UNIVERSITY OF ILLINOIS

December 14 1988

THIS IS TO CERTIFY THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Charles W. Tolan

ENTITLED The Effect of Transgene T-Cell Receptors on Endogenous Delta Chain Transcripts

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

DEGREE OF Bachelor of Science in Biochemistry

David M. Kranz
Instructor in Charge

APPROVED: Robert L. Switzer

HEAD OF DEPARTMENT OF Biochemistry

O-1364

THE EFFECT OF TRANSGENE T-CELL RECEPTORS ON ENDOGENOUS DELTA GENE TRANSCRIPTS

BY

CHARLES W. TOLAN

THESIS

for the

DEGREE OF BACHELOR OF SCIENCE

IN

BIOCHEMISTRY

College of Liberal Arts and Sciences

University of Illinois

Urbana, Illinois

1988

TABLE OF CONTENTS

ACKNOWLEDGMENTS

The author would like to thank Dr. Mike Lacy for his willingness, and his ability to teach the author with an openness unavailable in any classroom. I would also like to extend my appreciation to the other members of the Krans group for their support during this project. Thank you Matt, William, Leslie, Eric, and Ben. I would like to thank Katie Thorpe for her help in preparing this manuscript. Last, I would like to sincerely thank Dr. David Krans for his advisory position on this project.

ABSTRACT

Most peripheral T lymphocytes express an antigen specific receptor composed of two subunits, alpha (α) and beta (β), whose germline DNA undergoes rearrangement during development in the thymus. A large percentage of cells in 16 day thymus are a rich source of a similar heterodimeric receptor composed of the subunits gamma (γ) and delta (δ) (2). The gene families of these subunits are similar in that their germline DNA consists of discontinuous genetic elements which rearrange, and form unique T cell receptor (TCR) DNA. In germline the δ-gene family lies entirely between two elements of the α-gene locus. Upon the rearrangement ontogeny of the α gene, assembly of these elements excises the entire δ locus.

The PCR reaction was used in this study to amplify endogenous lymph node δ message from transgenic mice, in order to analyse the effect of a transgenic $\alpha\beta$ TCR on an endogenous $\gamma\delta$ receptor. This is done to examine expression regulation of the $\gamma\delta$ receptor by the $\alpha\beta$ receptor in contrast to direct deletion during rearrangement.

A primer for one of the upstream δ-gene regions called $V_{\delta 4}$' and one for a downstream region called C_δ were used in the PCR, and an internal oligonucleotide probe ($V_{\delta 4}$) was

used to detect amplified δ message. Cloned δ gene transcript from lymph node resulted in three in frame sequences, and two out of frame sequences. All five sequences obtained used two diversity regions common to δ-gene rearrangements. All δ-gene sequences increased their diversity to different extents by addition or deletion of bases at the junctions of assembled elements called nucleotide addition regions.

The transgene αβ receptor did not appear to affect the rearrangement or expression of the endogenous δ genes.

INTRODUCTION

Two immune systems that are used to recognize and bind foreign antigens within the body are centered around the B cells and the T cells. Both systems are part of the immune system, and are found throughout the bloodstream and lymphoid tissue. Unlike the B cells, T cells possess surface receptor's that recognize surface bound antigen only when "presented" by self proteins. These self proteins are called the products of the major histocompatibility complex (MHC). T cells are able to distinguish foreign antigens from self antigens, and mediate the destruction of cells infected with foreign antigens.

The T cell receptor (TCR) responsible for this recognition is composed of two protein chains. To date four possible chains and their genes have been described: alpha (α), beta (β), gamma (γ), delta (δ). Only two partner polypeptide chains are disulfied linked together to form a heterodimeric receptor. Thus, the α and β chains form one receptor and the γ and δ form another (Fig.1). Only one of these two distinct receptors are expressed on a cell at any time. The $\gamma\delta$ cells predominate in the thymus of 15 day fetal mouse embryo, yet they are a distinctly minor cell population in the adult. The $\gamma\delta$ receptor is expressed on

less than 2% of thymocytes, and T cells elsewhere in the adult mouse. The expressed $\gamma\delta$ receptor appears approximately two days before the cell surface $\alpha\beta$ receptors on developing thymocytes (1). The ligand for which the $\gamma\delta$ receptor is specific is unknown at this time, and the function of $\gamma\delta$ cells is a mystery, although they have been shown to have cytotoxic capabilities (3,4,5,).

