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REGULATION OF T CELL RECEPTOR β CHAIN GENE EXPRESSION: DNA-PROTEIN INTERACTIONS

BY

THERESE WILLIAMS FULLER

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

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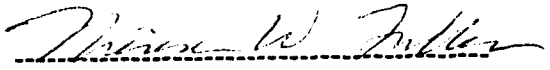

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

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submitted by THERESE W. FULLER

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE in the DEPARTMENT OF IMMUNOLOGY

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Preface

Parts of the work described in this thesis have since been published in the Journal of Immunology (Messier et al.,1992a). Results of the identification of the E4 motif have lead to the cloning of a transcription factor which binds this motif; these results have been submitted for publication Results of the identification and characterization of the DNA-protein interactions of the E3 motif have lead to the cloning of a transcription factor which binds this motif; these results have been submitted for publication.

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Abstract

T cell development within the thymus and mature T cell function rely on the surface expression of the T cell receptor. This expression is the result of coordinated rearrangement and transcription of the genes encoding the T cell receptor polypeptide chains. The mechanisms that control the transcription of the beta chain gene of the αβ T cell receptor heterodimer have been studied.

Specific binding of T cell nuclear proteins was observed within the TCR Vβ2 promoter. DNase I footprinting identified a protected region beginning at -90 and continuing downstream through an AP-1 site, an inverted repeat, and a conserved decamer motif. Another DNase I protected region mapped from -310 to -230 on the sense strand and from -287 to -258 on the antisense strand. These footprints correspond to functionally relevant promoter sequences.

DNase I footprinting of the TCR beta enhancer with T cell nuclear extracts identified four protected regions, E1 through E4. The E3 motif was suspected to be physiologically relevant because it was protected *in vivo*, conserved in both mouse and human β enhancers and is required for functional enhancer activity. Mobility shift assays demonstrated distinct specific binding of nuclear extracts from T cells, B cells and fibroblasts to overlapping oligos of the E3 motif. When the E3 motif was subdivided into the distinct oligos E3α, E3β and E3γ, differences in their abilities to compete for DNA-protein complexes were observed, suggesting that more than one protein interacts with this motif. UV crosslinking experiments with T cell nuclear extracts support this idea, identifying two protein complexes of 70 and 89 kd. The 70 kd protein complex was seen in UV crosslinking

experiments performed with nuclear extracts from T cells, pre-T cells, B cells, pre-B cells and fibroblasts. A cDNA clone corresponding to this protein has since been isolated by others, and has been found to be identical to the lupus autoantigen p70. The 89 kd protein was observed in T cells, pre-B cells and fibroblasts; however, in B cells and pre-T cells, a 60 kd protein complex was also observed.

Introduction

The immune system is a complex combination of cells and organs that develop and interact in a precisely organized and regulated fashion. A properly functioning immune system protects the organism from infection and disease and regulates itself to avoid self-reactivity. These functions are accomplished through communication between cells both directly via cell-cell contact and indirectly through the secretion of hormones. Lymphocytes develop from a common hematopoeitic stem cell within the bone marrow and can be distinguished based on their cell surface markers and their functions within the immune system. Two major classes of lymphocytes exist; B cells and T cells. B cells, which are central to the humoral immune system, recognize soluble foreign antigen and secrete antigen specific antibodies in response. In contrast, T cells are central to the cell mediated immune response. By virtue of a cell surface T cell receptor (TCR), T cells recognize foreign antigen presented in the context of the self major histocompatability complex (MHC) on the surface of antigen presenting cells. T cells are critical for the generation of cell mediated immune responses and are the primary regulatory cells in all immune responses.

The T cell receptor is a heterodimer composed of either the α and β polypeptides producing an $\alpha\beta$ TCR or the γ and δ polypeptides producing a $\gamma\delta$ TCR. Mature T cells express either the $\alpha\beta$ heterodimer or the $\gamma\delta$ heretodimer on the cell surface (Pardoll et al., 1987). The $\alpha\beta$ subset of T cells comprises the helper and cytotoxic T cells, the CD4+CD8- MHC Class II restricted and CD4-CD8+ MHC class I restricted T cell lineages

respectively. The functional importance of the γδ subset of T cells, sing only 1-10% of mature T cells, is an area of intensive study.

The immunoglobulin heavy (H) and light (κ and λ) chain genes as well as the T cell receptor α, β, γ, and δ chain genes are arranged to generate a huge degree of diversity in the antigen specificities of the resultant antigen receptor. Mature B and T cells are the result of multiple stages of differentiation that have been characterized and defined by changes in both the somatic arrangement and the expression patterns of their antigen receptor genes. The regulation of these genes is representative of the control mechanisms of genes of most higher eukaryotes.

Gene expression in higher eukaryotes is controlled at multiple levels, including the accessibility of genes to regulatory factors in condensed chromatin, the state of DNA methylation, and modulation at accessible promoters of the rate of transcriptional initiation (Gross and Garrard, 1987; Lewis and Bird, 1991). Post-transcriptional processes (e.g., pre-mRNA splicing, RNA transport, translational efficiency, and mRNA half-life) also play important roles in determining the level of gene expression (Leff et al., 1986; Fink, 1986; Brawerman, 1987).

Regulation of transcriptional initiation is clearly one of the most important and intensively studied of these processes. Regulatory DNA sequences that control the accuracy and rate of transcription initiation have been operationally divided into promoter and enhancer elements. Although there is undoubtedly a great deal of functional overlap between these cis-acting elements, promoter elements are generally located near the transcription initiation site and are required for a basal level of accurate

initiation of transcription. Enhancer elements, on the other hand, are often found clustered together further upstream or downstream of the transcription start site and can be responsible for regulating gene expression as a function of cell type, stage in the cell cycle, and in response to extracellular stimuli (Jones et al., 1988). These cis-acting control elements are composed of multiple sequence motifs which are the binding sites for transcription factors. While each enhancer or promoter element independently may not be able to confer absolute tissue specificity, in combination, these motifs can confer tissue specificity (Voss et al., 1986)

The way in which transcription is regulated by a promoter will depend upon the organization of its cis-acting elements in the DNA and upon the trans-acting protein factors which are present in the cell. The number and types of cis-acting elements in a regulatory region will determine how many and which trans-acting factors can interact. Because of stereospecific constraints, the positions of the regulatory elements relative to one another may also influence the way in which the transcription factors interact with each other, ultimately affecting transcription rates (Jones et al., 1988; Mitchell and Tjian, 1989; Johnson and McKnight, 1989).

Many mammalian transcription factors belong to families that contain multiple members which bind to highly related or identical DNA sequence motifs. In these cases, differential gene expression can be regulated in part by the tissue specific expression of transcription factors or by the formation of protein-protein complexes which can alter the DNA binding specificities of transcription factors (Frankel and Kim, 1991). An example of a protein-protein interaction which alters DNA binding specificity is the interaction of

the transcription factors c-fos and c jun (Nakabeppu et al., 1988); their heterodimerization is required for effective DNA binding.

The modular nature of transciption factors has been demonstrated by domain swap experiments in which the DNA binding domains and activation domains of transcription factors have been shown to confer their respective activities to a resulting chimeric transcription factor (Brent and Ptashne, 1985). The domains of transcription factors have been discovered to possess distinct structural motifs, allowing their classification on the basis of their structural homologies. These include the leucine zipper motif for protein dimerization (Landschulz et al., 1988), the zinc finger (zinc clusters and zinc twists; Vallee et al., 1991) and helix-turn-helix motifs involved in DNA-protein binding. Less ordered modules possess general 'acidic activation domains' involved in interactions with adjacent transcription factors after DNA binding (Ma and Ptashne, 1987). Sequence analysis of transcription factors has facitlitated their grouping into 'families' of proteins based on common 'domains', such as the homeodomain and the POU domain (Herr et al., 1988).

Changes in the level of transcription from a given promoter can occur by several different mechanisms. For example, the number or the specific activity of transcription factors can be controlled by covalent modification (such as phosphorylation or glycosylation) or by interaction with other factors including regulatory subunits, hormones, or other ligands. This complexity of control permits the precise regulation of transcription for developmental or tissue specific gene expression (Maniatis et al., 1987; Mitchell and Tjian, 1989).

During early lymphocyte development, the immunoglobulin heavy and light chain genes and the T cell receptor genes, each of which is composed of multiple variable (V), joining (J) and in some cases diversity (D) gene segments, undergo somatic rearrangement upstream of a constant (C) gene segment (Kronengerg et al., 1986 and Tonegawa, 1983). The rearrangements are mediated by conserved recombination signal sequences which flank each rearranging segment and consist of a palindromic heptamer and an A-T -rich nonamer separated by a spacer of either 12 or 23 base pairs (Hesse et al., 1989). Many of the rearrrangement events follow the looping/excision model of gene rearrangement (Alt et al., 1984). This model can be documented by the presence of closed circular DNA which represent excision products (Okazaki et al., 1987 and Okazaki and Sakano, 1988).

The generation of antibody and T cell receptor diversity is the result of three basic mechanisms: germline, combinatorial and junctional diversity, as well as somatic hypermutation for immunoglobulin genes only. Germline diversity is determined by the various gene segments which form the variable region of the resulting antigen receptor. Combinatorial diversity results from the random rearrangement of the different germ line gene segments. The amount of potential diversity that can be created by this mechanism is different for each polypeptide gene because it is dependent upon the number of V, D, and J gene segments, all of which differ for each gene. Junctional diversity is the result of many mechanisms involved in the joining of gene segments and includes the imprecise joining between gene segments and N-region diversification. (Kronenberg et al., 1986).

Through the use of artificial recombination substrates containing unrearranged V, D, or J segments, two lymphoid specific genes called the recombination activation genes, RAG-1 and RAG-2 were identified (Schatz and Baltimore, 1988, Schatz et al., 1989 and Oettinger et al., 1990). The identification of these genes supports the model which suggested that a common recombinase is active in both pre-T and pre-B cells and is responsible for mediating the rearrangement events in each of these cell types (Alt et al., 1986). Most recent support for this model comes from research on mice which are defective for either RAG-1 or RAG-2. In these mice, no mature B and T lymphocytes are seen due to their inability to perform V(D)J rearrangements (Mombaerts et al., 1992 and Shinkai et al., 1992).

The rearrangement events of both the TCR and immunoglobulin genes are restricted to lymphocytes and occur in an ordered sequence during lymphocyte development. Although Ig and TCR D-J rearrangements and sterile transcripts can be observed in both B cells and T cells, B cells never completely rearrange TCR genes, nor do T cells completely rearrange Ig genes. The rearrangement occurs in ordered stages; D-toJ rearrangement occurs first and is followed by V-to-DJ rearrangement. In pre-B cells, V(D)J rearrangement at the Ig heavy-chain (IgH) locus precedes rearrangement at the Ig light-chain (IgL) locus (Alt et al., 1987). In an analogous manner, rearrangement at the TCRβ locus precedes rearrangement at the TCRα locus in pre-T cells (Born et al., 1985).

The rearrangements of both the Ig and TCR gene segments are mediated in part by the interactions of the products of the RAG-1 and RAG-2 genes early in development, supporting a model which suggests that the

accessibility of germ line gene segments to a common recombinase plays an important role in the regulation of the temporal and tissue specific assembly of these genes in the respective lymphocytes. This model has also been termed the 'accessibility model' for gene regulation and rearrangement. It proposes that the transcription of germline gene segments establishes an open or accessible conformation of the chromatin, thus allowing it to be accessible to rearrangement events (Yancopoulos and Alt, 1986). The detection of sterile germline transcripts from unrearranged V_H segments at the IgH locus prior to or during V_H-to-DJ_H rearrangement as well as the disappearance of these transcripts once productive rearrangement has occurred supports the accessibility model for gene regulation (Lennon and Perry, 1990). It has been shown in the IgL κ locus that the appearance of unrearranged Jκ-Cκ transcripts precedes Vκ to Jκ rearrangement, again suggesting that these sterile transcripts somehow facilitate the rearrangement process (Oettinger et al., 1990). Similar events have been demonstrated for both the TCR β locus, in which incompletely rearranged Dβ-Jβ transcripts are apparent during T cell development (Snodgrass et al., 1985) and for the TCR α locus, in which a direct correlation has been demonstrated between transcriptionally active Vα gene segments and ongoing Vα-to-Jα gene rearrangements (Fondell and Marcu, 1992).

The rearrangement events that occur in the immunoglobulin heavy chain gene bring the H enhancer into closer proximity to the V_H promoter. Transcription from the V_H promoter is then increased, presumably due to its location with respect to the H enhancer (Gilles et al., 1983). This has been demonstrated to be the case for the increase in the transcription of sterile

DJ_H transcripts following D-to-J_H rearrangement. The increase in DJ_H transcripts has been shown to be directly correlated to the approximation of the D promoter and the Ig heavy chain enhancer (Alessandro and Desiderio, 1991).

The mouse immunoglobulin heavy and light chain enhancers were the first examples of cellular enhancers and have served as useful models for enhancer function regarding tissue specificity and developmental regulation. As was seen for the SV40 enhancer, the IgH enhancer is composed of multiple, distinct DNA sequence motifs, each of which is recognized by nuclear factors. Multiple B cell nuclear proteins were found to interact with the immunoglobulin enhancer *in vivo* (Ephrussi et al., 1985). Many nuclear factors which bind within immunoglobulin enhancers have been cloned including, TFE-3 which interacts with the IgH enhancer (Beckmann et al., 1990), Oct-1 and Oct-2, and NF-κB (Kawakami et al, 1988).

The Ig enhancers display the typical enhancer characteristics, including distance and orientation independence and cell-type specificity, mediating transcriptional activation preferably in lymphoid cells (Gilles et al, 1983, Picard and Schaffner, 1984, and Spandidos and Anderson, 1984). The immunoglobulin promoters act in combination with their homologous enhancer elements to confer B cell specific activity (Garcia et al., 1986).

As has been demonstrated for the immunoglobin genes, regulatory regions of T cell receptor genes have been identified and partially characterized with respect to tissue specificity and interactions with nuclear proteins. The enhancers of the TCR α (Winoto and Baltimore, 1989), β (Krimpenfort et al., 1988 and McDougall et al., 1988) and γ (Spencer et al.,

1991 and Kappes et al., 1991) genes are located at the 3' end of their respective genes, and the TCR δ enhancer (Gill et all, 1991; Redondo et al, 1990) is located within the J and C gene segments. The locations of these enhancers, being 3' of the J gene segments as is seen for the immunoglobulin genes, agree with the model that nonrearranged variable gene segments need to be brought into closer proximity to a downstream enhancer for full transcriptional activity (Yancopoulos and Alt, 1985). The enhancers of all four TCR genes identified have been found to be lymphoid specific and, in the case of the α chain, required by V region promoters to confer lymphoid specific activity. The TCRα chain gene enhancer is the best characterized of all the TCR genes (see Leiden, 1992 for review). This enhancer has been localized to a region 4.5 kb downstream of the α-chain gene (Ho et al., 1989 and Winoto and Baltimore, 1989a), with the core enhancer motif being localized to a 100 bp region. This core enhancer has been shown to bind several proteins, including a member of the cAMP-responsive transcription factor family, or CREB/ATF (Ho et al, 1989), the lymphoid specific factor LEF-1 (Travis et al., 1991), the T cell specific factor, TCF-1α (Waterman and Jones, 1990), and TCF-2α (Ho et al, 1990), the latter of which may be a member of the c-*ets* family of transcription factors.

The coordinated expression of T cell receptor genes has been shown to be important in the expression of a single type of T cell receptor on its cell surface. This expression is regulated in part at the level of transcription. For example, the TCR α enhancer has been shown to be active in αβ but not in γδ T cells due to the presence of negative cis-acting elements flanking the TCR α enhancer which are able to silence enhancer activity in γδ but not in αβ T cells (Winoto and Baltimore, 1989). A silencer

element is also thought to be associated with the TCR γ gene which represses the expression of productively rearranged γ and δ genes in γδ transgenic mice and allows for the production of a normal number of αβ T cells (Ishida et al., 1990).

After pre-T cells seed the thymus, each of the TCR genes displays time- and tissue- specific gene rearrangement and expression. The transcription of TCR genes occurs in a developmentally regulated fashion, with the g and d genes first, followed by the b gene and then the a gene (Royer et al., 1984). The thymus provides the necessary microenvironment for differentiation of T lymphocytes from pre-T cells to immature T cells to mature T cells. Various stages of T cell development occur in various thymic compartments during which differential surface expression of thymocyte antigens and antigen receptors occurs (for reviews see Strominger, J.L., 1989 and Davis, M.M., 1990).

