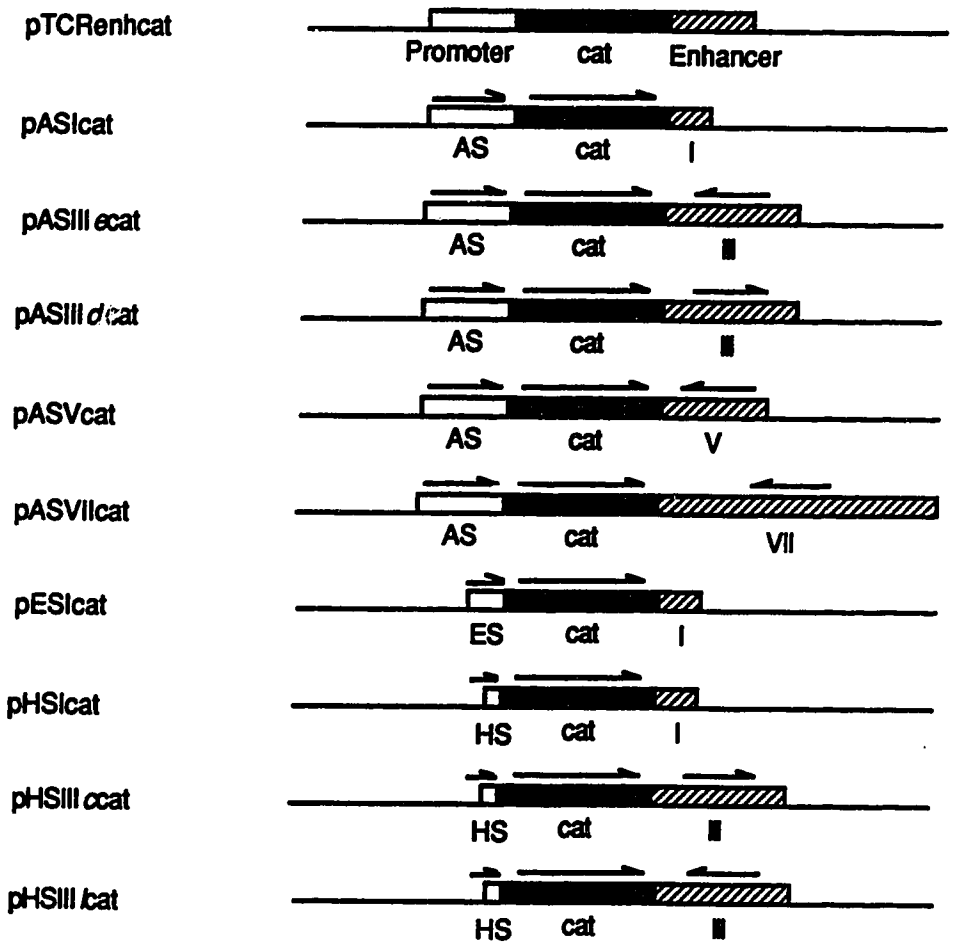
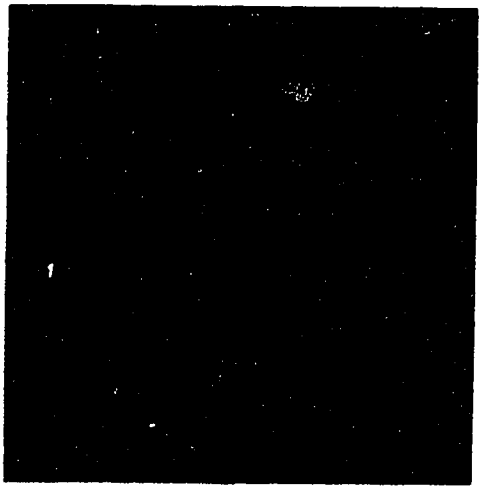


Figure 12 The map of pTCRenhcat constructs. The AS, ES and HS fragments of TCR promoter and fragment #I, #III, #V, #VII are described in Figures 2 and 11 respectively. The arrows indicate the orientations of the promoter and enhancer relative to chloramphenicol acetyl trarsferase gene.

Promoter	-	SV2	SV2	SS	BS	XS	AS	AS	ES	ES	HS	HS
Enhancer	-	SV2	SV2	-	-	-	-	I	-	I	-	I



Promoter	HS	HS	HS	HS	dell
Enhancer	-	I	III*d*	III*e*	-

Figure 13 CAT expression of TCRenhcat in EL-4 cells. The constructs are described in Figures 2 and 11.

Promoter – SV2 BS XS AS ES HS

Figure 14 CAT expression of pTCRcat in MOPC-21 cells. The constructs are described in Figure 2.

Promoter	–	AS	AS	AS	ES	ES
Enhancer	–	–	I	VII	–	I

Figure 15 CAT expression of pTCRenhcat in NIH3T3. The constructs are described in Figures 2 and 11.

TABLE 3

INTERACTION OF TCR β CHAIN PROMOTER AND ENHANCER IN EL-4 T CELLS

CONSTRUCTS[1]			% CONVERSION[2]	
Name	Promoter	Enhancer	Exp.#1	EXp.#2
SV0cat	-	-	0.4	0.3
SV2cat	SV40	SV40	76.5	66.7
AScat	AS	-	2.08	2.40
ASIcat	AS	I	26.70	32.0
ASIII*d*cat	AS	III	ND[3]	14.7
ASIII*e*cat	AS	III	ND	28.63
ASVcat	AS	V	12.59	ND
EScat	ES	-	13.1	8.97
ESIcat	ES	I	25.2	22.43
HScat	HS	-	1.58	0.64
HSIcat	HS	I	34.19	23.68
HSIII*c*cat	HS	III	16.22	12.02
HSIII*l*cat	HS	III	23.5	ND

1. The orientation of the enhancer fragment in the constructs were explained in Figure 12. The symbols of TCR promoter and enhancer followed Figure 2 and Figure 11.

2. Transfection efficiencies were monitored by cotransfecting the test plasmids with pCH110, the β-galactosidase expression vector. The % CAT conversion values were normalized to the level of β-galactosidase level. These experiments have been repeated in different transfection experiments at different time (n=10) and gave similar results. CAT assays were done for 18 hours. Under such assay condition, the substrate was not limiting if the % CAT conversion was less than 60%.

3. Not done.

TABLE 4

PROMOTER ACTIVITY OF THE 5' DELETION CONSTRUCTS OF TCR β CHAIN PROMOTER IN MOPC-21 B CELLS

CONSTRUCT[1]			% CONVERSION[2]	
Name	Promoter	Enhancer	Exp. #1	Exp #2
SV0cat	-	-	0.1	0.1
SV2cat	SV40	-	40.54	35
SScat	SS	-	0.1	0.1
XScat	XS	-	0.1	0.1
AScat	AS	-	0.1	0.1
BScat	BS	-	0.1	0.1
EScat	ES	-	0.1	0.1
HScat	HS	-	0.1	0.1

1. pSV0cat was derived from pSV2cat. All the other vectors have TCR β chain promoter fragments driving CAT expression. The symbols are explained in Figure 2.

2. Transfection efficiencies were monitored by cotransfecting the test plasmids with pCH110, the β-galactosidase expression vector. The % CAT conversion values were normalized to the level of β-galactosidase level. These experiments have been repeated in different transfection experiments at different time (n=10) and gave similar results.

TABLE 5

PROMOTER ACTIVITY OF THE TCR β CHAIN PROMOTER IN FIBROBLASTS

Name	CONSTRUCT[1] Promoter	Enhancer	%CONVERSION[2]
Balb3T3			
SV0cat	-	-	0.1
SV2cat	SV40	SV40	92.0
SScat	SS	-	8.8
XScat	XS	-	7.5
EScat	ES	-	11.7
del1cat	del1	-	ND[3]
NIH3T3			
SV0cat	-	-	0.2
SV2cat	SV40	SV40	82.0
SScat	SS	-	3.9
HScat	HS	-	2.3
del1cat	del1	-	0.3
PA317			
SV0cat	SV40	-	0.2
SV2cat	SV40	SV40	71.0
SScat	SS	-	16.0
EScat	ES	-	14.0
HScat	HS	-	14.0
del1cat	del1	-	ND

1. pSV0cat was derived from pSV2cat. All the other vectors have TCR β chain promoter fragments driving CAT expression. The symbols are explained in Figure 2.

2. Transfection efficiencies were monitored by cotransfecting the test plasmids with pCH110, the β-galactosidase expression vector. The %CAT conversion values were normalized to the level of β-galactosidase level. These experiments have been repeated in different transfection experiments at different time (n=10) and gave similar results.

3. not done

TABLE 6

ENHANCER ACTIVITY OF THE TCR β CHAIN ENHANCER IN FIBROBLASTS

	CONSTRUCT[1]		%CAT CONVERSION[2]
Name	Promoter	Enhancer	
BALB/c 3T3			
AScat	AS	-	1.5
ASIcat	AS	I	31.0
ASVIIcat	AS	VII	22.0
SV0cat	SV	-	0.7
SV2cat	SV	SV	91.0*
NIH3T3			
AScat	AS	-	0.2
ASIcat	AS	I	38
ASVIIcat	AS	VII	20
SV0cat	SV	-	0.1
SV2cat	SV	SV	82.0*
PA 317			
AScat	AS	-	2.3
ASIIIcat	AS	III	4.7
ASVIIcat	AS	VII	12.0
SV0cat	SV	-	0.2
SV2cat	SV	SV	71.0*

1. The orientation of the enhancer fragment in the constructs were explained in Figure 12. The symbols of TCR promoter and enhancer followed Figures 2 and 11.

2. Transfection efficiencies were monitored by cotransfecting the test plasmids with pCH110, the β-galactosidase expression vector. The %CAT conversion values were normalized to the level of β-galactosidase level. These experiments have been repeated in different transfection experiments at different time (n=10) and gave similar results.

* The % CAT conversion was underestimated as it was not in the linear range.

C. Mapping of TPA Responsive Element in Vβ2 Promoter.

Effect of TPA on the Vβ2 Promoter.

In this section we inquire whether the TCR Vβ2 promoter is TPA inducible. We decided to address this issue because TCR β transcript levels are elevated when T cells are treated with phorbol ester (236-239) and from our work described in Section A, a conserved AP-1 motif (145) is found in the minimal Vβ2 promoter region. The AP-1 motif acts as a TPA inducible element in many genes (140-142). In order to address the question whether the Vβ2 TCR promoter was TPA inducible, 5'nested deletion contructs, pEScat (-343 to +31), pHScat (-85 to +31) and pdel1cat (-42 to +31) were transfected into EL-4 by the DEAE-dextran method and 24 hours later induced with either DMSO alone or TPA dissolved in DMSO. Data in Figure 16 and Table 7 clearly demonstrated that while the -343 to +31 and -85 to +31 fragments were TPA inducible, the -42 to +31 was not, suggesting that the Vβ2 promoter was TPA inducible and the TPA inducible element mapped between -85 and -42. The demonstration that this region of the Vβ2 promoter controls TPA inducibility suggests that an inducible transcription regulating transacting factor binds to this element. This was shown by other people in the lab by doing footprinting

and gel shift assays. A footprint extending through the conserved AP-1, inverted repeat and TCR decamer was found (Fuller and Fotedar, unpublished results). Similar transfection result was also obtained in NIH 3T3, S49, YAC-1, PEER and Jurkat (data not shown).

