

DETERMINATION OF CIS-ACTING REGULATORY ELEMENTS OF THE
IL-7R α GENE USING FLOW CYTOMETRIC REPORTER ASSAYS

by
ŞAFAK İŞİL NALBANT

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Master of Science

Sabancı University
February 2006

© Şafak Işıl Nalbant

ALL RIGHTS RESERVED

ABSTRACT

Interleukin 7 (IL-7) is a cytokine that has an essential role in the development and survival of lymphocytes. IL-7 signaling through its receptor complex IL-7R, has diverse effects in the early stages of development of T-and B-lymphocytes. IL-7 also plays a role in the maintenance of T-cell homeostasis and survival of memory T-cells. IL-7R is a heterodimer comprised of the common gamma chain (γ_c) and IL-7R alpha chain (IL-7R α). As γ_c is expressed ubiquitously on lymphocytes, IL-7 responsiveness in different developmental stages is controlled by stage specific IL-7R α gene transcription. Although two different transcription factors, PU.1 and GABP were identified as transcriptional regulators of IL-7R α in earlier studies, the complete transcriptional mechanism still remains to be elucidated. The aim of this study was to determine cis-acting regulatory regions of IL-7R α . Conserved noncoding sequences in the upstream region of IL-7R α were identified using bioinformatic tools. Three of the seven identified regions were cloned into a GFP reporter vector and transfected into EL-4 mouse T lymphoma cells. Transcriptional enhancer activities of these regions were assessed using flow cytometry to record GFP levels in live cells. Flow cytometric analyses revealed that one of the constructs showed a higher level of expression compared to background indicating that this CNS contains a potential transcription enhancer element. This study also demonstrated that the constructed GFP reporter vectors and flow cytometric analysis of reporter expression were convenient to identify and characterize putative cis-acting regulatory regions acting on a particular gene of interest.

ÖZET

İnterlökin-7 (IL-7), lenfositlerin gelişimi ve sağkalımında önemli role sahip sitokinlerden biridir. Kendi reseptörü (IL-7R) üzerinden IL-7 sinyal iletimi, T- ve B-lenfosit gelişimlerinin erken evrelerinde farklı etkilere sahip olup, aynı zamanda hafıza T hücrelerinin sağkalımı ve T hücresi homeostazında da önemli role sahiptir. IL-7R, ortak gama zinciri (γ c) ve interlökin reseptör alfa zincirinden (IL-7R α) oluşan bir heterodimerdir. Lenfositlerde gama zincirinin transkripsiyonunun her zamana gerçekleşiyor olması nedeniyle farklı IL-7 yanıtları, IL-7R α gen transkripsiyonunun kontrolü sonucunda oluşur. Önceki çalışmalarda IL-7R α 'nın transkripsiyonel kontrolünde etkili olan iki farklı transkripsiyon faktörü PU.1 ve GABP tanımlanmış olmasına karşın, bu genin transkripsiyon mekanizmasının tamamı bilinmemekte ve aydınlatılmayı beklemektedir. Bu çalışmada amaç IL-7R α geninin transkripsiyon kontrol bölgelerini belirlemek olup, IL-7R α geninin upstream bölgesindeki kodlanmayan korunmuş diziler, temel biyoinformatik araçları kullanılarak tanımlanmıştır. Tanımlanan 7 bölgeden 3'ü GFP-reporter vektöre klonlanmış ve EL-4 fare T-lenfositlerine aktarılmıştır. Transkripsiyonel aktivite, canlı hücrelerdeki GFP seviyelerini belirlemek için flow sitometri kullanılarak test edilmiştir. Flow sitometre analizlerinin sonucunda konstraklardan birinin bazal seviyeye göre yüksek ekspresyon gösterdiği belirlenmiştir. Bu veriler, olası enhancer elementinin bu bölgede olabileceğini göstermektedir. Tüm bunlara ek olarak bu çalışma, oluşturulmuş GFP-reporter vektörlerin ve reporter ifadesini gösteren flow sitometrik analizlerin ilgilenilen gendeki olası cis-acting düzenleyici bölgelerin karakterize edilmesi ve tanımlanması için uygun olduğunu göstermektedir.

To my family...

ACKNOWLEDGMENTS

First of all, I would like to express my gratitude to my supervisor Batu Erman for his guidance and advices, support and patience throughout the whole study. As a prospective scientist, I feel extremely lucky for having such a role model like him. I would like to express my appreciation to the members of my thesis committee Hüveyda Başağa and Alpay Taralp for their advices, helpful criticisms and support not only for the thesis and in the dissertation but also during the hard times. I would also like to express my sincere thanks to Uğur Sezerman, for his continuous encouragement as a whole and for his creative ideas particularly in relation to the bioinformatics part of this project.

I would like to thank Sema Kurtuluş and Halil İbrahim Aksoylar for their technical support throughout the study in the lab. I should also mention Alper Arslan, Özgür Gül, Pınar Önal, Tuğsan Tezil and Onur Gökçe, and thank them all for sharing their experience and knowledge during the studies, also for their support during the times we spent together. There is no way I can appreciate the overall support of my dearest friends Aylin Aydın and Eren Şimşek. I thank my office mates Burcu Köktürk and Özge Cebeci for cheering me up during writing the thesis.

Last but certainly not least, I would like to acknowledge my wonderful family. My father Levent Nalbant and my mother Dilek Nalbant had always encouraged me to pursue a higher education and to aspire to be a successful scientist. I could not be the person and daughter whom they are proud of now, without the support, love and trust I received from them. I should also mention the great contribution of my father on the matter of improving the technical parts of this thesis. My beautiful and smart sister Başak has been the best friend and little comrade that one could ever have. My special thanks to my aunt Demet Nalbant for being another great model and for her valuable contributions and outstanding advices not only in this study but also throughout my life. I would also like to thank Oytun Çevik for understanding and respecting my desire to pursue my dreams and for extending his constant love and patience.

TABLE OF CONTENTS

ABSTRACT	iii
ÖZET	iv
ACKNOWLEDGMENTS	vi
LIST OF SYMBOLS AND ABBREVIATIONS	xiii
1 INTRODUCTION	1
1.1 INTERLEUKIN-7 AND ITS RECEPTOR	1
1.1.1 INTERLEUKIN-7	1
1.1.2 IL-7 RECEPTOR	2
1.1.3 IL-7 RECEPTOR SIGNALING	4
1.2 B CELL DEVELOPMENT AND IL-7	5
1.3 T LYMPHOCYTES AND IL-7	8
1.3.1 DEVELOPING T CELLS	9
1.3.2 MATURE T CELLS	11
1.3.3 T CELL HOMEOSTASIS	12
1.4 IL-7 RECEPTOR EXPRESSION AND TRANSCRIPTIONAL CONTROL	12
1.5 MECHANISM OF TRANSCRIPTIONAL REGULATION	14
1.5.1 GENE EXPRESSION CONTROL AT DNA LEVEL	14
1.6 COMPARATIVE GENOMICS AND IDENTIFICATION OF REGULATORY REGIONS ON DNA	16
1.6.1 CROSS-SPECIES SEQUENCE COMPARISONS	17
1.7 REPORTER ASSAY SYSTEMS	18
1.7.1 LUCIFERASE REPORTER ASSAY SYSTEM	18
1.7.2 FLUORESCENCE REPORTER ASSAY SYSTEMS	19
2 PURPOSE OF THE STUDY	21
3 MATERIALS AND METHODS	22
3.1 MATERIALS	22
3.1.1 CHEMICALS	22
3.1.2 EQUIPMENT	23
3.1.3 BUFFERS AND SOLUTIONS	24

3.1.4	GROWTH MEDIA.....	25
3.1.5	MOLECULAR BIOLOGY KITS.....	25
3.1.6	ENZYMES.....	26
3.1.7	CELLS, PLASMIDS, OLIGOS.....	26
3.1.8	COMPUTER BASED PROGRAMS.....	28
3.2	METHODS	28
3.2.1	CULTURE GROWTH	28
3.2.2	IDENTIFICATION AND AMPLIFICATION OF CONSERVED NON-CODING SEQUENCES.....	29
3.2.3	VECTOR CONSTRUCTION.....	30
3.2.4	CLONING OF AMPLIFIED REGIONS INTO REPORTER VECTORS	33
3.2.5	TRANSIENT TRANSFECTION OF MAMMALIAN CELLS.....	33
3.2.6	FLOW CYTOMETRIC ANALYSIS	33
4	RESULTS	34
4.1	IDENTIFICATION AND AMPLIFICATION OF THE CONSERVED NON-CODING REGIONS.....	34
4.1.1	DETERMINATION AND RETRIEVAL OF GENOMIC SEQUENCES	34
4.1.2	ALIGNMENT OF THE SEQUENCES.....	35
4.1.3	DETERMINATION OF BAC CLONE.....	38
4.1.4	AMPLIFICATION OF MOUSE CONSERVED NON-CODING SEQUENCES	38
4.2	CONSTRUCTION OF LUCIFERASE REPORTER VECTOR.....	39
4.3	CONSTRUCTION OF pGL3.Fos-SV40	43
4.4	CONSTRUCTION OF GFP REPORTER VECTOR.....	45
4.5	CONSTRUCTION OF pTurboGFP-fos-ESV40	48
4.6	CONSTRUCTION OF pDSRedExpress-pSV40.....	52
4.6.1	CONSTRUCTION OF pDSREXPRESS-PSV40-ESV40.....	54
4.7	CONSTRUCTION OF pCDNA3-EGFP	56
4.8	CLONING OF AMPLIFIED REGIONS INTO REPORTER VECTORS	56
4.8.1	CLONING TO THE LUCIFERASE REPORTER VECTOR- pGL3.FOS	56

4.8.2	CLONING TO THE GFP REPORTER VECTOR-pTURBOGFP.Fos.....	60
4.9	TRANSIENT TRANFECTION OF EL-4 CELLS.....	63
4.9.1	OPTIMIZATION OF ELECTROPORATION CONDITIONS.....	63
4.10	FLOW CYTOMETRIC REPORTER ANALYSIS.....	66
4.10.1	REPORTER ASSAY.....	66
5	DISCUSSION.....	70
6	CONCLUSION.....	76
7	FUTURE STUDIES.....	78
8	REFERENCES.....	79
	APPENDIX-PLASMID MAPS.....	90

LIST OF FIGURES

Figure 1.1: Type-1 cytokine receptor family	3
Figure 1.2: B cell development.....	6
Figure 1.3: T cell development in thymus	9
Figure 1.4: Schematic representation of distant enhancer activity on transcription.....	15
Figure 4.1: IL-7R α gene locus and upstream sequences of a) mouse and b) human from USCS Genome Browser	34
Figure 4.2: The regions taken and corresponding numbers given through analysis for a)mouse and b)human.....	35
Figure 4.3: The AVID alignment results,visualized with VISTA , window length=50bp, minimum percent identity= 85%	37
Figure 4.4: The BAC clone determined using USCS Genome Browser	38
Figure 4.5: Amplification of K1 to K7.	38
Figure 4.6: Strategy for constructing the pGL3.Fos luciferase reporter plasmid.	39
Figure 4.7: Construction of pGL3.Fos.....	40
Figure 4.8: The restriction map of pGL3.Fos showing only the relative locations of the restriction enzymes used for diagnosis and confirmation.....	41
Figure 4.9: Diagnostic digest of pGL3.Fos.....	41
Figure 4.10: The digests for confirmation of pGL3.Fos plasmid (colony 2)	42
Figure 4.11: Strategy for constructing the pGL3Fos-SV40 positive control plasmid....	43
Figure 4.12: The BamH1 and Nco1 digested pGL3.Fos and pGL3Enhancer.	44
Figure 4.13: Restriction map of pGL3Fos-SV40 positive control plasmid showing only the relative locations of restriction enzyme recognition sites used for diagnosis and confirmation.....	44
Figure 4.14: Confirmation of pGL3Fos-SV40 positive control plasmid.....	45
Figure 4.15: Strategy for constructing the pTurboGFP-fos reporter plasmid.....	46
Figure 4.16: Restriction map of pTurboGFP-fos plasmid, showing only the relative locations of restriction enzyme sites used for diagnosis and confirmation.	47
Figure 4.17: Pvu2 digest for identification of pTurboGFP.Fos plasmid.	47
Figure 4.18: The confirmation of pTurboGFP.Fos plasmid.	48
Figure 4.19: Strategy for constructing the pTurboGFP-fos-ESV40 positive control plasmid.....	49

Figure 4.20: pTurboGFP-fos plasmid and SV40 PCR product.	49
Figure 4.21: Determination of amount of SV40 enhancer PCR product to be used for ligation.	50
Figure 4.22: The Sal1 digests for identification of pTurboGFP-fos-ESV40.	50
Figure 4.23: Restriction map of pTurboGFP-fos-SV40 plasmid showing only the relative locations of restriction enzyme sites used for confirmation.	51
Figure 4.24: Confirmation of positive control plasmids pTurboGFP.Fos-ESV40 (G5 and G9).	52
Figure 4.25: Strategy for constructing the pDSRedExpress-pSV40 plasmid.	53
Figure 4.26: pDSRedExpress-pSV40 construction.	53
Figure 4.27: Diagnostic digests of three different colonies of pDSRec-pSV40.	54
Figure 4.28: Sal1 digestion for identification of pDSRedExpress-pSV40-ESV40.	55
Figure 4.29: Further characterization of pDSRedExpress-pSV40-ESV40 plasmids.	56
Figure 4.30: Determination of amount of K1 to K7 PCR product to be used for ligation.	57
Figure 4.31: Colony PCR results of ligations of regions K1 to K7 into Sal1 digested pGL3.Fos plasmid.	58
Figure 4.32: Restriction map of pGL3fos and inserted regions.	59
Figure 4.33: Confirmation of K1, K2 and K3 cloned into pGL3.Fos plasmid.	59
Figure 4.34: Confirmation of K6 and K7 cloned into pGL3.Fos plasmid.	60
Figure 4.35: The regions K1, K2, K3 and K6, K7 digested out from pGL3.Fos.	60
Figure 4.36: Colony PCR results of identified colonies containing inserts K3, K6 and K7.	61
Figure 4.37: Restriction map of the pTurboGFP.Fos and inserted regions.	62
Figure 4.38: Diagnostic digests of pTurboGFP.Fos plasmid containing the inserted regions K3, K6 and K7.	63
Figure 4.39: Flow cytometric analysis of untransfected cells.	64
Figure 4.40: Flow cytometric analysis of six different conditions of electroporation showing GFP versus number of events.	65
Figure 4.41: Flow cytometric analysis of untransfected cells to define the viable EL4 population and P2 gate of GFP fluorescence.	67
Figure 4.42: The flow cytometry data of reporter assays showing GFP versus number of events in each histogram.	68

LIST OF TABLES

Table 3.1: List of Enzymes	26
Table 3.2: List of Plasmids	27
Table 3.3: List of primers used for amplification of seven CNS.....	27
Table 3.4: List of primers used for amplification of SV40 enhancer from pGL3Control plasmid.....	28
Table 3.5: PCR Conditions	30
Table 4.1: Regions determined to be tested K1 to K7.....	36
Table 4.2: Optimization of electroporation conditions for EL-4 cells.....	63
Table 4.3: The quantitative data obtained from flow cytometric analysis of six different electroporation conditions.....	66
Table 4.4: The quantitative data obtained from flow cytometric analysis reporter assay constructs.	69

LIST OF SYMBOLS AND ABBREVIATIONS

-/-	Knock-out
α	Alpha
γ	Gamma
BAC	Bacterial Artificial Chromosome
BCR	B Cell Receptor
bp	Basepair
CIAP	Calf Intestinal Alkaline Phosphatase
CNS	Conserved Noncoding Sequence
Da	Dalton
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DN	Double Negative
DNA	Deoxyribonucleic Acid
DP	Double Positive
EDTA	Ethylenediaminetetraaceticacid
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GABP	GA Binding Protein
GFP	Green Fluorescent Protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-7R	Interleukin-7 Receptor
JAK	Janus Kinase
Luc	Luciferase
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Protein
MODC	Mouse ornithine decarboxylase
NCBI	National Center for Biotechnology Information

NK	Natural Killer Cells
nm	Nanometers
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PI-3	Phosphatidylinositol 3-kinase
RAG	Recombinase Activating Genes
rpm	Revolution per minute
SCID	Severe Combined Immunodeficiency Disease
SOCS	Suppressors of Cytokine Signaling
SP	Single Positive
STAT	Signal Transducer and Activator of Transcription
SV40	Simian Virus 40
TBE	Tris Borate EDTA
TCR	T Cell Receptor
TGF	Transforming Growth Factor
TN	Triple negative
TSLP	Thymic Stromal Derived Lymphopoietin
USCS	University of California at Santa Cruz

1 INTRODUCTION

1.1 INTERLEUKIN-7 AND ITS RECEPTOR

Cytokines are small protein molecules that regulate communication among immune system cells and those of other tissue types. Cytokines, actively secreted by immune cells as well as other cell types in response to external stimuli, act by binding to their cell-specific receptors. These receptors are located in the cell membrane, and each allows a distinct signal transduction cascade to start in the cell, that eventually leads to biochemical and phenotypical changes in the target cell.¹ Cytokines have key roles in controlling the growth, development and functional differentiation of lymphocytes and as effector molecules of activated T cells. The action of cytokines are said to be pleiotropic, meaning that the same cytokine can have different effects on a cell depending on the type or state of the cell.²

1.1.1 INTERLEUKIN-7

Interleukin-7 (IL-7), previously termed ‘lymphopoietin 1’ and ‘pre-B cell factor’, is classified as a type 1 short-chain cytokine of the hematopoietin family that is essential for the development and the survival of lymphocytes. IL-7 was first discovered in 1998 as a factor that promoted murine B cell precursor growth in a bone marrow culture system.³ It was then shown that IL-7 injection into mice induces increase in number of both T and B lymphocytes.⁴ IL-7 is known to play an essential non-redundant role in the development of T and B cells in the mouse and of T cells in humans.

There is an important requirement of IL-7 for lymphocytes. In the bone marrow the IL-7 mediated signaling is required for T- and B-precursor cell maturation and survival.⁵⁻⁷ In the thymus the earliest stem cells require IL-7 for survival, proliferation and rearrangement of genes coding for T cell receptor.^{8,9} IL-7 found to be a critical cytokine for regulation of T-cell receptor gamma (TCR γ) and immunoglobulin heavy

chain (IgH) locus accessibility to the V(D)J recombinase.¹⁰ At a later stage, IL-7 is involved in positive selection of CD8 cells.⁹ More recently, IL-7 was also shown to be required for survival and peptide-induced homeostatic proliferation of mature and naive peripheral T cells.¹¹⁻¹³ Furthermore, circulating levels of IL-7 increase in response to T cell depletion, suggesting a role in T cell regeneration.^{14, 15} It is evident that IL-7 is one of the important factors that determine the size of peripheral T cell pool. Besides its important role in T lymphocyte development, IL-7 is also required for the generation of murine B lymphocytes, but not the human B cell.

IL-7 was shown to be produced by the non-lymphoid cells in lymphoid organs. Production of IL-7 has been detected from multiple stromal tissues, including epithelial cells in thymus and bone marrow.¹⁶ The mechanism of IL-7 production is not well defined, especially in stromal cells that are considered the main production site of this cytokine. In human peripheral monocytes IL-7 also induces the synthesis of some inflammatory mediators such as IL-1, IL-6 and macrophage inflammatory protein, MIP.¹⁰ Transforming growth factor beta (TGF- β) and IL-7 share an antagonistic relationship, wherein they reciprocally can down-regulate each others production. Although the mechanism and implications of this relationship are yet to be elucidated, in macrophages this function of IL-7 has been suggested as an inhibitor of the antitumor immune response.¹⁷

1.1.2 IL-7 RECEPTOR

IL-7 receptor is a type I cytokine receptor that is a heterodimer, composed of IL-7 receptor alpha chain (IL-7R α) (CD127) and a common cytokine gamma chain (γ c) that is shared by IL-2, IL-4, IL-9, IL-15 and IL-21 receptors (Figure 1.1), also involved in lymphocyte maturation, homeostasis and proliferation. Each of these chains is independently expressed on the cell surface.^{2,16,18,19} The γ c chain was originally cloned as a third component of the IL-2 receptor.²⁰ It was shown that the mutations in the chain results in diverse non-functional lymphoid cells thus the chain is common for interleukins that are important in lymphocyte development. γ c is expressed ubiquitously on lymphoid cells. All six of the γ c-dependent cytokines were shown to have the ability to act, at least in vitro, as T-cell growth factors.^{2,21}

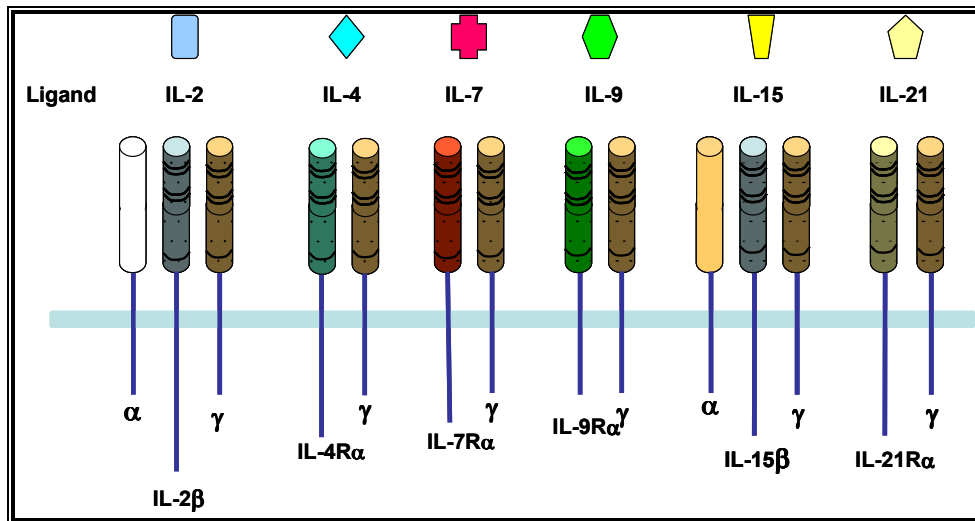


Figure 1.1: Type-1 cytokine receptor family

The cells expressing only γc do not bind IL-7.^{18,19} When the binding affinity of IL-7 to the receptor was tested by reconstitution studies, the cells showed intermediate IL-7 binding affinity when transfected with IL-7R α alone and showed high IL-7 binding affinity when transfected with complex of IL-7R α and γc chain.²² The findings suggest the existence of an IL-7 binding site on IL-7R α chain that is γc dependent. When the downstream signaling of IL-7 receptor was examined, it was found that the homodimerization of IL-7R α was not sufficient for signaling.^{19,23} In addition the intact cytoplasmic domains of each chain were shown to be required for downstream STAT5 activation.^{24,25} Together these data indicate that both IL-7R α and γc are essential for the biologically important effects of IL-7.

The IL-7R α gene is localized to human chromosome 5²⁶ and murine chromosome 15.^{2,18,26} Both human and mouse genes span eight exons and seven introns with a size of 19.79 kb in the human and 22.17 kb in mouse.^{2,19} IL-7R α is a type 1 membrane glycoprotein folded to accommodate the binding of alpha helical cytokines. The 220 amino acid extracellular domain contains major regions of homology with other members of this family. In addition, there is a single 25 amino acid transmembrane region and a 195 amino acid cytoplasmic tail that is important for recruitment of intracellular signaling molecules.^{27,28} The mature form of IL-7R α consisting of 439 amino acids has a calculated molecular weight of 49.5 KDa.^{19,29}

IL-7R α is expressed on hematopoietic cells, especially of the lymphoid lineage. IL-7R α can be identified on fetal NK/dendritic precursors, bone marrow macrophages, common T/NK/B lymphoid precursors, immature B cells through the early pre-B stage, on thymocytes, and on most mature T cells with transient down-regulation upon activation.^{29,30} IL-7R α is also a component of the high-affinity receptor complex of thymic stromal-derived lymphopoietin (TSLP) which heterodimerizes with the unique TSLP receptor chain that has some homology to the γ c chain and the erythropoietin receptor.³¹

1.1.3 IL-7 RECEPTOR SIGNALING

IL-7 signaling involves a number of non receptor tyrosine kinase pathways that associate with the cytoplasmic tail of the receptor. These include the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, phosphatidylinositol 3-kinase (PI3-kinase), and Src family tyrosine kinases. IL-7 shares intracellular signaling molecules with a number of other cytokines, and the exact mechanism responsible for signaling specificity remain unclear.^{16,19,32,33}

JAK-STAT dependent signaling pathway initiates when IL-7 binds IL-7R α that leads to dimerization of IL-7R α with γ c that also has a weak IL-7 binding site.^{34,35} Jak3, associated with γ c, phosphorylates tyrosine residues in the cytoplasmic portion of IL-7R α that leads to recruitment of Jak1 and of STAT molecules. Phosphorylation of STATs by JAK1 and JAK3 induces their dimerization, translocation to the nucleus and activation of specific genes.^{24,25,32,33,35,36} Signaling ends when IL-7 induces negative feedback on its own signaling by down regulating JAK1 activation. Suppressor of cytokine signaling (SOCS) proteins can bind and inhibit JAKs. The negative feedback loop involves SOCS expression induced by IL-7^{24,32,37,38} and inhibition of STAT5 phosphorylation by SOCS1.

PI3 kinase pathway that plays a central role in promoting the survival and growth of a wide range of cell types has been demonstrated to be important for B and T cell development. Developmental and functional defects in B cells and reduced thymic cellularity are observed when regulatory subunit or catalytic subunit of PI3 kinase is

disrupted respectively.^{39,40} IL-7 has been shown to activate PI3 kinase pathway and activation of PI3 kinase has been found to be essential for the IL-7 mediated survival and proliferation of human T cell precursors.^{41,42} In murine B cells PI3 kinase is activated after its regulatory subunit is recruited by a residue of IL-7R α chain and mutation of this residue shown to block the PI3 kinase activity, PI3 kinase components are viable candidates for IL-7 downstream mediators.⁴³ The downstream events in PI3 kinase pathway in response to IL-7 have not yet been fully determined and it constitutes a hot topic of research.¹⁹ Src family of non-receptor tyrosine kinases are also activated by IL-7⁴⁴ although none of the members of the family have been shown to be uniquely required for IL-7. In T lymphocytes and in a pre-B cell line, two members of family, Lck and Fyn are associated with IL-7R α and they undergo phosphorylation leading to the successive phosphorylation of exogenous substrates in response to IL-7.¹⁹ Although the IL-7 receptor is very similar to the IL-2, IL-4, IL-9, IL-15 and IL-21 receptors, it is uniquely required for lymphocyte development and homeostasis. Therefore it is possible that there is a unique yet to be determined another signaling pathway through IL-7R α , which distinguishes it from these related receptors.

1.2 B CELL DEVELOPMENT AND IL-7

IL-7 was first identified based on its capacity to induce the growth of immature B lymphocytes.^{3,6} The mice deficient in IL-7 or IL-7R α and monoclonal antibody blocking experiments demonstrate the requirement of IL-7 for B cell development in mice. B cell development proceeds from a common lymphoid progenitor that is characterized by the expression of IL-7R α expression but lacks lineage-specific markers. The first progenitor that is committed to the B lineage is the pre-pro-B cell. The committed cells then transit to the pro-B-cell stage involving a period of proliferation and the initiation of immunoglobulin heavy chain rearrangement. Productive rearrangement of heavy chain, completed at the late pro-B-cell stage, leads to the expression of an intact μ heavy chain, that is the hallmark of next main stage of development, the pre-B cell stage. At the large pre-B-cell stage the cell express its BCR composed of μ and a surrogate light chain on the cell surface and divide several times before giving rise to small pre-B cells, in which light-chain rearrangements begin. Once

a light-chain is assembled and a complete immunoglobulin molecule is expressed on the cell surface, the cell is defined as an immature B cell. Immature B cells then undergo selection for self tolerance; B cells that survive in the periphery undergo further differentiation to become mature B cells.¹ (Figure 1.2)

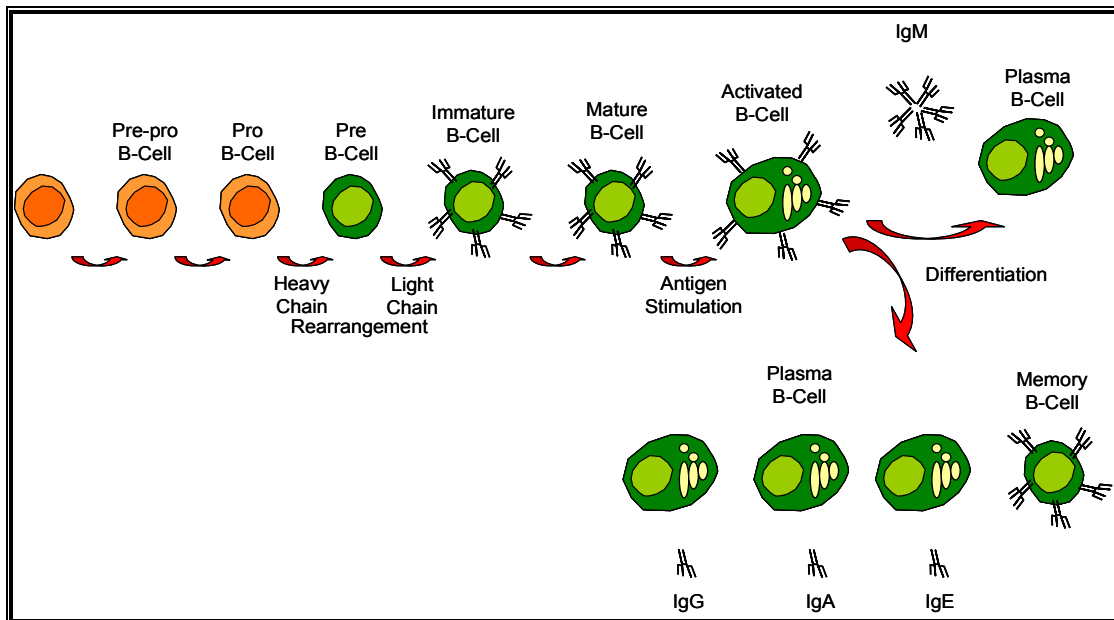


Figure 1.2: B cell development

In $IL-7R\alpha^{-/-}$ mice, a block in B cell development occurs at the pre-pro-B cell stage^{45,46}, whereas in the $IL-7^{-/-}$ mice, the block in development occurs later at the transition from pro-B-cell to the pre-B cell.⁴⁷ These results indicated the presence of a second ligand of the $IL-7R\alpha$, a molecule that regulates B-cell development at the pre-pro-B cell stage. This second molecule is found to be TSLP.^{31,48} Transgenic $IL-7$ expression in lymphoid cells using other immunoglobulin promoter results in the dramatic expansion of immature and mature B-cells.⁴⁹ Furthermore, the administration of exogenous $IL-7$ to normal mice leads to significant expansion of pre-B cells and mature B cells in normal and lymphocyte depleted mice.⁵⁰⁻⁵²

Although it is particularly clear that levels of $IL-7$ potentially expand B cell progenitors in mice, leading to the expansion of the entire B cell population, it is evident that murine B cells show significant changes in the capacity and threshold for $IL-7$ induced proliferation, depending on the exact stage in development. Pre-pro B cells

display a high threshold for IL-7 induced proliferation, followed by a diminished threshold at the pro-B-cell state with a return of higher threshold at the pre-B-cell stage.⁵³ Moreover, it is also shown that there is a requirement of stromal contact for the IL-7 induced proliferation of pre-pro B cells (before immunoglobulin rearrangement) but not of pro-B cells(D-J rearranged).^{29,54,55}

Immunoglobulin variable regions are encoded in several so called gene segments and these segments are assembled in the developing lymphocyte by somatic DNA recombination named gene rearrangement that is mainly mediated by recombinase activating gene-RAG.¹ It was shown that, pro-B cells from RAG2^{-/-} mice which lack a pre-B cell receptor, have an increased threshold for IL-7 responsiveness at the pro-B cell stage and a failure to shut down IL-7 responsiveness at the pre-B cell stage.^{53,56} These data lead to the argument that assembly of the B cell antigen receptor (BCR) complex regulates IL-7-induced proliferation.⁷ Corcoran et al.⁵⁷ found impaired immunoglobulin gene rearrangements in IL-7R α ^{-/-} mice, but D-J and VDJ rearrangements of the heavy chain locus were detectable in IL-7^{-/-} mice⁵⁵ which is possibly due to another putative factor that uses IL-7R α . Indeed, by transferring mutated forms of the gene for IL-7R α into IL-7R α ^{-/-} mice, it was found that even the mutation at site that abrogated proliferation retained the ability to mediate immunoglobulin gene rearrangement. Therefore, it appears that signaling through IL-7R α might play a role in immunoglobulin gene rearrangement and it remains possible that TSLP or another molecule to be identified can induce these effects on immunoglobulin rearrangement.