The potential of the T cell receptor to recognize a vast array of antigens is a result of the rearrangement of the germline DNA that encodes these polypeptide chains. TCR germline DNA consists of discontinuous genetic elements corresponding to various regions of the antigen receptor such as the V (variable) region, the D (diversity) region, the J (joining) region, and the C (constant) region. The germline DNA rearranges to form a continuous V-J sequence or V-D-J sequence thereby assembling the cell's unique TCR DNA. Delta chains increase their diversity by using two D regions. In addition enzymatic processes responsible for joining the genetic elements often add or delete bases at junctions such as V-D, D-J, or V-J. These junctional regions are called the N (nucleotide addition) regions (Fig.2).

The germline δ gene family lies entirely within the α gene locus, and a rearrangement of a V_α element to a J_α element will completely delete the entire δ locus (Fig 3). It can be inferred that this may be a novel means of feedback inhibition used by the $\alpha\beta$ receptor upon the $\gamma\delta$ receptor. That is cells that have rearranged their α-chain genes can not express δ-chains.

Transgenic mice provided a method to analyze regulation of the TCR. The transgenic mice used in this study have had the genes that encode a single α and a single β chain inserted into their genome. These mice were prepared by microinjection of cloned DNA into a fertilised egg. This results in the insertions of the exogenous DNA, and in appropriate cells, the transcription of the cloned DNA (7). When DNA of an $\alpha\beta$ TCR is incorporated into a cell that would otherwise delete δ in order to obtain α expression, both α and δ could be expressed potentially. Are the endogenous $\gamma\delta$ genes of these cells prevented from rearranging? Using DNA of a specific $\alpha\beta$ TCR from the cell line 2C (12), transgenic mice were obtained (7). These mice provided a means to investigate whether $\alpha\beta$ gene expression regulates δ-gene rearrangement.

To perform these experiments we used the polymerase chain reaction (13) to amplify δ-chain cDNA of total RNA obtained from lymph nodes of transgenic mice. To analyze the issue, the lymph nodes from a tra' enic animal were examined to determine whether endogenous δ gene transcript could be detected. Several of these were sequenced to show that they could encode protein (ie. they were in-frame).

MATERIALS AND METHODS

Oligonucleotides. Oligonucleotides were manufactured at the University of Illinois Genetic Engineering Facility. A δ-chain constant region primer 5'-GACGTCAGATGGTTTGGCCGGAGGC-3' with a PstI restriction site (GACGTC) (referred to as the C_δ primer) was used to produce cDNA for amplification reactions. A variable region primer 5'-GCTGGGGGATCCTGCGACG-3' with a Bam HI restriction site (GGATCC) (referred to as $V_{\delta 4}$' primer) was used in the amplification reactions. An oligonucleotide 5'-TATTCTGTGCTCTATC-3' (referred to as $V_{\delta 4}$) was used to identify $V_{\delta 4}$ DNA after amplification and to detect any $V_{\delta 4}$ positive inserts during cloning.

RNA Preparation and cDNA synthesis. The polymerase chain reaction (PCR) was performed as described (9,13) and shown in figure 4. Total RNA was obtained from intestinal lymph nodes of transgene mice by the guanidinum thiocyanate/CsCl method (10).

Dot Blot Analysis. Five μl of amplified material was denatured in 0.25 M NaOH, diluted 10^{-3} to 10^{-1} into 0.375 M NaCl, 0.125 M NaOH, 0.0375 M sodium citrate (0.15 x SSC), and various dilutions were added to Gene Screen Plus (Dupont) in a 96-well apparatus. Filters were prehybridized

in 1% bovine serum albumin, 7% $NaDodSO_4$, 1.5 mM EDTA, 250 mM NaCl, 0.52 M sodium phosphate, ph 7.2, at 52-54°C and were hybridized overnight with the ^{32}P labeled $V_{\delta 4}$ oligomer. Blots were washed in 2 x SSC and 0.5% SDS at 52-54°, and exposed to film for 1-2 days.

Cloning. Twenty-five μl of PCR-amplified cDNA was digested with BamHI and PstI restriction enzymes, (BRL) and ligated into puc19 (cut with BamHI and PstI) plasmids with T4 DNA ligase (BRL). Recombinant plasmids were transformed into E.coli cells (RR1). RR1 cells were made competent overnight in $CaCl_2$. Puc19 plasmids contain an ampicillin resistance gene which was used to select for recombinants by growing them up on Luria agar (Difco) plates that contained 50 $^{\mu g}/_{ml}$ ampicillin (Sigma).

The $V_{\delta 4}$ probe was radioactively labeled with ^{32}P ATP (ICN) by T4 Kinase, (BRL) and purified on the Sep-Pak C_{18} cartridges (Millipore). For screening, colonies of ampicillin resistant transformants were lifted onto nylon membranes (Gene Screen Plus, Dupont) lysed and probed with ^{32}P-$V_{\delta 4}$ in hybridization buffer. The hybridization solution contained 1% bovine serum albumin, 7% $NaDodSo_4$, and the hybridization was incubated overnight at 52°-54°C. After

washing, filters were exposed to film for 1-2 days. Positive colonies were picked, replated, and restarted.