Identification of TPA Responsive Element in Vβ2 Promoter

To help identify the critical TPA motifs in the Vβ2 promoter, we cloned double stranded oligonucleotides corresponding to different parts of the -85 and -42 region upstream of the TPA uninducible and inactive -42 to +31 Vβ2 promoter in the pdelcat vector (see Materials and Methods; pX/delcat). These oligonucleotides include the sequences between -85 to -73, -84 to -62, -75 to -62 of the TCR Vβ2 promoter and the conserved sequence of TCR decamer. The constructs were transfected into EL-4 and then induced with TPA. Table 8 shows that basal expression was increased above the del1 background by the TCR conserved decamer oligonucleotide, and dramatically increased by three copies in the sense orientation of the -84 to -62 oligonucleotide. The -84 to -62 oligonucleotide contained the conserved AP-1 and inverted repeat motifs. TPA inducibility was only conferred by three copies of the -84 to -62 oligonucleotide. The inability of a single copy of the -84

to -62 oligonucleotide to increase either basal or TPA induced expression is among other reasons probably due to spacing requirements of the TCR promoter.

To address this issue, the same series of double stranded oligonucleotides were cloned upstream of the TPA uninducible herpes simplex thymidine kinase promoter in TKcat vector (pX/TKcat, see materials and methods). This would also test if this element could confer TPA inducibility to a heterologous TPA uninducible promoter. These constructs were transfected into EL-4 cell and TPA inducibility monitored by CAT assays. Results shown in Table 9 indicate that while the -85 to -62 oligonucleotide clearly conferred TPA inducibility, the -85 to -73 oligonucleotide was barely able if at all to confer TPA inducibility to TKcat. One copy of the -85 to -73 oligonucleotide was not inducible while five copies were weakly inducible. On the other hand, a single copy of the -84 to -62 oligonucleotide was clearly inducible while three copies were dramatically inducible, suggesting that the AP-1 conserved motif and some flanking sequences were required for optimal TPA inducibility in the TCR Vβ2 promoter.

A number of studies have investigated TPA inducibility of AP-1 containing transcription regulating sequences in

the human collagenase, rat stromelysin and human metallothionin, MTIIA genes (140-141). They were able to confer TPA inducibility to the TCR promoter by the core AP-1 sequences (145). Our studies, on the other hand, indicated the additional involvement of flanking sequences. These discrepancy could be due to the differences in cell types studied or the differences in the core AP-1 motif used. The first possibility was tested by transfection studies in EL-4 cells. A series of pDNAs generated by Peter Angle and Michael Karin were used. These pDNAs include Col-TREx3/TKcat (145), Col-TREx1/TKcat (145), TREΔ-72/TKcat and pBLcat2. pBLcat2 carries the TK promoter (-109 to +57) driving CAT gene expression. Col-TREx1/TKcat, Col-TREx3/TKcat and TREΔ-72/TKcat are BLcat2 derivatives in which a single copy, three copies and a mutant (three bases substitution) AP-1 sequence from the human collagenase gene respectively are inserted upstream of the TK promoter. Figure 17 shows that human collagenase AP-1 sequence (Col-TREx1/TKcat and Col-TREx3/TKcat) but not the mutant (TREΔ-72/TKcat) can confer the TPA inducibility in EL-4. This rules out the possibility of differences in cell types.

A single base difference in the core sequences of collagenase and Vβ2 AP-1 motif was observed (Figure 18). Single base change in the core sequence is critical for the

function of many enhancer elements as shown by saturation mutagenesis (217). It is possible that the G/T base substitution in Vβ2 AP-1 motif weakens the functional DNA protein interaction and the flanking sequences help compensate for it. To help unambigously demonstrate this, we cotransfected a c-jun expression plasmid with our CAT constructs in EL-4 cells. The c-jun cDNA is driven by the RSV LTR, which is very active in T cells. The result in Figure 19 clearly shown that 5 copies of the -85 to -73 oligo (containing the core Vβ2 AP-1 motif) was not transactivated by c-jun. On the other hand, 3 copies of the -84 to -62 oligo (containing the Vβ2 AP-1 motif and flanking sequences) was transctivated by c-jun. The c-jun/AP-1 motif interaction has been extensively analyzed. Our results in this context can be interpreted to suggest that a substitution of G to T in the Vβ2 promoter weakens the functional interactions which is compensated for by flanking sequences which seems to restore TPA inducibility to the core motif and transactivating by c-jun. The sequences of the Vβ2 promoter both upstream and downstream of the core AP-1 site shares some base homologies to the flanking sequences in the human collagenase, rat stromelysin and SV40 AP-1 motif, suggesting that these nucleotides may play a role in the interaction at the Vβ2 AP-1 binding site. It should be noted that although the Vβ2

AP-1 motif is identical to those of SV40, mapping of these regions in the SV 40 promoter have only been implicated by sequence comparisons. Saturation mutagenesis analysis in the -85 to -62 will be required to precisely map the critical bases.

Discussion

Analysis of TCR promoter in this study indicates that the Vβ2 promoter is TPA inducible and this element mapped between -85 and -62 which contained the conserved AP-1 and inverted repeat motifs. The TCR decamer motif, although helping to increase the strength of TCR promoter, is not involved in TPA inducible gene expression. Similar observations have also been made in the study of human Vβ8.1 (191) in that the decamer motif may play a role in TCR gene transcription but its requirement is not absolute. Sequence between -75 to -62 carrying an inverted repeat motif cannot confer TPA inducibility but is required with the AP-1 motif for the successful induction as well as to increase the basal level expression of both TCR and TK promoters. DNaseI footprinting analysis supports this finding as the entire region is protected when the EL-4 extract is used (Fuller and Fotedar, unpublished data).

A number of groups have investigated TPA inducible gene expression of the human collagenase, rat stromelysin

and human metallothionin genes (140-141). The conserved AP-1 motif was identified as the target DNA sequence which binds transacting factors and confers TPA inducibility to heterologous promoters (145). The similarity in the amino acid sequences of the avian oncogene v-jun (151) and GCN4, a yeast transactivator (154) with a DNA binding specificity very similar to the AP-1 motif (155) laid down the rationale for cloning the c-jun gene (153). The c-jun expression plasmid was found to transactivate via the AP-1 motif (155). In the TCR Vβ2 promoter, we have found that additional flanking sequences were critical in conferring TPA inducibility and transactivation by c-jun to probably help compensate for a G/T substitution (as compared to the classic collagenase motif) in the core AP-1 motif.

The c-jun transactivator forms a heterodimer with c-fos via the respective leucine zipper motifs which bind to the DNA sequence with a log fold higher affinity as compared to c-jun homodimers (163,164). A number of other members of the c-jun family like junB (156), junD (147) and the c-fos family like fosB (243) and fra1 (242) have been cloned. They show different patterns of tissue expression (143) and more recently differential activation requirement. Future studies should help determine the

correct c-jun/c-fos family members involved in TCR gene induction in T cells by TPA.

The finding that TPA enhances TCR gene transcription is in agreement with studies reported by others on increased β transcript level after phorbol ester treatment. Transcript levels of α and β TCR genes are increased when protein kinase C is activated by phorbol esters in a human T cell tumor, Jurkat (238), human thymocytes (239), and a murine T cell tumor, EL-4 (237). An elevated cytosolic free Ca^{++} induced by ionophores, on the other hand, increased γ and δ chain transcripts but decresed the TCR α and β mRNA levels (239).

Both the 1.3 kb (VβDβJβCβ) and 1 kb (DβJβCβ) transcripts were inducible (238) suggesting the presence of additional TPA inducible elements near the Cβ gene. This is consistent with the observation of a NF-KB TPA inducible element in the Jβ2Cβ2 intron (236). What is not clear at the present time is at what stage in T cell ontogeny are the TPA inducible elements active. The 2 TPA inducible elements, one reported by us upstream of the 5' most Vβ2 gene (800 kb from Cβ2) and the second near the Cβ2 gene (236) could be involved in either inducing gene expression prior to rearrangement or in the context of the

accessibility of the region to nuclear factors after rearrangement.

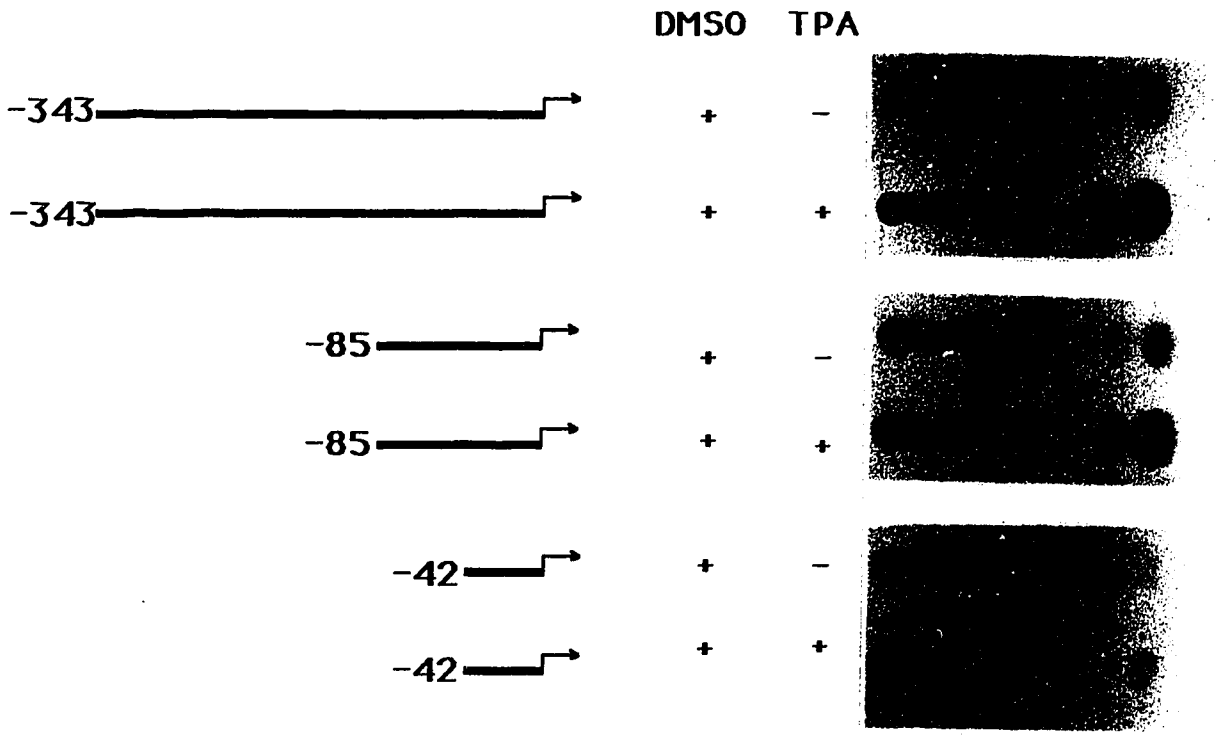


Figure 16 Basal and TPA induced CAT expression of pTCRcat in EL-4. The constructs are described in Figure 2.