The cell surface expression of the T cell receptor is required for the selective events termed 'negative' and 'positive' selection which occur within the thymus. Thymocytes which express a TCR that has a high affinity for self-MHC are eliminated by negative selection, however, those thymocytes which express TCR that have a low affinity to self MHC are 'positively selected' and allowed to mature (Sprent et al, 1988 and Schwartz, 1989). The result of negative and positive selection events within the thymus is a peripheral T cell population that is able to recognize foreign antigen in association with self-MHC and yet remains tolerant to self antigens.

Because the expression of the T cell receptor gene is crucial for the selection events which take place during the development of T cells, proper

TCR gene regulation is a prerequisite to the formation of a mature, peripheral T cell population. The transcriptional regulation of the TCR β chain gene has been the focus of this thesis. By studying the regulation of TCR gene expression, I hope to understand the steps which lead to the T cell lineage and the processes by which TCR gene expression is restricted to this cell type.

T cell receptor β chain gene regulation

The Vβ promoter has been shown to be critical for the specific expression of the TCR Vβ chain gene and to be required for rearrangement and expression of the TCR β chain in cultured cells and transgenic mice (Ferrier et al., 1990). The Vβ promoter has also been shown to act in a tissue specific manner in transfection analyses (Diamond et al., 1989 and Ratanavongsiri et al., 1990). Within the Vβ promoters, many potential sites for interaction with transcription factors have been identified (Ratanavongsiri et al., 1990 and Diamond et al., 1989) including CAAT boxes (Siu et al., 1986), inverted repeats, direct repeats, as well as a conserved decanucleotide sequence (5'AGTGACATCA3') (Anderson et al., 1988). This decamer has sequence similarity to the AP-1 and cyclic AMP response element binding protein (Atf/CREB) binding sites and has been shown to be important in TCR gene transcription (Anderson et al., 1989). This motif is now known to be bound by the transcription factor CREB as well as the recently cloned factors TCR-ATF1 and TCR-ATF2 (Lee et al., 1992).

The β enhancer is localized to a region 4.5 kb downstream of Cβ2 and has been shown to play an important role in TCR gene expression in T cells in transient expression assays and in transgenic mice (Krimenfort et

al., 1988 and McDougall et al., 1988). The smallest functional fragment of the enhancer was localized to a 500 bp fragment. This element displays the typical enhancer characteristics upregulating promoter activity in a distance and orientation independent manner. In addition, Ratanavongsiri et al (1990) have shown that the TCR β enhancer can overcome the suppressive effect of a negative regulatory element upstream of the Vβ2 promoter.

Background and Rationale

Previously, multiple cis-acting elements were demonstrated to be important for the transcription of the rearranged Vβ2 gene (Ratanavongsiri et al., 1990) as shown in Figure 1. The TCR β gene studied was derived from a T cell hybridoma B.1.1, which is specific for the polypeptide poly-18 $(EYK(EYA)^5)$ in the context of IA^d (Fotedar et al., 1985). The β gene was cloned and sequenced and was found to utilize Vβ2. The promoter region of the TCR Vβ2 gene was mapped functionally using transient expression assays to -85 to +31. Sequences from -42 to +31 were transcriptionally silent. An upregulatory sequence was mapped to -343 to -86 by virtue of its ability to enhance transcription from this promoter when present upstream. In addition, an upstream silencer element was identified, by virtue of its ability to decrease promoter activity.

Within the TCR Vβ2 promoter region are sequences which are putative binding sites for transcription factors. These include an AP-1 sequence (-82 to -75), inverted repeats (-73 to -63 and -149 to -139), decamer homologous regions (-63 to -54 and -257 to -248), a direct repeat (-170 to -151) and a CAAT box homology (-288 to -294) (Ratanavongsiri et al., 1990).

The tissue specificity of these elements has also been addressed. The Vβ2 promoter has been shown to be active in T cells but not B cells, but it is active in fibroblasts. The β enhancer has been shown to be able to enhance transcription from the Vβ2 promoter in T cells in transient expression systems. Interestingly, the enhancer decreases transcription from the TK promoter in fibroblasts, but increases transcription from the TK promoter in T cells (Ratanavongsiri et al., 1990).

Because TCR β expression has been well characterized and its cis-acting regulatory regions identified, I chose to study where within these regions DNA-protein interactions were occuring and how they might be involved in T cell specific expression of the β chain gene. To do this, I performed DNA-protein binding assays using multiple regions of the TCR Vβ2 promoter and the β enhancer. I have found several regions within these regulatory sequences that interact with nuclear extracts from T cells and, in the case of a DNA sequence within the β enhancer, nuclear extracts from fibroblasts, B cells, pre-T and pre-B cells as well. These results provide the basis for furthering our understanding of the molecular basis for T lineage differentiation and contribute to the identification of nuclear factors that are important in the expression of the TCR β chain gene.

Methods

General Molecular Biology Techniques

Trapping DNA inserts using agarose gel electrophoresis

DNA regions of interest which had been cloned into the pUC18 vector were cut out with appropriate enzymes within the polylinker before separating them from vector DNA by agarose gel electrophoresis. The inserts were trapped with DE-81 paper (Whatman) by inserting the paper ahead of the DNA in the electrophoretic field and continuing electrophoresis until the DNA was transferred to the paper. The DE-81 paper was washed three times in 10 mM Tris pH 7.4, 0.1 mM EDTA, 0.1 M NaCl. To elute the DNA, the paper was washed with 10 mM Tris pH 7.4, 0.1 mM EDTA, 1 M NaCl. The resulting DNA was precipitated after the addition of an equal volume of water and then four volumes of ethanol.

Blunt Ending inserts

Before cloning DNA into the pUC18 vector, the overhangs generated by restriction enzymes when the insert was cut out of the previous vector were blunted using T4 DNA polymerase. DNA (1ug) was incubated with 2.5 units of T4 polymerase and 200 uM dNTPs in 33 mM Tris-acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol and 100 ug/ml BSA at 37°C for 15 minutes (reaction volume of 20 ul). To stop the reaction, 1 ul of 0.5 M EDTA was added and then the mixture was phenol/chloroform (1:1) extracted and ethanol precipitated.

Preparation of Vector DNA

The plasmid DNA pUC18 was digested with either SmaI before the cloning of blunt ended inserts, or HindIII before the cloning of inserts cut

with HindIII (see below). After digestion, pUC18 DNA was dephosphorylated with bacterial alkaline phosphatase (BAP) (USB) prior to ligation in order to prevent self-ligation. To do this, 1 ug of DNA was incubated with 1 unit of BAP in 50 mM Tris (pH 8.0) at 65^{o}C for 30 minutes (reaction volume of 20 ul) and the reaction was then stopped by the addition of 1 ul of 0.5 M EDTA, after which the DNA was phenol/chloroform (1:1) extracted twice and then ethanol precipitated.

Ligation Reations

Both blunt and sticky end ligations were performed by incubating vector and insert DNA at a 1:1 molar ratio in 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 5% polyethylene glycol (PEG), 1 mM ATP, 1 mM DTT and 1 unit T4 ligase in a reaction volume of 10 to 25 ul. The reaction was incubated at room temperature for at least 4 hours before it was stopped by adding 1 ul of 0.5 M EDTA.

Annealing Oligos

All oligos were annealed by incubating sense and antisense oligos at a concentration of 0.1 ug/ul in 100 mM NaCl, 10 mM Tris (pH 7.9), 0.1 mM EDTA, and allowing slow cooling to room temperature over 1-2 hours from 85^{o}C.

End-labeling DNA inserts

DNA of interest which was cloned into a vector such as pUC 18 was cut with a restriction enzyme present within the polylinker to give a 5' overhang. The DNA was then precipitated and washed once with 80% Ethanol. 5 ug of linearized plasmid DNA containing the relevant DNA insert was then incubated with 25 uM dNTPs (excluding dCTP), α^{32}P dCTP and Klenow in a final volume of 25 ul and was allowed to incubate for 20

minutes at room temperature. Unincorporated nucleotides were removed by passing the reaction over a 1 ml column containing Sephadex G-50. The DNA was then digested with an appropriate enzyme which allowed the removal of the insert of interest from the plasmid. To purify the labeled insert from vector DNA, the reaction was run on a 6% non-denaturing polyacrylamide gel and then visualized by autoradiography. The end-labeled insert was then cut out of the gel and allowed to elute into 0.5 ml TE overnight at 37°C. The probe was phenol-chloroform extracted and ethanol precipitated before use.

Kinase labeling oligos

5 ng of both the sense and antisense strands of DNA were incubated with 2 ul of 10X kinase buffer (500 mM Tris-Cl, 100 mM $MgCl_2$, 50 mM DTT, 1 mM spermidine, 1 mM EDTA) in a final volume of 20 ul and heated at 85°C for 2 minutes before chilling on ice. 10 uCi of ^{32}PdATP and 10 U T4 polynucleotide kinase were added to the DNA and then the reaction was allowed to incubate at 37°C for 30 minutes. 100 ul of 2.5 M ammonium acetate was then added and the reaction was incubated at 70°C for 15 minutes before cooling slowly to room temperature. 10 ug of total yeast RNA was then added and the reaction was ethanol precipitated. The DNA was resuspended in TE and then phenol-chloroform extracted once before being reprecipitated.

Preparation of Competent Host Cells

A 500 ml bacterial culture was grown by inoculating 5 ml of an overnight TB-1 culture into TB broth (for 1 L: 2.31 g KH_2PO_4, 12.5 g K_2HPO_4, 12 g Tryptone, 4 ml glycerol) and shaken vigorously at 37°C until the OD_{610} was between 0.4 and 0.6. The culture was centrifuged at 4,000

rpm for 10 minutes, after which the pellet was resuspended in 250 ml sterile, ice cold 50 mM $CaCl_2$, left on ice for 30 minutes, and then spun at 4,000 rpm for 10 minutes. The bacteria were then resuspended in 50 ml of cold 50 mM $CaCl_2$/ 15% glycerol and frozen in small aliquots at -70°C.

Transformation of Competent Bacteria

DNA from a ligation mix (10 to 100 ng) was added to 50 ul of competent host cells and incubated on ice for 1 hour. The bacteria were then heat shocked at 42°C for 2-3 minutes and plated on agar plates containing ampicillin , X-Gal (5-bromo-4-chloro-3-3indolyl-β-D-galactoside) and IPTG (Isopropyl-β-D-thiogalactopyranoside). The plates were incubated overnight at 37°C.

Rapid Plasmid Preparation and Double Stranded Sequencing

An overnight culture of TB-1 (10 ml) grown in TB media was centrifuged at 4,000 rpm for 5 minutes after which the pellet was resuspended by vortexing in 250 ul of 250 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA. To the suspension, 500 ul of 0.2 M NaOH and 1 % SDS was added and then mixed by inversion for 3 minutes before adding 250 ul of 5 M potassium acetate (equal volume of 3 M potassium acetate and 2 M acetic acid). The sample was then centrifuged at 4,000 rpm for 5 minutes and then the supernatant was transferred to a new eppendorf tube in which the plasmid DNA was precipitated by the addition of 0.5 ml of isopropanol. The resulting DNA pellet was dissolved in 20 ul of TE (10 mM Tris pH 8.0, and 1 mM EDTA) and treated with RNase A (40 ug/ml) at 37°C for 30 minutes. An equal volume of 13% PEG in 1.6 M NaCl was added to precipitate the DNA. The DNA pellet was dissolved in 100 ul of TE, phenol-chloroform extracted (1:1), chloroform extracted, and ethanol precipitated in

the presence of 0.5 M ammonium acetate. The pellet was rinsed and resuspended in 10 ul of TE and then stored at 4°C.

Before sequencing, the plasmid DNA (2-4 ug) was treated with 0.4 M NaOH at room temperature for 5 minutes, ethanol precipitated with 0.3 M sodium acetate, allowed to dry and then dissolved in 8 ul distilled water.

Dideoxy sequencing was performed using the Sequenase (US Biochemicals) enzyme. The alkali denatured plasmid DNA (in 7 ul) was mixed with 1 ul primer (5 ng/ul) and 2 ul of 5X sequencing buffer (200 mM Tris pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), incubated at 65°C for 2 minutes and then allowed to cool slowly to room temperature over 30 minutes to 1 hour. To this mixture, 2 ul of diluted labeling-mix (1:5; labeling mix: 7.5 uM dGTP, 7.5 uM dCTP, 7.5 uM dTTP), 1 ul 0.1 M DTT, 0.5 ul $\alpha^{35}S$ dATP (1,000 Ci/mmole), 2 ul of diluted sequenase (1:8) and incubated at room temperature for 5 minutes. After incubation, 3.5 ul aliquots of the labelling reaction were added seperately to 2.5 ul of either ddATP, ddTTP, ddCTP, or ddGTP termination mix. These reactions were then incubated at 37°C for 15 minutes and then stopped with 4 ul of sequencing dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) before being heat denatured and run on a 8% denaturing polyacrylamide gel (8% polyacrylamide (19:1 acrylamide:bisacrylamide), 8 M urea) in 1X TBE (50 mM Tris base, 50 mM boric acid, 1 mM EDTA). After the gel was run, it was dried and exposed to X-ray film overnight.

Large scale plasmid prep

250 ml of transformed bacteria were grown overnight in TB broth with 50 ug/ml ampicillin. The cells were pelleted at 5K in a GSA rotor for 10 minutes. The cell pellets were resuspended in 7.2 ml glucose solution (50

mM glucose, 25 mM Tris(pH 8), 10 mM EDTA), lysed by the addition of 1.2 ml lysozyme solution (20 mg/ml lysozyme in glucose solution) and allowed to shake gently for 10 minutes at room temperature. 16.5 ml alkaline SDS (0.2 N NaOH, 1% SDS) was added to the cells and they were put on ice for 5 minutes. 8.4 ml 5M potassium acetate (pH 4.8) was then added and then the cells were put back on ice for 15 minutes. The bacterial debris was removed by a 10K, 10 minute spin (SS34 rotor). The supernatant was then extracted with phenol-chloroform (1:1) and the resulting aqueous phase was precipitated with isopropanol. The pellet was resuspended in 2.5 ml TE and 4 g cesium chloride was added and dissolved before 0.2 ml of EtBr (10 mg/ml) was added. This mix was then added to a quick seal ultracentrifuge tube below 8 ml of a cesium chloride solution of a density of 1.47 g/ml. This was spun at 65,000 rpm in a Ti80 rotor (Beckman) for 5 hours at 22°C. The plasmid band at the interphase of the two CsCl solutions was removed and extracted with water saturated butanol to remove the ethidium bromide. The DNA was then dialyzed against TE (pH 8) to remove the cesium chloride.

Cells

The TCR αβ positive mouse T cell lymphomas EL-4, YAC-1 and S49.1 were obtained from ATCC, as was the immunoglobulin secreting mouse myeloma MPC-11 and the mouse embryo fibroblast NIH/3T3. The pre-B cell BASCI/C2 was obtained from Dr. John Elliott at the University of Alberta. The pre-T cells 2052C and 2017 obtained from Whitehead Institute. TPA induced cells were prepared by addition of TPA (dissolved in DMSO at 10^{-2}M) at a concentration of 60-100 ng/ml eight to ten hours prior to harvesting.

Nuclear Extracts

Nuclear extracts were prepared by the method of Dignam et al., 1983. Cells were harvested at a concentration of 0.4 to 0.8 x 10^6 cells per ml and then washed once in 5 packed cell volumes of PBS (4°C) and then centrifuged 2,000 rpm for 10 minutes at 4°C before being resuspended in 5 packed cell volumes of Buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT) and allowed to swell for 10 minutes at 4°C. The cells were centrifuged for 10 minutes at 2,000 rpm, resuspended in 2 volumes of Buffer A and lysed by douncing with a Kontes all glass dounce homogenizer (lysis was checked by light microscopy). After centrifugation, the supernatant was removed and the pellet was spun at 25,000 x G for 20 minutes before being resuspended (3 ml per 10^9cells) in Buffer C (20 mM Hepes pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, and 2 mg/ml each of: aprotinin, leupeptin, pepstatin A, trypsin inhibitor, benzamide), dounced 10 times, stirred gently 30 minutes and centrifuged for 30 minutes at 25,000 x G. The supernatant was dialysed against 50 volumes of buffer D (20 mM Hepes pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) for 5 hours and then centrifuged for 20 minutes at 25,000 x G. The supernatant was aliquoted and frozen in liquid nitrogen and stored at -80°C.