Mature B cells are generally incapable of responding to IL-7. However, recent work has demonstrated that a subset of peripheral B cells can become transiently IL-7 responsive. As RAG expression was thought to cease in peripheral B cells, it was originally assumed that no further immunoglobulin rearrangement does occur in mature B cells. It is now known that the immunization of mice results in the re-expression of RAG genes, it is shown that IL-7R α is also re-expressed in lymphoid germinal center B cells and that IL-7 could induce the observed re-expression of RAG.⁵⁸

IL-7 has also found to act as a trophic factor, providing a survival signal in a number of cell types, including developing B cells. This effect of IL-7 to maintain developing B cells by providing a survival signal appears to involve bcl-2 family members that includes both pro-apoptotic and antiapoptotic members.⁵⁹ Although this mechanism has been well defined for T cells (as will be discussed), the exact role of this pathway in developing B cells is yet to be elucidated.

Humans with severe combined immunodeficiency disease (SCID) caused by IL-7R α mutations show normal number of B cells and IL-7 is found not to be absolutely required for B cell development in humans.⁶⁰ In an in-vitro system, the generation of immature B cells from bone-marrow derived stem cells did not require the presence of IL-7.⁶¹ Although human B-cell development does not appear to require IL-7, immature human B cells do proliferate in response to IL-7, according to some previous reports.^{16,62,63}

1.3 T LYMPHOCYTES AND IL-7

T lymphocytes also develop from common lymphoid progenitor in the bone marrow and continue in thymus following migration. Like previously explained developing B lymphocytes, T lymphocytes also pass through series of distinct phases depending on the changes in the status of T cell receptor genes, expression of T cell receptor on the cell surface and by changes in expression of cell surface proteins and the co receptor proteins CD4, CD8, through development.⁶⁴

Defects in T cell development are highly susceptible to a broad range of infectious agents as neither specific T cell dependent antibody responses nor cell-mediated immune responses are made; resulting in SCID phenotype. Several different defects, including IL-7R can lead to the SCID phenotype as IL-7R mediated signaling is necessary for the development, survival and homeostasis of T cells.^{5,60}

1.3.1 DEVELOPING T CELLS

T cell precursors arriving in the thymus from bone marrow enter a phase of intense proliferation after they spend up to a week differentiating. The supporting cells within the thymus include epithelial cells, dendritic cells, fibroblasts and a variety of other cell types ; providing a network of cytokines and growth factors that are critical for T cell development. Production of IL-7 and SCF¹⁰, that is shown to synergize with IL-7 in thymocyte proliferation, from a subset of epithelial cells within thymus is observed coincident with the first wave of thymocyte expansion.⁶⁵ The thymocytes then bear distinctive markers of T cell lineage but do not express any of the three cell surface markers that define mature T cells that are TCR:CD3 complex, co receptors CD4 and CD8. Such cells are called double negative-DN- (or sometimes triple negative-TN-) thymocytes.^{1,66,67} (Figure 1.3)

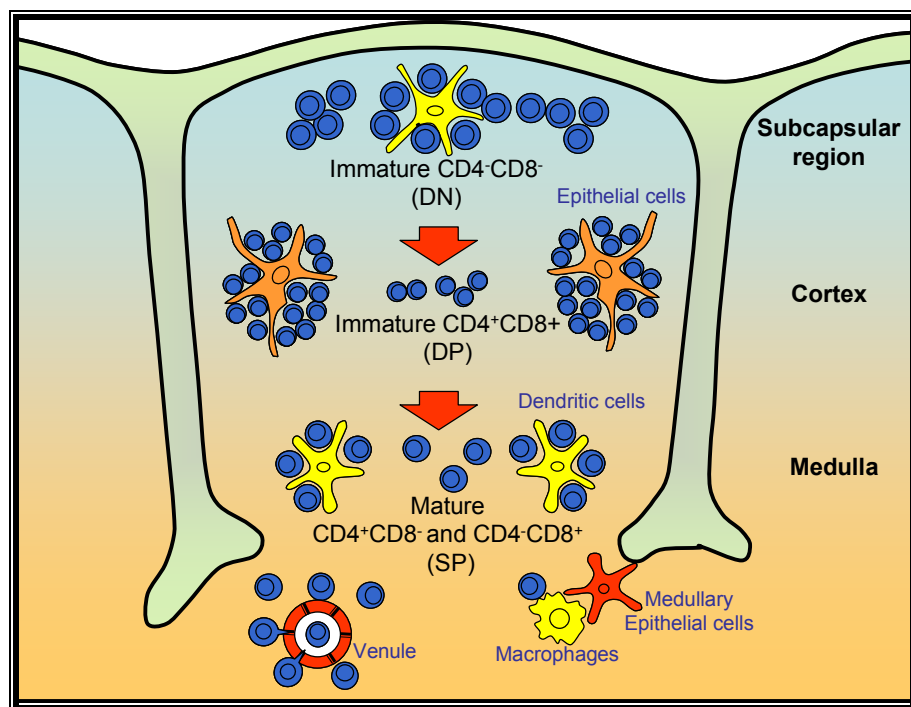


Figure 1.3: T cell development in thymus

Although IL-7 was first identified as a growth factor for developing B cells, soon after it was recognized that IL-7 could also induce the survival and proliferation of immature thymocytes in culture.⁶⁸ As in B cell development, differences between the IL-7^{-/-} and IL-7R α ^{-/-} mice have suggested the existence of other molecules using the IL-

7R α chain that are important for early T cell development.^{65,69-71} In IL-7^{-/-} mice analysis of thymocyte subsets showed a partial inhibition in DN differentiation with an accumulation of DN thymocytes.^{68,72-74} IL-7 was also shown to maintain the survival of early thymocytes during the DN stage of development through the modulation of apoptosis by altering anti-apoptotic bcl-2 and pro-apoptotic bax proteins.^{19,59,75} It is demonstrated that the forced expression of IL-7R α beyond the DN stage also results in diminished size of DN pool, and have suggested that this may occur as a result of IL-7 consumption, which results in reduced supply of IL-7 available for DN thymocytes.⁷⁶ As a result of the effects of IL-7 on DN stage of development, sufficient numbers of T cell precursors undergo the next TCR rearrangement involving stage of development and a lack of IL-7R α signaling severely curtails this process, leading to a consequent reduction in T cell export.^{46,57}

As this immature double negative thymocytes mature further, rearrangement of T cell receptor β -chain locus occurs.¹ IL-7 has been found to sustain the expression of genes known to control rearrangement of the T-cell receptor beta gene and to be a cofactor for such rearrangements during early T-cell development in mice.^{10,77-79} IL-7 also appears to be directly involved in the induction of TCR rearrangement. The exact contribution of IL-7 to the process of rearrangement is still unclear but it has recently been demonstrated that IL-7 regulates accessibility of the TCR γ locus by affecting histone acetylation through STAT5.^{10,25,79,80} and also the IL-7R α generated survival signals(bcl-2 family members) appear to be important for this stage.^{14,16}

The β -chain expressed combined with surrogate pre-T cell receptor α chain forms a pre-T cell receptor that is expressed on the cell surface in a complex with the CD3 molecules that provide the signaling components of the receptor. The expression of pre-TCR serve to maintain IL-7R α expression, perhaps allowing responsiveness to limiting concentrations of IL-7 and successful cell proliferation, arrest of further β -chain gene rearrangement and the expression of both CD4 and CD8 co-receptors and called double positive –DP- thymocytes.⁸¹⁻⁸⁴(Figure 1.3)

Decreased expression of IL-7R α chain is observed on DP thymocytes, suggesting that IL-7 mediated survival signals may be less important at this stage.⁶⁴ These double-positive thymocytes lacking the survival and proliferation signals then cease to proliferate and enter small-double positive cell stage during which the rearrangement of

the α -chain locus occurs.^{82,85} Small double positive thymocytes initially express low levels of TCR and depending on these receptors ability to recognize Major Histocompatibility Complexes (MHCs) and self antigens they undergo positive selection and negative selection that involves apoptosis.¹ The survivors of positive and negative selection go on to maturation and express higher level of TCR on their surface, until when they cease to express one or other of the two co-receptor molecules, becoming either CD4 or CD8 single positive thymocytes.⁸³ When the role of IL-7 during the differentiation from DP to SP thymocytes which is a critical step for lineage commitment as it involves the loss of expression of either CD4 or CD8 co-receptor, is examined; it is suggested that IL-7 mediates the suppression of CD4 transcription in thymocytes destined to become CD8 SP cells.⁸⁶⁻⁸⁹ The mature single positive T cells (CD4 or CD8) are then exported from thymus to periphery and become functional for the immune response. It is not really clear which cells supply the essential IL-7 to pro-T cells and peripheral T cells, nor is it clear whether synthesis of IL-7 by these cells is regulated or constitutive. Currently thinking is that T cells compete for a limited amount of IL-7 whose production is constitutive and in a way the major checkpoint of the competition is the receptor of the IL-7.^{82,90,91}

1.3.2 MATURE T CELLS

Besides the important functions of IL-7 and IL-7R α mediated signaling events during the development of both B and T cells, IL-7 potently modulates mature T cell function and thymic aging.^{92,93} IL-7 not only costimulates for T cell activation by enhancing proliferation and cytokine production through upregulation of the IL-2R α gene^{84,94}, it is also found to have a tendency to induce type 1 immune responses by upregulating IFN- γ .⁹⁵ IL-7 also enhances expression of the chemokine receptor CXCR4, which is expressed on a subset of memory CD4⁺ T cells and may be important in T cell homing to lymphoid tissues.⁹⁶ The combination of enhanced costimulation and programmed cell death inhibition as explained previously by IL-7 is likely responsible for the role of IL-7 in facilitating memory T cell differentiation in-vivo.^{12,64,97,98}

1.3.3 T CELL HOMEOSTASIS

Despite of the previous reports identifying capacity of IL-7 to costimulate for TCR activation in mature T cells, the central role for IL-7 in peripheral T cell homeostasis remained largely unsolved as the animals lacking IL-7 had negligible numbers of peripheral T cells (due to the need of IL-7 in the previous steps). Several studies especially in the last couple of years focused on this area and have established IL-7 as a critical modulator of peripheral T cell homeostasis, the effects of which are most pronounced during T cell lymphopenia.^{11,14,64,99}

Following thymic export of SP CD4 or CD8 T cells, this recently emigrated T cells are preferentially incorporated into the periphery regardless of the size of the existing pool. Once in periphery, these recent emigrants continue to undergo cycling which contributes to efficient post-thymic T cell differentiation and maintenance of a diverse T cell repertoire that is highly correlated with IL-7 responsiveness. Synergy of concomitant TCR and IL-7 signaling was demonstrated with the observation that IL-7 mediated expansion of antigen specific T cell populations was much greater than the expansion of non antigen specific populations when exogenous IL-7 was administered with a cellular vaccine.^{100,101} Activation of T cells results in down regulation of IL-7R α , but there is re-expression on the resting memory T cell pool. As mentioned above IL-7R α expression is up regulated especially CD8 SP cells after thymic export and it is demonstrated this expression identifies subset of cells destined to differentiate into true immunological memory cells and IL-7 therapy augments the size of the CD8 memory pool generated following immunization.¹⁰⁰ There are reports demonstrating an important role of IL-7 in the generation of CD4 memory cells.^{10,102,103} IL-7 has also shown to effect the expression of lung Kruppel-like factor that is proposed to be a 'quiescence factor' critical in naïve and resting memory cells.⁶⁴

1.4 IL-7 RECEPTOR EXPRESSION AND TRANSCRIPTIONAL CONTROL

IL-7R α is expressed on several different cells especially in the lymphoid lineage as explained previously. Its expression level is tightly regulated rising and falling at

various stages in the development, activation and differentiation of lymphocytes. This regulation is what brings the several postulates to explain the different and important biological roles of IL-7 signaling in immune cells. IL-7 being the survival signal brings out the concept that receptor expression level regulation is what determines the cells to receive more survival signals and therefore to persist as memory cells.¹⁰⁴ On the other hand for the downregulation of the expression level of the receptor chain, an 'altruistic' role has been proposed that a T cell that has been exposed to and received sufficient IL-7 for survival, would cease consuming IL-7 by this downregulation and thus allow other cells to receive the survival signal.⁷⁶ During B cell development, IL-7R α would block expression of genes required for further maturation steps during development and it is down-regulated as expected.¹⁹

Although there is a significant importance of the differential regulation of the IL-7R α gene for the lymphocyte development, until now the mechanism regulating gene expression of IL-7R α have only been partially characterized. It was shown that Ets family transcription factor PU.1, mainly functioning in the development of the lymphoid system, directly regulates transcription of the IL-7R α gene in pro-B cells.¹⁰⁵ With a computer based algorithm the DNA sequence spanning the transcriptional start site was examined and a high affinity PU.1 binding site that lies between the transcriptional and translational start sites was found in the murine IL-7R α gene.⁶⁰ when different cell types were examined for the effect of PU.1, although it was found that mutation of the PU.1 gene results in a block to B cell development¹⁰⁶, it impairs or delays but does not completely block $\alpha\beta^+$ T cell development. Although control of IL-7R α expression in early lymphoid development is a function uniquely provided by PU.1 it was noted that in later stages of B and T cell development, expression of PU.1 no longer correlates with IL-7R α expression.^{105,106} Therefore other transcription factors must regulate IL-7R α gene expression in these later stages of lymphocyte development.

In T cells another Ets family transcription factor, GABP, essential in the regulation of IL-7R α gene expression was detected in T cells, where PU.1 is not expressed. GABP, binds to GGAA motif between positions -185 and -182, same as the binding site for PU.1.¹⁰⁷ Besides these two identified transcription factors and their respective binding sites in upstream region of IL-7R α gene; more recently

transcriptional regulation of mouse IL-7R α promoter by glucocorticoid receptor was also reported in T cells.^{108,109}

1.5 MECHANISM OF TRANSCRIPTIONAL REGULATION

Regulation of gene expression is a crucial step in the maintenance of cellular homeostasis. The control of gene expression that is highly regulated can occur in multiple steps. The devastating majority of regulatory events occur at the level of transcription. Transcription factors involving in the initiation of eukaryotic transcription bind to regulatory sequences organized in a series of regulatory modules along the DNA. Thus the molecular basis for transcriptional regulation of gene expression is the binding of trans-acting proteins (transcription factors) to cis-acting sequences.^{26,110}

1.5.1 GENE EXPRESSION CONTROL AT DNA LEVEL

Transcription is initiated at promoter regions of the genes by assembly of a transcription initiation complex composed of RNA polymerase and associated general transcription factors. This step, in principle, is sufficient to initiate transcription but many classes of transcriptional regulators, including DNA-binding transcriptional factors, coactivators, corepressors, DNA modifying proteins and nucleosomal histones combinatorially influence the function of minimal promoters by binding to different regulatory elements.

Cis-regulatory sequences play a determinative role in the tissue and developmental specificity of gene expression. Interplay of cis-acting sequences and trans-acting factors in establishing the patterns of gene expression is a topic of long-standing interest in the studies of gene regulation.¹¹⁰ In contrast to promoter the position and orientation of the cis-acting elements are variable with respect to the genes.²⁶ These elements are called enhancers if the specific factors that bind to the elements activate transcription and silencers if they repress transcription. The importance of a particular cis-acting element may vary greatly in different cell types or in response to different

physiological stimuli as the transcription factors that bind to these elements vary in abundance or in ability to function in different cell types and under different circumstances.^{110,111}

Enhancers were initially identified and characterized in mammalian viruses and cultured cells.¹¹² They were shown to be composed of multiple binding sites for different trans regulatory proteins. Subsequent analyses in transgenic animals revealed that enhancers integrate different regulatory inputs, such as those produced by multiple signaling pathways, to direct tissue specific or developmental stage specific patterns of gene expression. The tissue-specific enhancers are shown to work over distances of 100 kb or even more in flies and mammals.^{111,113,114} A typical animal gene is likely to contain several enhancer regions located either in 5' or 3' regions of the gene. There are also several enhancers found so far that are located even in the intronic sequences of the gene. Each enhancer has a function in expression pattern of the gene either in different cell types and tissues or during different developmental stages. Animal enhancers are around 500 bp in length and contain binding sites for different sequence specific transcription factors either activator or repressor.¹¹⁵(Figure 1.4)

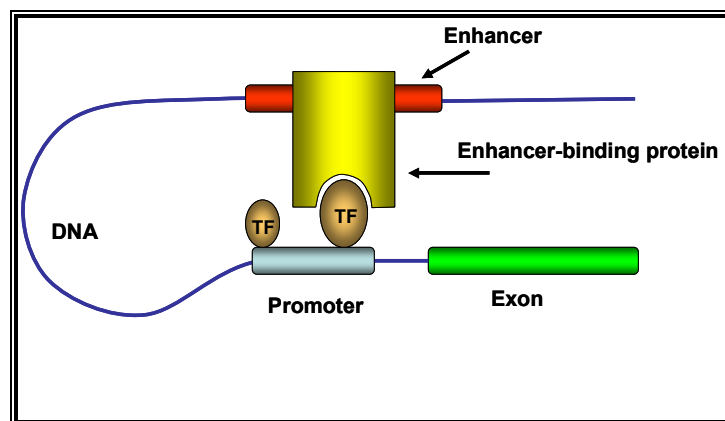


Figure 1.4: Schematic representation of distant enhancer activity on transcription.

It is worth to mention that many genes also contain binding sites for proximal regulatory factors located just 5' of the core promoter. These regulatory factors binding do not always function as classical activators or repressors but instead, they function as 'tethering elements' that recruit distal enhancers to the core promoter.¹⁰ These cis-regulatory elements, like enhancers, are scattered over distance of roughly 100 kb in

mammals. This sophisticated and modular organization of the regulatory DNA permits the tight control of the gene expression. In fact, the complex and detailed regulation of the metazoan genomes arises from the fact that multiple enhancers, silencers and promoters generally control the activity of a single transcription unit. As the cis-regulatory DNAs can map far from their target promoter and sometimes closer to the wrong promoter, there are insulator DNAs for ensuring that the right enhancer interacts with the right promoter. Insulator DNAs selectively block the long-range interaction of a distant enhancer with a proximal target promoter by positioning between the two. Gene competition concept that arises from the fact that shared enhancer prefers to interact with just one of the linked promoters depending on the cis-elements within the core promoter and also the previously mentioned tethering elements also account for the correct interaction of the enhancer promoter couple.¹¹¹

1.6 COMPARATIVE GENOMICS AND IDENTIFICATION OF REGULATORY REGIONS ON DNA

The combination of bioinformatics and biological approaches constitutes a powerful method for identifying gene regulatory elements. Multiple sequence alignments represent the fundamental basis for comparative analysis aimed at identification and characterization of functional elements.¹¹⁶⁻¹¹⁸ Comparing the DNA sequences of different species is a powerful method for decoding genomic information, because functional sequences tend to evolve at a slower rate than non functional sequences. Modular genomic regulatory elements have been focus of investigation for about 30 years, but the availability of fully sequenced genomes simplifying functional analyses become the starting point of most current investigations. As mentioned, DNA based regulatory modules can be widely dispersed in the genome even near the gene or distal from the gene in 3' or 5' regions.^{111,119}

1.6.1 CROSS-SPECIES SEQUENCE COMPARISONS

High-quality genome sequences are available in public databases for several vertebrate species. Comparative cross-species sequence analysis of these genomes shows considerable conservation of non coding sequences in DNA. Genome sequence comparisons are now used to detect noncoding genomic regions that have been evolutionarily conserved, presumably to maintain some critical biological function.¹¹⁸ Such regions are called conserved noncoding sequences (CNSs) and they often correspond to dispersed regulatory elements.^{117,118} The potential functions of conserved non-coding sequences are numerous, and include roles in chromosomal assembly and replication as well as transcriptional regulation. Compelling support for the conservation of sequence based regulatory information across species comes from a diverse set of experimental approaches. Most importantly, this support includes the DNA sequence conservation of experimentally defined regulatory elements among mammals^{26,59,120}

The strategy for comparative genome analyses can be outlined as follows: First the orthologue of the gene for study in another species should be identified. While doing so the criterion is that flanking genes of the gene of interest are conserved, in the same order, in the species being compared. Genome browsers available can be used to determine orthology and to visualize of genes chromosomal context. After the identification of the orthologue the extent of DNA to cover with functional assays should be determined. The two sequences must be aligned then in the determined region and considering the exons the CNS should be determined. Sequence comparison alignment programs use local or global alignment or the combination of two, for the sequences to be aligned.^{117,118,121} Global alignment programs produce a single optimum alignment that is optimized along the entire length of two sequences. Local alignment programs find all of the high quality alignments between sub regions of the two sequences, regardless of order or orientation. For the detection of conserved regions of functional importance, both global and local alignment programs should be used and the results should be compared.

Once CNS regions have been identified, the next step is to assess their functions by conducting biological experiments. Without wet-lab experiments, the biological functions of modules that have already been identified cannot be predicted, nor it can be confirmed that a specific module actually affects a given gene.

1.7 REPORTER ASSAY SYSTEMS

The mechanism by which a given regulatory region influences transcription through its target promoter can be investigated with cell based assays. A variety of reporter assays using cell lines have been used to assess whether putative cis acting element influence gene expression or not. Genetic reporter systems represent an extensive toolbox for the study of regulatory promoter and enhancer sequences.

1.7.1 LUCIFERASE REPORTER ASSAY SYSTEM

Firefly Luciferase is a nearly ideal reporter gene for plant cells. The first luciferase gene cloned was from the North American firefly *Photinus pyralis*. The active enzyme is a single polypeptide with a mass of 62kDa. The luciferase reaction emits yellow-green light (560nm) and requires only the enzyme, ATP, Mg_2^+ , O_2 and the substrate luciferin. Most frequently the detection of the luciferase activity is performed in a photon measuring device such as a luminometer or a scintillation counter.

The glow is widely used as an assay for luciferase activity to monitor regulatory elements that control its expression.¹²² *Luc* is particularly useful as a reporter gene since it can be introduced into living cells and into whole organisms such as plants, insects, and even mammals. *Luc* expression does not adversely affect the metabolism of transgenic cells or organisms. In addition, the *luc* substrate luciferin is not toxic to mammalian cells, but it is water-soluble and readily transported into cells. Since *luc* is not naturally present in target cells the assay is virtually background-free. Hence, the *luc* reporter gene is ideal for detecting low-level gene expression.

1.7.2 FLUORESCENCE REPORTER ASSAY SYSTEMS

A system that is sensitive enough to observe the difference among different reporter constructs even under low transfection efficiency is suitable to use especially when the mammalian cells in suspension are being used for transfection. Over the past 30 years, fluorescence activated cell sorting (FACS) machines have evolved to become powerful tools for analyzing and isolating single cells at very high rates.¹²³ Flow cytometry of reporter cell populations also constitutes a powerful quantitative analytical system in which each individual cell is in effect, a microplate well.^{123,124} With its capacity to interrogate and sort more than 50 000 cells per second shows great potential to speed up efficient clone characterization and isolation.^{125,126}

Flow Cytometry involves the use of a beam of laser light projected through a moving fluid stream that contains cells, or other particles, which when struck by the focused light give out intrinsic and evoked signals. By the aid of detectors, light scattering and emitted fluorescence are then measured for each individual particle that passes the excitation source. These signals are converted for computer storage and data analysis, and provide information about a variety of biochemical, biophysical and molecular aspects of particles.¹²⁵ Quantification of reporter gene expression of transiently transfected cells by flow cytometry is therefore a powerful method to characterize *cis*- and *trans*-acting elements overcoming the obstacle due to low transfection efficiency of suspension cells.

The green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria* is a widely used reporter protein for monitoring gene expression and protein localization in a variety of cells and organisms. The green fluorescent protein from the *Aequorea victoria* jellyfish emits green fluorescence without the need for any enzyme or co-factors and therefore can be easily detected. The fluorescence of GFP is dependent on the key sequence Ser-Tyr-Gly that undergoes spontaneous oxidation to form a cyclized chromophore.¹²⁷⁻¹²⁹

Applications using GFP reporters have expanded due to the availability of mutants with altered spectral properties. Now there are a number of GFP variants known with excitation maxima ranging from blue to red excitation. Normally the compact structure of GFP makes it very stable under a variety of conditions that limits its application in some studies including transcriptional studies. Addition of PEST sequence, from the C terminus of mouse ornithine decarboxylase -MODC- , correlated with protein degradation, to the C terminus of EGFP, half life of GFP is decreased leading to an destabilized variant of GFP and makes it more suitable for genetic reporter assays. Destabilized version of GFP can be used as a transient reporter in the study of cis-acting regulatory elements or transcriptional induction.^{10,130}

2 PURPOSE OF THE STUDY

The regulation of the expression of Interleukin-7 receptor α chain (IL-7R α) is found to be essential for T cell development in both humans and mice and for B cell development in mice. IL-7 mediated signals through the receptor are firmly regulated throughout the development and survival of lymphocytes. Selective survival of lymphocytes that receive IL-7 signals is regulated by differential expression of the IL-7R α . Transcriptional control mechanism underlying the expression of IL-7R α has not yet been clearly explained. The previous data on regulation of transcription in different developmental stages of different lymphocytes suggests the existence of a putative distant regulatory element important for differential expression of reporter gene.

The aim of this study was to determine any possible enhancer region in the upstream region of IL-7R α coding gene in mouse T lymphocyte cell line using both bioinformatics tools and reporter assay systems. Determination of the conserved noncoding sequences that has a high probability of containing the regulatory regions, in the upstream of the IL-7R α gene by using basic bioinformatics tools was the first step taken. The conserved regions were then assayed for the presence of the transcriptional control activity by luciferase reporter system and later by a more effective and convenient flow cytometric reporter system.

The conserved non-coding regions were determined by comparative genome analysis between mouse and human genomic sequences. The determined regions were amplified from the BAC clone of corresponding sequence by PCR and then cloned into the upstream of the weak promoter in the constructed luciferase reporter vector. The same regions were also cloned into the constructed GFP reporter vector. Following the transfection of IL-7R α positive mouse CD4 T-lymphoma cell line EL-4 with these reporter vectors, fluorimetric reporter assays using flow cytometer was carried out particularly in this study.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 CHEMICALS

The chemicals used in the study are listed below:

6X Loading Dye	Fermentas, Lithuania
Acetic Acid	Riedel-de Haen, Germany
Agarose	AppliChem, Germany
Ampicillin	Sigma, Germany
Boric Acid	Riedel-de Haen, Germany
CaCl ₂	Merck, Germany
DMEM	Sigma, Germany
DMSO	Sigma, Germany
EDTA	Riedel-de Haen, Germany
Ethanol	Riedel-de Haen, Germany
Ethidium Bromide	Merck, Germany
FBS	Biological Industries, Israel
Glycerol	Riedel-de Haen, Germany
HEPES	AppliChem, Germany
HCl	Merck, Germany
Isopropanol	Riedel-de Haen, Germany
Kanamycin	Sigma, Germany
L-glutamine	Merck, Germany
Liquid Nitrogen	Karbogaz, Turkey
Luria agar	Sigma, Germany
Luria Broth	Sigma, Germany
MgCl ₂	Sigma, Germany
Penicillin-Streptomycin	Biological Industries, Israel
PIPES	Sigma, Germany

KCl	Fluka,Switzerland
RPMI 1640	Biological Industries,Israel
NaCl	Riedel-de Haen,Germany
NaOH	Merck,Germany
Tris Base	Fluka,Switzerland
Trypan Blue	Sigma,Germany
Trypsin-EDTA	Biological Industries,Israel

3.1.2 EQUIPMENT

The equipment used in the study are listed below:

Autoclave	Hirayama,Hiclave HV-110,Japan Certoclav, Table Top Autoclave CV-EL-12L, Austria
Balance	Sartorius, BP221S, Germany Schimadzu, Libror EB-3200 HU, Japan
Cell Counter	Cole Parmer, USA
Centrifuge	Eppendorf, 5415D, Germany Hitachi, Sorvall RC5C Plus, USA
CO ₂ Incubator	Binder,Germany
Deepfreeze	-80 ⁰ C, Forma,Thermo ElectronCorp.,USA -20 ⁰ C,Bosch,Turkey
Distilled Water	Millipore, Elix-S, France
Electrophoresis Apparatus	Biogen Inc., USA Biorad Inc., USA
Electroporation Cuvettes	Eppendorf, Germany
Electroporator	BTX·ECM630, Division of Genetronics, Inc, USA
Filter Membranes	Millipore,USA
Flow Cytometer	BDFACSCanto,USA
Gel Documentation	UVITEC,UVIdocGel Documentation System, UK Biorad, UV-Transilluminator 2000, USA

Heater	Thermomixer Comfort,Eppendorf,Germany
Hematocytometer	Hausser Scientific,Blue Bell Pa.,USA
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert, Modell 300, Germany Memmert, Modell 600, Germany
Laminar Flow	Kendro Lab. Prod., Heraeus, HeraSafe HS12, Germany
Liquid Nitrogen Tank	Taylor-Wharton,3000RS,USA
Magnetic Stirrer	VELP Scientifica, ARE Heating Magnetic Stirrer, Italy
Microliter Pipettes	Gilson, Pipetman, France Eppendorf, Germany
Microscope	Olympus CK40,Japan Olympus CH20,Japan Olympus IX70,Japan
Microwave Oven	Bosch,Turkey
pH meter	WTW, pH540 GLP MultiCal, Germany
Power Supply	Biorad, PowerPac 300, USA
Refrigerator	Bosch,Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	Schimadzu, UV-1208, Japan Schimadzu, UV-3150, Japan
Thermocycler	Eppendorf, Mastercycler Gradient, Germany
Vortex	Velp Scientifica,Italy

3.1.3 BUFFERS AND SOLUTIONS

Standard buffers and solutions used in cloning and molecular manipulations were prepared according to the protocols in Sambrook *et al.*, 2001³⁵. Trypan blue dye used for exclusion of dead cells under hematocytometer was prepared at a final concentration of 0.4%(w/v) by dissolving 40 µg of trypan blue in 10 ml PBS. Calcium Chloride (CaCl₂) Solution used for competent cell preparation contained 60mM CaCl₂ (diluted

from 2.5M stock), 15% Glycerol, 10mM PIPES (pH=7) and the solution prepared was autoclaved at 121⁰ C for 15 min and stored at 4⁰ C.