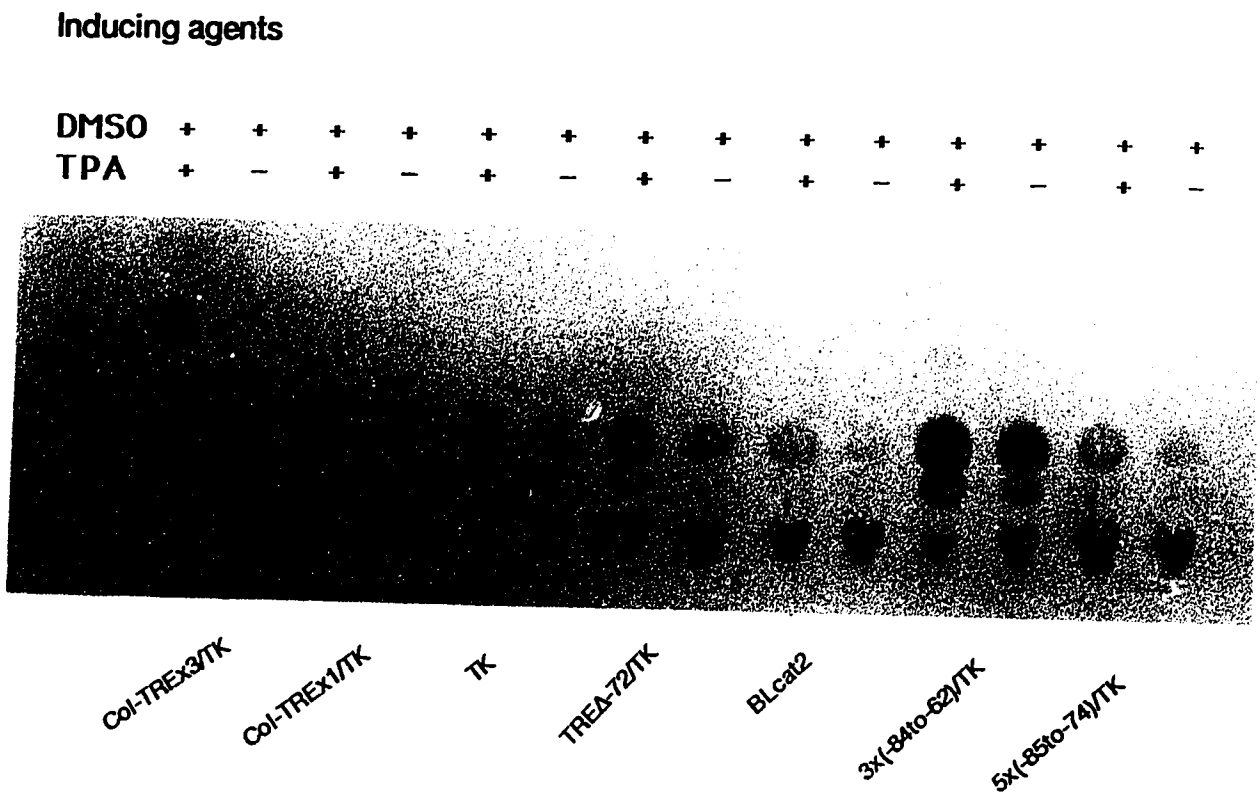


Figure 17 Basal and TPA induced CAT expression of pBLcat2 and its derivatives.

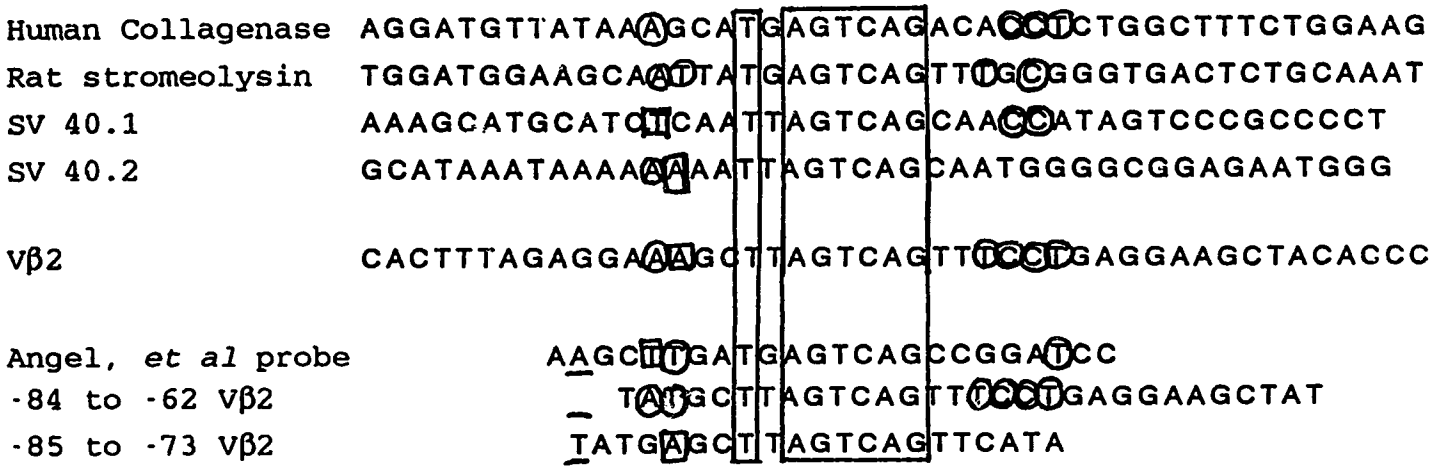


Figure18 Sequence comparisons between different AP-1 binding site.

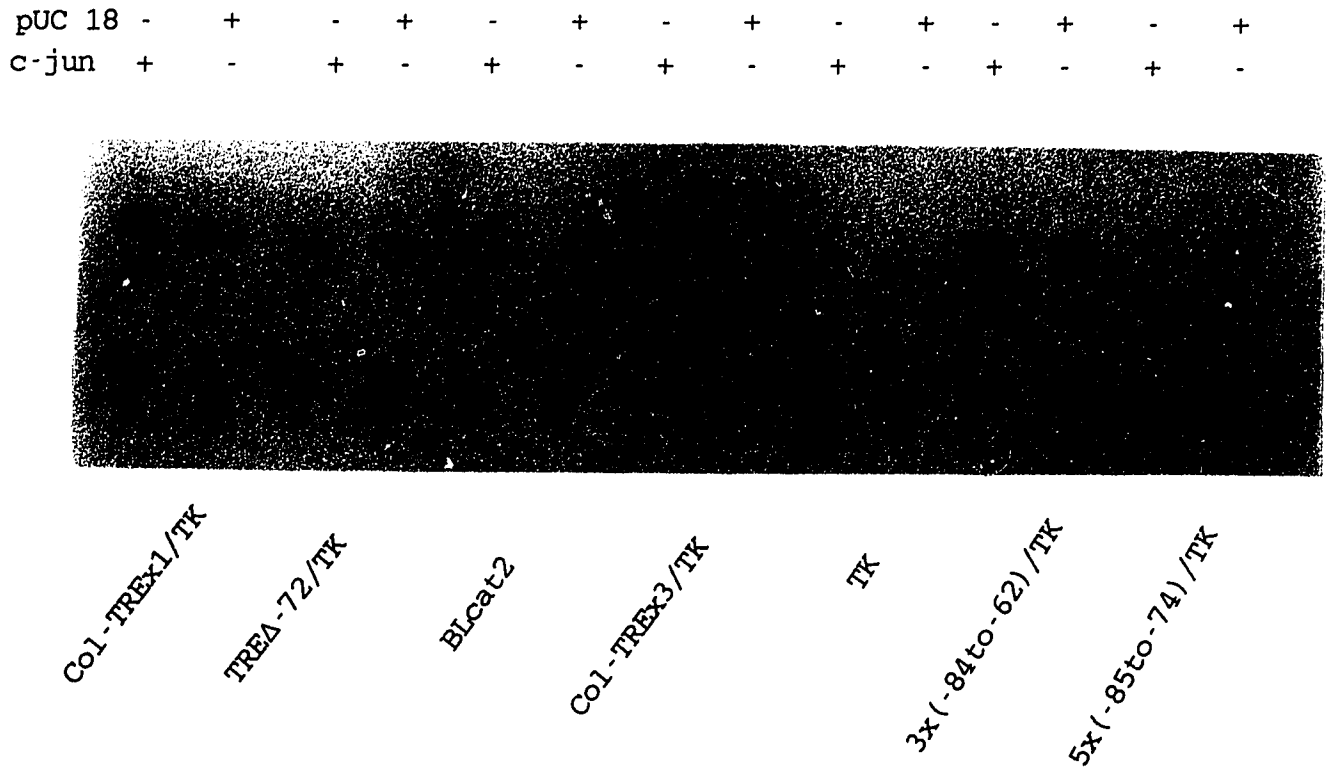


Figure 19 CAT expression of the CAT constructs cotransfected with the c-jun expression vector.

TABLE 7

BASAL AND TPA INDUCED ACTIVITY OF pTCRcat.[1]

CONSTRUCT[2]	TREATMENT		%CAT CONVERSION	
	DMSO	TPA	EL-4	NIH 3T3
EScat	+	-	1.45	1.0
EScat	+	+	6.0	4.36
HScat	+	-	1.39	0.6
HScat	+	+	5.51	2.06
del1cat	+	-	0.69	0.3
del1cat	+	+	0.44	0.2

1. The experiments have been repeated in different transfection experiments at different times (n=10) and gave similar results.
2. See Table 1.

TABLE 8

BASAL AND TPA INDUCED ACTIVITY OF pX/delcat CONSTRUCTS IN EL-4 CELLS

CONSTRUCT	TREATMENT		%CAT CONVERSION2
	DMSO	TPA	EL-4
dellcat	+	-	2.1
dellcat	+	+	2.0
1x(-85 to -73)dell	+	-	2.8
1x(-85 to -73)dell	+	+	3.6
2x(-85 to -73)dell	+	-	1.7
2x(-85 to -73)dell	+	+	1.1
1x(-84 to -62)dell	+	-	0.4
1x(-84 to -62)dell	+	+	0.2
3x(-84 to -62)dell	+	-	62.3
3x(-84 to -62)dell	+	+	94.7*
1x(-75 to -62)dell	+	-	1.1
1x(-75 to -62)dell	+	+	0.9
1xDecamer dell	+	-	5.2
1xDecamer dell	+	+	3.0
EScat	+	-	54.2
EScat	+	+	90.5*

1. The sequences of each oligonucleotides was shown in materials and methods.

2. Transfection efficiencies were monitored by co-transfecting the test plasmids with pCH110, the β-galactosidase expression vector. The % CAT conversion values were normalized to the level of β-galactosidase level. These experiments have been repeated in different transfection experiments at different times (n=10) and gave similar results.

* The fold induction is underestimated as %CAT conversion is not in the linear range.

TABLE 9

BASAL AND TPA INDUCED ACTIVITY OF pX/TKcat CONSTRUCTS IN EL-4 CELLS

CONSTRUCT[1]	TREATMENT		%CAT CONVERSION[2]
	DMSO	TPA	
SV0cat	+	-	0.8
SV0cat	+	+	0.6
TKcat	+	-	9.4
TKcat	+	+	3.7
1x(-85 to -73)TKcat	+	-	1.6
1x(-85 to -73)TKcat	+	+	2.2
5x(-85 to -73)TKcat	+	-	2.0
5x(-85 to -73)TKcat	+	+	7.8
1x(-84 to -62)TKcat	+	-	4.0
1x(-84 to -62)TKcat	+	+	14.0
3x(-84 to -62)TKcat	+	-	13.8
3x(-84 to -62)TKcat	+	+	98.1*
1x(-75 to -62)TKcat	+	-	3.8
1x(-75 to -62)TKcat	+	+	3.8
1xDecamer TKcat	+	-	4.4
1xDecamer TKcat	+	+	2.5

1. The sequences of each oligonucleotides was shown in materials and methods.

2. Transfection efficiencies were monitored by co-transfecting the test plasmids with pCH110, the β-galactosidase expression vector. The % CAT conversion values were normalized to the level of β-galactosidase level. These experiments have been repeated in different transfection experiments at different times (n=10) and gave similar results.

* The fold induction is underestimated as %CAT conversion is not in the linear range.