When extracts were made with 16 - 32 litres of cells, a modified version of this protocol was used. The cells were harvested by centrifugation at 4 K for 15 minutes in a Sorvall TC-3. The cells were then washed with 1XPBS containing 10 mM $MgCl_2$ and then spun at 3 K for 10 minutes. The pellet was then resuspended in 4 packed cell volumes of Buffer H (10 mM Tris HCl, pH 7.9, 10 mM KCl, 1 mM DTT, 0.75 mM

spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA) and allowed to swell 15 minutes on ice. The cells were then dounced 8 times on ice with a B pestle and lysis was checked by microscopy. The burst cells were then spun at 2,000 xG for 8 minutes in polycarbonate tubes in an SS34 rotor and the supernatant was carefully decanted. The nuclei were resuspended in buffer H and spun at 4,000 xG for 7 minutes after which they were stored at -70°C. Nuclei were then resupended in 4 PCV of Buffer D (50 mM Tris-HCl, pH 7.5, 10% sucrose, 0.42 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol, 2 mM DTT, 0.1 mM PMSF, with 2 mg/ml each of: aprotinin, leupeptin, pepstatin A, trypsin inhibitor, benzamidine) and stirred on ice for 30 minutes before spinning at 25,000 xG for 60 minutes. The supernatant was then precipitated with 30% ammonium sulfate and then the pellet resuspended in TM 0.1 (20 mM HEPES pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) and then dialyzed against TM 0.1.

A smaller scale nuclear extract protocol was also used. Cells were washed with 10 ml cold 1X PBS three times. For adherent cells, 2 ml 1X PBS with 10 mM EDTA was then added to each plate and allowed to stand 3 - 5 minutes before being scraped off with a rubber policeman. Cells were then pelleted by spinning 7 minutes at 1.2 K rpm before being resuspended in 1X PBS with 10 mM $MgCl_2$. The packed cell volume was determined after pelleting the cells. Buffer H was added (large scale protocol above) and cells were homogenized using a minidounce before being transferred to 1.7 ml eppendorf tubes and spun at 5500 rpm for 8 minutes at 4°C. The supernatant was then decanted and the nuclei were resuspended in Buffer D (Large scale protocol above) and rotated for 30 minutes at 4°C. The

extract was then spun at 15,000 xG for 30 minutes at 4°C and the supernatant was dialyzed against TM 0.1.

The protein concentrations of nuclear extracts were determined using Bio-Rad's Protein assay with BSA as a standard.

DNase I Footprinting Assay

50,000 to 100,000 cpm of single end labeled DNA was allowed to bind with 50 - 100 ug of nuclear extract in the presence of poly dI-dC in binding buffer (see gel shift protocol) for 20 minutes. DNAse I (Worthington), which had been diluted in ice cold water just before use, was added. After one minute, 100 ul of DNAse I stop solution was added (20 mM EDTA (pH 8), 1% SDS, 0.2 M NaCl, 250 ug/ml carrier tRNA). The reaction was then phenol-chloroform extracted, ethanol precipitated, washed and resuspended in formamide dye. The sample was then resolved on an 8% sequencing gel. Control reactions included end-labeled probe digested with DNase I in the absence of nuclear extract and Maxam-Gilbert reactions.

Maxam Gilbert sequencing was performed by spotting 100,000 - 200,000 cpm of end-labeled probe on to Hybond M&G membranes (Amersham) and carrying out reactions specific for G cleavage and G+A cleavage (Maxam and Gilbert, 1980).

Mobility Shift Assays

Mobility shift assays were performed by binding 25,000 - 50,000 cpm of end-labeled probe with 5 - 15 ug of nuclear extract in a binding buffer used by Singh et. al. (1986) (10 mM Tris, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol) and in the presence of 25ng - 2 ug of poly dI-dC. The binding reactions took place at room temperature for 15 minutes in the

absence of the endlabeled probe and then another 15 minutes after the addition of the probe. If a specific competitor DNA was included in the binding reaction, it was added with the radioactive probe after the nuclear extract had preincubated with cold poly dI-dC. The optimal concentration of poly dI-dC was titrated for each binding reaction.

The binding reaction was then resolved by 4% nondenaturing PAGE in 1X TBE. The gels were run at 20-30 mA and then exposed to X-Ray film overnight at -70^{o}C.

The optimum conditions for binding and electrophoresis varied with the DNA probes used and are indicated in the figure legends.

UV Crosslinking Assay

This assay utilized a bromodeoxyuridine substituted radiolabeled probe that was created by annealing a 25 to 30 base 'template' DNA with a 10 base primer that was complementary to the 3' terminal 10 bases of the template and then synthesizing the complementary strand incorporating radiolabeled and cold nucleotides and the thymidine analogue 5'-bromo-2'-deoxyuridine-5'-triphosphate (Boehringer Mannheim Cat. #103080). The synthesis reaction mixed 10 pmoles of template with 500 pmoles of primer in a volume of 1 - 5 ul at 10 to 50 mM NaCl. The template and primer were heated at 80^{o}C for 3 minutes and allowed to anneal with slow cooling over at least one hour. After annealing, 5 ul of each of the two chosen α^{32}P-dNTPs (eg., G and A), 1 ul each of 100 mM stock of the two other unlabeled dNTPs, 1 ul of 100mM BrdU, and 5 U of Klenow were added in a reaction volume of 20 ul with the salt concentration at 50 mM NaCl. This reaction was then incubated at 16^{o}C for 2 hours and then chased with 1 ul of unlabeled dNTP mix containing each nucleotide at 25 mM and 5 U of

Klenow and incubated at 16°C for another 30 minutes. 80 ul of TE was added and then the reaction was phenol extracted once. Unincorporated nucleotides were removed by running the reaction mixture over a Push Column (Stratagene), after which 20 ug of tRNA was added and the reaction was ethanol precipitated.

When the binding reaction was set up using the substituted probe, 10^6 cpm of probe were used per reaction, nuclear extract concentration was increased to approximately 20 ug, the same binding buffer was used as in gel shift assays and the poly dI-dC was titrated as in gel shift assays. After binding, the reaction was resolved on a 4% nondenaturing PAGE in 1X TBE. After the gel had been run, it was exposed to X-ray film and then to UV light for 30 minutes using a Fotodyne transilluminator (305nm). The gel was aligned with the film and the shifted bands were cut out and placed in microfuge tubes which contained 100 ul of 2X SDS-PAGE sample buffer (Laemmli SDS buffer) containing DTT or 2-ME. The gel slice was then incubated at 100°C for 5 to 10 minutes and then resolved by 10% SDS-PAGE along with ^{14}C molecular weight markers (Amersham Rainbow protein molecular weight markers).

A slight modification of this procedure, termed 'In Tube' UV crosslinking allows the observation of UV crosslinked proteins without prior resolution on a 4% polyacrylamide gel. This method also can demonstrate competition for protein binding with cold oligos. The binding reaction was exposed to the UV light of the transilluminator after binding by inverting the reaction tube over the transilluminator for 30 minutes and then adding SDS buffer, boiling and running on 10% SDS-PAGE as above.

Results

Identification of DNA-protein interactions within the TCR Vβ2 Promoter

The T cell receptor Vβ2 promoter has been shown in transient expression assays to extend from -85 to +31. The -343 to -86 region of the Vβ2 promoter has been shown to upregulate the transcriptional activity of the Vβ2 promoter. While transient expression assays are useful in delineating the regulatory regions of a gene, they do not demonstrate directly the interaction of nuclear transcription factors within these regions. In order to assess if T cell nuclear proteins can bind these transcriptionally active regions, DNA-protein binding assays were undertaken. A series of DNA fragments within the -343 to +31 region of the Vβ2 promoter were generated (Figure 2) in order to facilitate the mapping of DNA sequences bound by nuclear proteins.

Nuclear protein binding studies of the TCR Vβ2 Minimal Promoter

In order to assess if T cell nuclear proteins can bind the minimal promoter, mobility shift assays were performed using the -85 to +31 fragment. The mobility shift assay, or gel shift assay, relies on the ability of a DNA-binding protein to alter the mobility of a ^{32}P end-labeled DNA probe in an electrophoretic field. The specificity of the DNA-protein interaction can be assessed by including in the binding reaction, unlabeled DNA of either a specific or nonspecific regulatory region of the gene. The interaction is termed 'specific' if excess unlabeled DNA of the same region can compete for the DNA-protein interaction. The competition is evidenced as a decrease in intensity of the shifted band on autoradiograms.

Mobility shift assays using the -85 to +31 probe demonstrate that when this probe is incubated with T cell nuclear extract derived from EL-4 cells in the presence of the nonspecific competitor poly dI-dC, a shifted band appears (Figure 3). This shifted band can be competed out by the addition of 80 ng of unlabeled -85 to +31 DNA but cannot be competed out with 80 ng poly dI-dC. To determine where nuclear proteins bind within this promoter region, DNase I footprinting experiments were performed. The DNase I footprinting assay utilizes a DNA probe which is single end-labeled on either the sense or antisense strand and then allowed to bind with nuclear extracts in the presence of the non-specific competitor DNA poly dI-dC. After binding, the reaction is digested with the double stranded specific DNase, DNase I. The concentration of DNase I is chosen to give an average of one cut per DNA molecule, resulting in an entire panel of sizes of digested DNA molecules. When this mixture is run on an 8% denaturing polyacrylamide gel, the region of the DNA that is not digested by DNase I, having been protected by a DNA-binding protein(s), is evident as a 'footprint'. The DNase I footprint is compared to probe DNA that had been digested with DNase I in the absence of protein and is mapped to specific base pairs by comparing with Maxam-Gilbert sequencing reactions.

DNase I footprinting analysis of the -174 to +31 probe of the Vβ2 promoter is shown in Figure 4. When this probe was end-labeled on the antisense strand and incubated with T cell nuclear extracts, a protected region mapping from -90 downstream through the AP-1 site, the inverted repeat and the 5' half of the decamer motif was apparent after digestion with DNase I (Figure 4). When this probe was labeled on the sense strand and digested with DNase I after binding with EL-4 nuclear extract, the AP-1

motif, inverted repeat sequence and the entire decamer motif were found to be protected (data not shown).

It has been previously shown that TCR β chain transcripts are elevated when T cells are treated with phorbol esters. In addition, the TCR Vβ2 minimal promoter element had been shown to be TPA inducible in transient expression assays. While the AP-1 motif has been shown to be the TPA inducible element in many genes, this motif alone (which corresponds to -85 to -73) could not confer TPA inducibility in transient expression systems. One copy of the -84 to -62 region however, was clearly inducible, suggesting that other flanking sequences are required to confer TPA inducibility. To address this possibility, DNase I footprinting experiments were performed with the TCR Vβ2 minimal promoter element using nuclear extracts that had been prepared from T cells which had been treated with TPA for 8 hours. When end-labeled -174 to +31 probe was bound with nuclear extract from TPA treated T cells and then digested with DNase I, the DNase I protected footprints appeared the same as those seen with nuclear extracts from untreated T cells (Figure 4).

Nuclear Protein Interactions with the TCR Vβ2 Upregulatory promoter element

The -286 to -85 region of the promoter was shown to upregulate transcription from the Vβ2 minimal promoter, suggesting that there are positively acting transcription factors which bind within this region. To examine this, mobility shift assays were performed with the -286 to -85 probe. When this probe was endlabeled and bound with T cell nuclear extract in the presence of poly dI-dC, two shifted bands appeared after electrophoresis on a nondenaturing polyacrylamide gel (Figure 5). When

competition experiments were performed with specific unlabeled -286 to -85 DNA, both bands are competed out, as assessed by a decrease in intensity of the shifted bands. In contrast to the competition observed with cold -286 to -85 DNA, unlabeled -85 to +31 DNA was not able to compete out these shifted bands when included at the same concentration (Figure 5). These results were obtained by perfoming this experiment at least four times.

To determine where T cell nuclear proteins were binding within the upregulatory TCR Vβ2 promoter region, DNase I footprinting experiments were performed with the -343 to -174 probe (Figure 2). When this probe was labeled on the antisense strand, bound with 50 ug of T cell nuclear extract and then digested with DNase I, a protected region was apparent (Figure 6). This footprint maps from -287 to -258 when aligned with Maxam-Gilbert G and G+A reactions and overlaps consensus sequences for a decanucleotide homology sequence and a CAAT box sequence. The protected region was not seen when the probe was digested by DNase I in the absence of nuclear extract. In addition, the DNase I protected region was not visible when excess cold specific DNA was included in the binding reaction before DNase I digestion. When the -343 to -174 region was labeled on the sense strand and digested with DNase I after binding with T cell nuclear extract, a footprint mapping from -310 to -230 was protected (data not shown).

TCR β Enhancer

DNase I Footprinting

The TCR β enhancer region has been shown to be crucial in the regulation of TCR β gene transcription in experiments done with both transgenic mice and in transient expression systems. One of the ways the enhancer can regulate transcription is through the binding of sequence specific transcription factors. Because this had not been shown for the β enhancer, DNase I footprinting experiments were performed in order to identify the sequences within the β enhancer which bind such factors. Overlapping fragments of the TCR β enhancer as shown in Figure 7 were used as probes in DNase I footprinting experiments.

When the 694-760 subfragment was end-labeled and digested with DNase I after binding with 100 ug EL-4 extract in the presence of poly dI-dC, a 14 bp protected region was apparent, mapping from 728 to 742 (Figure 8). This footprint, termed E1 was not seen when either the probe alone or the probe incubated with 100 ug BSA was digested with DNase I.

As with all DNase I footprinting results, these experiments were performed an average of five to six times. The criteria used to judge the actual footprints observed are the result of a compilation of all of the resulting autoradiographs. Protected regions observed with crude nuclear extracts as have been used in this case will not be as clear as those performed with even partially purified nuclear extracts. By observing the relative intensities of the bands from the DNase I digested free probe and comparing them to the DNase I probe bound with nuclear extract, an approximate region of protection can be designated after several experiments have been performed. To obtain a more detailed readout of

the changes in intensities of the bands, the resulting gels or autoradiograms from several replicates of the experiment could be scanned and the average densities or cpm of specific bands could be compared.

The second fragment of the TCR β enhancer studied for binding of nuclear factors was the 600-772 subfragment. DNase I footprinting analysis of this probe identified two protected regions after binding with 100 ug EL-4 nuclear extract (Figure 9). These footprints map from 680 to 692 and from 618 to 636 and have been designated E2 and E3 respectively. When this probe was digested with DNase I either in the absence of nuclear extract or when incubated with 100 ug of BSA prior to digestion, protected regions were not observed.

The 5'-most region of the TCR β enhancer used as a probe was the 350-600 subfragment. When DNase I footprinting experiments were performed with this probe, a protected region was observed after binding with 100 ug of EL-4 nuclear extract which mapped from 557 to 564 (Figure 10). This protected region, termed E4, was not seen when DNase I footprinting experiments were performed with either the probe alone, or probe which had been incubated with 100 ug BSA.

When the 772-910 subfragment was used in DNase I footprinting experiments, no DNase I protected regions were observed (data not shown).

Characterization of the TCR β Enhancer E3 motif

After identifying four DNase I footprints in the TCR β enhancer, it was crucial to evaluate their physiological relevance. While DNase I footprinting allows the identification of regions of DNA which bind sequence specific DNA binding proteins, it does not demonstrate if the observed DNA-

protein interaction is occuring *in vivo*. A method which allows the identification of protected regions of DNA while the DNA is still arranged in the physiological chromatin configuration within the cell is called *in vivo* footprinting. *In vivo* footprinting uses DMS to treat cells directly, causing methylation of the DNA in the cells' chromatin. After the '*in vivo*' methylation has taken place, the genomic DNA is isolated and cleaved with piperidine. As a control, genomic DNA isolated from untreated cells is treated with DMS *in vitro* before piperidine cleavage. After piperidine cleavage, the DNA is amplified by the polymerase chain reaction within the region of interest and then electrophoresed on a 8% denaturing polyacrylamide gel. Using this method, three *in vivo* footprints were mapped in our lab using T cells (S. Mangal). One of the footprints reproducibly mapped to the E3 *in vitro* DNase I footprint on both sense and antisense strands.

The mapping of the E3 motif by footprinting both *in vivo* and *in vitro* suggests that it is a physiologically important DNA sequence. A closer examination of the E3 motif within the TCR β enhancer substantiates this. The sequence of the E3 motif is conserved in the TCR β enhancer 100% between mouse and man. In addition, this exact motif has since been shown to be protected in DNase I footprinting analyses from two separate studies of the mouse and one in human (Takeda et al., 1990 and Gottschalk and Leiden, 1990). Finally, functional studies on the human enhancer have shown that if this region is mutated, enhancer function is reduced by 70%.

Because of this information available concerning the E3 motif as well as the fact that it had been protected on both strands by *in vivo* footprinting, this motif became the focus of subsequent studies.