3.1.4 GROWTH MEDIA

Bacterial Growth Media: Luria Broth from Sigma was used for liquid culture of bacteria.. 20 g of LB Broth was dissolved in 1 L of distilled water and autoclaved at 121⁰ C for 20 min. For selection Kanamycin at a final concentration of 50 µg/ml and ampicilin at a final concentration of 100 µg/ml were added to liquid medium after autoclave. LB Agar from Sigma was used for preparation of solid medium for the growth of bacteria. 40 g of LB Agar was dissolved in 1 L distilled water and autoclaved at 121⁰ C for 20 min. Autoclaved medium was poured to sterile Petri plates (~20 ml/plate) after cooling down to room temperature. For selection, Kanamycin at a final concentration of 50 µg/ml and ampicilin at a final concentration of 100 µg/ml were added to medium before pouring the medium.

Mammalian Growth Media: Suspension cell lines (EL-4 mouse T-Lymphoma) were grown in RPMI 1640 cell culture medium that is supplemented with 5% fetal bovine serum, 2mM L-Glutamine , 100 unit/ml penicillin and 100 unit/ml streptomycin. The cells were frozen in medium containing DMSO added into fetal bovine serum at a final concentration of 10% (v/v) and stored at 4⁰ C.

3.1.5 MOLECULAR BIOLOGY KITS

- ✓ Luciferase Assay System, Promega, USA
- ✓ QIAGEN Plasmid Midi Kit, QIAGEN, Germany
- ✓ Qiaprep Spin Miniprep Kit, QIAGEN, Germany
- ✓ Qiaquick Gel Extraction Kit, QIAGEN, Germany
- ✓ Qiaquick PCR Purification Kit, QIAGEN, Germany
- ✓ DNA Blunt and Ligation Kit, Fermentas, Lithuania

3.1.6 ENZYMES

Enzymes and their corresponding reaction buffers used for cloning are :

Enzyme	Buffer	Company
BamH1	E	Promega
Bgl2	D	Promega
CIAP (Alkaline Phosphatase)	CIAP Buffer	Promega
EcoR1	H	Promega
EcoRV	D	Promega
Hind3	E	Promega
Klenow Fragment	Klenow Buffer	Promega
Kpn1	J	Promega
Nco1	D	Promega
Pst1	H	Promega
Pvu2	B	Promega
Sal1	D	Promega
Sma1	J	Promega
Sph1	K	Promega
Taq Polymerase	Taq Buffer	Promega
T4 DNA Ligase	T4 Buffer	Promega
Xba1	D	Promega
Xho1	D	Promega

Table 3.1: List of Enzymes

3.1.7 CELLS, PLASMIDS, OLIGOS

DH-5 α competent cells were used for bacterial transformation of plasmids. For the reporter assays, mouse T lymphoma cell line EL-4 was used for transfection. Plasmids used in the study and basic features of these plasmids are given in Table 3.2. Primers used in the study for the amplification of seven determined CNS regions and SV40 Enhancer are given in Table 3.3 and 3.4 respectively.

Plasmid	Company	Feature-Use	Selective Marker
pCDNA3	Invitrogen	Cloning plasmid	Amp
pDSRedExpress-DR	Clontech	DSRed-repoter	Kan
pGL3Basic	Promega	Luc-reporter	Amp
pGL3Control	Promega	Luc,SV40Pomoter and SV40Enhancer	Amp
pGL3Enhancer	Promega	Luc,SV40Enhancer	Amp
pMaxGFP	Amara	EGFP	Kan
pTurboGFP-PRL-Dest	Evrogen	GFP- reporter	Kan

Table 3.2: List of Plasmids

Name	Location (VISTA)	Start	Sall recognition site	Sequence	Product Length
K1F	160200-	99	GACGCGTCGAC	GGCAAAGACTCTCCAAATGC	1298
K1R	161600	1396	GACGCGTCGAC	CATTTTGGCCTTTACCTACCTG	
K2F	161500-	29	GACGCGTCGAC	TTTTGACACAGAGAACTACCTACCA	750
K2R	162300	778	GACGCGTCGAC	CACATTGGCAGCTTTGAGAA	
K3F	162000-	22	GACGCGTCGAC	GAGAGATTGCGAGGCTGTTT	858
K3R	162900	879	GACGCGTCGAC	GTCTTTTGTGTTTGAGGCAAAG	
K4F	180900-	13	GACGCGTCGAC	GGTCTCTTCCACTAAGACATTTC	626
K4R	181550	638	GACGCGTCGAC	CACAAGGAAGGCCTGACAGT	
K5F	186600-	8	GACGCGTCGAC	CAGTTATGAAGCCACCACCA	865
K5R	187500	872	GACGCGTCGAC	TTGAAGGAAGTTGCCTGCTT	
K6F	189000-	1	GACGCGTCGAC	CCTCTTGGCTGGTTGCATA	574
K6R	189600	574	GACGCGTCGAC	TTCCACATCAACAGTCTGC	
K7F	191600-	2	GACGCGTCGAC	GGCTCCAAAGCTAACCAAAA	430
K7R	192200	431	GACGCGTCGAC	GAACGTGGTCCTCTCACACA	

Table 3.3: List of primers used for amplification of seven CNS.

Name	Location	Start	Sequence	Product Length
SV40F	2183-	2183	GACGCGTCGACGTAAAATCGATAAGG	270 bp
SV40R	2470	2470	GGCTCTCAAGGGCATCGGT	

Table 3.4: List of primers used for amplification of SV40 enhancer from pGL3Control plasmid.

3.1.8 COMPUTER BASED PROGRAMS

The software and online programs used are listed in alphabetical order:

AVID-VISTA	http://genome.lbl.gov/vista/index.shtml
NEB CUTTER V2.0	http://tools.neb.com/NEBcutter2/index.php
PIP-MAKER	http://pipmaker.bx.psu.edu/pipmaker/
PRIMER3	http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Spidey	http://www.ncbi.nlm.nih.gov/spidey/
UCSC Genome Browser	http://genome.ucsc.edu/cgi-bin/hgGateway
VectorNTI 9.1.0	Software

3.2 METHODS

3.2.1 CULTURE GROWTH

Bacterial Culture Growth: Bacterial cells were grown overnight (12-16h) at 37 °C shaking at 270 rpm in Luria Broth prior to any application. Depending on the application selective antibiotics were added to the media. Bacterial cells either spreaded or streaked, were grown on LB Agar Petri plates overnight (12-16h) at 37 °C. For the glycerol stock preparation glycerol was added to the overnight grown cultures to a final concentration of 15%. Cells were frozen first in liquid nitrogen and then stored at -80°C.

Mammalian Culture Growth: Suspension cell lines (EL-4 mouse T-Lymphoma) were grown in a humidified atmosphere of 5%CO₂ at 37 °C in RPMI 1640 Medium supplemented with 10% fetal bovine serum, 2mM L-Glutamine , 100 unit/ml penicillin and 100 unit/ml streptomycin. Cell cultures were splitted in every 2-3 days by 1/10 dilutions. For the preparation of frozen stock of EL-4 cells, cells at mid to late growth phase were resuspended in freezing medium (10%DMSO in fetal bovine serum) and stored at -80°C for 48 hours. Then, the cells were stored in liquid nitrogen tank. After thawing the cells were immediately washed with growth medium to get rid of DMSO.

3.2.2 IDENTIFICATION AND AMPLIFICATION OF CONSERVED NON-CODING SEQUENCES

The mouse and human IL-7R α gene locus were downloaded from USCS genome browser. After finding the corresponding gene loci in these genomes the upstream region was obtained by changing the display properties. Coding regions, exon files were generated by using the Spidey program available at the NCBI website. The sequences were aligned first by global alignment using PIP Maker and then by local alignment using AVID. Similarity plot of the local alignment was generated by VISTA program using 50 base pair window length and 85% minimum similarity parameters. (Details are given in section 4.1)

BAC clone database on USCS genome browser was searched for the corresponding upstream region. The identified BAC clone was ordered from Children's Hospital Oakland Research Institute, USA cloned in pBACe3.6 vector. BAC plasmids were transformed and isolated as described in Sambrook *et al.*, 2001³⁵ Conserved non-coding sequences were amplified from RP23-365P6 BAC vector by PCR using the designed primers. Primers to amplify the defined conserved regions were designed using Primer3 program. The parameters were arranged so that the T_m values were close among the primer pairs. SalI restriction sites were added to the 5' ends of both the forward and the reverse primers for further restriction and cloning into the reporter vectors. Primers were ordered from Operon Sciences. Primer sequences and data are given in Table 3.3.

The PCR conditions were determined based on Promega technical bulletin. Gradient PCR was first done to find the optimal annealing temperature. Optimized PCR reaction and thermal cycle conditions are shown in Table 3.5

PCR Reaction	Volume Used	Final Concentration
Template DNA		
10X Polymerase Buffer	2.5 μ l	1X
MgCl ₂ (25mM)	2 μ l	2 mM
dNTP Mix (10mM)	0.5 μ l	0.2 mM
Primer forward(10mM)	2 μ l	0.8 μ M
Primer reverse(10mM)	2 μ l	0.8 μ M
Taq Polymerase(5u/ μ l)	0.125 μ l	0.025u/ μ l
ddH ₂ O	12.875 μ l	
Total	25 μ l	

Step	Temperature	Duration	Cycle
Initial Denaturation	95°C	2min.	1
Denaturation	95°C	0.5min	30
Annealing	56°C	1min	
Extension	72°C	2min	
Final Extension	72°C	5min	1
Hold	4°C	∞	1

Table 3.5: PCR Conditions

3.2.3 VECTOR CONSTRUCTION

The following general procedures were used for vector construction: *DH5 α* competent cells were prepared starting from a single colony of previously streaked *DH5 α* . Single colony from the plate was grown overnight at 37°C, 270 rpm in 50ml LB. Next day 4 ml from the overnight grown culture was diluted with 400ml LB and incubated at 37°C, 270 rpm until the OD₅₉₀ reaches 0.375. Then the CaCl₂ solution (as in 3.1.3) was used for the successive centrifuge steps and isolation of competent cells. The cells prepared were frozen immediately in liquid nitrogen and then stored at -80°C. The competency of the prepared cells were tested by transforming 5/10 ng of a known plasmid.

Transformation of bacterial cells : CaCl_2 treated competent cells were taken out of -80°C and the plasmids to be transformed were added (no more than 50ng in a volume of 10 μl or less) to each tube before complete thaw of the cells. The tubes were then stored on ice for 30 minutes. The cells were then incubated at 42°C for 90 seconds and transferred back on the ice rapidly to chill. 800 μl of autoclaved Luria Broth was added and the cultures were incubated for 45 minutes in a 37°C water bath to allow the bacteria recover and express the antibiotic resistance marker encoded by the plasmid. Transformed cells were spreaded on Luria Agar plates containing the appropriate antibiotic. The plates were then incubated overnight at 37°C to observe the colonies.

Plasmid DNA was isolated either by the alkaline lysis protocol in Sambrook *et al.*, 2001³⁵ or by the Qiaprep Spin Miniprep or QIAGEN Plasmid Midi Kits. Single colony of *E coli* from Luria Agar plate or glycerol stock was grown overnight at 37°C , 270 rpm in liquid culture containing the selective antibiotic, prior to the plasmid isolation. A portion of every culture grown overnight for was used to make glycerol stock as explained previously. The concentration of the plasmid DNA isolated was determined by UV Spectrophotometry and quantitatively on an agarose gel. Several dilutions of plasmid DNA mini- or midiprep was prepared and using quartz cuvettes UV absorbance was measured at 260nm. The concentration was then calculated as follows:

$$\text{Concentration} = \text{Absorbance} \times \text{Dilution Factor} \times 50$$

Restriction Enzyme Digestion: Digestion reactions mixtures were set by the addition of the DNA, the enzyme and the compatible buffer, and incubated at the optimum temperature of the enzyme used for 2.5-3 hrs. Agarose gels to observe the DNA according to their size were prepared in different concentrations ranging from 0.7% to 2% depending on the size of the fragments to be separated. In order to separate large fragments the percentage was low and vice versa for small fragments. Gel was prepared by dissolving determined amount of agarose powder in 0.5X TBE and heating 3-5 minutes in a microwave, after the gel cools to room temperature ethidium bromide was added to a final concentration of 0,001(v/v) and then the gel was poured in the gel apparatus. 0.5X TBE buffer was also used in the tank that was used to run the gel. When applying samples to the gel 6X loading dye was added to the samples. Gels were run at 100-110 volts for 40-60 minutes , and the bands were observed under UV.

Ligation: The ligation reactions were carried out using T4 Ligase (Promega), in a 3:1 vector to insert ratio, using 100 ng vector. 5' overhangs of the vectors were dephosphorylated by calf intestinal alkaline phosphatase (CIAP) prior to the ligation. Ligation reaction mixtures were kept at 16 ° C overnight. The first identification of correct ligation colonies were made via colony PCR.

Colony PCR: The single colonies from the plates were dissolved in 8.5 µl distilled water in autoclaved PCR tubes by the aid of sterile toothpick, after twirling the toothpick in the distilled water the toothpick was then used to streak the colony just by touching, onto a fresh replicate agar plate using a numbered template. The tubes were then incubated at 94 ° C for 8 minutes and the PCR master mix and the primers (the same primers used for the amplification of the inserts from BACs) were added to the tube and normal PCR reaction as in section 3.2.2 was carried out. The PCR products were run on agarose gel to see if there was any positive colony with an amplification of the inserted fragment. Whenever there was a positive colony we go to the replica agar plate on the same day and set up miniprep cultures of the likely candidate colonies.

Reporter vectors: As the luciferase reporter vector pGL3.Fos vector was constructed from backbone of pGL3Basic (Promega) plasmid and fos promoter from Δ56fosCAT plasmid. As a positive control the SV40 enhancer containing region from pGL3Enhancer backbone was digested out and ligated to constructed pGL3.Fos vector. The formed luciferase positive control vector was named pGL3.Fos-SV40.

As the GFP reporter vector pTurboGFP.Fos vector was constructed by inserting fos promoter from pGL3.Fos plasmid into pTurboGFP-PRL-dest1 (Evrogen) plasmid. As a positive control the SV40 Enhancer region amplified by PCR using primers in Table 3.4, from pGL3Control plasmid was inserted into constructed plasmid and the formed vector is named pTurboGFP.Fos-ESV40.

As the reference plasmid for flow cytometry analysis pDSRedPro-ESV40 was constructed. SV40 promoter from pGL3Control plasmid and SV40 Enhancer amplified via PCR from pGL3Control was inserted into originally promoterless pDSRedExpressDR vector (Clontech).

3.2.4 CLONING OF AMPLIFIED REGIONS INTO REPORTER VECTORS

For luciferase assays; the CNS regions amplified by PCR were digested with SalI and ligated to constructed pGL3.Fos vector. For the flow cytometric reporter assays; the CNS regions were digested with SalI from pGL3.Fos vector and ligated to constructed pTurboGFP.Fos vector. Correct plasmids in each case were diagnosed first by colony PCR and then confirmed by diagnostic digests.

3.2.5 TRANSIENT TRANSFECTION OF MAMMALIAN CELLS

EL-4 cells were grown to $1-2 \times 10^6$ cells/ml as explained in section 3.2.1. About 10^7 cells were centrifuged at 1000 rpm for 5 minutes at 25°C . Pelleted cells were then washed with serum-free RPMI 1640 and centrifuged again. Cells were then resuspended in 500 μl serum-free RPMI 1640 and mixed with 20 μg of plasmid DNA, incubated at room temperature for 10 minutes before transferring into 4mm electroporation cuvettes. Electroporation was carried out at 290 V, 1400 μF and 100 Ω . After electroporation, cells were transferred to cell culture plates and 4 ml of complete RPMI 1640 was added. The luciferase assay of the cells transfected with luciferase reporter vectors were carried out in our lab using Promega Luciferase Assay System according to the instructor's manual.

3.2.6 FLOW CYTOMETRIC ANALYSIS

The transfected and untransfected EL-4 cultured cells were counted by the aid of hemacytometer. 10 μl Trypan blue solution was mixed with 10 μl of culture of cells to exclude dead cells. About 10^6 cells were centrifuged at 1000 rpm for 5 minutes at 25°C . Pelleted cells were washed with PBS, centrifuged and resuspended in 1 ml PBS. Resulting 10^6 cells/ml solution was directly used for flow cytometric analysis on a BD FACSCanto flow cytometer. GFP fluorescence emission, excited by the argon laser was detected using a FITC 530/30 nm band pass filter.

4 RESULTS

4.1 IDENTIFICATION AND AMPLIFICATION OF THE CONSERVED NON-CODING REGIONS

4.1.1 DETERMINATION AND RETRIEVAL OF GENOMIC SEQUENCES

The sequence corresponding to the IL-7 α gene in mouse and human genome was found in USCS genome browser according to May 2004 assembly. The region to cover was chosen to start from 1000 bp upstream of the first exon of the gene closer in the upstream of IL-7 α and end with the last exon of IL-7 α gene. The orientation was chosen to be the orientation of coding strand of IL-7 α gene.

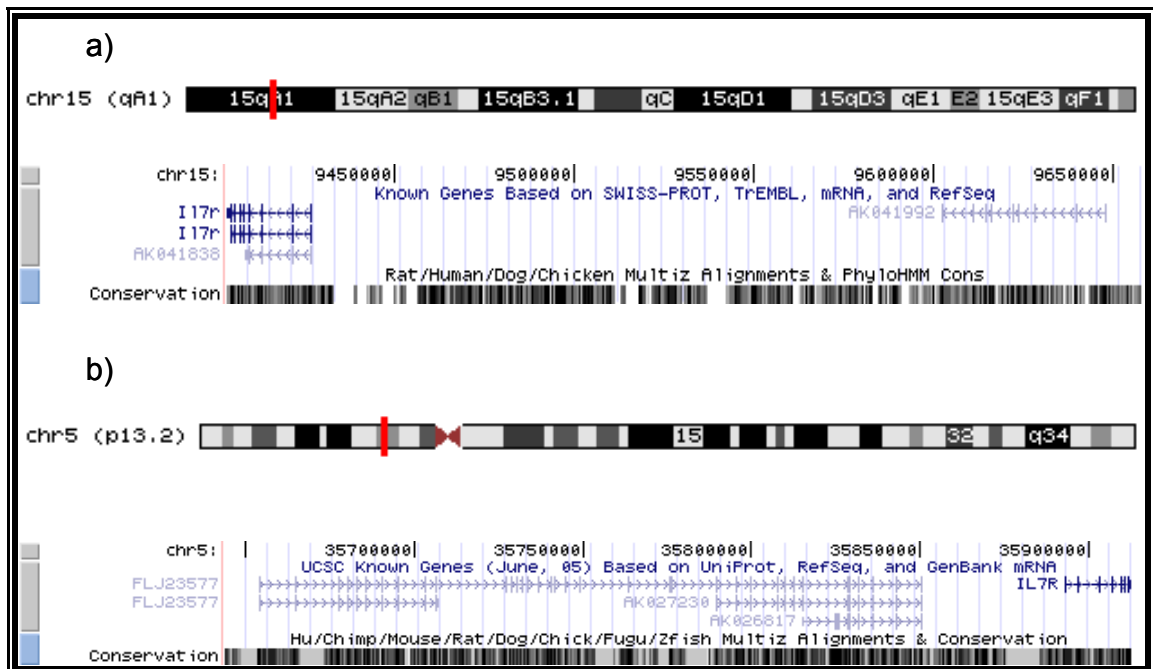


Figure 4.1: IL-7 α gene locus and upstream sequences of a) mouse and b) human from USCS Genome Browser

4.1.2 ALIGNMENT OF THE SEQUENCES

The sequences retrieved from USCS Genome Browser were aligned using AVID program after the generation of exon files with Spidey, and visualized by VISTA. For the alignment reverse complement of mouse DNA region was taken so that the orientation will be same for both human and mouse DNA and be same with the orientation of IL-7R α gene. The conversion table of the numbers given for the alignment and corresponding locations in human and mouse are explained in Figure 4.2.

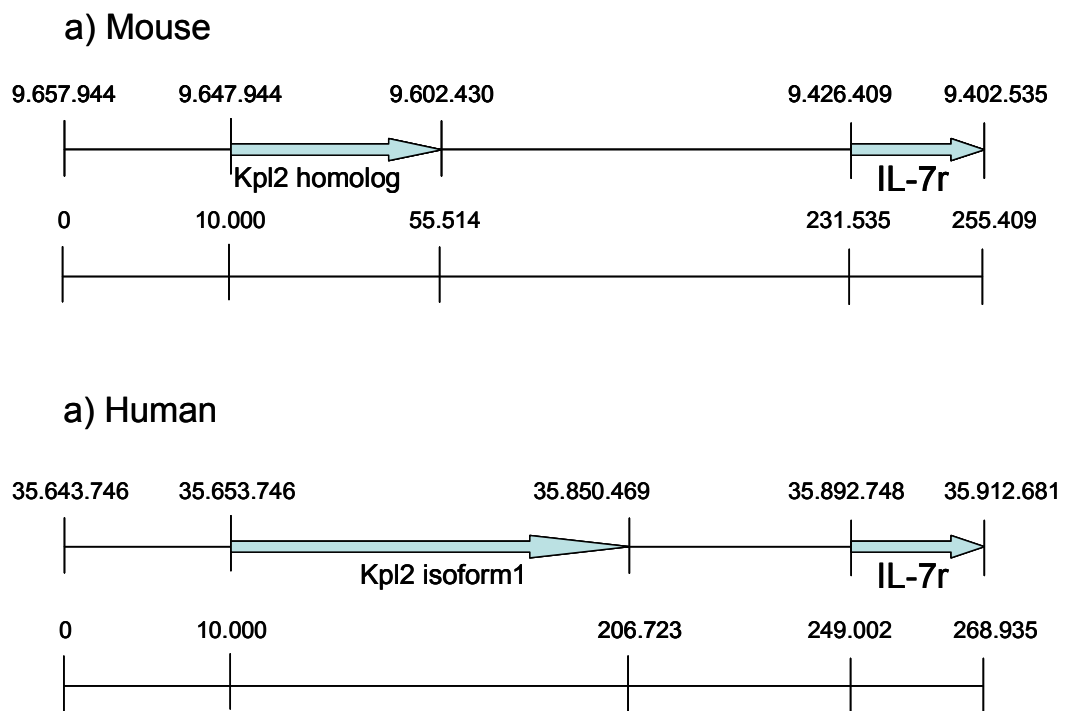


Figure 4.2: The regions taken and corresponding numbers given through analysis for a)mouse and b)human

The VISTA parameters were set so that the minimum percent identity was 85%, meaning that the resulting conservation was due to at least 85% sequence identity; window size was 50 bp that leads results to contain the CNS of at least 50 base pairs. According to the VISTA output (Figure 4.3) the pink color indicates minimum 85% identity of at least 50 bp long sequences. Among the CNS regions given out in the result of VISTA, the ones corresponding to coding regions of gene model or exons of the longer kpl2 isoform gene upstream of the IL-7R α in human were neglected and final outcome of the CNS regions far upstream of IL-7R α gene are given below:

Mouse	(Human)		Mouse	(Human)	=	Length	at	Identity%	Defined
160386	(180066)	to	160499	(180179)	=	<u>114bp</u>	at	86.0%	noncoding
160515	(180195)	to	160586	(180266)	=	<u>72bp</u>	at	84.7%	noncoding
160644	(180324)	to	161134	(180814)	=	<u>491bp</u>	at	86.4%	noncoding
161157	(180840)	to	161209	(180892)	=	<u>53bp</u>	at	84.9%	noncoding
161320	(181003)	to	161481	(181164)	=	<u>162bp</u>	at	87.0%	noncoding
161755	(181449)	to	161806	(181500)	=	<u>52bp</u>	at	86.5%	noncoding
161905	(181606)	to	162133	(181834)	=	<u>229bp</u>	at	87.3%	noncoding
162209	(181913)	to	162281	(181985)	=	<u>73bp</u>	at	86.3%	noncoding
162421	(182129)	to	162484	(182192)	=	<u>64bp</u>	at	87.5%	noncoding
162625	(182345)	to	162685	(182405)	=	<u>61bp</u>	at	90.2%	noncoding
181160	(203362)	to	181263	(203464)	=	<u>104bp</u>	at	86.5%	noncoding
186910	(214725)	to	187012	(214827)	=	<u>103bp</u>	at	87.4%	noncoding
187110	(214910)	to	187178	(214978)	=	<u>69bp</u>	at	87.0%	noncoding
189257	(217823)	to	189323	(217889)	=	<u>67bp</u>	at	86.6%	noncoding
191846	(220336)	to	191900	(220390)	=	<u>55bp</u>	at	89.1%	noncoding
194658	(222942)	to	194858	(223139)	=	<u>201bp</u>	at	86.6%	noncoding

By comparing these results with local alignment data and merging continuous CNS regions, we identified seven different regions to be amplified and tested for enhancer activity (Table 4.1).

Name given	VISTA Input Locations		Corresponding Mouse Locations	
	Start	End	Start	End
K1	160200	161600	9496344	9497744
K2	161500	162300	9495644	9496444
K3	162000	163900	9494044	9495944
K4	180900	181550	9476394	9477044
K5	186600	187500	9470444	9471344
K6	189000	189600	9468344	9468944
K7	191600	192200	9465744	9466344

Table 4.1: Regions determined to be tested K1 to K7.

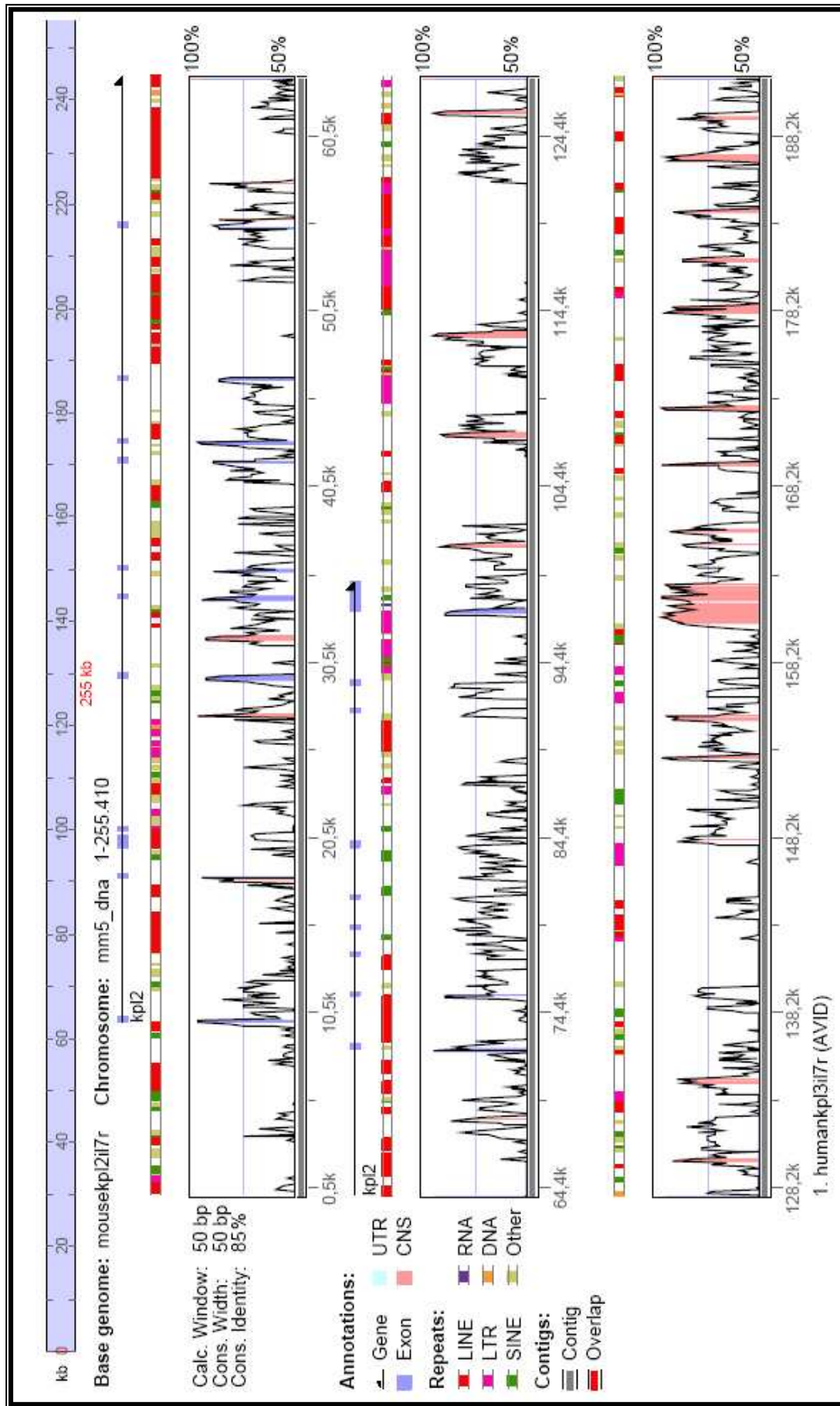


Figure 4.3: The AVID alignment results, visualized with VISTA, window length = 50 bp, minimum percent identity = 85%

4.1.3 DETERMINATION OF BAC CLONE

After the regions to be cloned and checked for enhancer activity was determined, the BAC clone containing the regions to be amplified was chosen using USCS Genome Browser (Figure 4.4). Chosen RP23-365P6 BAC Clone was obtained from Children's Hospital Oakland Research Institute (CHORI).