IV General Discussion and Future Direction

In this thesis, control of TCR gene transcription by cis-acting elements was investigated. Multiple cis-acting elements were identified from the murine Vβ2 promoter. These include the minimum promoter region, the upstream positive regulatory region and the upstream negative regulatory region. The role of Vβ2 promoter and β enhancer in the tissue specific expression was also analysed. In addition, the effect of a phorbol ester, TPA, on the TCR gene transcription was studied. The findings reported in this thesis have provided the new information about the TCR gene expression and suggested that regulation of TCR gene transcription may be more complicated than what was originally conceived. Among the Vβ genes that have been so far studied (murine Vβ2 (this thesis), human Vβ8.1 (191) and murine Vβ8.3 (192)), a few differences have been observed and are summarized as follow:

1) The upstream positive regulatory region was identified in Vβ2 and Vβ8.1 but not in the Vβ8.3 promoter. Whether these positive regulatory elements in the murine Vβ2 and the human Vβ8.1 promoters are the same awaits a detailed analysis. The negative regulatory region was unique for the Vβ2 promoter. Functional data of the human Vβ8.1 promoter indicated that there might be a negative

control element although it was not pointed out by the investigators. It is not known why some of the Vβ promoters carry the negative control elements and other, such as Vβ8.3 does not. Base on my functional data that the upstream negative regulatory element can be overruled by the TCR enhancer, it is not unreasonable to speculate that these elements function to maintain the transcriptional quiescent stage of the unrearranged Vβ genes. Suppression of the unrearranged V gene transcription may in turn prevent the TCR gene rearrangement in B cells since it has been shown that the unrearranged Ig transcript can trigger the Ig gene rearrangement. One might also predict that these elements would work over a long distance. In the case of Vβ2 gene, which is the 5' most Vβ gene in the β gene locus, this negative control element might suppress the transcription of not only the unrearranged Vβ2 gene, but also other downstream unrearranged Vβ genes. The presence of some similar negative regulatory elements in other Vβ genes would ensure this function. It would also be interesting to find out if the Vβ14 located downstream of the Cβ2 gene segment in the reverse orientation also has a similar negative control element.

2) The decanucleotide sequence reported by Anderson (189) is the only conserved sequence found among the Vβ

promoters. The initial speculation that the TCR decamer motif may serve as a T cell specific control element, i.e., binds to the T-cell specific *trans*-acting factor and mediates the tissue specific gene transcription of TCR genes is still controversial. DNaseI footprinting data of the Vβ2 and Vβ8.3 promoters indicated that this region specifically bound to the nuclear proteins. However, the functional significance of this motif is only demonstrated in the murine Vβ8.3 promoter. The authors show that a CREB (cAMP responsive element binding protein) like transactivator was the active protein. In the human Vβ8.1 promoter, the role of the decamer motif is not at all clear. It seems to have no function. We found that the decamer motif is mildly active while an AP-1 like motif is the dominant positive *cis*-acting element regulating Vβ2 promoter activity (both basal and inducible transcription).

3) The role of Vβ promoter in regulating tissue specific expression is still not clear. The murine Vβ8.3 (192) and Vβ2 promoter are active in both T cells and fibroblasts while the human Vβ8.1 promoter is expressed only in T cells but not in fibroblasts (191). The results from Vβ2 (this thesis) and human Vβ8.1 show that the TCR β promoter is not active in B cells. This is in contrast to the Ig gene promoters that were only active in B cells and

not in other cell types. More functional studies on other Vβ promoters will be required to help understanding the nature of the TCR β chain promoter.

4) The β chain enhancer can enhance gene transcription of the Vβ2 promoter in both T cells and fibroblasts. However, enhancement of the heterologous (TK) promoter by β enhancer is only observed in T cells (Messier and Fotedar, unpublished data). Other studies have only analyzed the interaction between the β enhancer and the heterologous promoters, such as pim1 and SV40 and concluded that the β enhancer is tissue specific. Such conclusions may have to be reconsidered since our study show that the interaction between the β enhancer and its cognate promoter was different from the interaction between the β enhancer and the heterologous promoter.

Although the Ig and TCR share a number of similarities, such as the molecular structure, the genomic arrangement/rearrangement, and the generation of diversity, the above findings suggest that they are quite different in the regulatory mechanisms that control their tissue specific expression. In human and murine kappa and heavy chain Ig promoters, the octamer motif is the critical element (248-250,252). Mutational or deletional analysis

studies indicate that this motif is sufficient to direct lymphocyte specific RNA synthesis (88). Other elements such as the heptamer and pyrimidine rich region in heavy chain and deca- and pentadecanucleotide sequences in the κ, λ and IgH are also important for the promoter function (246,247,253,245). None of these elements, however, are present in the transcriptionally competent murine Vβ2, Vβ8.3 and human Vβ8.1 promoters, suggesting that the elements regulating expression in the β promoters are different from those of Ig genes.

The β chain enhancer that is located downstream of the Cβ2 gene segment are also quite different from the κ, λ and IgH enhancers that are located in the J-C intron (246,248,251,214). The Ig enhancers are tissue specific regardless of the promoters used (246-248). Nevertheless, the Ig enhancers prefer to cooperate with their cognate promoters relative to the heterologous promoters (214). In short, there are significant differences between TCR and Ig gene regulation and a consensus picture for Vβ promoter controlling elements is not yet emerging.

There are a number of interesting issues emerging from this work. One of this is to explore the variety of effects the negative regulatory element in the Vβ2 promoter

mediates. It would be important to know which of these silencer elements are active and if the suppression is the additive effect of these silencer elements. One such approach would involve the internal deletional analysis, site specific mutagenesis and DNaseI footprinting analysis. Transfection experiments of the plasmid constructs in which these negative regulatory region is inserted upstream of other Vβ promoters will influence our ideas if this element is actually involved in the control of transcriptional quiescent stage of other unrearranged Vβ genes. An alternative way will be to use the transgenic mice in which the negative regulatory region is deleted.

In this thesis, I have demonstrated that the Vβ2 promoter is TPA inducible. The TPA induction will be another way for TCR genes to regulate the level of expression. The TRE (TPA responsive element) maps to the AP-1 and inverted repeat motifs (-85 to -62) in the Vβ2 minimum promoter, an observation that does not agree with the human collagenase TRE (AP-1 alone). The questions arising from this study are what role does the inverted repeat motif play? What are the transactivators that bind to the Vβ2 TRE? At what stage in the thymic maturation is TPA induction involved? Does TPA induced TCR gene transcription relate to T cell activation? Is TPA induction

involved in inducing gene expression prior to rearrangement of unrearranged Vβ genes? Clearly, some of these questions may not be easy to answer. The availibility of new approaches will resolve the puzzle of developmentally regulated expression of T cell receptor genes.

V BIBLIOGRAPHY

1. Zinkernagel, R.M., and Doherty, P.C. (1975). H-2 compatibility requirement for T cell mediated lysis of target infected with lymphocytic choriomeningitis virus. Different cytotoxic T cell specificities are associated with structures in H-2K or H-2D. J. Exp. Med. 141, 1427-1437.

2. Kronenberg, M., Siu, G., Hood, L.E., and Shastri, N. (1986). The molecular genetics of the T cell antigen receptor and T cell antigen recognition. Ann. Rev. Immunol. 4, 529-541.

3. Bevan, M.J. (1975). The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. J. Exp. Med. 142, 1349-1364.

4. Kappler, J.W., and Marrack, P.C. (1976). Helper T cells recognise antigen and macrophage surface components simultaneously. Nature 262, 797-799.

5. Sprent, J. (1978). Restricted helper function of F1 hybrid T cells positively selected to heterologous erythrocytes in irradiated parental strain mice. II. Evidence for restrictions affecting helper cell induction and T-B collaboration, both mapping to the K end of the H-2 complex. J. Exp. Med. 147, 1159-1174.

6. Kappler, J.W., Skidmore, B., White, J., and Marrack, P.C. (1981). Antigen-inducible, H-2 restricted, interleukin-2 producing T cell hybridomas, lack of independent antigen and H-2 recognition. J. Exp. Med. 153, 1198-1214.

7. Dembic, Z., Haas, W., Weiss, J., McCubrey, H., Kiefer, H., von Boehmer, H., and Steinmetz, M. (1986). Transfer of specificity by murine α and β T cell receptor genes. Nature 320, 232-238.

8. Gabert, J., Langlet, C., Zamoyska, R., Parnes, J.R., Schmitt-Verhulst, A.M., and Malissen, B. (1987). Reconstitution of MHC Class I specificity by transfer of the T cell receptor and Lyt-2 genes. Cell 50, 545-554.

9. Saito, T., Weiss, A., Miller, J., Norcross, M.A., and Germain, R.N. (1987). Specific antigen-Ia activation

of transfected human T cells expressing murine Ti αβ-human T3 receptor complexes. Nature 325, 125-130.

10. Buus, S., Sette, A., Colon, S.M., Jenis, D.M., and Grey, H.M. (1986). Isolation and characterization of antigen-Ia complexes involed in T cell recognition. Cell 47, 1071-1077.

11. Allison, J.P., McIntyre, B.W., and Bloch, D. (1982). Tumor-specific antigen of murine T lymphoma defined with monoclonal antibody. J. Immunol. 129, 2293-2300.

12. Haskins, K., Kumbo, R., White, J., Pigeon, M., Kappler, J., and Marrack, P. (1983). The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. J. Exp. Med. 157, 1149-1169.

13. Lanckin, D.W., Lorber, M.I., Loken, M.R., and Fitch, F.W. (1983). A clone-specific monoclonal antibody that inhibits cytolysis of a cytolytic T cell clone. J. Exp. Med. 157, 921-935.

14. McIntyre, B.W., and Allison, J.P. (1983). Mouse T cell receptor: Structural heterogeneity of molecules of normal T cells defined by xenoantiserum. Cell 34, 739-746.

15. Meuer, S.C., Fitzgerald, K.A., Hussey, R.E., Hodgdon, J.C., Schlossman, S.F., and Reinherz, E.L. (1983). Clonotypic structures involved in antigen-specific human T cell function. Relationship to the T3 molecular complex. J. Exp. Med. 157, 705-719.

16. Samelson, L.E., Germain, R.M., and Schwartz, R.H. (1983). Monoclonal antibodies against the antigen receptor on a cloned T cell hybrid. Proc. Natl. Acad. Sci. USA 80, 6972-6976.

17. Haars, R., Rohowsky-Kochan, C., Reed, E., King, D.W., and Sucia-Foca, N. (1984). Modulation of T cell antigen receptor on lymphocyte membrane. Immunogenetics 20, 397-405.

18. White, J., Haskin, K.M., Marrack, P., and Kappler, J. (1983). Use of I region restricted, antigen specific T cell hybridomas to produce idiotypically specific antigen-receptor antibodies. J. Immunol. 130, 1033-1037.

19. Kappler, J., Kubo, R., Haskins, K., White, J., and Marrack, P. (1983). The mouse T cell receptor: comparison of MHC-restricted receptors on two T cell hybridomas. Cell 34, 727-737.

20. Acuto, O., Hussey, R.E., Fitzgerald, K.A., Protentis, J.P., Meuer, S.C., Schlossman, S.F., and Reinherz, E.L. (1983). The human T cell receptor: appearance in ontogeny and biochemical relationship of α and β subunits on IL-2 dependent clones and T cell tumors. Cell 34, 717-726.

21. Meuer, S.C., Acuto, O., Hercend, T., Schlossman, S.F., and Reinherz, E.L. (1984). The human T cell receptor. Ann. Rev. Immunol. 2, 23-50.

22. Hedrick, S., Nielsen, E., Kavaler, J., Cohen, D., and Davis, M.M. (1984). Sequence relationships between putative T cell receptor polypeptides and immunoglobulins. Nature 308, 153-158.

23. Yanagi, Y., Yoshikai, Y., Leggett, K., Clark, S., Aleksander, I., and Mak, T.W. (1984). A human T cell specific cDNA clones encodes a protein having extensive homology to immunoglobulin chains. Nature 308, 145-149.

24. Yanagi, Y., Chan, A., Chin, B., Minden, M., and Mak, T. (1985). Analysis of cDNA clones specific for human T cells and the α and β of the T cell receptor heterodimer from a human T cell line. Proc. Natl. Acad. Sci. USA 82, 3430-3434.

25. Chien, Y., Becker, D., Lindsten, T., Okamura, M., Cohen, D., and Davis, M.M. (1984). A third type of murine T cell receptor gene. Nature 312, 31-35.