Mobility Shift Assays with E3 Oligos

In order to carefully study the binding characteristics of this region of the enhancer, the E3 motif was subdivided into two overlapping oligos termed E3A and E3B (Figure 11). These oligos were end-labeled and used in mobility shift assays with T cell nuclear extracts. Binding reactions were performed with excess cold specific and nonspecific competitor DNA to demonstrate the sequence specificity of the interaction(s).

When binding reactions were performed with the E3A probe using 5 ug of EL-4 (T cell) nuclear extract in the presence of the non-specific competitor DNA, poly dI-dC, two dominant shifted bands of differing mobilities were seen (Figure 12). When the E3A probe was incubated in a binding reaction which also contained 5 ng and 25 ng of cold E3A, both bands were competed out. When 5 ng and 25 ng of cold E3B was included in the E3A binding reaction, both bands are competed out and a third, faster migrating band faintly appears.

When gel shift experiments were performed with the E3B probe in the absence of specific competitor DNA, a pattern of three shifted bands was observed, the third lower band appearing faintly (Figure 12). When competition experiments were performed with 5 and 25 ng of cold E3A oligo, the upper most band disappeared most completely, and the fastest migrating band became more intense. Cold E3B oligo competed for the upper two bands, while the lower band became more intense. To confirm the sequence specificity of these interactions, unlabeled DNA corresponding to the -84 to -62 region of the Vβ2 promoter (which represents the AP-1 site and the inverted repeat motif) was included in the binding reaction of E3B and T cell nuclear extract. As shown in Figure 13,

the -84 to -62 region was not able to compete for binding of nuclear factors, contrasting with the ability of unlabled E3B DNA to compete.

When the enhancer oligos E3A and E3B were used as probes in gel shift experiments prepared from the T cell lines YAC-1 and S49.1, similar patterns of shifted bands were seen as in the gel shift experiments performed with EL-4 nuclear extracts (data not shown).

When the E3 region was subdivided into three distinct oligos termed E3α, E3β, and E3γ as shown in Figure 11, differences were seen in their ability to compete for shifted complexes as unlabeled competitors. When E3A was used as a probe in gel shift experiments and unlabeled E3α oligo was included in the binding reaction, neither upper nor lower bands are competed out, contrasting with the ablilty of cold E3A to compete for both bands (Figure 14). When either unlabeled E3γ or E3β oligos were included in an E3A binding reaction, competition was observed for the lower band but not the upper band. The third, faster migrating band was not observed when competition experiments were performed with any of these E3 oligos.

UV Crosslinking Analysis of the TCR β Enhancer E3 motif

It is important to note that while mobility shift assays are useful in ascertaining the specificity of DNA-protein interactions, they do not give direct information concerning the physical properties of DNA binding proteins. To gain a more direct look at the sorts of interactions occuring between the E3 region and proteins found in nuclear extracts of T cells, UV crosslinking assays were performed. The UV crosslinking assay utilizes a bromodeoxyuridine substituted DNA probe in a scaled-up binding reaction similar to that used in gel shift assays. After the binding reaction is electrophoresed on a nondenaturing polyacrylamide gel, the entire gel is

exposed to UV light using a transilluminator. The UV light induces the formation of bromine free radicals, causing covalent crosslinking of protein(s) to DNA. The region(s) of the gel that corresponds to the resulting shifted band(s) is then excised from the gel, boiled in SDS sample buffer containing DTT and resolved by SDS-PAGE. A slight modification of this procedure can also be performed, in which the whole binding reaction is exposed to UV light while in the tube in which the binding reaction took place and then resolved by SDS-PAGE. This method, termed 'in tube' UV crosslinking, allows competition experiments to be performed by including cold competitor oligos in the binding reaction.

When either the E3A or E3B oligo was synthesized as bromodeoxyuridine substituted probe, allowed to bind with T cell nuclear extracts in the presence of poly dI-dC and then resolved on a non-denaturing polyacrylamide gel, two shifted bands appeared for each oligo in a pattern reminiscent to that seen with gel shifts using the unsubstituted oligos with the exception that the third faster migrating band never appeared (data not shown). After exposing the gel to UV light over a transilluminator the regions of the gel corresponding to the shifted bands (as determined by autoradiography) were cut out and treated as described in Methods section. As shown in Figure 15, the upper and lower crosslinked bands correspond to a molecular weight of approximately 70 kD and 89 kD respectively after SDS-PAGE. When the shifted complexes were not UV crosslinked prior to treatment for SDS-PAGE, no complexes were visible (Figure 15), demonstrating the requirement of UV light for covalent crosslinking.

In Figure 16, the products of 'in tube' UV crosslinking experiments with E3A probe are shown from a binding reaction using EL-4 nuclear extracts . The resulting complexes, which appear 'merged together', are competed out when excess cold E3A probe is included in the binding reaction, but not when 10 fold more poly dI-dC is included in the binding reaction. Comparable competition experiments using E3B as a probe yielded identical results to those seen with E3A (data not shown).

Tissue Specificity

The T cell receptor is expressed on the surfaces of T cells exclusively. Current understanding of gene expression predicts that this tissue specific expression may be due in part to the binding of tissue specific trans-acting factors to cis-acting regulatory regions of the TCR genes. In the case of the TCR β gene enhancer, in particular the E3 motif, it was of interest to determine if nuclear factors from other tissue types would recognize and bind specifically to this regulatory region.

In order to determine if nuclear factors from B cells and fibroblasts bound the E3 region of the TCR β enhancer, mobility shift experiments were performed with the overlapping E3 oligos, E3A and E3B. When labeled E3A was incubated with nuclear extract prepared from the B cell line MPC11 in the presence of nonspecific DNA poly dI-dC, two major shifted bands appeared after electrophoresis (Figure 17). When either cold E3A or E3B was included in the binding reaction, both bands were competed out. A third, faster migrating band was apparent when E3B is used as a competitor, reminiscent to that seen in mobility shift experiments with T cell nuclear extracts. When E3B was used as a probe and bound with MPC11 nuclear extract, two distinct shifted bands were apparent (Figure 17), with

an additional 'super-shifted' complex which is also seen with E3A probe. Unlabeled E3A or E3B were able to compete for these bands as well as trigger the formation of the third, faster migrating band.

Mobility shift assays were performed with nuclear extracts derived from the fibroblast cell line, NIH 3T3 and are shown in Figure 18. The binding reaction of the labeled E3A oligo and fibroblast nuclear extract generated two dominant shifted bands after electrophoresis. When either cold E3A or E3B oligos were present in the binding reaction, competition for both bands was seen. In addition, when E3B was used as a competitor, a third faster migrating band appeared. When the E3B oligo was labeled and bound with fibroblast nuclear extract, three shifted bands were visible. When either cold E3A or E3B were included in the binding reaction, both the slower migrating complexes disappeared, but the third, faster migrating band remained.

UV crosslinking tissue specificity

To extend the analysis of the tissue specificity of interactions between nuclear proteins and the E3 motif of the TCR beta enhancer, UV crosslinking experiments were performed with nuclear extracts derived from the T cells; EL-4, YAC-1, S49.1, the RAG-1, RAG-2 positive pre-T cell lines 2017 and 2052C, the pre-B cell line BASCI/C2, the B cell line MPC 11, and fibroblast cell line NIH 3T3. When binding reactions were performed using the bromodeoxyuridine substituted E3A probe then electrophoresed, two distinct bands were present for all nuclear extracts tested (data not shown). After UV crosslinking and resolution by SDS-PAGE, band 1 migrated at the molecular weight of approximately 70 kD for all nuclear extracts studied (Figure 19). When band 2 was resolved by SDS-PAGE, it migrated at

approximately 89 kD for all nuclear extracts tested (Figure 20), with the exception of nuclear extracts derived from the B cell line, MPC 11 and the pre-T cells, 2017 and 2052C, in which a band of approximately 60 kD was also present. While the possibility of 'spill-over' from the MPC 11 lane into the pre-T cell lanes is a possibility (thus resulting in the appearance of the 60 kd band in the pre-T cell lanes), this pattern of crosslinked proteins was observed even when the actual samples were loaded on the gel in different orders; also, the 60 kd band is not present in the pre-B lane. The highest band in lane 3 represents the actual well of the gel. When 'in tube' UV crosslinking assays were performed with extracts from B cells, fibroblasts and T cells, and unlabeled E3A competitor was included in the binding reaction, the resultant complexes were competed out (Figure 21).

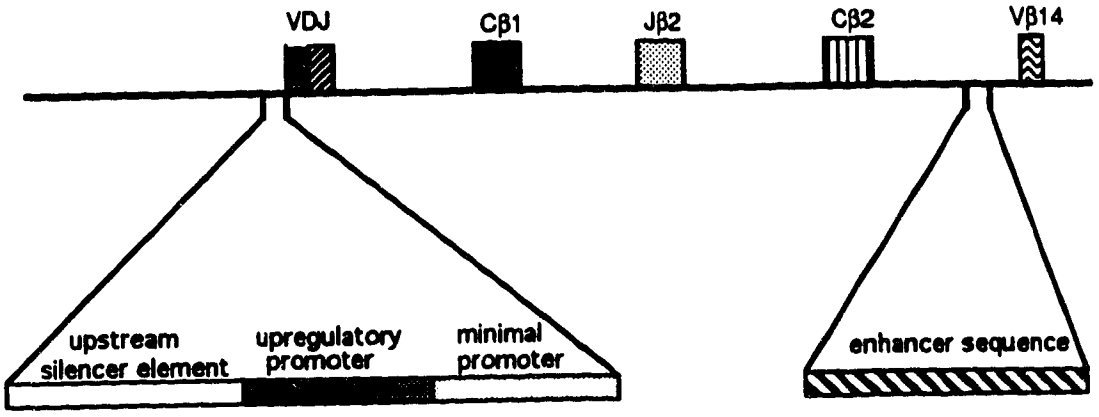


Figure 1. Schematic organization of the rearranged mouse T cell receptor Vβ2 gene of the mouse hybridoma, B.1.1. . Identified cis-acting regulatory sequences are indicated as defined by Ratanavongsiri et. al., 1990.

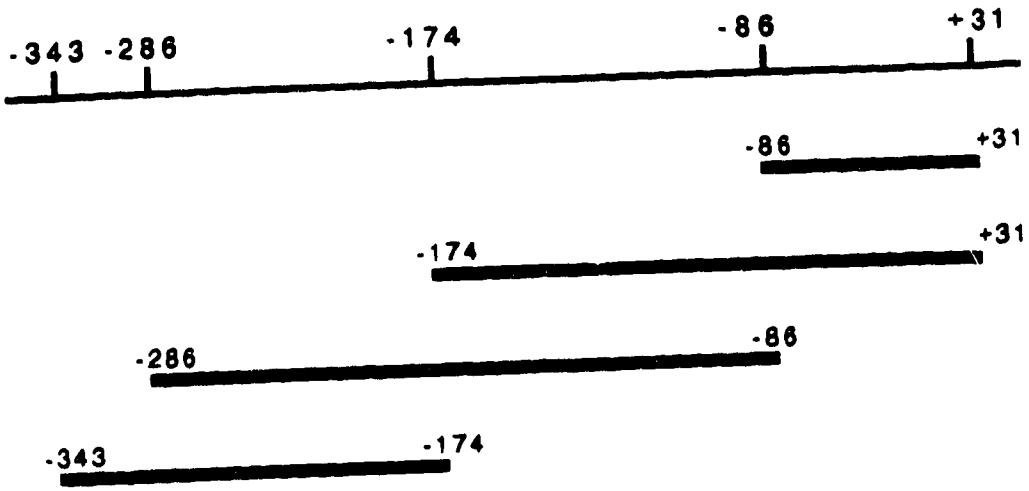


Figure 2. DNA probes of the of the TCR Vβ2 promoter used for DNA-protein binding assays.

Figure 3. Mobility shift experiment using the -85 to +31 probe of the TCR Vβ2 promoter; competition analysis. Lane 1, unbound probe; lane 2, probe bound with 15 ug EL-4 nuclear extract; lane 3, probe bound with nuclear extract with the addition of 2 ug poly dI-dC; lane 4, competition with 80 ng cold -85 to +31 DNA; lane 5, competition with 80 ng poly dI-dC.

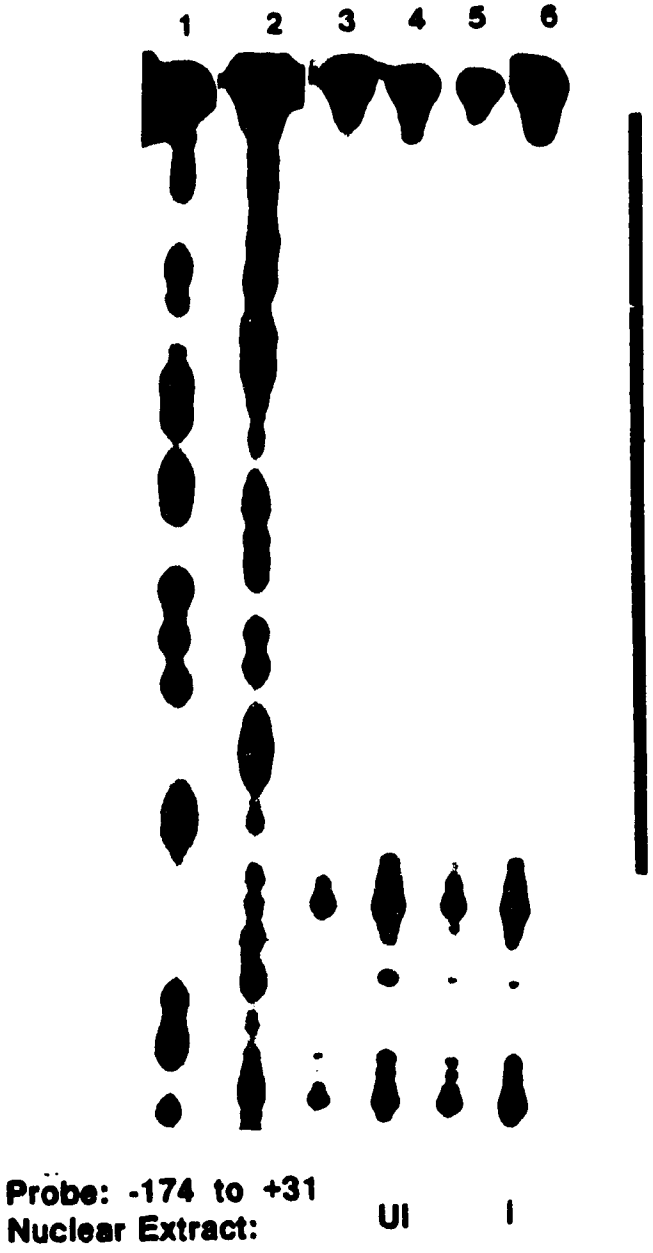


Figure 4. DNase I footprinting of the -174 to +31 probe of the TCR Vβ2 promoter. Lane 1, Maxam-Gilbert G reaction; lane 2, DNase I digested end-labeled probe; lanes 3, 4, 5 and 6, DNase I digested probe incubated with 100 ug and 50 ug EL-4 nuclear extract, and 100 ug and 50 ug TPA induced EL-4 nuclear extract respectively.