Sequencing. Plasmid mini preps of confirmed positives were produced by alkaline lysis (10). Both strands of the plasmid inserts were sequenced by the dideoxy method using Sequenase (U.S. Biochem. Corp.). Approximately 150 nucleotides from the 5' V to the 3' C region of the amplified δ gene were sequenced.

Transgenic Mice. Transgenic mice were the generous gift of Dennis Y. Loh, Washington University School of Medicine, St. Louis, MO. DNA that encodes a functionally rearranged $\alpha\beta$ antigen receptor from a cytotoxic T lymphocyte (2C) was introduced into the genome of these mice. In one experiment a control littermate, that did not express the $\alpha\beta$ transgene was used.

RESULTS

In order to examine endogenous δ-chain expression, the PCR was used to amplify δ-chain transcripts cDNA from lymph node RNA. Dot blot analysis using the internal $V_{\delta 4}$ probe showed that amplified cDNA from the transgene lymph node (TL) contained a δ genetic element. In fact, the level of expression, as judged by the PCR and blotting was similar to that of control lymph node (CL) (Fig.5). This suggests that the transgene $\alpha\beta$ TCR did not have an effect on the quantity of endogenous $\gamma\delta$ receptors.

In order to determine whether the δ-chain transcripts, were potentially functional (ie. in-frame sequences) amplified material was cloned and sequenced. Transformation efficiencies ranged 10^3 to 10^4 colonies/$_{\mu g}$ puc. The quantity of recombinants colonies selected was very large. Approximately 3,500 ampicillin-resistant colonies were screened with the ^{32}P-$V_{\delta 4}$ probe. Seventy-five colonies, (~2%) that initially screened positive with the $V_{\delta 4}$ probe were pooled on a single ampicillin plate and restarted. Eighteen partial sequences were obtained. Nine clones turned out to have identical sequences. Five clones were verified by sequencing both of their strands.

Nucleotide sequencing of the cloned endogenous δ-chain inserts yielded three in-frame δ sequences, and two out of frame δ sequences (Fig.6). Transgene lymph node (TLN) clone TLN 25 utilized the $J_{\delta 2}$ gene segment. The other four clones used the $J_{\delta 1}$ gene segment. All five sequences utilized both diversity regions (D_1,D_2). The uses of the three possible nucleotide addition regions (N_1,N_2,N_3,) were unique in each sequence, and ranged in length from zero to seven nucleotides. These regions increase diversity by enzymatically adding or deleting bases at the $V_{\delta 4}$-$D_{\delta 1}$, $D_{\delta 1}$-$D_{\delta 2}$, and $D_{\delta 2}$-J_{δ} junctions.

The amino acids sequences of the polypetide chains translated from these δ-genes would normally be paired with γ-gene products as functional cell surface receptors. Three of the amino acid sequences from the transgene were in-frame. Thus, they could yield functional subunits of a heterodimeric receptor. Because of the three possible reading frames the conserved repeating amino acids motifs of the C region (CALMER) and V region (FGXG) are used to judge the reading frames across the arranged D and N segments. The two in-frame sequences which utilized $J_{\delta 1}$ are shown in figure 7.

DISCUSSION

The ligand that binds the γδ receptor has not been identified, and the function of γδ bearing cells remains unknown. Nevertheless the potential for great diversity in the δ-chain suggests the γδ receptor could bind a variety of different ligands. In normal mice, T cells express either an αβ receptor or a γδ receptor, but not both. It is thought that one means of regulating such expression is that rearrangement of the α-gene deletes the δ-gene from the cell's DNA. In the study reported here, an exogenous α and β-gene (transgenes) had been introduced into a mouse's germline. We attempted to determine whether the presence of these functional TCR genes would affect the expression of the endogenous TCR genes. Thus, we examined the level of expression and the sequences of endogenous δ-chain transcription in these transgenic mice. Our results, using PCR and dot blot analysis, showed that both the transgene and the control mice expressed equivalent levels of the δ-chain transcript. In addition, nucleotide sequences of five δ-chain transcripts confirmed that the δ-genes had undergone rearrangement ($V_{\delta 4}$ to $D_{\delta 1}$ to$D_{\delta 2}$ to C_{δ}). Three of these sequences would yield an in-frame δ-chain.