26. Sim, G., Yague, J., Nelson, J., Marrack, P., Palmer, E., Augustin, A., and Kappler, J. (1984). Primary structure of human T cell receptor α chain. Nature 312, 7715-775.

27. Brenner,M.B., McLean, J., Dialynas, D.P., Strominger, J.L., Smith, J.A., Owen, F.L., Seidman, J.G., Ip.S., Rosen, F., and Krangel, M.S. (1986). Identification of a putative second T cell receptor. Nature 322, 145-149.

28. Lanier, L.L., Federspiel, N.A., Ruitenberg, J.J., Philips, J.S., Allison, J.P., Littman, D., and Weiss, A. (1987). The T cell antigen receptor complex expressed on normal peripheral blood $CD4^-$, $CD8^-$ T lymphocytes. J. Exp. Med. 165, 1076-1094.

29. Lanier, L.L., and Weiss, A. (1986). Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. Nature 324, 268-270.

30. Pardoll, D.M., Fowlkes, B.J., Bluestone, J.A., Kruisbeek, A., Maloy, W.L., Coligan, J.E., and Schwartz, R.H. (1987). Differential expression of two distinct T cell receptors during thymocyte development. Nature 326, 79-81.

31. Maeda, K., Nakanishi, N., Rogers, B.L., Haser, W.G., Shitara, K., Yoshida, H., Takagaki, Y., Augustin, A.A., and Tonegawa, S. (1987). Expression of the T cell receptor γ-chain gene products on the surface of peripheral T cells and T cell blasts generated by allogeneic mixed lymphocyte reaction. Proc. Natl. Acad. Sci. USA 84, 6535-6540.

32. Cron, R., Koning, F., Maloy, W., Pardoll, D., Coligan, J., and Bluestone, J. (1988). Peripheral murine $CD3^+$, $CD4^-$, $CD8^-$ T lymphocytes express novel T cell receptor γδ receptor structures. J. Immunol. 141, 1074-1082.

33. Nakanishi, N., Maeda, K., Ito, K.I., Heller, M.,and Tonegawa, S. (1987). Tγ protein is expressed on murine fetal thymocytes as a disulfide-linked heterodimer. Nature 325, 720-723.

34. Lew, A.M., Pardoll, D.M., Maloy, W.L., Fowlkes, B.J., Kruisbeek, A., Cheng, S-F., Germain, R.N., Bluestone, J.A., Schwatrz, R.H., and Colligan, J.E. (1986) Characterization of T cell receptor gamma chain expression in a subset of murine thymocytes. Science 234,1401-1405.

35. Yoshik i, Y., Reis, M.D., and Mak, T.W. (1986). Athymic mice express a high level of functional γ-chain but greatly reduced level of α and β chain T cell receptor messages. Nature 324, 482-485.

36. Koning,F., Stingl, G. , Yokoyama, W.M., Yamada, H., Maloy, W.L., Tschahler, E., Shevach, E.M., and

Coligan, J.E. (1987). Identification of a T3 associated γδ T cell receptor on thy-1$^+$ dendritic epidermal cell lines. Science 236, 834-837.

37. Moingeon, P., Ythier, A., Goubin, G., Faure, F., Nowill, A., and Delmon, L., Rainaud, M., Forestier, F., Daffos, F., Bohuon, D., and Hercend, T. (1986). A unique T cell receptor complex expressed on human fetal lymphoctyes displaying natural killer-like activity. Nature 323, 638-640.

38. Moingeon, P., Jitsukawa, S., Faure, F., Troalen, F., Triebel, F., Graziani, M., Forestier, F., Bellet, D., Bohuon, C.,and Hercend, T. (1987). A γ-chain complex forms a functional receptor on cloned human lymphocytes with natural killer like activity. Nature 325, 723-726.

39. Nixon-Fulton, J.L., Hackett, J., Lewis, J., Bergstresser, P.R., Kumar, V., and Tigelaar, R.E. (1987). Phenotypic heterogeneity and cytoxic activity of ConA and IL-2 stimulated cultures of mouse Thy-1$^+$ epidermal cells. J.Invest.Dermotol. 91, 62-68.

40. Bank, I., DePinho, R.A., Brenner, M.B., Cassimeris, J., Alt, F.W., and Chess, L. (1986). A function T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. Nature 322, 179-181.

41. Ferrini, S., Bottino, C., Biassoni, R., Poggi, A., Sekaly, R.P., Moretta, L., and Moretta, A. (1987). Characterization of CD3$^+$, CD4$^-$, CD8$^-$ clones expression the putative T cell receptor γ gene product. J.Exp.Med. 166, 277-282.

42. Bluestone, J.A., Cron, R.Q., Cotterman, M., Houlden, B.A., and Matis, L.A. (1988). Structure and specificity of TCR γ/δ receptors on major histocompatibility complex antigen specific CD3$^+$, CD4$^-$, CD8$^-$ T lymphocytes. J. Exp. Med. 168, 1899-1916.

43. O'Brien, R.L., Happ, M.P., Dallas, A., Palmer, E., Kubo, R., and Born, W.K. (1989). Stimulation of a major subset of lymphocytes expressing T cell receptor γδ by an antigen derived from *Mycobacterium tuberculosis*. Cell 57, 667-674.

44. Reilly, E. B., Kranz, D.M., Tonegawa, S., and Eisen, H.N. (1986). A functional gene formed from known γ-gene segments is not necessary for antigen specific responses of murine cytotoxic T lymphocytes. Nature 321, 878-880.

45. Heilig, J.S., and Tonegawa, S. (1987). T-cell γ gene is allelically but not isotypically excluded and is not required in known functional T-cell subsets. Proc. Nalt. Acad. Sci. USA. 84, 8070-8074.

46. Winoto, A., and Baltimore, D. (1989). Separate lineages of T cells expressing the αβ and γδ receptors Nature 338, 430-432.

47. Hayday, A.C.,Saito, H., Gillies, S.D., Kranz, D.M., Tanigawa, G., Eisen, H.N., and Tonegawa, S. (1985). Structure, organization and somatic rearrangement of T cell gamma genes. Cell 40, 259-269.

48. Hedrick, S.M., Cohen, D.I., Nielsen, E.A., and Davis, M.M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. Nature 308, 149-153.

49. Saito, H., Kranz, D.M., Takagaki, Y., Hayday, A., Eisen, H.N., and Tonegawa, S. (1984). A third rearranged and expressed gene in a clone of cytotoxic T lymphocytes. Nature 312, 36-40.

50. Caccia, N., Kronenberg, M., Saxe, D., Haars, R., Bruns, G., Goverman, J., Malisen, M., Willard, H., Simon, M., Hood, L., and Mak, T.W., (1984). The T-cell receptor β chain genes are located on chromosome 6 in mice and chromosome 7 in humans. Cell 37, 1091-1099.

51. Barth, R., Kim, B., Lan, N., Hunkapiller, T., Sobieck, N., Winoto, A., Gershenfeld, H., Okada, C., Hansburg, D., Weissman, I., and Hood, L. (1985). The murine T-cell receptor employs a limited repertoire of expressed V gene segments. Nature 316, 517-523.

52. Behlke, M.A., Spinella, D.G., Chou, H.S., Sha, W., Hartl, D.L., and Loh, D.Y. (1985). T-cell receptor β-chain expression: Dependence on relatively few variable region genes. Science 229, 566-570.

53. Concannon, P., Pickering, L.A., Kung, P., and Hood, L. (1986). Diversity and structure of human T-cell receptor β-chain variable region genes. Proc. Natl. Acad. Sci. USA 83, 6598-6602.

54. Kimura, N., Toyonaga, B., Yoshikai, Y., Du, R.P., and Mak, T.W. (1987). Sequences and repertoire of the human α and β chain variable region genes in thymocytes. Eur. J. Immunol. 17, 375-383.

55. Siu, G., Clark, S., Yoshikai, Y., Malissen, M., Yanage, Y., Strauss, E., Mak, T.W., and Hood, L. (1984). The human T cell antigen receptor is encoded by variable, diversity, and joining gene segments that rearranged to generate a complex gene. Cell 37, 393-401.

56. Malissen, M., McCoy, C., Blanc, D., Trucy, J., Devaux, C., Schmitt-Verhulst, A., Fitch, F., Hood, L., and Malissen, B. (1986). Direct evidence for chromosomal inversion during T-cell receptor β-gene rearrangements. Nature 319, 28-33.

57 Gascoigne, N., Chien, Y., Becker, D., Kavaler, J., and Davis, M. (1984). Genomic organization and sequence of T cell receptor β-chain constant and joining region genes. Nature 310, 387-391.

58. Malissen, M., Minard, K., Mjolsness, S., Kronenberg, M., Goverman, J., Hunkapiller, T., Prystowsky, M.B., Yoshikai, Y., Fitch, F., Mak, T.W., and Hood, L. (1984). Mouse T-cell antigen receptor: Structure and organization of constant and joining gene segments encoding the beta polypeptide. Cell 37, 1101-1110.

59. Toyonaga, B., Yoshikai, Y., Vadasz, V., Chin, B., and Mak, T.W. (1985). Organization and sequences of the diversity, joining and constant region genes of the human T cell receptor β chain. Proc. Natl. Acad. Sci. USA 82, 8624-8628.

60. Caccia, N., Brulns, G.A.P., Kirsch, I.R., Hollis, G.R., Bertness, V., and Mak, T.W. (1985). T cell receptor α chain genes are located on chromosome 14 at 14q11-14q12 in humans, J. Exp. Med. 161, 1255-1260.

61. Dembic, Z., Bannworth, W., Taylor, B.A., and Steinmetz, M. (1985). The gene encoding the T cell

receptor α-chain maps close to the Np-1 locus on mouse chromosome 14. Nature 314, 271-273.

62. Yoshikai, Y., Kimura, N., Toyonaga, B., and Mak, T.W. (1986). Sequences and repertoire of human T cell receptor α chain variable regions in mature T lymphocytes. J. Exp. Med. 164, 90-103.

63. Arden, B., Klotz, J.L., Siu, G., and Hood, L. (1985). Diversity and structure of genes of the α family of mouse T cell antigen receptor. Nature 317, 783-787.

64. Yoshikai, Y., Clark, S.P., Taylor, S., Sohn, V., Wilson, B., Minden, M., and Mak, T.W. (1985). Organization and sequences of the variable, joining, and constant region genes of the human T cell receptor α chain. Nature 316, 837-840.

65. Winoto, A., Mjolsness, S., and Hood, L. (1985). Genomic organization of genes encoding mouse T cell receptor α chain. Nature 316, 832-836.

66. Elliott, J.F., Rock, E.P., Patten, P.A., Davis, M.M., and Chien, Y. (1988). The adult T-cell receptor δ chain is diverse and distinct from that of fetal thymocytes. Nature 331, 627-631.

67. Raulet, D.H. (1989). The structure, function, and molecular genetics of the γ/δ T cell receptor. Ann. Rev. Immunol. 7, 175-207.

68. Tonegawa, S. (1983). Somatic generation of antibody diversity. Nature 302, 575-581.

69. Yoshikai, Y., Anatoniou, D., Clark, S.P., Yanagi, Y., Sangster, R., Van den Elsen, P., Terhorst, C., and Mak, T.W. (1984). Sequence and expression of transcripts of the human T-cell receptor β-chain genes. Nature 312, 521-524.

70. Jones, N., Leiden, J., Dialynas, D., Fraser, J., Clabby, M., Kishimoto, T., Strominger, J.L., Andrews, D., Lane, W., and Woody, J. (1985). Partial primary structure of the alpha and beta chains of human tumor T-cell receptors. Science 227, 311-314.

71. Rupp, F., Acha-orbea, H., Hengadrtener, H., Zinkernagel, R., and Joho, R. (1985). Identical Vβ T-

cell receptor genes used in alloreactive cytotoxic and antigen plus I-A specific helper T cells. Nature 315, 425-427.