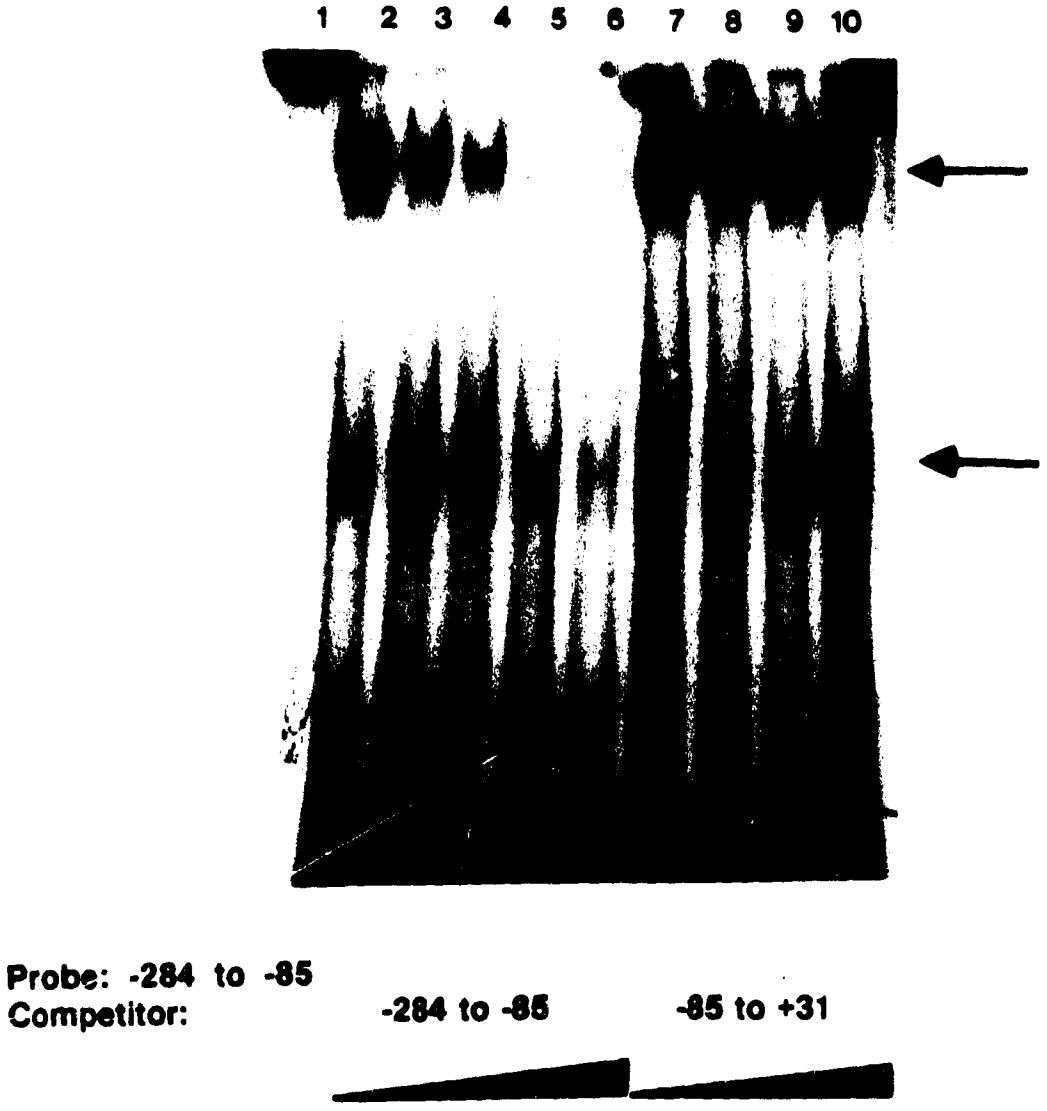


Figure 5. Mobility shift experiment of the -284 to -85 DNA probe of the TCR Vβ2 promoter; competition analysis. Lane 1, probe bound with 5 ug EL-4 nuclear extract; lane 2, binding reaction with the addition of 1 ug poly dI-dC; lanes 3-6, binding reaction as in lane 3 with the addition of 10, 20, 40 and 80 ng of unlabeled -284 to -85 DNA respectively; lanes 7-10, binding reaction with the addition of 10, 20, 40, and 80 ng of unlabeled -85 to +31 DNA respectively.

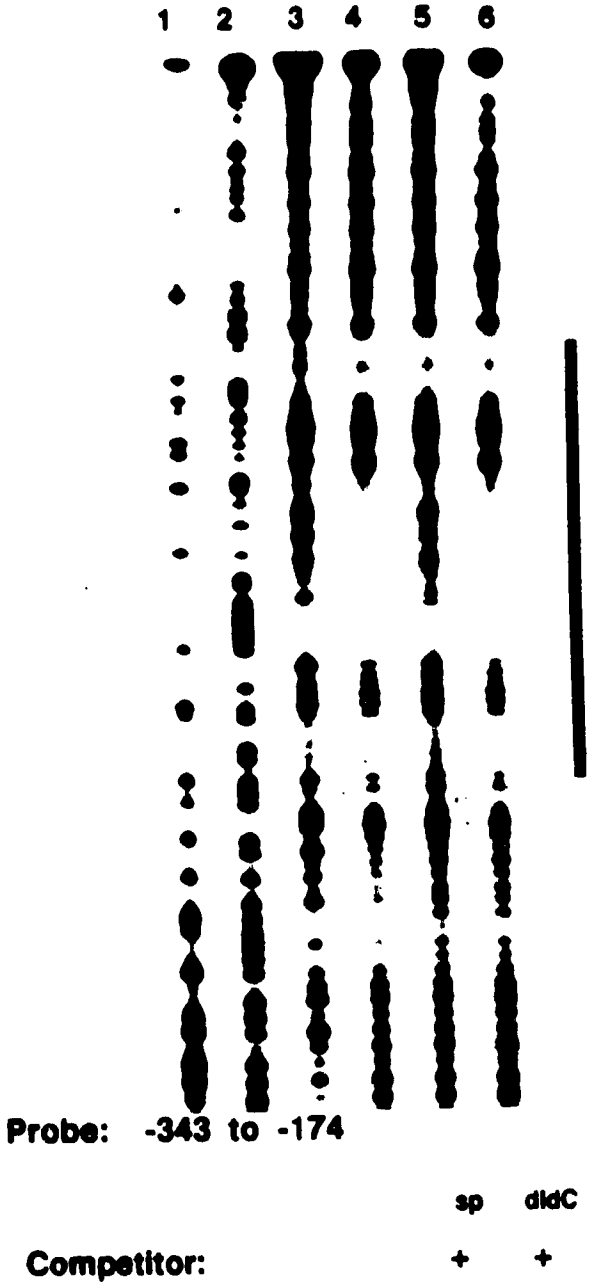
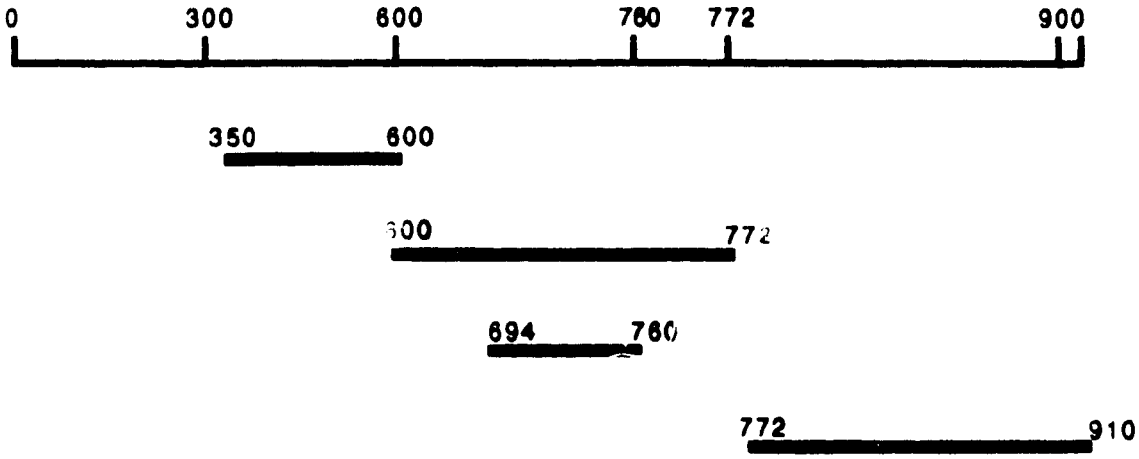


Figure 6. DNase I footprinting of the -343 to -174 region of the TCR Vβ2 promoter. Lanes 1 and 2, Maxam-Gilbert G and G+A reactions respectively; lane 3, free end-labeled probe digested with DNase I; lane 4, DNase I digested probe bound with 50 ug EI-4 nuclear extract in the presence of poly dI-dC; lanes 5 and 6, DNase I digested probe bound as in lane 4 with the addition of 100 ng unlabeled -343 to -174 DNA and 100 ng nonspecific poly dI-dC DNA respectively.



Figue 7. DNA fragments of the TCR β core enhancer used as probes to map and characterize their interactions with nuclear proteins.

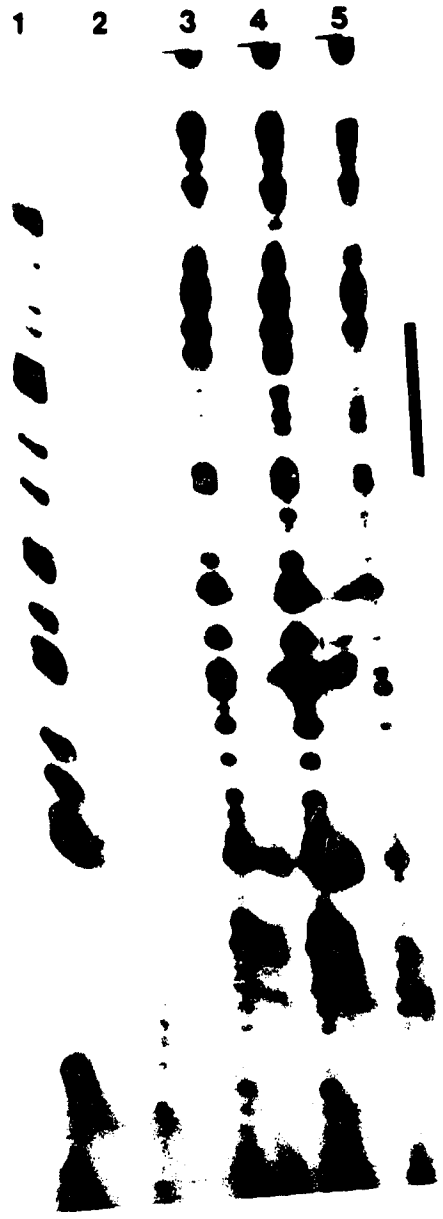


Figure 8. DNase I footprinting of the 694-760 region of the TCR β enhancer. Lanes 1 and 2, Maxam-Gilbert G and G+A reactions respectively; lane 3, DNase I digested probe; lanes 4 and 5, DNase I digested probe after incubation with 100 ug BSA and 100 ug EL-4 nuclear extract respectively.

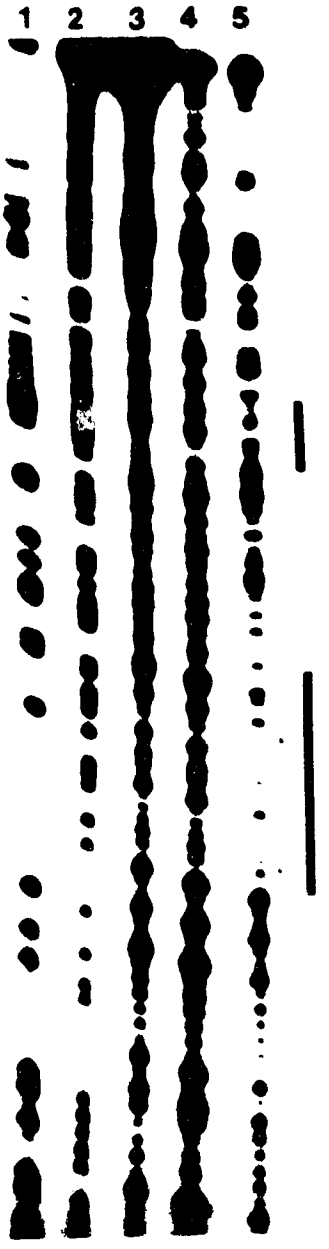


Figure 9. DNase I footprinting of the 600-772 region of the TCR β enhancer. Lanes 1 and 2, Maxam-Gilbert G and G+A reactions; lane 3, DNase I digested probe; lanes 4 and 5, DNase I digested probe after binding with 100 ug BSA and 100 ug EL-4 nuclear extract respectively.

Probe: 350-600

BSA NE

Figure 10. DNase I footprinting of the 350-600 region of the TCR β enhancer. Lanes 1 and 2, Maxam-Gilbert G and G+A reactions; lane 3, DNase I digested probe; lanes 4 and 5, DNase I digested probe after binding with 100 ug BSA and 100 ug EL-4 nuclear extract respectively.

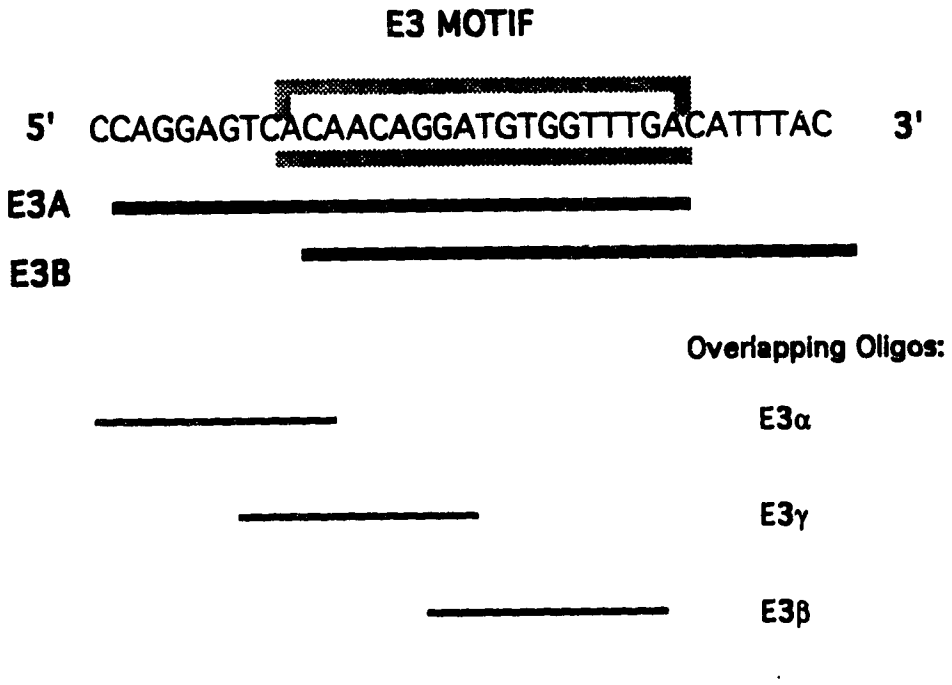


Figure 11. Overlapping oligos of the E3 motif of the TCR β enhancer used to characterize their interactions with nuclear extracts.

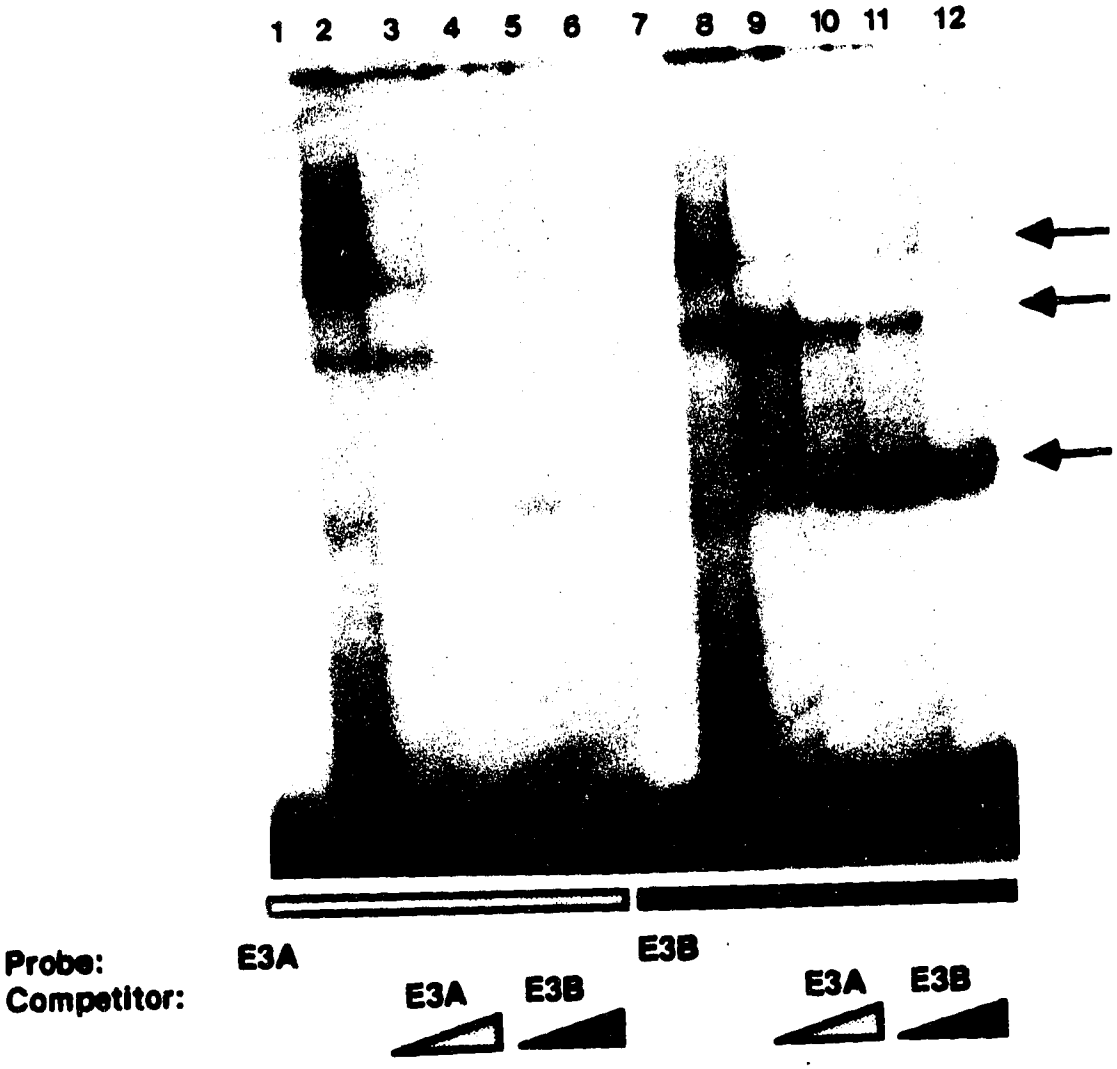


Figure 12. Mobility shift and cross-competition experiment of the E3 oligos E3A (lanes 1-6) and E3B (lanes 7-12) of the TCR β enhancer. Lane 1, E3A unbound probe; lane 2, probe bound with 5 ug EL-4 nuclear extract in the presence of 25 ng poly dI-dC, lanes 3 and 4, competition with 5 and 25 ng respectively of E3A oligo; lanes 5 and 6, competition with 5 and 25 ng respectively of E3B oligo; lane 7, E3B free probe; lane 8, probe bound with 5 ug EL-4 nuclear extract, lanes 9 and 10, competition with 5 and 25 ng respectively of E3A oligo; lanes 11 and 12, competition with 5 and 25 ng respectively of E3B oligo.