Two of the sequences (TLN 25 and TLN 12) were identical to sequences derived from fetal thymus and spleen found earlier in our lab (BDN-1,BDN-21) (8). The probability of finding the same sequences in two different mice is unlikely given random addition or deletion of bases at gene segment junctions. Although the basis of this finding is unclear, it will be of interest in the future to determine if there is a mechanism that has selected this particular δ-chain.

REFERENCES

1. Chien, Y., Iwashima, M., Kaplan, K. B., Elliott, J.F., and Davis, M. M. (1987) Nature 327, 677-682.
2. Tonegawa, S. (Nobel Lecture), Angew Chem. Int. Ed. Engl. 27 (1988), 1028-1039
3. Borst, K., Van de Griend, R. J., van Oostveen, J. W., Ang, S. L., Melief, C.J., Seidman, J. G., and Bolhuis, R. L. H. (1987) Nature(London) 325, 683-688.
4. Matis, L. A., Cron, R., and Bluestone, J. A. (1987) Nature (London) 330, 262-264.
5. Moingeon, P., Jitsukawa, S., Faure, F., Troalen, F., Triebel, F., Graziani, M., Forestier, F., Bellet, D., Bouuon, C., and Hercend, T. (1987) Nature(London) 325, 723-726.
6. Pardoll, D. M., Fowlkes, B. J., Luestone, J. A., Kruisbeek, A., Maloy, W. L., Coligan, J. E., and Schwartz, R. E. (1987) Nature 326, 79-81.
7. Sha, W. S., Nelson, C. A., Newberry, R. D., Kranz, D. M., Russel, J. H., and Loh, D. Y. (1988) Nature 335, 271-273.
8. Lacy, M.J., Klis McNeil, L., Roth, M. E., and Kranz, D. M. (1988) P.N.A.S. in press.

9. Roth, M. E., Lacy, M. J., McNeil, L. K., and Kranz, D. M. (1988) Science 241, 1354-1358.

10. Elliot, J. F., Rock, E. P., Patten, P. A., Davis, M. M., and Chein, Y-h. (1988) Nature 331, 627-631.

11. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloaning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

12. Marx, L. J., (1987) Science 326, 1187-1188.

13. Sitkovsky, M. V., Pasternack, M. S., and Eisen, H. N., (1982) J. Immunol. 129, 1372-1376.

14. Saiki, R. K., et al., (1985) Science 230, 1350.

Figure 1. Diagram showing the subunit structures of T-cell receptors $\alpha\beta$ (left) and $\gamma\delta$ (right) as deduced from the nucleotide sequences of cDNA clones. The $\alpha\beta$ receptor is from an alloreactive cytotoxic mouse thymocyte hybridoma T-cell clone (2C), and the $\gamma\delta$ receptor from a mouse thymocyte hybridoma (KNS) prepared by Osami Kanagawa of Lilly Research Laboratories, La Jolla, California. Intra- and interchain disulfide bonds are indicated. The receptors are thought to be anchored on the membrane lipid bilayer by transmembrane peptides. The invariant CD3 complex associated with the heterodimers is not shown (1).

Figure 2. Classical immunoglobulin-like rearrangement of the δ locus. The germline locus is shown at the top. Unknown recombinases in the thymus cause the germline rearrangement and selection of the particular gene from the V, D_1, D_2, and J regions. These are assembled with the enzymatic addition or subtraction of bases at their junctions. RNA splicing of this primary message results in the mRNA of the protein product subunit of the receptor.

Figure 3. The germline DNA of the α and δ genes showing the complete germline δ locus between the V_αs and the J_αs.

Figure 4. (Top) Oligonucleotides and their positions used in the amplification the δ chain transcripts. The constant region primer (C_δ, 5'-GACTGCAGATGGTTTGGCCGGAGGC-3') which contained the restriction site (GACGTC) for cloning was used as a primer for cDNA synthesis and subsequent PCR. The variable region oligomer ($V_\delta 4'$, 5'-GCTGGGGGATCCTGCGACG-3') contained a BamHI site for cloning was used as a primer in the PCR. An oligomer ($V_\delta 4$, 5'-TATTTCTGTGCTCTCATG-3') internal to C_δ and the $V_\delta 4'$ was used for detection of amplified transcripts. (Bottom) The flow chart diagram of the polymerase chain reaction as performed on the total RNA from T cells from the intestinal lymph nodes of the transgenic mice.

Figure 5. Dot Blot analysis of the amplified δ chain transcript. All RNA was amplified by the PCR ($V_{\delta 4}$'→ C_{δ}) and 3.2 fold dilutions of cycle 0 (prior to polymerase addition) and cycle 30 material was blotted and probed with ^{32}P-labeled $V_{\delta 4}$. (from top) control thymus, control lymph node, transgene thymus, transgene lymph node. Across the top the dilutions are listed.