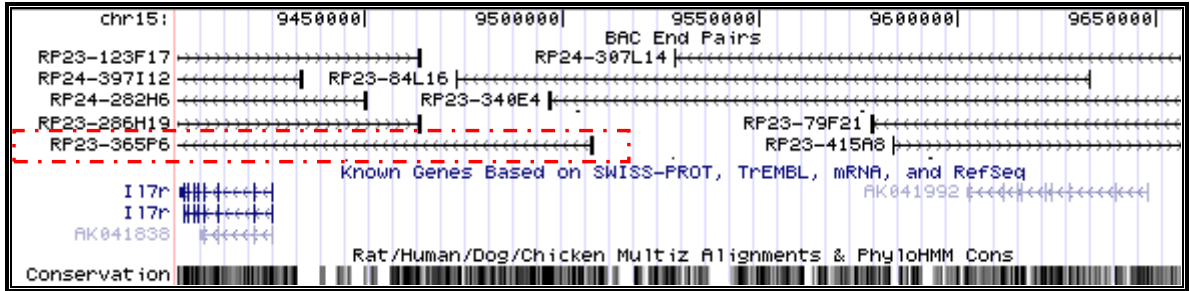


Figure 4.4: The BAC clone determined using USCS Genome Browser

4.1.4 AMPLIFICATION OF MOUSE CONSERVED NON-CODING SEQUENCES

The 7 identified CNS were amplified from RP23-365P6 BAC by PCR using primers listed in Table 3.3. The expected sizes of the PCR products are also given in Figure 4.5.

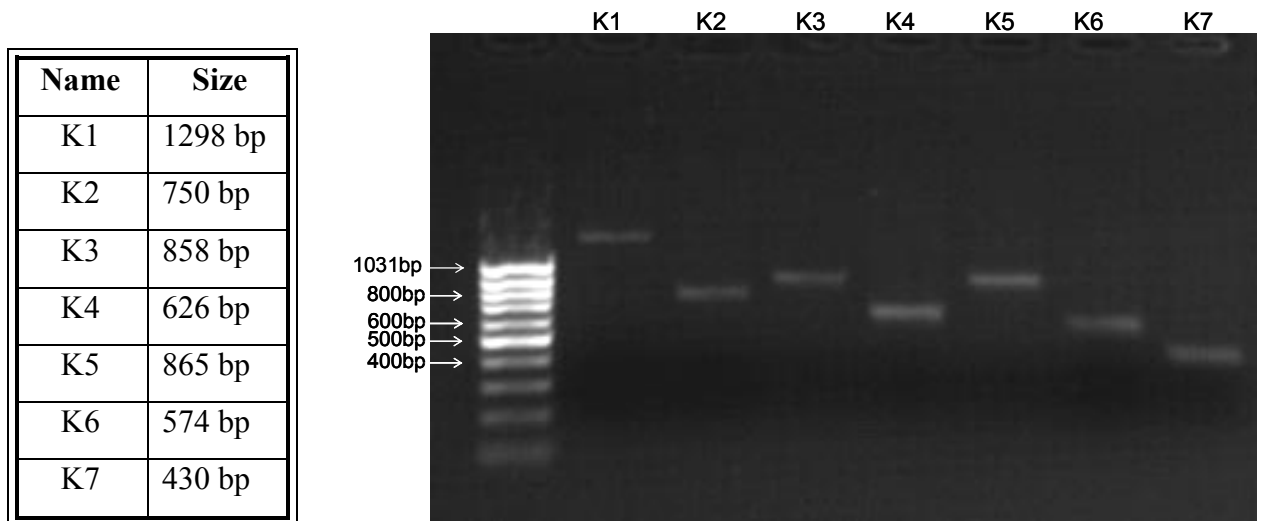


Figure 4.5: Amplification of K1 to K7.

4.2 CONSTRUCTION OF LUCIFERASE REPORTER VECTOR

To minimize background transcription activity in the absence of a transcriptional enhancer we constructed a luciferase reporter vector containing a weak promoter. A weak promoter is necessary to detect even small changes in enhancer activity provided by putative enhancer elements that were introduced into the transcription reporter vectors. We cloned the fos promoter from a pre-existing $\Delta 56$ fosCAT plasmid into the promoterless pGL3Basic luciferase reporter vector. The construction strategy of this luciferase reporter vector named pGL3.Fos is shown in Figure 4.6.

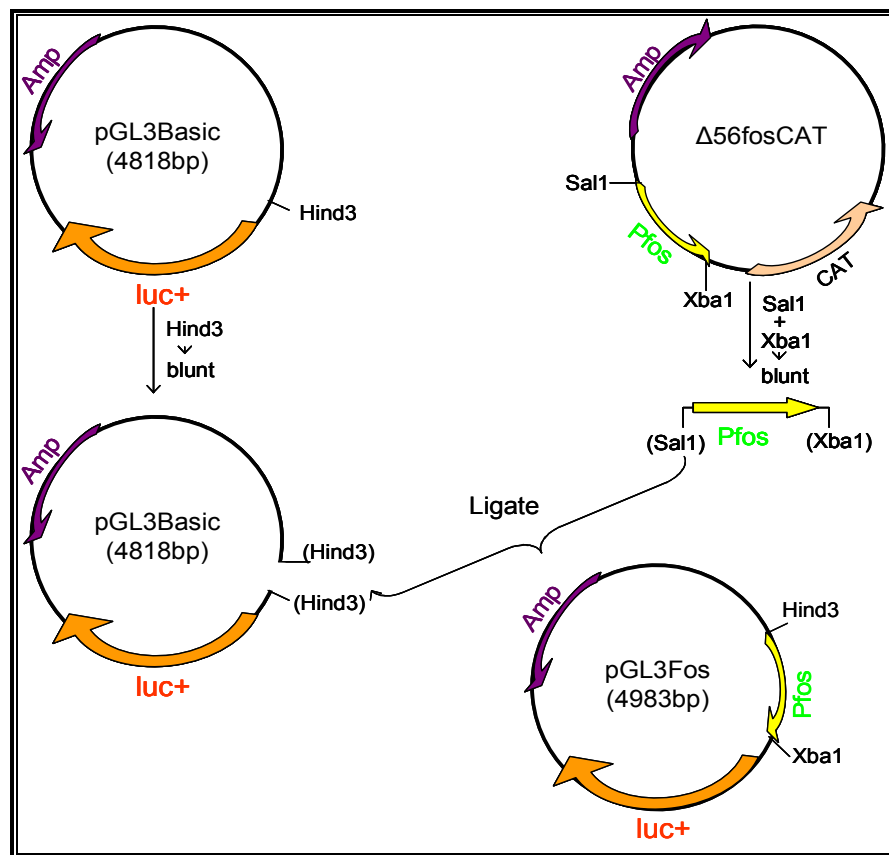


Figure 4.6: Strategy for constructing the pGL3.Fos luciferase reporter plasmid.

pGL3Basic (Promega) vector was digested with HindIII enzyme that had a recognition site at position 54, blunted and dephosphorylated. A fragment containing the fos promoter from the $\Delta 56$ fosCAT plasmid was cut with Xba1 and Sal1 and the formed sticky ends were treated with Klenow enzyme to generate blunt ends (Figure 4.7). These blunt ended DNA molecules were ligated to generate the pGL3.Fos plasmid.

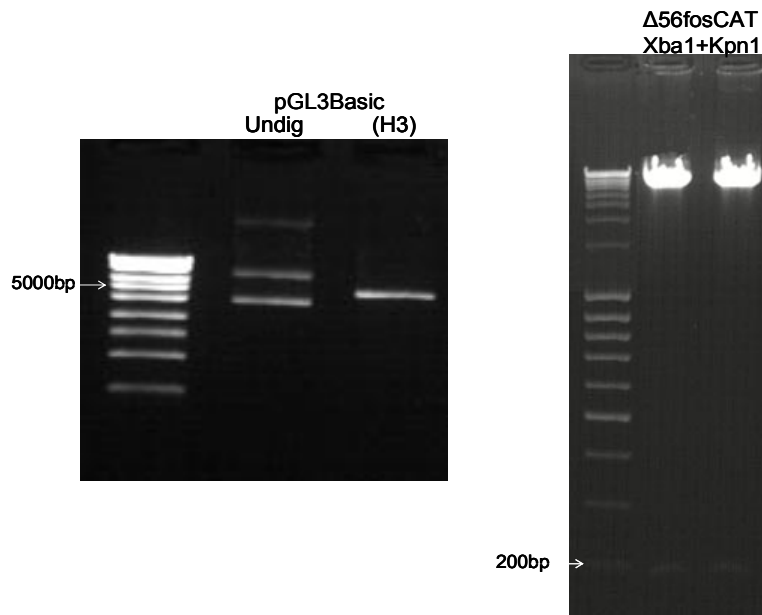


Figure 4.7: Construction of pGL3.Fos.

pGL3Basic(Promega) plasmid digested with HindIII and blunted resulting in linearized plasmid and $\Delta 56$ fosCAT plasmid digested with Xba1 and Kpn1 resulting in the fos promoter of length 165bp.

To confirm the identity of this plasmid miniprep DNA from 5-10 colonies was digested using several restriction enzymes with recognition sites in the vector and the insert according to the restriction map of the pGL3.Fos vector (Figure 4.8).

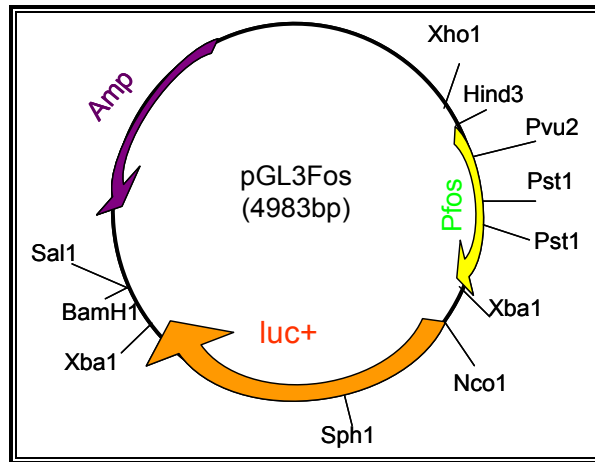


Figure 4.8: The restriction map of pGL3.Fos showing only the relative locations of the restriction enzymes used for diagnosis and confirmation.

First digest was with Xba1 that the vector pGL3Basic had a unique recognition site and the insert (fos promoter) introduced a second site by the ligation of blunted Xba1 and HindIII. Second digest was HindIII and Nco1 double digest so that if there was an insert (fos promoter) in between these sites it would be possible to see the inserted region.

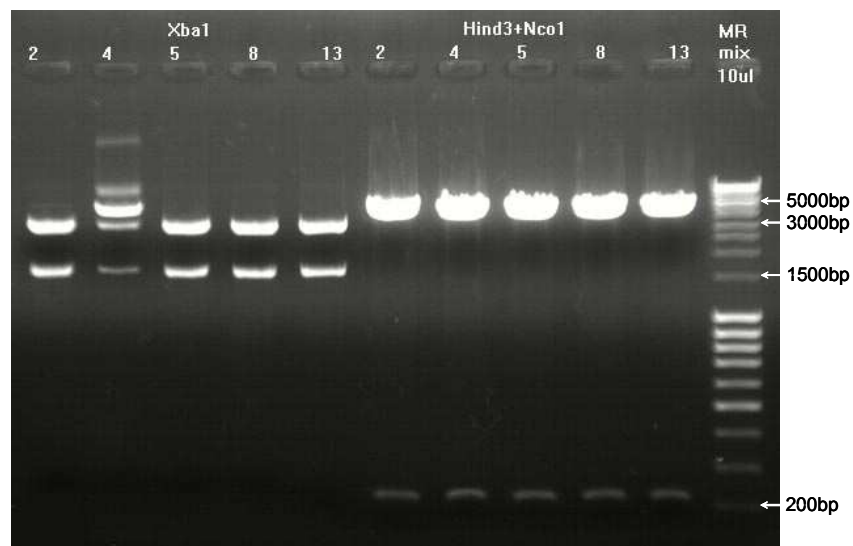


Figure 4.9: Diagnostic digest of pGL3.Fos.

The colonies with indicated numbers were first digested with Xba1 and all resulted in expected fragments of length 3301bp and 1693 bp. Second digest was with HindIII and Nco1 that gave the insert containing small fragment of size 211 bp.

Figure 4.9 shows that all the colonies contained the correct plasmid. Only the colony number 4 seemed not to be digested completely but still the sites exist in the expected regions. Colony number 2 was arbitrarily selected for the further cloning steps and midprep DNA of colony 2 was prepared as our luciferase reporter vector, pGL3.Fos. The identity of midprep DNA was further confirmed by digesting it with restriction enzymes that have recognition sites in the fos promoter and the pGL3Basic plasmid. First digest was Sph1 and Pvu2 double digest and the second was HindIII and Xba1 double digest. Figure 4.10 shows the expected size of the fragments and the resulting fragments.

Expected	pGL3Basic	pGL3fos
Sph1+Pvu2 digest	4818bp linear	831bp, 4163bp
HindIII+Xba1 digest	1689bp, 3129bp	174bp, 1693bp, 3129bp

Expected	pGL3fos
Pst1+Sal1 digest	81bp, 1975bp, 2938bp
HindIII+Nco1 digest	209bp, 4785bp

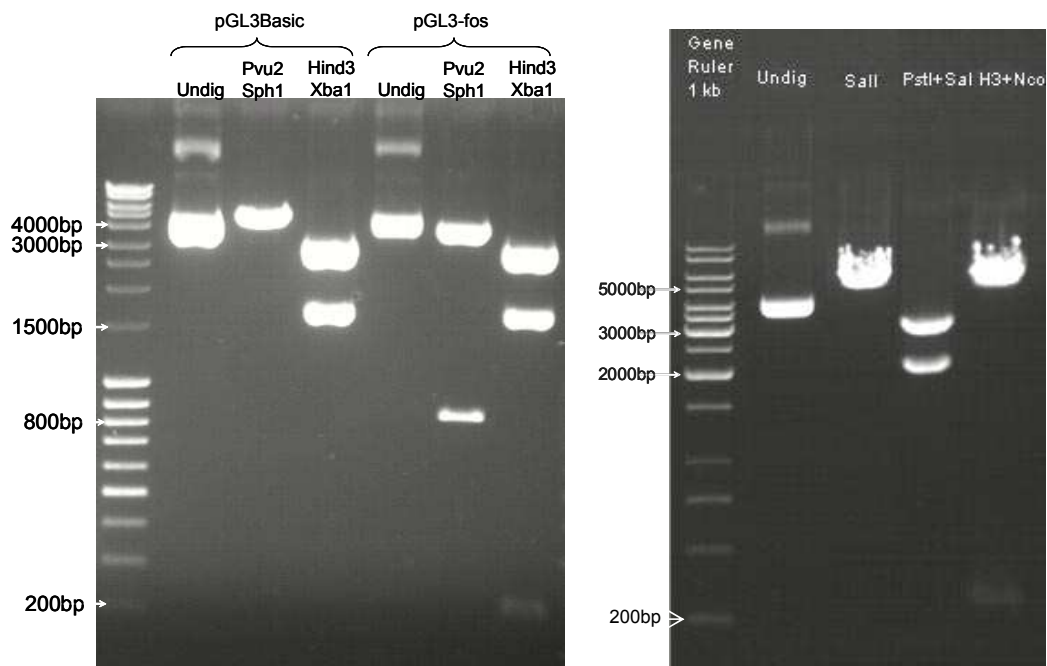


Figure 4.10: The digests for confirmation of pGL3.Fos plasmid (colony 2) .

4.3 CONSTRUCTION OF pGL3.Fos-SV40

A positive control vector named pGL3.Fos-SV40 that has transcription activity due to the presence of a strong transcriptional enhancer was constructed. A region of the pGL3Enhancer plasmid (Promega) containing SV40 enhancer and luc gene was purified and inserted into the pGL3.Fos plasmid replacing the luc gene in this plasmid. (Figure 4.11 and 4.12)

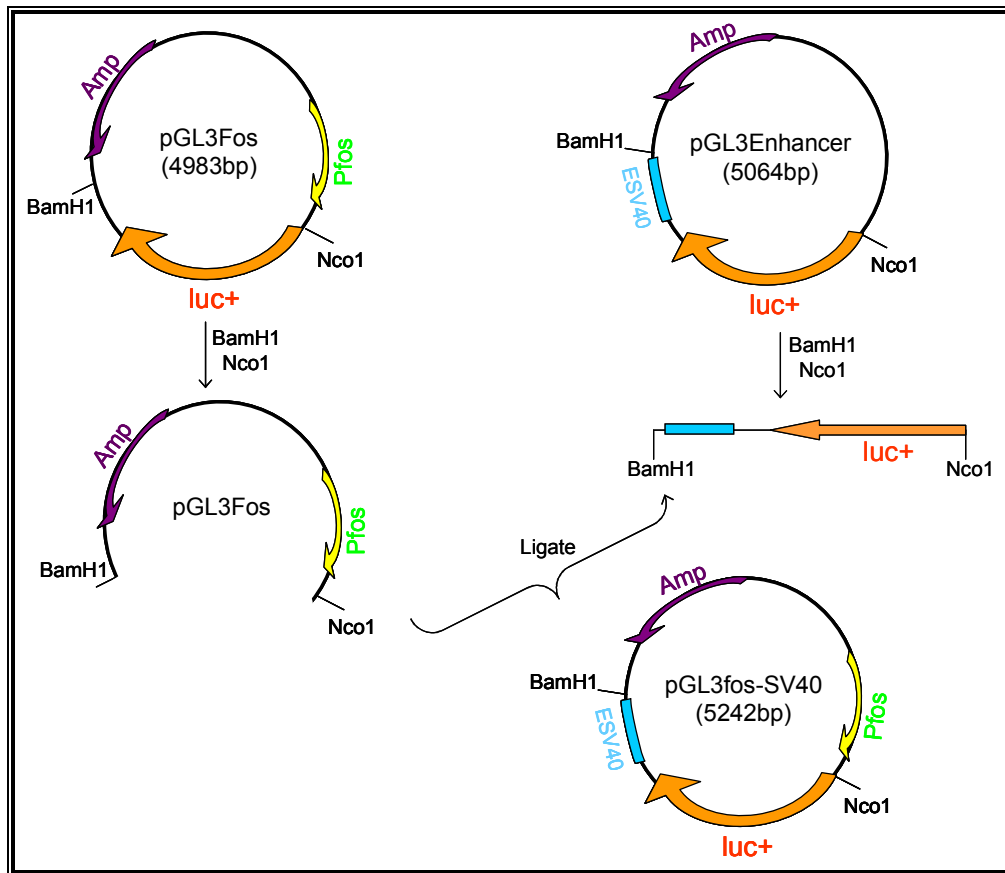


Figure 4.11: Strategy for constructing the pGL3Fos-SV40 positive control plasmid.

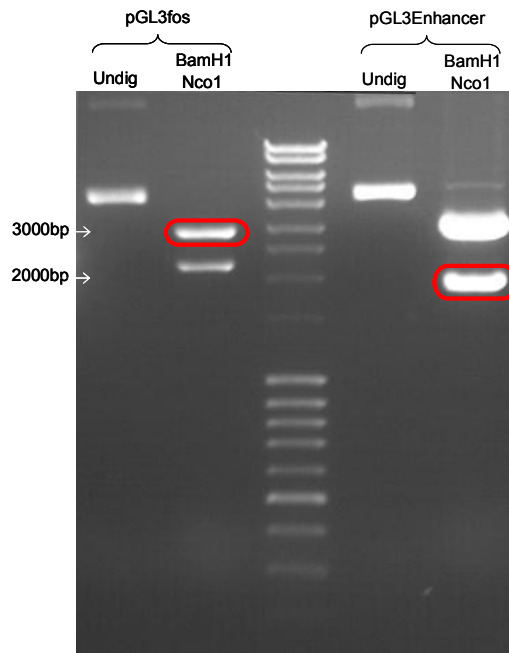


Figure 4.12: The BamH1 and Nco1 digested pGL3.Fos and pGL3Enhancer.

According to the restriction map (Figure 4.13) ,for the confirmation of pGL3fos-SV40 positive control vector Sph1 and Pvu2 digest was repeated as the inserted SV40 enhancer contains two successive Sph1 recognition sites. The second digest was again with HindIII and Xba1 to confirm the presence of Xba1 site at the end of luciferase coding region.

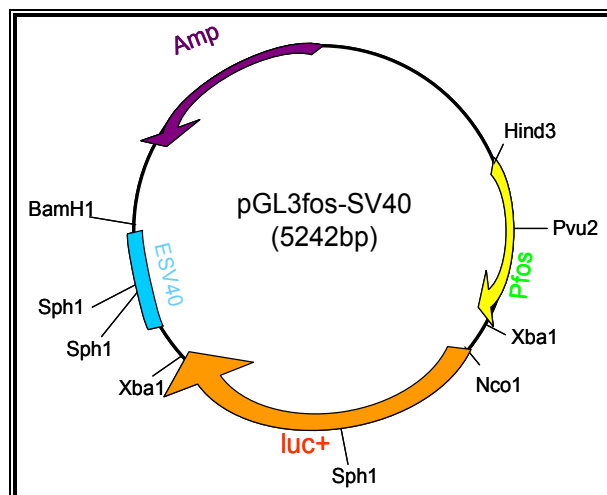


Figure 4.13: Restriction map of pGL3Fos-SV40 positive control plasmid showing only the relative locations of restriction enzyme recognition sites used for diagnosis and confirmation.

The first Sph1 and Pvu2 double digest resulted in the expected fragments listed in the chart except that 72bp fragment was not observed as it was too small. The double digest with Hind3 and Xba1 resulted in the expected length of fragments as in the table (Figure 4.14).

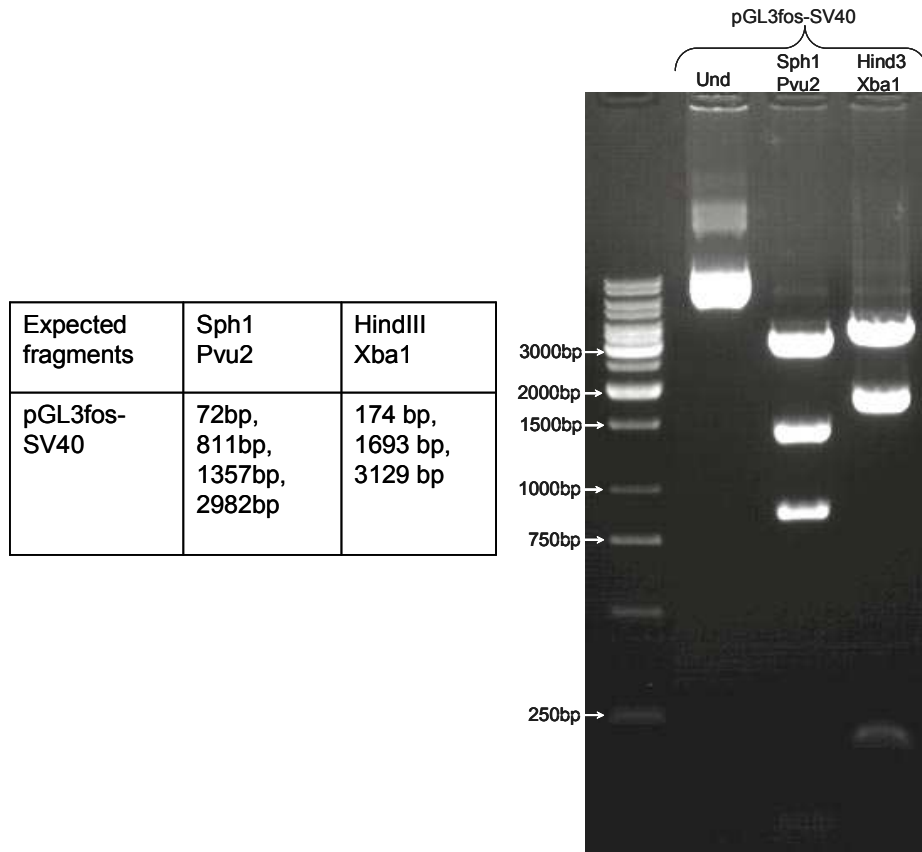


Figure 4.14: Confirmation of pGL3Fos-SV40 positive control plasmid.

4.4 CONSTRUCTION OF GFP REPORTER VECTOR

In order to construct a GFP reporter vector to detect any possible enhancer activity of the regions to be cloned, we inserted the weak fos promoter upstream of the turboGFP and downstream of the Sal1 site that the regions will be inserted in the originally promoterless pTurboGFP-PRL-dest1 plasmid (Evrogen). The construction strategy of this GFP reporter vector named pTurboGFP.Fos is given in Figure 4.15.

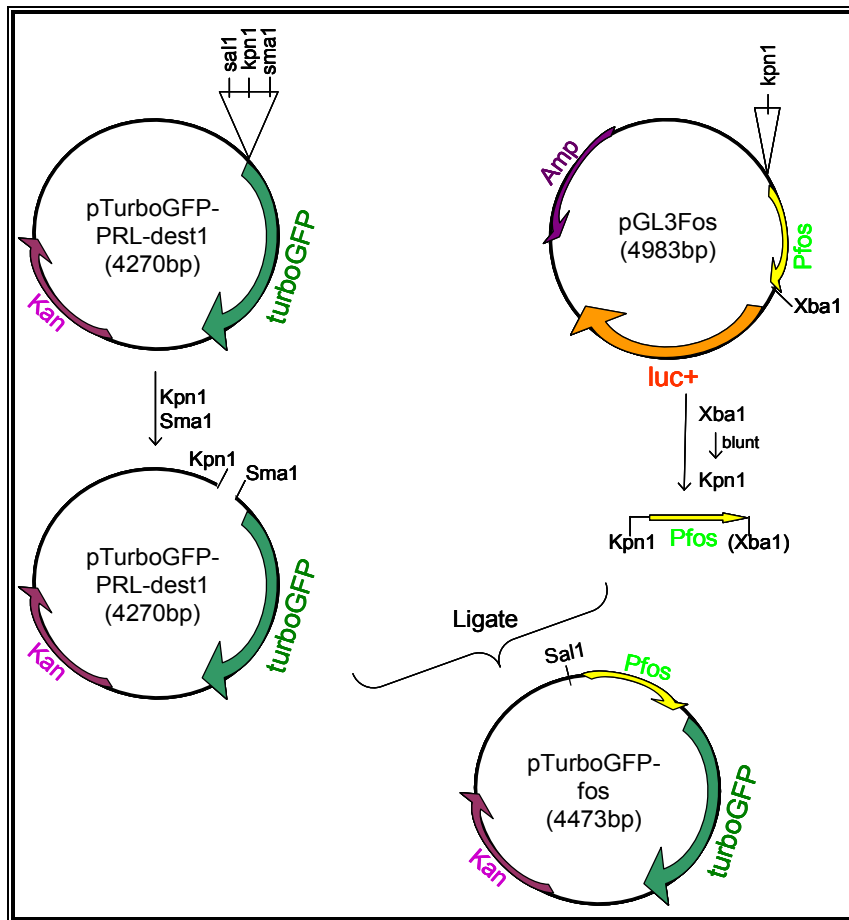


Figure 4.15: Strategy for constructing the pTurboGFP-fos reporter plasmid.

pTurboGFP-PRL-dest1 (Evrogen) vector was cut with Kpn1 at position 68 and Sma1 at position 77, linearized and dephosphorylated by alkaline phosphatase treatment. Fos promoter containing region of the pGL3.Fos plasmid (constructed as in section 4.2) was digested with Xba1 at position 226 first and blunted then purified via PCR purification. The 3' end of the fos promoter containing region was digested with Kpn1 at position 6. The resulting fos promoter and linearized pTurboGFP-PRL-dest1 vector were extracted from the gel and ligated.

To confirm the identity of this plasmid, miniprep DNA from 6 colonies was digested using Pvu2 that the vector has two sites and the insert (fos promoter) introduces a third site (Figure 4.16). The colonies except 1-4 and 1-5 gave positive results according to the table of expected fragment sizes.(Figure 4.17)

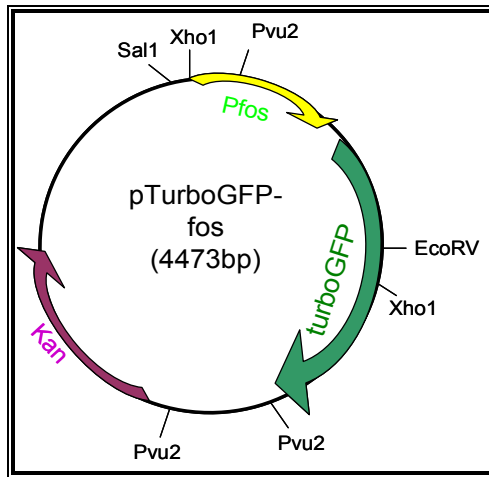


Figure 4.16: Restriction map of pTurboGFP-fos plasmid, showing only the relative locations of restriction enzyme sites used for diagnosis and confirmation.

Expected	pTurboGFP	pTurboGFP-fos
Pvu2 sites	1789,2397	161,2004,2612
Pvu2 digest products	608bp,3662bp	608bp,1841bp,2024bp

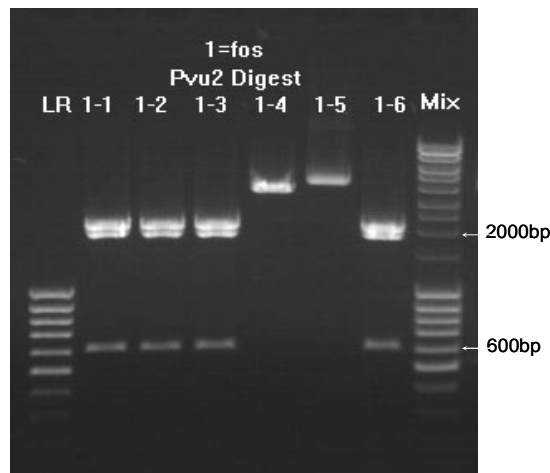


Figure 4.17: Pvu2 digest for identification of pTurboGFP.Fos plasmid.