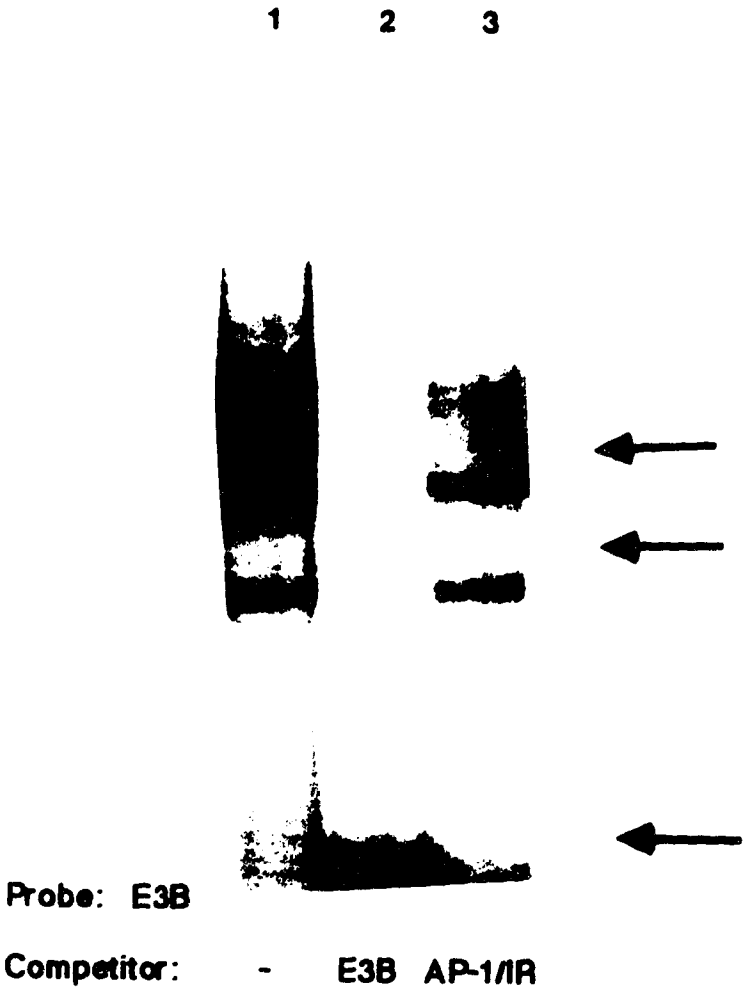


Figure 13. Mobility shift experiment of the E3B probe with competition with specific and nonspecific competitors. Lane 1, probe bound with 5 ug EL-4 nuclear extract in the presence of 25 ng poly dI-dC; lanes 2 and 3, competition with 25 ng of unlabeled E3B probe and Vβ2 promoter oligo -84 to -64 DNA respectively.

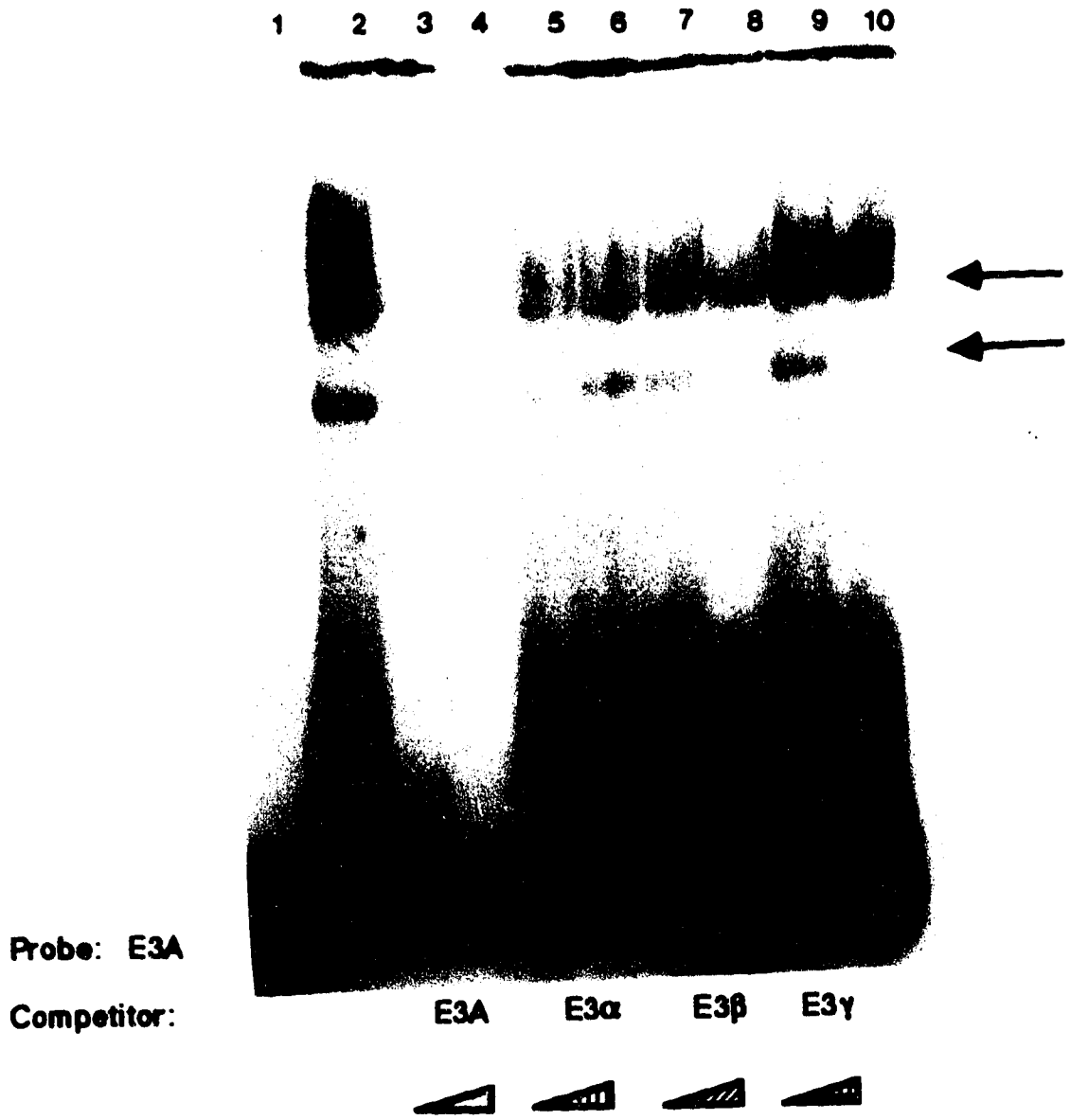


Figure 14. Mobility shift experiment of the E3A probe with competition by cold, competitor oligos E3A, E3α, E3β and E3γ. Lane 1, unbound E3A probe; lane 2, probe bound with 5 ug EL-4 nuclear extract and 25 ng poly dI-dC; lanes 3 and 4, binding reaction as in lane 2 with the addition of unlabeled E3A at 5 ng and 25 ng respectively; lanes 5 and 6, competition with unlabeled E3α at 5 ng and 25 ng respectively; lanes 7 and 8, competition with unlabeled E3β at 5 ng and 25 ng respectively; lanes 9 and 10, competition with unlabeled E3γ at 5 ng and 25 ng respectively.

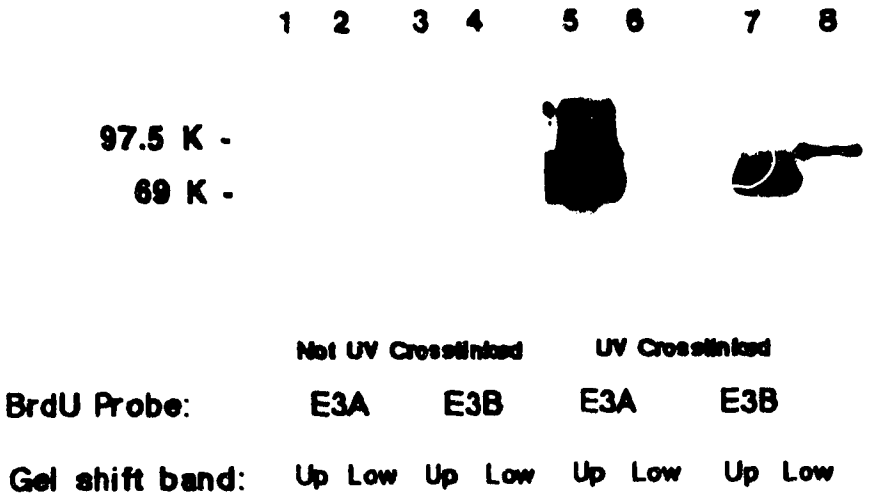


Figure 15. Characterization of the proteins which bind to BrdU substituted E3 probes by UV crosslinking and SDS-PAGE. Lanes 1-4 are the resultant products of the upper (lanes 1 and 3) and lower (lanes 2 and 4) bands from gel shift assays using the substituted E3A and E3B probes respectively without being UV crosslinked prior to SDS-PAGE. Lanes 5-8 are the UV crosslinked products of the upper (lanes 5 and 7) and lower (lanes 6 and 8) bands from gel shift assays using substituted E3A and E3B probes respectively.

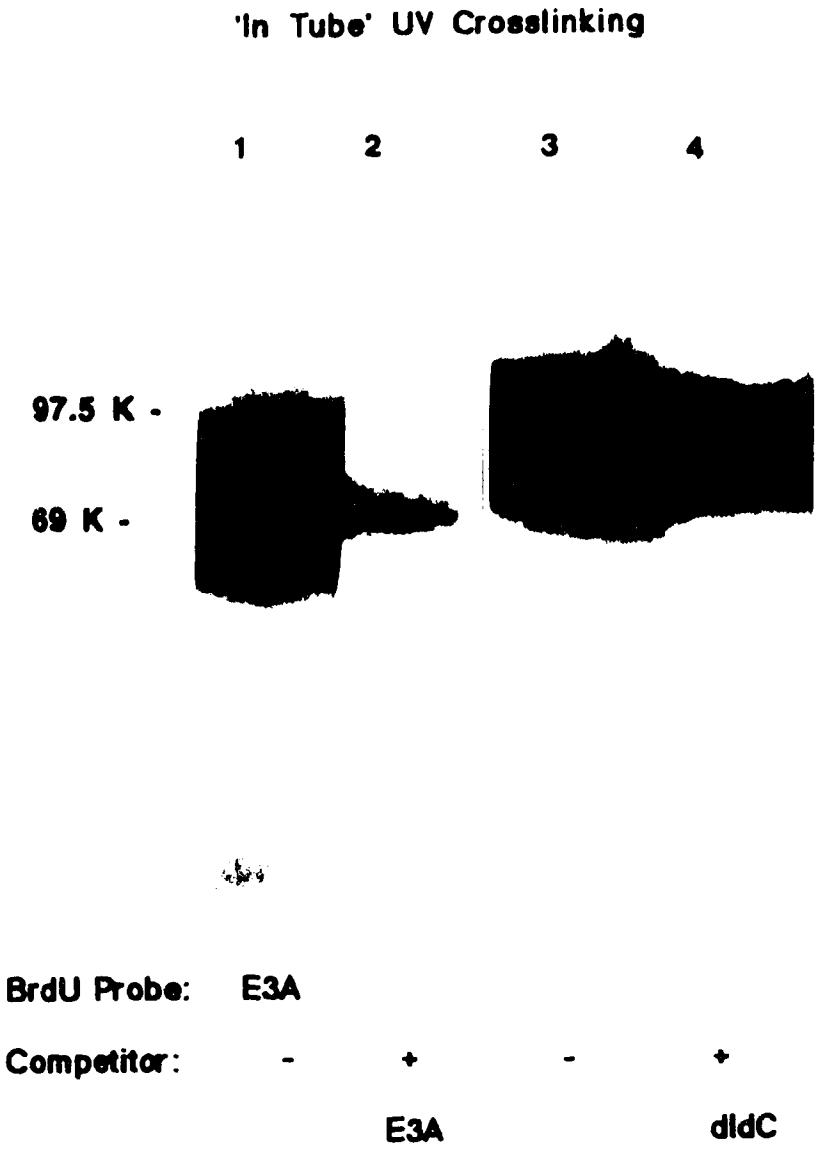


Figure 16. 'In Tube' UV crosslinking of E3A with EL-4 nuclear extracts: demonstration of sequence specificity. Lane 1, Binding reaction of BrdU substituted E3A probe and EL-4 nuclear extract; lane 2, binding reaction as in lane one with the addition of 100 ng unlabeled E3A DNA; lane 3, binding reaction as in lane 1; lane 4, lane 3 binding reaction with the addition of 1 ug poly dI-dC.

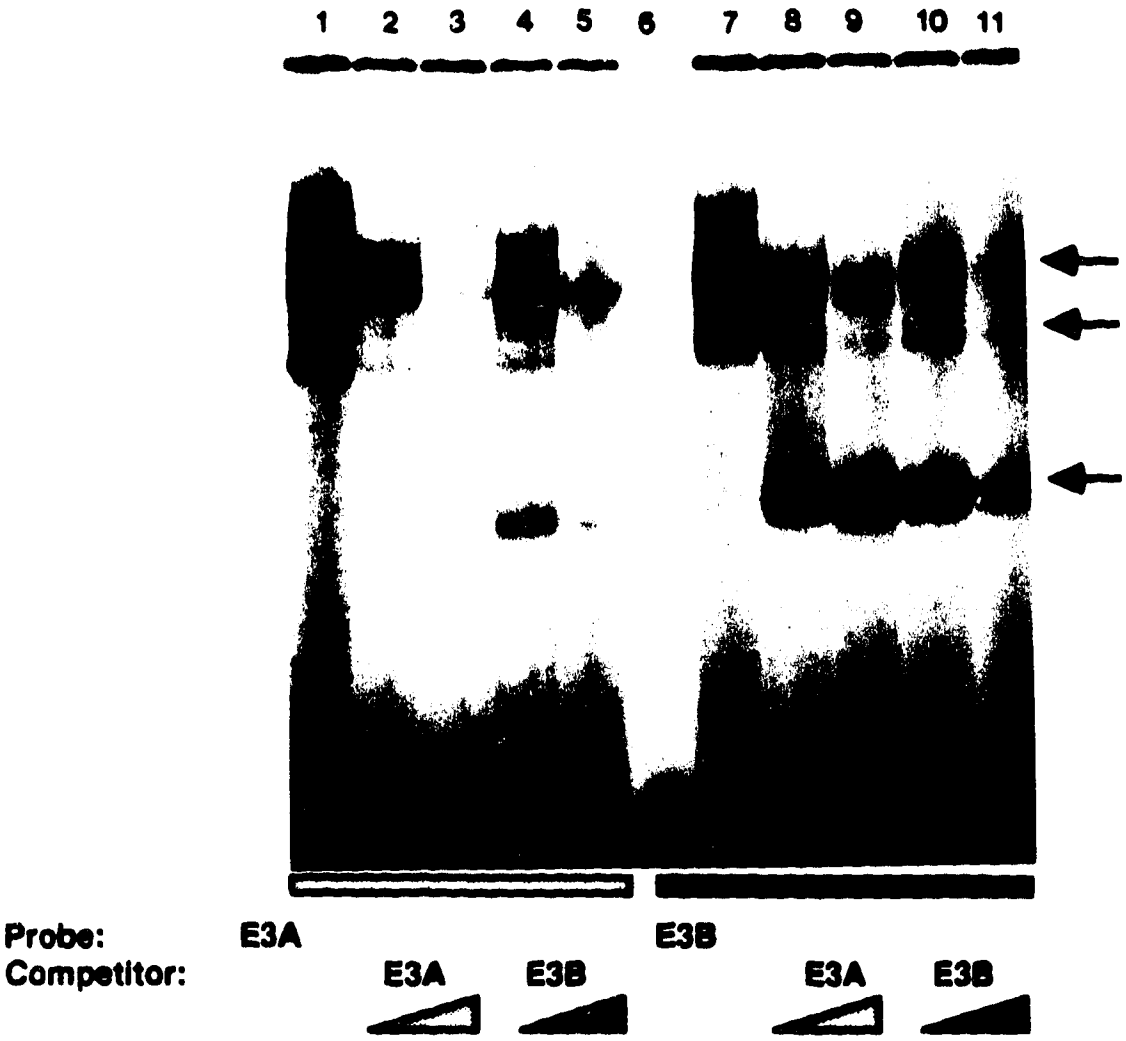


Figure 17. Mobility shift and cross competition experiment with the E3A (lanes 1-5) and E3B (lanes 6-11) oligos of the TCR β enhancer using B cell nuclear extracts prepared from MPC 11 cells. Lane 1, E3A probe bound with 5 ug EL-4 nuclear extract in the presence of 25 ng poly dI-dC, lanes 2 and 3, competition with 5 and 25 ng respectively of unlabeled E3A oligo; lanes 4 and 5, competition with 5 and 25 ng respectively of unlabeled E3B oligo; lane 6, labeled E3B probe; lane 7, E3B probe bound with 5 ug EL-4 nuclear extract, lanes 8 and 9, competition with 5 and 25 ng respectively of unlabeled E3A oligo; lanes 10 and 11, competition with 5 and 25 ng respectively of unlabeled E3B oligo.