Figure 6. Nucleotides sequences of transgene δ chain transcripts. DNA sequences were aligned with published germline sequences (8). The top four sequences use the $J_{\delta 1}$ gene region. The bottom sequence utilizes the $J_{\delta 2}$ gene region. Transgene lymph node (TLN) clones 12, 24, and 25 were in frame and could be expressed with an in-frame γ partner as a functional cell surface receptor. TLN clones 35 and 46 were out of frame. The three enzymatic addition or subtraction of nucleotides regions are denoted N_1, N_2, and N_3.

Figure 7. Partial Amino Acid sequences of the in-frame δ gene transcripts from amplified transgene lymph node. Amino acid residues from the TLN clones 12 and 24 utilized the $J_{\delta 1}$ region, and are aligned together under the regions that encode them. The repeating motifs of the $V_{\delta 4}$ (CALMER) and the J_{δ} (FGXG) are shown.

Figure 1.

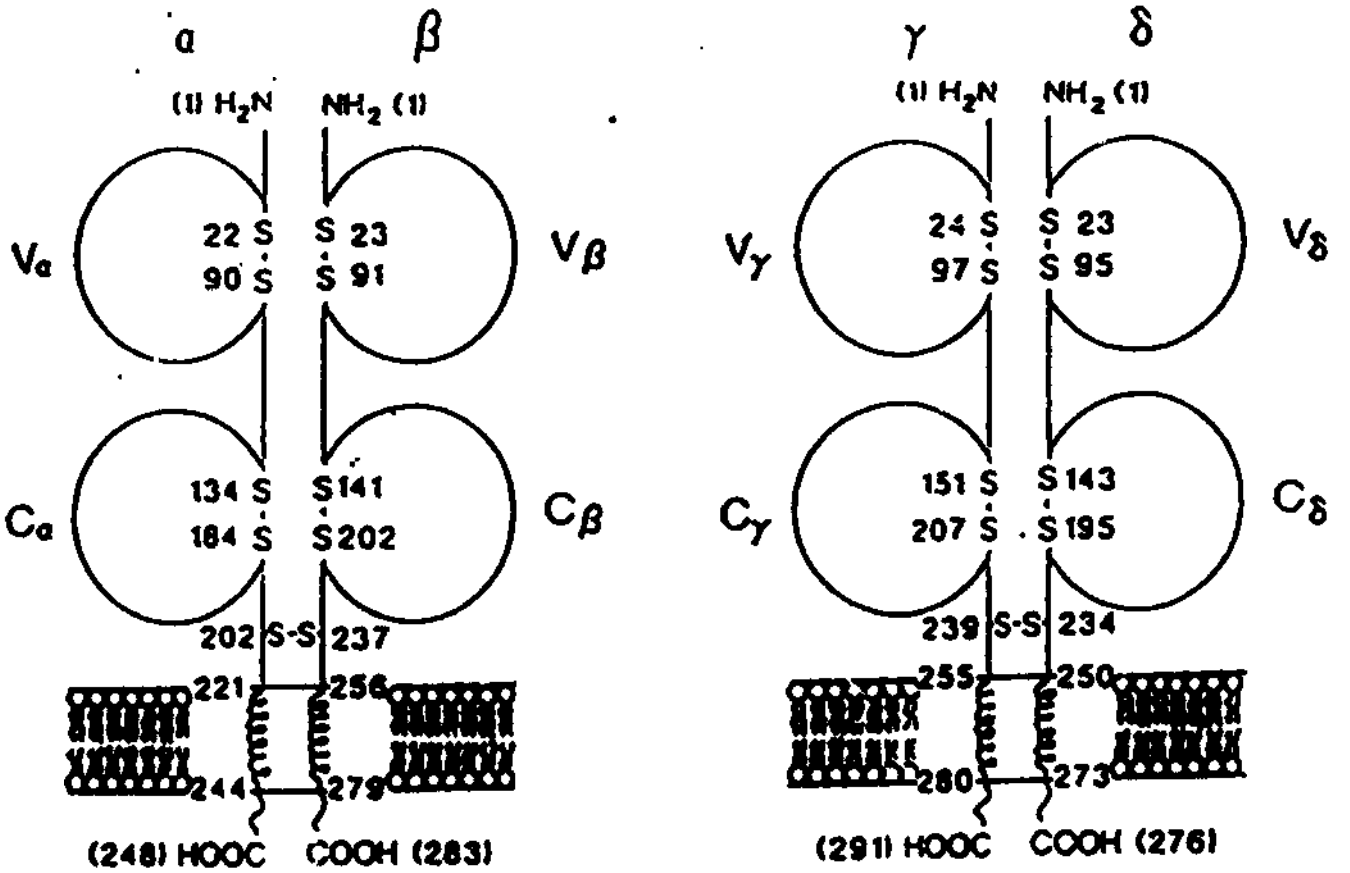

α
β
(1) H2N
NH2 (1)
Vα
Vβ
22 S
90 S
S 23
S 91
Cα
Cβ
134 S
184 S
S141
S202
202 S-S 237
221
256
244
279
(248) HOOC
COOH (283)
γ
δ
(1) H2N
NH2 (1)
Vγ
Vδ
24 S
97 S
S 23
S 95
Cγ
Cδ
151 S
207 S
S 143
S 195
239 S-S 234
255
250
280
273
(291) HOOC
COOH (276)

Figure 2.