The colonies 1-1 and 1-2 that gave positive results from the Pvu2 digests were confirmed with Sal1 and EcoRV and Xho1 digests both of which were used to see the different size of the small digestion product formed. The formation of a larger size fragment around 950 bp and 907 bp respectively indicates the presence of the insert between the two restriction sites of the plasmid. (Figure 4.18) The shift of the smaller band in Sal1+EcoRV digests and Xho1 digests imply the presence of insert and the

length of corresponding bands were as in the table of expected fragments. The midiprep DNA from colony 1.1 was used as pTurboGFP-fos vector for the further cloning steps.

Expected	pTurboGFP (V)	pTurboGFP-fos (1-1,1-2)
Sal1+EcoRVdigest products	747bp,3523bp	950bp,3523bp
Xho1 digest products	766bp,3504bp	907bp,3523bp

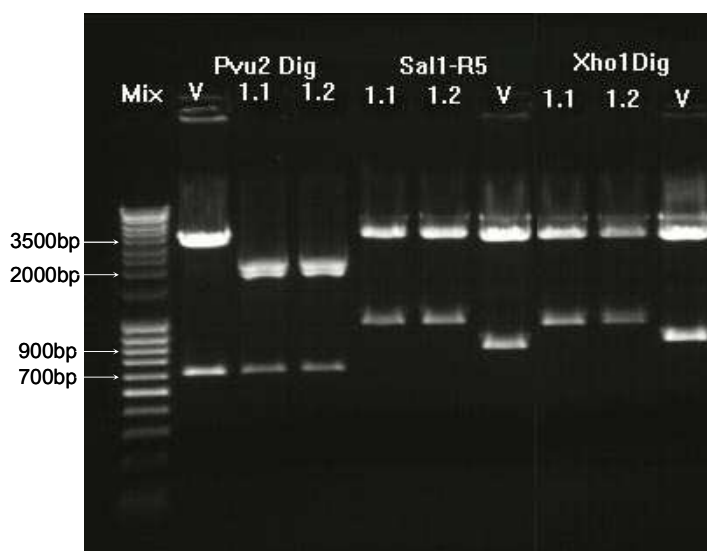


Figure 4.18: The confirmation of pTurboGFP.Fos plasmid.

4.5 CONSTRUCTION OF pTurboGFP-fos-ESV40

For the positive control vector of pTurboGFP.Fos, the SV40 enhancer from pGL3Control vector was amplified with PCR using designed primers listed in Table 3.4. Following the Sal1 digestion of the PCR products and PCR purification of the digestion mixture, the SV40 Enhancer was ligated to the Sal1 digested and dephosphorylated pTurboGFP.Fos vector (Figure 4.19 and 4.20).

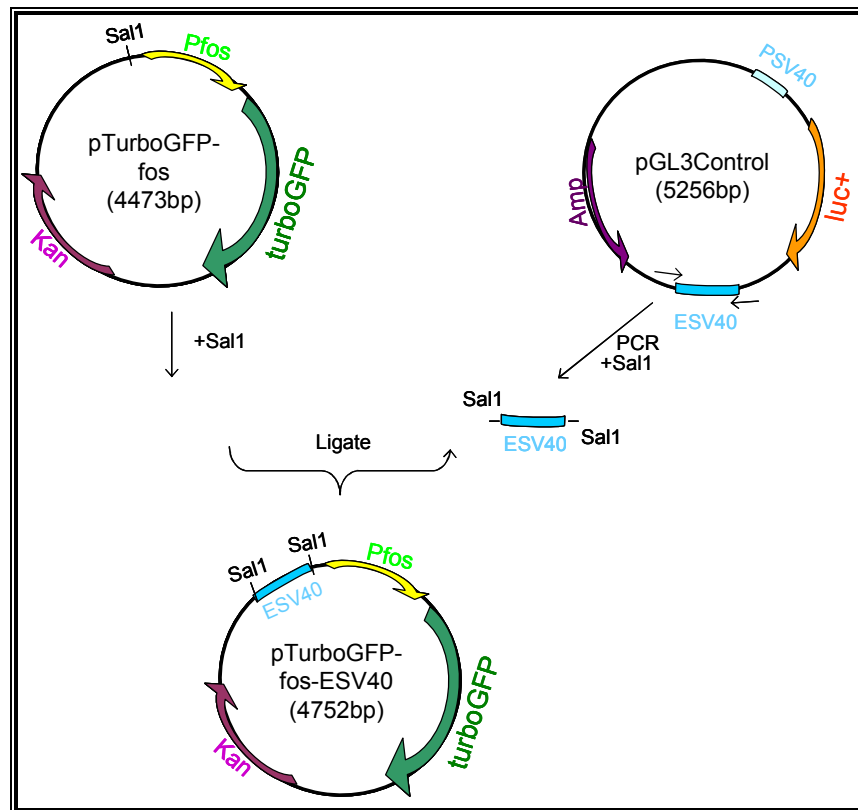


Figure 4.19: Strategy for constructing the pTurboGFP-fos-ESV40 positive control plasmid.

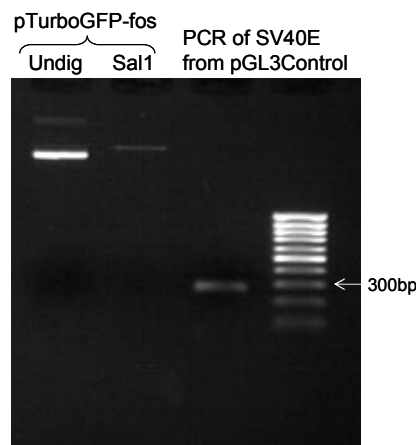


Figure 4.20: pTurboGFP-fos plasmid and SV40 PCR product.

In order to find the relative amount of PCR product to be used for the ligation reaction several dilutions of the known concentration Sal1 digested vector were run on the same gel with three different volumes of Sal1 digested PCR products. By comparing the intensity of the bands the amount of Sal1 digested PCR product to be used with 100 ng vector was found to be around 5 μ l (Figure 4.21).

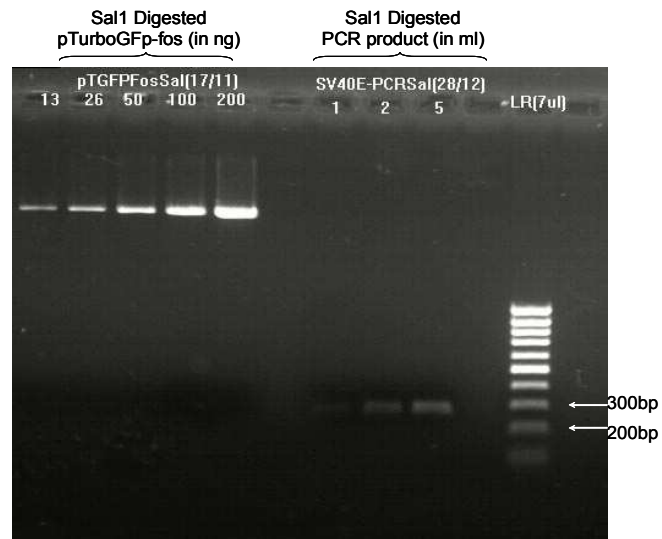


Figure 4.21: Determination of amount of SV40 enhancer PCR product to be used for ligation.

To confirm the identity of ligation products, miniprep DNA from 10 colonies was digested with Sal1 at the beginning to eliminate the incorrect ligation products, to see if there was any insert to pop-out from Sal1 site of the vector. (Figure 4.22)

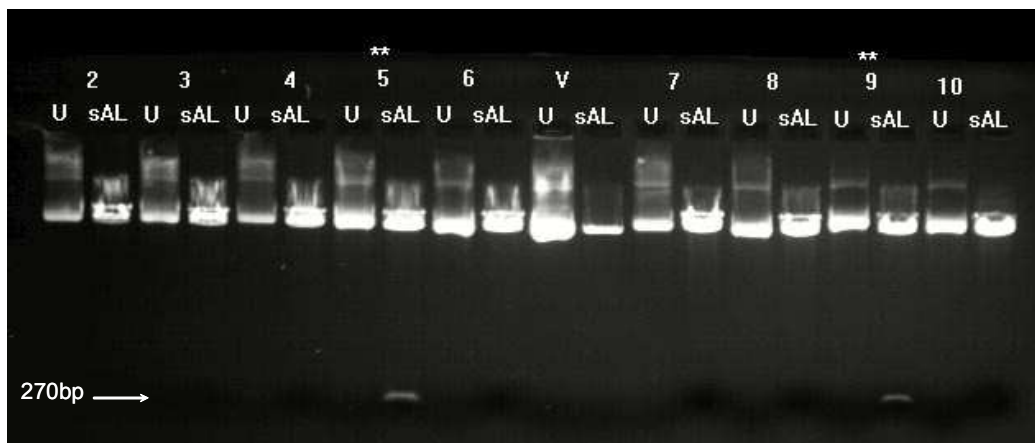


Figure 4.22: The Sal1 digests for identification of pTurboGFP-fos-ESV40.

The colonies 5 and 9, had inserts popping out at the size of 270bp, the size of PCR product of SV40 enhancer. These colonies were further digested for the number and the orientation of the inserts according to the restriction map of pTurboGFP.Fos-ESV40. (Figure 4.23)

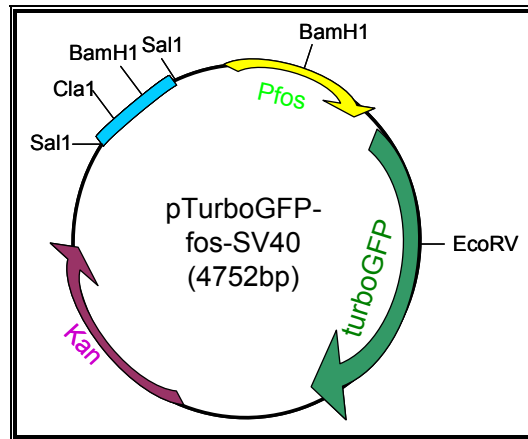


Figure 4.23: Restriction map of pTurboGFP-fos-SV40 plasmid showing only the relative locations of restriction enzyme sites used for confirmation.

First digest was with BamH1 that the vector has one site and the insert (SV40 enhancer) introduces a second site. The second digest was Cla1 and EcoRV double digest, the vector has the EcoRV site and the insert introduces a Cla1 site. Looking at the products of digests and the table of expected fragments, G5 seems to have the insert but in reverse orientation as the BamH1 digestion gives a band around 500 bp. The G9 on the other hand seems to have two inserts in the vector one reverse and one direct oriented forming a concatamer (Figure 4.24). DNA from both of the colonies were used for further flow cytometric analysis to see the possible difference in transcriptional activity due to the orientation of the present transcriptional enhancer.

Expected	pTurboGFP	pTurboGFP.Fos	
		+ orientation	- orientation
BamH1 digest products	4481bp linear	242bp, 4510bp	501bp, 4251bp
Cla1+EcoRV digest products	4481bp linear	1221bp, 3531bp	974bp, 3778bp

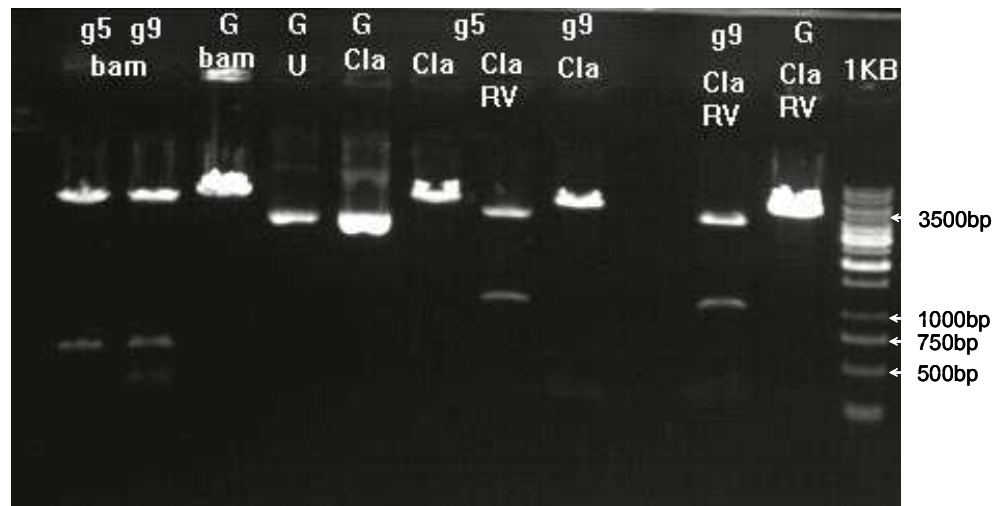


Figure 4.24: Confirmation of positive control plasmids pTurboGFP.Fos-ESV40 (G5 and G9).

4.6 CONSTRUCTION OF pDSRedExpress-pSV40

To normalize a reporter activity to the efficiency of transfection an independent fluorescent reporter plasmid was constructed. In our study we decided to use a DSRed expressing vector as the reference plasmid and inserted a SV40 promoter and enhancer sequentially into the promoterless pDSRedExpress-DR plasmid (Clontech) for this purpose.

First, we constructed a plasmid named pDSRedExpress-pSV40 by inserting SV40 promoter from pGL3Control plasmid into the promoterless pDSRedExpress-DR plasmid (Clontech) upstream of DSRedExpress coding region (Figure 4.25).

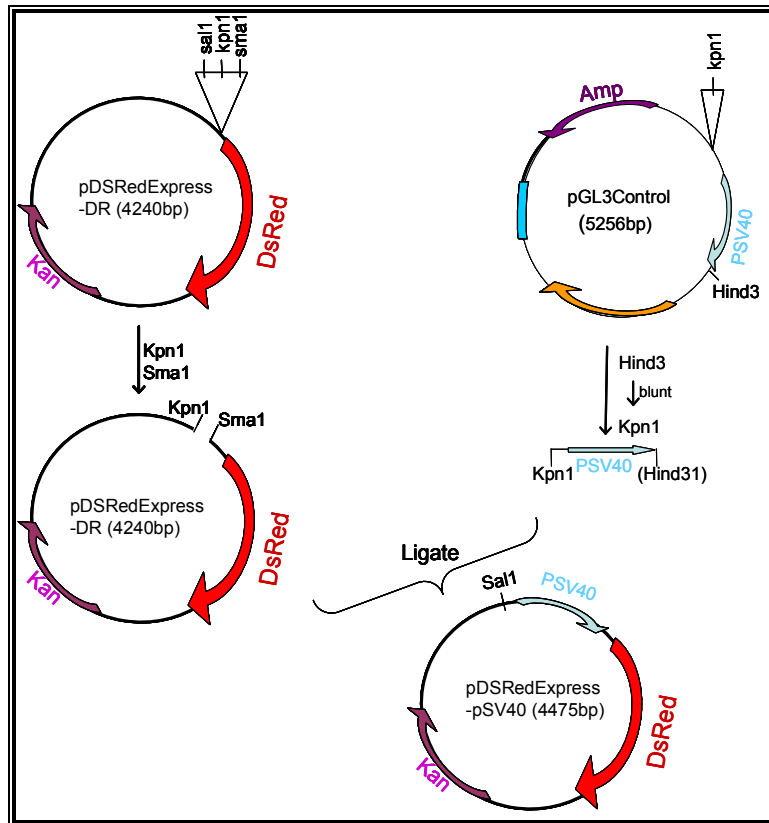


Figure 4.25: Strategy for constructing the pDSRedExpress-pSV40 plasmid.

The pDSRedExpressDR vector (Clontech) was digested with Kpn1 at position 68 and Sma1 at position 77, linearized and dephosphorylated. The SV40 promoter containing region of the pGL3Control plasmid was digested with HindIII at position 245 first, blunted and purified via PCR Purification. The 3' end of the SV40 promoter containing region was digested with Kpn1 at position 5 (Figure 4.26). The resulting SV40 promoter and linearized pDSRedExpressDR vector were extracted from the gel and ligated.

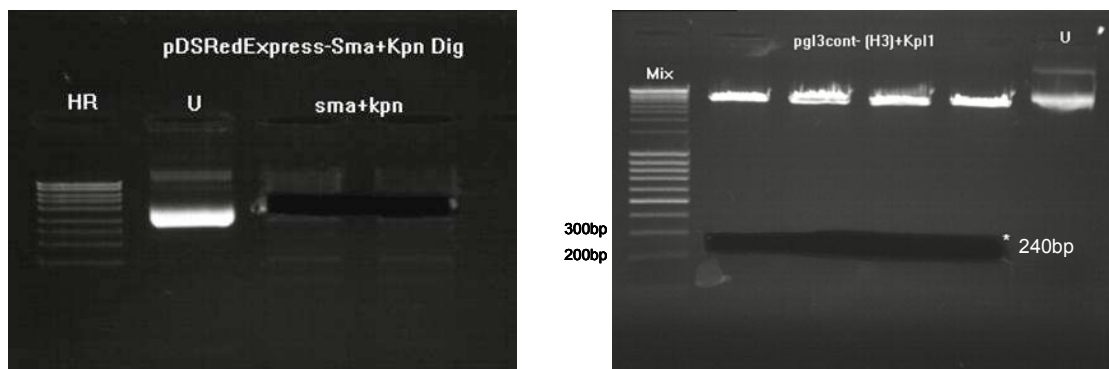


Figure 4.26: pDSRedExpress-pSV40 construction.

To confirm the identity of correct ligation products miniprep DNA from several colonies were digested for confirmation with restriction enzymes that have sites in the vector that are downstream and upstream of the site of insert and band shift was observed for confirmation. Both the Sal1 EcoR1 and Bgl2 EcoRV digests were for the detection of the change in length of the small fragment produced and the expected bandshift was observed in both of the digests (Figure 4.27). Among the positive 2.1 and 4.1, DNA from colony 2.1 was used for the further steps.

Expected fragments	Sal1+EcoRV	Bgl2+EcoRV
pDSRedExpress-DR	723bp, 3752bp	753bp,3722bp
pDSRedExpress-pSV40	953bp,3752bp	912bp,3722bp

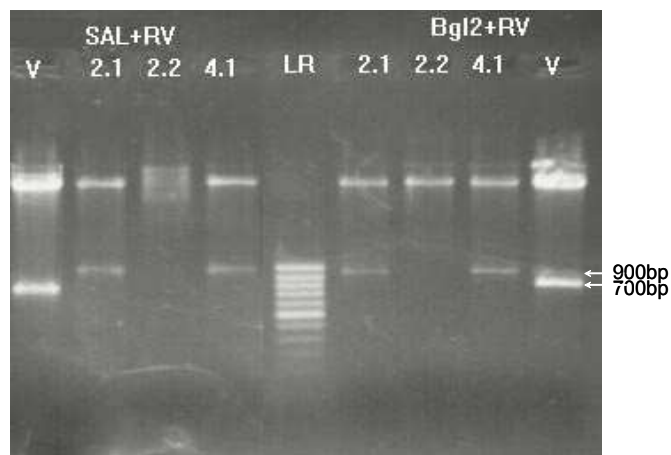


Figure 4.27: Diagnostic digests of three different colonies of pDSRec-pSV40.

4.6.1 CONSTRUCTION OF pDSREDEXPRESS-PSV40-ESV40

In order to observe enhanced expression of DSRed as reference fluorescence marker for the flow cytometer analysis, strong enhancer SV40 was inserted into pDSRedExpress-pSV40 plasmid into the Sal1 site of the plasmid through the same strategy used for the construction of pTurboGFP.Fos-ESV40(as in section 4.5.1). This constructed plasmid was named pDSRedExpress-pSV40-ESV40.

The enzymes used for confirmation and the expected fragments of the digestions were same as the section 4.5.2, confirmation of pTurboGFP-fos-ESV40. Again at the beginning to eliminate the incorrect ligation products SalI digest was carried out to see if there was any insert to pop-out from SalI site of the vector. Colonies 2,3,4,6,7 and 9 seemed to have an insert at SalI site (Figure 4.28).



Figure 4.28: SalI digestion for identification of pDSRedExpress-pSV40-ESV40.

The colonies bearing the plasmids with inserts were further digested in order to find the orientation and number of the inserts using the same enzymes used for confirmation of pTurboGFP.Fos-SV40 (Figure 4.29). Looking at the digestion reaction products and the table of expected fragments; two colonies D7 and D9 ,having one inverse and one direct oriented insert successfully ligated to the vector respectively, were chosen for further high quality DNA preparation and transfection.

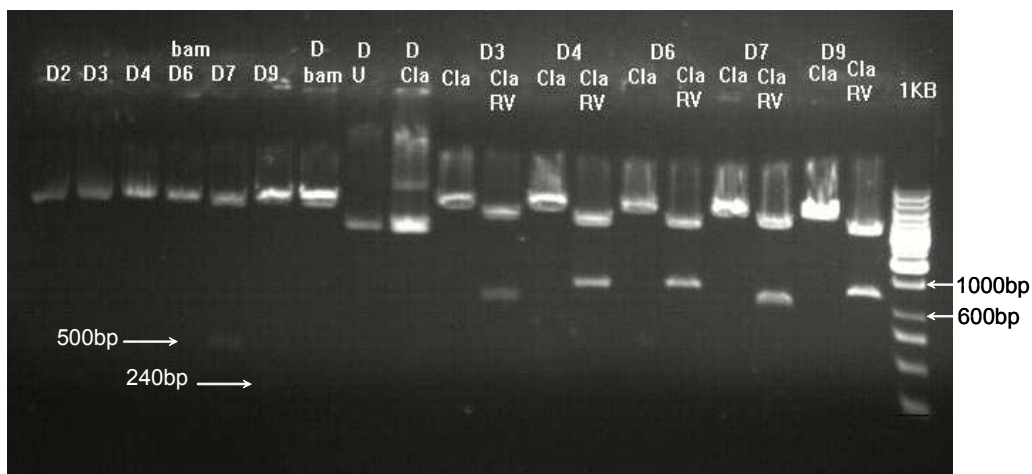


Figure 4.29: Further characterization of pDSRedExpress-pSV40-ESV40 plasmids.

4.7 CONSTRUCTION OF pCDNA3-EGFP

In order to optimize transfection efficiency and to test the applicability of flow cytometry for studying the increase in GFP expression, EGFP from pMaxGFP was digested out and cloned into pCDNA3 (Promega). EGFP containing region of pMaxGFP was digested out by Kpn1 and Xho1 enzymes and following the digestion of the pCDNA3 plasmid by the same enzymes found in the multiple cloning site, the EGFP containing region was directionally cloned into pCDNA3 plasmid. The constructed plasmid was named pCDNA3-EGFP.

4.8 CLONING OF AMPLIFIED REGIONS INTO REPORTER VECTORS

4.8.1 CLONING TO THE LUCIFERASE REPORTER VECTOR-pGL3.FOS

PCR reactions for the amplification of determined seven CNS regions were carried out (Figure 4.5) The PCR products were extracted from the gel, the PCR products were then digested with Sal1 by the aid of the Sal1 sites added to the primers, forming sticky ends. After digestion the purified DNA was ligated to Sal1 digested and dephosphorylated pGL3.Fos plasmid.

The amounts to be used for ligation reactions were determined by running several dilutions of the vector on the same agarose gel with the PCR products, as explained previously (Figure 4.3).

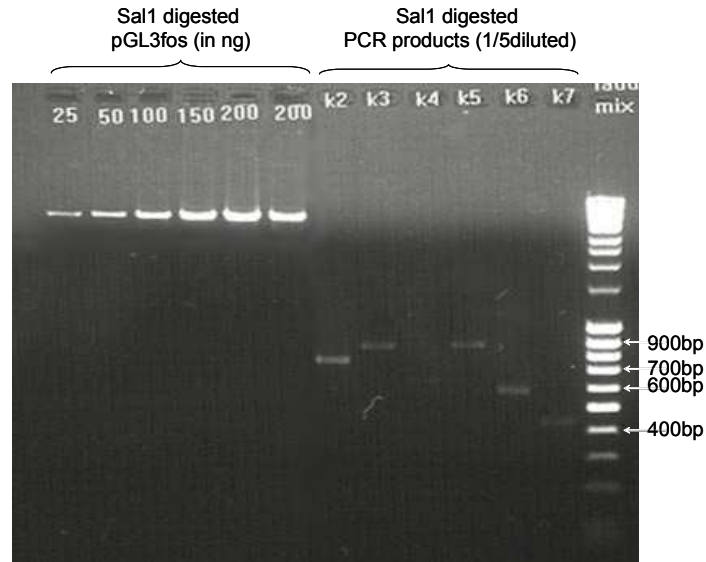
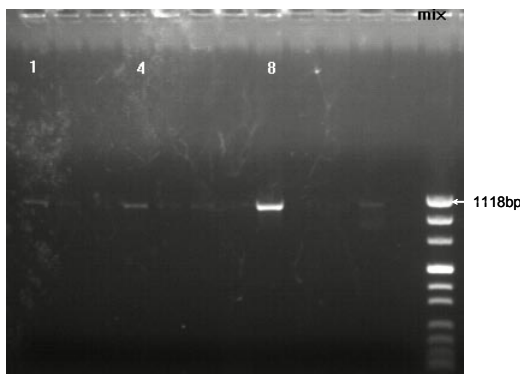


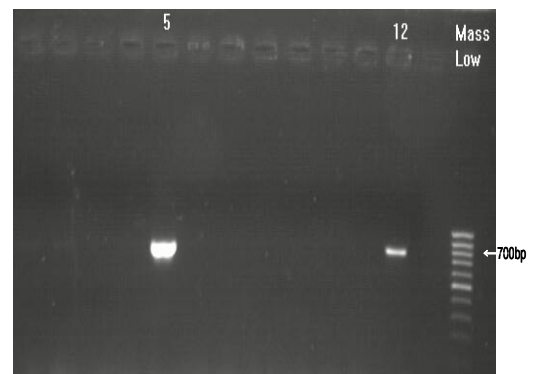
Figure 4.30: Determination of amount of K1 to K7 PCR product to be used for ligation.

The identity of correct ligation products were diagnosed first by colony PCR and then by diagnostic digests. In order to identify the colonies having the correct plasmids with correct inserts colony PCR was the first technique used. The presence of products having same size with the original CNS indicated the presence of insert in the plasmid isolated. The colonies were analyzed in tens until a positive colony was identified. The regions K1, K2, K3, K6 and K7 were determined to be successfully cloned into the luciferase reporter plasmid (Figure 4.31).

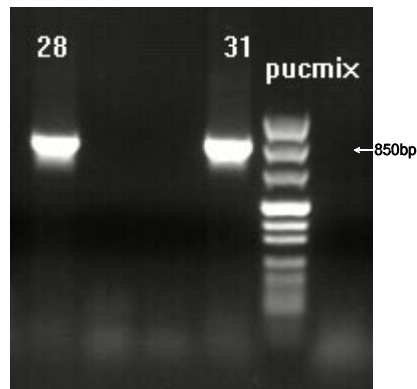
K1



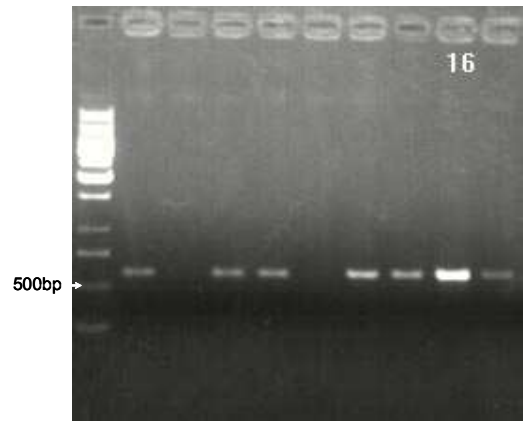
K2



K3



K6



K7

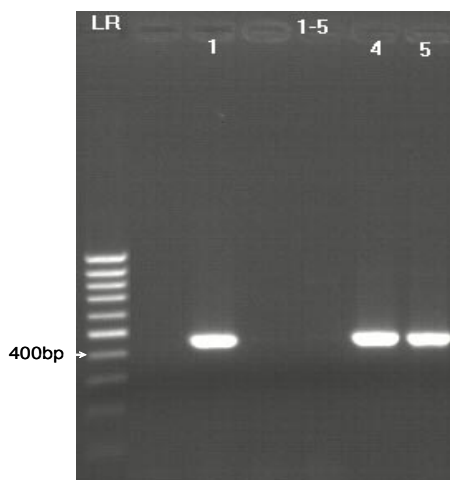


Figure 4.31: Colony PCR results of ligations of regions K1 to K7 into SalI digested pGL3.Fos plasmid.

The miniprep DNA from candidate colonies, that gave positive result from the colony PCR, were digested further to confirm the presence of insert and also to determine the orientation of insert. At first SalI digestion was made for all the colonies to see the presence of insert in the SalI site of the plasmid. Then the other restriction enzymes used for confirmation were selected among the ones having sites located in the inserted regions and the ones in the plasmid sequence. Restriction enzymes used and their recognition sites in the plasmid and the inserts are given in Figure 4.32. In region K6 there was no restriction enzyme recognition site present.

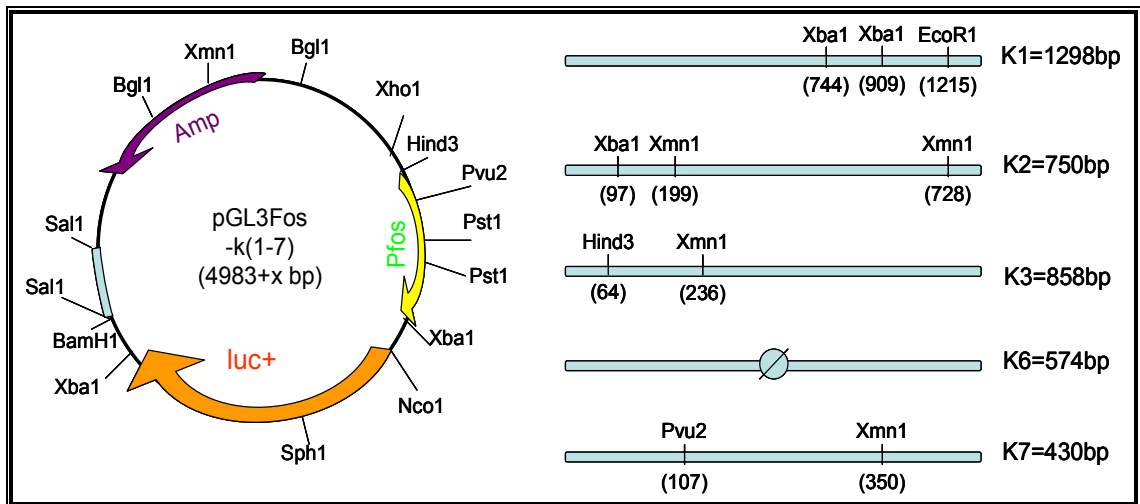


Figure 4.32: Restriction map of pGL3fos and inserted regions.