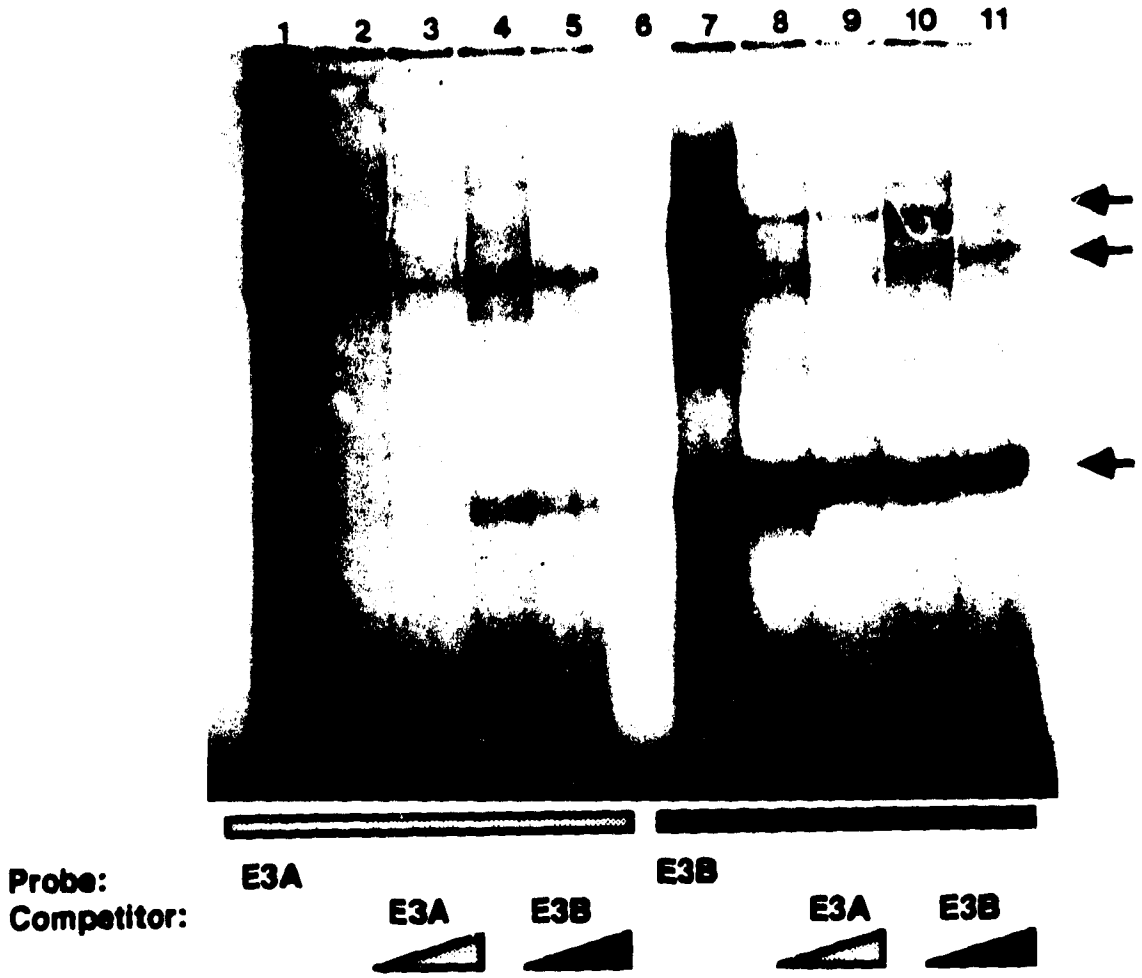


Figure 18. Mobility shift and cross competition experiment with the E3A (lanes 2-5) and E3B (lanes 6-11) oligos of the TCR β enhancer using fibroblast nuclear extracts prepared from NIH 3T3 cells. Lane 1, E3A probe bound with 5 ug EL-4 nuclear extract in the presence of 25 ng poly dI-dC, lanes 2 and 3, competition with 5 and 25 ng respectively of unlabeled E3A oligo; lanes 4 and 5, competition with 5 and 25 ng respectively of unlabeled E3B oligo; lane 6, labeled E3B probe; lane 7, E3B probe bound with 5 ug EL-4 nuclear extract, lanes 8 and 9, competition with 5 and 25 ng respectively of unlabeled E3A oligo; lanes 10 and 11, competition with 5 and 25 ng respectively of unlabeled E3B oligo.

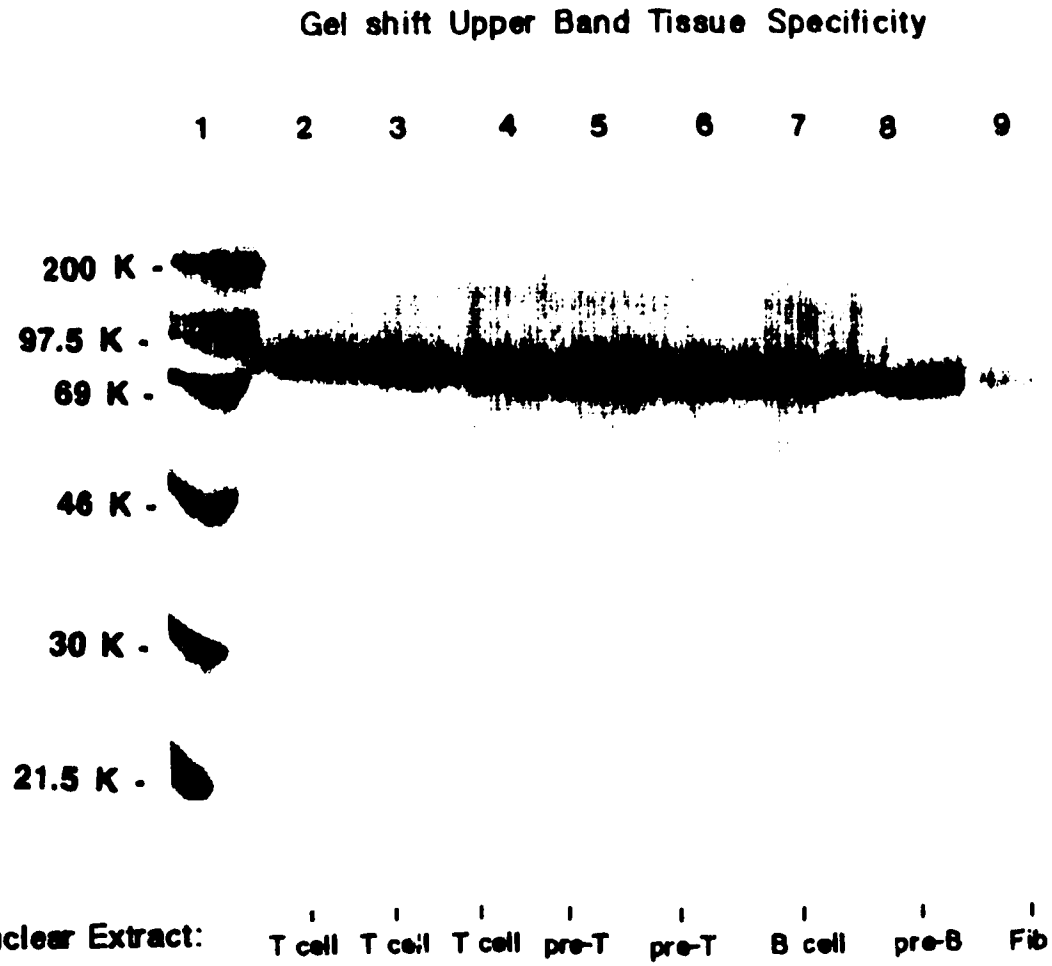


Figure 19. UV crosslinking analysis of proteins bound to the E3A oligo of the TCR β enhancer; demonstration of tissue specificity of the upper band from mobility shift assays with the bromodeoxyuridine substituted E3A probe. Lane 1, ^{14}C molecular weight markers; lane 2, E3A probe bound with EL-4 nuclear extract; lane 3, S-49 nuclear extract; lane 4, YAC-1 nuclear extract; lane 5, pre-T cell extract from 2052C cells; lane 6, pre-T cell nuclear extract from 2017 cells; lane 7, B cell nuclear extract from MPC 11 cells; lane 8, pre-B cell nuclear extract from BASCI/C2 cells; lane 9, NIH 3T3 nuclear extract.

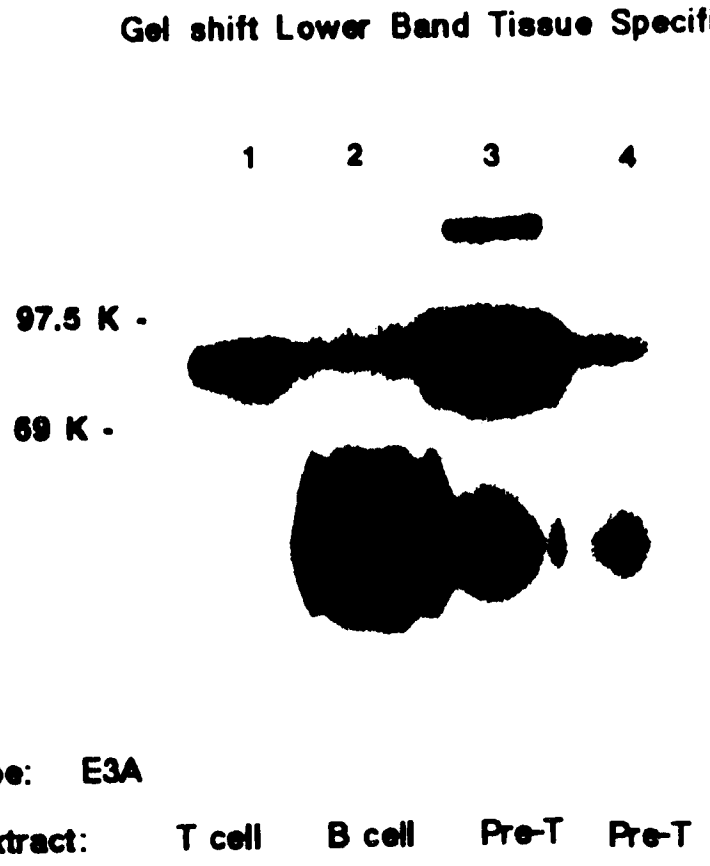


Figure 20. UV crosslinking analysis of proteins bound to the E3A oligo of the TCR β enhancer; demonstration of tissue specificity of the lower band from mobility shift assays. Lane 1, E3A probe bound with EL-4 nuclear extract; lane 2, B cell nuclear extract from MPC 11 cells; lane 3, pre-T cell extract from 2052C cells; lane 4, pre-T cell nuclear extract from 2017 cells.

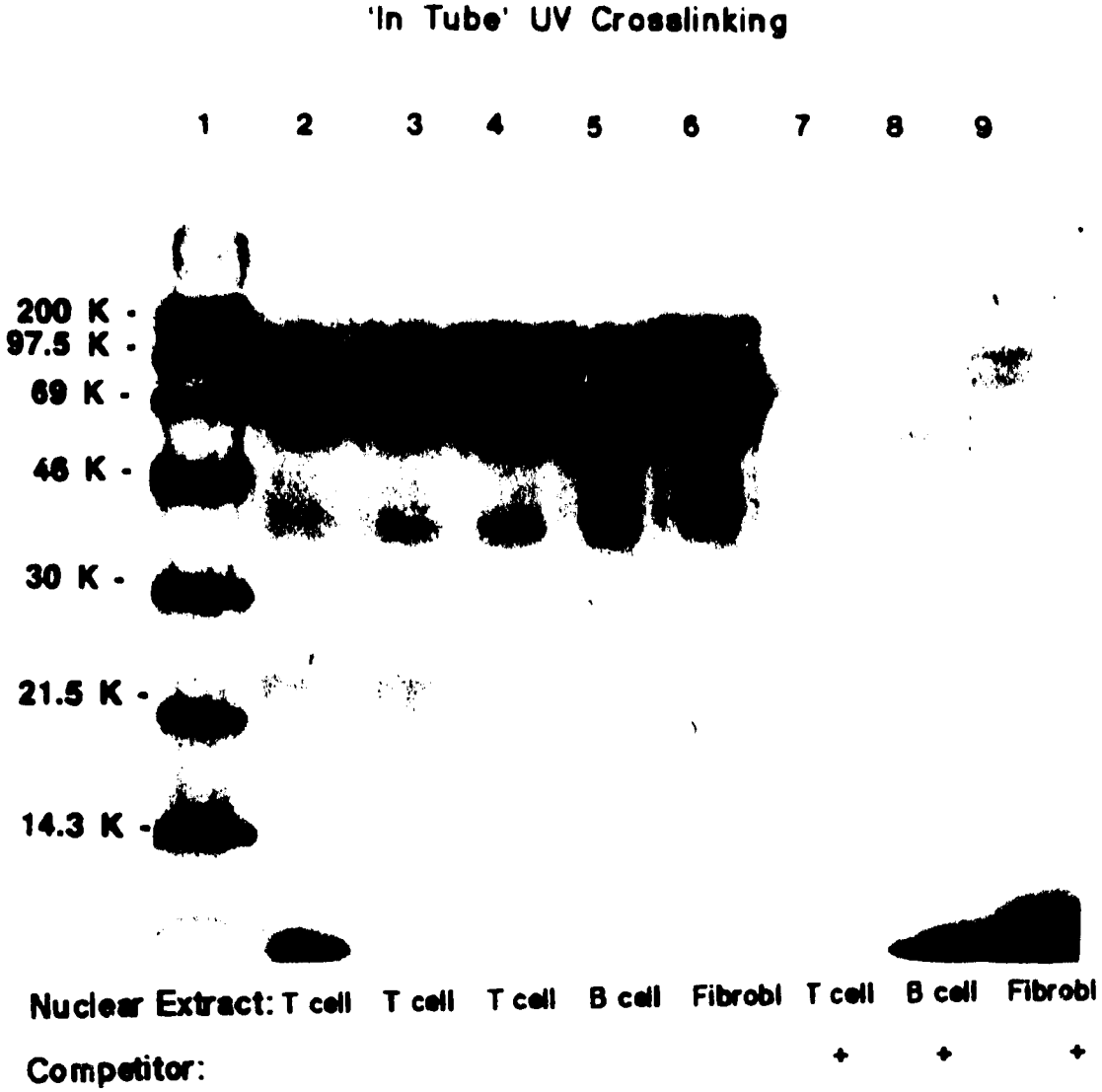


Figure 21. UV crosslinking experiment of the E3A oligo of the TCR β enhancer using the 'In Tube' method, demonstrating both tissue and sequence specificity. Lane 1, ^{14}C molecular weight markers; lanes 2 - 6, in tube UV crosslinking of the E3A probe with nuclear extracts from EL-4 cells, S-49 cells, YAC-1 cells, MPC 11 cells and NIH 3T3 cells respectively; lanes 7 - 9, competition experiment in which 100 ng unlabeled E3A is added to binding reactions with nuclear extracts from EL-4 cells, MPC 11 cells and NIH 3T3 cells respectively.