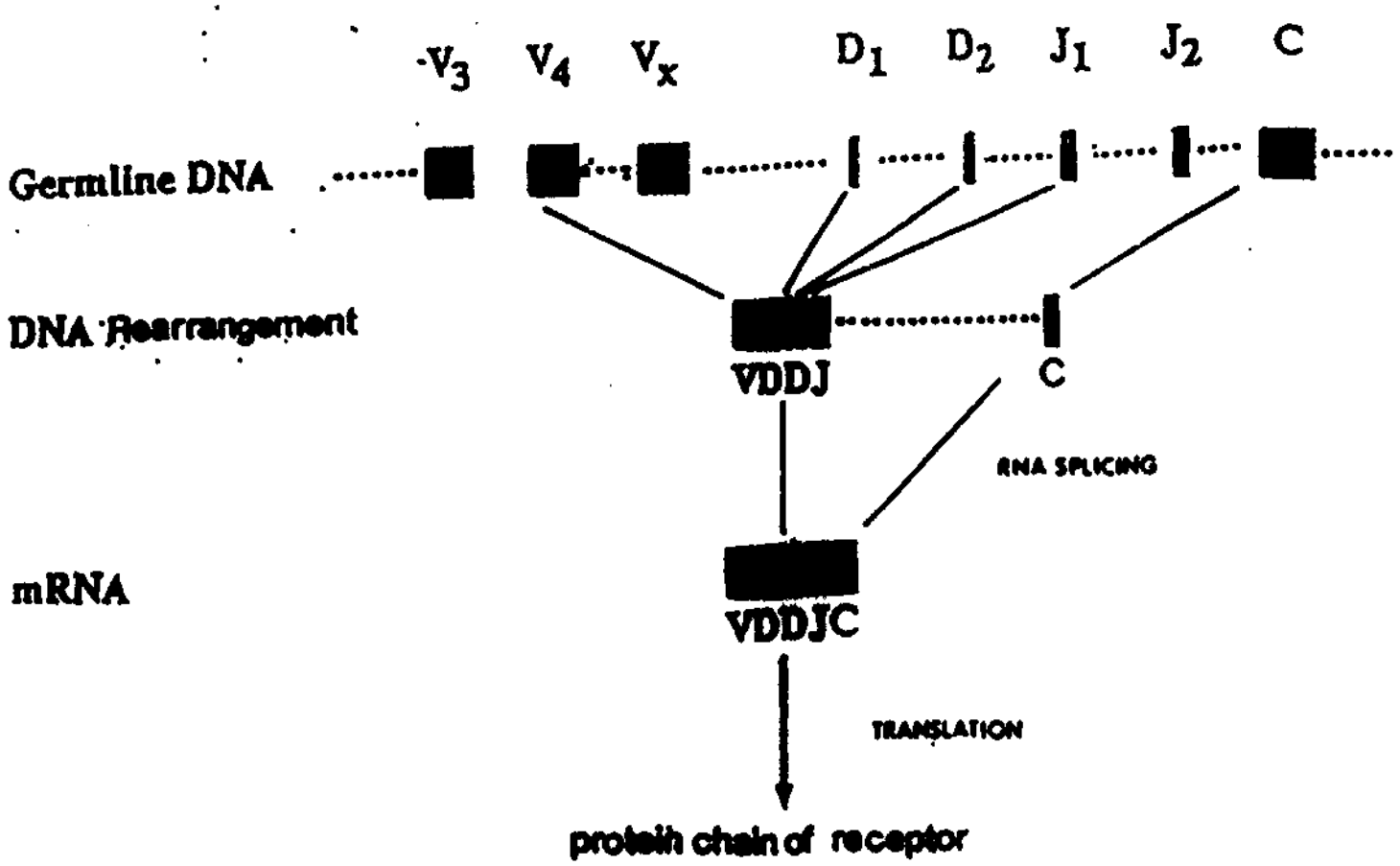

V_3
V_4
V_x
D_1
D_2
J_1
J_2
C
Germline DNA
DNA Rearrangement
VDDJ
C
RNA SPLICING
mRNA
VDDJC
TRANSLATION
protein chain of receptor

Figure 3.