The first digest for confirmation was with Sal1 to see whether the insert would pop-out. Subsequent digests were with the restriction enzymes indicated in Figure 4.32. All the digests confirmed the presence of the inserts cloned successful into the pGL3.Fos luciferase reporter plasmid (Figure 4.33 and 4.34).

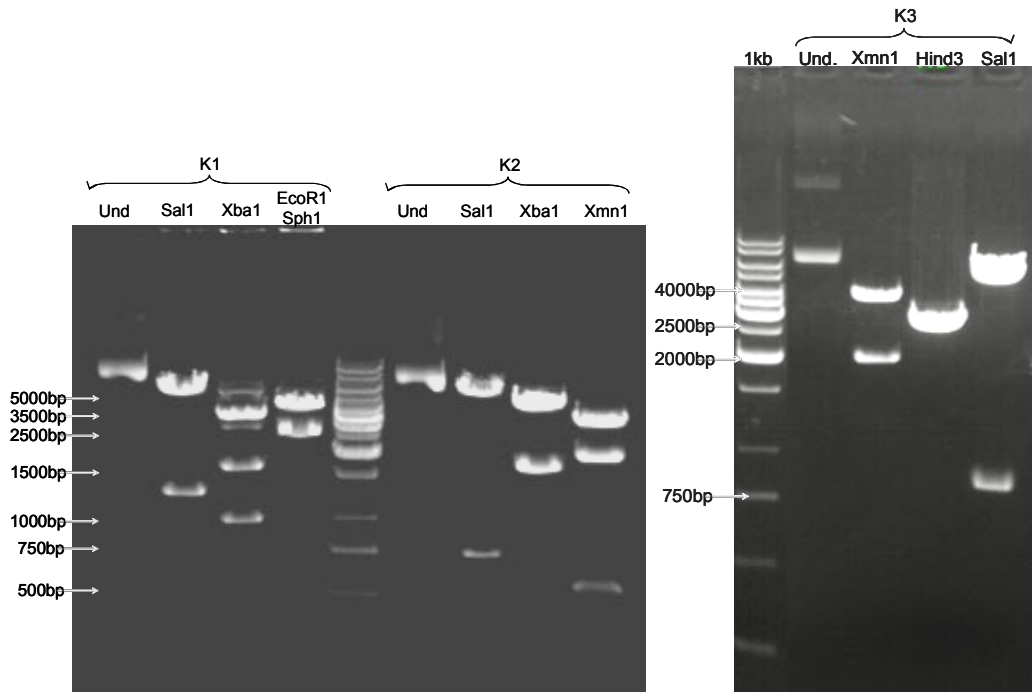


Figure 4.33: Confirmation of K1, K2 and K3 cloned into pGL3.Fos plasmid.

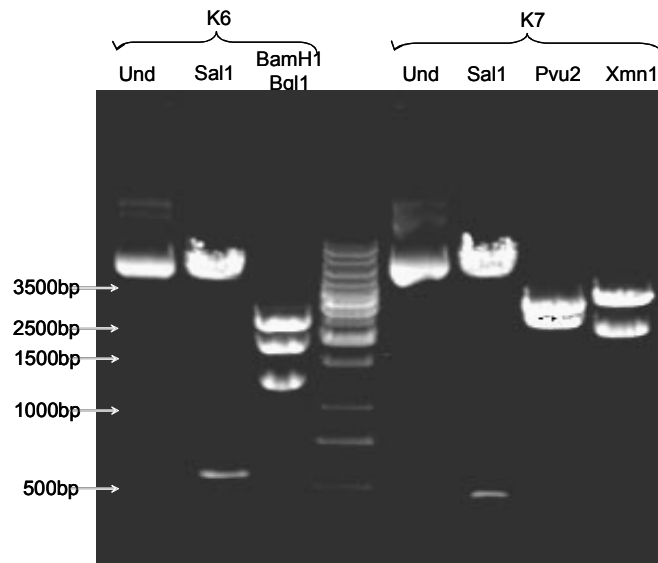


Figure 4.34: Confirmation of K6 and K7 cloned into pGL3.Fos plasmid..

4.8.2 CLONING TO THE GFP REPORTER VECTOR-pTURBOGFP.Fos

For the second and the main reporter assay of this study the regions were cloned into the GFP reporter plasmid to be assayed in flow cytometry. For this purpose identified CNS regions successfully cloned into pGL3fos luciferase reporter vector in the first part of the study, were digested out by the Sal1 restriction sites that were previously used for cloning (Figure 4.34).

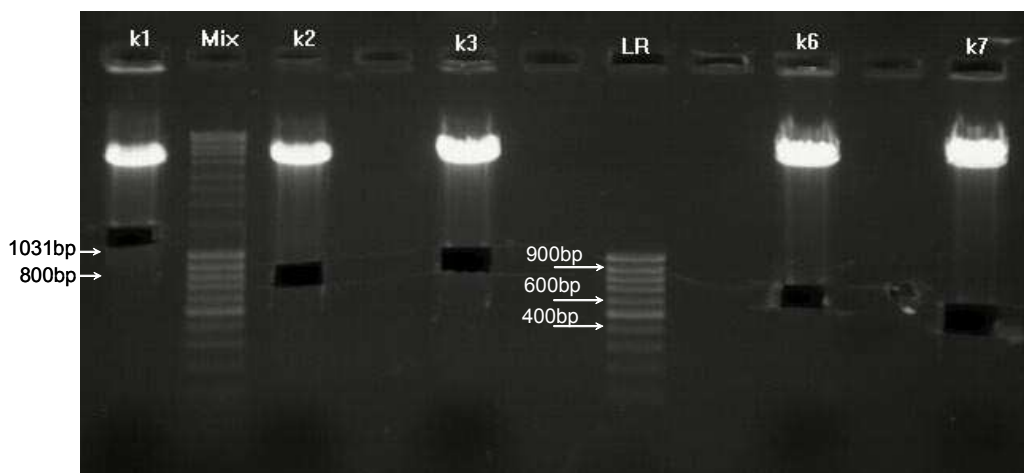


Figure 4.35: The regions K1, K2, K3 and K6, K7 digested out from pGL3.Fos.

SalI digested, dephosphorylated pTurboGFP.Fos vector was ligated to three molar excess SalI digested and gel extracted insert. The amount to be used for ligation was determined as the concentrations of the pGL3.Fos ligated inserts were previously calculated by UV Spectrophotometry.

The confirmation of identity of correct ligation products were made by colony PCR and diagnostic digests. Colony PCR from the colonies of ligation products were carried out as explained previously. K3, K6 and K7 were shown to be successfully cloned into pTurboGFP.Fos plasmid (Figure 4.36)

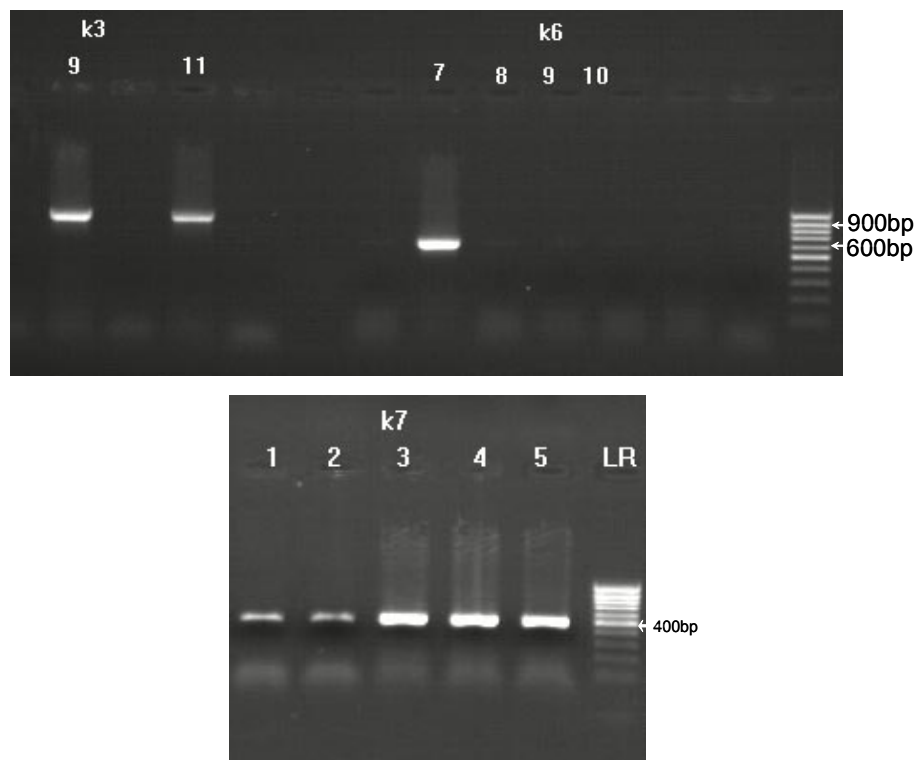


Figure 4.36: Colony PCR results of identified colonies containing inserts K3, K6 and K7.

The miniprep DNA from candidate colonies that gave positive result from the colony PCR; K3 colony 9 and colony 11, K6 colony 6 and colony 7, K7 colony 3 and colony 4 were digested for detection of the presence of insert and also the orientation of insert. The restriction enzymes used and the respective recognition sites on the plasmid and the inserts are given in Figure 4.37.

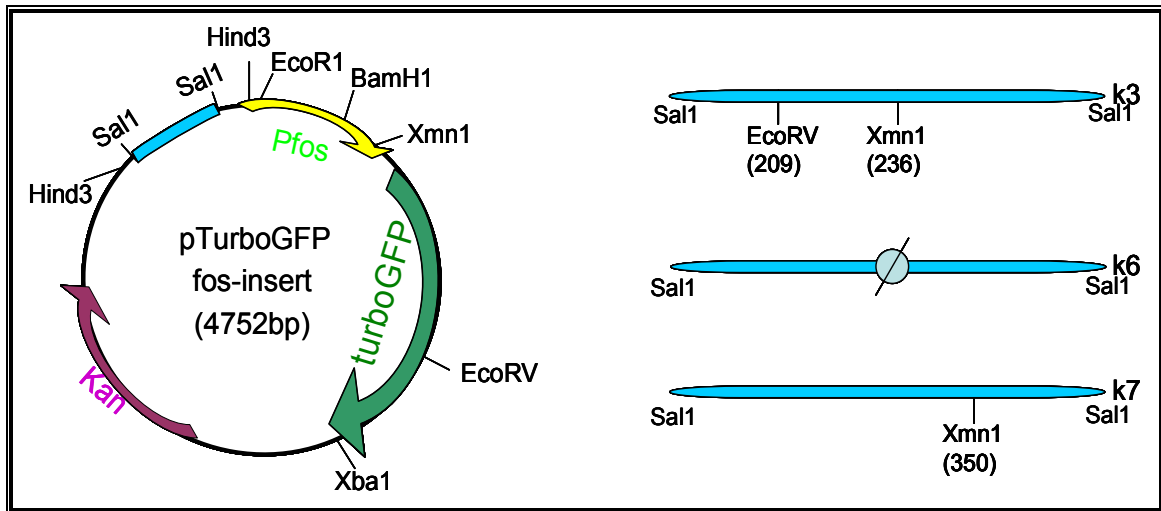


Figure 4.37: Restriction map of the pTurboGFP.Fos and inserted regions

According to the digests the colony 3.9 had a reverse oriented insert and 3.11 had a direct oriented insert. Analyzing the HindIII digest 6.7 had a correct oriented insert. The seemingly wrong result of EcoR1 and Xba1 double digest of the colony 6.7 made us to further investigate the Xba1 site on the vector backbone, and the digests revealed that the Xba1 site in the backbone of the pTurboGFP-PRL-dest1 vector gets methylated and the DH-5 α used as the competent cell methylates DNA. Both colonies 7.3 and 7.4 had a direct oriented insert according to the results of both digests (Figure 4.38). After all, the colonies 3.9, 3.11, 6.7 and 7.4 were chosen for further transfection. The reason of using both 3.9 and 3.11 was to be able to see the effect of orientation of the enhancer element on the activity of transcription, if there exists any in this region.

Expected Fragments	pTurboGFP-fos	pTurboGFP-fos-k3	pTurboGFP-fos-k6	pTurboGFP-fos-k7
EcoRV	4473bp,linear	1602bp, 3729bp	-	-
Xmn1	4473bp,linear	1100bp,4231bp	-	558bp,4345bp
HindIII	75bp, 4398bp	-	649bp, 4398bp	505bp,4398bp
EcoR1+ Xba	1093bp,3380bp	-	1667bp, 3380bp	-

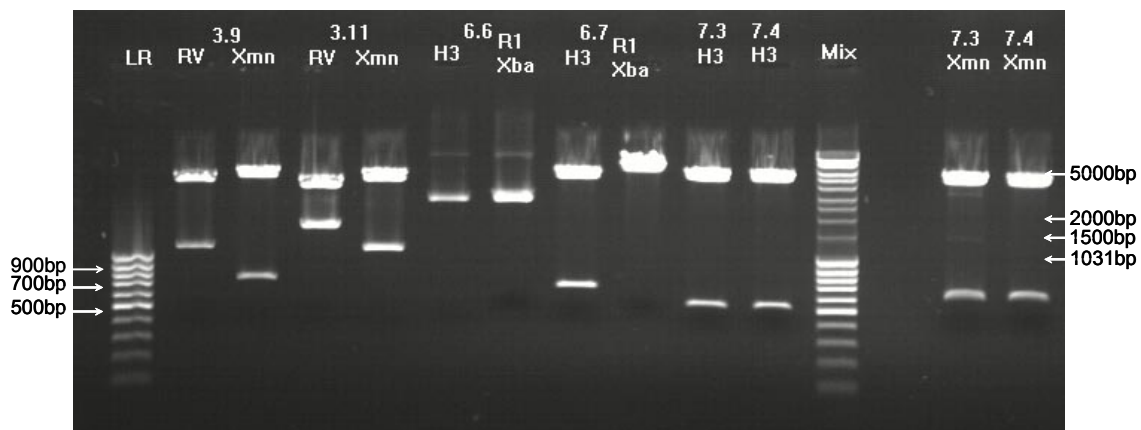


Figure 4.38: Diagnostic digests of pTurboGFP.Fos plasmid containing the inserted regions K3, K6 and K7.

4.9 TRANSIENT TRANFECTION OF EL-4 CELLS

4.9.1 OPTIMIZATION OF ELECTROPORATION CONDITIONS

Previous studies indicated that the transfection efficiency of EL-4 cells was low regardless of the method used for transient transfection of cells. In order to optimize the transfection efficiency, six different electroporation conditions to electroporate EL-4 cells (Table 4.2). Transfection efficiency was determined by assaying for fluorescence due to the expression of EGFP from the transfected pcDNA3-EGFP plasmid. The flow cytometric analyses were made 24 hrs after transfection.

Condition	Volt(v)	Resistance(Ω)	Capacitance(μ f)	DNA (μ g)
1	290	100	1400	10
2	290	100	1400	15
3	290	100	1400	20
4	300	725	500	10
5	300	960	725	10
6	300	960	100	15

Table 4.2: Optimization of electroporation conditions for EL-4 cells.

In order to find the viable cells during the flow cytometric analysis a forward scatter versus side scatter dot plot was generated and viable EL-4 cells were gated according to previous propidium iodide viability tests. The population corresponding to viable EL-4 cells was placed in a gate named as P1. The size distribution of these cells in P1 gate was confirmed by forward scatter plots. The cells that fluoresce in the P2 gate in untransfected samples were defined as background. In untransfected samples only 0.4% of the parent P1 population fell into the P2 GFP positive gate (Figure 4.39). In figure 4.39, the top dot plot (a) shows forward scatter versus side scatter data of all events. Viable EL-4 cells were further analyzed by electronic gating as P1. The middle histogram (b) shows forward scatter values of P1 gated EL-4 cells. the size distribution of selected viable EL-4 cells ,gated as P1, showed normal distribution as expected. Bottom histogram (c) shows GFP fluorescence P1 gated viable EL-4 cells. Positive fluorescence was arbitrarily set to fall into the P2 electronic gate.

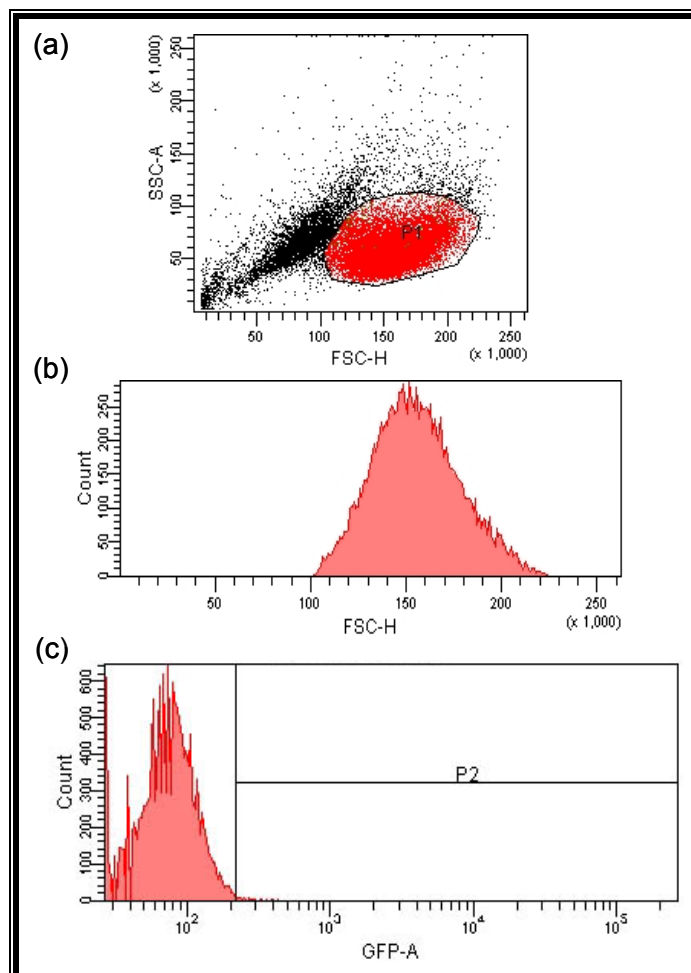


Figure 4.39: Flow cytometric analysis of untransfected cells.

All samples corresponding to different electroporation conditions were examined using identical flow cytometer settings and voltage parameters (Figure 4.40). Histograms of GFP fluorescence revealed that the percentage of cells that fluoresce in the P2 gate varies with different transfection conditions. Transfection efficiency was determined by examining the percentage of parent cells that appear in the P2 gate. The experiment demonstrates that conditions 2 and 3, of electroporation at 290 V, 100 Ω , 1400 μ F using 15 μ g and 20 μ g respectively, yielded the highest transfection efficiency of approximately 29% (Figure 4.39 and Table 4.3). These conditions were used for the following transfections in this study.

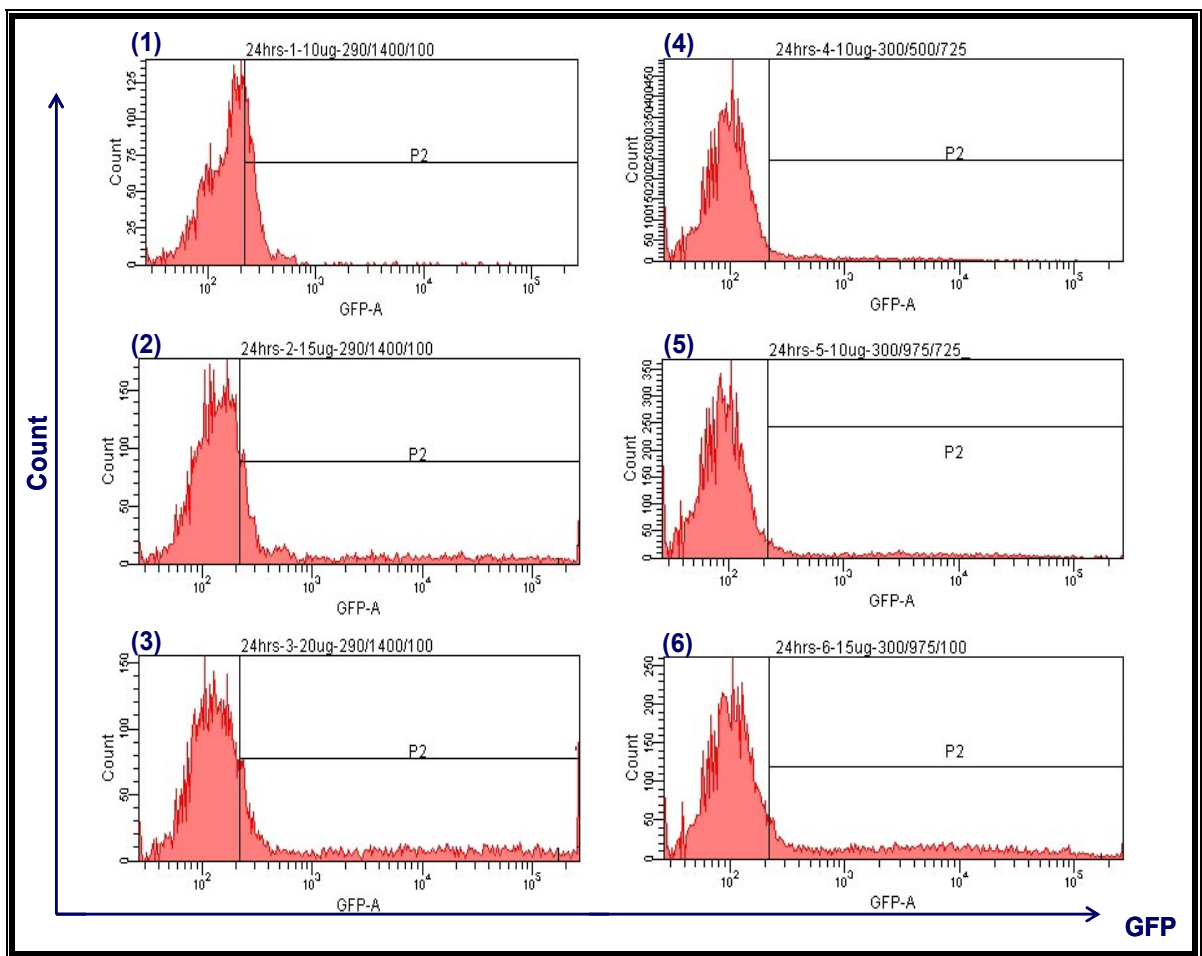


Figure 4.40: Flow cytometric analysis of six different conditions of electroporation showing GFP versus number of events.

The quantitative data showing the percentage of parent cells in populations P1 and P2 are given in Table 4.3.

SAMPLE	POPULATION	%PARENT
Untransfected	P1	72
	P2	0.4
1	P1	18.1
	P2	26,5
2	P1	29,5
	P2	30,3
3	P1	26,7
	P2	29,2
4	P1	51,9
	P2	7
5	P1	45,9
	P2	11,2
6	P1	42,6
	P2	12,7

Table 4.3: The quantitative data obtained from flow cytometric analysis of six different electroporation conditions.

4.10 FLOW CYTOMETRIC REPORTER ANALYSIS

4.10.1 REPORTER ASSAY

The population of viable EL-4 cells was gated in untransfected cells based on pre-determined forward and side scatter values. Population of P1 corresponding to the viable untransfected EL-4 cells was used to set background GFP fluorescence levels that fall into the P2 region. The P2 region contained 0.5% of the parent cell population that fall into the P1 gate -88% of all events- (Figure 4.41).

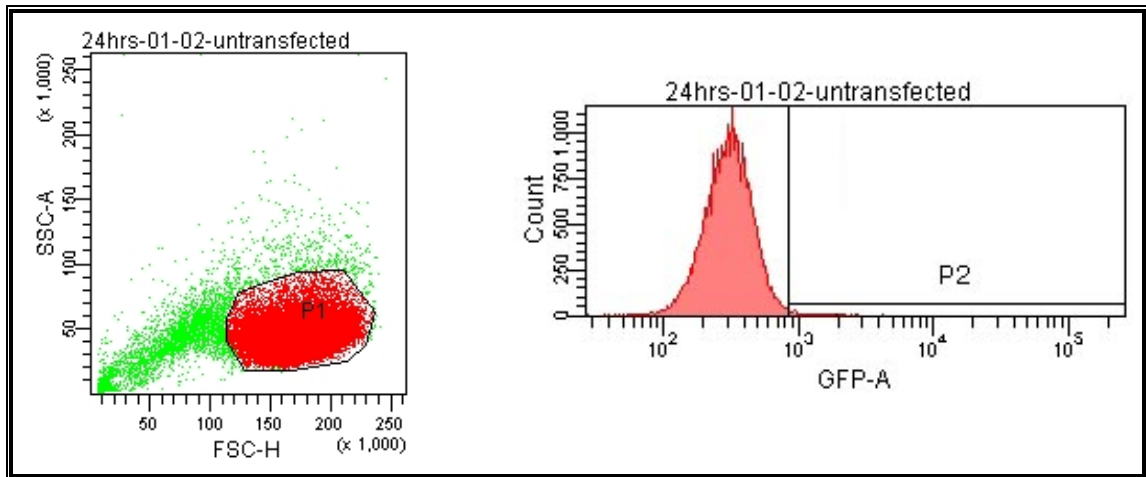


Figure 4.41: Flow cytometric analysis of untransfected cells to define the viable EL4 population and P2 gate of GFP fluorescence.

Next, the EL4 cells were transfected with various plasmids using conditions outlined in section 4.9.1. Figure 4.42 shows GFP fluorescence values of untransfected cells (a) in addition to cells transfected with pTurboGFP.Fos plasmid (promoter only plasmid)(b), pTurboGFP.Fos-ESV40.G5 (positive control plasmid having one reverse oriented SV40 enhancer)(c), pTurboGFP.Fos-ESV40.G9 (positive control plasmid having one reverse and one direct oriented SV40 enhancer)(d) and experimental constructs pTurboGFP.Fos-K3(e), pTurboGFP.Fos-K6(f), pTurboGFP.Fos-K7(g).

EL-4 cells transfected with the promoter only plasmid pTurboGFP.Fos showed an increase in the percentage of cells in the P2 gate from 0.5% to 15%. This increase is due to an increase in the transcription of TurboGFP resulting from the presence of the fos promoter (Table 4.4). Thus, the basal level of TurboGFP expression in the absence of any enhancer activity was set to 15%. When the samples 'c' and 'd' corresponding to different positive control plasmids containing an SV40 enhancer were analyzed, the percentage of cells in the P2 gate was found to be 30.5% for c and 32% for d (Table 4.5). This increase in percentage revealed the enhanced transcription of TurboGFP, likely due to the presence of an SV40 enhancer. The percentage of cells in the P2 gate were used to assess the enhancer activity of the K3, K6 and K7 regions. The region resulting in the largest increase in the percentage of cells in P2 was K3. 30.8% of EL-4 cells transfected with the pTurboGFP.Fos-K3 plasmid fell in the P2 gate. This increase indicates the presence of a putative enhancer element in region K3.

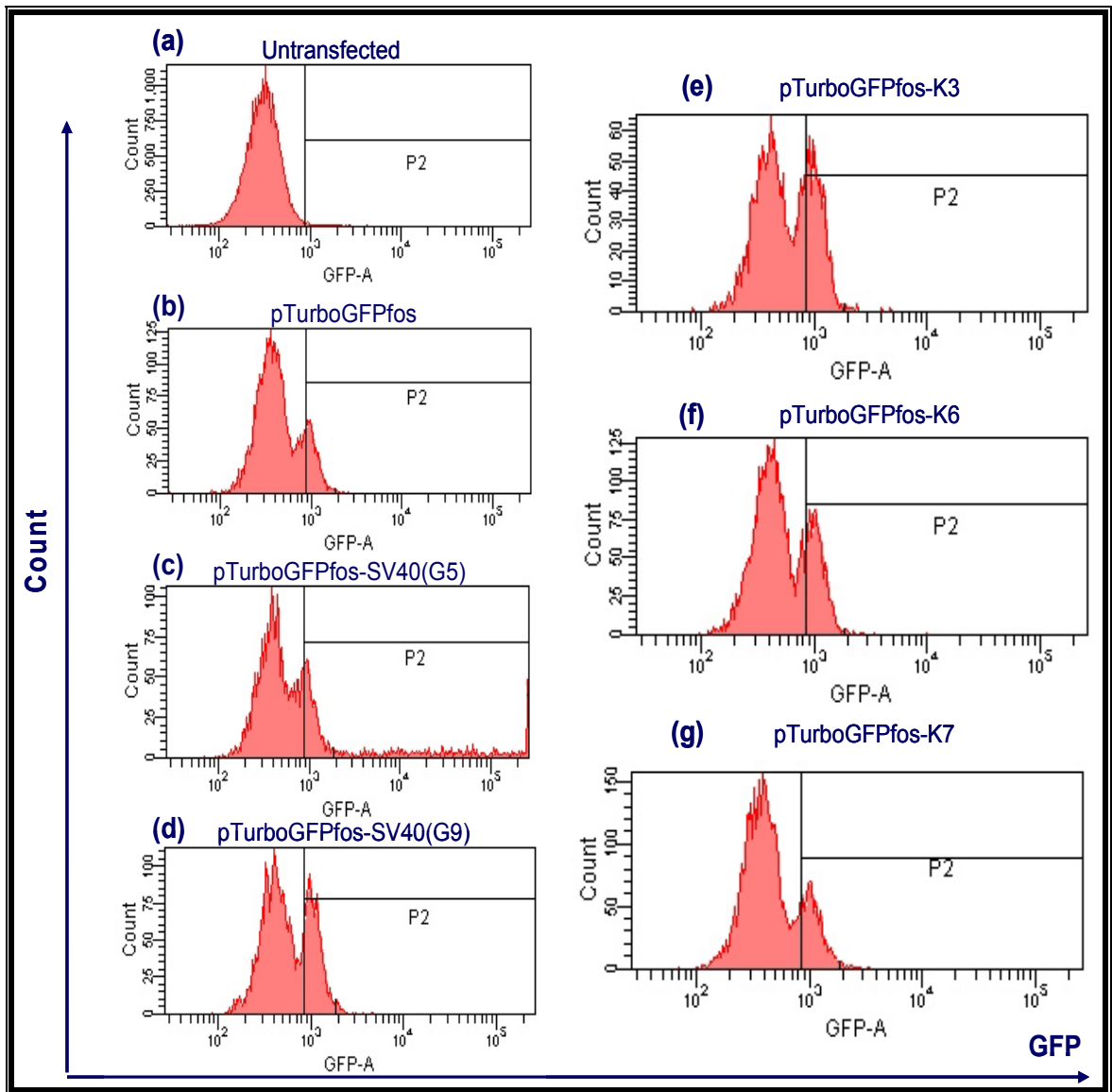


Figure 4.42: The flow cytometry data of reporter assays showing GFP versus number of events in each histogram.