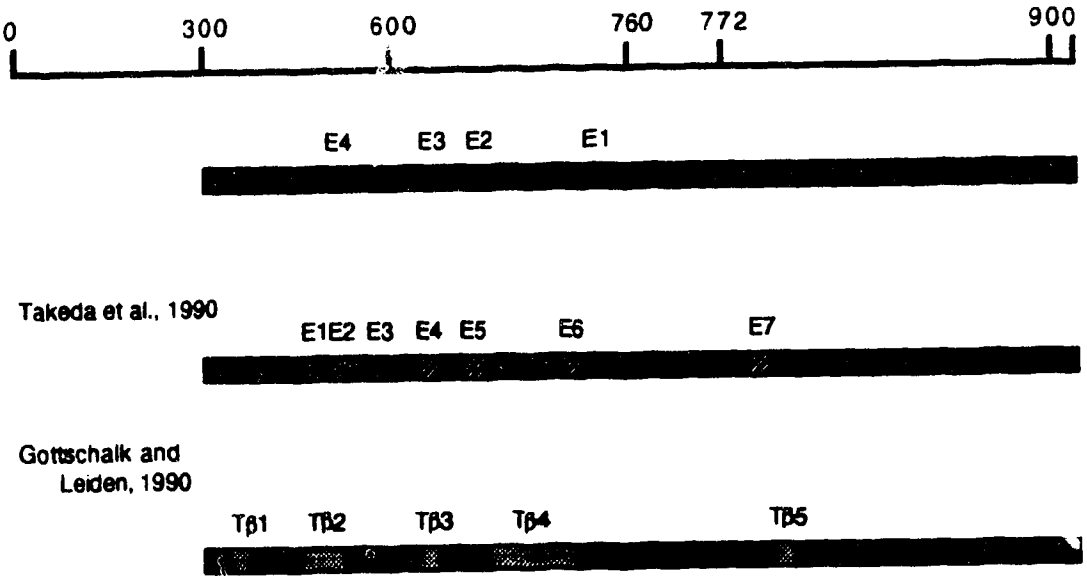


Figure 22. TCR β enhancer DNase I footprinting summary.

Discussion

In this thesis, multiple regions known to be functionally important for TCR β gene transcription have been identified as sites of interaction with nuclear proteins.

TCR Vβ2 promoter

The mouse Vβ2 promoter of the TCR gene has been demonstrated to interact in a sequence specific manner with T cell nuclear proteins. DNase I footprinting experiments identified two large, sequence specific protected regions. The protected region identified in the Vβ2 minimal promoter overlaps several previously identified consensus sequences including a conserved AP-1 site, the inverted repeat motif and the decanucleotide motif (the 5' half on the antisense strand but the entire motif on the sense strand). The size of this protected region was not increased when footprinting experiments were performed with nuclear extracts from TPA induced T cells. DNase I footprinting of the upregulator Vβ2 promoter demonstrated a large asymmetric protected region which overlapped a decanucleotide sequence, and a CAAT box sequence.

The decanucleotide motif has been implicated as being important for high level, T cell specific expression (Anderson et al., 1989). This motif is found to be conserved in either the promoter or enhancer regions of several other developmentally regulated T cell specific genes including the TCR α chain (Winoto and Baltimore, 1989), CD3 δ chain (Georgopoulos et al, 1990) and CD8 (Nakauchi, et al, 1987), suggesting that it may be crucial for the tissue specific and developmentally regulated expression of these

genes. The decamer motif is homologous to the cyclic AMP response element (CRE) and has been shown to be bound by the nuclear proteins TCR-ATF1 and TCR-ATF2 (Lee at al., 1992). These proteins are leucine zipper proteins and they show a high homology to CREB, c-Jun and c-Fos within both the leucine zipper domain and the DNA binding domain. TCR-ATF1 and TCR-ATF2 have not been shown to be T cell specific factors, implicating other DNA sequences in addition to the decamer motif for conferring tissue specificity.

The AP-1 site in the minimal promoter has been demonstrated to confer TPA inducibility when present in the context of the inverted repeat motif. This AP-1 site has been shown to be bound by the transcription factors c-Fos and Jun-B, but at a lower affinity than for the collagenase AP-1 site. The inverted repeat has been shown to be bound by the ets-2 transcription factor suggesting that there is cooperativity between these transcription factors to confer inducibility (Messier et al., 1992a).

The size of the protected regions observed in the promoter region, as well as the presence of more than one consensus binding sequence within the protected regions suggests that more than one protein is binding within these footprinted areas. In addition, the size of DNase I protected regions by known DNA binding proteins are approximately 20 bp (Dynan and Tjian, 1988), reinforcing the idea that more than one protein is binding within these regions. In support of this hypothesis are studies that have also demonstrated asymmetrical protected regions, as well as 'merged' footprints (Royer and Reinherz, 1987). The demonstration of DNase I footprints which are larger on one DNA strand than the other may be due to

asymmetric binding of nuclear proteins to these regions, allowing increased DNase I sensitivity on one strand as compared to the other.

The fact that no protected regions were observed further downsteam of the decanucleotide motif (downstream of -54) agrees with functional studies which were used to map the TCR minimal promoter. In these studies, the -85 fragment of the promoter was shown to have promoter activity, whereas the -42 fragment was silent (Ratanavongsiri et al., 1990).

TCR β Enhancer

Through the use of DNAse I footprinting assays, I have demonstrated that the TCR β enhancer is a site for the binding of multiple nuclear proteins derived from T cells (EL-4). Four enhancer motifs, termed E1 through E4 have been identified using nuclear extracts derived from EL-4 cells. Since demonstrating these four DNase I footprints within the mouse TCR β enhancer, other labs have documented these and other DNase I footprints within the human and mouse TCR β enhancers (Gottschalk and Leiden, 1990 and Takeda et al., 1990). Each motif identified will be discussed and related to the published findings (summarized in Figure 22) as well as to functional data and, if available, tissue specificity.

Gottschalk and Leiden (1990) localized the minimal human β enhancer to a 393 bp region which contains the motifs Tβ2, Tβ3 and Tβ4. The location of these identified motifs correlates very well to the regions of the murine β enhancer which I identified as being binding sites for T cell nuclear factors. The murine and human Tβ2, Tβ3 and Tβ4 motifs share 72 out of 88 bp and are required for full enhancer activity as demonstrated by either deletion or site directed mutagenesis (Gottschalk and Leiden, 1990).

The motifs Tβ1 and Tβ5 are present outside of the core enhancer and were not found to be required in transient expression assays in mature T cells. As indicated by the authors, however, this can not rule out the possibility that these 2 sites may play a functional role either during earlier stages of T cell development or during T cell activation.

The most 3' probe used for mapping DNase I protected regions was the 772-910 region. While I did not detect any footprints within this region, observations in the mouse identified the βE7 motif (Takeda et al., 1990) and in the human identified the Tβ5 motif (Gottschalk and Leiden, 1990). Deletion of Tβ5 from the human core enhancer did not affect enhancer function; however, deletion of the murine βE7 motif, which is just 5' to the human Tβ5 motif, from the core murine enhancer motif, reduced enhancer activity to only 20%. The functional significance of this region in the murine enhancer is supported by the observations that while the 772-910 region of the murine β enhancer cannot enhance transcription in T cells, it can inhibit transcription from a TK promoter in NIH 3T3 cells (Messier and Fotedar, unpublished data). This data suggests that this region does play a role in TCR gene expression although no protein binding sites were detected.

The Tβ5 motif, present outside of the core enhancer, has since been shown to bind the T cell specific transcription factor TCF-1 (Oosterwegel et al., 1991). TCF-1 is a member of the family of transcription factors which share a homology termed the HMG-box. In addition, TCF-1 has been shown to have binding specificity for a motif in the CD3 ε enhancer, the TCR α enhancer, and the Tδ7 element in the TCR δ enhancer. The fact that TCF-1 recognizes motifs in so many different T cell specific genes and is itself T cell specific, implicates it as being involved in the control of several T cell

specific genes and having an important role in the development and maintenance of mature T cells.

The 5' most region of the enhancer studied was the 350-600 probe, within which the E4 motif was found to be protected from DNase I digestion. The E4 motif, corresponds to 557-564 and overlaps with the two footprints βE1 and βE2 identified in the mouse β enhancer (Takeda et al., 1990) and the motif Tβ2 identified in the human β ehancer (Gottschalk et al., 1990). The protected sequences are conserved approximately 90% between mouse and man. The documented binding of nuclear factors to this region of the enhancer correlates well with the functional importance of this motif. Transient expression assays performed in our lab have demonstrated that this subfragment is able to enhance transcription in EL-4 cells when cloned upstream of the TK promoter, but is not able to enhance transcription from the TK promoter in NIH 3T3 cells. In addition, when the 5' end of the β enhancer is deleted, enhancer activity is reduced by more than 90% (Messier and Fotedar, unpublished results). These findings agree with those of Gottschalk and Leiden (1990), who found that the human Tβ2 motif is required for full enhancer activity, and those of Takeda et al (1990), who showed that the deletion of the mouse βE1 and βE2 motifs reduced enhancer activity to only 24%, lending further support to the sigificance of this region.

The human Tβ2 motif, which contains an AP-1 and a CRE motif, was shown by competition experiments with gel shift assays to bind a set of cyclic AMP response element-binding proteins that are bound by the Tα1 element of the human TCR α enhancer and the decamer element present in

a large number of human and murine TCR β promoters (Gottschalk and Le on, 1990 and Takeda et al., 1990).

At the 5' end of the E4 motif is a conserved binding site for the T cell specific transcription factor, GATA-3. This protein has been implicated in binding the Tβ2 motif by using gel shift assays (Marine and Winoto, 1991). It has also been shown to bind to the GATA-3 recognition site in the TCRγ, δ (Joul et al., 1991) and α enhancers, implicating it as being a potentially import cell specific transcription factor.

Since the identification of the E4 motif by DNase I footprinting assays, a novel, sequence specific DNA-binding protein has been identified by screening a T cell λgt11 library with multimerized E4 probe (Fotedar, unpublished data). This protein, termed TCFβ1 has a molecular weight of 45kd and a POU homeodomain homology in the carboxyl end of the protein as is seen in the immunoglobulin transcription factors Oct-1 and Oct-2.

Within the β enhancer 350-600 region, Gottschalk and Leiden (1990) identified an additional DNase I footprint termed Tβ1 located at 392-406 which did not correspond to previously identified enhancer motifs. This motif was not observed in the murine β enhancer by either Takeda et al (1990) or myself, and therefore may be specific to the human β enhancer. When this motif was deleted from the human β enhancer, no effect was seen on enhancer function (Gottschalk and Leiden, 1990).

The E2 motif identified within the 600-772 region of the enhancer maps to the βE5 footprint identified by Takeda et al (1990). The 660-772 region was found not to enhance transcription from the TK promoter in either EL-4 cells or in NIH 3T3 cells in transient expression assays (Messier and Fotedar, unpublished data). This data suggests that although this

probe alone is nonfunctional in transient expression systems, it does bind T cell nuclear proteins and may require other motifs in order to demonstrate functional activity in *in vitro* assays.

The E3 motif identified is present in the 600-772 region of the β enhancer. This motif corresponds to the βE4 motif identified in the murine β enhancer (Takeda et al., 1990) and to the Tβ3 motif in the human β enhancer (Gottschalk and Leiden, 1990). The importance of this motif is suggested by several lines of evidence; the protection of this motif in *in vivo* footprinting experiments, the 100% sequence conservation between mouse and man, and functional studies which demonstrated that deletion of this motif affected the function of the β enhancer. More recently, substitutional mutagenesis of the E3 motif has demonstrated that this motif is critical for the β enhancer activity in transient expression assays (Messier et al, 1992b).

The overlapping oligos E3A and E3B were shown to bind nuclear proteins from T cells, B cells and fibroblasts in a sequence specific manner, generating two distinct shifted complexes. Gel shifts with the competitors E3α, E3β and E3γ, demonstrated differences in specificity of regions within the E3 motif. The 5' most oligo, E3α was not able to compete for either s 'ted complex, while E3β and E3γ were able to compete only for the lower complex. The differential competition by these oligos suggests that more than one protein binds to the E3 motif.

In all of the gel shift experiments performed with the E3 oligos, a distinct, faster migrating band is apparent, and is more pronounced when competitor oligos are included in the binding reaction, in particular when the E3B oligo is used as a probe, and when either E3A or E3B is used as a

competitor. Because these gel shifts were performed with very little of the non-specific competitor DNA, poly dI-dC, the addition of competitor DNA would be able to drive the formation of a DNA-protein complex between the probe and a more abundant protein which has a weaker affinity for the DNA sequence (Dr. C Murre, U.C.S.D. Dept. of Biology, personal communication). This could be proven by two methods: 1) binding reactions could be performed with larger amounts of poly dI-dC (100 - 1000 ng) in order to more successfully 'compete out' proteins with weaker affinity for the DNA; or 2) much greater amounts of competitor DNA could be included in the binding reaction in order to compete for a very highly abundant protein. Evidence in support of this theory lies in the methodology used for gel shifts performed with BrdU substituted E3A or E3B probes. In these gel shifts, the third lower band was never observed, even when cold competitor was included in the binding reactions. This may be due to the fact that all binding reactions with BrdU substituted probes were performed with 100 - 1000 ng of poly dI-dC as opposed to 25 ng, and competition experiments were performed with 100 ng of cold competitor as opposed to 25 ng. Another possible explanation could be that the cold E3B oligo contributes to the formation of the third band by creating an allosteric interaction with a protein, which then can bind to the E3 probe (with greater affinity for E3B than E3A).

UV crosslinking experiments performed with nuclear extracts from T cells demonstrate two distinct proteins crosslinked to the E3 motif, one which is 70 kd and the other 89 kd. Competition experiments with specific and non-specific oligos confirmed that these proteins were binding specifically to the E3 motif. These two dominant complexes were also

observed when UV crosslinking experiments were performed with fibroblasts and pre-B cells. In contrast, while UV crosslinking using nuclear extracts from pre-T cells and B cells did demonstrate a 70 kd complex, a sequence specific 60 kd complex was also observed.

The 89 kd protein has a similar size to a UV crosslinked protein complex termed NF-δE3A that has been observed to bind to the core TGTGGTTT within the E3 sequence homologue in the TCR δ enhancer termed δE3 (Redondo et al., 1991). This complex is preferentially expressed in T lymphocytes as opposed to B lymphocytes. The smaller complex observed in B cells and pre-T cells could be an *ets* like protein as the 5' end of the E3 motif does possess a purine rich sequence 5' to the TGTGG motif. The size of *ets*-1 (53 kd) is consistent with such a postulation as the oligonucleotide used for UV crosslinking probably only contributes about 10 kd to the size of the resulting complex (in Redondo et al., 1991). In addition, the lymphoid specific tissue distribution of *ets* is consistent with a role of such a factor in recognizing a TCR enhancer motif (Chen, 1985).

Since this work has been performed, the p70 lupus autoantigen has been demonstrated to specifically bind to the E3 motif. The carboxyl terminus of the protein represents the DNA-binding region of the p70 lupus autoantigen and contains a leucine zipper motif with an adjacent basic region. (Messier et al., 1992b).

To conclude, multiple regions of the TCR promoter and enhancer have been characterized with respect to their interactions with nuclear proteins. Several transcription factors have been cloned by others that are able to interact with regulatory regions of the TCR β gene, such as GATA-3, *ets*-2, TCR-ATF 1 & 2, and more recently the E4 binding protein, TCFβ1 and

the E3 binding protein, p70. Many of these proteins are not T cell specific, such as *ets*-2, whereas others, such as GATA-3, are. Also, many of the motifs identified do not function exclusively in T cells when analysed in isolation, *in vitro*; for example, the core TCR β enhancer is active in B cells, but the entire 4 kb enhancer displays T cell specific activity. These observations stress the importance of multiple regulatory elements as well as the chromatin structure *in vivo* for the regulation of tissue specific gene expression. These DNA sequence and conformation requirements, combinated with the presence of both ubiquitous and tissue specific transcription factors ultimately result in proper gene expression.

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