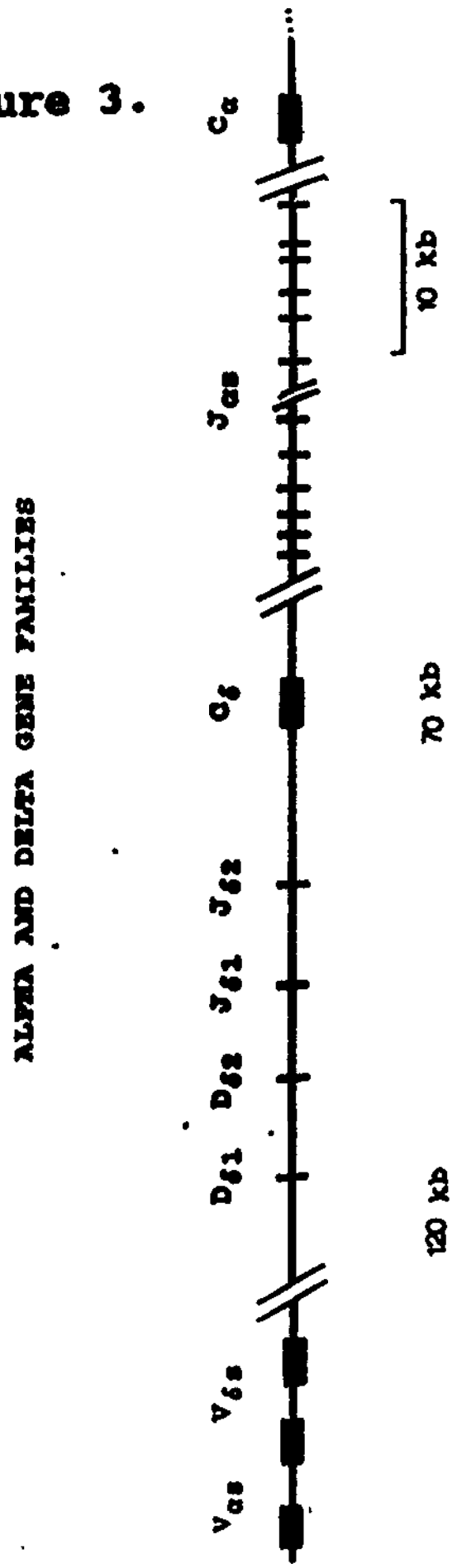
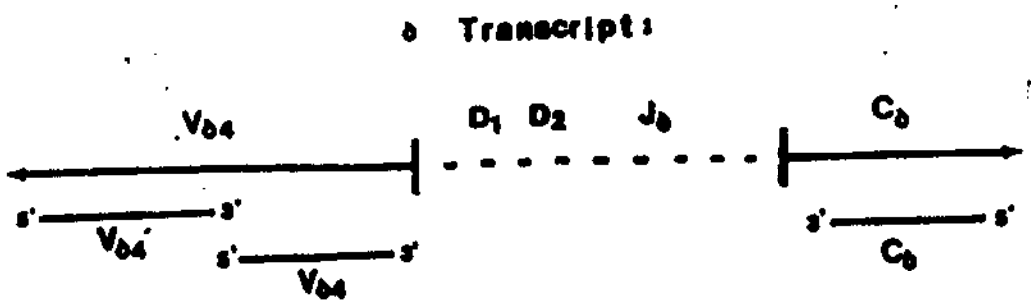

ALPHA AND DELTA GENE FAMILIES
C_α
$J_{\alpha 8}$
C_δ
$J_{\delta 2}$
$D_{\delta 1}$
$D_{\delta 2}$
$J_{\delta 1}$
$V_{\delta 8}$
$V_{\alpha 8}$
10 kb
70 kb
120 kb

Figure 4.