72. Alt, F.W., Yancopoulos, G.D., Blackwell, T.K., Wood, C., Thomas, E., Bos, M., Coffman, R., Rosenberg, N., Tonegawa, S., and Baltimore, D. (1984). Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J. 3, 1209-1219.

73. Okazaki, K., Davis, D.D., and Sakano, H. (1987). T cell receptor β gene sequences in the circular DNA of thymocyte nuclei: Direct evidence for intramolecular DNA deletion in V-D-J joining. Cell 49, 477-485.

74. Okazaki, K., and Sakano, H. (1988). Thymocyte circular DNA excised from T cell receptor α-δ gene complex. EMBO J. 7, 1669-1674.

75. Akira, S., Okazaki, K., and Sakano, H. (1987). Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 238, 1134-1138.

76. Duby, A.D., Klein, K.A., Murre, C., and Seidman, J.G. (1985). A novel mechanism of somatic rearrangement predicted by a human T cell antigen receptor β-chain complementary DNA. Science 228, 1204-1206.

77. Caccia, N., Toyonaga, B., Kimura, N., and Mak, T.W. (1989). The α and β chains of the T cell receptor. In The T-cell receptors, T.W. Mak, ed. (New York and London, Plenum Press), pp. 9-52.

78. Chien, Y-H, Gascoigne, N.R.J., Kavaler, J., Lee, N.E., and Davis, M.M. (1984). Somatic recombination in a murine T cell receptor gene. Nature 309, 322-326.

79. Pardoll, D.M., Fowlkes, B.J., Bluestone, J.A., Kruisbeek, A., Maloy, W.L., Coligan, J.E., and Schwartz, R.H. (1987). Differential expression of two distinct T-cell receptors during thymocyte development. Nature 326, 79-81.

80. Haars, R., Kronenberg, M., Gallatin, W.M., Weissman, I.L., Owen, F., and Hood, L. (1986). Rearrangement and expression of T-cell antigen receptor and γ chain genes during thymic differentiation. J. Exp. Med. 164,1-24.

81. Raulet, D.H., Germain, R.D., Saito, H., and Tonegawa, S. (1985). Developmental regulation of T-cell receptor gene expression. Nature 314, 103-107.

82. Snodgrass, H.R., Kisielow, P., Kiefer, M., Steinmetz, M., and von Boehmer, H. (1985). Ontogeny of the T-cell antigen receptor within the thymus. Nature 313, 592-595.

83. Finkel, T.H., and Cambier, J.C. (1989). The thymus has two functionally distinct populations of immature $\alpha\beta^+$ T cells: one population is deleted by ligation of $\alpha\beta$ TCR. Cell 58, 1047-1054.

84. Benoist, C., and Mathis, D. (1989). Positive selection of the T cell repertoire: where and when does it occur? Cell 58, 1027-1033.

85. Berg, L.J., Pullen, A.M., Groth, B.F., and Davis, M.M. (1989). Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. Cell 58, 1035-1046.

86. von Bohmer, H. (1988). The developmental biology of T lymphocytes. Ann. Rev. Immunol. 6, 309-326.

87. Schwartz, R.H. (1989). Acquisition of immunologic self-tolerance. Cell 57, 1073-1081.]

88. Wirth, T., Staudt, L., and Baltimore, D. (1987). An octamer oligonucleotide upstream of a TATA motif is sufficient for lymphoid-specific promoter activity. Nature 329, 174-178.

89. Kappler, J.W., Wade, T., White, J., Kushnir, E., Blackman, M., Bill, J., Roehm, N., and Marrack, P. (1987). A T cell receptor $V\beta$ segment that imparts reactivity to a class II major histocompatibility complex product. Cell 49, 263-271.

90. Kappler, J.W., Roehm, N., and Marrack, P. (1987). T cell tolerance by clonal elimination in the thymus. Cell 49, 273-280.

91. MacDonald, H.R., Schneider, R., Lees, R.K., Howe, R.C., Acha-Orbea, H., Festenstein, H., Zinkernagel, R.M., and Hengartner, H. (1988). T cell receptor $V\beta$

use predicts reactivity and tolerance of Mls^c encoded antigens. Nature 332, 40-45.

92. Kappler, J.W., Staerz, U., White, J., and Marrack, P. (1988). Self tolerance eliminates T cell specific for Mls-modified products of the major histocompatibility complex. Nature 332, 35-40.

93. Davidson, E.H., and Britten, R.J. (1973). Organization, transcription and regulation in the animal genome Quart. Rev. Biol. 48, 565-613.

94. Axel, R., Cedar, H., and Felsenfeld, G. (1973). Chromatin template activity and chromatin structure. Cold Spring Harb. Symp. Quant. Biol. 38, 773-783.

95. Elgin, S.C.R. (1981). DNaseI hypersensitive sites of chromatin. Cell 27, 413-415.

96. Labhart, P., and Koller, T. (1982). Structure and the active nucleolar chromatin of *Xenopus laevis* oocytes. Cell 28, 279-292.

97. Pfluzfelder, G.O., and Sonnenbichler, J. (1982). Oligonucleosomes as a model system for chromatin transcription. J. Mol. Biol. 158, 365-389.

98. Solomon, M.J., Larsen, P.L., and Varshavsky, A. (1988). Mapping protein-DNA interactions *in vivo* with formaldehyde: Evidence that histone H4 is retained on a highly transcribed gene. Cell 53, 937-947.

99. Buus, S., Sette, A., Colon, S.M., Jenis, D.M., and Grey, H.M. (1986). Isolation and characterization of antigen-Ia complexes involved in T cell recognition. Cell 47, 1071-1077.

100 Kovesdi,I., Reichel, R., and Nevins, J.R. (1987). Role of adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control Proc. Natl. Acad. Sci. USA 84, 2180-2184.

101. Harrington, M.A., Jones, P.A., Imagawa, M., and Karin, M. (1988). Cytosine methylation does not affect binding of transcription factor Sp1. Proc. Natl. Acad. Sci. USA 85, 2066-2070.

102. Hoeller, M., Westin, G., Jiricny, J., and Schaffner, W. (1988). Sp1 transcription factor binds DNA and activates transcription even when the binding site is CpG methylated. Genes Dev. 2, 1127-1135.

103. Antequera, F., Macleod, D., and Bird, A.P. (1989). Specific protection of methylated CpGs in mammalian nuclei. Cell 58, 509-517.

104. Meehan, R.R., Lewis, J.D., Mckay, S., Kleiner, E.L., and Bird, A.P. (1989). Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell 58, 499-507.

105. Maniatis, T., Goodbourn, S., and Fischer, J.A. (1987). Regulation of inducer and tissue-specific gene expression. Science 236, 1237-1244.

106. Dynan, W.S., and Tjian, R. (1985). Control of eukaryotic mRNA synthesis by sequence specific DNA binding proteins. Nature (London) 316, 774-778.

107. McKnight, S., and Tjian, R. (1986). Transcriptional selectivity of viral genes in mammalian cells. Cell 46,795-805.

108. Fried, M., and Crothers, D.M. (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. 9, 6505-6525.

109. Galas, D.J. and Schmitz, A. (1978). DNase footprinting; a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Res. 5, 3157-3170

110. Tadahashi, K. Vigneron, M., Matthes, H., Wildeman, A., Zenke, M., and Chambon, P. (1986). Requirement of stereospecific alignment for initiation from the simian virus 40 early promoter. Nature (London) 319, 121-126.

111. Buratowski, S., Hahn, S., Guarente, L., and Sharp, P.A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II. Cell 56, 549-561.

112. Sopta, M., Burton, Z.F., and Greenblatt, J. (1989). Structure and associated DNA-helicase activity of a general transcription initiation factor that binds to RNA polymerase II. Nature 341, 410-414.

113. Horikoshi, M., Wang, C.K., Fujii, H., Cromlish, J.A., Weil, P.A., and Roeder, R.G. (1989). Cloning and structure of a yeast gene encoding a general

transcription initiation factor TFIID that binds to the TATA box. Nature 341, 299-303.

114. Cavallini, B., Huet, J., Plassat, J.L., Sentenac, A., Egly, J.M., and Chambon, P. (1988). A yeast activity can substitute for the Hela cell TATA box factor. Nature 334, 77-80.

115. Buratowski, S., Hahn, S., Sharp, P.A., and Guarente, L. (1988). Function of a yeast TATA element-binding protein in a mammalian transcription system. Nature 334, 37-42.

116. Hahn, S., Buratowski, S., Sharp, P.A., and Guarente, L. (1989). Isolation of the gene encoding the yeast TATA binding protein TFIID: A gene identical to the SPT15 suppressor of Ty element insertions. Cell 58, 1173-1181.

117. Horikoshi, M., Wang, C.K., Fujii, H., Cromlish, J.A., Weil, P.A., and Roeder, R.G. (1989). Purification of a yeast TATA box-binding protein that exhibits human transcription factor IID activity. Proc. Natl. Acad. Sci. USA 86, 4843-4847.

118. Ahearn, J.M., Jr., Bartolomei, M.S., West, M.L., Cisek, L.J., and Corden, J.L. (1987). Cloning and sequence analysis of the mouse genomic locus encoding the largest subunit of RNA polymerase II J. Biol. Chem. 262, 10695-10705.

119. Allison, L.A., Moyle, M., Shales, M., and Ingles, C.J. (1985). Extensive homology among the largest subunits of eukaryotic and prokaryotic RNA polymerases. Cell 42, 599-610.

120. Zehring, W.A., Lee, J.M., Weeks, J.R., Jokerst, R.S., and Greenleaf, A.I. (1988). The C-terminal repeat domain of RNA polymerase II largest subunit is essential *in vivo* but is not required for accurate transcription initiation *in vitro*. Proc. Natl. Acad. Sci. USA 85, 3698-3702.

121. Bartolomei, M.S., Halden, N.F., Cullen, C.R., and Corden, J.L. (1988). Genetic analysis of the repetitive carboxyl-terminal domain of the largest subunit of mouse RNA polymerase II. Mol. Cell. Biol. 8, 330-339.

122. Nonet, M., Sweetser, D., and Young, R.A. (1987). Functional redundancy and structural polymorphism in

the largest subunit of RNA polymerase II. Cell 50, 909-915.

123. Bartholomew, B., Dahmus, M.E., and Meares, C.F. (1986). RNA contacts subunits II_o and II_c in HeLa RNA polymerase II transcription complexes. J. Biol. Chem. 261, 14226-14231.

124. Little, J.W., and Green, M.R. (1989). Activator's target in sight. Nature 341, 279-280.

125. Horikoshi, M., Hai, T., Lin, Y., Green, M.R., and Roeder, R.G. (1988). Transcription factor ATF interacts with the TATA factor to facilitate establishment of a preinitiation complex. Cell 54, 1033-1042.

126. Hai, T., Horikoshi, M., Roeder, R.G., and Green, M.R. (1988). Analysis of the role of the transcription factor ATF in the assembly of a functional preinitiation complex. Cell 54, 1043-1051.

127. Benoist, C., and Chambon, P. (1981). *In vivo* sequence requirements of the SV40 early promoter region. Nature 290, 304-310.

128. Gruss, P., Dhar, R., and Khoury, G. (1981). Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc. Natl. Acad. Sci. USA 78, 943-947.

129. Wasylyk, B. (1988). Enhancers and transcription factors in the control of gene expression. Biochimical et Biophysica Acta 951, 17-35.

130. Khoury, G., and Gruss, P. (1983). Enhancer elements. Cell 33, 313-314.

131. Serfling, E., Jasin, M., and Schaffner, W. (1985). Enhancer and eukaryotic gene transcription. TIG,

132. Ondex, B., Gloss, L., and Herr, W. (1988). The SV40 enhancer contains two distinct levels of organization. Nature 333, 40-45.