SAMPLE	POPULATION	%PARENT	GFP MEAN	GFP MEDIAN
Untransfected (a)	P1	88,7	328	305
	P2	0,5	1266	1043
pTurboGFP.Fos (b)	P1	18,1	507	400
	P2	15,0	1107	1041
pTurboGFP.Fos- SV40(G5) (c)	P1	16,6	11327	506
	P2	30,5	36159	1394
pTurboGFP.Fos- SV40(G9) (d)	P1	17,9	667	516
	P2	32,0	1163	1086
pTurboGFP.Fos- K3 (e)	P1	10,5	656	530
	P2	30,8	1116	1057
pTurboGFP.Fos- K6 (f)	P1	19,0	600	471
	P2	24,5	1122	1060
pTurboGFP.Fos- K7 (g)	P1	21,9	544	417
	P2	19,1	1161	1074

Table 4.4: The quantitative data obtained from flow cytometric analysis reporter assay constructs.

5 DISCUSSION

When a typical metazoan genome is considered, the protein coding sequences account only for a small fraction. On the other hand the fraction of regulatory regions of DNA is as much as a third of the human genome. It is not easy to estimate the cis-regulatory content of metazoan genomes awaiting discovery.¹¹¹

The focus on the identification of regulatory elements for individual genes up to date has included several experimental approaches other than reporter assays including the generation of deletion constructs to determine the minimal sequences necessary for transcription in cell-culture-based systems; DNase1 hypersensitivity studies to identify sequences potentially available for transcription factor binding; and *in vitro* approaches, such as DNA footprinting and gel shifts, to determine sequences that bind various regulatory proteins. The common disadvantage of these studies is the lack of preliminary determination of the putative regions to check the activity that leads to either an unguided long journey of studies or small scale experiments spanning only a short region of DNA.

Use of computational sequence analysis to identify the conserved non coding sequences prior to the experimental approaches to identify the putative cis regulatory elements makes it possible to scan long distance regions as well as increasing the possibility of coming up with the functional regulatory regions.¹³¹ The CNS determined by cross species sequence comparison have a great reasonable probability of having gene regulatory properties¹³²⁻¹³⁴ as the sequences that mediate gene expression tend to be conserved between species, evolving at a slower rate than the non functional counterparts. The main assumption while using comparative genome analysis is that the functional conservation will be reflected in similar nucleotide sequences. Both global and local alignment of sequences is important for the determination of conserved regions in the compared genomes. Therefore both methods were used in the study and the non coding conserved regions to further study for the regulatory activity were determined according to the data from both of the analyses although the local alignment data did not change the resulting profile.

Prediction of regulatory regions based on bioinformatics of course could not be more than a start point for biological experiments and the regions identified by cross species comparison should be validated by further biological experiments for activity. The most widely used biological experiments to assess the regulatory function to a sequence of DNA include reporter systems that makes it possible to indirectly observe the effect of regions of interest by the aid of the reporter expression being used.

The previous studies to identify the upstream regulatory regions of IL-7R α included only 5 kb upstream of exon 1, and could identify two different Ets family transcription factor (PU.1 and GABP) functioning on the promoter of the gene. Among the identified transcription factors PU.1 was found to be essential in regulation of gene in pro-B cells and not expressed in T cell development.¹⁰⁵ The GABP on the other hand, was suggested as a regulator of transcription of IL-7R α gene in T cells but fail to explain the strict regulation in different developmental stages of T cells.¹⁰⁷ The concluding remark of both of these studies was that there could be an unidentified far upstream enhancer region controlling the expression of the gene that would facilitate the understanding of the tight regulation of transcription of IL-7R α . In this current study by comparative genome analysis between mouse and human genomic sequences, we identified seven CNS between 40kb and 70 kb upstream of exon1 in order to determine any cis-acting enhancer element far upstream of the gene. The names given to these seven regions were K1 to K7 as the gene upstream of the region was a Kpl2 isoform both in mouse and human.

To amplify regions K1 to K7, we selected RP23-365P6 BAC clone by using USCS Genome browser that contained the sequences corresponding to all the seven regions to be amplified. While designing the primers that will be used for the amplification, we used online Primer3 program and we added Sal1 restriction sites to the 5' ends of the primers for the following digestion of the PCR products prior to cloning into the reporter vectors.

The first reporter system chosen for our study was luciferase assay system. The basic promoterless luciferase vector pGL3 basic was used as the backbone and a weak promoter was inserted into this basic vector to be able to detect even a low enhanced activity due to the inserted region. After the ligation of the inserts with the constructed

pGL3.Fos vector, the colonies bearing correct ligation products were selected first by colony PCR and then by diagnostic digests.

The EL-4, mouse single positive CD4 T cell line was used for transfection as IL-7R α is expressed in these cells and consequently the cells contain the factors binding to putative enhancer regions. The EL-4 cell lines are suspension cells and they are not easily transfected using general transfection methods. Among the methods tried so far and also in our study electroporation was an effective strategy for the transfection of EL-4 cells with the plasmids. The transfection efficiency was first determined under fluorescence microscope following the transfection of the cells with GFP expressing positive control vectors. The preliminary transfection efficiency calculated was around 15 % and these low transfection efficiency made it difficult for us to carry out luciferase assay. The luciferase assays following optimization were carried out in our lab and no transcriptional activity was observed even in the positive control vector pGL3.Fos-ESV40 transfected EL-4 cells, indicating that the transfection efficiency of EL-4 cells was low and it was therefore not possible to study the expression level using the luciferase assay reporter system with the luminometer used in the experiments.

The search for an alternative reporter system that triggered the current project lead us to come up with the idea of using GFP as the reporter and flow cytometry as the scanning and detection method to determine cis-regulatory regions. The fluorescence intensity of GFP is a more direct measure of the promoter and enhancer activity that drives its expression than are assays for enzymes or protein binding, since there is no amplification of signal with substrates or multiple fluorophores. Analysis with flow cytometry makes it possible to determine reporter expression even when the transfection efficiency of used cell line is low, as it permits the quantification of expression level of reporter constructs as well as that of a reference plasmid at the single cell level.

Reporter vectors with the destabilized variant of GFP as the fluorescent protein to be assayed, was constructed by inserting the same weak promoter fos to the basic promoterless pTurboGFP-PRL-dest plasmid. TurboGFP found in this plasmid is an improved variant of the green fluorescent protein and reveals bright green fluorescence and noticeably faster maturation than GFP when expressed in eukaryotic cells. The destabilized variant of GFP has a rapid turnover rate resulting in less accumulation in

cells leading to lower toxicity and also it functions better as a transient reporter in the study of cis-acting regulatory elements or transcriptional induction.

The amplified regions cloned into the luciferase reporter vectors were digested out and religated to this constructed pTurboGFP.Fos vector. The EL-4 cell line in this study was transfected with this GFP reporter vectors containing the regions to be analyzed again using electroporation. The optimization of transfection was carried out this time by quantifying in a more reliable way via flow cytometry. For optimization the parameters tested were the ones that have been used in literature for transfection of EL-4 cells. The previously used condition with 290 V, 1400 μ F and 100 Ω using 15 to 20 μ g DNA to be transfected was found to be the optimum. The EL-4 cells transfected with pTurboGFP.Fos, pTurboGFP.Fos-ESV40 and the constructed K3, K6, K7 inserted pTurboGFP.Fos plasmids via electroporation. Flow cytometric analyses were carried out 24 hours after transfection.

The transfected cells in this study were analyzed on the BDFacsCanto flow cytometer. Using forward scatter (FSC) that is an indicator of the relative size and side scatter (SSC) that is an indicator of relative granularity of internal complexity, we eliminated dead cells and cell debris from the analysis via gating. The use of light scattered parameters for estimating viability could be used as a reasonable surrogate for more direct viability measurements as Propidium Iodide staining, as the EL-4 cell line showed distinctive changes in light scatter on loss of viability. To determine convenience of using light scatter data to estimate viability in EL-4 cells flow cytometric analysis were made for the same cell population with or without addition of propidium iodide and the population of viable cells determined in each case was compared.(Data not shown). GFP was excited by an argon laser and fluorescence was detected using a 530/30nm bandpass filter as the emission wavelength for TurboGFP is 502nm. The histograms of population P1 showing number of cells versus GFP fluorescence intensity was plotted to be able to compare the difference in expression of TurboGFP reporter in different constructs.

The pTurboGFP.Fos transfected cells showed a higher GFP fluorescence intensity when compared to the untransfected cells as a result of the activity of fos promoter on the expression. In this step a second gate was used in order to differentiate between the increases in GFP fluorescence due to promoter activity or inserted putative enhancer

activity. To test the effectiveness of the system used including the constructed pTurboGFP.Fos and the flow cytometer parameters selected, positive control plasmid pTurboGFP.Fos-ESV40 transfected cells were analyzed. We had two different positive control vector constructs, G5 having one reverse oriented SV40 enhancer and G9 having two SV40 enhancer one reverse one direct oriented. Data from both of them was useful as the difference between them would implicate the effect of the number and the orientation of enhancer on the activation of expression. As seen in the histograms, both of the positive control vectors showed an increased level of GFP compared to that of pTurboGFP.Fos transfected cells. The GFP amount not only increased in the mean value for the population, but also in G5 the intensity range increased leading to an increase in the percentage of cells in gate P3.

When the data obtained from the cells transfected with three constructs K3,K6 and K7 was analyzed, K7 showed almost no change in fluorescence indicating the absence of an enhancer element in this region. But for K3 the increase in GFP fluorescence and formation of the second higher peak falls into P2 gate, revealed that there could be an enhancer element in the region. The increase in GFP was close to the positive control vector G5. The region named K3 corresponds to 162000-163900 in vista input file, that is a 850bp length region 67kb upstream of IL-7R α exon1 in mouse(Figure 4.2). When the vista result of this region is observed it corresponds to a region containing three successive conserved noncoding sequences of 73,64 bp and 61 bp having 86.3, 87.5, 90.2% identity respectively (Table 4.1). 90.2% was the highest identity of conserved non coding sequences identified in the first place. Further studies should be done to elucidate the enhancer activity bearing region and to further characterize the enhancer. As the enhancers are cis-acting elements and can function in distance both in 5' and 3' direction, whether this enhancer functions on the expression of IL-7R α or the upstream Kpl2 homolog gene should further be determined.

Most previous systematic attempts to identify transcriptional enhancers utilized transient transfection assays; in this study of ours we employed the same strategy for identification of enhancer activity, through transient transfection of cells with our reporter constructs. However, in a study to find the enhancer region of the RAG gene, it was shown that a stable transfection construct was key to the discovery of the enhancer region identified and maybe of a value in other searches for enhancer activity.¹⁰ It was

stated that the activity of some cis acting regulatory regions including enhancers was demonstrable only when the reporter construct was chromosomally integrated. That is why use of stable transfections in order to identify enhancer regions might be of a great value. Further analysis of stably transfected EL-4 cells might change the obtained results or add up new regions previously failed to be identified.

After all the GFP reporter system used in this study and the flow cytometric analysis made were convenient to be used for further studies to reveal any cis-acting regulatory region.

6 CONCLUSION

IL-7 mediated cell signals through its receptor complex is central to lymphoid development. IL-7 receptor is a heterodimer composed of an ubiquitously expressed common gamma chain (γ_c) and IL-7R α that has a tightly regulated transcription. IL-7R α is abundantly expressed by fetal liver and bone marrow common lymphoid progenitors, early B cell precursors and cells of the T-lymphocyte lineage and important in responsiveness of cells to IL-7 mediated proliferation and survival signals. Mice lacking IL-7R α expression manifest substantial reductions in thymic and peripheral T cells as well as B cells. In humans mutations in IL-7R α gene results in a selective loss of T cells. Understanding the detailed mechanism controlling transcription of the IL-7R α gene is of great importance and until now only little is known about this regulatory mechanism.

In order to determine any cis acting element located upstream of IL-7R α gene, we constructed cross species sequence comparison between mouse and human genomic sequences and identified seven conserved non coding sequences. Flow cytometric reporter assay was chosen to study the enhancer activity of the sequences and for this purpose a GFP reporter vector by addition of a weak promoter fos to the upstream of TurboGFP in originally promoterless pTurboGFP-PRL-dest vector, was constructed. Three of the identified conserved non coding sequences were cloned into the constructed GFP reporter vectors and the presence of inserts in these constructed vectors was demonstrated via colony PCR and diagnostic digests.

EL-4 mouse T lymphoma cells were transfected with these constructs parallel with the positive control of reporter vector containing SV40 enhancer. The data obtained from flow cytometry was compared to that of empty reporter vector. The results indicated the presence of a putative enhancer region at least in one of the constructs that contains a highly conserved non coding sequence determined previously.

In conclusion, the studies presented herein provide the first step in the determination of cis regulatory elements of IL-7R α gene and elucidation of the mechanism controlling the IL-7R α transcription. The results not only enhanced our

understanding of flow cytometric reporter assay systems but also showed the effectiveness of the constructed GFP reporter vector and convenience of the strategy used to study the cis-acting regulatory regions regulating the transcriptional activity.

Additional studies are required to further characterize the putative enhancer element suggested in this study in order to show its effect on differential regulation of the IL-7R α expression. Moreover it is absolutely critical to carry out specific mutational experiments to show that the enhancer region identified acts on the downstream IL-7R α gene.

7 FUTURE STUDIES

Fully revealing the signaling and death pathways in the IL-7 system that are dependent on the generation of the signal and therefore the presence of the receptor, may indicate new molecular targets for chemotherapeutics and immune suppressants.

The potency and breadth of effects suggest that IL-7 administration or neutralization of IL-7 may allow the modulation of immune function in patients with lymphocyte depletion, vaccine administration, or autoimmunity. As shown previously IL-7 as an adjuvant increased the size of the CD8 memory pool generated following immunization.¹⁰⁰ The identification of the transcriptional control mechanism of IL-7R α gene might lead to the identification or design of putative therapeutic agents that might upregulate the level of immunological memory cells.

8 REFERENCES

1. Janeway, Charles. *Immunobiology : the immune system in health and disease*, xxiii, 823 p. (Garland Science, New York, 2005).
2. Ozaki, K. & Leonard, W. J. Cytokine and cytokine receptor pleiotropy and redundancy. *J Biol Chem* **277**, 29355-8 (2002).
3. Namen, A. E., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D. Y., Schmierer, A., Mosley, B., March, C. J., Urdal, D. & Gillis, S. Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature* **333**, 571-3 (1988).
4. Appasamy, P. M. Interleukin-7 and lymphopoiesis: biological and clinical implications. *Cancer Treat Res* **80**, 235-60 (1995).
5. Plum, J., De Smedt, M., Leclercq, G., Verhasselt, B. & Vandekerckhove, B. Interleukin-7 is a critical growth factor in early human T-cell development. *Blood* **88**, 4239-45 (1996).
6. Goodwin, R. G., Lupton, S., Schmierer, A., Hjerrild, K. J., Jerzy, R., Clevenger, W., Gillis, S., Cosman, D. & Namen, A. E. Human interleukin 7: molecular cloning and growth factor activity on human and murine B-lineage cells. *Proc Natl Acad Sci U S A* **86**, 302-6 (1989).
7. Stoddart, A., Fleming, H. E. & Paige, C. J. The role of the preBCR, the interleukin-7 receptor, and homotypic interactions during B-cell development. *Immunol Rev* **175**, 47-58 (2000).
8. Boesteanu, A., Silva, A. D., Nakajima, H., Leonard, W. J., Peschon, J. J. & Joyce, S. Distinct roles for signals relayed through the common cytokine receptor gamma chain and interleukin 7 receptor alpha chain in natural T cell development. *J Exp Med* **186**, 331-6 (1997).
9. Varas, A., Vicente, A., Jimenez, E., Alonso, L., Moreno, J., Munoz, J. J. & Zapata, A. G. Interleukin-7 treatment promotes the differentiation pathway of T-cell-receptor-alpha beta cells selectively to the CD8+ cell lineage. *Immunology* **92**, 457-64 (1997).
10. Hsu, L. Y., Lauring, J., Liang, H. E., Greenbaum, S., Cado, D., Zhuang, Y. & Schlissel, M. S. A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. *Immunity* **19**, 105-17 (2003).
11. Schluns, K. S., Kieper, W. C., Jameson, S. C. & Lefrancois, L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol* **1**, 426-32 (2000).

12. Schluns, K. S. & Lefrancois, L. Cytokine control of memory T-cell development and survival. *Nat Rev Immunol* **3**, 269-79 (2003).
13. Okamoto, Y., Douek, D. C., McFarland, R. D. & Koup, R. A. IL-7, the thymus, and naive T cells. *Adv Exp Med Biol* **512**, 81-90 (2002).
14. Fry, T. J., Connick, E., Falloon, J., Lederman, M. M., Liewehr, D. J., Spritzler, J., Steinberg, S. M., Wood, L. V., Yarchoan, R., Zuckerman, J., Landay, A. & Mackall, C. L. A potential role for interleukin-7 in T-cell homeostasis. *Blood* **97**, 2983-90 (2001).
15. Napolitano, L. A., Grant, R. M., Deeks, S. G., Schmidt, D., De Rosa, S. C., Herzenberg, L. A., Herndier, B. G., Andersson, J. & McCune, J. M. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. *Nat Med* **7**, 73-9 (2001).
16. Fry, T. J. & Mackall, C. L. Interleukin-7: from bench to clinic. *Blood* **99**, 3892-904 (2002).
17. Zhang, L., Keane, M. P., Zhu, L. X., Sharma, S., Rozengurt, E., Strieter, R. M., Dubinett, S. M. & Huang, M. Interleukin-7 and transforming growth factor-beta play counter-regulatory roles in protein kinase C-delta-dependent control of fibroblast collagen synthesis in pulmonary fibrosis. *J Biol Chem* **279**, 28315-9 (2004).
18. Park, L. S., Friend, D. J., Schmierer, A. E., Dower, S. K. & Namen, A. E. Murine interleukin 7 (IL-7) receptor. Characterization on an IL-7-dependent cell line. *J Exp Med* **171**, 1073-89 (1990).
19. Jiang, Q., Li, W. Q., Aiello, F. B., Mazzucchelli, R., Asefa, B., Khaled, A. R. & Durum, S. K. Cell biology of IL-7, a key lymphotrophin. *Cytokine Growth Factor Rev* **16**, 513-33 (2005).
20. Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Munakata, H., Nakamura, M. & Sugamura, K. Cloning of the gamma chain of the human IL-2 receptor. *Science* **257**, 379-82 (1992).
21. Leonard, W. J. Cytokines and immunodeficiency diseases. *Nat Rev Immunol* **1**, 200-8 (2001).
22. Noguchi, M., Nakamura, Y., Russell, S. M., Ziegler, S. F., Tsang, M., Cao, X. & Leonard, W. J. Interleukin-2 receptor gamma chain: a functional component of the interleukin-7 receptor. *Science* **262**, 1877-80 (1993).
23. Ziegler, S. E., Morella, K. K., Anderson, D., Kumaki, N., Leonard, W. J., Cosman, D. & Baumann, H. Reconstitution of a functional interleukin (IL)-7 receptor demonstrates that the IL-2 receptor gamma chain is required for IL-7 signal transduction. *Eur J Immunol* **25**, 399-404 (1995).
24. Lai, S. Y., Molden, J. & Goldsmith, M. A. Shared gamma(c) subunit within the human interleukin-7 receptor complex. A molecular basis for the pathogenesis

- of X-linked severe combined immunodeficiency. *J Clin Invest* **99**, 169-77 (1997).
25. Yao, Z., Cui, Y., Watford, W. T., Bream, J. H., Yamaoka, K., Hissong, B. D., Li, D., Durum, S. K., Jiang, Q., Bhandoola, A., Hennighausen, L. & O'Shea J, J. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc Natl Acad Sci U S A* **103**, 1000-5 (2006).
 26. Lynch, M. & Conery, J. S. The origins of genome complexity. *Science* **302**, 1401-4 (2003).
 27. He, Y. W., Adkins, B., Furse, R. K. & Malek, T. R. Expression and function of the gamma c subunit of the IL-2, IL-4, and IL-7 receptors. Distinct interaction of gamma c in the IL-4 receptor. *J Immunol* **154**, 1596-605 (1995).
 28. Trinchieri, G. Cytokines and cytokine receptors. *Immunol Rev* **202**, 5-7 (2004).
 29. Sudo, T., Nishikawa, S., Ohno, N., Akiyama, N., Tamakoshi, M., Yoshida, H. & Nishikawa, S. Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proc Natl Acad Sci U S A* **90**, 9125-9 (1993).
 30. Armitage, R. J., Ziegler, S. F., Beckmann, M. P., Idzerda, R. L., Park, L. S. & Fanslow, W. C. Expression of receptors for interleukin 4 and interleukin 7 on human T cells. *Adv Exp Med Biol* **292**, 121-30 (1991).
 31. Park, L. S., Martin, U., Garka, K., Gliniak, B., Di Santo, J. P., Muller, W., Largaespada, D. A., Copeland, N. G., Jenkins, N. A., Farr, A. G., Ziegler, S. F., Morrissey, P. J., Paxton, R. & Sims, J. E. Cloning of the murine thymic stromal lymphopoietin (TSLP) receptor: Formation of a functional heteromeric complex requires interleukin 7 receptor. *J Exp Med* **192**, 659-70 (2000).
 32. Hofmeister, R., Khaled, A. R., Benbernou, N., Rajnavolgyi, E., Muegge, K. & Durum, S. K. Interleukin-7: physiological roles and mechanisms of action. *Cytokine Growth Factor Rev* **10**, 41-60 (1999).
 33. Foxwell, B. M., Beadling, C., Guschin, D., Kerr, I. & Cantrell, D. Interleukin-7 can induce the activation of Jak 1, Jak 3 and STAT 5 proteins in murine T cells. *Eur J Immunol* **25**, 3041-6 (1995).
 34. Macchi, P., Villa, A., Giliani, S., Sacco, M. G., Frattini, A., Porta, F., Ugazio, A. G., Johnston, J. A., Candotti, F., O'Shea, J. J. & et al. Mutations of Jak-3 gene in patients with autosomal severe combined immune deficiency (SCID). *Nature* **377**, 65-8 (1995).
 35. Sambrook, Joseph & Russell, David W. *Molecular cloning : a laboratory manual*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001).
 36. Zeng, Y. X., Takahashi, H., Shibata, M. & Hirokawa, K. JAK3 Janus kinase is involved in interleukin 7 signal pathway. *FEBS Lett* **353**, 289-93 (1994).
 37. Chong, M. M., Cornish, A. L., Darwiche, R., Stanley, E. G., Purton, J. F., Godfrey, D. I., Hilton, D. J., Starr, R., Alexander, W. S. & Kay, T. W.

- Suppressor of cytokine signaling-1 is a critical regulator of interleukin-7-dependent CD8⁺ T cell differentiation. *Immunity* **18**, 475-87 (2003).
38. Trop, S., De Sepulveda, P., Zuniga-Pflucker, J. C. & Rottapel, R. Overexpression of suppressor of cytokine signaling-1 impairs pre-T-cell receptor-induced proliferation but not differentiation of immature thymocytes. *Blood* **97**, 2269-77 (2001).
 39. Fruman, D. A., Snapper, S. B., Yballe, C. M., Davidson, L., Yu, J. Y., Alt, F. W. & Cantley, L. C. Impaired B cell development and proliferation in absence of phosphoinositide 3-kinase p85alpha. *Science* **283**, 393-7 (1999).
 40. Sasaki, T., Irie-Sasaki, J., Jones, R. G., Oliveira-dos-Santos, A. J., Stanford, W. L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., Joza, N., Mak, T. W., Ohashi, P. S., Suzuki, A. & Penninger, J. M. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* **287**, 1040-6 (2000).
 41. Dadi, H. K., Ke, S. & Roifman, C. M. Interleukin 7 receptor mediates the activation of phosphatidylinositol-3 kinase in human B-cell precursors. *Biochem Biophys Res Commun* **192**, 459-64 (1993).
 42. Dadi, H., Ke, S. & Roifman, C. M. Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor is dependent on protein tyrosine kinase activity. *Blood* **84**, 1579-86 (1994).
 43. Venkitaraman, A. R. & Cowling, R. J. Interleukin-7 induces the association of phosphatidylinositol 3-kinase with the alpha chain of the interleukin-7 receptor. *Eur J Immunol* **24**, 2168-74 (1994).
 44. Seckinger, P. & Fougereau, M. Activation of src family kinases in human pre-B cells by IL-7. *J Immunol* **153**, 97-109 (1994).
 45. Miller, J. P., Izon, D., DeMuth, W., Gerstein, R., Bhandoola, A. & Allman, D. The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7. *J Exp Med* **196**, 705-11 (2002).
 46. Peschon, J. J., Morrissey, P. J., Grabstein, K. H., Ramsdell, F. J., Maraskovsky, E., Gliniak, B. C., Park, L. S., Ziegler, S. F., Williams, D. E., Ware, C. B., Meyer, J. D. & Davison, B. L. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *J Exp Med* **180**, 1955-60 (1994).
 47. von Freeden-Jeffry, U., Vieira, P., Lucian, L. A., McNeil, T., Burdach, S. E. & Murray, R. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *J Exp Med* **181**, 1519-26 (1995).
 48. Ray, R. J., Furlonger, C., Williams, D. E. & Paige, C. J. Characterization of thymic stromal-derived lymphopoietin (TSLP) in murine B cell development in vitro. *Eur J Immunol* **26**, 10-6 (1996).

49. Samaridis, J., Casorati, G., Traunecker, A., Iglesias, A., Gutierrez, J. C., Muller, U. & Palacios, R. Development of lymphocytes in interleukin 7-transgenic mice. *Eur J Immunol* **21**, 453-60 (1991).
50. Morrissey, P. J., Conlon, P., Charrier, K., Braddy, S., Alpert, A., Williams, D., Namen, A. E. & Mochizuki, D. Administration of IL-7 to normal mice stimulates B-lymphopoiesis and peripheral lymphadenopathy. *J Immunol* **147**, 561-8 (1991).
51. Damia, G., Komschlies, K. L., Faltynek, C. R., Ruscetti, F. W. & Wiltrout, R. H. Administration of recombinant human interleukin-7 alters the frequency and number of myeloid progenitor cells in the bone marrow and spleen of mice. *Blood* **79**, 1121-9 (1992).
52. Sudo, T., Ito, M., Ogawa, Y., Iizuka, M., Kodama, H., Kunisada, T., Hayashi, S., Ogawa, M., Sakai, K. & Nishikawa, S. Interleukin 7 production and function in stromal cell-dependent B cell development. *J Exp Med* **170**, 333-8 (1989).
53. Marshall, A. J., Fleming, H. E., Wu, G. E. & Paige, C. J. Modulation of the IL-7 dose-response threshold during pro-B cell differentiation is dependent on pre-B cell receptor expression. *J Immunol* **161**, 6038-45 (1998).
54. Corcoran, A. E., Smart, F. M., Cowling, R. J., Crompton, T., Owen, M. J. & Venkitaraman, A. R. The interleukin-7 receptor alpha chain transmits distinct signals for proliferation and differentiation during B lymphopoiesis. *Embo J* **15**, 1924-32 (1996).
55. Wei, C., Zeff, R. & Goldschneider, I. Murine pro-B cells require IL-7 and its receptor complex to up-regulate IL-7R alpha, terminal deoxynucleotidyltransferase, and c mu expression. *J Immunol* **164**, 1961-70 (2000).
56. Smart, F. M. & Venkitaraman, A. R. Inhibition of interleukin 7 receptor signaling by antigen receptor assembly. *J Exp Med* **191**, 737-42 (2000).
57. Corcoran, A. E., Riddell, A., Krooshoop, D. & Venkitaraman, A. R. Impaired immunoglobulin gene rearrangement in mice lacking the IL-7 receptor. *Nature* **391**, 904-7 (1998).
58. Hikida, M., Nakayama, Y., Yamashita, Y., Kumazawa, Y., Nishikawa, S. I. & Ohmori, H. Expression of recombination activating genes in germinal center B cells: involvement of interleukin 7 (IL-7) and the IL-7 receptor. *J Exp Med* **188**, 365-72 (1998).
59. Glusman, G., Rowen, L., Lee, I., Boysen, C., Roach, J. C., Smit, A. F., Wang, K., Koop, B. F. & Hood, L. Comparative genomics of the human and mouse T cell receptor loci. *Immunity* **15**, 337-49 (2001).
60. Puel, A. & Leonard, W. J. Mutations in the gene for the IL-7 receptor result in T(-)B(+)NK(+) severe combined immunodeficiency disease. *Curr Opin Immunol* **12**, 468-73 (2000).