Amplification of δ-Gene Transcripts



T CELL RNA

↓ $C_δ$ primer, XTPs

↓ Reverse Transcriptase

cDNA

$V_{δ4}$ primer, XTPs

↓ Taq Polymerase

↓ 92°C, 1 min

↓ 50°C, 1 min

↓ 70°C, 2 min

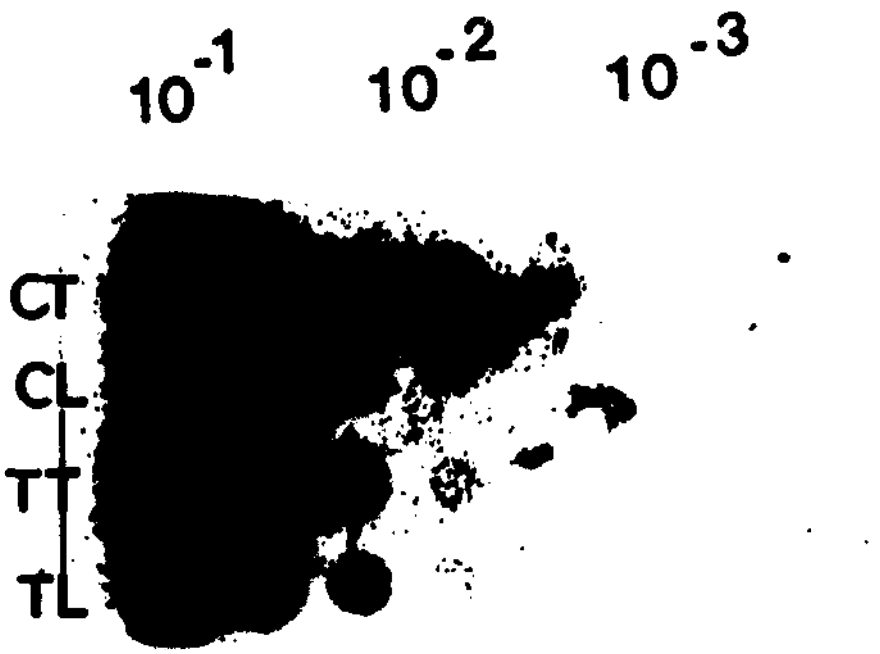
↓ 30 Cycles

AMPLIFIED $V_{δ4}C_δ$ TRANSCRIPTS

↙ BLOT

↘ CLONE AND SEQUENCE

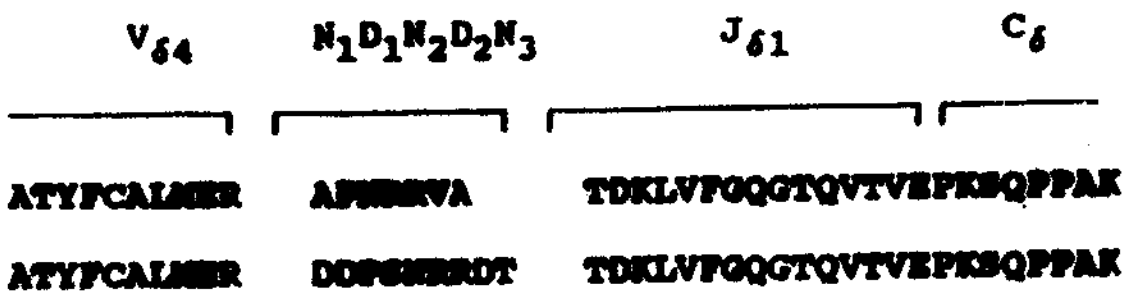
Figure 5.



	$V_{\delta 4}$	N_1	D_1	N_2	D_2	N_3	$J_{\delta 1}$
Germline			GTGGCATATCA		ATCCGACGGATACCAG		CTACCGACAAACTCGTCTTTGGACAACGAACCCAAGTC
TLN12	TGCGACGTATTTCTGTGCTCTCATGGACCCCG	CCCCTA			ATCCGACGG	TCG	CTACCGACAAACTCGTCTTTGGACAACGAACCCAAGTC
TLN24	TGCGACGTATTTCTGTGCTCTCATGGACCCCG	ACG	AT	CCCCTCTA	ATCCGACGGATACG		ACCGACAAACTCGTCTTTGGACAACGAACCCAAGTC
TLN35	TGCGACGTATTTCTGTGCTCTCATGGACCCCG	G			ATCCGACGGATACCAG	A	TACCAACGACTCGTCTTTGGACAACGAACCCAAGTC
TLN46	TGCGACGTATTTCTGTGCTCTCATGGACCG	GA	CCC		GACG		ACCGACAAACTCGTCTTTGGACAACGAACCCAAGTC
TLN25	TGCGACGTATTTCTGTGCTCTCATGGAG	ACAC	GTGGCAT	CT	ATCCGACCGATACCAG		CTCCTCCGACACCCGACAGATGTTTTTTCCAACTGCCA
Germline							CTCCTCCGACACCCGACAGATGTTTTTTCCAACTGCCA
							$J_{\delta 2}$

Figure 6

Figure 7.


V_{64}
$N_1D_1N_2D_2N_3$
J_{61}
C_6
ATYFCALSER ASSSNVA TDKLVFGQGTQVTVEPKSQPPAK
ATYFCALSER DDPGSSRDT TDKLVFGQGTQVTVEPKSQPPAK