133. Blumberg, P.M. (1981). In vitro studies on the mode of action of the phorbol esters, potent tumor promoters. CRC Crit. Rev. Toxicol. 9, 153-197.

134. Slaga, T.J. (1983). Cellular and molecular mechanisms of tumor promotion. Cancer Surv. 2, 595-612.

135. Weinstein, I.B., Lee, L.S., Fisher, P.B., Mufson, A., and Yamasaki, H. (1979). Action of phorbol esters in cell culture: mimicry of transformation, altered differentiation and effects on cell membrane. J. Supramol. Struct. 12, 195-208.

136. Greenberg, M.E., and Ziff, E.B. (1984). Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. Nature 311, 433-438.

137. Kelly, K., Cochran, B.H., Stiles, C.D., and Leder, P. (1983). Cell-specific regulation of the *c-myc* gene by lymphocyte mitogens and platelet derived growth factor. Cell 35, 603-610.

138. Colamanici, O.R., Trepel, J.B., Vidal, C.A., and Neckers, L.M. (1986). Phorbol ester induces *c-sis* gene transcription in stem cell line K-562. Mol. Cell. Biol. 6, 1847-1850.

139. Lerman, M.I., and Colburm, N.H. (1987). Pro genes: a novel class of genes that specify sensitivity to induction of neoplastic transformation by tumor promoters. In Tumor Promotion: Biological Approaches for Mechanistic Studies and Assay Systems, R. Langenbach, J.C. Barrett, and E. Elmore, eds. (New York: Raven Press).

140. Whitham,S.E., Murphy, G., Angel, P., Rahmsdorf, H.J., Smith, B.J., Lyons, A., Harris, T.J.R., Reynolds, J.J, Herrlich, P., and Doherty, A.J.P. (1986). Comparison of human stromelysin and collagenase by cloning and sequence analysis. Biochem. J. 240, 913-916.

141. Lee, W., Haslinger, A., Karin, M., and Tjian, R. (1987). Activation of transcription by two factors that bind promoter and enhancer sequences of the human metalothionein gene and SV40. Nature 325, 368.

142. Chiu, R., Imagawa, M., Imbra, R.J., Bockoven, J.R., and Karin, M. (1987). Multiple *cis*- and *trans*-acting elements mediate the transcriptional response to phorbol esters. Nature 329, 648-651.

143. Baeuerle, P.A., Lenardo, M., Pierce, J.W., and Baltimore, D. (1988). Phorbol ester-induced activation of the NF-kB transcription factor involves dissociation of an apparently cytoplasmic NF-KB inhibitor complex. Cold Spring Harbor Symp. Quant. Biol. 53, 789-798.

144. Moye-Rowley, W.S., Harshman, K.D., and Parker, C.S. (1988). YAP1 encodes a yeast homolog of mammalian transcription factor AP-1. Cold Spring Harbor Symp. Quant. Biol. 53, 711-717.

145. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P., and Karin, M. (1987). Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated trans-acting factor. Cell 49, 727-739.

146. Curran, T., and Franza Jr., B.R. (1988). Fos and Jun: The AP-1 connection. Cell 55, 395-397.

147. Hirai, S.I., Ryseck, P.P., Mechta, F., Bravo, R., and Yaniv, M. (1989). Characterization of *junD*: a new member of the jun proto-oncogene family. EMBO J. 8, 1433-1439.

148. Prywes, R., Fisch, T.M., and Roeder, R.G. (1988). Transcriptional regulation of *c-fos*. Cold Spring Harbor Symp. Quant. Biol. 53, 739-748.

149. Norman, C., and Treisman, R. (1988). Analysis of serum response element function *in vitro*. Cold Spring Harbor Symp. Quant. Biol. 53, 719-726.

150. Ryder, K,, Lau, L.F., and Nathan, D. (1988). A gene activated by growth factor is related to the oncogene *v-jun*. Proc. Natl. Acad. Sci. USA. 85, 1487-1491.

151. Lee, W., Mitchell, P., and Tjian, R. (1987). Purified transcription factor AP-1 interacts with TPA inducible enhancer elements. Cell 49, 741-752.

152. Maki, Y., Bos, T.J., Davis, C., Stabuck, M., and Vogt, P.K. (1987). Avian sarcoma virus 17 carries the *jun* oncogene. Proc. Natl. Acad. Sci. 84, 2848-2852.

153. Ball, R.R., Jr., Bos, T.J., Loliger, C., Nagata, L.P., Nishimura, T., Su, H., Tsuchie, H., and Vogt, P.K. (1988). jun: oncogene and transcriptional regulator. Cold Spring Harbor Symp. Quant. Biol. 53, 687-693.

154. Vogt, P.K., Bos, T.Y., and Doolittle, R.F. (1987). Homology between the DNA-binding domain of the GCN4 regulatory protein of yeast and the carboxy-terminal region of a protein coded for by the oncogene jun. Proc. Natl. Acad. Sci. 84, 3316-3319.

155. Bohmann, D., Bos, T.J., Admon, A., Nishimura, T., Vogt, P.K., and Tjian, R. (1987). Human protoncogene c-jun encodes a DNA-binding protein with structural and functional properties of transcription factor AP-1. Science 238, 1386-1392.

156. Bohmann, D., Admon, A., Turner, D.R., and Tjian, R. (1988). Transcriptional regulation by the AP-1 family of enhancer-binding proteins: A nuclear target for signal transduction. Cold Spring Harbor Symp. Quant. Biol. 53, 695-700.

157. Short, N.J. (1988). Fraudulent promotion. Nature 331, 393-394.

158. Corsi, P.S., Lamph, W.W., Kamps, M., and Verma, I.M. (1988). fos-associated cellular p39 is related to nuclear transcription factor AP-1. Cell 54, 553-560.

159. Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Huter, T., and Karin, M. (1988). The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell. 54, 541-552.

160. Gentz, R., RauscherIII, Fj., Abate, C., and Curran, T. (1989). Parallel association of fos and jun leucine zippers juxtaposes DNA binding domains. Science 243, 1695-1699.

161. Kouzarides, T., and Ziff, E. (1989). Leucine zippers of fos, jun and GCN4 dictate dimerization specificity and thereby control DNA binding. Nature 340, 568-571.

162. Ransone, L., Visvader, J., Sassone-Corsi, P., and Verma, I.M. (1989). Fos-Jun interaction: mutational anlysis of the leucine zipper domain of both proteins. Genes Dev. 3, 770-781.

163. Kouzarides, T., and Ziff, E. (1988). The role of the leucine zipper in the fos-jun interaction. Nature 336, 646-651.

164. O'Shea, E.K., Rutkowski, R., Stafford III, W.F., and Kim, P.E. (1989). Preferential heterodimer formation by isolated leucine zippers from fos and jun. Science 245, 646-648.

165. Neuberg, M., Adamkiewicz, J., Hunter, J.B., and Muller, R. (1989). A Fos protein containing the Jun

leucine zipper forms a homodimer which binds to the AP1 binding site. Nature 341, 243-245.

166. Dynan, W.S. (1989). Modularity in promoters and enhancers. Cell 58, 1-4.

167. Ptashne, M. (1986). Gene regulation by protein acting nearby and at a distance. Nature 322, 697-701.

168. Wang, J.C., and Giaever, G.N. (1988). Action at distance along a DNA. Science 240, 300-304.

169. Plon, S.E., and Wang, J.C. (1986). Transcription of the human β-globin gene is stimulated by an SV40 enhancer to which it is physically linked but topologically uncoupled. Cell. 45, 575-580.

170. Johnson, P.F., and McKnight, S.L. (1989). Eukaryotic transcriptional regulatory proteins. Ann. Rev. Biochem. 58, 799-839.

171. Mitchell, P.J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245, 371-378.

172. Murre, C., Schonleber, McCaw, P., and Baltimore, D. (1989). A new DNA binding and demerization motif in immunoglobulin enhancer binding, daughterless, MyoD and myc proteins. Cell 56, 777-783.

173. Steitz, T.A., Ohlendorf, D.H., McKay, D.B., Anderson, W.F., and Matthews, B.W. (1982). Structural similarity in the DNA-binding domains of catabolite gene activator and cro repressor proteins. Proc. Natl. Acad. Sci. USA 79, 3097-3100.

174. Herr, W., Sturm, R.A., Clerc, R.G., Corcoran, L.M., Baltimore, D., Sharp, P.A., Ingraham, H.A., Rosenfeld, M.G., Finney, M., Ruvkun, G., and Horvitz, H.R. (1988). The POU domain: a large conserved region in the mammalian pit-1, oct-1, oct-2, and *Caenorahabditis elegans* unc-86 gene products. Genes Dev. 2, 1513-1516.

175. Sturm, R.A., Das, G., and Herr, W. (1988). The ubiquitous octamer-binding protein Oct-1 contains a POU domain with a homeobox subdomain. Genes Dev. 2, 1582-1599.

176. Miller, J., McLachlan, A.D., and Klug, A.(1985). Repetitive zinc binding domains in the protein

transcription factor IIIA from *Xenopus* oocytes. EMBO J. 4, 1609-1614.

177. Berg, J.M. (1986). Potential metal binding domains in nucleic acid binding proteins. Science 232, 485-487.

178. Evans, R.M., and Hollenberg, M. (1988). Zinc fingers: Gilt by association. Cell 52, 1-3.

179. Fairall,L. Rhodes, D., and Klug, A. (1986). Mapping of the sites of protection on a 5S RNA gene by the Xenopus transcription factorIIA. A model for the interaction. J. Mol. Biochem. 192, 577-591.

180. Kadonaga, J.T., Carner, K.R., Masiarz, F.R., and Tjian, R. (1987). Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell 51, 1079-1090.

181. Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1989). The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science 243, 1681-1688.

182. Landschulz, W., Johnson, P.F., and McKnight, S.T. (1988). The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. Science 240, 1759-1764.

183. Murre, C., McCaw, P.S., Vaessin, H., Caudy, M., Jan, L.Y., Jan, Y.N., Cabrera, C.V., Buskin, J.N., Hauschka, S.D., Lassar, A.B., Weintraub, H., and Baltimore, D. (1989). Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell 58, 537-544.

184. Krimpenfort, P., de Jong, R., Uematsu, Y., Dembic, Z., Ryser, S., von Boehmer, H., Steinmetz, M., and Berns, A. (1988). Transcription of T cell receptor β-chain genes is controlled by a downstream regulatory element. EMBO J. 7, 745-750.

185. McDougall, S., Peterson, C.L., and Calame, K. (1988). A transcriptional enhancer 3' of Cβ2 in the T cell receptor β locus. Science 241, 205-208.

186. Luria, S., Gross, G., Horowitz, M., and Givol, D. (1987). Promoter and enhancer elements in the

rearranged alpha chain gene of the human T cell receptor. EMBO J. 6, 3307-3312.

187. Winito, A. and Baltimore, D. (1989). A novel, inducible and T cell-specific enhancer located at the 3'end of the T cell receptor α locus. EMBO J. 8, 729-733.

188. Ho, I.C., Yang, L.H., Morle, G., and Leiden, J.M. (1989). A T-cell-specific transcriptional enhancer element 3' of Cα in the human T-cell receptor α locus. Proc. Natl. Acad. Sci. USA 86, 6714-6718.

189. Anderson, S.J., Chou, H.S., and Loh, D.Y. (1988). A conserved sequence in the T-cell receptor β-chain promoter region. Proc. Natl. Acad. Sci. USA 82, 3551-3554.

190. Lee, N.E., and Davis, M.M. (1990). T cell receptor β-chain genes in BW5147 and other AKR tumors: deletion order of murine Vβ gene segments and possible 5'regulatory regions. J. Immunol. 140, 1665-1675.

191. Diamond, D.J., Nelson, F.B., and Reinherz, E.L. (1989). Lineage-specific expression of a T cell receptor variable gene promoter controlled by upstream sequences. J. Exp. Med. 169, 1213-1231.