61. Prieyl, J. A. & LeBien, T. W. Interleukin 7 independent development of human B cells. *Proc Natl Acad Sci U S A* **93**, 10348-53 (1996).
62. Saeland, S., Duvert, V., Pandrau, D., Caux, C., Durand, I., Wrighton, N., Wideman, J., Lee, F. & Banchereau, J. Interleukin-7 induces the proliferation of normal human B-cell precursors. *Blood* **78**, 2229-38 (1991).
63. Dittel, B. N. & LeBien, T. W. The growth response to IL-7 during normal human B cell ontogeny is restricted to B-lineage cells expressing CD34. *J Immunol* **154**, 58-67 (1995).
64. Fry, T. J. & Mackall, C. L. The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *J Immunol* **174**, 6571-6 (2005).
65. Wiles, M. V., Ruiz, P. & Imhof, B. A. Interleukin-7 expression during mouse thymus development. *Eur J Immunol* **22**, 1037-42 (1992).
66. Amsen, D. & Kruisbeek, A. M. Thymocyte selection: not by TCR alone. *Immunol Rev* **165**, 209-29 (1998).
67. Ceredig, R. & Rolink, T. A positive look at double-negative thymocytes. *Nat Rev Immunol* **2**, 888-97 (2002).
68. Conlon, P. J., Morrissey, P. J., Nordan, R. P., Grabstein, K. H., Prickett, K. S., Reed, S. G., Goodwin, R., Cosman, D. & Namen, A. E. Murine thymocytes proliferate in direct response to interleukin-7. *Blood* **74**, 1368-73 (1989).
69. Chazen, G. D., Pereira, G. M., LeGros, G., Gillis, S. & Shevach, E. M. Interleukin 7 is a T-cell growth factor. *Proc Natl Acad Sci U S A* **86**, 5923-7 (1989).
70. Costello, R., Imbert, J. & Olive, D. Interleukin-7, a major T-lymphocyte cytokine. *Eur Cytokine Netw* **4**, 253-62 (1993).
71. Murray, R., Suda, T., Wrighton, N., Lee, F. & Zlotnik, A. IL-7 is a growth and maintenance factor for mature and immature thymocyte subsets. *Int Immunol* **1**, 526-31 (1989).
72. Crompton, T., Outram, S. V., Buckland, J. & Owen, M. J. Distinct roles of the interleukin-7 receptor alpha chain in fetal and adult thymocyte development revealed by analysis of interleukin-7 receptor alpha-deficient mice. *Eur J Immunol* **28**, 1859-66 (1998).
73. Napolitano, L. A., Stoddart, C. A., Hanley, M. B., Wieder, E. & McCune, J. M. Effects of IL-7 on early human thymocyte progenitor cells in vitro and in SCID-hu Thy/Liv mice. *J Immunol* **171**, 645-54 (2003).
74. Moore, T. A., von Freeden-Jeffry, U., Murray, R. & Zlotnik, A. Inhibition of gamma delta T cell development and early thymocyte maturation in IL-7 ^{-/-} mice. *J Immunol* **157**, 2366-73 (1996).

75. Khaled, A. R., Li, W. Q., Huang, J., Fry, T. J., Khaled, A. S., Mackall, C. L., Muegge, K., Young, H. A. & Durum, S. K. Bax deficiency partially corrects interleukin-7 receptor alpha deficiency. *Immunity* **17**, 561-73 (2002).
76. Munitic, I., Williams, J. A., Yang, Y., Dong, B., Lucas, P. J., El Kassar, N., Gress, R. E. & Ashwell, J. D. Dynamic regulation of IL-7 receptor expression is required for normal thymopoiesis. *Blood* **104**, 4165-72 (2004).
77. Candeias, S., Muegge, K. & Durum, S. K. IL-7 receptor and VDJ recombination: trophic versus mechanistic actions. *Immunity* **6**, 501-8 (1997).
78. Durum, S. K., Candeias, S., Nakajima, H., Leonard, W. J., Baird, A. M., Berg, L. J. & Muegge, K. Interleukin 7 receptor control of T cell receptor gamma gene rearrangement: role of receptor-associated chains and locus accessibility. *J Exp Med* **188**, 2233-41 (1998).
79. Bertolino, E., Reddy, K., Medina, K. L., Parganas, E., Ihle, J. & Singh, H. Regulation of interleukin 7-dependent immunoglobulin heavy-chain variable gene rearrangements by transcription factor STAT5. *Nat Immunol* **6**, 836-43 (2005).
80. Ye, S. K., Agata, Y., Lee, H. C., Kurooka, H., Kitamura, T., Shimizu, A., Honjo, T. & Ikuta, K. The IL-7 receptor controls the accessibility of the TCRgamma locus by Stat5 and histone acetylation. *Immunity* **15**, 813-23 (2001).
81. Trigueros, C., Hozumi, K., Silva-Santos, B., Bruno, L., Hayday, A. C., Owen, M. J. & Pennington, D. J. Pre-TCR signaling regulates IL-7 receptor alpha expression promoting thymocyte survival at the transition from the double-negative to double-positive stage. *Eur J Immunol* **33**, 1968-77 (2003).
82. Park, J. H., Yu, Q., Erman, B., Appelbaum, J. S., Montoya-Durango, D., Grimes, H. L. & Singer, A. Suppression of IL7Ralpha transcription by IL-7 and other prosurvival cytokines: a novel mechanism for maximizing IL-7-dependent T cell survival. *Immunity* **21**, 289-302 (2004).
83. Erman, B., Feigenbaum, L., Coligan, J. E. & Singer, A. Early TCRalpha expression generates TCRalphagamma complexes that signal the DN-to-DP transition and impair development. *Nat Immunol* **3**, 564-9 (2002).
84. He, W. & Kabelitz, D. Differential effects of interleukin-2 and interleukin-7 on the induction of CD4 and CD8 expression by double-negative human thymocytes. *Scand J Immunol* **41**, 309-12 (1995).
85. Yu, Q., Park, J. H., Doan, L. L., Erman, B., Feigenbaum, L. & Singer, A. Cytokine signal transduction is suppressed in preselection double-positive thymocytes and restored by positive selection. *J Exp Med* **203**, 165-75 (2006).
86. Brugnera, E., Bhandoola, A., Cibotti, R., Yu, Q., Guinter, T. I., Yamashita, Y., Sharrow, S. O. & Singer, A. Coreceptor reversal in the thymus: signaled CD4+8+ thymocytes initially terminate CD8 transcription even when differentiating into CD8+ T cells. *Immunity* **13**, 59-71 (2000).

87. Yu, Q., Erman, B., Bhandoola, A., Sharrow, S. O. & Singer, A. In vitro evidence that cytokine receptor signals are required for differentiation of double positive thymocytes into functionally mature CD8⁺ T cells. *J Exp Med* **197**, 475-87 (2003).
88. Erman, B., Guinter, T. I. & Singer, A. Defined alphabeta T cell receptors with distinct ligand specificities do not require those ligands to signal double negative thymocyte differentiation. *J Exp Med* **199**, 1719-24 (2004).
89. Bachmann, M. F., Wolint, P., Schwarz, K., Jager, P. & Oxenius, A. Functional properties and lineage relationship of CD8⁺ T cell subsets identified by expression of IL-7 receptor alpha and CD62L. *J Immunol* **175**, 4686-96 (2005).
90. Murphy, K. M. & Reiner, S. L. The lineage decisions of helper T cells. *Nat Rev Immunol* **2**, 933-44 (2002).
91. Germain, R. N. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol* **2**, 309-22 (2002).
92. Phillips, J. A., Brondstetter, T. I., English, C. A., Lee, H. E., Virts, E. L. & Thoman, M. L. IL-7 gene therapy in aging restores early thymopoiesis without reversing involution. *J Immunol* **173**, 4867-74 (2004).
93. Fry, T. J. & Mackall, C. L. Interleukin-7: master regulator of peripheral T-cell homeostasis? *Trends Immunol* **22**, 564-71 (2001).
94. Okazaki, H., Ito, M., Sudo, T., Hattori, M., Kano, S., Katsura, Y. & Minato, N. IL-7 promotes thymocyte proliferation and maintains immunocompetent thymocytes bearing alpha beta or gamma delta T-cell receptors in vitro: synergism with IL-2. *J Immunol* **143**, 2917-22 (1989).
95. Borger, P., Kauffman, H. F., Postma, D. S. & Vellenga, E. IL-7 differentially modulates the expression of IFN-gamma and IL-4 in activated human T lymphocytes by transcriptional and post-transcriptional mechanisms. *J Immunol* **156**, 1333-8 (1996).
96. Schmitt, N., Chene, L., Boutolleau, D., Nugeyre, M. T., Guillemard, E., Versmisse, P., Jacquemot, C., Barre-Sinoussi, F. & Israel, N. Positive regulation of CXCR4 expression and signaling by interleukin-7 in CD4⁺ mature thymocytes correlates with their capacity to favor human immunodeficiency X4 virus replication. *J Virol* **77**, 5784-93 (2003).
97. Seddon, B., Tomlinson, P. & Zamoyska, R. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat Immunol* **4**, 680-6 (2003).
98. Ku, C. C., Murakami, M., Sakamoto, A., Kappler, J. & Marrack, P. Control of homeostasis of CD8⁺ memory T cells by opposing cytokines. *Science* **288**, 675-8 (2000).
99. Lee, S. K. & Surh, C. D. Role of interleukin-7 in bone and T-cell homeostasis. *Immunol Rev* **208**, 169-80 (2005).

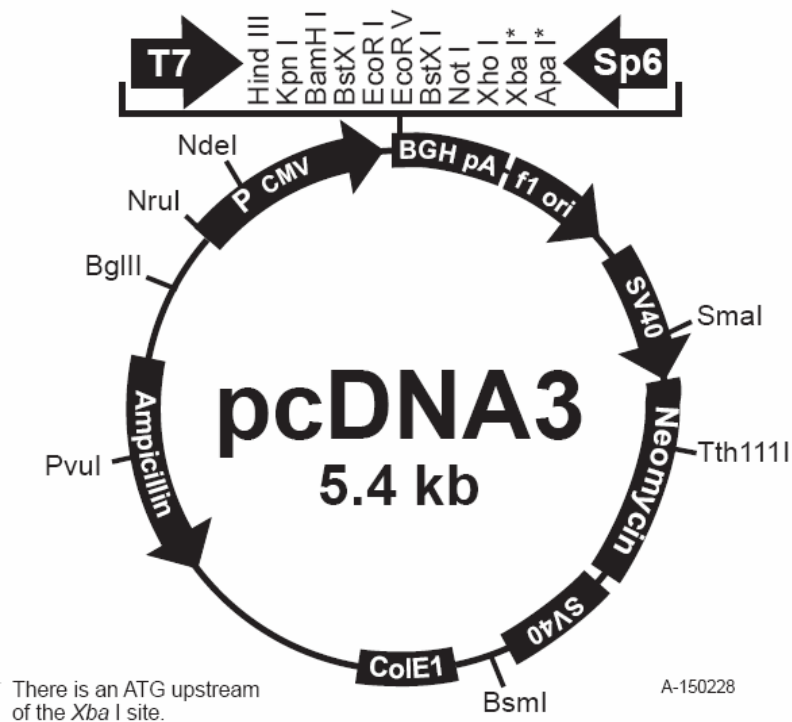
100. Melchionda, F., Fry, T. J., Milliron, M. J., McKirdy, M. A., Tagaya, Y. & Mackall, C. L. Adjuvant IL-7 or IL-15 overcomes immunodominance and improves survival of the CD8⁺ memory cell pool. *J Clin Invest* **115**, 1177-87 (2005).
101. Moniuszko, M., Fry, T., Tsai, W. P., Morre, M., Assouline, B., Cortez, P., Lewis, M. G., Cairns, S., Mackall, C. & Franchini, G. Recombinant interleukin-7 induces proliferation of naive macaque CD4⁺ and CD8⁺ T cells in vivo. *J Virol* **78**, 9740-9 (2004).
102. Bradley, L. M., Harbertson, J., Freschi, G. C., Kondrack, R. & Linton, P. J. Regulation of development and function of memory CD4 subsets. *Immunol Res* **21**, 149-58 (2000).
103. Kondrack, R. M., Harbertson, J., Tan, J. T., McBreen, M. E., Surh, C. D. & Bradley, L. M. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J Exp Med* **198**, 1797-806 (2003).
104. Kaech, S. M., Tan, J. T., Wherry, E. J., Konieczny, B. T., Surh, C. D. & Ahmed, R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* **4**, 1191-8 (2003).
105. DeKoter, R. P., Lee, H. J. & Singh, H. PU.1 regulates expression of the interleukin-7 receptor in lymphoid progenitors. *Immunity* **16**, 297-309 (2002).
106. Colucci, F., Samson, S. I., DeKoter, R. P., Lantz, O., Singh, H. & Di Santo, J. P. Differential requirement for the transcription factor PU.1 in the generation of natural killer cells versus B and T cells. *Blood* **97**, 2625-32 (2001).
107. Xue, H. H., Bollenbacher, J., Rovella, V., Tripuraneni, R., Du, Y. B., Liu, C. Y., Williams, A., McCoy, J. P. & Leonard, W. J. GA binding protein regulates interleukin 7 receptor alpha-chain gene expression in T cells. *Nat Immunol* **5**, 1036-44 (2004).
108. Lee, H. C., Shibata, H., Ogawa, S., Maki, K. & Ikuta, K. Transcriptional regulation of the mouse IL-7 receptor alpha promoter by glucocorticoid receptor. *J Immunol* **174**, 7800-6 (2005).
109. Franchimont, D., Galon, J., Vacchio, M. S., Fan, S., Visconti, R., Frucht, D. M., Geenen, V., Chrousos, G. P., Ashwell, J. D. & O'Shea, J. J. Positive effects of glucocorticoids on T cell function by up-regulation of IL-7 receptor alpha. *J Immunol* **168**, 2212-8 (2002).
110. Mitchell, P. J. & Tjian, R. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science* **245**, 371-8 (1989).
111. Levine, M. & Tjian, R. Transcription regulation and animal diversity. *Nature* **424**, 147-51 (2003).
112. Banerji, J., Rusconi, S. & Schaffner, W. Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences. *Cell* **27**, 299-308 (1981).

113. Webber, A. L. & Tilghman, S. M. The absence of enhancer competition between Igf2 and H19 following transfer into differentiated cells. *Mol Cell Biol* **18**, 1903-10 (1998).
114. Dorsett, D. Distant liaisons: long-range enhancer-promoter interactions in *Drosophila*. *Curr Opin Genet Dev* **9**, 505-14 (1999).
115. Morley, M., Molony, C. M., Weber, T. M., Devlin, J. L., Ewens, K. G., Spielman, R. S. & Cheung, V. G. Genetic analysis of genome-wide variation in human gene expression. *Nature* **430**, 743-7 (2004).
116. Dubchak, I. & Frazer, K. Multi-species sequence comparison: the next frontier in genome annotation. *Genome Biol* **4**, 122 (2003).
117. Frazer, K. A., Elnitski, L., Church, D. M., Dubchak, I. & Hardison, R. C. Cross-species sequence comparisons: a review of methods and available resources. *Genome Res* **13**, 1-12 (2003).
118. Nardone, J., Lee, D. U., Ansel, K. M. & Rao, A. Bioinformatics for the 'bench biologist': how to find regulatory regions in genomic DNA. *Nat Immunol* **5**, 768-74 (2004).
119. Ovcharenko, I., Loots, G. G., Giardine, B. M., Hou, M., Ma, J., Hardison, R. C., Stubbs, L. & Miller, W. Mulan: multiple-sequence local alignment and visualization for studying function and evolution. *Genome Res* **15**, 184-94 (2005).
120. Kirschner, M. & Gerhart, J. Evolvability. *Proc Natl Acad Sci U S A* **95**, 8420-7 (1998).
121. Chapman, M. A., Donaldson, I. J., Gilbert, J., Grafham, D., Rogers, J., Green, A. R. & Gottgens, B. Analysis of multiple genomic sequence alignments: a web resource, online tools, and lessons learned from analysis of mammalian SCL loci. *Genome Res* **14**, 313-8 (2004).
122. Williams, T. M., Burlein, J. E., Ogden, S., Kricka, L. J. & Kant, J. A. Advantages of firefly luciferase as a reporter gene: application to the interleukin-2 gene promoter. *Anal Biochem* **176**, 28-32 (1989).
123. Wittrup, K. D. The single cell as a microplate well. *Nat Biotechnol* **18**, 1039-40 (2000).
124. Ducrest, A. L., Amacker, M., Lingner, J. & Nabholz, M. Detection of promoter activity by flow cytometric analysis of GFP reporter expression. *Nucleic Acids Res* **30**, e65 (2002).
125. Jaroszeski, Mark J. & Heller, Richard. *Flow cytometry protocols*, x, 274 p. (Humana Press, Totowa, N.J., 1998).
126. Mazda, O., Teshigawara, K., Fujimoto, S., Hattori, N., Dou, Y. M. & Katsura, Y. A reporter system using a flow cytometer to detect promoter/enhancer activity in lymphoid cell lines. *J Immunol Methods* **169**, 53-61 (1994).

127. Gerdes, H. H. & Kaether, C. Green fluorescent protein: applications in cell biology. *FEBS Lett* **389**, 44-7 (1996).
128. Phillips, G. N., Jr. Structure and dynamics of green fluorescent protein. *Curr Opin Struct Biol* **7**, 821-7 (1997).
129. Cody, C. W., Prasher, D. C., Westler, W. M., Prendergast, F. G. & Ward, W. W. Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. *Biochemistry* **32**, 1212-8 (1993).
130. Zhao, X., Duong, T., Huang, C. C., Kain, S. R. & Li, X. Comparison of enhanced green fluorescent protein and its destabilized form as transcription reporters. *Methods Enzymol* **302**, 32-8 (1999).
131. Pennacchio, L. A. & Rubin, E. M. Genomic strategies to identify mammalian regulatory sequences. *Nat Rev Genet* **2**, 100-9 (2001).
132. Duret, L. & Bucher, P. Searching for regulatory elements in human noncoding sequences. *Curr Opin Struct Biol* **7**, 399-406 (1997).
133. Hardison, R. C., Oeltjen, J. & Miller, W. Long human-mouse sequence alignments reveal novel regulatory elements: a reason to sequence the mouse genome. *Genome Res* **7**, 959-66 (1997).
134. Hardison, R. C. Conserved noncoding sequences are reliable guides to regulatory elements. *Trends Genet* **16**, 369-72 (2000).

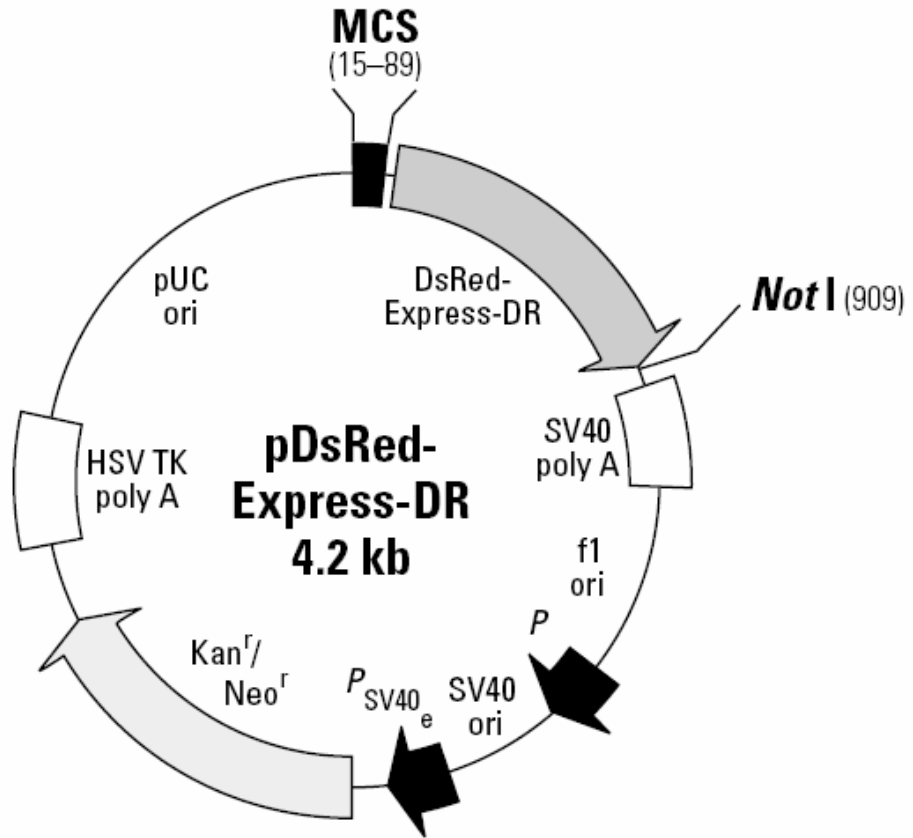
APPENDIX-PLASMID MAPS

pcDNA3



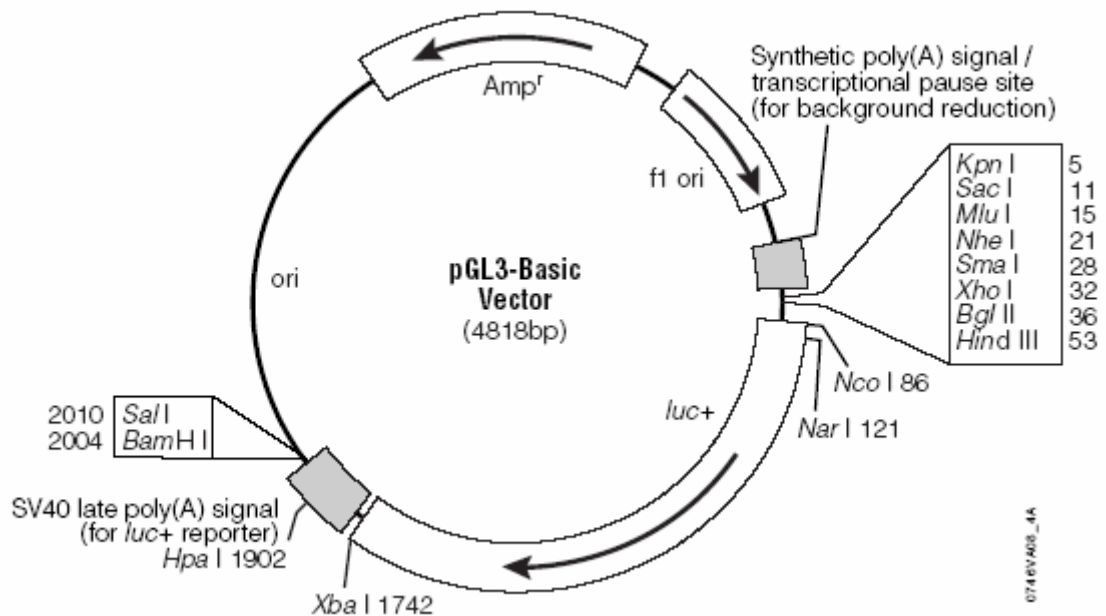
Features	Location (bases)
CMV Promoter	209-863
T7 Promoter	864-882
Polylinker	889-994
Sp6 Promoter	999-1016
BGH poly A	1018-1249
SV40 Promoter	1790-2115
SV40 Origin of replication	1984-2069
Neomycin ORF	2151-2945
SV40 polyA	3000-3372
ColE1 origin	3632-4305
Ampicillin ORF	4450-5310

pDSRedExpress-DR



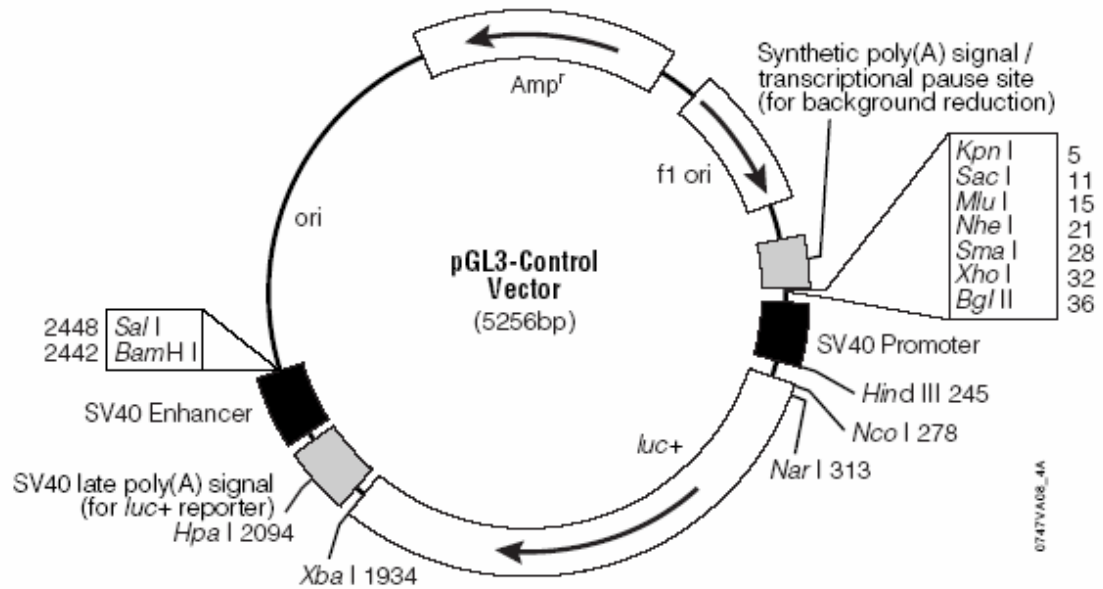
Features	Location (bases)
MCS	15-89
DSRedExpress	97-784
PEST	784-906
SV40 early poly A	1059-1109
F1 Origin of Replication	1018-1249
SV40 Origin of replication	1952-2087
Kan/Neo Resistance gene	2136-2930
HSV poly A	3166-3184
pUC plasmid replication origin	3515-4158

pGL3Basic



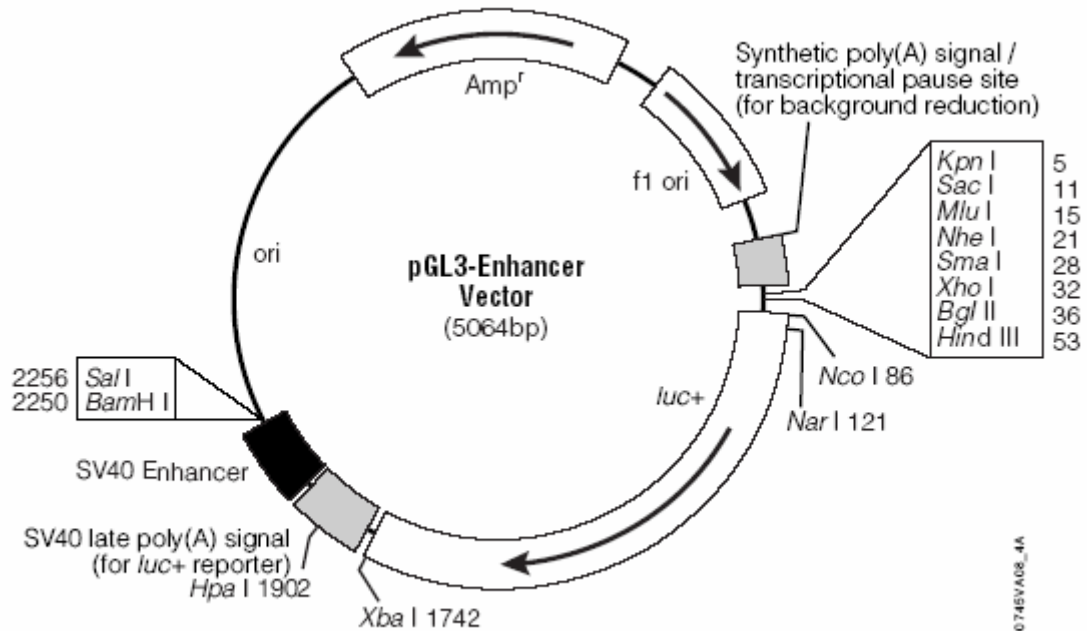
SV40 Promoter	(none)
SV40 Enhancer	(none)
Multiple cloning region	1-58
Luciferase gene (<i>luc+</i>)	88-1740
GLprimer2 binding site	89-111
SV40 late poly(A) signal	1772-1993
RVprimer4 binding site	2080-2061
<i>ColE</i> 1-derived plasmid replication origin	2318
β -lactamase gene (<i>Amp^r</i>)	3080-3940
f1 origin	4072-4527
Synthetic poly(A) signal	4658-4811
RVprimer3 binding site	4760-4779

pGL3Control



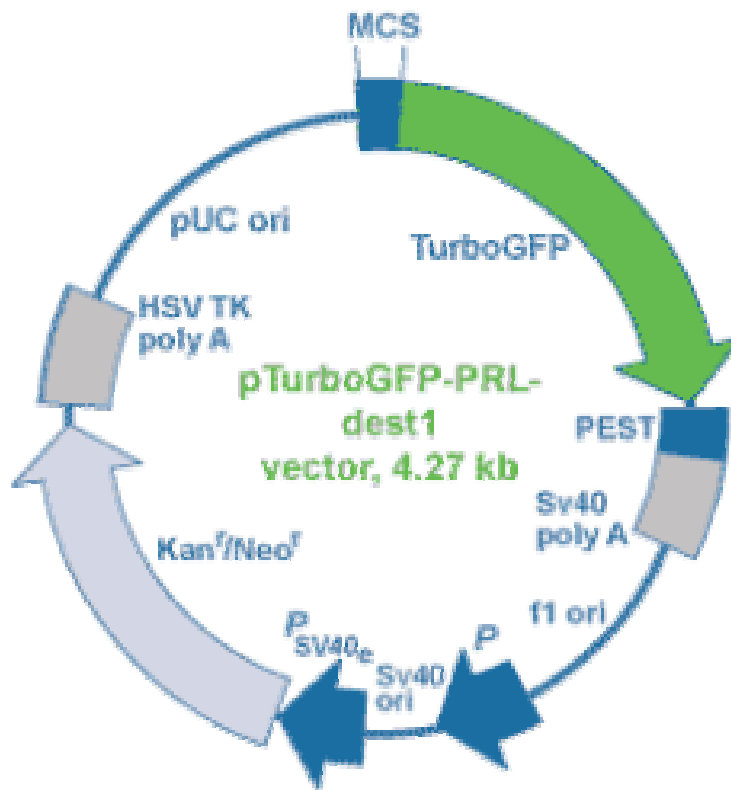
Multiple cloning region	1-41
SV40 Promoter	48-250
Luciferase gene (<i>luc+</i>)	280-1932
GLprimer2 binding site	281-303
SV40 late poly(A) signal	1964-2185
SV40 Enhancer	2205-2441
RVprimer4 binding site	2499-2518
<i>ColE</i> 1-derived plasmid replication origin	2756
β -lactamase gene (<i>Amp^r</i>)	3518-4378
f1 origin	4510-4965
Synthetic poly(A) signal	5096-5249
RVprimer3 binding site	5198-5217

pGL3Enhancer



SV40 Promoter	(none)
Multiple cloning region	1–58
Luciferase gene (<i>luc+</i>)	88–1740
GLprimer2 binding site	89–111
SV40 late poly(A) signal	1772–1993
SV40 Enhancer	2013–2249
RVprimer4 binding site	2307–2326
<i>ColE1</i> -derived plasmid replication origin	2564
β -lactamase gene (<i>Amp^r</i>)	3326–4186
f1 origin	4318–4773
Synthetic poly(A) signal	4904–5057
RVprimer3 binding site	5006–5025

pTurboGFP-PRL-DEST



Features	Location (bases)
MCS	12-89
TurboGFP	90-792
MODC PEST	808-930
SV40 early poly A	1085-1119
F1 Origin of Replication	1182-1637
SV40 Origin of replication	1978-2113
Kan/Neo Resistance gene	2162-2956
HSV poly A	3166-3184
pUC plasmid replication origin	3541-4184