192. Anderson, S.J., Miyake, S., and Loh, D.Y. (1989). Transcription from a murine T-cell receptor Vβ promoter depends on a conserved decamer motif similar to the cyclic AMP response element. Mol. Cell. Biol. 9, 4835-4845.

193. Parslow, T., and Granner, D. (1982). Chromatin changes accompany immunoglobulins κ gene activation: a potential control region within the gene. Nature 299, 449-451.

194. Weischet, W., Glotov, B., Schnell, H., and Zachau, H. (1982). Difference in the nuclease sensitivity between the two allelles of the immunoglobulin κ light chain genes in mouse liver and myeloma nuclei. Nucleic Acids Res. 10, 3627-3645.

195. Chung, S-Y., Folsom, V., and Wooley, J. (1983). DNaseI hypersensitive sites in the chromatin of

immunoglobulin κ light chain genes. Proc. Natl. Acad. Sci. 80, 2427-2431.

196. Mills, F., Fisher, L., Kuroda, R., Ford, A., and Gould, H. (1983). DNaseI hypersensitive sites in the chromatin of human μ immunoglobulin heavy chain genes. Nature 306, 809-812.

197. Miyazaki, J., Appella, E., and Ozato, K. (1986). Negative regulation of the major histocompatibility class I gene in undifferentiated embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 83, 9537-9541.

198. Bier, E., Hashimo, Y., Greene, M.I., and Maxam, A.M. (1985). Active T-cell receptor gene have intron deoxyribonuclease hypersensitive sites. Science 229, 528-534.

199. Hashimoto, Y. (1989). T cell receptor β gene has two downstream DNase-I hypersensitive regions. J. Exp. Med. 169, 2097-2107.

200. Doerfler, W. (1983). DNA methylation and gene activity. Ann. Rev. Biochem. 52, 93-124.

201. Fukunaga, R., Matsuyama, M., Okamura, H., Nagata, K., Nagata, S., and Sakawa, Y. (1986). Undermethylation of interferon-gamma gene in human T cell lines and normal T lymphocytes. Nucleic Acids Res. 14, 4421-4436.

202. Sakamoto, S., Ortaldo, J.R., and Young, H.A. (1988). Methylation patterns of the T cell receptor β-chain gene in T cells, large granular lymphocytes, β cells, and monocytes. J. Immunol. 140, 654-660.

203. Sakamoto, S., and Young, H. (1988). Modification of T cell receptor β-chain gene. The Jβ2 region but not the Jβ1 region of the T cell receptor β-chain gene is hypomethylated in human B cells and monocytes. Nucleic Acid Res. 16, 2149-2163.

204. Fotedar, A., Boyer, M , Smart, W., Widtman, J., Fraga, E., and Singh, B. (1985). Fine specificity of antigen recognition by T cell hybridoma clones specific for poly-18: A synthetic polypeptide antigen of defined sequence and conformation. J. Immunol. 135, 3028-3033.

205. Dusty Miller, A., and Buttimore, C. (1986). Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Mol. Cell. Biol. 6, 2895-2902.

206. Blin, N. and Stafford, D.W. (1976). Isolation of high molecular weight DNA. Nucleic Acids Res. 3: 2303.

207. Feinberg, A.P., and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6-13.

208. Yamamoto, K.R., Alberts, B.M., Benziger, R., Lawhorne, L., and Treiber, G. (1970). Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. Virology 40, 734-744.

209. Glisin, V., Crkvenjakov, R., and Byus, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry 13, 2633-2637.

210. Maniatis, T., Firtsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

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

212. Gorman, C. (1985). High efficiency gene transfer into mammalian cells. In DNA Cloning, Vol.II, Glover, D.M. (ed.), IRL Press, Oxford, England, pp. 143-190.

213. Miller, J.H. ed. 1972. Experiments in molecular gentics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

214. Carcia, V. Bich-Thuy, L., Stafford, J., and Queen, C. (1986). Synergism between immunoglobulin enhancers and promoters. Nature 322, 383-385.

215. Siu, G., Strauss, E, C., Lai, E., and Hood, L.E. (1986). Analysis of human Vβ gene subfamily. J. Exp. Med. 164, 1600-1614.

216. Royer, H.D., and Reinherz, E.L. (1987). Multiple

promoter region of a T-cell recptor β-chain variable-region gene: evidence for tissue specificity. Proc. Natl. Acad. Sci. USA 84, 232-236.

217. Myers, R.M., Tilly, K., and Maniatis, T. (1986). Fine structure genetic analysis of a β-globin promoter. Science 232, 613-618.

218. Chadosh, L.A., Baldwin, A.S., Carthew, R.W., and Sharp, P.A. (1988). Human CCAAT-binding proteins have heterologous subunits. Cell 53, 11-24.

219. Charnay, P., Mellon, P., and Maniatis, T. (1985). Linker scanning mutagenesis of the 5'-flanking region of the mouse β major globin gene: sequence requirements for transcription in erythroid and nonerythroid cells. Mol. Cell. Biol. 5, 1498-1511.

220. Baniagmad, A.,.Muller, M., Steiner, C.H., and Renkawitz, R. (1987). Activity of two different silencer elements of the chicken lysozyme gene can be compensated by enhancer elements. EMBO J. 6, 2297-2303.

221. Borrelli, E., Hen, R., and Chambon, P. (1984). Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. Nature. 312, 608-612.

222. Cao, S.X., Gutman, P.D., Dave, H.P.G., and Schechter, A.N. (1989). Identification of a transcriptional silencer in the 5'-flanking region of the human ε-globin gene. Proc. Natl. Acad. Sci. USA 86, 5306-5309.

223. Goodbourn, S., Burstein, H. and Maniatis, T. (1986). The human β-interferon gene enhancer is under negative control. Cell 45, 601-610.

224. German, C.M., Rigby, P.W.J. and Lane, D.P. (1985). Negative regulation of viral enhancers in undifferentiated embryonic stem cells. Cell 42, 519-526.

225. Gnez,M., Land, H., Giesecke, K., Schutz, G.,Jung, A. and Sippel, A.E. (1981). Multiple mRNAs are generated from the chicken lysozyme gene. Cell 25, 743-752.

226. Kadesch, T., Zerros, P. and Ruezinsky, D. (1986). Functional analysis of murine IgH enhancer: evidence

for negative control of cell type specificity. Nucleic Acids Res. 14, 8290-8221.

227. Laimin, S.L., Holmgren-Konig,M., and Khoury, G. (1986). Transcriptional silencer element in rat repetitive sequences associated with the rat insulin 1 gene locus. Proc. Natl. Acad. Sci. USA. 83, 3151-3155.

228. Larsen, P.R., Harney, J.W., and Moore, D.D. (1986). Repression mediates cell-type specific expression of the rat growth hormone gene. Proc. Natl. Acad. Sci. USA. 83, 8283-8287.

229. Muglia, L., and Rothman-Denes, L.B.(1986). Cell type specific negative regulatory element in the control region of the rat α-fetoprotein gene. Proc. Natl. Acad. Sci. USA., 83, 7653-7657.

230. Nir, U., Walker, M.D., and Rutter, W.J. (1986). Regulation of rat insulin 1 gene expression: Evidence for negative regulation in nonpancreatic cells. Proc. Natl. Acad. Sci. USA. 83, 3180-3184.

231. Rosen, C.A., Sodroski, J.G., and Haseltine, W.A. (1985). The location of cis-acting regulatory sequences in the human T cell lymphotropic virus Type III (HTLV/LAV) long terminal repeat. Cell 41, 813-823.

232. Steiner, C., Muller,B., Baniahmad, A., and Renkawitz, R. (1987). Lysozyme gene activity in chicken macrophages is controlled by positive and negative regulatory elements. Nucleic Acids Res. 15, 4163-4178.

233. Stumph, W.E., Hodgson, C.P., Tsai, M.J. and O'Malley, B.W. (1984). Genomic structure and possible retroviral origin of the chicken CR1 repetitive DNA sequence family. Proc. Natl. Acad. Sci. USA. 81, 6667-6671.

234. Wasylyk, K.C., and Wasylyk, B. (1986). The immunoglobulin heavy chain B-lymphocyte enhancer efficiently stimulates transcription in non lymphoid cells. EMBO J. 5, 553-560.

235. Sullivan, K.E., Calman, A.F., Nakanishi, M., Tsang, S.Y., Wang, Y., and Peterlin, B.M. (1987). A model for the transcriptional regulation of MHC class II genes. Immunol. Today. 289-293.

236. Jamieson, C., Mauxion, F., and Sen, R. (1989). Identification of a functional NF-κB binding site in

the murine T cell receptor β2 locus. J. Exp. Med. 170, 1737-1743.

237. Noonan, D.J., Isakov, N., Theofilopoulos, A.N., Dixon, F.J., and Altman, A. (1987). Protein kinase C activating phorbol esters augment expression of T cell receptor genes. Eur. J. Immunol. 17, 803-807.

238. Lindsten, T., June, C.H., and Thompson, C.B. (1988). Transcription of T cell antigen receptor genes is induced by protein kinase C activation. J. Immunol. 141, 1769-1774.

239. Martinez-Valdez, H., Doherty, P.J., Thompson, E., Benedict, S.H., Gelfand, E.W., and Cohen, A. (1988). Antagonistic effects of calcium ionophores and phorbol esters on T cell receptor mRNA levels in human thymocytes. J. Immunol. 140, 361-366.

240. Lamph, W.W., Wamsley, P., Sassoni-Corsi, P., and Verma, I.M. (1988). Induction of protooncogene JUN/AP-1 by serum and TPA. Nature 3344, 629-631.

241. Saltzman, A.G., and Weinmann, R. (1989). Promoter specificity and modulation of RNA polymerase II transcription. FASEB J. 3, 1723-1733.

242. Cohen, C.R., Ferreira, P.C.P., Gentz, R., Franza Jr., B.R., and Curran, T. (1989). The product of a fos-related gene, fra-1, binds cooperatively to the AP-1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes. Genes & Dev. 3, 173-184.

243. Zerial, M., Toschi, L., Ryseck, R.P., Schuermann, M., Muller, R., and Bravo, R. (1989). The product of a novel growth factor activated gene, fosB, interacts with Jun proteins, encoding their DNA binding activity. EMBO J. 8, 805-813.

244. Franza Jr., B.R., Raucher III, F.J., Josephs, S.F., and Curran, T. (1988). The Fos complex and Fos-related antigens recognize sequence elements that contain AP-1 binding sites. Science 239, 1150-1153.

245. Falkner, F.G., and Zachau, H.G. (1984). Correct transcription of an immunoglobulin κ gene requires an upstream fragment containing conserved sequence elements. Nature 310, 71-74.

246. Banerji, J., Olson, L., and Schaffner, W. (1983). A lymphocyte specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell 33, 729-740.

247. Gillies, S.D., Morrison, S.L., Oi, V.T., and Tonegawa, S. (1983). A tissue specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell 33, 717-728.

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

249. Foster, J., Stafford, J., and Queen, C. (1985). An immunoglobulin promoter displays cell-type specificity independently of the enhancer. Nature 315, 423-425.

250. Mason, J.O., Williams, G.T., and Neuberger, M.S. (1985). Transcription cell type specificity is conferred by an immunoglobulin VH gene promoter that includes a functional consensus sequence. Cell 41, 479-487.

251. Neuberger, M.S. (1983). Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells. EMBO J. 2, 1373-1378.

252. Billard, D.W., and Bothwell, A. (1986). Mutational analysis of the immunoglobulin heavy chain promoter region. Proc.Natl.Acad.Sci. USA 83, 9626-9630.

253. Eaton, S., and Calame, K. (1987). Multiple DNA sequence elements are necessary for the function of an immunoglobulin heavy chain promoter. Proc.Natl.Acad.Sci. USA 84, 7634-7638.